Improving the clinical pathway for Crohn's perianal fistulas - novel approaches to aetiopathogenesis, treatment and outcome measurement

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Declaration

That this thesis, and all material presented herein, is the result of my own work and original ideas, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.

Surgical procedures and treatment were undertaken by the clinical staff at St Mark's Hospital.

Dedication

To my God, the young family you've given me (Simisola, Olayinka) and the ones who have supported my journey in delivering this piece of work; Dad, Mum, Tosin, Anu, I love you all dearly and as insufficient as the words are… thank you.

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Abstract

Crohn's perianal fistulas are often complex and represent a challenging and disabling disease phenotype with unknown optimal treatment strategy. There are paucities in understanding of disease aetiopathogenesis, paucity in real word data on long-term outcomes in the biologic era, and the best medical / surgical treatment strategies remain unknown. There is limited ability to measure robust and comparative outcomes due to heterogeneity in outcome measurement with uncertain relevance to patients.

Metabonomics (metabolic profiling/metabolomics) is a rapidly advancing field in systems biology that generates disease-relevant micro-molecular information downstream of the genome and proteome. Metabolic profiling studies utilising nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) have demonstrated early promise in inflammatory bowel disease research. In this thesis, I have implemented a metabolic profiling strategy using mass spectrometry for evaluation of fresh frozen fistula tissue from idiopathic and Crohn's fistula patients and in so doing have distinguished these two groups of fistula patients by their fistula metabolic phenotype, developing corroborative hypotheses on factors involved in pathogenesis.

Using real world data from a single institution I have catalogued the disease course for a cohort of patients with Crohn's perianal fistula on anti-TNF therapy over an 11 year period, representing one of the longest follow-up durations / largest cohorts investigated. I have also investigated and chronicled the disease course and natural history for a cohort of patients refractory to anti-TNF, who inevitably have limited options in management. I have also characterised the disease burden in this group, using novel disease states to model transition probabilities between these over time.

In order to attempt to streamline patients into avoiding futile treatments and stemming the burden of futile treatment and potential side effects in refractory patients, I have investigated the potential of tissue levels as a biomarker of treatment response. I demonstrate the absence of tissue anti-TNF (infliximab and adalimumab) in a small cohort of Crohn's fistula patients on maintenance anti-TNF therapy for Crohn's perianal fistula. Further work is required to corroborate this interesting finding and relate it to clinical outcome as well as develop the search for such a biomarker to facilitate personalised treatment pathways for these patients.

I have explored novel minimally invasive surgical treatment options for perianal fistulas, describing early success with FiLaC™, VAAFT and OTSC® for idiopathic fistulas but with even more limited evidence in Crohn's fistulas. This thesis introduces the concept of symptom amelioration for symptom refractory Crohn's perianal fistulas, demonstrating patient reported benefit in amelioration of symptoms of pain and discharge. However, this was limited by the absence of a control arm and the lack of a validated patient reported outcome measure.

To address the latter issue I have developed a new patient reported outcome measure, the Crohn's Anal Fistula Quality of Life (CAF-QoL) questionnaire, using a qualitative exploration into the lives of patients with Crohn's perianal fistulas as well as a multidisciplinary nationwide consensus exercise to inform a process of item generation and psychometric testing, and investigating stability, reliability, construct and content validity and sensitivity to change.

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Abbreviations

AGA	American Gastroenterology Association
Anti-TNF(α)	Anti-tumour necrosis factor-alpha
ASC	Adipose-derived stem cells
AZA	Azathioprine
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
Cer	Ceramide
CI	Confidence Interval
COS	Core Outcome Set
CT	Computer Tomography
CTVC	Computer Tomography Virtual Colonography
DG	Diglyceride (diacylglycerol)
EAS	External Anal Sphincter
ECCO	European Crohn's & Colitis Organisation
ENiGMA	Evaluating goal-directed management of fistulising perianal Crohn's disease
ERAF	Endorectal advancement flaps
EUA	Examination under anaesthesia
EUS	Endoanal Ultrasound
FC	Faecal Calprotectin
FDA	Fistula drainage assessment
FDR	False Detection Rate, pFDR = adjusted p-value
FiLAC™	Fistula tract laser closure
GI	Gastrointestinal
HexCer	hexosylceramide

IAS	Internal Anal Sphincter
IBD	Inflammatory Bowel Disease
IBDQ	Inflammatory Bowel Disease Questionnaire
IQR	Inter quartile range
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LIFT	Ligation of the intersphincteric fistula tract
LIS	Lateral Internal Sphincterotomy
MDT	Multidisciplinary team
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal stem cell
MYMOP	Measure Your Medical Outcome Profile
m/z	mass-to-charge ratio
netCDF	Network Common Data Form
NHS	National Health Service
OR	Odds Ratio
OTSC®	Over-the-scope-clip
PACS	Picture archiving and communication system
PC	Phosphatidylcholine
pCD	Perianal Crohn's disease (includes non-fistulising manifestations)
pCf	Perianal Crohn's fistula
PDAI	Perianal Disease Activity Index
PE	phosphatidylethanolamine
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
QoL	Quality of Life
R&D	Research and Development
RCT	Randomised Control Trial
RVF	Rectovaginal fistula

SD	Standard Deviation
SE	Standard Error
SM	sphingomyelin
STL	Stereolithography
TG	Triglyceride (triacylglycerol)
TNF	Tumour necrosis factor
UC	Ulcerative Colitis
UK	United Kingdom
UK-IBDQ	United Kingdom - Inflammatory Bowel Disease Questionnaire
VAAFT	Video assisted anal fistula treatment

List of Publications and presentations from this thesis

1. **Adegbola SO,** Sahnan K, Tozer PJ, Strouhal R, Hart AL, Lung PF, Phillips RK, Faiz O, Warusavitarne J. Symptom amelioration in Crohn's perianal fistulas using video assisted anal fistula treatment (VAAFT). J Crohns Colitis. 2018 May 25. doi: 10.1093/ecco-jcc/jjy071
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Chapter 1. Introduction

1.1 Background and historical perspective on perianal fistulas

Perianal fistulas are abnormal tunnels or tracts connecting the inside of the anus with the skin of the buttock nearby. They represent a pathological connection between the anorectal mucosal surface and the perianal skin often causing pain and discharge amongst other secondary impacts of the condition on the individual's wellbeing (for example on mobility, cleanliness, social, sporting and professional activities, etc).

History documents a wide range of publications on fistulas, dating at least back to the times of Hippocrates, when in 400BC he described potential strategies for treating fistulas[1]. A wide range of treatments have since been undertaken over the years in an attempt to treat this problem, with no one particular treatment accepted as a gold standard. Another notable historical author on the topic was John of Arderne (14th century AD) who published a treatise on fistula. Interestingly a relative of his, William Shakespeare, also brought fistulas into the popular cultural domain, with the help of his play "All's Well that Ends Well", where the tale of fistula is featured in the central plot[2]. Extensive documentation of the treatment of fistula for King Louis XIV of France further solidified fistula disease in historical documentation[3]. Treatment of fistulas even stemmed a dedicated hospital, "*The Infirmary for the Relief of the Poor Afflicted with Fistula and Other Diseases of the Rectum" (now St Mark's Hospital)* following the founder (Frederick Salmon's) successful treatment of Charles Dickens[1].

Despite their long history and many attempts at management, the search for a gold-standard cure without sacrificing continence remains elusive, and fistulas remain a recognised and persistent challenge in the field of coloproctology. Fistulas may be seen in association with several conditions including tuberculosis, pilonidal disease, hidradenitis suppurativa, lymphogranuloma venereum, presacral dermoids, rectal duplication, actinomycosis, trauma and foreign bodies. Treatments in these situations are often targeted at the underlying cause. However, the vast majority of fistulas in the UK occur with no specific association and are termed idiopathic or cryptoglandular fistulas. The other significant group are seen in association with Crohn's disease and these two types (in particular the latter) will form the focus of this thesis.

1.1 Demographics of idiopathic and Crohn's perianal fistulas

1.1.1 Idiopathic perianal fistula

Accurate incidence of idiopathic fistulas is unknown and likely underestimated. Earlier reports ascertaining incidence from operating room data in Helsinki estimated single institution figures of 8.6 per 100,000 population/year (12.6 for males and 5.6 for females) between 1969 and 1978. More recently in 2007, Zanotti demonstrated a higher incidence

extrapolating from single year data. They reviewed European databases and described higher incidences of 1.84/10,000 in the United Kingdom (UK) (2003-4) and of 2.32/10,000 in Italy (2002)[4]. The fistula research unit at St Mark's performed more recent epidemiological analysis, using data from the Hospital Episode Statistics (HES) database, an administrative data set with almost complete capture of all hospital episodes in England since its inception in 1987.

Analysis of the database from April 1997 to March 2012, extrapolating data from incidence of abscess, found that an annual incidence of 10,708 *new* diagnoses of abscesses occur in a population of 53.01 million.[5] From the *new* abscess diagnoses, an overall incidence (incl. CD) of fistula of 3.4/100,000 was derived.[6]

1.1.2 Crohn's perianal fistula

Crohn's disease (CD) affects 115,000 people in the UK and the incidence and consequent burden on health services are increasing. Crohn's is a disease with an unpredictable clinical course and is increasingly thought to encompass multiple possible phenotypes. Its description was popularized by Burrill B Crohn[7], who also (in co-authorship with a colleague) initially (1938) described the common complication of perianal fistula, accounting for a 14% prevalence in a case series of 50 patients with terminal ileal Crohn's disease[8] Subsequent studies have since demonstrated that this prevalence is closer to a third of patients with Crohn's disease [6,9]. Perianal fistulas are the most common fistulas associated with CD (54%, others include entero-enteric (24%) and recto-vaginal (9%)). Perianal fistulas are not specific for CD, but are the initial manifestation and the presenting complaint in 10% of Crohn's diagnoses[10]. Other causes include infection, hidradenitis suppurativa and malignancy. Patients diagnosed with Crohn's before the age of 40 are at an increased risk of penetrating Crohn's disease, including anal fistula.[11] Anal fistulas are more common in men, non-Caucasians and Sephardic (as opposed to Ashkenazi) Jews.[11–14] Patients with colonic disease but specifically those who have active rectal disease are at greatest risk of developing fistulas[15,16].

1.2 Risk factors for acquiring idiopathic and Crohn's perianal fistulas

1.2.1 Perianal abscess / sepsis

Perianal abscess is a term used to describe a localised collection of infected fluid in the perianal region. The term is often used to encompasses a range of strict anatomical definitions for the different anorectal abscesses (classified according to their relation to the sphincters), albeit with similar initial management. Anorectal fistula and abscesses often coexist. Published data have reported that in about a third of patients, a fistula is found either at the time or subsequent to abscess drainage[17–20]. The majority of fistulas arise on the background of a pre-existing abscess[21], but may also appear spontaneously and less commonly in the context of inflammatory bowel disease, tuberculosis, trauma, iatrogenic/complication of local surgical procedures (e.g. haemorrhoidectomy, episiotomy) and sexually transmitted infection. The 'cryptoglandular theory' (section 1.3.1)is thought to account for the majority of perianal abscesses, and it is thought that subsequent to abscess drainage, there may be residual septic foci, which persist, and maturation of the resultant tract (via granulation / epithelialization) may occur, leading to a chronic fistula-in-ano[22]. In fact, the overlap in aetiology, assessment, classification, pathophysiology and management has led to the concept of an abscess as the acute and a fistula as the chronic state of anorectal suppuration[23]. It is thus of paramount importance for abscesses to be sought and drained when managing fistulas, and similarly the assessment and management of anorectal abscesses warrants consideration of the presence of coexistent or subsequent fistula formation. There is no definitive means of preventing or predicting fistula occurrence/formation, however, microbiological analysis from the abscess cavity can often have a predictive role[24]. Isolation of enteric organisms in the perianal abscess confers a significantly higher risk of subsequent fistula formation in comparison to skin flora[25].

The annual incidence of perianal abscess is estimated between 10 000 and 20 000 people in the UK, resulting in about 12 500 operations in the NHS each year[6,26]. Sainio et al. in a Swedish

cohort study estimated the incidence at 16.1 per 100 000[27]. The true incidence may be higher, since many patients are treated with antibiotics in the community and some abscesses spontaneously regress or discharge[26]. A UK population-based study with a 15 year follow-up (April 1997 – March 2012) analysis of the HES dataset revealed an overall incidence rate of abscess of 20.2 per 100 000. The rate of subsequent fistula formation following an abscess was 15.5% (23 012 of 148 286) in idiopathic cases and 41.6% (4337 of 10 427 in patients with inflammatory bowel disease (IBD) (26.7 per cent of patients coded concurrently as ulcerative colitis; 47.2 per cent of patients coded as Crohn's disease). Of all patients who developed a fistula, 67.5 per cent did so within the first year. Independent predictors of fistula formation included IBD, and in particular Crohn's disease (hazard ratio (HR) 3.51; P<0.001), ulcerative colitis (HR 1.82; P<0.001).[6]

Perianal abscess and Crohn's disease

Perianal abscesses are a common complication in Crohn's disease and one study found up to 62% of Crohn's patients will develop at least one during the course of the disease.[28], Indeed, anal sepsis has been found to be the initial manifestation and the presenting complaint in 10-25% of Crohn's diagnoses.[10,13] A more recent population based cohort study found that up to a fifth of patients develop perianal disease at least 6 months prior to luminal disease.[29] The above mentioned HES study analysed over 7,000 patients presenting with a new abscess on a background of CD. Subsequent presentation with anal fistula occurred in 47%. In this sub-group Cox regression analysis revealed independent predictors of fistula formation were ischiorectal abscess (HR 1.30 vs. perianal abscess, CI: 1.18- 1.42, p<0.001) and female gender (HR 1.11, CI: 1.04- 1.19, p<0.001). Diabetes was not predictive whether type 1 (HR 0.41, CI 0.21- 078, p=0.007) or type 2 (HR 0.87, CI 0.68- 1.13, p=0.308)[6]. The study also demonstrated that approximately three in every 100 patients presenting with an anorectal abscess will go on

to a diagnosis of Crohn's disease. The median time to diagnosis is just over a year, and half of these patients who are not diagnosed at the time of their abscess admission develop a subsequent fistula. In luminal disease, there is a benefit in initiating earlier biologic therapy in appropriate patients[30,31] and this has been shown to increase the remission rate.[32] In perianal disease, the impact on outcomes of a delay in diagnosis is not known but is likely to be detrimental given that perianal Crohn's disease represents a severe phenotype[6].

1.2.2 Sex and hormones

Current understanding suggests that the male population has a higher incidence of abscess and fistula[6,33] with the male: female ratio in the order of 2:1 and this has been corroborated in a prospective randomised control trial (RCT) of 307 patients (OR = 3.11; CI, 1.31–7.38 and p = 0.010).[34] In neonates, the gender difference in fistula incidence is even more striking, with an overwhelming male preponderance with limited reports of fistulas in female infants under two[35]. In the population study by Sahnan and colleagues[6], there was a higher incidence of fistulas in men, however, females were found to be (18%) more likely to develop a fistula following abscess formation. Similar findings were reported in the single centre retrospective data from the same authors, whereby men were less likely to develop a fistula following abscess formation than women (OR 0.7, 95% CI: 0.5-0.9, p=0.005)[36], suggesting that men are at a greater risk of abscess formation, but not of persistent fistula following the event. Suggested hypotheses for this were that men either develop abscesses that are of an aetiology less likely to progress to fistula (superficial, skin based sepsis rather than cryptoglandular sepsis, for example), or that they are protected from persistence by an absence of factors that drive this, compared to women[36]. There have been few studies examining this apparent gender difference. In order to investigate the possibility of the effect of sex hormone influences in fistula-in-ano, Lunniss and colleagues[35] examined a range of circulating sex hormones in 15 male and 12

female patients with anal fistulas, comparing them with those in equal numbers of age-matched controls. There were no differences in levels of any measured hormones between male patients and healthy controls. There was no evidence of increased androgenisation of female patients.

1.2.3 Other risk factors

There have been a few other risk factors reported in association with fistulas. Hamadani et al.[37] followed 148 patients with first-time perianal abscesses and examined age, gender, smoking, antibiotic use, diabetes and human immunodeficiency virus status as risk factors for recurrent disease (perianal sepsis or chronic fistula). They reported that patients less than 40 years old and non-diabetics were at risk on univariate analysis, but only age less than 40 remained a risk factor on multivariate analysis. Wang et al.[38] compared 1342 patients with anal fistulas to matched controls with other anorectal complaints. They reported several independent risk factors for anal fistula on multi- variate analysis including several lifestyle factors: body mass index greater than 25 kg/m2, high daily salt intake, diabetes, hyperlipidaemia, dermatosis, anorectal surgery, history of smoking and alcohol intake, sedentary lifestyle, excessive intake of spicy/greasy food, infrequent participation in sports, and prolonged sitting on the toilet for defecation. Further studies are needed to corroborate the findings from these studies and to discover if any modifiable risk factors can be adjusted to reduce the incidence of anal fistulas.[39]

1.3 Aetiology of perianal fistula – are Crohn's fistula a different entity to idiopathic perianal fistula

1.3.1 idiopathic fistula and the cryptoglandular theory

As early as the 19th century it was [39,40] hypothesized that some cases of anal fistula may be caused by anal glands penetrating deep from the lower rectal mucous membrane into the perianal tissue. It has been proposed that up to 90% of idiopathic perianal sepsis occurs due to infection of the anal glands.[41,42]These anal glands are distributed circumferentially at the level of the dentate line and communicate with the anal canal via the crypts (of Morgagni) or ducts[43,44]. The cryptoglandular hypothesis indicates that infection of an intersphincteric gland via an internal opening leads to fistulation out to the perianal skin. These fistulas occurring following anorectal sepsis are often termed 'cryptoglandular', due to this theory of pathogenesis that was popularized by Parks[44] in 1961. In 21/30 consecutive fistula cases Parks demonstrated histological evidence of infection within anal glands located in the intersphincteric space [44]. In the 30 patients that were analysed following fistula excision, anal-gland epithelium was found in all but two. Gross cystic dilatation of the anal gland with pus was found in eight cases; abscess or tract lined with anal-gland epithelium in thirteen and anal gland in proximity in seven. He proposed that the initial abscess formation subsides but persists deep to the internal sphincter and leaves behind the 'now-diseased' gland, which becomes the seat of chronic infection. Consequent to this, is the formation of a granulation tissue lined tract or fistula. He suggested that the intersphincteric component of the infected system is fed with bacteria from the gastrointestinal tract which leads to persistence of the tract, however this point is debated later in the section on fistula pathogenesis (Section 1.4)[40]. The majority of abscesses occur posteriorly and in the intersphincteric space, where the anal glands are located[22]. Furthermore, most internal openings are found within the crypt at the dentate line. Abscesses are classified as superficial or deep in relation to the anal sphincter. If the infection spreads beyond the external sphincter then it results in an ischiorectal abscess and if it spreads laterally on both sides it can form a collection (or 'horseshoe') of sepsis around the sphincters. Superior extension (supralevator abscess) beyond the puborectalis or the levators is rare and

may represent iatrogenic injury. The presence of gut-specific bacteria in an anorectal abscess can predict the development of a fistula. Eykyn and Grace[45] found a significantly higher rate of E.coli and gut-specific Bacteroides in patients with fistulas (>85%) compared to those without (<30%), following the culture of pus from these patients. Toyonaga et al.[46] substantiated these findings by culturing pus from 514 patients with anorectal abscesses. They reported a significant association of fistulas with gut-derived (rather than skin derived) microorganisms, with several other studies corroborating this finding[20,25,47,48]. The question remains whether gut-derived organisms contribute to the development of anal fistulas or whether they are simply a marker of a fistulous process in a patient with an anorectal abscess[39].

Anorectal abscesses certainly play a major role in the development of idiopathic fistulas, however, the evidence is not conclusive and there are some data that do not support the cryptoglandular theory[49]. Several characteristic aspects of anal fistulas cannot
be explained by the cryptoglandular theory. Furthermore, almost all fistulas are preceded by an abscess, but many abscesses do not result in a fistula and the reasons why some fistulas persist are less clear[39]. Oliver and colleagues found that 83% of a series of 200 abscesses had an internal opening when examined by an experienced proctologist. They found treating fistulas in the acute phase yielded a reduced recurrence rate (5% vs. 29%),[50] demonstrating that not all of the abscesses identified to have an internal opening then presented with a fistula. Hamadani and co-workers[37] conducted a retrospective cohort study in 148 patients who underwent incision and drainage of their first-time perianal abscess. During a mean follow-up of 38 months the cumulative incidence of recurrent abscess or anal fistula was only 36.5%. Abcarian and colleagues similarly found that 40% of abscesses progress to fistula formation but other studies have found this rate to range from 26% to 87%[17,19,51–55]. Even in studies in which all included patients had an internal opening, i.e. acute fistula, not all cases resulted in

fistula formation[56,57]. This allows the conclusion that some patients with abscesses associated with an internal opening never go on to develop persistent fistula and the reasons why some do are unclear[49,58].

1.3.2 Multifactorial aetiology of Crohn's fistulas

Penner and Crohn described the first case of perianal fistula in a patient with regional enteritis in 1938[8], in a paper describing a cohort of eight out of 50 cases of regional ileitis exhibiting anal fistulas as a complication (i.e. 14%). Thoughts on aetiology around the time centred on an underlying infective process, perhaps related to the contaminated ileal contents (secondary to Crohn's disease) causing local infection and fistulation originating locally in the anal crypts of Morgagni[8]. Whilst Park's et al. demonstrated objective evidence (above) for the cryptoglandular theory, no similar studies have assessed pathology of anal glands and sepsis in relation to Crohn's disease. More theories stemmed in 1972 when Hughes published concepts on potential underlying causes based on an experience of approximately 400 cases[59]. He proposed a theory in which primary lesions (fissures, ulceration) closely related to Crohn's disease activity and which tended to improve with medical therapy, were thought to predispose to fistula formation due to mechanical forces (pressure of defaecation) forcing faecal material into subcutaneous tissue, leading to sinus and subsequent fistula formation. The process was thought to be unrelated (or inversely so) to CD activity, but to persistent faecal contamination of the fistula tract secondary to defaecation pressure. This theory does not however explain the non-epithelialised and complex fistulas with multiple tributaries that are often seen in Crohn's disease, and several studies have since built on this rather simplistic theory, with improved understanding that the aetiology of this phenomenon is much more complicated. The current knowledge points to a much more multifactorial process albeit with no definite single theory on the exact cause or explanation for why they occur as a common entity within the

context of Crohn's disease. Multifactorial causation is thought to involve an interplay between genetic, microbiological and immunological factors.

1.3.2.1 *Genetic factors:*

A multitude of genetic polymorphisms conferring increased risk of CD have been reported in recent years, and some of these have been related specifically to perianal fistula in CD. Variants on a susceptibility locus on chromosome 5 (IBD5) have been described[60,61]. This locus codes for a carnitine/organic cation transporter (OCTN), variants of which may confer impaired / dysfunctional activity, and may reduce carnitine transport. This is thought to potentially contribute to defects in handling and neutralization of bacteria / pathogens and play a role in CD pathogenesis[40,61]. Another susceptibility locus on chromosome 5 (5q33.1) has also been reported, and similarly is involved in eliminating intracellular organisms (via autophagy pathway)[62]. It is noted however, that these susceptibilities are not unique to perianal fistulas alone and were linked with non-perianal fistulas as well. The reports are also conflicting as other studies have reported no discovered links between OCTN and perianal CD[12,63].

1.3.2.2 *Microbiological factors*

The cryptoglandular theory has been used in the context of perianal Crohn's fistulas, as a potential causative factor leading to perianal sepsis / abscess and fistula formation (section1.4.2). Proposed support for this included reports of improvement conferred by surgical diversion of the faecal stream [64,65]. However, surgical diversion also improves luminal CD, so the improvement seen in patients with perianal CD may reflect a decrease in Crohn's activity rather than a direct effect on the perianal fistula[40]. There is weak evidence for antibiotic use in Crohn's perianal fistula. Metronidazole (targeting anaerobic bacteria), has been used both topically and orally[66,67]. In a study using metronidazole on 21 consecutive patients with

perianal CD 10 of the 18 patients who remained on therapy, were considered to have complete healing of their fistulas at 10 weeks[68]. However, after stopping metronidazole, 78% had symptomatic recurrence[69]. There is also some limited evidence for ciprofloxacin (broad spectrum) use[40], but symptomatic benefit is usually transient and recurrence occurs when treatment is stopped[70].

1.3.2.3 *Immunological factors*

CD results in mucosal injury and inflammation, whereby the epithelial barrier is breached as a primary or secondary event, and the luminal microflora stimulates a proinflammatory immune response. Mucosal injury and damage is associated with dysbiosis, which potentially perpetuates the inflammatory cascade [71,72]. The available data demonstrate that CD- associated fistulas originate from an epithelial injury/ defect that occurs during chronic inflammation[73], which may often be paralleled by infection[74]. They are then thought to undergo a process of *epithelial-to-mesenchymal transition* (EMT) which allows intestinal epithelial cells to penetrate into deeper tissue layers causing tissue damage and a connection to other organs or the body surface[73].

The effect of immunosuppressants, and most notably anti-TNF antibodies (see section 1.8.2.1), suggests that immune-mediated mechanisms play a role in Crohn's fistula formation and also it implies that a purely infective pathogenic process is unlikely in Crohn's fistulas. The latter is due to the fact that although perianal abscesses have been reported in trials of immunosuppressants and anti-TNF antibodies, this does not appear to be a major problem[40], suggesting that the use of immunosuppressants to treat Crohn's fistulas does unveil / worsen a process of infective origin.

1.3.3 Limitations in understanding of aetiology

Limitations in our understanding of aetiology of this disease is mainly due to the fact that there are a small number of studies which consider the aetiological aspects of idiopathic perianal fistula, and even fewer considering that of Crohn's perianal fistulas. A common limitation they tend to suffer from is heterogeneity of included patients in terms of fistula duration and classification, as well as in terms of their luminal and perianal CD in the case of Crohn's perianal fistulas. In the case of the latter, this is often complicated by a mix of medical therapies at the time of sampling and differences in the sampling and experimental methods themselves[70]. Also, most aetiological studies of anal fistula are produced by tertiary centres which probably see (and sample) a subset of fistula which are more likely to be complex, refractory and recurrent than those found in secondary care, which form the majority of fistula overall.

1.4 Pathogenesis of Crohn's perianal fistula

1.4.1 Aetiology vs persistence in the pathogenesis of perianal fistulas

The pathogenesis of perianal fistulas is poorly understood. The reasons why some fistulas resolve, and some go on to persist have been debated, with several theories and none unifying. Published data and current evidence point to an interaction of several concepts which will be discussed. These include, microbiological, histological and immunological factors, including inflammation and failure of wound repair.

1.4.2 Infection / microbiology in perianal fistula pathogenesis

Chronic infection and microbiological factors (particularly relating to bowel-derived organisms)[25,45,48,75] have long since been thought to be at the heart of fistula persistence in both Crohn's and idiopathic fistulas. Studies date back to the 20th century, using detection

techniques of the time (culture / histology). The analyte was often exudate, pus or debris from the fistula. Results suggested a paucity of organisms grown with the requirement of enrichment techniques to detect any organisms[7677]. One study suggested that whilst Gram positive organisms predominate in Crohn's anal fistulas, Gram negative enteric organisms were more common in those with idiopathic fistulas, albeit with an overall paucity in bacteria [12,70]. Modern molecular techniques (e.g. targeting bacterial 16S rRNA, i.e., using polymerase chain reaction techniques, Fluorescent in situ Hybridisation) offer alternative and more sensitive means for the study of bacterial flora and have since been used to assess the bacteriology of persistence of anal fistulas. Tozer et al.[78] used fluorescent in situ hybridization, Gram staining, and scanning electron microscopy to analyse obtained biopsy specimens from patients with both idiopathic and Crohn's fistulas aiming to characterize the microbiota within the fistula tracts. Surprisingly, only 1 of the 32 fistula specimens was found to contain bacteria associated with the wall of the tract. This led the authors to conclude that anal fistula tracts do not harbor high levels of mucosa-associated bacteria, suggesting that alternative explanations were needed to explain the persistence of anal fistulas[39,78]. Another study using molecular techniques (16S rRNA) to study 10 idiopathic (cryptoglandular) fistulas also found a paucity of bacteria[45]. The authors demonstrated the presence of peptidoglycan (in 90% of the fistulas), a major component of bacterial cell walls known to stimulate inflammation (being involved in the secretion and processing of proinflammatory cytokine aka 'fever cytokine' interleukin 1beta) [79–81]. The authors introduced the notion of bacterial remnants (as opposed to live bacteria) potentially contributing to the ongoing inflammation in the remaining fistula tract. Bacterial cell wall components (endotoxin) have also been demonstrated in Crohn's anal fistulas[70]. It is now the thinking that live bacteria do not in fact play a significant role in the persistence of fistulas, given their absence in chronic anal fistulas. However, the products of

bacterial degradation may well perpetuate ongoing inflammation and fistula persistence, representing an overreaction to the products of a normal host defence[39,70].

1.4.3 Histological and immunological factors in perianal fistula pathogenesis

Histological features in Crohn's fistulas have been poorly studied and are largely non-specific. The limited data suggests[82] they are lined by granulation tissue and/ or squamous epithelium and typically filled with debris, erythrocytes and acute inflammatory cells. Whilst granulomas may occur, most perianal samples from CD patients[83,84] do not contain granulomas, and neither are they specific to CD. In non-epithelialized areas there was a lining of myofibroblast-like cells (termed 'transitional cells' in later studies), which could form a new basal membrane. Fistulas in CD, but not control fistulas, had areas with disordered myofibroblasts and fragmented basal membrane, raising the possibility that mechanisms of fistula formation in CD differ from those in other settings. [82,83]

1.4.3.1 *Epithelialisation*

Tissue analysis of idiopathic chronic anal fistulas reveals a tract surrounded by dense fibrous tissue and chronic inflammatory cells[39]. Published literature has assessed and hypothesised on the impact of epithelialisation seen in fistula tracts on pathogenesis of the disease. In 1995, Lunniss et al.[77] observed epithelium lining the intersphincteric fistula tract in 13 out of 18 patients with a low perianal fistula. Though the effect of this epithelialisation on fistula healing was not assessed, the authors suggested its potential implication in the persistence of fistulas. In 2004 Bataille[82] reported the presence of epithelialisation in 31% of non-CD fistulas and 27% of CD fistulas. Although it is worth noting that perianal fistulas where only a tiny minority of the cohort of fistulas included (which were largely enteric, i.e. colonic or small bowel fistulas). More recently, van Koperen et al.[85] also assessed the presence of epithelium in fistula tracts.

In 18 patients with a low transsphincteric fistula they took biopsies at three different locations, on the side of the internal opening, in the middle of the fistula tract, and near the distal end close to the external opening. They found that epithelialization was present in at least 1 of the biopsy sites in 15 patients (80%), and this was most often near the internal opening, and the degree of epithelialization was unrelated to fistula duration. Epithelialization was found to be rare in the other parts of the fistula tract and none of the tracts were fully epithelized. The authors concluded similarly that epithelialization might con- tribute to the failure of healing seen with persistent fistulas, although no mechanistic theories were offered. Mitalas et al. [86] gave more detailed analysis of epithelization, documenting it in 11 of 44 (25%) patients with presumed idiopathic (or cryptoglandular) fistulas. It is not known why epithelialization does not occur in every fistula at the same stage. It has been suggested that perpetuation of inflammation prevents the migration and arrangement of myofibroblasts, which are the key cells in the events of tissue repair[86]. However, most studies have sampled a portion of the fistula track and of these portions, limited sections have been examined. It is therefore possible that a sampling error would lead to an incorrectly low proportion of fistula tracks being found to contain discontinuous epithelialisation. Indeed, epithelium has been found in all cryptoglandular fistula track specimens when obtained by full fistulectomy[70], leading to the suggestion that even limited islands of epithelialisation, likely to be found in all cryptoglandular fistula, and probably Crohn's anal fistula too, may be sufficient to prevent healing if inadequately removed. However, in the study Mitalas et al. [86] patients in the study went on to undergo surgical repair of their fistulas (by advancement flap) with similar healing rates found in patients with and those without epithelial lining. This led the authors to conclude that whilst epithelialisation may be related to persistence it doesn't necessarily affect healing.

1.4.3.2 *Immune cells and inflammation*

Although the inflammatory nature of Crohn's anal fistulas is well recognized[70], there are sparse data exploring quantitative and qualitative changes in immune cells in perianal Crohn's fistulas. One such study[82] revealed that approximately a third of intestinal or perianal CD fistula tracts are covered by flattened intestinal epithelial cells or squamous epithelial cells. A majority (70%) of CD fistula tracts were also found to be covered by a thin layer of myofibroblast-like cells, so-called "transitional cells", forming a new basal membrane.CD fistulas show a central infiltration by CD45R0+ T-cells, an underlying band of CD68+ macrophages and a dense CD20+ B-cell infiltrate in the outer wall[82]. By contrast, control fistulas typically had a dense macrophage infiltrate and sparse CD20+ B cells or CD45R T cells[82,83]. In these studies however, only a small number of patients with perianal CD were included and it is not clear whether the described changes, which represent the entire cohort of patients, also apply to those with perianal disease specifically.[40] Accumulation of CD4+ CD161+ T-cells with a Th17, Th17/Th1, and Th1 phenotype has also been described in perianal fistulas[74].

Local immunological microenvironment and cytokine expression patterns have been studied in the epithelial lining of the fistula tract[73,83]. TNF-α appears to be a principal component, being highly expressed by the lining epithelium but also by immune cells that surround the fistula, as well as by epithelial cells of the adjacent crypts[87]. TNF receptor-1 is also expressed by epithelialized fistulas. IL-13 and its α1 receptor (IL-13R1) are also abundantly expressed in the fistula lining and this is in contrast to the absence of IL-13 expression both in the healthy intestine and in non-fistulizing lesions of IBD, irrespective of the inflammatory state[88]. There is also an increase in CD fistula track pro- inflammatory cytokines IL-8, IL-1β and IL-6 (compared to the rectal mucosa of the same patients), and a decrease in fistula track wall IL17α (compared to idiopathic fistula and normal rectum)[70]. An Italian group published a study of the serum concentrations of pro-inflammatory cytokines in four groups: CD patients with perianal

fistulas and inactive luminal disease; patients with active intestinal CD without perianal manifestations; patients with perianal complications after restorative proctocolectomy; and healthy controls[89]. They reported that the presence of fistulas correlated with serum elevations of TNF-α and IL-6 but not of IL-12 or IL-1β, and also reported that mucosal expression of IL-1β and IL-6 was higher in those with perianal CD than in those with small bowel CD and in healthy controls[89]. However, whether the TNFα seen in Crohn's-related or idiopathic fistulas represents a pathologically elevated level or not is unknown[70] and a study has previously reported no difference in levels of TNFα in Crohn's anal fistula tissue compared to idiopathic (or cryptoglandular) fistulas or the rectal mucosa of healthy individuals[70]. In summary, the evidence supports a disordered process of inflammation which drives fistula persistence.

1.4.3.3 *Inflammation and Idiopathic fistulas*

Perpetuation of inflammation has also been described as a potential factor for persistence in idiopathic (or cryptoglandular) fistulas. In a cohort of 18 patients with analyzed fistula tract specimens, van Koperen et al.[85] reported findings of 'active inflammation' in 17/18. However, histological/molecular level characteristics weren't described. As described above, Mitalas et al. [86]also proposed a reason for the lack of epithelialization in the majority of our patients being an expression of ongoing inflammation. Kiehne et al.[90] compared levels of cytokines and antimicrobial peptides between non-Crohn's fistula tissue, perianal skin, and normal rectal mucosa and found that fistulas express high levels of antimicrobial defensins. In addition, they found that the distal parts of the fistula strongly express IL-1b and the proximal parts IL-8, both proinflammatory cytokines. This finding has been corroborated by Ratto et al.[91] who also reported increased expression of IL-1b and IL-8 in the fistula tissue (compared to anal mucosa) in a pilot study on 12 patients with chronic cryptoglandular anal fistulas undergoing fistulectomy. Van Onkelen et al.[92] used immunohistochemistry to quantify the expression of 8 cytokines in the distal portion of the fistula tract in 27 patients undergoing sphincter-sparing

repair. They reported 4 proinflammatory cytokines being expressed in their specimens; IL-1b (93%), IL-8 (70%), IL-12p40 (33%), and TNF-a (30%). No correlation of the differences in cytokine expression with clinical outcomes was described.

1.4.3.4 *Epithelial-to-mesenchymal transition (EMT)*

EMT describes a process of tissue transformation (i.e. 'differentiated epithelial cell' to 'mesenchymal-type cell') that is associated with embryogenesis, organ development and wound repair and also with pathological fibrosis, tumour growth and metastasis.[88] There is evidence to support EMT being a crucial process in fistula pathogenesis in CD and idiopathic fistulas[39,91]. It confers the ability of cells to migrate and to penetrate into adjacent layers, characteristics seen in cancer cells phenotypically similar to stem cells[83]. Several pathological hallmarks of EMT have been described in fistula tracts of Crohn's patients, including the detection of epithelial markers (e.g. Cytokeratin 8 and 20) in submucosal cells, and upregulation of factors (e.g. TGF-1 and 2 in the lining of fistula tracts)[73,83,93]. Furthermore, known inducers of EMT include TGF-β and TNF-α [83], both of which are markers that have been demonstrated in CD and idiopathic perianal fistulas[9]. EMT-associated transcription factors, such as SNAIL, SLUG, and ETS-1, as well as markers for cell proliferation and migration, such as β6-integrin have also been shown to be expressed in or around CD fistula tracts[74,87].

1.4.3.5 *Matrix metalloproteinases (MMPs)*

This group of molecules consists of a number of enzymes that degrade all components of the extracellular matrix and in so doing remodel the intercellular matrix. The expression of EMT-associated molecules results in enhanced activation of matrix remodelling enzymes such as MMP-3 and MMP-9 causing further tissue damage and inflammation[73]. There have been reported imbalance between these MMPs and their tissue inhibitors (TIMP-1,2,3) resulting in

aberrant extracellular matrix degradation[74,94] in Crohn's fistulas[94]. MMPs are also involved in the tissue repair process which is also implicated in fistula pathogenesis.

1.4.4 Failure of wound healing

The pathophysiology of chronic mucosal wound healing and the late events of repair have not really been explored in fistulas. In CD it is likely that intestinal inflammation is an important initiating event, which can either be followed by normal restitution, or by pathologic fibrosis and/or fistula formation. It remains unclear which signals and pathways initiate chronic wound healing abnormalities in late healing, rather than normal restitution and resolution. If normal restitution and pathologic healing after inflammation are distinct pathways, these could be separately targeted, allowing selective therapy for the wound healing abnormalities seen in IBD[72]. The balance between MMPs and TIMPs appears to be disturbed in chronically impaired wound healing in IBD[95,96], although it is unclear exactly how they are involved[72]. MMPs are secreted by myofibroblasts, which are a key cell type in intestinal wound healing[40,72] that have also been implicated in fistula pathogenesis. Myofibroblasts become activated, proliferate and then migrate to the site of injury in order to facilitate repair. However, a malfunction in their normal activity which results in increased proliferation and a failure to migrate results in excessive secretion of MMPs with ongoing tissue destruction without wound healing[70]. In fistulas from patients with CD, there is an abnormal arrangement and distribution of myofibroblasts compared to control myofibroblasts[40]. It is important to note that several molecules derived from multiple cell types involved in IBD can activate myofibroblasts[72].

1.4.5 Summary

The aetiopathogenesis of Crohn's perianal fistula remains unclear. Available data suggest origination from an epithelial defect in the context of chronic inflammation, which may be

perpetuated by bacterial products. EMT is important for tissue penetration and results in enhanced activation of MMPs (such as MMP-3 and MMP-9) which cause further tissue damage and inflammation. Soluble mediators such as TNF and IL-13 promote their own expression in an autocrine manner and enhance expression of molecules being associated with cell invasiveness. The process is thought to be perpetuated into to a chronic inflammatory state in a cycle of increased proinflammatory mediators with defective tissue repair mechanisms. Subsequently, fistula formation and "growth" is constantly promoted and further sup- ported by the presence of EMT-inducers, such as TGF, and PAMPs[73] . However, our understanding of the sequence of events and key steps and mediators remains limited with the possibility of 'unknown unknown' factors at play. This thesis will explore a novel approach to improve our understanding, in light of newer available investigative tools that improve characterisation of the molecular / biochemical status of bio-samples (blood, tissue, faeces etc) as a means of understanding function in health and disease since, intuitively, an improved understanding of causation is necessary to identify an optimal treatment strategy.

1.5 Crohn's perianal fistula presentation and disability

Anal fistulas are the initial manifestation and the presenting complaint in 10% of Crohn's diagnoses[10]. There are substantial data suggesting that perianal disease within the context of CD represents a distinct, more aggressive phenotype[97], with associated higher rates of recurrence following treatment and a shorter median time to recurrence[98,99]. This is reflected in the Montreal classification of 2005, in which perianal CD obtained a separate sub-classification[100]. Common complaints include perianal pain, swelling, discharge and bleeding from the perineum or anus, skin excoriation or pruritus and often a perineal external opening or subcutaneous lump is visible/palpable. Fistulas may also present as abscesses with acute

pain in the perianal region as a localized, erythematous swelling. Symptoms of Crohn's anal fistula have been reported to reduce quality of life[101], although there have been only limited qualitive explorations of symptoms beyond those described. The latter will be the focus of a section of this thesis (section 1.12.5). Clinical examination often reveals the location of an abscess, and also often the site of the external opening and a subcutaneous tract, but in more complex presentations examination under anaesthetic (EUA) is a useful diagnostic adjunct with a specificity of up to 91%[102,9]. Certain anatomical locations, such as intersphincteric abscesses [103], are more difficult to assess clinically, and factors like this as well as the absence of an external opening of a fistula warrant imaging such as MRI or endoanal ultrasound[104] . This in combination with an EUA may be needed to identify the fistula/abscess. Examination is also important in delineating the height of the internal fistula opening in relation to the anorectal junction, which has consequences for surgical options particularly relating to continence preservation.

1.6 Assessment and classification of perianal fistulas

A number of classification systems exist for fistulas including Hanley's[105] and perhaps the most widely known, by Parks[106]. In a study involving 400 consecutive patients with perianal fistulas, he documented the morphology of each patient's fistula in relation to the internal opening and the anal sphincters. In particular the primary tract is described in reference to the external sphincter, of which he described 4 types i.e. intersphincteric, transsphincteric, suprasphincteric and extrasphincteric. Superficial fistulas were excluded as they were thought not to arise from the anal gland[106]. Fistulas can also be classed as 'low' (involving the distal sphincter) or 'high' (involving the middle/upper third of the sphincter). The American Gastroenterological Association (AGA)[107] classification

relates largely to Crohn's perianal fistulas, and describes fistulas as either simple or complex. Simple fistulas have one external opening and are low (below the dentate line; superficial, inter- or trans-sphincteric). Complex fistulas may have more than one external opening, be high (above the dentate line; inter-, trans-, supra-, or extra-sphincteric) or have extensions (a term denoting abscess/secondary tracts). Patients with Crohn's disease can present with either, but complex fistulas are more common in Crohn's than non-Crohn's patients. They have a tendency to recur and often patients require multiple operations[108]. With repeated surgery and episodes of sepsis the fistulas become harder to assess both clinically and radiologically due to an increase in scar tissue and distortion of the surrounding anatomy.[109] Assessment includes a focused history, examination including proctosigmoidoscopy followed by imaging, ideally with MRI or endoanal ultrasound to delineate the anatomy of the tracts themselves and their relation to the sphincter and levator plate, examination under anaesthetic with drainage of sepsis and seton placement and endoscopy to map the luminal disease where this is unknown[110].

1.7 Imaging of perianal fistulas

MRI is the gold standard for imaging anal fistula and has been shown to be superior to examination under anesthesia[108] and endoanal ultrasound[111]. A technical review by the American gastrointestinal association[107] found a diagnostic accuracy of 76-100% in complex Crohn's fistula[102,112–117]. However, the location of the internal opening can be difficult to ascertain on MRI[118]. Schwartz et al, demonstrated in 32 patients with perianal Crohn's fistula that diagnostic accuracy could be improved if two modalities (MRI, endoanal ultrasound, EUA) were used in combination.[115] Establishing the presence or absence of proctitis is fundamental and influences both treatment and prognosis. Proctosigmoidoscopy or formal endoscopy should be performed to determine this[119].

Imaging is also used for monitoring the effect of treatment. Established treatment principles involve draining the sepsis and aggressively managing proctitis whilst treating the fistula medically, usually with a combination of thiopurines and anti-TNF therapies. Medical therapy trials have usually used the Fistula Drainage Assessment as their clinical endpoint, which defines clinical response as a 50% decrease in the number of external openings and/or a lack of discharge from these openings over two consecutive clinic visits[120] and clinical remission or 'healing' as complete cessation of drainage despite gentle finger pressure or the healing of all external openings, measured on two separate occasions. This subjective, clinical drainage assessment has been criticised for failing to appreciate the natural history of anal fistulas and failing to assess residual tracts which have been radiologically shown to heal a median of a year after clinical 'healing' by Tozer et al.[110] Clinical assessment alone is inadequate to identify fistula healing and radiological confirmation of deep tissue healing is required.

Previous attempts to standardize radiological response, such as the van Assche score [121] have been criticised for insensitivity to change in the long term[122] and weak correlation with PDAI ($r=0.371$, $p= 0.036$)[121]. Currently, there is no universally accepted or reliable method of monitoring long-term radiological response to treatment. Clinical scoring systems such as the Perianal Disease Activity Index (PDAI) have been used, though they are not specific to fistulous disease and currently no widely accepted scoring system exists[123].

1.8 Treatment of perianal fistulas

1.8.1 Idiopathic perianal fistulas

Most idiopathic fistulas are treated surgically, with the goal of curing the fistula and preserving adequate sphincter function. Multiple surgical interventions and approaches are available, suggesting that none offers both, particularly in the context of more complex fistulas (with multiple extensions) and those involving a significant proportion of the sphincter complex. Treatment for low perianal fistulas usually consists of fistulotomy, resulting in closure rates between 80 and 100 %[124] whereas high fistulas are by definition complex with an increased risk of continence impairment with treatment. Group consensus statements have advocated that complex fistulas should be treated by an experienced surgeon, particularly when they are associated with Crohn's disease[125]. Established techniques for complex or higher fistulas include the mucosal advancement flap[126,127] and ligation of intersphincteric tract[128–130], as well as more novel sphincter sparing techniques (utilizing laser technology[131] and endoscopic techniques[132,133]) as well as cell based therapies, e.g. plasma / fat injection[124]. The latter are still in their development and evaluation phases with limited data available. Some of the newer, minimally invasive approaches to surgical treatment that have emerged will be reviewed in this thesis and assessed in the context of their use in treatment of Crohn's anal fistulas.

1.8.2 Crohn's perianal fistulas

The principles of treatment of Crohn's perianal fistulas are to drain the underlying sepsis, aggressively manage proctitis and medically treat the fistulas with a combination of antibiotics, immunosuppressants and anti-TNF therapy[110]. Anti-TNF therapies are the standard treatment for complex fistulas and should be initiated once sepsis is controlled, usually in combination with a thiopurine. Simple fistulas, in the absence of proctitis, are sometimes managed by experienced surgeons in a similar fashion to those of cryptoglandular aetiology but the risks of impairment of continence, recurrence and poor wound healing, heightened in the Crohn's

patient, lead to a preference for sphincter preserving techniques. Complex fistulas are notoriously difficult to manage with high rates of recurrence and wound failure. Bell et al., working in the pre-anti TNF era, found complex fistulas in their cohort required a median of six procedures to heal and 50% of patients ultimately went on to proctectomy[134]. Patients need to be assessed in an individualized fashion but a multi-disciplinary team and clear communication with the patient and management of expectations are fundamental[134].

1.8.2.1 *Medical Treatment of Crohn's anal fistulas*

Antibiotics alone have failed to demonstrate long-term benefit.[107] Adjunctive antibiotic therapies in the form of ciprofloxacin or metronidazole are used in pCf with some success. West et al. compared combined infliximab + ciprofloxacin treatment with infliximab alone, in a double blind randomized control trial (RCT) and found 73% vs. 39% (p=0.12) fistula response respectively[135]. The ADAFI trial, a multicenter, double-blind RCT, demonstrated that clinical response was observed in 71% of patients treated with adalimumab plus ciprofloxacin compared with 47% treated with adalimumab plus placebo (p=0.047)[136]. Although both metronidazole and ciprofloxacin have demonstrated a benefit[137], the side effect profile of ciprofloxacin is preferable.

Despite widespread use, the evidence for immunomodulators (such as azathioprine and 6-mercaptopurine) inducing fistula healing is limited. A recent Cochrane review assessed immunomodulators vs. placebo for fistula healing and found a non-significant benefit (RR 2.0; 95% CI 0.67-5.93). However, the review only had a small number of patients and the studies included were over 40 years old. A recent meta-analysis by Jones et al. found anti-TNF monotherapy was equivalent to concomitant use of immunomodulators and anti-TNF therapy[138]. As such the role of these agents needs addressing in prospective trials.

Anti-TNF therapies have enhanced our management of pCf and a number of different agents exist[139]. The ACCENT II trial, a double blind RCT, demonstrated the benefit of infliximab maintenance treatments in fistulising Crohn's disease[140]. A partial response was shown in 64% at 54 weeks and a complete response was shown in 36% vs. 19% in the placebo group. In addition, there was a 50% reduction in the rate of hospitalization, surgery and there is increasing evidence that infliximab improves health related quality of life[141,142]. In general the better the initial response to infliximab the lower the fistula recurrence rate[143] and monitoring drug levels and proactive dosing are advocated. The combination of anti-TNF therapy and seton insertion has been assessed recently in a multicenter observational study[144]. Both utilization of infliximab prior to surgery[145,146] and seton insertion prior to infliximab have demonstrated benefit[147].

The search for clinical factors which can predict response to therapy and relapse rate is a focus for current research[148]. The role of antibodies and trough levels of the anti-TNF agents have also been explored and both may guide therapy[149].

Adherence to the first anti-TNF agent is usually recommended unless there is a loss of response. Van Assche et al. performed an open labeled study to assess the benefit of switching between infliximab and adalimumab. They found that electively switching from infliximab to adalimumab was detrimental and associated with a decreased tolerance and efficacy at 1 year[150]. The CHARM study showed benefits to both the anti-TNF naïve patients and those who had electively switched to adalimumab. Fistula response was found in 41% of patients at 56 weeks[151] of all those patients who had fistula response (including those in the placebo group)

90% had maintained response following 1 year of open-label adalimumab therapy, for at least an additional year [152].

The role of adalimumab following lack of response to infliximab has been considered in several studies. The GAIN study found no difference between adalimumab and placebo as a second line agent[153]. The CHOICE trial found complete fistula closure in 34/88 (39%) treated with adalimumab after loss of response to infliximab[154]. Echarri et al. reported complete response in 50% (n=8) after four weeks of whom 87.5% remained in remission after 48 weeks of treatment[155]. A recent retrospective review of 46 patients assessed the role of adalimumab in anti-TNF naïve patients and found complete remission in 41% at 12 months, which was defined clinically and radiologically with good correlation between both[10].

The only other anti-TNF therapy used thus far in pCf is Certolizumab Pegol. This was compared against placebo and assessed at 26 weeks in the PRECISE 1 and 2 trials. Of the 55 patients with perianal fistula the closure rate was superior with Certolizumab compared to placebo (36% vs. 17%, p=0.038). However the difference was not statistically significant (54% vs. 43%, p=0.069) for the protocol definition of fistula closure (≥50% closure at two consecutive post-baseline visits ≥3 weeks apart) [156]. Trials are ongoing with newer monoclonal agents such as Vedolizumab which may prove to be a viable alternative.

1.8.2.2 *Surgical treatment of Crohn's anal fistulas*

Due to the risks of impairment of continence, recurrence and poor wound healing, fistulotomy is rarely (if ever) appropriate in Crohn's disease. The surgeon's role is predominantly to assess and drain the fistula complex prior to medical management. Sphincter preserving treatments may be considered and data on drainage procedures (long term loose seton), disconnection

procedures (advancement flaps, the LIFT procedure), infill procedures (glues, plugs) and ablative procedures (VAAFT, FiLaC), whilst mostly limited to case series, have shown feasibility in Crohn's disease.

In the context of rectal inflammation and a complex fistula, a long term loose seton in combination with medical therapies can be very effective[157,158]. Cutting setons are less widely used and many argue that they cause pain and sphincter injury [159]. A long–term loose seton is an acceptable management strategy in some patients and may only need to be changed in the case of persistent inflammation (which suggests they are not fulfilling the function of a conduit for suppuration and that further drainage may be needed), snapping or calcification. More commonly the loose seton is placed to ensure full drainage of all perianal sepsis prior to anti-TNF therapy. Traditionally and empirically seton removal has been after the second or third infliximab infusion however more recent evidence suggests decreased fistula recurrence rates when setons are left in place longer[160,161]. There is no consensus as to the optimum time to remove the seton. Setons may be used as a bridge between draining the initial sepsis and optimizing the patient medically before definitive surgical treatment.

'Infill strategies' such as glues and plugs have been employed with varying success. The closure rate was 57% at 23.4 months in one study of fourteen patients[162]. A systematic review found a 55% fistula closure rate in a pooled analysis of 42 patients[163]. The initial success for infill strategies has not been replicable in other centres and limited long-term data are available in pCf[163]. There are however potential uses for these materials as scaffolds for newer therapies such as stem cells and local pharmaceuticals. Indeed a phase II multicentre study of complex fistula (14 patients out of 49 had pCf), compared glue vs. glue and expanded adipose-derived

stem cells (ASCs) and reported healing in 16% vs. 71% respectively, with a one year recurrence of 17.6% in the ASCs group[164].

Endorectal advancement flaps (ERAF) for anorectal and rectovaginal fistula (RVF) have been used in Crohn's disease with variable success. The surrounding mucosa in the rectum must be healthy and creating a tension free anastomosis is key[165]. Patients with perineal descent and/or internal intussusception are often better suited to advancement flaps and surgeons may choose between mucosal, partial or full thickness flaps. In a series of 36 Crohn's fistulas, there was an 11% primary failure rate and recurrence rate of 31%[166]. Solanti et al, performed a review of the literature in which 10 studies featured pCf with a 64% success rate and a 9.4% incontinence rate[127] but multiple attempts at advancement flap were permitted. A retrospective review from the Cleveland clinic identified 28 patients with pCf and a higher recurrence rate was found compared to idiopathic fistula (57.1% vs. 33.3%, $p<0.04$).

'Ligation of the intersphincteric tract' (LIFT) is a procedure used to treat transsphincteric fistula. Many of the earlier studies excluded pCf however Gingold et al. found in 8 out of 12 patients (67%) had LIFT site healing at 12 months[167], Kaminski et al.[168] reported healing in (11/23, 48%) Crohn's fistula patients at median follow-up of 10.5months. In patients without perineal descent it is an alternative to an advancement flap. Complexity in the intersphincteric space and high fistula (difficult the surgical access) are relative contraindications.

Novel treatments such as 'Fistula tract laser closure' (FiLaC) and 'Video assisted anal fistula treatment' (VAAFT) have shown some promising results in cryptoglandular fistula but prospective trials in Crohn's perianal fistula are needed.

Intra-fistula injection of stem cells has been shown to be safe and a promising area of research and has shown benefit in previously refractory complex fistula. De la Portilla et al. performed an open-label, single-arm clinical trial in six Spanish hospitals of 24 patients with pCf. At 24 weeks, 56.3% of patients achieved complete closure of the tract, with 30% achieving closure of all existing tracts; defined clinically and radiologically as the absence of collections.[169] A Phase II trial in Korea reported complete fistula healing (defined clinically) in 27/33 patients (82%) at 8 weeks[170] and the same group have recently reported their 2 year outcomes in which in modified per protocol analysis 75% had complete healing at 2 years defined clinically[171]. A longer follow up was found by Ciccocioppo et al. who found a fistula free survival of 88% at 1 year, 50% at 2 years, and 37% at 4 years[172].

Faecal diversion can be a useful adjunct to complex pCf operations either in the form of defunctioning colostomy or ileostomy. A recent meta-analysis of 16 cohort studies (556 patients) found that 63.8% (95% CI: 54.1-72.5) of patients had early clinical response after faecal diversion for refractory pCf. Whilst most often the decision to defunction is regarded as temporary, the same study reported that restoration of bowel continuity was only attempted in 34.5% (95% CI: 27.0-42.8) of patients, and was successful (without relapse of symptoms or need for additional surgery) in only 17% of patients[173].

In severe and refractory pCD proctectomy can be considered. A combination of immunosuppressive medications and chronic disease often mean the rates of poor perineal wound healing are high. Yamamoto and colleagues, found a persistent perineal sinus in 33 out of 145 patients (28%) after proctocolectomy for Crohn's disease.[174] Other post-operative complications include recurrent abscess and fluid collections in the ‘dead space’ created and damage to pelvic nerves. Proctectomy is often seen as a failure of management but in a few

patients with very severe perianal disease and/or proctitis, it may be a life changing intervention. Careful discussion and counselling are crucial.

1.8.2.3 *Rectovaginal fistulas*

Rectovaginal fistula (RVF) most commonly occur due to complications of childbirth with obstetric injury to the rectovaginal septum but also occur secondary to Crohn's disease, radiation, trauma and iatrogenic injury. RVF occur in 10% of women with Crohn's disease and are classed as complex anal fistulas[175]. The majority occur in the middle of the rectovaginal septum and are secondary to an anterior rectal ulcer which erodes into the vagina[165]. As with perianal fistula they can also occur secondary to an infected anal gland[13], but also following Bartholin's abscess[176], malignancy or radiotherapy[177]. A more benign course is attributed to those originating from an infected anal gland[13]. RVF which are higher, associated with active rectal disease or originating from a Bartholin's abscess are associated with worse symptoms and prognosis[15,175,176].

Symptoms include intermittent vaginal flatus, discharge[178] and, rarely, faecal incontinence[165]. Examination under anaesthesia, proctoscopy and vaginoscopy can be used in conjunction with contrast enemas or methylene blue infusion per vaginal tampon to identify the tract. MRI may miss the fistula but provides information about inflammatory changes, ongoing sepsis and may delineate fistula morphology.

Management of RVF is dependent on anatomical location, complexity and whether there is active inflammation in the distal colon. A multidisciplinary team should determine management options and reference must be made to severity of luminal as well as perianal disease, the presence of localized sepsis, sphincter function (through anorectal physiology

studies) and treatment goals of the patient. Patients should be appropriately counseled that there is often no 'panacea' treatment and the risks of sphincter injury and recurrence, sometimes with worsening of symptoms due to a larger aperture, should be explained.

The aim of immediate treatment is to control proctitis and drain any underlying sepsis, although the latter is often unnecessary as the 'tract' may simply be a hole. Surgical management is the mainstay but medical adjuncts are often used to alleviate active inflammatory processes and control pre/post-operative bowel habit[179]. The ACCENT II trial included 25 patients with Crohn's RVF who had infliximab infusions. Following induction infusions at zero, two and six weeks 44% of fistulas were closed at 14 weeks. The duration of closure was longer in the infliximab than the placebo group (median 46 vs. 31 weeks).[180] Lichtenstein and colleagues demonstrated that infliximab reduced hospitalisation and operations in fistulizing disease including a subgroup of RVF patients[141], but it is less likely to be curative in RVF than perianal fistulas, despite the low healing rates in the latter group[107].

Surgical options are indicated when there is endoscopic evidence of healed rectosigmoid mucosa[107] and include fistulotomy (rarely) if very superficial, trans- anal/vaginal advancement flaps, Martius flaps,[181] gracilis interposition and direct repair with sphincter repair.[182] Mucosal advancement flaps have been found to have highly variable rates of success ranging from 28% to 92%[166,183,184]. Makoweic et al, found that recurrence rates were higher following advancement flaps for Crohn's RVF compared to anal fistulas (70%; 25%) at 2 year follow up[166]. In cases where proctitis cannot be managed medically, options for treatment include anocutaneous flaps[185,186] or protectomy[175]. Full sleeve/Soave-type advancements have a role if anal stenosis[187] is present or in cases where the fistula has occurred much higher or at the site of an anastomosis[188]. Refractory fistulas can be managed with a diverting stoma with the aim

of reducing vaginal discharge, whilst others may opt for a proctectomy. Whilst patients may need only one operation, closure rates of 50% may be a reasonable expectation and women should be counseled appropriately, particularly where their symptoms are minimal.

1.8.2.4 *Pregnancy*

A recent retrospective review of all deliveries from 1998-2009 found patients with pCD were more likely to have a caesarean section compared to those patients with non-CD perianal fistula (83.1% vs. 38.9%, p<0.001). Multivariate analysis was performed to identify independent risk factors of 4th degree lacerations. Crohn's disease alone was not a risk factor (OR 1.18; 95% CI, 0.8-1.8, p 0.4), perianal disease was (OR, 10.9; 95% CI, 8.3–4.1; $p < 0.001$) but a distinction between pCf and non-CD perianal disease was not made.[189] The European Crohn's and Colitis Organisation (ECCO) guidelines recommend normal vaginal delivery as safe for all patients except those with active perianal disease, and to avoid episiotomy where possible[190].

1.8.2.5 *Conclusion*

The overall aim of surgery in Crohn's fistula is to provide high rates of closure without significant impairment of continence whether by definitive surgical intervention or, more commonly, by preparing a fistula tract for medical treatment[191]. However, despite advances in diagnosis, medications and surgical techniques, surgery for complex pCf remains challenging[192,193,194,145,195] and medical treatment rarely leads to durable remission. Recurrence and reoperation rates are high and proctectomy may ultimately be required in 10-18% of cases[157,28,196,158]. Complex fistulous disease may never be cured and for some patients a palliative approach is currently the only option. This thesis will explore minimally invasive

treatments (section 1.12.4) as well as outcome measures in the context of elusive cure (section 1.12.5).

1.9 Outcome measurement and Scoring tools

The literature on treatment of Crohn's perianal fistula is limited by heterogeneity in outcome measurement. Sustained healing and fistula closure are often difficult to ascertain due to length of follow-up and lack of objective measurements to define these, but in general high rates of fistula healing are not seen, and some treatments carry significant adverse effects and complications. As such, there is a need for robust and uniform outcomes and measures to objectively assess the effect of interventions particularly in refractory cases or situations when sustained healing is elusive, and improvement in quality of life is the best outcome most patients can hope for[197]. There are a number of questionnaires that are used to determine a patient's quality of life with Crohn's disease such as the Crohn's disease activity index *and IBDQ*. However, these are ineffective at capturing the degree of morbidity or the impairment in quality of life specific to perianal disease. They are therefore of limited use in assessing clinical progress/response to treatment in pCf [198]. The Cardiff classification described in 1978 by Hughes[59] assigned a score of 0 to 2 for each manifestation of perianal Crohn's disease: ulceration, fistula, and stricture, and also classified fistula location with respect to the dentate line. A later modification added a score for proximal intestinal Crohn disease[199].

In 1995, Irvine proposed the Perianal Disease Activity Index (PDAI)[123]. This sought to address the deficiencies of existing activity indices at the time to assess adequately perianal complications of CD[198,200]. The score was thus designed to aid physicians and researchers in measuring pCf severity both in clinical practice and in the context of clinical trials. The PDAI

focuses on five areas which assess quality of life (restriction of sexual activity, pain/restriction of activities) as well as disease severity (type of perianal disease, fistula discharge and degree of induration). In the study validation, it demonstrated good correlation between the score and patient global assessment[123,201].

A more recent scoring system proposed by Pikarsky et al[200] is useful in attempting to predict the outcome following surgical intervention in patients with perianal Crohn's disease. The index consists of 6 items that are ascertained during history taking and physical examination, i.e. abscess, fistula, fissure and/or ulcer, stenosis, incontinence. Each feature was rated on a point scale according to severity and complexity. A score of zero indicates the absence of that feature. Uniformly, a score of 1 represented de novo acute disease; 2, chronic disease; and 3, recurrent disease[200]. An incontinence score of 1 to 6 was graded as 1 point, 7 to 14 as 3 points, and more than 14 as 5 points using an incontinence score described by Jorge and Wexner[202]. The scoring system has correlated well with short-term outcomes of surgical intervention[201]. However, despite their ability to document the severity of symptoms objectively, none of the above classification systems has gained widespread acceptance owing to their lack of impact on clinical decision-making. For example, useful factors such as measures of change in score / absolute score that correlate with clinical response, remission or loss of response remain unknown. This is particularly relevant when assessing the effect of therapies in complex fistulising perianal Crohn's disease, when remission is not often achievable.

There have been other scoring systems described but these have essentially been within the context of clinical studies and have failed to break into routine clinical use[203,204]. The most widely used instrument for assessing treatment outcomes in clinical trials is the Fistula Drainage Assessment[120], described above. It was introduced as the primary end point in the

trial of Infliximab for the treatment of fistulas in CD. Fistulas are classified as open (i.e. purulent material is expelled with gentle pressure) or closed. A fistula has to remain closed for 2 consecutive visits (at least 4 weeks apart) to be considered closed. If half of all external openings are closed the patient has responded. If they are all closed, they are in remission, at least for that 4-week period. The Fistula Drainage Assessment does not consider changes in anal pain, which is also an important marker of treatment response. No perianal disease scoring system, either for symptoms or for assessing healing, has proved entirely successful. Furthermore, none has adopted a patient centred approach that fulfil criteria required for patient reported outcome measurement. The lack of a PROM for Crohn's perianal fistula is a significant weakness in interventional studies as there is often no validated capture of the crucial stakeholder's (patient's) evaluation of intervention. This area is explored in this thesis (section 1.12.5).

1.10 Prognosis of Crohn's perianal fistula

The natural history of a given Crohn's perianal fistula can be difficult to predict, however complex anal fistulas (particularly in the context of proctitis) tend to carry a poor prognosis with a disabling disease course[97], repeated attempts at medical and surgical intervention, seton treatment and frequent flare-ups. In general, perianal fistulas in the absence of rectal inflammation have a better outlook than disease associated with rectal inflammation.

The increasingly routine use of immunosuppressants and particularly anti-TNF agents has improved outcomes of complex Crohn's perianal fistulas with induction of response seen in >50% and maintenance of remission in a third of patients at 1year[140,151]. These medical treatments often have to be used in conjunction with careful drainage of sepsis to offer the best

outcomes[205]. As well as drainage, surgery includes bridging treatments (seton insertion, temporary faecal diversion) and definitive fistula surgery. There is however limited data on the long-term outcomes of patients on biologic therapy and it is uncertain whether the majority of patients achieve sustained fistula closure and true healing or what the disease burden is for patients on long-term treatment. This thesis will explore these areas in section 1.12.3).

A proportion of patients go on to have multiple attempts at attenuating symptomatic disease, and despite these some patients remain refractory to treatment and require faecal diversion or proctectomy[173]. Diversion is usually in the form of a loop ileostomy/colostomy and may allow control of the inflammatory process and optimisation of the patient's nutritional status and general condition prior to attempting definitive surgical treatment. A quarter of patients require re-diversion after restoration of bowel continuity and just under half of all patients requiring temporary diversion go on to eventual proctectomy[173]. Interestingly, the anti-TNF era does not seem to have had a significant impact on these rates, with 'absence of rectal involvement' being the only significant factor associated with restoration of bowel continuity[206–209].

1.11 Thesis Hypothesis, Aims & Objectives

1.11.1 Hypothesis

Management of Crohn's perianal fistula is challenging, and the optimum treatment strategy for patients with Crohn's perianal fistula is unknown. Several factors contribute to this including the limitations in our understanding of the disease aetiopathogenesis. Achieving the best outcome for patients is hindered by a lack of treatment which produce sustained closure/cure, and a limited understanding of how to assess quality of life following intervention in the face of this elusive cure. Improving these factors would optimise the clinical pathway and outcomes for patients with Crohn's perianal fistula, whether or not closure of fistulas is achieved.

1.11.2 Aims

The aims of this thesis are to improve our understanding of fistula aetiopathogenesis and discover potential biomarkers of disease, improve our understanding of the natural history of pCf in the biologic era of treatment, assess novel minimally invasive treatment strategies and develop a new patient reported outcome for Crohn's perianal fistulas.

1.11.3 Objectives

1.11.3.1 *Investigate Crohn's perianal fistula aetiopathogenesis using novel approach*

- Pilot 'metabonomics profiling' study of Crohn's perianal fistulas and idiopathic (cryptoglandular) perianal fistulas
    - Are Crohn's and idiopathic perianal fistula distinct entities?
    - Are there biomarkers of different disease processes?

- Are there means of in-vitro modelling of disease?

1.11.3.2 *Establish the burden of disease / natural history of Crohn's perianal fistula patients on biologic treatment*

- Depict natural history of Crohn's perianal fistulas following commencement of biologic therapy – real world data from single tertiary centre
- Assess response in terms of sustained fistula closure (radiological), proctectomy rates
- Assess natural history in those who fail biologic therapy
- Characterise disease course in the event of elusive cure
- Pilot study investigating potential biomarker (tissue levels of anti-TNF) of treatment response

1.11.3.3 *Evaluate minimally invasive treatment strategies*

- Assess efficacy and safety of novel therapies (FiLaC, VAAFT, OTSC) in the treatment of Crohn's perianal fistulas
- Assess safety and efficacy of local injection of anti -TNF for perianal fistulising Crohn's disease

1.11.3.4 *Development of a patient reported outcome measure (PROM)*

- Assess patient impact of living with Crohn's perianal fistula – qualitative approach
- Development and validation of the first patient reported outcome measure for Crohn's perianal fistula, i.e. the Crohn's Anal Fistula Quality of Life (CAF-QoL) questionnaire
- Involve patient in conception, design, testing and refinement

1.12 Thesis Structure

This thesis has been structured into four main sections (sections A-D).

1.12.1 Chapter 1 – Introduction

1.12.2 Section A – Improving understanding of Crohn's fistula aetiopathogenesis using novel platforms

1.12.2.1 *Chapter 2 Different metabonomic profiles between Crohn's perianal fistulas and idiopathic (cryptoglandular) perianal fistulas may offer clues to underlying pathogenesis.*

- Metabonomics profiling study of Crohn's perianal fistulas vs. idiopathic perianal fistulas – a step towards biomarkers of disease?

1.12.3 Section B – Natural history of Crohn's perianal fistulas on biologic treatment

1.12.3.1 *Chapter 3: Anti-TNF therapy in Crohn's disease (review article published)*

1.12.3.2 *Chapter 4: Long-term outcomes in Crohn's perianal fistula on biologic treatment – 11 year real world experience from a tertiary centre*

1.12.3.3 *Chapter 5: Disease course of Crohn's perianal fistula patients following failure of biologic therapy - real world lessons on impact of refractory disease from a tertiary centre cohort*

1.12.3.4 *Chapter 6: Lack of anti-TNF drugs levels in fistula tissue - a reason for non-response in Crohn's perianal fistula?*

1.12.4 Section C – Evaluating novel / minimally invasive surgical treatment strategies in Crohn's perianal fistulas

1.12.4.1 *Chapter 7: Systematic review of 3 novel sphincter sparing techniques in anal fistula surgery (published – Short term efficacy and safety of three novel sphincter-sparing techniques for anal fistulas: a systematic review (published)*

1.12.4.2 *Chapter 8: Review of Local injection of anti -TNF for perianal fistulising Crohn's disease (published)*

1.12.4.3 *Chapter 9: Video-assisted anal fistula treatment (VAAFT) in patients with Crohn's perianal fistulas – patient reported outcomes of symptomatic treatment (published)*

1.12.5 Section D - Development of a patient reported outcome measure for Crohn's perianal fistula

1.12.5.1 *Chapter 10: Burden of disease and adaptation to life in patients with Crohn's perianal fistula – a qualitative exploration*

1.12.5.2 *Chapter 11: Development and validation of a patient reported outcome measure for Crohn's perianal fistula - Crohn's Anal Fistula Quality of Life questionnaire (CAF-QoL)*

1.12.6 Chapter 12 – Thesis discussion and direction of future work

Section A - Novel tools in investigating fistula aetiopathogenesis

Chapter 2. Differences in amino acid and lipid metabolism distinguish Crohn's from idiopathic perianal fistulas by tissue metabonomic profiling and may offer clues to underlying pathogenesis.

2.1 Abstract

Introduction:

The pathogenesis of perianal fistulas is poorly understood. The reasons why fistulas originate have been explained in idiopathic cases, with the cryptoglandular theory being widely accepted. However, in Crohn´s disease, it is thought to involve interplay between microbiological, immunological and genetic factors. It remains unclear why the fistula persists. Novel investigative tools in systems biology are improving our understanding of pathogenesis, and one such tool is metabonomic profiling, whereby spectrometry is used to assess metabolic responses of complex systems in health and disease. These changes are mapped using analytical and statistical techniques. To our knowledge, these methodologies have not been applied to aid understanding of the pathogenesis of Crohn's perianal fistula persistence.

Aims and Methods:

We undertook a pilot study with the aim of defining how metabolic profile varies in Crohn´s perianal fistula tissue, using samples from idiopathic (cryptoglandular) patients of perianal fistula as a comparator. Fistula tissue biopsy was obtained from the fistula tract of 31 patients with idiopathic perianal fistula and 20 patients with Crohn´s anal fistula. Two analytical platforms were attempted to achieve broad metabolome coverage; a chromatographic tool, hydrophilic interaction liquid chromatography/mass spectrometry (HILIC-MS) was used to

generate polar metabolites, and lipids were generated using ultra-high performance liquid chromatography/mass spectrometry (UPLC-MS) profiling. Univariate (student t-test) and multivariate statistical data analyses were performed. From the latter principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) models were built to find metabolites that could predict tissue samples from patients with either Crohn's or idiopathic anal fistula. Metabolite putative identification was conducted by matching accurate mass:charge ratio (m/z) measurements of detected chromatographic features to theoretical value from in-house databases and on-line databases and previous publications.

Results:

Significant OPLS-DA predictive models (validated with CV-ANOVA p-value <0.05) differentiated metabolites from tissue samples from Crohn's versus idiopathic anal fistula patients using both metabonomic profiling platforms (HILIC / Lipid profiling). A total of 22 differentiating metabolites were identified from the HILIC profiling and 19 from the lipid profiling analysis. Mapped pathway analysis was performed for metabolites identified using online database searches.

Conclusion:

This study highlights the ability of metabonomic profiling to differentiate Crohn's from idiopathic anal fistula tract tissue and represents an exciting foray into a systems biological approach to understand fistula aetiopathogenesis. Forty-one differentiating metabolites were discovered belonging to various classes of lipids, amino acids and nucleotides. Pathways involved included amino acid metabolism, phospholipid metabolism and glycerolipid metabolism amongst others. Further work in larger numbers is required to validate these findings as well as cross correlation with microbiome work to understand the impact of host gut interactions in the pathophysiology of Crohn's and idiopathic perianal fistulas.

2.2 Introduction

The reasons why Crohn's perianal fistulas develop and persist remain poorly understood. Evidence from groups who have studied fistula aetiology and extrapolation from interventional studies, support a multifactorial hypothesis with several pathophysiological elements[40]. There is thought to be a genetic predisposition, immune dysregulation and an abnormal microbiome-induced response[210]. Particular elements which are considered to be important in promoting or facilitating perianal fistulation in Crohn's disease include persistent or poorly regulated inflammation, wound repair failure, infection, epithelialisation and physical connection/high pressure zones[70]. There have been a limited number of comparative studies of Crohn's perianal fistulas and more commonly encountered fistulas occurring in the absence of CD. idiopathic fistulas[78,82,93,94,211] The latter are the more common type of fistulas, accounting for up to 90% of fistulas encountered in the general population. They occur following anorectal sepsis and are often termed 'cryptoglandular', due to a theory of pathogenesis that was popularized by Parks[44] in 1961. In 21/30 consecutive fistula cases Parks demonstrated histological evidence of infection within anal glands located in the intersphincteric space[44]. Although this may account for the development of cryptoglandular fistulas, the evidence is not conclusive and there are some data that do not support the cryptoglandular theory[49]. Furthermore the reasons why these fistulas persist are less clear[39] and the term 'idiopathic' fistula has been used by some to represent the chronic phase after development of a 'cryptoglandular' fistula[49] (which is usually the presenting stage). Infection has long since been considered the cause of persistence, however studies have refuted this[47,76,78], suggesting disordered inflammation, with bacterial endotoxin[212] or bacterial cell wall products (peptidoglycan) driving this[81]. In fact, some of the factors associated with CD fistula persistence (poorly regulated inflammation)

have been reported to be involved in pathogenesis of both idiopathic and CD perianal fistulas, e.g. epithelial to mesenchymal transition [91,93].

The goals of treatment for fistulas is to eradicate it with minimal injury to the sphincter complex to avoid a risk to continence. Broadly speaking, the treatment of these two groups of fistulas (CD perianal fistulas and idiopathic fistulas) are significantly different. The primary treatment for idiopathic fistulas is surgery; fistulotomy for simple fistulas with little sphincter involvement, which minimizes risk to continence, and sphincter-sparing surgical procedures for complex fistulas with greater sphincter involvement. The treatment of CD fistulas is targeted at the molecular level with biological medication and immunomodulation. This suggests a differing pathogenic basis, however, the pathophysiology underpinning the creation and persistence of perianal Crohn's disease anal fistula tracts is poorly understood.

This limited understanding of the pathophysiology of both Crohn's and idiopathic fistulas highlights the need for further studies evaluating this area especially in light of huge strides in novel investigative platforms. Most of the existing knowledge of the aetiological factors in these perianal fistulas derive from traditional research methodologies; forming a hypothesis and using clinical/scientific experimentation to evaluate theory. However, recent advances in molecular biology allow derivation of complete sets of physiological variables to compare states of disease and health without prior hypothesis. Using systems biology, the processes involve building on genome knowledge to understand transcription, the resulting protein activity, and elucidating the absolute extents of physiological pathways. Metabonomics serves as a tool in this process and is defined by pioneers as ‘‘the quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification.[213] By studying end-point metabolites (lipids, amino acids,

nucleotides, etc.), it bears a collective ambition of uncovering the complete spectrum of biochemical function and has the potential to elucidate disease mechanisms and to identify biomarkers for diagnosis and monitoring disease activity[214]. The latter being particularly useful in inflammatory bowel diseases (IBD) which consists of multiple disease phenotypes / treatment. Classical diagnostic tools provide a snapshot of a few aspects of a very complex picture, with the low sensitivity and variability of currently employed biomarkers, seldom distinguishing between phenotypes of the disease[215]. The end metabolites reflect interaction of the genome with its environment in the cells and analyzing metabolic differences between pathological and normal conditions could derive biomarkers of Crohn's perianal fistula and also provide scientific insight into the underlying disease pathology [216]. It is this ability that stimulates the interest in application of this tool in perianal fistulas to aid understanding of disease specific pathogenesis of Crohn's disease fistulas and whether this differs from that of idiopathic fistulas. An individual's metabolomic profile reflects their unique genomic, proteomic, and transcriptomic alterations, and can thus provide a global system analysis offering greater insight compared to other approaches[217].

It remains unknown what metabolic alterations can be detected in fistula tissue of patients with CD and idiopathic perianal fistula using a highly sensitive, metabolomics platform. In this study, we describe a non-targeted metabonomic profiling analysis using mass spectrometry techniques in order to characterise a wide metabolome coverage for fistula tract biopsy samples from patients with Crohn's disease and idiopathic anal fistulas. Biopsy samples (i.e. tissue) was chosen as the bio-sample to investigate as it has been previously demonstrated to provide a useful parameter that is less easily altered by dietary intake, and using a metabonomics analytical pipeline that has previously been reported in our laboratory on cardiovascular tissue[218]. The pipeline has the potential to cover a wide range of metabolic pathways and

biological functions and the ultimate aim is to identify if there exist alterations in those with CD fistula as compared with non-Crohn's (idiopathic fistula) and explore any potential clues to underlying pathogenesis.

Fig. 1 Metabonomic workflow

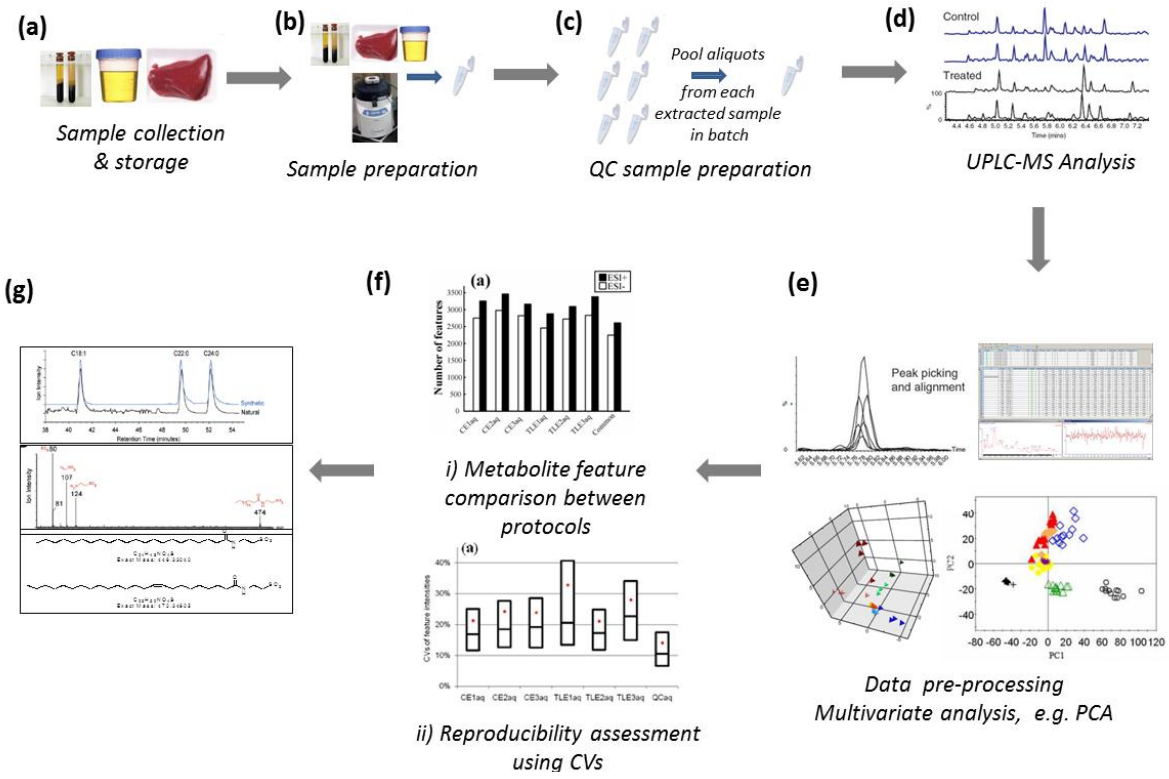

Figure 1: Schematic depicting workflow involved in metabolic profiling -
Patient bio-fluid samples are collected/stored/prepared for metabonomic analysis (a-c). These are analysed using options from a variety of spectroscopic platforms (d) The spectra generates thousands of data-points and require computational pre-processing and modelling to identify meaningful chemical structure within the spectral data (e-g). Pathology-related signatures are extracted from the models and bioinformatics approaches applied to enhance molecular identification and to map discriminatory metabolites to biochemical networks with the aim of improving mechanistic knowledge of pathological processes (Adapted from Holmes et al.[219])

2.3 Material and methods

2.3.1 Sample collection / Recruitment strategy

Two groups of patients were approached to take part in this study: patients with cryptoglandular (or idiopathic) anal fistulas (IPD) and patients with Crohn's anal fistulas (CPD). The patients with idiopathic fistulas, served as a comparator group. Patients were deemed eligible if they were adults (i.e. >18 years of age) with idiopathic or Crohn's disease perianal fistula undergoing an examination under anaesthesia (EUA) as part of their treatment for anal fistula. Included patients had at least one transsphincteric fistula (main tract) with or without other fistulas / secondary extensions. For recruited patients, demographic and disease related data were noted including duration and extent of disease, smoking status and previous medical and surgical therapy at time of recruitment. Baseline characteristics of all patients are shown in Table 1. All patients included had chronic persistent fistulas with a duration of at least 6 months. Each patient group (i.e. Crohn's versus idiopathic) served as control for the other, since there exists no true control group for fistulas traumas, they are by definition pathological, and have no equivalent in health.

For all participants, demographics including age, gender, medical history, and medication use were ascertained via discussion with patients and review of online medical records on the day of examination under anaesthesia / fistula biopsy. Intraoperatively, full-thickness fistula tract biopsies were taken from the central aspect of fistula (provided the fistula tract was non-epithelialised) using sterile endoscopic biopsy forceps or using scalpel excision. Tissue biopsies were deposited in cryovials, snap frozen with liquid nitrogen and stored at −80°C until utilized.

Follow up biopsies were not performed on a longitudinal basis in this study. Fresh biopsies were taken from 30 Crohn's and 20 idiopathic fistula patients.

An overview of the material and methods are outlined below and further described in *the Supplementary Methods. (Appendix 1- Chapter 13- section13.1)*

2.3.2 Metabonomic profiling:

Two analytical platforms were utilised in order to achieve broad metabolome coverage; a chromatographic tool, hydrophilic interaction liquid chromatography/mass spectrometry (HILIC-MS) [220], was used to generate polar metabolites, and lipids were generated using ultra-high performance liquid chromatography/mass spectrometry (UPLC-MS) profiling[218,221]. Profiling analyses of metabolites was performed on fistula tract biopsies from Crohn's and idiopathic patients. A detailed description of sample preparation is presented in the Supplementary Methods (Figure 38).

2.3.2.1 *Data pre-processing using "XCMS"* [222]

The UPLC-MS raw data were converted to netCDF format using MassLynx™ software (Waters Corporation, Milford, USA). Data extraction and processing were implemented using the XCMS package in R programming software (see supplementary Table S3)[222]. An in-house script was used incorporating various programs and algorithm combinations in order to process the data. Peak detection was implemented using centWave (algorithm for chromatographic peak detection for high resolution MS data in centroid mode) [223] with application of various filters (including, ppm, signal-to-noise threshold, number of scans, peak width and signal intensity). Peaks were grouped across the samples according to mass to charge (m/z) ratios for given retention times. They were also screened to ensure a predefined minimum of required fraction of samples in which peaks for the peak group were identified (minimum fraction filter).

2.3.2.2 *Univariate and multivariate and statistical data analysis*

Multivariate data analysis was performed using the SIMCA package (v.13.0.2, Umetrics, Umeå, Sweden). Multivariate Pareto scaled data were modelled using principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). These linear regression techniques relate spectroscopic features (metabolites) to class membership (i.e. Crohn's and idiopathic) of the sample, allowing the identification and visualization of metabolites responsible for differences between classes. Principal components analysis (PCA) was used as an unsupervised MVDA method to visualise data. PCA can provide a simplified overview of all the features detected for each sample and therefore uncover differential metabolic patterns. The OPLS-DA parameters (R^2X, R^2Y, Q^2Y and cross validated CV ANOVA p-value) aimed to assess the inherent metabolic variation and robustness of each model. R^2/ Q^2 values acts as model validatory tools aimed to assess the inherent metabolic variation and robustness of each model. R^2 value signifying the total variance explained by the model and Q^2 signifying the amount of variance that is predictable by the model. Q^2 varies from $-\infty$ to 1; whereas R^2 varies from 0 to 1. As a general rule,

The discriminatory power of each model was validated through the use of sevenfold and leave-one-out cross-validation, and calculation of the cross-validation parameter Q^2. Calculation of cross-validation ANOVA (CV-ANOVA) was then performed[224]. Markers were identified by selection from a coefficient correlation plot using the SIMCA package and these were defined as markers from the statistically significant OPLS-DA model that served as predictive of either CD or idiopathic fistulas. The student two-tailed t-test was applied to the main discriminatory features of each model (Office Excel 2007). The p-values associated with the both HILIC and Lipid profiling models were adjusted for multiple testing using the method of Benjamini and

Hochberg[225] to control the false detection rate at 0.05 level. A Python script was developed to plot the receiver operating characteristic (ROC) and calculate the area under the curve (AUC) with confusion matrix parameters (accuracy, sensitivity and specificity) of each marker [226].

2.3.2.3 *Metabolite assignment*

Markers list was retrieved from significant OPLS-DA model, and inspection of spectra was performed for each marker feature in order to select the base peak (i.e. the peak from the most abundant and often most stable ion). Metabolite identification by MS was conducted by matching accurate *m/z* measurements of detected chromatographic peaks to theoretical values from in-house databases and on-line databases such as the human metabolite database (HMDB, http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/ligand.html), and METLIN (http://metlin.scripps.edu/) , LIPID MAPS (http://www.lipidmaps.org/tools/index.html). For the HILIC data, spearman correlations were applied to identify ions (e.g. [M+H]+, [M+Na]+, [M+NH_4]) that were adducts in the spectra and hence related to the same marker. For the lipid profiling data, ions that were adducts included [M+K]+, [M+H-H2O]+, in addition to those seen in the HILIC data. Tandem MS fragmentation patterns were obtained for further structural elucidation and confirmed with an authentic standards matching for retention time and *m/z.* Metabolite assignment techniques were similar to those reported in the literature[227].

2.4 Results

Table 1 below demonstrates the demographic and disease characteristics for the total study group. A total of 50 patients (20 CD, 30 idiopathic) were included in this study. Median ages were 31 (range 22 – 58) for CD and 46 (range 25-77) for idiopathic fistula patients. Median duration of fistulas were 5 years (range 0.6 – 16years) for CD and 2 years (0.6 – 34) for idiopathic fistulas. CD patients had a median number of 1 prior operative procedure for their

perianal fistula whereas those with idiopathic fistulas had a median number of 2 prior procedures.

Table 1: Demographic and disease details for patients recruited to the study

	Crohn's Perianal Fistulas N = 20	**Idiopathic Perianal Fistulas N = 30**
Age in years Median (Range)	**31 (22-58)**	**46 (25-77)**
Female sex n (%)	**10 (50)**	**21 (70)**
Smokers n (%)	**4 (20)**	**8 (27)**
Stoma n (%)	**2 (10)**	**0**
Duration of Perianal fistula, median (range)	**5 (0.6-16)**	**2 (0.6-34)**
No. of previous fistula operations Median (range)	**1 (0-15)**	**2 (1-9)**
Seton n (%)	**16 (80)**	**13 (43)**
Disease Location **L1 (n, %)** **L2 (n, %)** **L3 (n, %)** **p**	 **1 (5%)** **3 (15%)** **5 (25%)** **11 (55%)**	**N/A**
Immuno-modulatory Medication n (%)	**Infliximab 2 (10)** **Adalimumab 6 (30)** **Thiopurine 5 (25)** **5 ASA 1 (5)**	**N/A**

2.4.1 Differences in metabolic profiles observed between CD fistula patients and idiopathic fistula patients using UPLC-MS approaches

2.4.1.1 *Polar metabolite analysis (HILIC UPLC-MS profiling) of fistula tract biopsies*

Figure 2A depicts a PCA plot of the HILIC UPLC MS analysis and demonstrates stability of the analytical process with good aggregation of QCs thus enabling the assumption that the method was performed in a sufficiently reproducible fashion for the data from the study samples to be valid.

In order to assess robustness of predictive model and potential influence (or confounding factor) of age and gender on the model, patients were stratified according to age and gender (Figure 3) respectively and the predictive model was assessed for grouping / clustering according to both of the above. It was noted that the model was unaffected by age and gender as seen in Figure 3, with no trends visible when the OPLS models were reconfigured according to disease group and a trend (for age) or colour coding (for gender) was applied, allowing the conclusion that any clustering of Crohn's and Idiopathic samples in the predictive HILIC or lipid profiling models were not driven by age / gender. Similar results were obtained with the lipid profiling analysis.

Robust OPLS-DA models, as assessed by the QC sample distribution and model validation statistics (Q^2Y, permutation testing)[228] were built from polar metabolites profiles (via HILIC assay). When comparing CD patients to idiopathic fistula patients' metabolic profiles generated across the HILIC UPLC-MS profiling assays of tissue biopsy samples showed clear discrimination of sample groups with a R^2Y of 0.78 and a Q^2Y of 0.385 (Figure 2B). Based on the OPLS-DA correlation coefficient plots (Figure 5), the features (mass-to-charge ratios / retention times, etc) that contributed to the significant predictive power (P-value: 0.0015644) between samples from CD fistula patients and idiopathic fistula patients were identified (see supplementary Table 38).

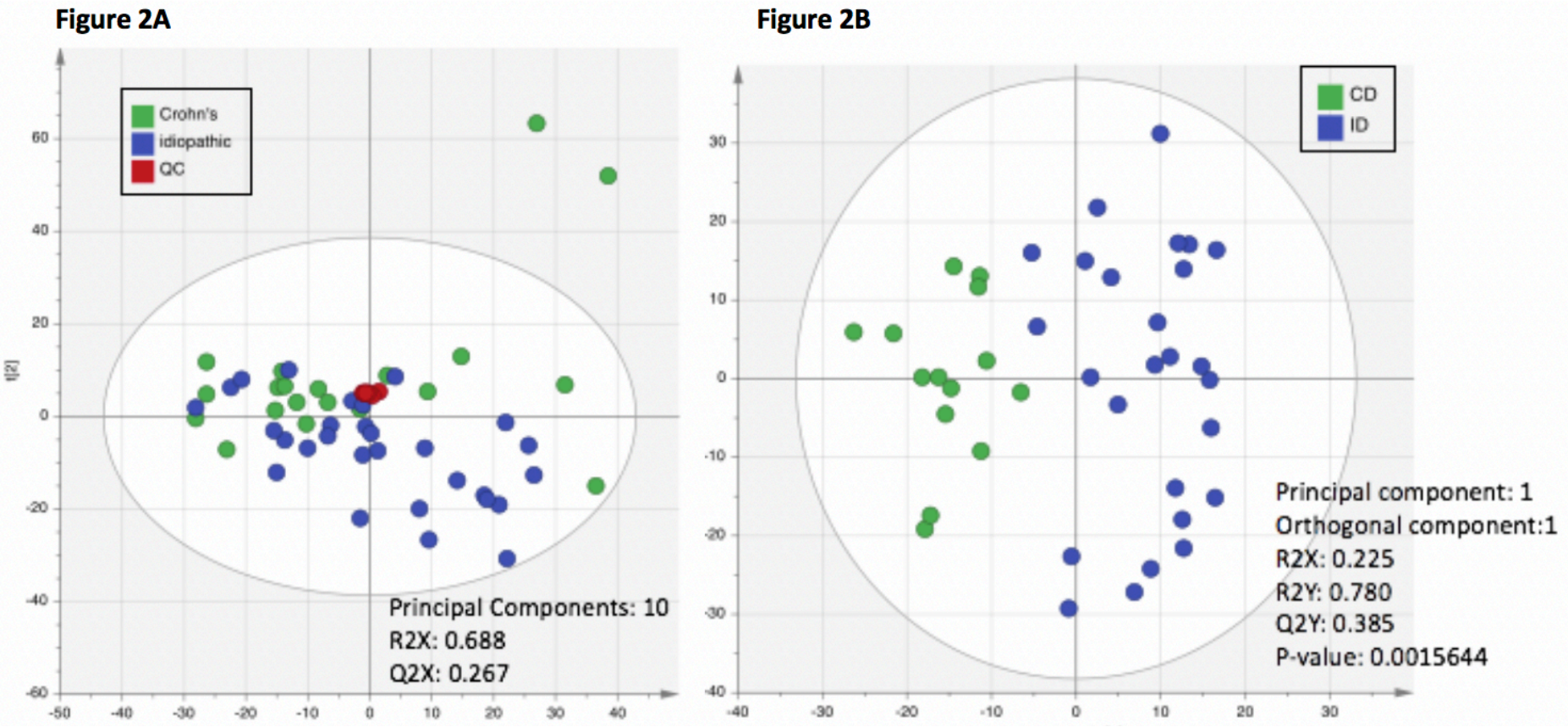

Figure 2: HILIC profiling results analysis

2A. PCA score plots from model of HILIC profiling of fistula biopsy tissue. Demonstrates samples of idiopathic and Crohn's fistula as well as good aggregation of QCs signifying stability of the UPLC MS analysis **2B.** OPLS-DA score plots of Hilic Profiling of fistula biopsy tissue demonstrates statistically significant predictive separation of Crohn's and idiopathic fistula tissue samples (Model validated with CV-ANOVA test p value = 0.0016)

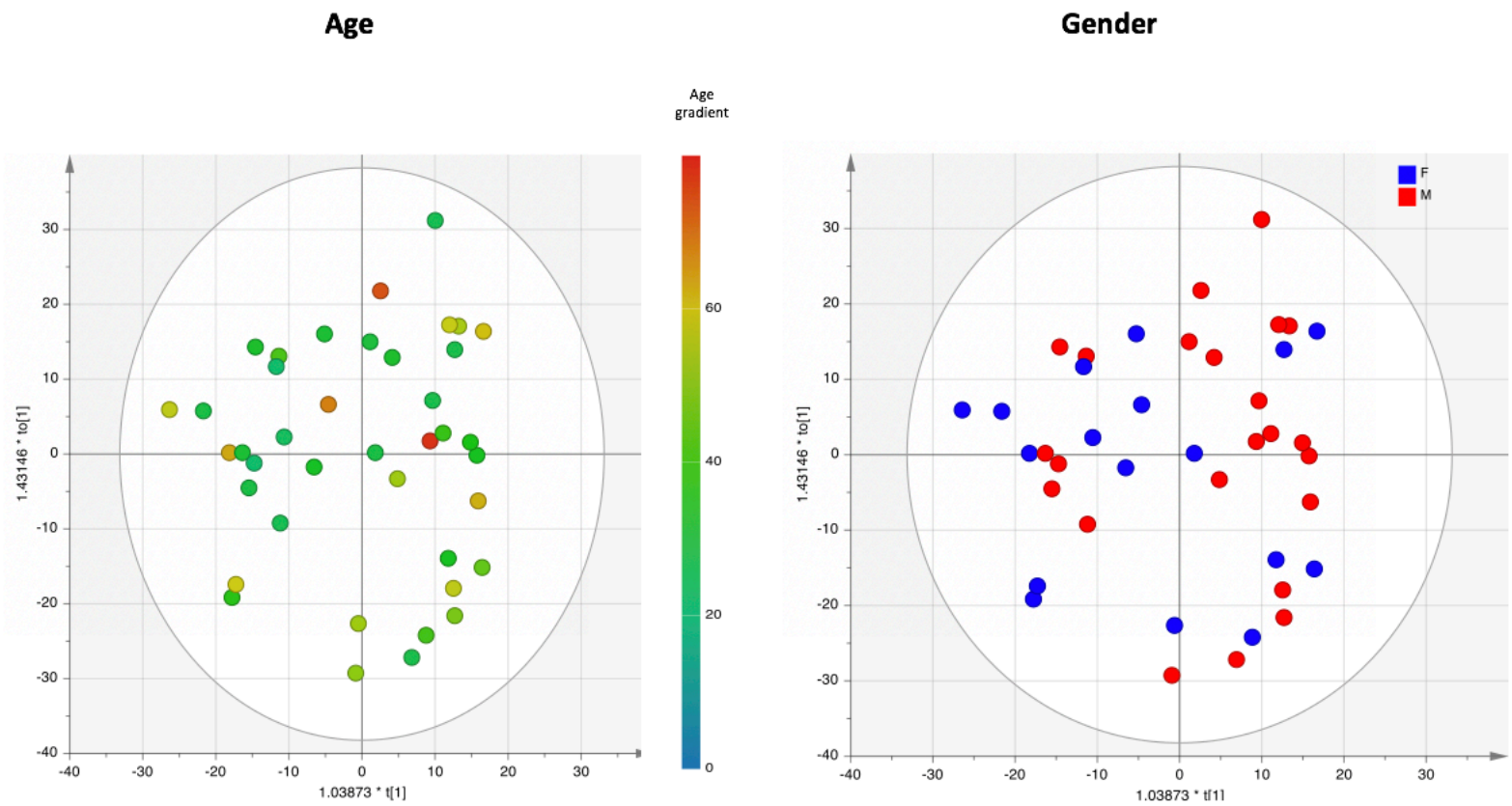

Figure 3: Age and sex distribution following HILIC profiling fistula biopsy tissue
Demonstrates data from samples of idiopathic and Crohn's fistula with non-clustering according to "age" (using the trend-bar on the right) or "gender" (as classified according colour)

2.4.1.2 *Lipidomic analysis of fistula tract biopsies*

Figure 3A demonstrates the PCA plot for the lipid profiling analysis, and again the QCs demonstrates good aggregation justifying the validity of the assay. OPLS-DA correlation coefficient score plots were comparing Crohn's and idiopathic fistula plots were generated, revealing discrimination between sample groups with a R^2Y of 0.836 and a Q^2Y of 0.297 (Figure 4B). Based on the OPLS-DA correlation coefficient plots (Figure 7), the features that contributed to the significant differentiation (P-value: 0.00914881) between samples from CD fistula patients and idiopathic fistula patients were identified (supplementary Table 39).

A final statistical multivariate analysis was performed using the different sub-phenotypes collected in patient's demographic details (Table 1), i.e. duration of perianal fistula (greater or less than one year), immuno-modulatory medication use (yes / no), presence of seton (yes/no). None of these models were significant, which decreased the chance of them being potential confounders.

2.4.2 Metabolic predictors of fistula type using UPLC-MS approaches to fistula tract biopsy

Forty-one distinguishing marker metabolites were identified using the HILIC and Lipid profiling platforms (Table 2). Correlation box plots highlighting whether each metabolite marker was positively / negatively correlated with Crohn's or idiopathic fistula tissue, is depicted in Figure 5 and Figure 7.

2.4.2.1 *HILIC UPLC-MS Profiling*

The OPLS-DA models that were built from patients with CD and idiopathic fistula were validated with a p-value of 0.0016 in the HILIC assay. This was subsequently classified

according to metabolite type and the identification process resulted in a total of 22 markers identified on the HILIC assay (Table 2). Metabolite identification revealed various marker metabolites belonging to several chemical classes including amino acids, nucleosides and lipid molecules (Table 2 / Supplementary Table 38). Univariate analysis (student t-test) revealed statistically significant differences ($p<0.05$) in expression between Crohn's and idiopathic fistula samples in all but three of the 22 metabolites (supplementary Table 38).

Twelve of the 22 metabolite markers were upregulated in Crohn's fistula tissue biopsies as demonstrated by positive correlation and seven of the markers were upregulated in idiopathic fistula tissue biopsies (Figure 5). Three marker metabolites (phosphocholine, guanine and palmitoyl L-carnitine) did not meet the threshold (coefficient 0.3) for consideration of correlation. Metabolic pathway analysis for the differentially expressed metabolites is demonstrated in Figure 6. This was done using Metscape to map the 22 significant marker metabolites on to KEGG pathway order to identify the pathways that may be altered as a result of the differences in metabolites between the CD and idiopathic fistula samples.

2.4.2.2 *Lipid UPLC-MS profiling*

The OPLS-DA models that were built from patients with CD and idiopathic fistula were validated with a p-value of 0.009 in the lipid profiling assay. Metabolite structural identification revealed 19 differentially expressed marker metabolites. This included several lipid molecule marker metabolites (Table 2/ supplementary Table 39) including diacylglycerols (3), Ceramides (1), diacylglycerophosphocholines (6), Triacylglycerols (3), glycosphingolipids (3), Glycerophosphoethanolamines (2) sphingomyelin (1). Univariate analysis (student t-test) revealed statistically significant differences ($p<0.05$) in expression between Crohn's and idiopathic fistula samples in all but four of the 19 metabolites (supplementary Table 39). Following application of the Benjamini-Hochberg statistical calculation, the number of

statistically significant distinguishing metabolites was reduced from nineteen to two statistically significant metabolites (Hex Cer d18:1/16:0, diglyceride DG 38:5)

Seventeen of the total 19 markers were upregulated in Crohn's anal fistula tract biopsies and showed positive correlation (i.e. coefficient > 0.3, Figure 7). Of the remaining two markers metabolites, triglyceride 56:4 showed a trend towards correlation in Crohn's fistula biopsies (but did not meet the threshold of 0.3) and Sphingomyelin (SM d18:2/24:0) showed a trend towards positive correlation in idiopathic fistula tract biopsies but did not meet threshold (Figure 7). A pathway analysis for the differentially expressed metabolites is demonstrated in Figure 8.

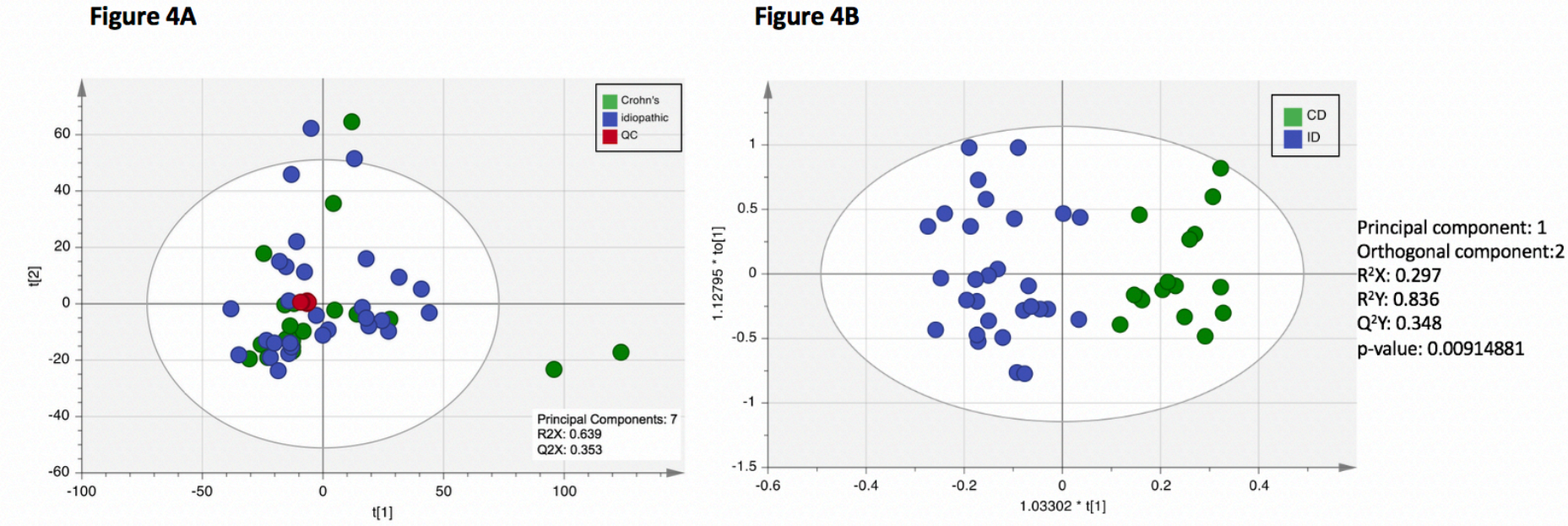

Figure 4: Lipid profiling results analysis

4A. PCA correlation coefficient score plots from the statistical model of results of lipid profiling of fistula biopsy tissue. Demonstrates samples of idiopathic and Crohn's fistula as well as good aggregation of QCs signifying stability of the lipid UPLC MS analysis **4B.** OPLS-DA score plot of lipid profiling of fistula biopsy tissue demonstrates statistically significant predictive separation of Crohn's and idiopathic fistula tissue samples (Model validated with CV-ANOVA test p value = 0.00915).

Table 2: List of differentially expressed metabolite markers and chemical class following HILIC and Lipid profiling assays

HILIC profiling differentially expressed marker metabolites	Chemical class	Trend in CD anal fistula tissue	Lipid profiling differentially expressed marker metabolites	Chemical class	Trend in CD anal fistula tissue
Arginine	Amino acid	**Increased**	***Cer (d18:1/24:1)***	Ceramides	**Increased**
Threonine	Amino acid	**Increased**	***DG 36:1***	Diacylglycerols	**Increased**
Tyrosine	Amino acid	**Increased**	***DG 36:3***	Diacylglycerols	**Increased**
Lysine	Amino acid	**Increased**	***DG 38:5***	Diacylglycerols	**Increased**
Citrulline	Amino acid	**Increased**	***DG 40:4***	Diacylglycerols	**Increased**
Proline	Amino acid	**Increased**	***HexCer (d18:1/16:0)***	Glycosphingolipids	**Increased**
Methionine	Amino acid	**Increased**	***HexCer (d18:1/23:0)***	Glycosphingolipids	**Increased**
Dimethylarginine	Amino acid	**Increased**	***HexCer (d18:1/24:0)***	Glycosphingolipids	**Increased**
Phenylalanine	Amino acid	**Increased**	***LPE (18:0)***	Monoacylglycero-phosphoethanolamines	**Increased**
Homoarginine	Amino acid	**Increased**	***PC 38:2***	Diacyl-glycerophosphocholines	**Increased**
Guanine	Nucleoside	**Increased**	***PC 38:3***	Diacyl-glycerophosphocholines	**Increased**
O acetyl L-carnitine	acylcarnitine	**Increased**	***PC 38:4***	Diacylglycero-phosphocholines	**Increased**
N-acetyl lysine	Amino acid	**Increased**	***PC 38:6***	Diacylglycero-phosphocholines	**Increased**
Octanoyl L-Carnitine	Acyl carnitine	**Increased**	***PC O-34:1 or PC P-34:0***	Diacylglycero-phosphocholines	**Increased**
Phosphocholine PC 34:2	lipid	**Decreased**	***PE P:40:5 or O-40:6***	Diacylglycero-phosphoethanolamines	**Increased**
Adenosine	Nucleoside	**Decreased**	***SM (d18:2/24:0)***	Sphingomyelins	**Decreased**
Creatinine	UNCLASSIFIED	**Decreased**	***TG 53:7***	Triacylglycerols	**Increased**
Hexanoyl L-Carnitine	acylcarnitine	**Decreased**	***TG 46:3***	Triacylglycerols	**Increased**
Monoacylglycerol 18:3	lipid	**Decreased**	***TG 56:4***	Triacylglycerols	**Increased**
S-methyl-5-thioadenosine	Nucleoside	**Decreased**			
Decanoyl L-Carnitine	Acylcarnitine	**Decreased**			
Palmitoyl L-Carnitine	Acylcarnitine	**Decreased**			

Cer – Ceramide; HexCer (hexosylceramide); PC- Phosphatidylcholine; PE- phosphatidylethanolamine; SM – sphingomyelin; DG – diglyceride (diacylglycerol) TG – triglyceride (triacylglycerol)

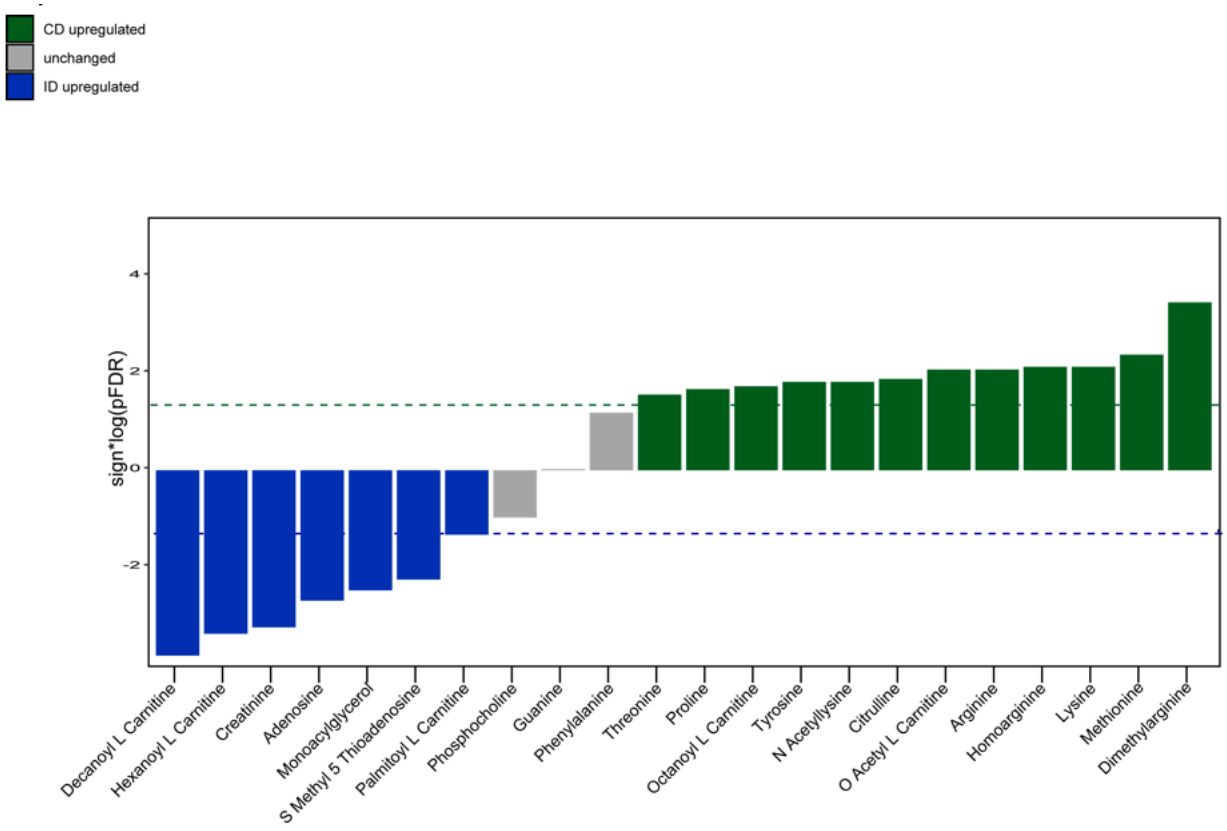

Figure 5: Visualization of the correlation plots for each of the distinguishing marker metabolites from the HILIC profiling assay (n = 22)
Bar plot demonstrates the Benjamini-Hochberg [225]corrected statistical significance (–log10 (pFDR) x sign of beta coefficient of each marker). Markers corresponding to green bars (n=12) were positively correlated with Crohn's fistula tissue biopsies; blue bars (n=7) signified – positive correlation with idiopathic fistula tissue biopsies; grey bars (n=3) – marker correlation was below a cut-off threshold of (+/-0.3), i.e. the grey coloured markers had sufficient variations between samples such that the trends of the markers (i.e. upregulation / downregulation relative to CD) showed only weak correlation.

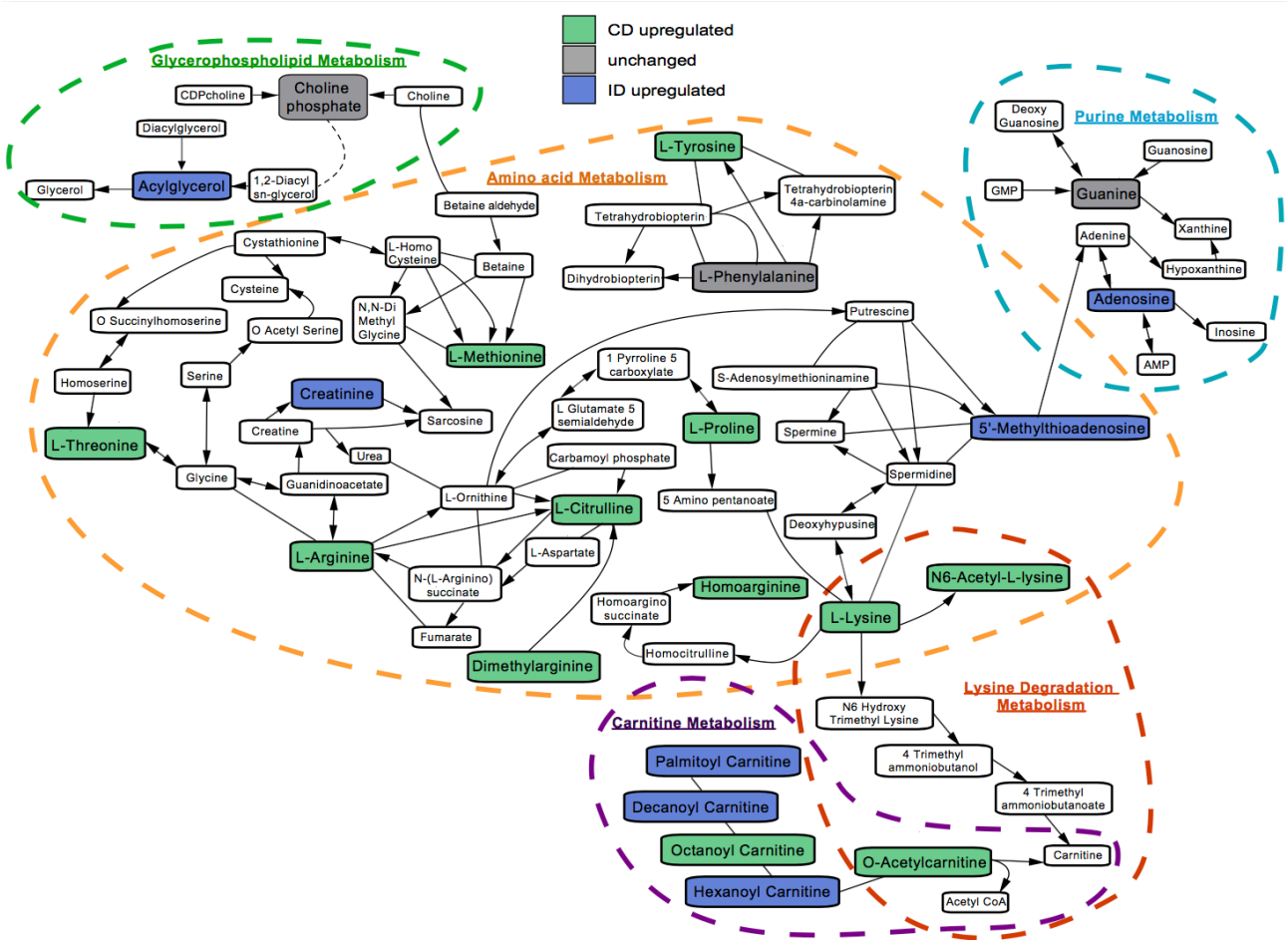

Figure 6: Representation of the biochemical relationships between the differentially expressed metabolite markers following HILIC profiling of CD and idiopathic fistula tissue

In total 22 of the marker metabolites were mapped onto the KEGG pathways using Metscape. 12 of these show positive correlation with CD fistula tract biopsies (i.e. upregulated in Crohn's disease anal fistulas and are highlighted green; those upregulated in idiopathic fistula tissue (7), are highlighted blue. Those in grey showed no correlation / upregulation with either CD/ idiopathic fistula tissue.

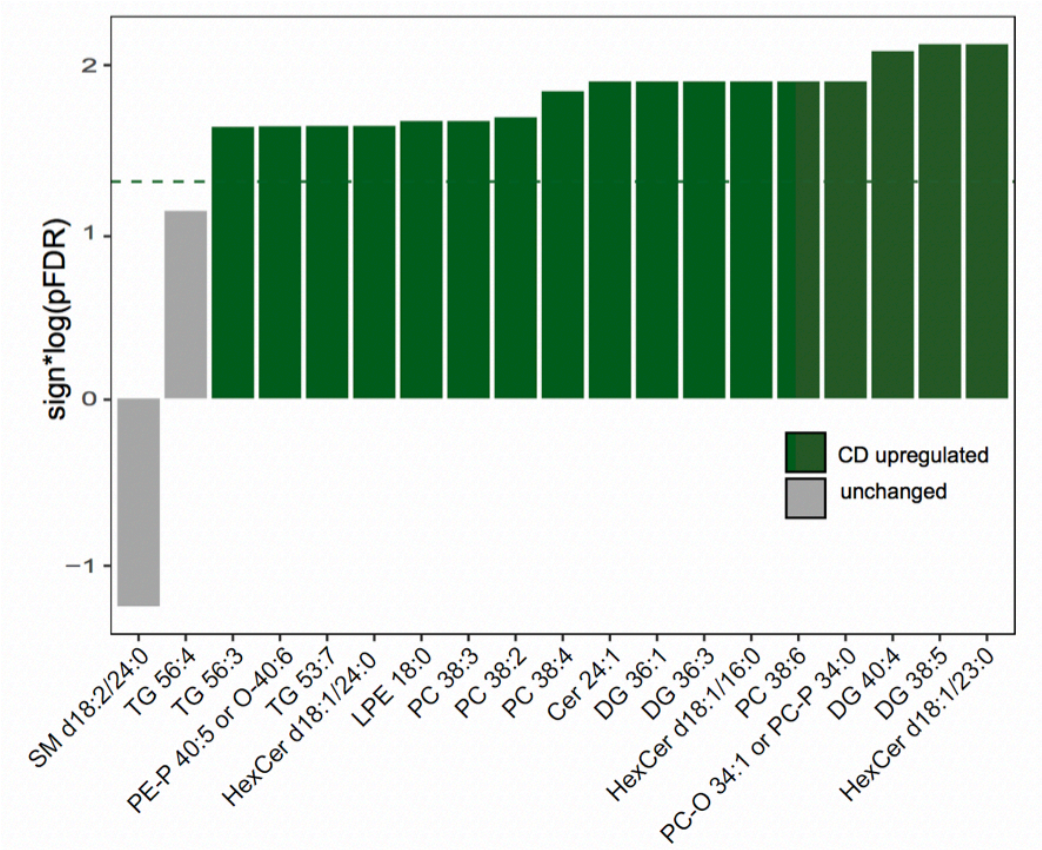

Figure 7:Visualization of the correlation plots for each of the distinguishing marker metabolites from the lipid profiling assay
Bar plot demonstrates the Benjamini-Hochberg corrected statistical significance (–log10 (pFDR) x sign of beta coefficient of each marker). Markers coloured in green were positively correlated with Crohn's fistula tissue biopsies and grey if correlation was below a cut-off threshold of (+/-0.3), i.e. the grey coloured markers had sufficient variations between samples such that the trends of the markers (i.e. upregulation / downregulation relative to CD) did not meet the cut off threshold.

2.5 Discussion

Our study demonstrates a UPLC-MS characterisation of fistula tissue metabonome that is differentially expressed in Crohn's perianal fistulas when compared to idiopathic perianal fistulas (figure 8). HILIC profiling revealed 22 differentially expressed metabolites whilst lipid profiling yielded 19 lipid metabolites. The differences in marker metabolites occur in pathways involved in amino acid metabolism, carnitine metabolism, phospholipid metabolism, lysine degradation metabolism, sphingolipid metabolism, glycerophospholipid metabolism, and purine metabolism. Hilic profiling revealed twelve metabolite markers were found to be upregulated in Crohn's perianal fistula tract biopsies. The majority of these upregulated metabolites in Crohn's disease fistula tracts were involved in amino acid metabolism (Figure 5 & Figure 6) and three of the marker metabolites upregulated in CD fistula tract biopsy samples were contributors to the lysine degradation metabolism pathway in addition. Upregulated marker metabolites for idiopathic fistula were involved in carnitine metabolism (3) glycerophospholipid metabolism (1), and purine metabolism (1), as well as some being also involved in amino acid metabolism (3). Various markers belonging to different classes of lipids were also found to be upregulated in Crohn's fistulas (Figure 7 & Figure 8), including diacylglycerols (3), Ceramides (1), diacylglycerophosphocholines (6), Triacylglycerols (3), glycosphingolipids (3), Glycerophosphoethanolamines (2).

Previous studies have reported on several alterations in amino acids in CD patients, with decrease in **serum** amino acids suggestive of their utilisation as a catabolic energy source in the inflammatory process[229,230]. Amino acids and their metabolites are involved as both

substrates and regulators in many metabolic pathways[230,231]. In our study, alterations in amino acids metabolism between the two groups was evidenced by increased **tissue** levels of several amino acids (Figure 9) in CD compared to idiopathic fistula tissue. Levels of dimethyl arginine were most notably increased in CD fistula tissue, but levels of arginine and homoarginine were also increased. Dimethylarginine, is closely related to L-arginine (a precursor of nitric oxide) and interferes with the latter in the production of nitric oxide (important in normal endothelial function). This may well have an effect on blood supply and a role in ongoing inflammation and impaired healing of fistulas. A decrease in L-arginine has also previously been reported in colonic tissue of inflammatory bowel disease patients and this has been inversely proportional to the disease activity / level of inflammation (albeit in ulcerative colitis) [229]. Methionine and Lysine were also increased and reported functions include involvement in angiogenesis and several metabolic processes, protein synthesis, and fatty acid metabolism. Several metabolites were decreased in Crohn's anal fistulas, and these included those involved in energy-related / lipid metabolism. Decreased levels of decanoyl L-carnitine and hexanoyl l-carnitine were found in CD fistula tissue. These belong to a different class of compounds to amino acids, acylcarnitines, which are more lipid-like organic compounds. They are involved in lipid and fatty acid metabolism as well as cell signalling and play a role in membrane stability and integrity and thus may well play a role in fistula persistence. The previously reported decrease in serum levels of carnitine and acylcarnitine species in CD are thought to provide evidence for decreased beta-oxidation of fatty acids in CD[229]. One current hypothesis suggests that epithelial-to- mesenchymal transition (EMT) is the driving force behind the development of fistulas in CD patients[93,210]. In this process, differentiated epithelial cells transform to mesenchymal-type cells and acquire the ability to migrate and penetrate adjacent tissues[232]. Recently, an EMT-generated metabolome was described using LC-MS metabolomics profiling of cells that have undergone EMT and their epithelial counterparts, with reports of a common

set of metabolites which increase/decrease in abundance relative to the control[233]. Of note, some of the differentiating metabolites associated with EMT (e.g. carnitine precursor deoxycarnitine; lysine) are closely related to some found in our study (acylcarnitines and lysine were both differentiating in our study). There is a paucity in the literature with regard to the other metabolites found and their potential significance in the context of perianal fistula of Crohn's or idiopathic origin. Furthermore, studies with correlation of data across various platforms are lacking, with future need for integration of multi-omics platforms (metabonomic, genomic, transcriptomic, microbiome data) to make meaningful in-roads in understanding pathogenesis[234].) in CD compared to idiopathic fistula tissue. Levels of dimethyl arginine were most notably increased in CD fistula tissue, but levels of arginine and homoarginine were also increased. Dimethylarginine, is closely related to L-arginine (a precursor of nitric oxide) and interferes with the latter in the production of nitric oxide (important in normal endothelial function). This may well have an effect on blood supply and a role in ongoing inflammation and impaired healing of fistulas. A decrease in L-arginine has also previously been reported in colonic tissue of inflammatory bowel disease patients and this has been inversely proportional to the disease activity / level of inflammation (albeit in ulcerative colitis) [229]. Methionine and Lysine were also increased and reported functions include involvement in angiogenesis and several metabolic processes, protein synthesis, and fatty acid metabolism. Several metabolites were decreased in Crohn's anal fistulas, and these included those involved in energy-related / lipid metabolism. Decreased levels of decanoyl L-carnitine and hexanoyl l-carnitine were found in CD fistula tissue. These belong to a different class of compounds to amino acids, i.e. acylcarnitines, which are more lipid-like organic compounds. They are involved in lipid and fatty acid metabolism as well as cell signalling and plays a role in membrane stability and integrity and thus may well play a role in fistula persistence. Previously reported decrease in serum levels of carnitine and acylcarnitine species in CD are

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The strengths of this study include the untargeted approach facilitating a broad and detailed view of the perianal fistula tissue metabolome. The use of tissue allowed for an initial investigation of metabonomics of perianal fistula whilst limiting the fluctuations in metabolites that may be seen with the use of serum, urine, or in response to dietary intake etc.

There are several limitations and considerations to be borne in mind when considering these data, including the fact that there is no true healthy control for the CD group which leads to difficulty in hypothesising on the overall metabotype for fistulas in comparison to healthy states, and hence we decided on a comparative study approach. Also, we took one central

biopsy of the fistula in non-epithelialised fistula tracts, however for further detail it may be interesting to biopsy multiple sites along the fistula tract (fistula internal opening vs. distal tract) to identify any intra-fistula variations due to biopsy site. Bataille et al.[82] described electron microscopy findings of non-epithelialised CD fistulas being lined in sections with myofibroblasts, with gap junctions to each other. Some areas of the tract had a new basement membrane, localised between the myofibroblasts and the underlying granulation tissue, although the underlining basement membrane was in some cases fragmented which may have implications if only one biopsy is taken [82]. Other limitations of our study include the size and the different durations of perianal fistula between groups. The longer median duration of fistulas in the Crohn's group, metabolic changes due to drug therapy, previous operative interventions and long-term seton use may all represent potential confounders. Furthermore, the sample size limits the ability to adjust for these. Also, the results in general need to be interpreted in light of potential confounding factors, such as medication and changed dietary strategies during active and inactive disease, which was not accounted for in the current study. There is also a potential selection bias, as many patients seen in our tertiary centre have complex and long standing fistulas. This may make these results less generalisable outside of our institution. Finally, the detected metabolite list does not represent all the metabolites detectable by the analysis platforms used (lipid / HILIC) in this study and some of the ions were not assignable due to ambiguity of their mass-to-charge (m/z) ratio and in some cases due to absence from online databases.

Conclusion

Our pilot study applies metabonomics techniques are applicable to perianal fistula tissue. We have demonstrated the ability to distinguish Crohn's perianal fistulas from idiopathic fistulas on a molecular basis (Figure 9), with identification of differentiating metabolites of lipid and

amino acid metabolism. Further studies are required to validate these findings and more work in correlating omics research (including correlating microbiota with metabonomic data) would help in the improved understanding of why fistulas persist in a chronic unhealed state. Mechanistic explanation of underlying processes remains rudimentary, due to limited studies in aetiopathogenesis in this specific disease phenotype (Crohn's perianal fistula). Future studies should include larger numbers of patients (e.g. via collaborations with tissue biobank), potentially increasing the bio-samples (blood, urine, faeces, tissue) and ideally at the moment of early diagnosis of the fistula which allows for assessment prior to potential influence of immunomodulators / medication on the biochemical milieu

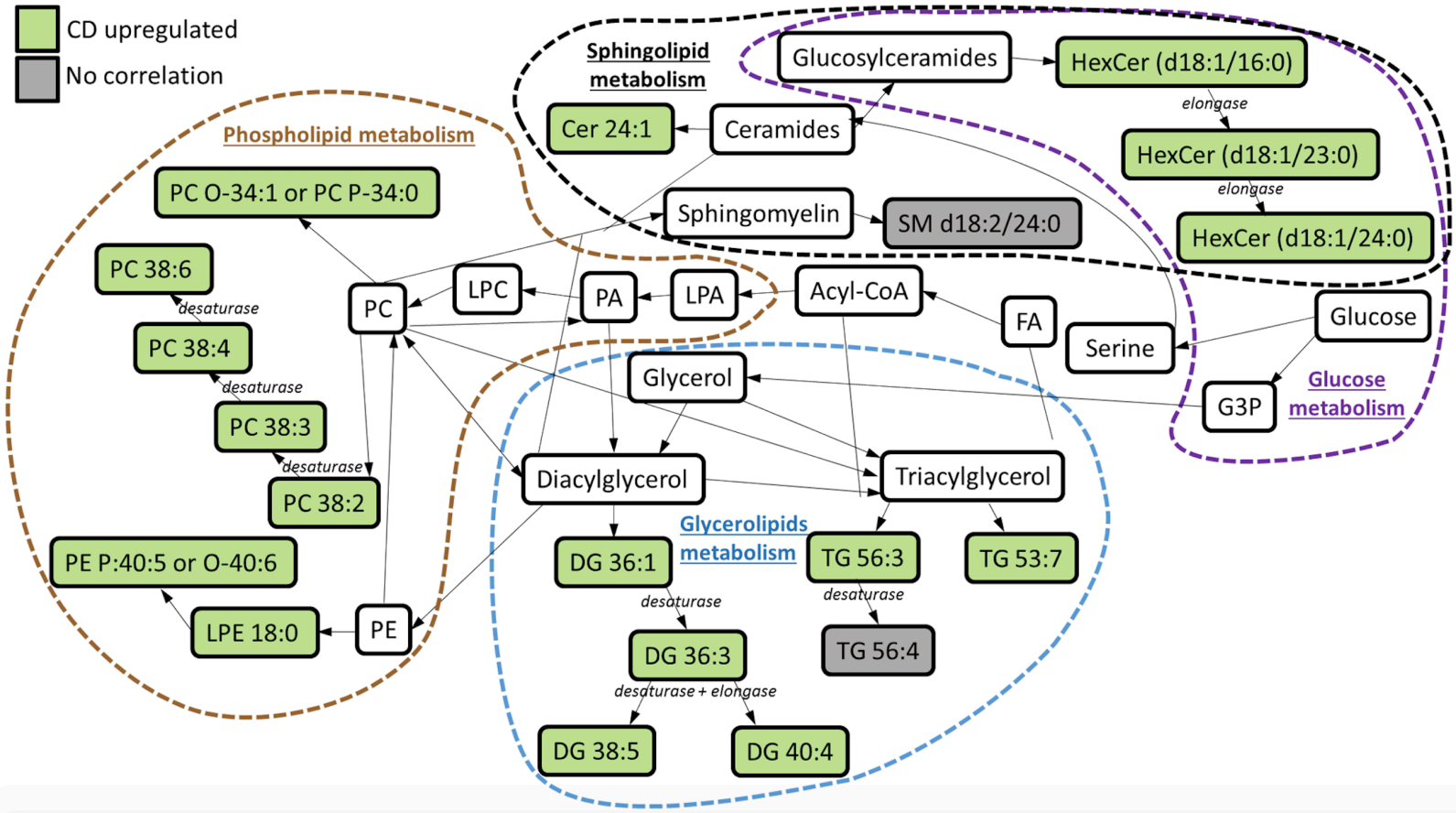

Figure 8:Representation of the biochemical relationships between the differentially expressed metabolite markers on lipid profiling of the anal fistula biopsy samples

19 lipid marker metabolites were mapped onto the KEGG pathways using Metscape. 17 of which showed positive correlation with CD fistula tissue (highlighted in green), whereas two lipid markers (in grey) showed no correlation with either CD/idiopathic fistula tissue. A correlation coefficient cut-off of 0.3 was used as the distinguishing level.

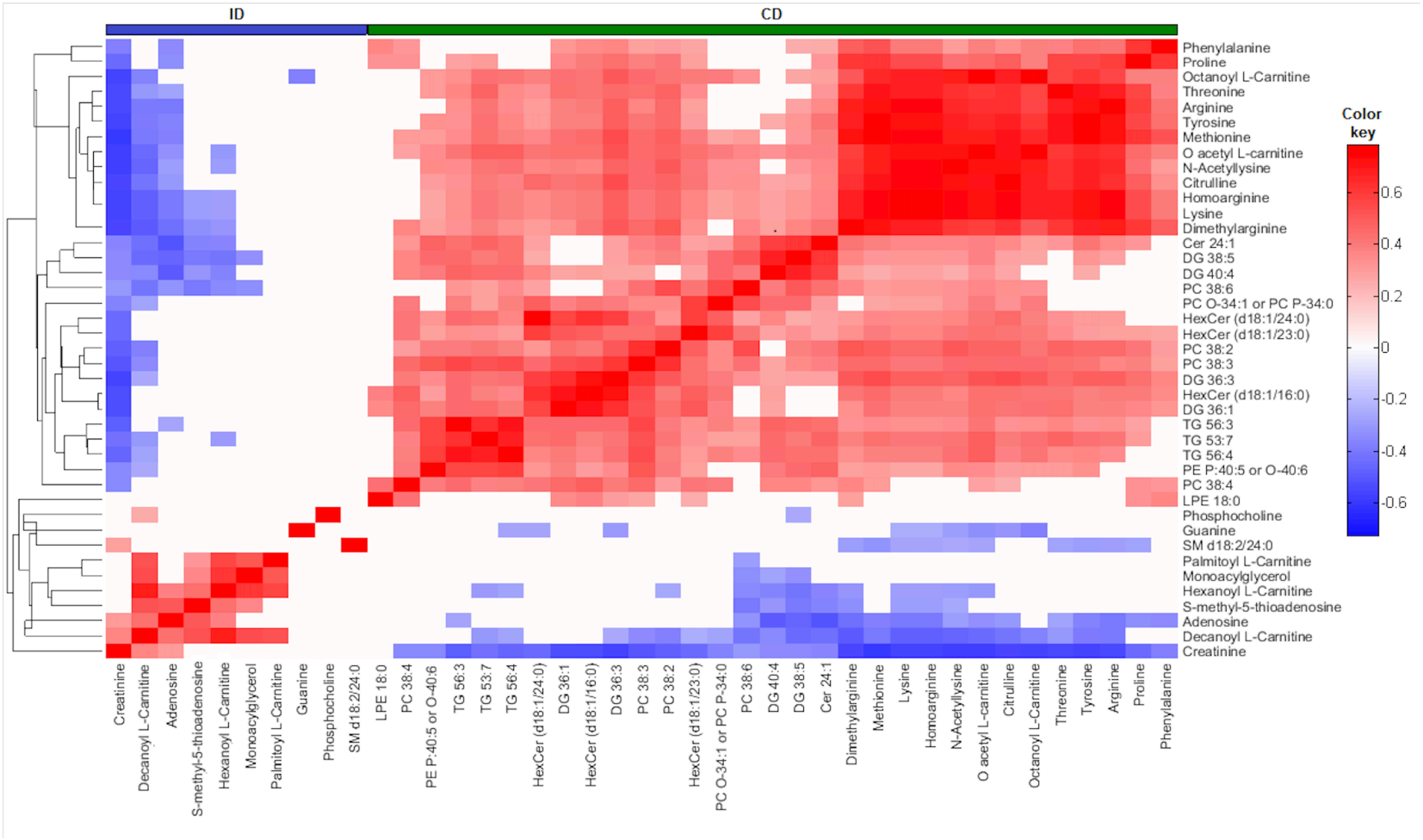

Figure 9: Heatmap plot of the differentially expressed metabolites using hierarchical clustering technique[235]

The 41 differentially expressed metabolites correctly cluster the fistula tract biopsy samples into Crohn's and idiopathic groups. The 'Color Key' corresponds to the magnitude of correlation with the corresponding marker: red cell – positive correlation between the metabolites; blue cell – anti-correlation between the metabolites; white cell – no correlation. The hierarchical clustering represented by a dendrogram shows the partitioning of the data between the two groups

2.6 Other novel platforms for future direction on Crohn's fistula aetiopathogenesis

As demonstrated in sections 2.1-2.5, analysis of fistula biopsy tissue by histology or molecular profiling provides a snapshot of the status of the tissue but it reveals limited information about the development of imbalances in the number and function of cells. In vitro models may aid the investigative process by providing tissue sources for identifying molecular patterns at varying stages of disease[236,237]. One such model is the intestinal epithelial organoid units, which are 3-dimensional epithelial structures derived from intestinal stem cells near the base of small intestinal crypts. The intestinal organoid as a 3D culture (which faithfully mimics the in vivo tissue from both intestinal crypts as well as single isolated Lgr5+cells) was first established by Sato et al[238,239]. Several studies have since developed organoids from different mammalian organs[240–242]. Aside from providing insight into disease development, organoid units have the added advantage of creating more accurate cellular models than cell lines which serve as useful tools for identifying novel therapeutic agents or testing drugs and assessing effect[243].

2.6.1 Intestinal organoid culture

Intestinal crypt organoid (stem cell) cultures can be perpetuated indefinitely. This allows for extensive characterisation of cell production and testing hypotheses with molecules probing receptors or signalling pathways. The ability to harvest stem cells from diseased and healthy

individuals offers a comparative assessment of their role in health and disease and may offer novel treatment targets. This is of particular interest in Crohn's perianal fistula whereby a regenerative medicine approach to healing fistulas is showing efficacy similar to current available treatment of the more complex anal fistulas. This involves intra/peri-fistula injection of stem cells generated from allogeneic (non-self) fat tissue. These adipose-derived mesenchymal stem cells (Cx601) are thought to offer benefit due to their inherent anti-inflammatory and immunomodulatory potential. Results of a recent Phase III double blind clinical trial indicate that Cx601cell therapy initiates tissue repair and regeneration of Crohn's anal fistulas with 50% of anal fistulas healed with Cx601 treatment (about the same as surgery) compared to 35% healing in the comparator group[244]. Other studies have also reported its use[245164], as well as reported use of autologous fat injections for fistula treatment[246247], with variable (up to 75%) success rates[246]. Proposed mechanisms of action include the potential of cells with regenerative properties (e.g. adipocytes, preadipocytes, mesenchymal stem cells, endothelial cells, fibroblasts and hematopoietic-lineage cells) to encourage wound healing processes including neo-angiogenesis, tissue growth, anti-inflammatory and immunomodulatory properties in the target area[248,249246].

An interesting factor from the study by Panes and colleagues is the relatively high healing rate in the comparator group. This suggests that an aggressive fistula preparation protocol as used in the study (i.e. examination under anaesthesia, fistula curettage and seton drainage) may suffice for success. A question raised from this is whether there is a role for activation

of a Crohn's fistula patient's own stem cells to more effectively manage inflammation and repair tissues. Intestinal crypt organoid (stem cell) cultures can be perpetuated indefinitely. This allows for extensive characterisation of cell production and testing hypotheses with tool molecules probing receptors or signalling pathways. A regenerative medicine approach to healing fistulas is showing efficacy similar to current available treatment of the more complex anal fistulas. This involves intra/peri-fistula injection of stem cells generated from allogeneic (non-self) fat tissue. These adipose-derived mesenchymal stem cells (Cx601) are thought to offer benefit due to their inherent anti-inflammatory and immunomodulatory potential. Results of a recent Phase III double blind clinical trial indicate that Cx601cell therapy initiates tissue repair and regeneration of Crohn's anal fistulas with 50% of anal fistulas healed with Cx601 treatment (about the same as surgery) compared to 35% healing in the placebo control group[244]. Other studies have also reported its use[245164], as well as reported use of autologous fat injections for fistula tratment[246247], with variable (upto75%) success rates[246] Proposed roles include the potential of cells with regenerative properties (e.g. adipocytes, preadipocytes, mesenchymal stem cells, endothelial cells, fibroblasts and hematopoietic-lineage cells) to encourage wound healing processes including neo-angiogenesis, tissue growth, anti-inflammatory and immunomodulatory properties in the target area[248,249246]. An interest factor from the study by Panes and colleagues is the relatively high healing rate in the placebo control group. This suggests that an aggressive fistula preparation protocol as used in the study (i.e. examination under anaesthesia, fistula curettage and seton drainage) may suffice for success. A question raised from this is whether there is

a role for activation of a Crohn's fistula patient's own stem cells to more effectively manage inflammation and repair tissues.

2.6.2 Modelling Crohn's perianal fistula using anorectal transition zone organoids

2.6.2.1 *Introduction*

For Crohn's perianal fistulas, a proposed tissue of interest would be the anorectal transitional zone tissue (ATZ). This region contains the dentate line and is connected to the excretory ducts of the anal glands which have reported implications in perianal fistula development (see cryptoglandular theory above). The ATZ like other transition zones (TZs) in humans are defined by the junction between two types of epithelia[250] (the simple columnar epithelium of the rectum and much of GI tract and the stratified squamous epithelium of the anus[251]). As the ATZ represents a merger of two distinct epithelia, there is likely be to an assortment of different signalling clues present, which results in the cells of the TZ having unique properties as well as providing a potential niche for stem cells[250].For Crohn's perianal fistulas, a proposed tissue of interest would be the anorectal transitional zone tissue (ATZ), this region contains the dentate line and is connected to the excretory ducts of the anal glands / located close to the anal glands which have reported implications in perianal fistula development. The ATZ like other transition zones (TZs) in humans are defined by the junction between two types of epithelia[250] (i.e. simple columnar epithelium of rectum and much of GI tract vs. stratified squamous/ columnar rectum of the transition zone / anus[251]). As the ATZ represents

a merger of two distinct epithelium, there is likely be to an assortment of different signalling clues present, which results in the cells of the TZ having unique properties as well as providing a potential niche for stem cells[250].

2.6.2.2 *Methods*

An ability to perpetuate anal / rectal organoids would allow for interesting tissue analyses in patients with Crohn's perianal fistulas with healthy controls. In particular the evaluation of the health of mucosal stem cells in the anorectal region and to test the role of defective or ineffective stem cells in anal fistula formation and perpetuation. Preliminary work in the porcine anorectal region has demonstrated some ability to grow organoids from anorectal biopsies. Anorectal tissue was resected from healthy Landrace / Cross pigs (females, aged 4-6 months) within 1 hour of termination. Biopsies taken from anal, anorectal transitional zone (ATZ), and rectal tissues were transferred to petri dishes where they were cleaned and minced. Tissues were exposed to enzymatic digestion with collagenase P at 37ºC for 1 hour on a shaker to release crypts and single cells.[4] Tissue fragments from enzymatic digestion were removed by sequential filtration and centrifugation.

2.6.2.3 *Results*

Organoids were initiated in human culture medium with crypts isolated from rectum and ATZ tissues and single cells isolated from anal tissue.[5] Cultured cells formed grape-like structures that progressed to ring structures and then to organoids within a week (Figure 10). Rectal organoids formed branched tubular structures, similar to the phenotype reported for

human small intestine organoids.[5] ATZ organoids formed branched spherical structures. Anal single cells formed ring structures that did not subsequently progress to branched structures (Figure 10 & Figure 11).

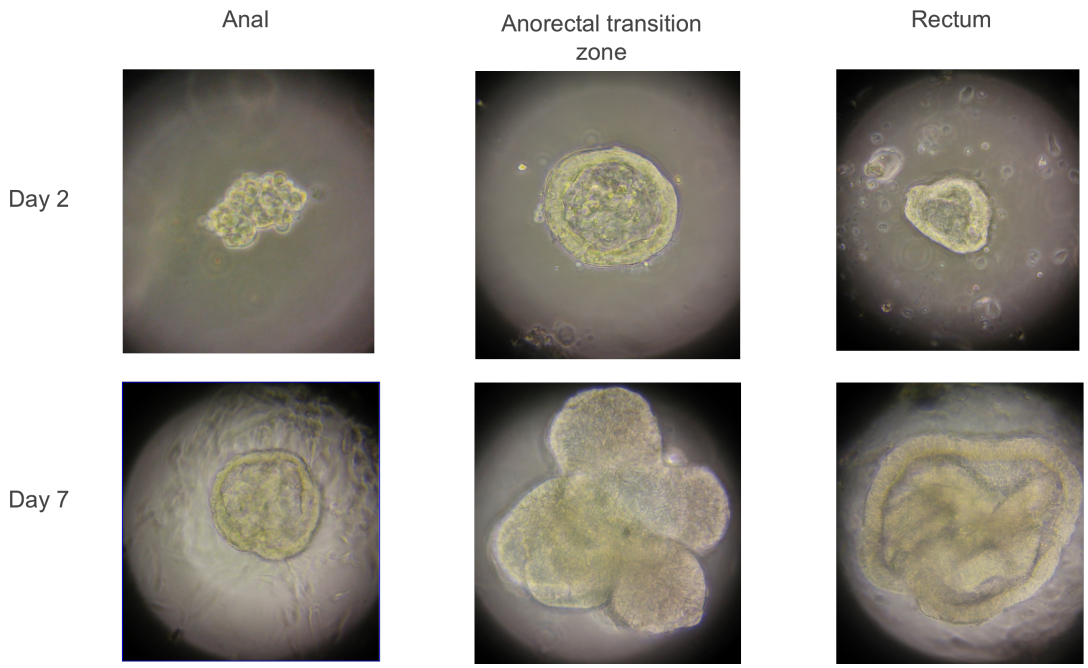

Figure 10: Porcine anorectal organoid development in 3D *in vitro cultures*

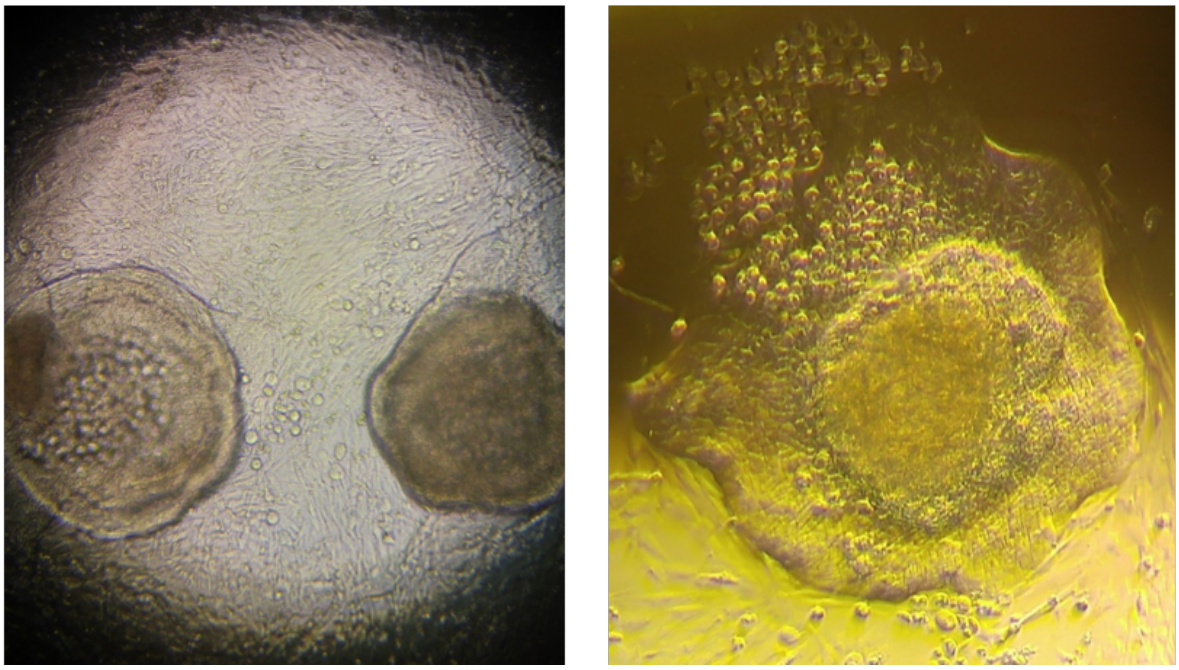

Figure 11: Organoids from porcine anal biopsies (2weeks following organoid initiation)

2.6.2.4 *Conclusion*

Whilst reports for intestinal organoids (small bowel, large bowel) exist, there are no reports of anal organoids[252]. Further work is underway in order to establish the possibility of defining human anal organoids and studying the transition zone using a combination of novel tools in molecular phenotyping / classification (via omics platforms) to better understand their roles health and disease e.g. in fistula persistence. This would open up exciting new opportunities to gain valuable insights into disease aetiology and progression, and more importantly, a route to understand and identify the role of stem cells in fistula wound resolution. The characterisation of healthy versus Crohn's disease rectal organoids alongside healthy anal and 'fistula organoids' provides a closely associated surrogate tissue for developing molecular biomarkers which could lead to a new range of therapeutic targets in the long run for this debilitating condition.

Section B – Natural history of Crohn's perianal fistulas on biologic treatment

Chapter 3. Anti-TNF therapy in Crohn's disease

3.1 Abstract

Crohn's disease (CD) accounts for a variety of clinical manifestations or phenotypes which stem from chronic inflammation in the gastrointestinal tract. Its worldwide incidence is increasing including younger patients and childhood-onset of disease. The natural history of Crohn's disease is characterized by a remitting and relapsing course that progresses to complications and surgery in most patients. The goals of treatment are to achieve clinical and endoscopic remission, to avoid disease progression and minimise surgical resections. Medical treatment usually features antibiotics, corticosteroids and immunomodulators (thiopurines, methotrexate). Anti-TNF therapy was approved for use in Crohn's disease in 1998, and has changed the paradigm of treatment, leading to improved rates of response and remission in patients. There are significant considerations that need to be borne in mind, when treating patients including immunogenicity, safety profile and duration of treatment. This section reviews the evidence for anti-TNF treatment in CD.

3.2 Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease that can affect any part of the gastrointestinal (GI) tract. Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and CD which are characterised by chronic, uncontrolled and relapsing inflammation of the GI tract. The incidence and prevalence of IBD is increasing with the highest annual incidence of CD being in North America (20.2 per 100,000 person-years) [253]. CD is regarded as a broad diagnosis with various phenotypes of immune mediated inflammatory disease of the GI tract, with T cell mediated tissue destruction [254,255]. It is associated with elevated pro-inflammatory cytokines such as tumour necrosis factor (TNF or TNF-α) as well as interferon gamma and interleukin 12 [255,256]. The phenotypic manifestations of CD are relevant clinically, due to differences in disease behaviour and treatment options between these phenotypes, defined by the[255,256]. The phenotypic manifestations of CD are relevant clinically, due to differences in disease behaviour and treatment options between these phenotypes, defined Montreal Classification [100]. The clinical features of CD vary according to the disease phenotype (location and behaviour: inflammatory/stricturing/fistulising) but commonly include chronic diarrhoea, abdominal pain and weight loss. The course of CD is typified by periods of relapse and remission with recurrent cycles of inflammation leading to development of complications such as strictures and intestinal fistulas. Perianal Crohn's disease represents a severe disease phenotype that affects a third of patients with CD [197]. It encompasses a range of

manifestations that include perianal fistulas, anal stenosis, skin tags, fissures and ulceration, imposing a considerable burden on patients with CD. The therapeutic goal in CD is to induce and maintain remission, heal the mucosa and optimize quality of life for the patient [257]. Consensus guidelines recommend "step-up" treatment with initial trials of immunosuppressive treatment (corticosteroid and steroid sparing immunomodulators) prior to starting anti-TNF agents [258,259], although there is increasing evidence advocating for early institution of anti-TNF treatment [260], the "top-down" approach, especially in severe disease phenotypes [261]. In this review, we compile the evidence in the literature on the use of anti-TNF therapy in Crohn's disease, its mechanism of action, pharmacokinetics and treatment strategy, outcomes, adverse effects, including analysing failure of treatment, as a means of understanding its role and efficacy as well as future directions in therapeutic use.

3.3 Methods

Evidence to support this review was gathered via the PubMed database. Studies which discuss anti-TNF treatment in Crohn's disease particularly (but not exclusively) in Crohn's perianal fistula were reviewed and cross-referenced for additional reports. Search terms used were combinations of "Crohn's disease", "anal fistula", "perianal fistula", "rectal fistula", "treatment", "anti-TNF therapy", "adverse effects" "loss of response" "drug monitoring", "therapeutic monitoring", "dosing", "primary non-response", "secondary non-response" and "withdrawal".

3.4 Biology of TNF

Human TNF is a member of a large family of proteins and receptors that are involved in immune regulation [262]. The secreted form of TNF is a 17 kD, non-glycosylated protein, which is cleaved from its cell-surface bound precursor by TNFα converting enzyme (TACE). Both the transmembrane precursor and the soluble TNF have biological activity, with soluble/secreted TNF acting at sites remote from TNF producing cells, whereas the transmembrane form acts as a receptor via cell-to-cell contact, transmitting signals to targets when cells are directly in contact with TNF receptor expressing cells. This form of transmission of biological activity is known as "outside-to-inside signal" or ''reverse signal" [263–265]. TNF is mainly produced by monocytes, macrophages and T lymphocytes, but also by mast cells, granulocytes, fibroblasts and several other cell types [262]. Various stimuli result in its release and these include bacteria, viruses, immune complexes, super-antigens, tumour cells, radiation, and stress [266]. TNF is a highly pro-inflammatory cytokine that is involved in the induction of fever, insulin resistance, bone resorption, anaemia, the activation of granulocytes/T cells, and in sepsis. TNF reacts with two distinct receptors (TNF receptor 1 expressed on all nucleated cells; and TNF receptor 2 preferentially expressed on endothelial and haematopoietic cells [265,267]) through which they exert their biologic effect. These effects are pro-inflammatory in nature and occur through increased production of proinflammatory cytokines, including interleukin (IL)-1β and IL-6, expression of adhesion molecules, proliferation of fibroblasts and procoagulant factors, as well as initiation of acute-phase

responses, and inhibition of apoptosis of inflammatory cells [268,269]. It is involved in key processes in inflammation including the activation of coagulation and fibrinolytic responses, promoting the neutrophil-endothelial adhesion necessary for recruitment to sites of inflammation [270–272], and promoting granulomatous inflammation through its role in recruitment of component cells (T lymphocytes, monocytes and macrophages) [266,273,274 275]. Human TNF is a member of a large family of proteins and receptors that are involved in immune regulation [262]. The secreted form of TNF is a 17 kD, non-glycosylated protein, which is cleaved from its cell-surface bound precursor by TNF α converting enzyme (TACE). Both the transmembrane precursor and the soluble TNF have biological activity, with soluble/secreted TNF acting at sites remote from TNF producing cells, whereas the transmembrane form acts as a receptor via cell-to-cell contact, transmitting signals to targets when cells are directly in contact with TNF receptor expressing cells. This form of transmission of biological activity is known as "outside-to-inside signal" or ''reverse signal" [263–265]. TNF is mainly produced by monocytes, macrophages and T lymphocytes, but also by mast cells, granulocytes, fibroblasts and several other cell types [262]. Various stimuli result in its release and these include bacteria, viruses, immune complexes, super-antigens, tumour cells, radiation, and stress [266]. TNF is a highly pro-inflammatory cytokine that is involved in the induction of fever, insulin resistance, bone resorption, anaemia, the activation of granulocytes/T cells, and in sepsis. TNF reacts with two distinct receptors (TNF receptor 1 - expressed on all nucleated cells; and TNF receptor 2–preferentially expressed on endothelial and haematopoietic cells [265,267]) through which they exert their biologic effect. These effects

are pro-inflammatory in nature and occur through increased production of proinflammatory cytokines, including interleukin (IL)-1β and IL-6, expression of adhesion molecules, proliferation of fibroblasts and procoagulant factors, as well as initiation of acute-phase responses, and inhibition of apoptosis of inflammatory cells [268,269]. It is involved in key processes in inflammation including the activation of coagulation and fibrinolytic responses, promoting the necessary neutrophil-endothelial adhesion necessary for recruitment to sites of inflammation [270–272], and promoting granulomatous inflammation through its role in recruitment of component cells (T lymphocytes, monocytes and macrophages) [266,273,274]. It activates leukocytes and induces acute-phase reactants and metalloproteinases and also inhibits apoptosis of inflammatory cells [275].

3.5 Role of TNF in the Aetiopathogenesis of CD

The underlying cause of CD is not fully understood. However, the aetiopathogenesis is thought to involve an interplay between environmental triggers, dysbiosis, aberrant immune responses/immunoregulation and genetic susceptibility [255]. CD results in mucosal injury and inflammation whereby the epithelial barrier is breached as a primary or secondary event, and the luminal microflora stimulates a proinflammatory immune response. Mucosal injury and damage are associated with dysbiosis, which potentially perpetuates the inflammatory cascade [71]. CD is associated with a T-cell mediated response, and the hallmark of pathogenesis is transmural inflammation, which is facilitated by increased proinflammatory

cytokines, interferon gamma and interleukin 12 [255,256] as well as TNF. Increased secretion of TNF from lamina propria mononuclear cells has been found in the intestinal mucosa and TNF positive cells have been found deeper in the lamina propria and in the submucosa [276,277]. Studies have demonstrated that TNF is increased in both the stool of patients with active CD compared to controls (i.e., those with inactive disease or absence of CD), although serum concentrations have been less distinctive [278–280]. It is an early potent pro-inflammatory cytokine in the inflammatory process underlying CD [276,281] and has been demonstrated, in vitro, to be involved in the pathological processes (including neutrophil accumulation, granuloma formation, increased epithelial permeability) [282–284] seen in CD.

3.6 Mechanism of Action of Anti-TNF Therapy in CD

Antibody neutralization studies implicated a significant role for TNF in the pathogenesis of Crohn's disease [255]. These were initially done in animal models, before the first administration of an anti-TNF-α antibody cA2 (which later became infliximab, IFX) in a Crohn's patient, followed by the first case series [285,286]. The results spurred on multicentre studies and led to the approval of anti-TNF agents in the treatment of CD by the Food and Drug Administration in 1998. Anti-TNF antibodies are thought to have multiple mechanisms of action including neutralization of TNF-α, reverse signalling, apoptosis, and cytotoxicity [287] and they have a predilection and efficiency for distribution into inflamed tissue [265]. They are able to deplete overexpression of TNF-α, by binding soluble and transmembrane TNF-α and inhibiting binding to its receptors, resulting in blockage of proinflammatory signals or

molecules that are upregulated by TNF-α. Anti-TNF treatment has also been shown in vitro to induce cytokine suppression via reverse signalling [263,288]. This interesting phenomenon occurs when the cell-surface bound precursor to TNF binds to anti-TNF and acts as a ligand and triggers cell activation, cytokine suppression or apoptosis of the cell bearing the cell-surface bound precursor [263,288,289]. It is thought that this is done via exhaustion of common signalling products during simultaneous endotoxin/lipopolysaccharide signalling pathway activation [287]. Anti-TNF also induces apoptosis of activated lamina propria T lymphocytes [290], countering a proposed pathological mechanism in CD, where mucosal T cell proliferation exceeds T cell apoptosis [291]. Anti-TNF therapies with an Fc region (i.e., infliximab and adalimumab but not certolizumab) are also able to induce antibody-dependent cell mediated cytotoxicity and complement-dependent cytotoxicity [265].Antibody neutralization studies implicated a significant role for TNF in the pathogenesis of Crohn's disease [255]. These were initially done in animal models, before the first administration of an anti-TNF-α antibody cA2 (which later became infliximab, IFX) in a Crohn's patient, followed by the first case series [285,286]. The results spurred on multicentre studies and led to the approval of anti-TNF agents in the treatment of CD by the Food and Drug Administration in 1998. Anti-TNF antibodies are thought to have multiple mechanisms of action including neutralization of TNF-α, reverse signalling, apoptosis, and cytotoxicity [287] and have a predilection and efficiency for distribution into inflamed tissue [265]. They are able to deplete overexpression of TNF-α, by binding soluble and transmembrane TNF-α and inhibiting binding to its receptors, resulting in blockage of proinflammatory signals or molecules that are upregulated by TNF-

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3.7 Types of Anti-TNF Treatment and Efficacy in CD

3.7.1 Infliximab

Infliximab was the first biological response modifier to be used in the treatment of inflammatory bowel disease [292] and is a genetically engineered chimeric (mouse/human) immunoglobulin (Ig)G1 anti human tumour necrosis factor agent. It has the ability to fix complement and lyse cells expressing membrane-bound TNF-alpha and induce downregulation of the inflammatory mechanisms in the entire mucosal layer [293,294]. It is administered intravenously, typically on a maintenance schedule of every eight weeks after

an initial three-dose induction. Two landmark randomised controlled trials, the ACCENT I and II studies [140,295] (**A C**rohn's disease **C**linical study **E**valuating infliximab in a **N**ew long-term **T**reatment regimen) evaluated the efficacy of infliximab in patients with luminal as well as fistulising phenotypes of CD (see Table 3). These trials demonstrated the efficacy and safety of induction and maintenance therapy for moderate-to-severe CD as assessed by a disease activity grading system (CDAI—Crohn's Disease Activity Index). In luminal disease, an increased likelihood for short and longer term remission, as well as discontinuation of corticosteroids (which would otherwise have been required to dampen the immune response), was found [295]. Subsequent studies have demonstrated that Infliximab treatment not only results in a positive clinical response, but also in a significant endoscopic improvement and histological examination confirmed that a complete reduction in the inflammation infiltrate could only be seen in the patients treated with infliximab [293]. Outcomes in perianal fistulising disease (rectovaginal/perianal fistulas) included the closure of draining fistulas [296]. In the first placebo-controlled trial of 94 patients (mostly with perianal fistulas), closure of ≥50% of the fistulas (primary endpoint) occurred in 68% (compared with 26% in placebo, $p = 0.002$) and closure of all fistulas draining at baseline occurred in 55% (compared with 13% placebo, $p = 0.001$) of the 63 patients receiving infliximab at 0, 2, and 6 weeks [120]. Maintenance therapy resulted in around a third of patients remaining in remission at one year [140]. However, clinical remission does not always reflect true deep tissue healing, which has been demonstrated on imaging (magnetic resonance imaging/endoscopic ultrasound) [122].

Table 3: Summary of significant studies of anti-TNF in moderate to severe Crohn's disease patients.

Study	Drug	Patient Groups	Response (Where Reported)	Remission
Targan et al. [256] 1997 Multicentre Double-blind placebo-controlled trial	IFX	3 Treatment groups: Infliximab–5 or 10 or 20 mg/kg Placebo	At week 4: 5 mg/kg group 81% (22/27) 10 mg/kg group: 50% (14/28) 20 mg/kg group: 64% (18/28) Placebo: 17% (4/24)	At week 4: All dose treatment group–33% (27/83) Placebo group 4% (1/24)
ACCENT-I (Hanauer et al. [295]) 2002	IFX	3 treatment groups–same induction regimen (IFX 5 mg/kg at week 0) followed by: Group 1–Placebo at weeks 2 & 6 then every 8 weeks through to week 54 Group 2–IFX 5 mg/kg at weeks 2 & 6 then every 8 weeks through to week 54 Group 3–IFX 10 mg/kg at weeks 2 & 6 then every 8 weeks through to week 54	At week 2: For all participants receiving IFX 5 mg/Kg at week 0: 58% (335/573)	At week 30: (in those patients demonstrating clinical response at week 2) Group 1–21% (23/110) Group 2–39% (44/113) Group 3–45% (50/112)
ACCENT-II (Sands et al. [140]) 2004 Multicentre RCT	IFX	2 treatment groups–same induction regimen (IFX 5 mg/kg at week 0, 2, 6) followed by: Group 1–IFX 5 mg/kg every 8 weeks through to week 54 Group 2–Placebo every 8 weeks through to week 54	Median time to loss of response Group 1: >40 weeks Group 2: 14 weeks	At week 54–remission here refers to complete absence of draining fistulas Group 1–36% (50/138) Group 2–19% (27/144)
SONIC (Colombel et al. [297]) 2010 Multicentre RCT	IFX	3 treatment groups–all had IFX 5 mg/kg from weeks 8 through to week 50 In addition: Group 1: IFX 5 mg/kg at weeks 0, 2, 6 and azathioprine placebo daily Group 2: Placebo at weeks 0, 2, 6 and azathioprine 2.5 mg/kg daily Group 3: IFX 5 mg/kg at weeks 0, 2, 6 and azathioprine 2.5 mg/kg daily		*At week 26:* Group 1–44% (75/169) Group 2–30% (51/170) Group 3–57% (96/169) Mucosal healing in patients with ulcerations at baseline: Group 1 30% (28/93) Group 2 17% (18/109) Group 3 43.9% (47/107)
CLASSIC-I (Hanauer et al. [298]) Multicentre RCT 2006	ADA	4 treatment groups–initial dose (of ADA for groups 1–3 and placebo for group 4) at week 0, second dose at week 2, i.e., Group 1: ADA 160 mg/80 mg Group 2: ADA 80 mg/40 mg Group 3: ADA 40 mg/20 mg Group 4: Placebo/Placebo		At week 4: Group 1: 36% (27/76) Group 2: 24% (18/75) Group 3: 18% (13/74) Placebo: 12% (9/74)
CLASSIC-II (Sandborn et al. [299]) Multicentre RCT 2007	ADA	4 treatment groups– (1–3 were in remission at week 0, i.e., week 4 of CLASSIC-I, group 4 were not in clinical remission following treatment in CLASSIC-I) Group 1: ADA 40 mg fortnightly from 0 though to week 56 Group 2: ADA 40 mg weekly from 0 though to week 56 Group 3: Placebo through to week 56		At week 56: Group 1: 79% (15/19) Group 2: 83% (15/18) Group 3: 44% (8/18) Group 4: 46% (93/204)

		Group 4: ADA 40 mg fortnightly through to week 56 (with allowance for decreased interval, i.e., weekly if continued non-response/flare)		
CHARM (Colombel et al. [151]) 2007	ADA	3 treatment groups–all groups had ADA 80 mg at week 0, 40 mg at week 2 then: Group 1: 40 mg fortnightly through to week 56 Group 2: 40 mg weekly though to week 56 Group 3: Placebo through to week 56		At week 26: Group 1–40% (69/172) Group 2–47% (74/157) Placebo–17% (29/170) At week 56: Group 1 36% (62/172) Group 2: 41% (64/157) Placebo: 12% (20/170)
GAIN Sandborn et al. [153]] Multicentre RCT 2007	ADA	2 treatment groups Group 1: 160 mg at week 0, 80 mg at week 2 Group 2: Placebo at weeks 0,2	At week 4: Group 1: 52% (82/159) Group 2: 34% (56/166)	At week 4: Group 1: 21% (34/159) Group 2: 7% (12/166)
Schreiber et al. [300]] Multicentre RCT 2005	CZP	3 treatment groups–all with treatment at weeks 0, 4, and 8 weeks. However different drug dosing: Group 1: 400 mg CZP Group 2: 200 mg CZP Group 3: 100 mg CZP Group 4: Placebo	At week 12: Group 1: 44% (32/72) Group 2: 36.1% (26/72) Group 3: 36.4% (27/74) Placebo: 35.6% (26/73)	At week 12: Group 1: 26% (19/72) Group 2: 19% (14/72) Group 3: 27% (20/74) Placebo: 23% (17/73)
PRECISE I (Sandborn et al. [301]) Multicentre RCT 2007	CZP	2 treatment groups Group 1: 400 mg at week 0, 2, 4 then every 4 weeks through to week 26 Group 2: Placebo at week 0, 2, 4 then every 4 weeks through to week 26	At week 6: Group 1: 35% (115/327) Group 2: 27% (87/325) *At week 6 AND 26:* Group 1: 23% (75/325) Group 2: 16% (52/325)	At week 6: Group 1: 22% (71/329) Group 2: 17% (57/326) *At week 6 AND 26:* Group 1: 14% (47/327) Group 2: 10% (32/326)
Sandborn et al. [302] Multicentre RCT 2011	CZP	2 treatment groups: Group 1: 400 mg at 0, 2, 4 weeks Group 2: Placebo at 0, 2, 4 weeks		*At week 6:* Group 1: 32% (68/215) Group 2: 25% (53/209)
PRECISE-II (Schreiber et al. [303]) 2007 RCT	CZP	2 treatment groups: all received 400 mg at 0, 2, 4 weeks then following assessment of week 6 response: Group 1: 400 mgs at week 8, 12, 16, 20, 24 Group 2: Placebo at week 8, 12, 16, 20, 24	Maintenance of response at week 26: Group 1: 63% (135/215) Group 2: 36% (76/210)	At week 26 (i.e., remission data in those who demonstrated response at week 6): Group 1: 48% (103/215) Group 2: 29% (61/210)

3.7.2 Adalimumab

Adalimumab, a fully human antibody that also fixes complement and lyses cells expressing TNFα, is administered subcutaneously (via auto-injector pen) every two weeks. Crucial studies that demonstrated evidence for Adalimumab use in CD included the CLASSIC-I trial (Clinical Assessment of adalimumab Safety and efficacy Studied as Induction therapy in Crohn's disease) [298] (Table 2). CLASSIC-I demonstrated efficacy in induction of clinical response and remission for moderate to severe CD and a subsequent phase 3 study, CLASSIC II demonstrated maintenance of remission [299] in those with moderate to severe CD. The CHARM trial [151] (Crohn's trial of the fully Human Antibody Adalimumab for Remission Maintenance) also demonstrated safety and efficacy of adalimumab for maintenance of clinical remission following successful induction therapy and in the healing of draining perianal fistulas [152]. The GAIN (Gauging Adalimumab efficacy in Infliximab Nonresponders) trial assessed adalimumab efficacy in the context of loss of response or intolerance (secondary failure) to infliximab therapy [153]. The authors reported a significantly higher induction of clinical remission at week 4 in the adalimumab-treated group vs. the placebo-treated group. A meta-analysis including the CLASSIC-I, GAIN and CHARM studies, representing over 700 included participants with moderate to severe CD revealed a lower likelihood of failure to induce remission on adalimumab vs. placebo at weeks four and twelve [Relative risk (RR) 0.85, 95% confidence interval (CI) 0.79–0.91][139,304]. In these studies, the measure of efficacy used was the CDAI (Crohn's disease activity index) scores, with a decrease of 100 points signifying response and a decrease of 150 points signifying remission. The nature of the CDAI scoring system has been described as incorporating subjective symptoms, however, and a subsequent study went further in assessing efficacy using a more objective outcome measure; defining disease burden in terms of presence or absence of mucosal healing [305]. the Extend trial

found patients with moderate to severely active CD who continued to receive adalimumab were more likely to achieve mucosal healing. Adalimumab, a fully human antibody that also fixes complement and lyses cells expressing TNF-α, is administered subcutaneously (via auto-injector pen) every two weeks. Crucial studies that demonstrated evidence for Adalimumab use in CD included the CLASSIC-I trial (Clinical Assessment of adalimumab Safety and efficacy Studied as Induction therapy in Crohn's disease) [298] (Table 2). CLASSIC-I demonstrated efficacy in induction of clinical response and remission for moderate to severe CD and a subsequent phase 3 study, i.e., CLASSIC II demonstrated maintenance of remission [299] in those with moderate to severe CD. The CHARM trial [151] (Crohn's trial of the fully Human Antibody Adalimumab for Remission Maintenance) also demonstrated safety and efficacy of adalimumab for maintenance of clinical remission following successful induction therapy and in the healing of draining perianal fistulas [152]. The GAIN (Gauging Adalimumab efficacy in Infliximab Nonresponders) trial assessed adalimumab efficacy in the context of loss of response or intolerance (secondary failure) to infliximab therapy [153]. The authors reported a significantly higher induction of clinical remission at week 4 in the adalimumab-treated group vs. the placebo-treated group. A meta-analysis including the CLASSIC-I, GAIN and CHARM studies, representing over 700 included participants with moderate to severe CD revealed a lower likelihood of failure to induce remission on adalimumab vs. placebo at weeks four and twelve [Relative risk (RR) 0.85, 95% confidence interval (CI) 0.79–0.91][139,304]. In these studies, the measure of efficacy used was the CDAI (Crohn's disease activity index) scores, with a decrease of 100 points signifying response and a decrease of 150 points signifying remission. The nature of the CDAI scoring system has been described as incorporating subjective symptoms, however, and a subsequent study went further in assessing efficacy using a more objective outcome measure; defining disease burden in terms of presence or absence of

mucosal healing [305], the Extend trial found patients with moderate to severely active CD who continued to receive adalimumab were more likely to achieve mucosal healing.

3.7.3 Certolizumab

Certolizumab pegol is a chimeric humanized antibody fragment against soluble (secreted) TNF and transmembrane TNF, which is attached to polyethylene glycol, PEG ("pegylated"). It differs from IFX and ADA in that it does not contain the crystallisable fragment (Fc) region of typical antibody. It also differs in function in that it does not induce apoptosis as one of its mechanisms of action, nor does it activate the complement pathway, or result in cell or antibody mediated cytotoxicity [306,307]. It is however thought to have a higher binding affinity for TNF than adalimumab or infliximab [307]. Certolizumab is administered subcutaneously and has a longer half-life (due to the PEG addition), with maintenance dosing every four weeks (as opposed to adalimumab's two weeks) (Table 1). The PRECISE (Pegylated antibody fragment evaluation in Crohn's disease safety and efficacy) 1 and 2 [301,303] studies evaluated induction and maintenance of remission with certolizumab in patients with moderate to severe CD (Table 4). The PRECiSE 1 (Pegylated antibody fRagment Evaluation in Crohn's disease: Safety and Efficacy) study [301] did not find any significant difference in remission (at six weeks) between certolizumab and placebo groups; however, response rates were significantly improved with certolizumab vs. placebo (35% vs. 27% $p = 0.02$). The PRECiSE 2 trial [303] reported a significantly higher response rate (62% vs. 34%, $p < 0.001$) and remission rate (48% vs. 29%, $p < 0.001$) with maintenance certolizumab compared to placebo following positive response to induction therapy at 26 weeks. Certolizumab has also been evaluated using health related quality of life (QoL) as an outcome measure, by assessing patients' response to treatment using the Inflammatory Bowel Disease Questionnaire (IBDQ) [308]. Rutgeerts et al. [308] reported a significantly improved QoL in patients with certolizumab at all time points assessed compared

with placebo. The PRECiSE 3 trial assessed long-term outcome in patients successfully maintained on certolizumab at 26 weeks and reported remission rates of 63% at 80 weeks [309]. This was not statistically significantly different from those in whom the drug was stopped at 26 weeks (placebo). A meta-analysis [139] of four trials assessing certolizumab in over 800 patients found no statistically significant difference between certolizumab and placebo in inducing remission of active luminal CD (RR = 0.95; 95% CI 0.90–1.01). Maintenance therapy with certolizumab has demonstrated efficacy in perianal fistula closure. In a subgroup analysis of the PRECiSE 2 trial, 58 patients with draining fistulas who responded to induction with certolizumab were randomized to certolizumab or placebo every four weeks, with rates of clinical remission (100% closure of fistulas at baseline) at week 26 significantly higher in patients treated with certolizumab as compared to placebo (36 versus 17 percent $p = 0.038$)). The PRECiSE 1 (Pegylated antibody fRagment Evaluation in Crohn's disease: Safety and Efficacy) study [301] did not find any significant difference in remission (at six weeks) between certolizumab and placebo groups; however, response rates were significantly improved with certolizumab vs. placebo (35% vs. 27% $p = 0.02$). The PRECiSE 2 trial [303] reported a significantly higher response rate (62% vs. 34%, $p < 0.001$) and remission rate (48% vs. 29%, $p < 0.001$) with maintenance of certolizumab following positive response to induction therapy at 26 weeks, compared to placebo. Certolizumab has also been evaluated using health related quality of life (QoL) as an outcome measure, by assessing patients' response to treatment using the Inflammatory Bowel Disease Questionnaire (IBDQ) [308]. Rutgeerts et al. [308] reported a significantly improved QoL in patients with certolizumab at all time points assessed compared with placebo. The PRECiSE 3 trial assessed long-term outcome in patients successfully maintained on certolizumab at 26 weeks and reported remission rates of 63% at 80 weeks [309]. This was not statistically significantly different from those in whom the drug was stopped at 26 weeks (placebo). A meta-analysis [139] of four trials assessing certolizumab in over 800

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Table 4: Demonstrates the three available anti-TNF therapies in Crohn's disease (CD).

Anti-TNF	Dosing for Induction and Maintenance	Route	Properties	Indications
Infliximab (Remicade)	**Induction** 5 mg at weeks 0, 2 and 6 **Maintenance** 5 mg (or 10 mg/kg) every 8 weeks	IV	Chimeric monoclonal antibody	Induction and maintenance of remission
Adalimumab (Humira)	**Induction** 160 mg (or 80 mg) week 0, 80 mg (or 40 mg) week 2 **Maintenance** 40 mg every other week or weekly	SC	Humanized monoclonal antibody	Induction and maintenance of remission
Certolizumab (Cimzio)	**Induction** 400 mg at weeks 0, 2 and 4 **Maintenance** 400 mg every 4 weeks	SC	PEG-conjugated Fab fragment of recombinant humanised monoclonal antibody	Induction and maintenance of remission

IV—intravenous; SC—subcutaneous, TNF – tumour necrosis factor, PEG – polyethylene glycol, Fab – humanized antibody fragment

There are no trials comparing all three anti-TNF therapies, however there are suggestions that there are no significant differences in efficacy between infliximab and adalimumab [311]. They are both also thought to be superior to certolizumab in inducing remission [312–314]. In a pooled analysis of ten trials including over 2700 patients with Crohn's disease, patients who were treated with any of the three different anti-TNF agents discussed above were less likely to fail to achieve remission compared with placebo (RR 0.87, 95% 0.80–0.94) [139]. There are no trials comparing all three anti-TNF therapies, however there are suggestions that there are no significant differences in efficacy between infliximab and adalimumab [311]. They are both also thought to be superior to certolizumab in inducing remission [312–314]. In a pooled analysis of ten trials including over 2700 patients with Crohn's disease, patients who were treated with any of the three different anti-TNF agents discussed above were less likely to fail to achieve remission compared with placebo (RR 0.87, 95% 0.80–0.94) [139].

The efficacy of anti-TNF medication has led to the introduction of biosimilars, as the patents of older anti-TNF agents have either expired or are close to expiration. Biosimilars are synthesized versions of existing biological drugs with no perceived difference in safety or

efficacy. Several have been approved for treatment of CD in USA and Europe (with 19 products authorised at the end of 2015 [315]) and they are expected to gain a substantial portion of the market of biological therapy in the future [316]. They have the advantage of lower cost, reducing health-care spending and making them more accessible to a larger number of patients [315]. Biosimilar development requires selection of an appropriate reference biologic agent, understanding of the key molecular attributes of the reference product, development of a manufacturing process to match these attributes, and finally preclinical and clinical evaluation. This includes pharmacokinetic/pharmacodynamic studies, randomized controlled trials, etc. [317]

Preliminary data from real-life cohort studies across different countries may support the bioequivalence of infliximab biosimilars in IBD, as well as in rheumatology and dermatology [315,318]. Gecse et al. found a significant decrease in the clinical activity index and C-reactive protein (CRP) in the whole study population (inclusive of CD and UC), and only four allergic reactions in subjects previously exposed to infliximab. Similar preliminary results come from the large prospective study in Norway (the NOR-SWITCH study) [319]. It has reported that switching to a biosimilar infliximab was noninferior to continued treatment with the reference product in terms of efficacy, safety, and immunogenicity. However, the trial wasn't powered to demonstrate the noninferiority of the biosimilar in individual disease states, including CD and UC. The preliminary data suggest that little difference is anticipated from the use of biosimilars of infliximab compared to the originator. There are, however, contrasting data from an Irish cohort [315,318] that compared two groups of patients treated with infliximab originator or biosimilars, showing an increased surgery rate, less steroid-free remission, and less normalization of inflammatory markers (CRP).

Further appropriately designed observational studies and efficient pharmacovigilance programmes that improve biosimilars' safety profile are warranted [320] to address implications of these new drugs and whether existing techniques of drug monitoring, efficacy and safety application are relevant to these drugs. Furthermore, head-to-head trials to assess best treatment pathways [71], as well as close cooperation with regulatory authorities, scientific societies and the pharmaceutical industry would serve to improve knowledge and clinical practice guidelines to standardize the use of biosimilars [317,321].The efficacy of anti-TNF medication has led to the introduction of biosimilars, as the patents of older anti-TNF agents have either expired or are close to expiration. Biosimilars are synthesized versions of existing biological drugs with no perceived difference in safety or efficacy. Several have been approved for treatment of CD in USA and Europe (with 19 products authorised at the end of 2015 [315]) and they are expected to gain a substantial portion of the market of biological therapy in the future [316]. They have the advantage of lower cost, reducing health-care spending and making them more accessible to a larger number of patients [315]. Biosimilar development requires selection of an appropriate reference biologic agent, understanding of the key molecular attributes of the reference product, development of a manufacturing process to match these attributes, and finally preclinical and clinical evaluation. This includes pharmacokinetic/pharmacodynamic studies, randomized controlled trials, etc. [317]. Preliminary data from real-life cohort studies across different countries seem to support the bioequivalence of infliximab biosimilars in IBD, as well as in rheumatology and dermatology [315,318]. Gecse et al.[322] found a significant decrease in the clinical activity index and C-reactive protein (CRP) in the whole study population (inclusive of CD and UC), however, more allergic reactions were exhibited in those patients previously exposed to infliximab. Similar preliminary results come from the large prospective study in Norway (the NOR-SWITCH study) [319]. It has reported that switching to a biosimilar infliximab was noninferior to continued treatment with the reference product in terms of efficacy, safety,

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3.8 Loss of Response to Anti-TNF Therapy

Despite the paradigm shift seen over the last two decades with anti-TNF treatment in Crohn's disease, the response that an individual patient will have to a specific anti-TNF and dose is difficult to predict when compared with conventional (non-biologic) therapies. Up to 30% of patients do not respond to anti-TNF therapy (primary non-responders) and almost half of the patients who experience a benefit with these drugs will lose clinical benefits within the first year, requiring dose escalation or therapy change [153,323,324], termed "secondary loss of response". Several studies have demonstrated benefits in empiric switching to a different anti-TNF agent following loss of response. However, short-term remission rates were modest [320,325,326].Despite the paradigm shift seen over the last two decades with anti-TNF treatment in Crohn's disease, the response that an individual patient will have to a specific anti-TNF and

dose is difficult to predict when compared with conventional (non-biologic) therapies. Up to 30% of patients do not respond to anti-TNF therapy (primary non-responders) and almost half of the patients who experience a benefit with these drugs will lose clinical benefits within the first year, requiring dose escalation or therapy change [153,323,324], termed "secondary loss of response". Several studies have demonstrated benefits in empiric switching to a different anti-TNF agent following loss of response; however, short-term remission rates were modest [320,325,326].

The reasons why some patients do not respond or lose response after a successful course of therapy are not completely clear, but they are likely multifactorial and related to metabolism of the drug or to the development of antidrug antibodies [323]. This process is termed immunogenicity and results from exposure to anti-TNF, inducing cellular clonal expansion of lymphocytes that secrete specific antibodies that form immune complexes with anti-TNF agents. This often results in increased drug clearance via the reticuloendothelial system. Pre-treatment anti-drug antibodies have been reported in treatment-naïve patients, and levels were found to be higher in those who lost (primary and secondary) response when compared to responders [327]. Moreover, patients with secondary loss of response have been found to have higher anti-drug antibodies levels than those with primary loss of response. This process is not permanent, as anti-drug antibodies have been found to disappear within a year of discontinuing the anti-TNF treatment, and furthermore a subset of patients may spontaneously lose pre-existing anti-drug antibodies whilst on continuous anti-TNF treatment [327,328]. The latter makes the case for dose escalation following loss of response as well as consideration of a switch in anti-TNF agent following loss of response [324]. A systematic review of CD patients switching from infliximab to a second anti-TNF-α agent (i.e., adalimumab/certolizumab) revealed a clinical response rate of 63% and remission rate of 43% [329]. Concomitant therapy with

immunomodulators (i.e., azathioprine or methotrexate) reduces the risk of development of anti-drug antibodies [297,330]. The SONIC group (Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease) reported on a randomised controlled trial with azathioprine monotherapy, infliximab monotherapy or combination therapy in azathioprine and infliximab naive CD patients[297]. Formation of anti-drug antibodies was significantly less with scheduled infliximab combination therapy (0.9%) compared with infliximab monotherapy (14.6%). Combination therapy is also associated with higher infliximab serum levels and although the mechanism for this is unclear, infliximab serum levels at week 4 after the first infusion have been shown to be predictive of anti-drug antibody formation [331]. Measurement of serum anti-TNF trough levels and anti-drug antibodies (see therapeutic drug monitoring) are thus helpful in guiding treatment. Other potential therapeutic strategies to reduce the risk of anti-drug antibodies include the use of scheduled administrations of anti-TNF agents instead of episodic/'on-demand' treatment, which is associated with a higher rate of antibody formation, infusion reactions [332] and the risk of undergoing abdominal surgery [331,333].The reasons why some patients do not respond or lose response after a successful course of therapy are not completely clear, but they are likely multifactorial and related to metabolism of the drug or to the development of antidrug antibodies [323]. This process is termed immunogenicity and results from exposure to anti-TNF, inducing cellular clonal expansion of lymphocytes that secrete specific antibodies that form immune complexes with anti-TNF agents. This often results in increased drug clearance via the reticuloendothelial system. Pre-treatment anti-drug antibodies have been reported in treatment-naïve patients, and levels were found to be higher in those who lost (primary and secondary) response when compared to responders [327]. Moreover, patients with secondary loss of response have been found to have higher anti-drug antibodies levels than those with primary loss of response. This process is not permanent, as anti-drug antibodies have been found to disappear within a year of discontinuing the anti-TNF treatment, and

furthermore a subset of patients may spontaneously lose pre-existing anti-drug antibodies whilst on continuous anti-TNF treatment [327,328]. The latter makes the case for dose escalation following loss of response as well as consideration of a switch in anti-TNF agent following loss of response [324]. A systematic review of CD patients switching from infliximab to a second anti-TNF-α agent (i.e., adalimumab/certolizumab) revealed a clinical response rate of 63% and remission rate of 43% [329]. Concomitant therapy with immunomodulators (i.e., azathioprine or methotrexate) reduces the risk of development of anti-drug antibodies [297,330]. The SONIC group (Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease) reported on a randomised controlled trial with azathioprine monotherapy, infliximab monotherapy or combination therapy in azathioprine and infliximab naive CD patients[297]. Formation of anti-drug antibodies was significantly less with scheduled infliximab combination therapy (0.9%) compared with infliximab monotherapy (14.6%). Combination therapy is also associated with higher infliximab serum levels and although the mechanism for this is unclear, infliximab serum levels at week 4 after the first infusion have been shown to be predictive of anti-drug antibody formation [331]. Measurement of serum anti-TNF trough levels and anti-drug antibodies (see therapeutic drug monitoring) are thus helpful in guiding treatment. Other potential therapeutic strategies to reduce the risk of anti-drug antibodies include the use of scheduled administrations of anti-TNF agents instead of episodic/'on-demand' treatment, which is associated with a higher rate of antibody formation, infusion reactions [332] and the risk of undergoing abdominal surgery) [331,333].

Other factors that may result in loss of response are potential differences in pharmacokinetic properties, including tissue penetration and mode of action of the different anti-TNF drugs. The need to develop biomarkers that can predict response to therapies will become increasingly important for personalised medicine decisions in the future. Once loss of response is confirmed,

dose escalation/interval shortening and switching anti-TNF agents are recommended potential strategies [259].

3.9 Pharmacokinetics and Anti-TNF Monitoring in CD

The route of administration of anti-TNF influences its pharmcokinetics. Infliximab is administered intravenously, which allows for the usage of large volumes, rapid distribution, with peak serum concentrations shortly after infusion and low variability in bioavailability [334]. Adalimumab and certolizumab on the other hand, are subcutaneously injected, which improves the practicality and convenience of administration, but leads to higher variability in bioavailability (due to low injectable volumes), necessitating more frequent administrations. The subcutaneous route has been reported to be more likely to elicit an immunogenic response in part due to activation of dendritic cells present in skin [283].The route of administration of anti-TNF influences its pharmacokinetics. Infliximab is administered intravenously, which allows for the usage of large volumes, rapid distribution, with peak serum concentrations shortly after infusion and low variability in bioavailability [334]. Adalimumab and certolizumab on the other hand, are subcutaneously injected, which improves the practicality and convenience in administration, but leads to higher variability in bioavailability (due to low injectable volumes), necessitating more frequent administrations. The subcutaneous route has been reported to be more likely to illicit an immunogenic response in part due to activation of dendritic cells present in skin [283].

Antibody distribution is mainly within the extracellular fluid and occurs via cellular movement from circulation to tissue, diffusion or receptor mediated endocytosis [314,335]. For subcutaneously injected drugs, distribution can be slower, as it occurs through the lymphatic drainage and paracellular movement leading to assimilation into the vasculature [334]. Metabolism and clearance of the antibodies are not fully understood, but are thought to involve

various processes including degradation in lysosomes following immune complex (anti-TNF/trans-membrane TNF) formation and catabolism by phagocytic cells. The latter occurs via the reticuloendothelial system via proteolytic catabolism, which occurs at sites that are in rapid equilibrium with plasma [323]. Immunogenicity/development of anti-drug antibodies increases clearance rates and can contribute to a loss of response to the anti-TNF agent (see above). This varies between individuals and risk is increased with episodic or on demand anti-TNF treatment (see above—section 3.7.4). Furthermore, clearance rates can be affected by concomitant medication and disease state, with inflammatory tissue found to have significantly higher levels of TNF and anti-TNF, compared to matched uninflamed samples [334,336]. The serum concentrations of infliximab, adalimumab and certolizumab all have a linear relationship with administered dose and half-life is between 8–10 days for infliximab, 10–20 days for adalimumab, and 14 days for certolizumab. Serum drug concentration monitoring is used to ensure therapeutic dosing and avoid toxic concentrations (see below).Antibody distribution is mainly within the extracellular fluid and occurs via cellular movement from circulation to tissue, diffusion or receptor mediated endocytosis [314,335]. For subcutaneously injected drugs, distribution can be slower, as it occurs through the lymphatic drainage and paracellular movement leading to assimilation into the vasculature [334]. Metabolism and clearance of the antibodies are not fully understood, but is thought to involve various processes including degradation in lysosomes following immune complex (anti-TNF/trans-membrane TNF) formation and catabolism by phagocytic cells. The latter occurs via the reticuloendothelial system via proteolytic catabolism, which occurs at sites that are in rapid equilibrium with plasma [323]. Immunogenicity/development of anti-drug antibodies increases clearance rates and can contribute to a loss of response to the anti-TNF (see above). This varies between individuals and risk is increased with episodic or on demand anti-TNF treatment (see above—the Loss of response section). Furthermore, clearance rates can be affected by concomitant

medication and disease state, with inflammatory tissue found to have significantly higher levels of TNF and anti-TNF, compared to matched uninflamed samples [334,336]. The serum concentrations of infliximab, adalimumab and certolizumab all have a linear relationship with administered dose and half-life is between 8–10 days for infliximab, 10–20 days for adalimumab, and 14 days for certolizumab. Serum drug concentration monitoring is used to ensure therapeutic dosing and avoid toxic concentrations (see below).

3.9.1 Therapeutic Drug Monitoring

The principles of anti-TNF dosing regimens are to achieve steady-state range of serum or tissue drug concentrations that are adequate for the drug to neutralize surplus TNF, but not so high that it affects safety because of neutralization of homeostatic concentrations of TNF required for host defence. Conversely, tissue drug concentrations should not be so low as to impair efficacy as a result of suboptimal neutralization of TNF [262]. Within an individual patient, a linear relationship exists between anti-TNF dose and serum medication levels [337]. However, variability in the pharmacokinetics between patients makes it difficult to predict medication levels between patients. Measuring drug levels and determination of the presence of antidrug antibodies has the potential to identify those who will benefit from dose escalation and those who will be best served by switching to an alternate drug within or outside the class, for example in the event of loss of response. Therapeutic drug monitoring thus has the potential to help physicians to improve and personalize the management of Crohn's disease [257]. This could be done via serum level guiding dose escalation, or initiation of immunomodulators to maximise drug efficiency. This has been supported by studies assessing dosing based on symptoms/disease markers, versus dosing based on trough levels [337]. Patients with sub therapeutic trough levels were found to have significantly higher markers of inflammation (i.e., C-reactive protein) than those with optimal trough levels [337]. Analysis of patients from the

ACCENT 1 study also demonstrated that those who failed to respond to therapy had lower serum infliximab concentration than those with a sustained response [295]. Early therapeutic drug monitoring (TDM) could be used to prevent loss of response rather than to reverse it (although further research is required to corroborate this [320]), as well as to optimize treatment with anti-TNF antibodies based on actual exposure to the drug rather than according to a standard dosing regimen. The principles of anti-TNF dosing regimen are to achieve steady-state range of serum or tissue drug concentrations that are adequate for the drug to neutralize surplus TNF, but not so high that it affects safety because of neutralization of homeostatic concentrations of TNF required for host defence. Conversely, tissue drug concentrations should not be so low as to impair efficacy as a result of suboptimal neutralization of TNF [262]. Within an individual patient, a linear relationship exists between anti-TNF dose and serum medication levels [337]. However, variability in the pharmacokinetics between patients makes it difficult to predict medication levels between patients. Measuring drug levels and determination of the presence of antidrug antibodies has the potential of identifying those who will benefit from dose escalation and those who will be best served by switching to an alternate drug within or outside the class, e.g., in the event of loss of response. Therapeutic drug monitoring thus has the potential to help physicians to improve and personalize the management of Crohn's disease [257]. This could be done via serum level guiding dose escalation, or initiation of immunomodulators to maximise drug efficiency. This has been supported by studies assessing dosing based on symptoms/disease markers, versus dosing based on trough levels [337]. Patients with sub therapeutic trough levels were found to have significantly higher markers of inflammation (i.e., C-reactive protein) than those with optimal trough levels [337]. Analysis of patients from the ACCENT 1 study also demonstrated that those who failed to respond to therapy had lower serum infliximab concentration than those with a sustained response [295]. Early therapeutic drug monitoring (TDM) could be used to prevent loss of response rather than to reverse it (although

further research is required to corroborate this [320]), as well as to optimize treatment with anti-TNF antibodies based on actual exposure to the drug rather than according to a standard dosing regimen.

The use of TDM to optimise anti-TNF drug concentrations has a promising potential for dose optimisation in clinical practice, in view of the reported correlations between anti-TNF trough concentrations, anti-drug antibodies, and disease outcomes. However, studies in TDM are yet to demonstrate unequivocal benefit [338,339]. Two controlled trials, which investigated the clinical use of TDM based on drug concentration or symptoms, showed that trough-level-based dose intensification was not superior to dose intensification based on symptoms alone The use of TDM to optimise anti-TNF drug concentrations has a promising potential for dose optimisation in clinical practice, in view of the reported correlations between anti-TNF trough concentrations, anti-drug antibodies, and disease outcomes; however, studies in TDM are yet to demonstrate unequivocal benefit [338,339]. Two controlled trials, which investigated the clinical use of TDM based on drug concentration or symptoms, showed that trough-level-based dose intensification was not superior to dose intensification based on symptoms alone [71]. Concentration-based dosing was associated with fewer disease flares [338] but did not increase clinical, endoscopic or steroid-free remission in patients with active luminal CD [339]. The recent American Gastroenterological Association (AGA) guidelines in TDM [340] provide an overview and commentary on this evolving field and sets a framework for clinical management, albeit highlighting important gaps in current knowledge in this aspect of the care of individuals with IBD [341]. AGA guidelines specify target levels of anti-TNF drugs that are associated with clinical outcomes and also details issues related to the assessment of anti-drug antibody levels, for example, the inconsistency in results obtained with available assays. This raises the suggestion that anti-drug antibodies interpretation may need to be more regional and patient specific [341]. Based on the currently available evidence (often scanty), the suggested target

trough concentrations are >5 mg/mL for infliximab, >7.5 mg/mL for adalimumab, and >20 mg/mL for certolizumab pegol. These are proposed as guides to decide whether escalation of therapy may be beneficial (if trough is below this threshold) compared with switching therapy (to be considered if trough is above this threshold) to achieve clinical response in patients who are experiencing secondary loss of response on maintenance therapy [342]. For asymptomatic patients with ongoing endoscopic activity or with perianal disease [343] who undergo reactive TDM, target trough concentrations may be higher, such that escalating index therapy may be a preferable option before switching therapies in these settings. It is also important to note that these guidelines are for IBD and are not uniform trough levels that need to be targeted for all patients regardless of clinical status or disease phenotype [340]. Further studies are required to determine exactly when trough levels should be measured, whether or not in combination with anti-drug antibodies, what the optimal trough level concentrations should be and if the dose should be adapted to this target ("treat-to-trough" approach) as well as whether or not these strategies improve quality of life, and cost-effectiveness [331]. Furthermore, the role of combination therapy (an immunomodulator in combination with the biologic agent) and the effect on TDM need to be elucidated.

3.10 Withdrawal of Anti-TNF Therapy

Clinicians and patients are often faced with the question of whether it is possible to stop anti-TNF therapy once disease remission has been achieved. However, despite all the studies that have now addressed this issue in IBD, no conclusive strategy has yet emerged [344]. Data are[344]. Data is inconclusive due to varying reported rates of relapse on stopping anti-TNF, and conclusions are difficult to draw from the studies due to disease phenotype heterogeneity, with variable definitions of clinical remission and variable duration of remission before drug

withdrawal. It is not known the ideal duration of remission prior to stopping as well as in which phenotypes this is most likely to be successful. There was also a lack of control groups.

It is often empirically proposed not to routinely stop anti-TNF-α agents in IBD patients who respond, and especially in patients with disabling features of disease and/or at high-risk for relapse [345]. The STORI trial (Infliximab diSconTinuation in Crohn's disease patients in stable Remission on combined therapy with Immunosuppressors) was the pivotal study boosting clinical research in this topic, being thereafter followed by many studies. This was the first prospective multicentre study specifically designed to assess the risk of relapse, and to identify predictors of relapse following anti-TNF maintenance therapy withdrawal [148]. Among the 115 CD patients with luminal disease that were enrolled (perianal CD was excluded), there was a 43.9% (±5.0%) rate of relapse over one year and a 52.2% (±5.2%) rate of relapse over two years after stopping IFX. Relapse occurred after a median of 16.4 months. Other retrospective and prospective cohorts have also sought to address the issue of treatment withdrawal [319]. In most of the studies on withdrawal, patients had the anti-TNF discontinued while they were in clinical remission (with variable definitions of clinical remission and variable duration of remission before drug withdrawal). Relapse rates among those studies range from 21 to 56% at 12 months and from 47 to 64% at 24 month [148,346–350]. Further studies are still required in order to answer the question on whether maintaining the anti-TNF as opposed to reducing/discontinuing the drug is superior to maintain remission, as well as to define routine strategy in the future for long-term management of CD patients and to define the optimal withdrawal strategy [344,345]. Studies assessing withdrawal in circumstances such as early disease or in the context of monotherapy (as opposed to in combination with immunomodulator agent), and a large European trial (Biocycle Project) seek to answer some of these questions [344].It is often empirically proposed not to routinely stop anti-TNF-α agents in IBD patients who respond, and especially in patients with disabling features of disease and/or at high-risk for

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3.11 Adverse Effects of Anti-TNF Therapy in CD

Long-term therapeutic use of anti-TNF carries with it safety issues which include potential for development of skin lesions, immune reactions, peri-operative complications, infections, cancers and decreased fertility/adverse effects on pregnancy [320,351].

Infusion reactions relate to infliximab and can be categorized based on their timing, pathogenesis, and severity [352]. They are common within 1–2 h after an infusion and occur in about 20% of patients [275]. Symptoms include itching, flushing, breathlessness, chest pains, hypertension and headaches. Delayed reactions occur within 1–14 days following infusion and are rarer, occurring in about 2% of patients, usually in the form of headache, fever, fatigue, rash, myalgia and arthralgia [353,354]. Reactions have been associated with the presence of anti-drug antibodies [355,356], whereas concomitant immunomodulator use, and regular maintenance dosing have been shown to reduce the risk of infusion reactions by decreasing the incidence of anti-drug antibody formation [287,352]. Injection site reactions have also been reported [298] to occur following subcutaneous administration of anti-TNF therapy, (i.e., adalimumab/certolizumab) which can cause symptoms including burning sensation, pain, and pruritus.

Susceptibility to infection is a significant concern following instigation of treatment with anti-TNF. Anti-TNF treated patients are rendered immunocompromised through their treatment and this is often in the context of combination treatment with other immunosuppressant medication (e.g. corticosteroids, thiopurines, etc.). Infective complications vary from serious invasive bacterial/opportunistic infections to relatively milder cases (mild respiratory/urinary infections) [357]. Analysis from the CD TREAT registry (Crohn's Therapy, Resource, Evaluation and Assessment Tool), which incorporates data on more than 3000 patients on anti-TNF therapy, revealed that infliximab-treated patients displayed a significantly increased risk of serious infections compared with the 'other treatments-only' group (hazard ratio = 1.45, p = 0.008). However, multivariate analysis, demonstrated no significant increased risk of infection (OR 0.99, 95% CI 0.64–1.53) due to anti-TNF therapy after controlling for factors such as disease duration, severity and concurrent corticosteroid and immunomodulator use [358]. In a report of adalimumab safety including six clinical trials, 1.8% of patients had opportunistic infections (most commonly oral candidiasis), and 5.8% had serious infections (most commonly

abscess, gastrointestinal, pulmonary and viral infection) [359,360]. Anti-TNF therapy has also been implicated in the susceptibility to tuberculous infection, due to the role of TNF in the formation of granulomas. It is thought that suppression of TNF-α prevents adequate sequestration of Mycobacterium tuberculosis [361], which in turn leads to an increased risk that anti-TNF therapy could cause reactivation of latent tuberculosis [287]. In view of the above, consensus guidelines advocated vaccination and safety screening, with screening for risk of opportunistic infections and ascertainment of immunisation status prior to starting anti-TNF treatment [357].Susceptibility to infection is a significant concern following instigation of treatment with anti-TNF. Anti-TNF treated patients are rendered immunocompromised through their treatment and this is often in the context of combination treatment with other immunosuppressant medication (e.g., corticosteroids, thiopurines, etc.). Infective complications vary from serious invasive bacterial/opportunistic infections to relatively milder cases (mild respiratory/urinary infections) [357]. Analysis from the CD TREAT registry (Crohn's Therapy, Resource, Evaluation and Assessment Tool), which incorporates data on more than 3000 patients on anti-TNF therapy, revealed that infliximab-treated patients displayed a significantly increased risk of serious infections compared with the 'other treatments-only' group (hazard ratio = 1.45, p = 0.008). However, multivariate analysis, demonstrated no significant increased risk of infection (OR 0.99, 95% CI 0.64–1.53) due to anti-TNF therapy after controlling for factors such as disease duration, severity and concurrent corticosteroid and immunomodulator use [358]. In a report of adalimumab safety including six clinical trials, 1.8% of patients had opportunistic infections (most commonly oral candidiasis), and 5.8% had serious infections (most commonly abscess, gastrointestinal, pulmonary and viral infection) [359,360]. Anti-TNF therapy has also been implicated in the susceptibility to tuberculous infection, due to the role of TNF in the formation of granulomas. It is thought that suppression TNF-α prevents adequate sequestration of Mycobacterium tuberculosis [361], which in turn leads to an increased risk that anti-TNF therapy

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Anti-TNF therapy has been theoretically linked with a propensity for malignancy, due to the suppression of TNF, and a meta-analysis in 2006 reported a 3-fold increased risk of malignancy for patients with rheumatoid arthritis undergoing anti-TNF (infliximab/adalimumab) treatment [362]. However, this rate of malignancy is yet to be confirmed in Crohn's disease treatment. An analysis of the FDA's adverse event reporting system (AERS) database from 1968–2005 found a relatively strong signal for lymphoma, both in IBD and non-IBD patients, at the disproportionality analysis [363]. The risk of T-cell non-Hodgkin lymphoma was found to be increased in combination therapy use (95% confidence interval (CI) 4.98–354.09; $p < 0.0001$) and thiopurines alone (95% CI 8.32–945.38; $p < 0.0001$) but not with anti-TNF use alone (95% CI 0.13–10.61; $p = 1.00$) [360,364]. There are also data suggesting an increased risk of melanoma in patients on anti-TNF treatment for IBD, with a 1.5−2× increased risk compared to those not exposed [365], however this is also impacted by an increased risk of melanoma associated with IBD independent of the use of biologic therapy [366,367]. However, in the TREAT registry, the global incidence of neoplasia (malignant, benign, and unspecified) was similar between patients receiving infliximab and the other-treatments group (0.78 vs. 0.85 per 100 patients-years; RR = 0.90, CI 0.69–1.18, $p = 0.46$), with no differences seen for solid tumours, non-melanoma skin cancers, and lymphoma between the two groups [358]. In a meta-analysis of 21 studies enrolling 5356 CD patients, Peyrin–Biroulet and colleagues reported that anti-TNF therapy did not significantly increase the risk of death, malignancy, or serious infection when compared with placebo [368]. This has been corroborated in several other studies [369–371]. The evidence is mixed and this is unsurprising, as the risk of malignancy associated with treatment can be particularly difficult to evaluate in trials due to the rarity of cancer as an outcome and

resultant paucity of data. Furthermore, the multifactorial aetiopathogenesis of malignancy, predisposition to cancer from the underlying disorder as well as unquantifiable risk from other medication can contribute to difficulty in identification of an appropriate control group. The 2016 European Crohn's and Colitis organisation (ECCO) consensus statement concluded that there were currently insufficient data to suggest that anti-TNF agents alone increase the risk of lymphoproliferative disorders or solid tumours, and acknowledged the increased risk when used in combination with thiopurines, concluding that absolute rates of these malignancies remain low and risks and benefits of treatment should be discussed with the patient [259].Anti-TNF therapy has been theoretically linked with a propensity for malignancy, due to the suppression of TNF, and a meta-analysis in 2006 reported a 3-fold increased risk of malignancy for patients with rheumatoid arthritis undergoing anti-TNF (infliximab/adalimumab) treatment [362]. However, this rate of malignancy is yet to be confirmed in Crohn's disease treatment. An analysis of the FDA's adverse event reporting system (AERS) database from 1968–2005 found a relatively strong signal for lymphoma, both in IBD and non-IBD patients, at the disproportionality analysis [363]. The risk of T-cell non-Hodgkin lymphoma was found to be increased in combination therapy use (95% confidence interval (CI) 4.98–354.09; $p < 0.0001$) and thiopurines alone (95% CI 8.32–945.38; $p < 0.0001$) but not with anti-TNF use alone (95% CI 0.13–10.61; $p = 1.00$) [360,364]. There are also data suggesting an increased risk of melanoma in patients on anti-TNF treatment for IBD, with a 1.5−2× increased risk compared to those not exposed [365], however this is also impacted by an increased risk of melanoma associated with IBD independent of the use of biologic therapy [366,367]. However, in the TREAT registry, the global incidence of neoplasia (malignant, benign, and unspecified) was similar between patients receiving infliximab and the other-treatments group (0.78 vs. 0.85 per 100 patients-years; RR = 0.90, CI 0.69–1.18, $p = 0.46$), with no differences seen for solid tumours, non-melanoma skin cancers, and lymphoma between the two groups [358]. In a meta-analysis of 21

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All three anti-TNF agents have been deemed safe in pregnancy, not least because of the increased risk of complications, preterm delivery and low birth weight conferred by active uncontrolled disease [331]. However, most of the available data are from animal and retrospective studies, with no teratogenic effects observed [360]. Infliximab and adalimumab cross the placenta in the beginning of the second trimester, whereas certolizumab does not as it lacks the Fc fragment required for active transport to the foetus [331]. Serum concentrations of infliximab may be detected up to six months after delivery, and may be predisposed to opportunistic infection and, as such, live vaccines are not recommended for children of patients on anti-TNF therapy in the first six months after birth [330,372].

3.12 Conclusions

In summary, the introduction of anti-TNF agents has led to major shifts in the therapeutic paradigm that evolved from slow symptomatic clinical remission towards rapid, sustained and deep remission; short- and long-term clinical, and endoscopic endpoints can now be reached that were previously unachievable [331,334]. Anti-TNF treatment currently represents a central treatment modality in Crohn's disease and can be improved by rapid dose escalation and the use of combination therapy [344]. However, two decades on from their introduction, questions still remain about their use including timing, dosing, monitoring and issues surrounding loss of response such as risk factors, biomarkers, mechanism and strategies for prevention and treatment [320]. These will undoubtedly form the basis of research in the future.

Chapter 4. Long-term outcomes in Crohn's perianal fistula on biologic treatment – 11 year real world experience from a single tertiary centre.

4.1 Abstract

Introduction

A recent published expert global consensus group on perianal Crohn's disease recommended biological therapy as the current gold standard, with antibiotics and other immunosuppressants offering a role as adjunctive treatments[119]. Despite this, only a third of patients with Crohn's perianal fistulas remain in remission at 1 year on maintenance treatment[122]. There are limited real world data on long -term outcomes of Crohn's perianal fistula patients on biologic therapy. The aim of this study was to review long term outcomes of patients treated with biological therapy for perianal Crohn's fistula at our institution.

Methods:

A local database of Crohn's patients treated with biologic (anti-TNF) therapy at our tertiary institution from 2005-2016 was interrogated for those whose indication of treatment was perianal fistula. Data were extracted from electronic and paper records to include demographic and disease variables, time until starting anti-TNF therapy, duration of anti-TNF therapy, medical and surgical treatment.

Results:

The database identified 226 patients who had been treated with biologic therapy for perianal Crohn's fistula between January 2005 – December 2016. Patients were of either gender (male 53%) , median age at diagnosis was 23yrs (range 20-35yrs) and the majority had A2, L3, B1 disease. Over half (53%) of patients had proctitis. Median time to commencement of anti-TNF therapy was 6 months (IQR 0.0, 28) with a median duration of 4 yrs. once the anti-TNF was commenced. Some 37/202 (18%) had a healed fistula on magnetic resonance imaging (MRI) during the study period and 158 / 218 (73%) patients suffered failure (toxicity, primary / secondary loss of response) to at least 1 anti-TNF therapy during treatment course, whilst 47/202patients (23%) developed MRI detected collections whilst on anti-TNF therapy, requiring surgical drainage and subsequent treatment escalation (increase / switch in drug therapy). Twenty-three patients (23/226, 10%) underwent proctectomy,

Conclusion:

There is considerable delay between diagnosis / presentation with a perianal fistula and commencement of anti-TNF therapy, the effect of which, is currently unknown. Fewer than 20% of patients achieve radiological healing despite long-term treatment with anti-TNF, with the majority of patients undergoing failure of treatment and 10% patients undergo proctectomy.

4.2 Introduction:

Historically, Crohn's perianal fistula and other manifestations of perianal Crohn's disease have often been undertreated[373]. However, a change in the treatment paradigm for perianal fistulas with the introduction of anti-TNF and to immunosuppressive biological therapy may well be altering the natural history of perianal fistulas in CD[374]. Antibiotics (metronidazole and ciprofloxacin) and other immunomodulators (thiopurines) temporarily reduce symptoms but do not readily lead to sustained healing of anal fistulas and are considered adjunctive therapy to the biologic agents. The anti-TNF drugs, in particular infliximab, have been widely studied independently and have been demonstrated as effective in inducing and maintaining fistula closure. Two large randomized controlled trials demonstrated infliximab's (IFX) efficacy in inducing and maintaining perianal fistula closure in CD[120,140]. Adalimumab was also significantly more effective than placebo for inducing fistula closure in a subgroup analysis of the CHARM study.[152] Certolizumab has also been evaluated in the treatment of fistulas although no statistical difference was found in the rate of fistula response on treatment[156]. A meta-analysis of the anti-TNF agents in CD perianal fistulas demonstrated efficacy with infliximab possessing the most robust evidence[375,376]. These led to global recommendations of anti-TNF for the treatment of complex perianal fistulas in CD[119,320] and further biologic agents as well as biosimilars have since been introduced into the therapeutic arena.

The anti-TNFα therapies infliximab and adalimumab, have been shown to maintain clinical remission at one year in approximately a third of the patients in whom fistula remission is achieved at induction, and reduce hospitalisations and operations[141], however, recurrence of

fistula can occur, particularly if treatment is stopped prematurely after clinical remission. This is because radiological healing tends to lag behind clinical healing, and recurrence occurs at a rate of up to two-thirds of even fistulas which have been designated as healed clinically and radiologically, at 1 year after cessation of therapy[122,377]. Most trials of anti-TNF agents in pCf assess short term efficacy over the first year of treatment and there are few data that provide insight into the durability of CD treatment with anti-TNF therapy for periods longer than 12 months, as clinical trials have focused on efficacy and safety over the first year of treatment. However, infliximab is not typically discontinued at 12 months, and for many patients treatment is continued indefinitely, or rather until secondary loss of response occurs[326].The anti-TNFα therapies infliximab and adalimumab, have been shown to maintain clinical remission in approximately a third of patients after one year of treatment and associated reduced hospitalisations and operations[141], however, recurrence of fistula can occur, particularly if treatment is stopped prematurely after clinical remission. This is because radiological healing tends to lag behind clinical healing, and recurrence rates can occur with , at a rate of up to two-thirds of initially 'healed' fistulas at 1 year after cessation of therapy[122,377]. Most trials on anti-TNF assess short term efficacy over the first year of treatment and there are few data that provide insight into the durability of CD treatment with anti-TNF therapy for periods longer than 12 months, as clinical trials have focused on efficacy and safety over the first year of treatment. However, infliximab is not typically discontinued at 12 months, and for many patients, treatment is continued indefinitely[326]

Whilst clinicians may identify and consider patients who may benefit from early biologic therapy[320], data on natural history and long-term outcomes[122,193,378–381] of patients on anti-TNF medication are lacking, and the existing data are limited by small sample size and inadequate follow-up duration, making interpretation and summation of the studies difficult. In this study,

we aim to report retrospectively on a single centre experience of one of the largest series with longest follow-up of patients on biologic therapy for perianal Crohn's fistulas. By performing an audit of the Crohn's perianal fistula pathway, we aim to understand the course of disease of patients on biologic treatment with a particular focus on long-term outcomes of radiological healing and proctectomy. We also aim to report on the time lag of starting anti-TNF therapy from point of referral or diagnosis, rate and extent of healing (radiological), rates of and reasons for cessation of anti-TNF therapy, and proctectomy rates in the biologic era. Whilst clinicians may identify and consider patients who may benefit from early biologic therapy[320], there remains a paucity of data on natural course and long-term outcomes[122,193,378–381] of patients on anti-TNF medication and the existing data is limited largely by small sample size and limited follow-up, making interpretation and summation of the studies difficult. In this study, we to retrospectively report on a single centre experience on high volume of patients (one of the largest series with longest follow-up) with a database of patients on biologic therapy for perianal Crohn's fistulas. By performing an audit of the Crohn's perianal fistula pathway, we aim to understand the course of disease of patients on biologic treatment with particular focus on long-term outcomes of radiological healing and proctectomy. We also aim to report on time lag of starting patients on anti-TNF from point of referral or diagnosis, rate an extent of healing (radiological), rates and reasons of cessation of anti-TNF therapy as well as proctectomy rates.

4.3 Methods

A local database of patients diagnosed with complex perianal Crohn's fistula at our tertiary institution from January 2005- December 2016 was established for consecutive patients on biologic (anti-TNF) therapy. Data were extracted from electronic and paper records. The

Montreal classification was used to grade CD phenotype, and data on clinical course were collected including date of first presentation with symptomatic fistula within the study period, date of first anti-TNF dose, date and location of all IBD-related documented clinical interactions (outpatient/inpatient), the date and nature of any surgical procedures, date of MRI scans and absence or presence of radiological healing at any point during the study period. The definition of radiological healing was complete resolution of the previous high signal tract, or a subtle, narrow-calibre intermediate signal residual tract (frequently with low signal borders resembling train tracks).

All patients were treated on the basis of clinical need according to standard clinical care at our institution, on an open-label basis and according to licensed or published regimens. The study received departmental approval by the research lead of St Mark's Hospital, London, United Kingdom. The study was registered as a service evaluation (SE18.003) and all patients were treated only after full and informed clinical consent as part of routine care. As anonymized data were used, written consent was not required by our ethics committee. To help address interpretation of the retrospective data, 2 independent reviewers (SA and JM) collected the data and any discrepancies were resolved by consensus.

Data collected included demographic and disease details, including previous therapies, time to commencement of biologic therapy, surgical procedures performed at our institution, hospital interactions (outpatient/inpatient), follow-up period, date/report of any MRI scans. Statistical analyses were performed to examine the time to commencement of anti-TNF treatment. Two measurements were considered, firstly from the date of diagnosis of a fistula, and secondly from the date of presentation to St. Mark's (for the subgroup where this information was known). The length of time on the first anti-TNF treatment was examined. As not all patients

stopped treatment, survival analysis methods were used for the data analysis. Patients who had not stopped their first anti-TNF treatment were censored at the point of last known follow-up.

4.4 Results

4.4.1 Characteristics of the study population

Table 5 summarises the patient characteristics for Crohn's perianal fistula patients treated on biologic therapy at our institution. The retrospective database identified 236 patients who had been treated with biologic therapy for perianal Crohn's fistula, of which 53% were male, with median age at diagnosis of 26yrs (IQR 20 - 35) and the majority had A2, L3, B1 disease. Over half (54%) of patients had proctitis. The majority (84%) had infliximab as their first treatment, with all others receiving Adalimumab (Table 6). Demographic details included 53% (male),

4.4.2 Outcome Data

Mean duration of follow-up for the group was 6.7 years (SD 3.5). Time to commencement of anti-TNF was examined from date of initial diagnosis of Crohn's perianal fistula , with the results demonstrating a median time of 2.5yrs (IQR 0.8 - 7.3) from the date of fistula diagnosis (fig. 1 demonstrates time to starting anti-TNF therapy from date of fistula diagnosis as well as from date of presentation to St. Mark's Hospital, as some patients were referred from secondary care). A separate analysis revealed a median time 6months (IQR 0.0 - 28) from date of symptomatic presentation. These data are represented in the Kaplan-Meier plots below (Figure 12). The median time on the first anti-TNF agent was approximately 4 years, with 86% remaining on their first TNF after 1-year, 40% by 5 years, and 14% by 10-years. Table 7 demonstrates the frequency of surgical interventions during this period. A combination of

examination under anaesthesia, incision and drainage and seton insertion form the majority of procedures, however a third had other surgical interventions (which included a variety of procedures, Advancement Flap, VAAFT, Collagen plug, LIFT). Around 25% (59 patients had no procedures during the treatment period) whereas 13% (30 patients) underwent at least 1 surgical intervention every year of anti-TNF treatment (Table 8).

Table 5: Demographic details of patients on anti-TNF therapy for Crohn's perianal fistula

Variable	Category	N (of 226)	Summary
Age Crohn's	-	223	23 [17, 30]
Age at fistula Dx	-	218	26 [20, 34]
Gender	Male Female	226	120 (53%) 106 (47%)
Smoking	Current Ex Non	210	62 (30%) 33 (16%) 115 (55%)
Montreal (age)	A1 A2 A3	223	51 (23%) 150 (67%) 22 (10%)
Montreal (location)	L1 L2 L3	212	43 (20%) 73 (34%) 96 (45%)
Montreal (Behaviour)	B1 B2 B3	197	111 (57%) 31 (16%) 55 (27%)
Fistula complexity	Simple Complex	190	21 (11%) 169 (89%)
Proctitis	No Yes	158	73 (46%) 85 (54%)
Mode of presentation	Emergency Surgery Emergency Medicine IBD clinic Gastro clinic Surgical clinic Paediatric	224	36 (16%) 9 (4%) 95 (42%) 34 (15%) 49 (22%) 1 (1%)
Follow-up time (years)	-	225	6.8 ± 3.5

Summary statistics are: Mean ± Standard Deviation, Median [inter-quartile range] or Number (percentage)
*Emergency Surgical Assessment ** Emergency Medical Assessment

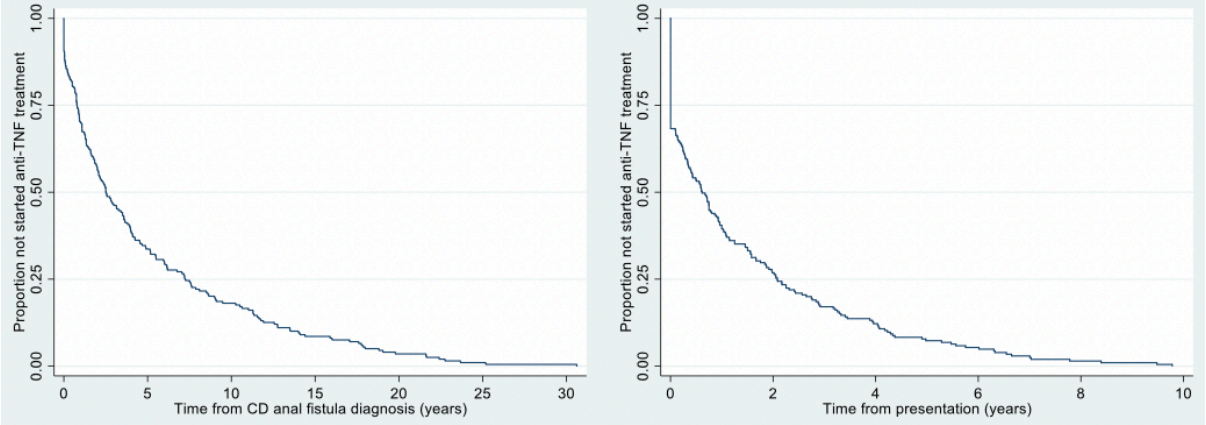


Figure 12: time to starting treatment from the time of fistula diagnosis and time of presentation

Table 6: Duration on anti-TNF agent

Number of patients	203
Concomitant Thiopurine	149 (73%)
1st TNF: Infliximab – N (%)	170 (84%)
Adalimumab – N (%)	33 (16%)
Median [IQR] years on 1st TNF	4.1 [1.7, 7.5]
% (95%) remaining on TNF at:	
1 year	86% (84%, 93%)
2 years	70% (63%, 76%)
5 years	40% (32%, 47%)
10 years	14% (8%, 22%)

Table 7: summary of procedures performed during patient treatment

Procedures	Number	Percentage
EUA	118	19%
Seton placement	66	10%
I&D	154	24%
Lay open	87	14%
Proctectomy (incl. panproctocolectomy)	27	4%
Others	180	29%
Total Number of Operations	632	100%

Table 8: overall surgical procedures per year

Procedures per year	Number of patients	Percentage
0	59	25%
0.01 – 0.25	51	22%
0.26 – 0.50	53	23%
0.51 – 1.00	42	18%
> 1.00	30	13%

4.4.2.1 *Fistula healing*

There were 202 patients with MRI imaging available during the study. Of these, 37 (18%) had a healed fistula at some point during their follow-up. The time to healing is demonstrated using a Kaplan-Meier plot (Figure 13). This shows a majority of fistulas (above 75%) persisting despite combination (medical including anti-TNF / surgical) treatment. Associations between various disease/demographic variables and fistula healing were assessed using hazard ratios from the Cox regression analyses with confidence intervals (Table 9). None of the variables examined were found to be significantly associated with healing. As no factors were found to be significant, multivariate analysis was not performed.

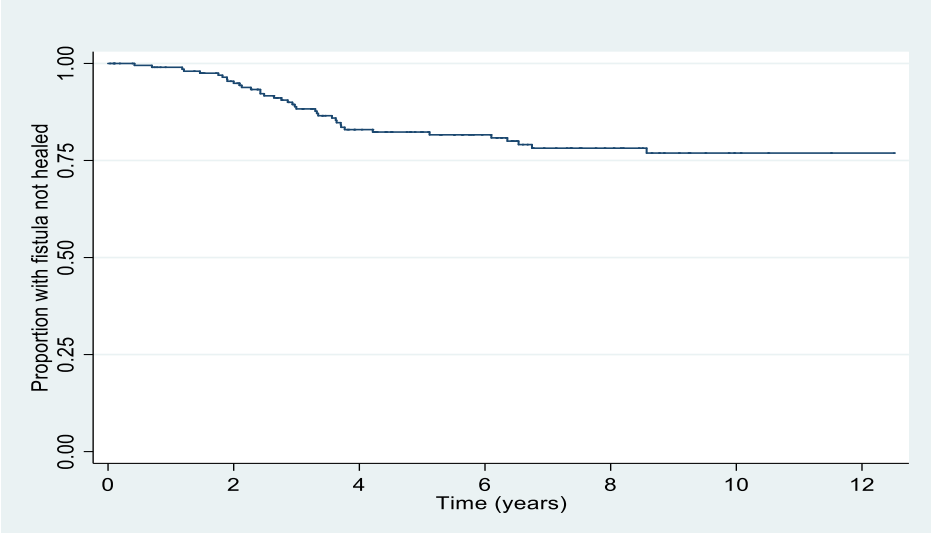


Figure 13: Kaplan-Meier plot of the proportion remaining persistent (i.e. no evidence of radiological healing) over time

Table 9: Univariate analyses of factors associated with healing

Variable	Category	Hazard Ratio (95% CI)	P-value
Age at presentation (*)	-	1.07 (0.82, 1.39)	0.62
Gender	Male	1	0.53
	Female	0.81 (0.42, 1.56	
Smoking status	Never smoked	1	0.12
	Current smoker	0.46 (0.17, 1.22)	
	Ex-smoker	1.37 (0.58, 3.24)	
Montreal age	A1	1	0.79
	A2	1.26 (0.55, 2.91)	
	A3	0.95 (0.24, 3.66)	
Montreal location	L1	1	0.33
	L2	0.82 (0.34, 1.96)	
	L3	0.53 (0.22, 1.31)	
Montreal behaviour	B1	1	0.22
	B2	1.37 (0.57, 3.29)	
	B3	0.53 (0.19, 1.42)	
Fistula complexity	Simple	1	0.80
	Complex	1.16 (0.36, 3.81)	
Proctitis	No	1	0.99
	Yes	0.99 (0.48, 2.07)	

(*) Hazard ratio given for 10-year increase in age

4.4.2.2 *Proctectomy*

The occurrence and time to undergoing proctectomy or panproctocolectomy for refractory perianal fistula was assessed. Of the 226 patients who received anti-TNFs, 23 patients (10%) underwent proctectomy at some point during the study period. The time to proctectomy from the date of diagnosis was evaluated using Kaplan Meier methods. Figure 14 shows a Kaplan-Meier plot of the proportion who had this procedure over time. Estimates from the plot suggest about a quarter of patients undergoing this procedure by 25 years after date of fistula diagnosis. Associations between various disease/demographic variables and proctectomy were assessed using hazard ratios from the Cox regression analyses with confidence intervals (Table 10). There was increased likelihood of proctectomy in females, those with colonic disease and

proctitis, however, none of the variables examined (including age, Montreal classification, etc.) were found to be statistically significantly associated (Table 10).

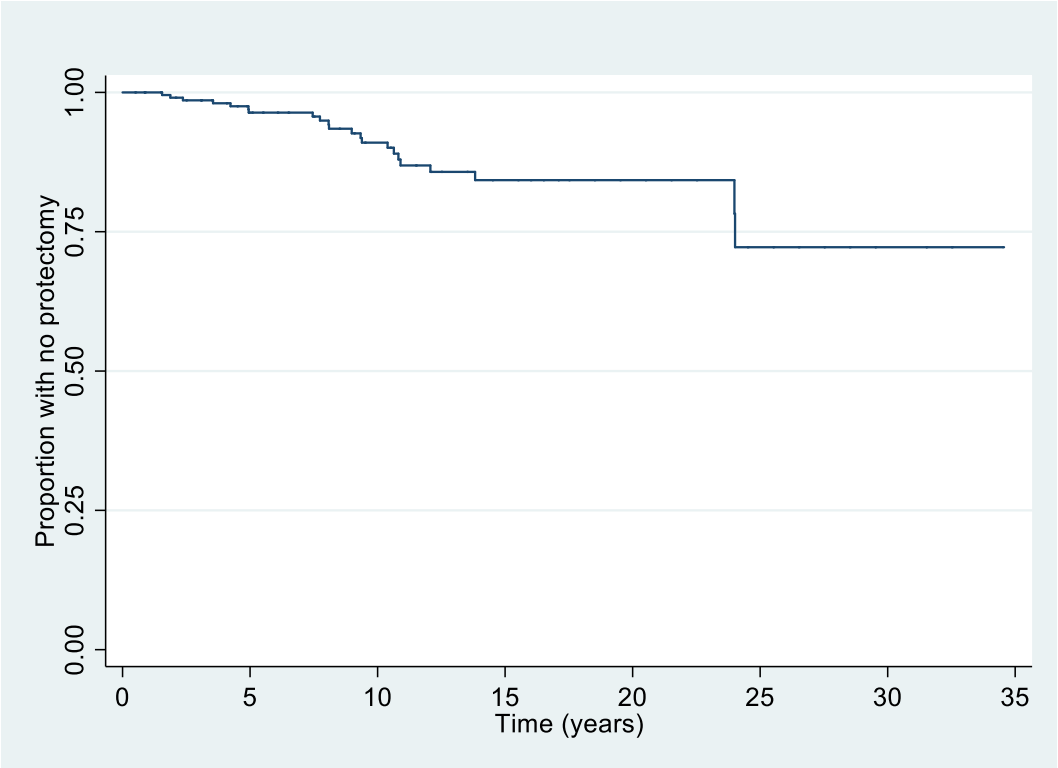


Figure 14: Kaplan-Meier graph of time to Proctectomy / Panproctocolectomy
Time to proctectomy is calculated from date of diagnosis of Crohn's perianal fistula (as opposed to date of commencement of anti-TNF / study start date)

Table 10: Univariate analyses of factors associated with time to Proctectomy / Panproctocolectomy

Variable	Category	Hazard Ratio (95% CI)	P-value
Age at diagnosis (*)	-	1.24 (0.85, 1.81)	0.25
Gender	Male	1	0.20
	Female	1.79 (0.74, 4.32)	
Smoking status	Never smoked	1	0.37
	Current smoker	0.47 (0.16, 1.43)	
	Ex-smoker	0.72 (0.21, 2.53)	
Montreal age	A1	1	0.33
	A2	0.49 (0.19, 1.24)	
	A3	0.80 (0.17, 3.88)	
Montreal location	L1	1	0.14
	L2	3.15 (0.70, 14.2)	
	L3	1.53 (0.32, 7.22)	
Montreal behaviour	B1	1	0.48
	B2	1.07 (0.34, 3.38)	
	B3	0.53 (0.17, 1.69)	
Fistula complexity	Simple	1	0.96
	Complex	1.04 (0.24, 4.51)	
Proctitis	No	1	0.46
	Yes	1.49 (0.52, 4.32)	

(*) Hazard ratio given for 10-year increase in age

4.4.2.3 *Anti-TNF treatment cessation*

Two-thirds of patients had anti-TNF therapy stopped at some point during the retrospective analysis period. Table 11 demonstrates the breakdown of cessation of anti-TNF therapy and reasons for this. The results suggested that loss of response was the single most common reason for stopping treatment (33%), followed by toxicity (27%). Of note were that 47/202 (23%) with had MRI confirmed collections whilst on therapy requiring temporary cessation where drainage of the collection was indicated.

Table 11: Reasons for cessation of anti-TNF therapy

Stop reasons	All TNFs (n=218)	Infliximab (n=146)	Adalimumab (n=71)
Planned cessation	43 (20%)	27 (18%)	16 (23%)
Toxicity	58 (27%)	43 (29%)	15 (21%)
Primary non-response	29 (13%)	19 (13%)	9 (13%)
Loss response	71 (33%)	48 (33%)	23 (32%)
Patient preference	17 (8%)	9 (6%)	8 (11%)

4.5 Discussion

Our study details real world data on one of the largest patient cohorts with Crohn's perianal fistula undergoing treatment with biologic (anti-TNF) therapy with a median follow up of approximately 7 years. We found that during the study period, 37/202 (18%) patients had radiological fistula healing [377]. Proctectomy rate for our study cohort was 10%. A majority of patients (75%) underwent surgical procedures during anti TNF treatment, with 13% undergoing more than one procedure per year on average. A third of patients lost response to treatment with 13% being primary non-responders. As this was a retrospective study, data on clinical remission and relapse were not reliably recorded. We have therefore not presented these data. Further, the lack of a high quality disease specific quality of life tool during the study period prevents robust measurement of subjective outcomes from the patient's point of view. We therefore chose to focus on robust, objective outcome measurements and radiological healing and proctectomy were chosen for this purpose. Radiological healing is the most robust method of determining deep tissue healing of the fistula, the stated objective of anti-TNF agents. Proctectomy is a consistent outcome measure in studies of pCf over the years and

identifying proctectomy rate in the biologic era is a useful marker of the overall efficacy of anti TNF agents. The problem with proctectomy as an outcome measure, in common with measure pouch excision/defunctioning for pouch failure, for example, is that a decision by surgeon and patient is required for the outcome to be reached, rather than a defined change in disease state or symptom profile. Some patients meet a reasonable threshold for proctectomy but decline it, preferring to live with difficult symptoms, whereas others might undergo the procedure with a much lower burden of symptoms because of a perceived quality of life improvement which will be achieved afterwards. Historically proctectomy represented final failure of all other options whereas newer thinking (admittedly mostly coming to the fore after the study period) suggests that proctectomy should be an option earlier in the disease course, rather than representing final failure.

A meta-analysis in 2011 by Ford and colleagues[139] assessed the effect of biologic therapies in inflammatory bowel disease. They performed a sub-analysis of the six trials using anti-TNF α antibodies in 453 patients with active fistulising CD. They reported risk of bias in all but one study and only one of the trials was specifically designed to assess efficacy in fistulising CD. This study demonstrated a clear benefit of infliximab over placebo (RR of fistulas remaining unhealed = 0.62; 95 % CI 0.48 – 0.81)[120]. However, when the studies are pooled, and results are compared at 26 weeks, there was no statistically significant difference detected in the RR of fistulas remaining unhealed with anti-TNFα agents vs. placebo (0.88; 95 % CI 0.73 – 1.05), although they note considerable heterogeneity between studies (I^2= 67 % , P = 0.01). Maintenance treatment with anti-TNF drugs monitored radiologically beyond 1 year has not been adequately described and studies using radiological assessment of follow up as an end point are lacking[122]. Data on long-term outcome beyond 1 year of fistulizing PCF

treated with IFX were scarce. Retrospective studies of small sample size reported that 29%, 18%, 75%, and 42% of patients treated with IFX achieved sustained complete fistula closure after a median follow-up ranging from 20–60 months[160,193,379–382]. Bouguen et al.[379] reported on the outcome of 156 patients treated with infliximab for Crohn's perianal fistulas at 2 tertiary centres. They reported the cumulative probability of fistula closure was 73% at 5 years and 88% at 10 years. After a median follow-up of 5 years, 72 of the 156 patients (46%) had 'sustained fistula closure'. There was no mention of radiological confirmation of fistula closure. St Mark's Hospital has previously published data on 34 patients with Crohn's anal fistulas treated with infliximab, adalimumab or thalidomide, assessed both clinically and using serial MRI scanning to determine deep tissue healing of the fistula tract[383]. At 1 year, clinical remission was seen in 53% of infliximab treated patients, and 29% of adalimumab treated patients. Radiological healing was seen in 28% of all anti-TNF treated patients and radiologically healed patients remained healed on maintenance treatment. Tozer et al.[122] followed the patients up and reported 58% of all patients, comprising 66% and 43% of infliximab and adalimumab-treated patients respectively, demonstrated remission or response at 3 years. Thirty-three percent of infliximab treated patients maintained clinical remission at 3 years. Radiological healing lagged behind clinical remission by a median of 12 months. Our study suggests that a significant number of patients fail to achieve radiological healing despite long term treatment. The burden of Crohn's perianal fistula treatment in these patients includes surgical interventions, risk of collections during treatments as well as the inherent side effects of long term treatment[320,351].

There is considerable delay between diagnosis / presentation with fistula and commencement of anti-TNF therapy, the effect of which is currently unknown. Reasons for the delay include delays in the service delivery infrastructure, but could also reflect changes in approach to

treatment. The "step-up option" was the standard approach, but data suggests that this strategy has not clearly changed the global outcome of CD[384385], and the "top-down" strategy[297] has gained increasing traction, although long-term risks and benefits of either strategy above the other are yet to be established[97,386,387]. Larger population studies with detailed medication data will be needed to confirm whether earlier and greater biological use is correlated with lower rates of surgical interventions for perianal Crohn's disease[388]. There is some evidence that early anti-TNF therapy correlates with slower progression of bowel damage in CD[32,389,390]. This may suggest that biological agents are capable of changing the natural history of disease and indeed some evidence from a Danish population study, suggests that this may be the case for the natural history of surgical interventions when biologic therapy is appropriately timed[391]. They reported a persistent significant decrease in surgery rates observed along with a significant decrease in use of 5-ASA and corticosteroids, which paralleled an increasing use of thiopurines and anti TNF-α agents in IBD over three decades. However, other studies suggest that there can be a delay in the indication for surgery after persistent medical therapy, leading to significant consequences, such as more extensive procedures owing to increased severity at the time of surgery and also increased postoperative complication rates[392,393]. These studies were done in luminal CD and are not necessarily translatable to perianal Crohn's fistula management, however, the duration of fistula persistence is a poor prognostic factor which suggests a benefit to a time based approach[394]. Suggested approaches for optimising this include streamlining the patient pathway, for example via fast track referral pathways between medical and surgical arms of the IBD service, including directly into surgical admissions units and then back to receive anti-TNF infusions, combined inpatient rounds and joint IBD clinics. Some of these (including the latter) have been instigated at our unit and are under evaluation.

Our study also suggested that the rate of fistula surgical approaches (e.g. Advancement Flap, VAAFT, Collagen plug, LIFT) was 29% in our study cohort and it may be that there is a case for increase, given the low rates of sustained fistula healing. Also, majority of these were due to symptomatic amelioration with VAAFT (as opposed to curative intent). There is potentially a role for more attempts at definitive surgical repair, particularly as the proctitis figure (which often precludes this approach) was 54% in our study cohort. Absence of proctitis is known to be an independent predictor for increased healing and reduced recurrence rates[209,395], so one would expect a higher percentage of surgery attempted in this group, although fistula morphology also restricts options, with more complex fistula being unsuitable for the LIFT procedure, for example. The combination of a lack of proctitis and the correct fistula morphology will always limit the pool of patients for whom reparative surgical procedures are suitable. Small retrospective studies have reported on surgical treatment approaches for Crohn's perianal fistulas, however, only a tiny minority of patients in these studies actually undergo definitive surgical interventions, but rather seton drainage procedures (EUA / seton insertion[160,396]. A systematic review in 2014[396] assessing combined surgical and medical treatment, corroborates this finding with the most common definitive procedure being fistulotomy (12%) and definitive sphincter sparing surgical techniques accounting for only 15% of all surgical treatments. Drainage procedures accounted for the majority (62%). Sauk et al. [388] reported a decline in the proportion of patients undergoing diversion, examination under anaesthesia, or fistulotomy since 2000, which some authors have attributed to better non-surgical management over time[388,395]. They also reported a plateau in decline in surgical interventions observed since about 2006, suggesting that there remains a subset of patients who require diversion procedures for perianal CD with a particularly poor prognosis. In our study, 4% of all surgical procedures were proctectomies, which occurred in 23/226 (10%) patients. No statistically significant predictive factors were identified as increasing its likelihood in this

study. Other studies have reported on the presence of colonic disease and anorectal strictures being associated with an increased risk of proctectomy in the presence of fistula[397]. Proctectomy is often done in the context of refractory disease and is usually preceded by temporary faecal diversion with the intention of disease amelioration. Sometimes diversion alone is enough to obtain improved quality of life and it can also be useful to delay the risks associated with deep pelvic dissection, such as sexual dysfunction and reduced fecundity. Perianal fistula, anorectal sepsis and severe proctitis are the leading causes of temporary faecal diversion[173]. A recent systematic review and meta-analysis highlighted that less than 20% undergo successful restoration of bowel continuity after temporary faecal diversion and about 42% of patients eventually require proctectomy (i.e. following primary or secondary non/loss of response to initial diversion, or relapse following restoration of continuity)[173]. Effective treatment strategies for such patients remain an important unmet medical need as they are also excluded from clinical trials of novel and promising agents (e.g. stem cell therapy[398]) despite poor response to existing therapies. Furthermore, biomarkers to identify this particularly refractory patient population deserves further investigation as more aggressive management early in the course of disease could potentially change the natural history of the disease, or at least an early ablative intervention which improves quality of life[388].

Regarding refractory disease, an important finding from this study is the proportion of those who stop treatment due to toxicity or as a result of non/loss of response to anti-TNF therapy. Combined, this figure represented 158/218 patients (72%); in other words, almost three quarters of patients are ultimately refractory to initial treatment and require a switch in therapy or consideration of more extensive surgery. This fits with the feeling of many IBD specialists who feel that the ever expanding list of biologic agents represents a buffet through which

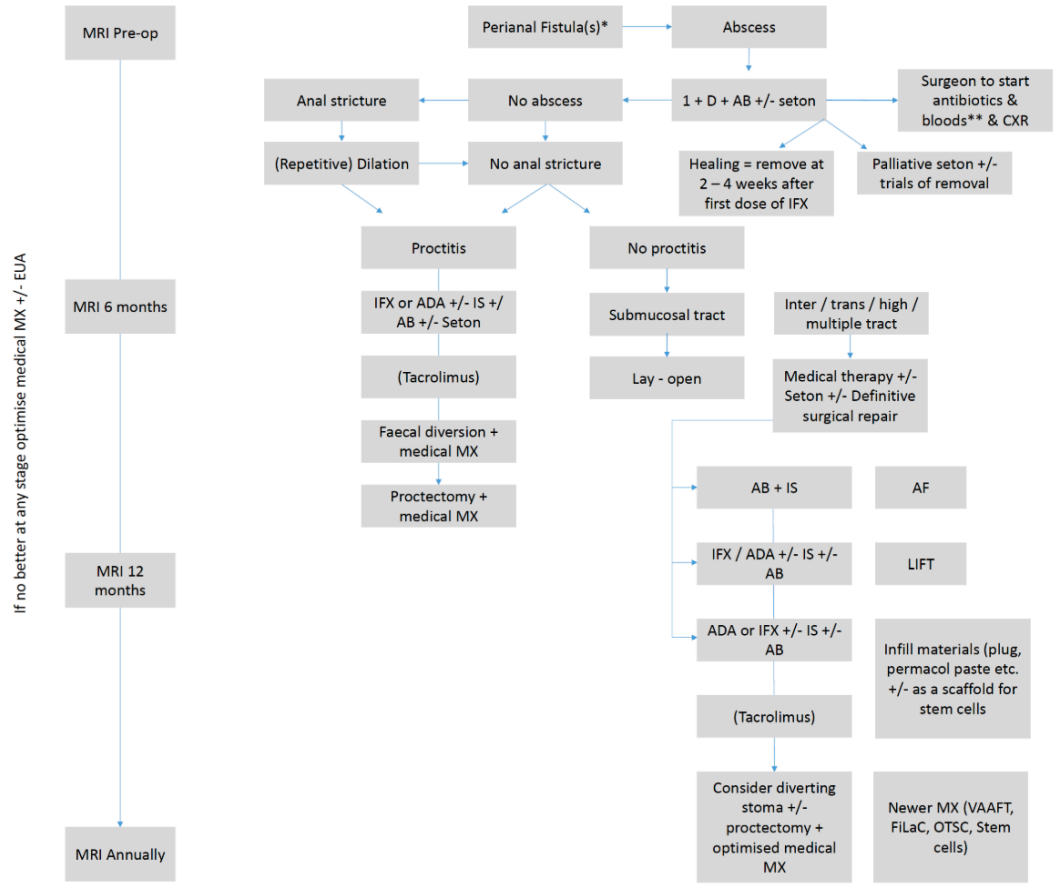
patients progress until they run out of options, rather than a menu from which the ideal course can be chosen.

Historically, a high proportion of patients underwent proctectomy for disabling disease (up to 40%[13,205]). This is particularly the case when the comparison is with older cohorts in the pre-biologic era[13]. Although our cohort proctectomy rate of 10% is on the lower end of quoted rates[9,13,374], it remains unclear whether this is truly an effect of improvements in medical therapy / multidisciplinary management over recent decades. Kaplan Meier analysis suggested just over a quarter of those undergoing proctectomy, had this within 25 years of fistula diagnosis. It is unclear what disease states these patients were in prior to proctectomy due to the absence of a widely accepted disease state classification and thus making it difficult to derive data regarding indication or effect of the timing of proctectomy. Further studies are necessary to assess the role of the latter on the effect on quality of life and patient perspectives on the 'willingness to trade' symptoms of debilitating or protracted perianal disease versus earlier proctectomy.

The main strength of our study lies in the real world data and large patient cohort with long follow-up relative to studies on biologic treatment of Crohn's perianal fistulas. Limitations include its retrospective nature, as well as the fact that a significant proportion of the patients were on open label treatments and didn't have scheduled imaging for robust, time-related assessment of radiological healing. This has since changed with an updated guideline for management of Crohn's perianal fistulas which includes regular imaging in the treatment algorithm(Figure 15). The guideline enforces the change in treatment paradigm to a top-down approach, with increased early use of biologic therapy. Our study is also limited by the lack

of defined and widely accepted disease states which hampers the true understanding of the natural disease course and the influence of anti-TNF agents on this in the absence of sustained healing. The definition of radiological healing is controversial and whilst MRI is the gold standard for identifying perianal morphology, it is not validated to evaluate response to treatment[377,395]. In this study we utilised a radiological healing definition that has previously been published by our group. Also, the implication of collections and their significance clinically is uncertain and their size and relation to fistula remission is also controversial[244]. Recently, a Crohn's perianal fistula study trial included collections measuring less than 2cm in at least two dimensions in their radiological remission definition. A further limitation of this study was the absence prospectively collected clinical healing data and patient reported QoL data, neither of which was sufficiently robust in this retrospective study to draws conclusions from, so we have not presented these data.

In summary, the real world data from our cohort of patients demonstrates limited long term healing in Crohn's perianal fistulas. In this context patients are on long term immunosuppressive medication and still require multiple surgical interventions in the context of relapse and remissions or chronic symptomatic disease, leading to proctectomy around 10% of the time. There is a need for more comprehensive classification of Crohn's perianal fistulas especially in terms of disease states, which would enable the better definition of disease course and biomarkers to identify the refractory patient population, for whom very limited treatment options exist and represent an unmet target for better care.



*Consider annual EUA/biopsies in patients with chronic complex perianal disease
**Bloods – routine + TB Quantiferon, Hep B surface antigen and core antibody, Hep c, HIV, VZV IgG, EBV, TPMT

Figure 15: St Mark's Algorithm for managing Crohn's perianal fistula (modified from Gecse et al. [119])

Chapter 5. Disease course and fate of Crohn's perianal fistula patients following failure of initial Biologic therapy – real world lessons on impact of refractory disease from a tertiary centre cohort

5.1 Abstract

Introduction: Current evidence demonstrates that anti-TNF therapies lead to clinical response rates of up to 68% and complete remission rates of 55% have been reported in the short-term. These remission rates decrease to about a third of patients who initially achieved remission, after three years. Our centre data revealed only 18% (37/210) with a radiologically healed fistula, with 73% (158/218) refractory to therapy during an 11year period in a cohort of Crohn's perianal fistula patients undergoing biologic therapy at a tertiary centre. Options are limited in cases refractory to biologic therapy, and there is limited knowledge of disease course in these cases. This is in part due to the limited disease states characterised for Crohn's perianal fistula in the absence of cure. The current study applies recently described disease states within the context of Crohn's perianal fistula to determine the fate of patients refractory to initial biologic therapy and quantitatively evaluate the health resource impact of loss of response to biologic treatment in this cohort.

Methods:

We undertook a retrospective review of records from patients undergoing anti-TNF treatment for Crohn's perianal fistula at St Mark's Hospital, identified those refractory to treatment and applied disease states defined from a recent vignette study of the general public and CD patients

in the UK. We obtained data on length of time patients spent in each disease state and the subsequent disease states they transitioned to and created a Markov model to assess transition probabilities. We also collected data on healthcare resource utilisation (MRI, hospital interactions, surgical procedures, medical therapy) within each disease state.

Results

Eight fistula disease states (including proctectomy, defunctioning surgery, remission and symptomatic states) were retrospectively ascribed to all clinical records / visits where documentation was available. The majority of refractory patients were switched on to alternate anti-TNF therapy and most reside in a 'mild chronic symptomatic fistula' disease state with a minority of patients subsequently transitioning into a severe disease state or remission state. Successful proctectomy disease state accounted for the longest period of continuous length of time in any one disease state (mean 4.4 years). The overall remission rate (of any duration) for our study cohort of 78 patients was 46%, however sustained (1year or more) remission rate was 27%. Some 19% patients underwent proctectomy. There was considerable healthcare resource utilisation amongst all disease states particularly with biologic therapy, which is the most expensive healthcare resource.

Conclusion

Whereas most patients reside in a mild chronic state following failure of at least one biologic therapy, a fifth of patients refractory to treatment undergo proctectomy which, when successful, accounts for the longest continuous length of time patients spend in a single disease state. There remains considerable healthcare resource utilisation and better treatment options are needed in refractory patients.

5.2 Introduction

The introduction of anti-TNF agents (such as infliximab and adalimumab) has heralded a significant addition to treatment of Crohn's perianal fistula and has since been adopted into international guidelines on management[119]. Current evidence demonstrates that anti-TNF therapies lead to clinical response rates of up to 68% and complete remission rates of 55% have been reported in the short-term[120]. These remission rates decrease to about a third of patients who achieved initial remission, after three years[122]. In fact, current existing pharmacological treatments for complex perianal fistulas have low efficacy in inducing remission (antibiotics 21–48%; thiopurines 20–40%; anti-tumour necrosis factor [TNF] treatment 23% 'complete responders' (i.e. 36% of 64% of patients who responded to induction treatment) [244]. Achieving complete fistula healing is often a long and usually elusive process accompanied by multiple relapses and despite medical and surgical therapies, durable remission rates remain low[399,400]. Failure of or intolerability to medical treatment can ultimately result in the need for permanent faecal diversion or proctectomy, rates of which remain significant, even in the biologic era[173]. Around 10–30%[139401] of patients do not respond to the initial treatment and 23–46%[402–404] of patients lose response over time. Furthermore, durability of maintenance therapy over multiple years has not been defined, and consequently, the true frequency of loss of efficacy and requirement of anti-TNF dose intensification in the long term is not fully understood[326]. Few treatment options exist for drug-treatment-refractory patients, and repeated surgical options can be associated with substantial morbidity (e.g. sphincter injury and continence impairment). Thus there is an unmet need for alternative treatments for those whose fistulas are refractory to management[244].

Management of refractory cases of Crohn's perianal fistulas is challenging and the optimal strategy remains unknown[395]. Quantitatively evaluating the burden and natural history in these cases is difficult. This is in part due to the fact that Crohn's perianal fistulas encompass a spectrum of disease states which may vary from a chronic quiescent fistula with minimal effect on lifestyle, to patients with multiple complex fistulas and multiple admissions secondary to fistula related sepsis. Currently there are no commonly agreed definitions for disease states in Crohn's perianal fistula as data capturing health-related utility associated with perianal fistulas in CD are scarce[405,406]. There are also limited reports on healthcare resource utilisation in this condition especially in the context of treatment failure. As a result, it is currently difficult to assess the disease course following failure of biologic therapy and the potential impact or comparative health/cost benefit of novel treatments in this context (e.g. new biologic agents, stem cell therapy, novel surgical interventions, etc).

Health-related utility measures are useful for capturing the value placed on different health effects, where utility is defined on a scale of 1 (full health) to 0 (dead), and it is routinely used in the economic evaluation of health technologies. They are used to calculate quality adjusted life years (QALYs), which serve as a measure of health benefit particularly of interventions for quality and length of patient life and favoured by National institute of health and care excellence (NICE) in appraisals[407]. Eliciting health-related utility values using a vignette approach has previously been published, describing health states for Crohn's perianal fistulas as assessed by the general public and patients with CD[405,407]. Health state descriptions associated with complex Crohn's perianal fistulas were developed following qualitative research with patients and validation by clinicians[405]. The current study aims to apply these health states relating to stages of Crohn's perianal fistula disease to quantitatively evaluate the impact of loss of response to biologic treatment in a cohort of Crohn's perianal fistula patients

refractory to initial therapy. We embarked on a single centre retrospective audit to assess the disease course and natural history in this cohort of patients.

5.3 Methods:

We undertook a retrospective review of records from patients undergoing anti-TNF treatment for Crohn's perianal fistula at St Mark's Hospital.

5.3.1 Patients and setting

A local database of patients diagnosed with complex perianal Crohn's fistula at our tertiary institution from January 2007 - December 2016 was established for patients on biologic (anti-TNF) therapy primarily for their fistula. St. Mark's Hospital is a tertiary referral centre for a variety of colorectal conditions including inflammatory bowel disease and colorectal cancer. As such, a variety of important variables can be collated such as patient demographics, comorbidities, operative procedures, etc from various established databases. This include the St Mark's Hospital database for patients with perianal fistula (on biologic treatment), IBD nurse patient database, hospital coding system, electronic patient records and, where necessary, hard copy patient case notes.

Consecutive patients that were refractory to initial biologic (anti-TNF) treatment for Crohn's perianal fistula were included in this study. This was defined as those with a diagnosis of CD prior to the index visit, with Crohn's perianal fistulas refractory to conventional therapies, following initiation for primarily Crohn's perianal fistula disease state. Hence patients must have received at least one anti-TNF agent and (or not including) combination treatment (with

antibiotics, steroids, thiopurines, methotrexate, surgery), that did not result in an adequate treatment response.

The Montreal classification was used to grade CD phenotype as inflammatory, stricturing or fistulising and location was defined by the CD findings in the gastrointestinal tract: ileal, colic, ileocolic and/or upper digestive tract CD, with perianal involvement (in all cases included). Each patient's clinical records were interrogated to collate drug and clinical course data following decision of failure/non-response to an anti-TNF drug. The date of the decision was taken as the baseline visit and the subsequent hospital interactions (scheduled / unscheduled documented visits with associated clinical entries) were interrogated for documented evidence of a disease state which was applied retrospectively based on criteria described in the section below.

5.3.2 Disease States

A consensus exercise was undertaken by of group of experts currently involved in managing this condition, including colorectal surgeons, gastroenterologists, radiologists, specialist nurses. Previously defined Health / Disease states from a vignette study were reviewed by the group (Table 12) and consensus was agreed on the disease states that were likely to encompass the cohort of patients encountered in the unit. The final disease states that were agreed upon are shown in Table 13. This mirrored the disease states from the previous study apart from abscess which was excluded as a disease state to avoid ambiguity (of terminology e.g. sepsis/collection etc) and instead collected as a separate data variable

. Table 12: Results of a vignette study ascribing disease states in Crohn's perianal fistula[405]

Health state	CD patients (N=162)		General population sample (N=835)	
	Mean	SD	Mean	SD
Remission	0.894	0.24	0.865	0.24
CSF with mild symptoms	0.657	0.41	0.578	0.44
CSF with severe symptoms	0.433	0.46	0.383	0.50
Defunctioning with positive outcome	0.541	0.51	0.567	0.46
Defunctioning with negative outcome	0.278	0.54	0.193	0.56
Proctectomy with positive outcome	0.568	0.52	0.564	0.50
Proctectomy with negative outcome	0.279	0.55	0.202	0.57
Abscess	0.342	0.50	0.223	0.55

CD - Crohn's disease; CSF – chronic symptomatic fistula, SD – Standard deviation
Values represent disease state utility values where utility is defined on a scale of 1 (full health) and 0 (dead)[405]

Table 13: Disease states and definitions

Disease States / definitions

1. Chronic symptomatic fistulae, with mild symptoms;
    a. mild discomfort and small amount of discharge from the fistula opening. daily activities are not restricted.
2. Chronic symptomatic fistulae, with severe symptoms;
    a. moderate or marked discomfort. moderate or substantial discharge from the fistula opening, which contains mucous, pus and/or faeces. daily activities are moderately to markedly restricted as a result of the fistulae. moderate or marked restrictions on sexual activity.
3. Remission;
    a. no pain associated with the fistulae and no anal discharge. daily activities are not restricted and there are no restrictions on sexual activity as a result of fistulae
4. Successful defunctioning surgery;
    a. operation was a success; no pain from the fistula. A small amount of discharge may sometimes leak from the fistula(s) but daily activities are not restricted.
5. Unsuccessful defunctioning surgery;
    a. operation has not completely relieved symptoms with persistence of pain and regular and substantial discharge from the fistula opening. daily activities are severely restricted as a result of the fistulae.
6. Successful proctectomy;
    a. operation was a success and no pain or discharge from the wound
7. Unsuccessful proctectomy;
    a. still experience discharge from the surgery wound which is not fully healed. may experience pain. daily activities are restricted. sexual activities are restricted
8. Death

*NB. Data on Perianal abscess was collected separately and not included as a disease state
a – explanation of disease state

The ‘disease/health state’ data were applied retrospectively to the refractory cohort of consecutive patients described above. On the baseline visit, an initial state was applied based on review of the case notes from that visit. Each patient’s clinical records were then reviewed in detail in order to estimate the length of time they spent in each disease state. Additionally, the following components of healthcare resource utilisation relating specifically to the

management of perianal Crohn's fistula (not luminal disease) were captured for each patient:

- Drug treatments
- Surgical operations such as examinations under anaesthesia, seton placement/removal/replacement, other surgical procedures (e.g. VAAFT, plug, FiLaC, etc), defunctioning stoma, proctectomy
- Hospital admissions: emergency and planned
- Outpatient visits
- Diagnostic imaging: MRIs
- Endoscopic procedures

Two independent researchers (SOA and JM) applied the disease states to all clinical interactions and any discrepancies were resolved with discussion or via senior team members (consultant colorectal surgeon / gastroenterologist). The primary outcome of the study was to allocate disease states to patients with Crohn's perianal fistulas refractory to an anti-TNF treatment (i.e. of loss of response to at least one anti-TNF agent) during their disease course. Once these had been established, we recorded data on length of time patients spend in each disease state and the subsequent disease states they transitioned to. This enabled assessment of healthcare resource utilisation and disease burden (including treatments undergone) following loss of response to an anti-TNF drug.

5.3.3 Data analysis

Quantitative variables were reported as median / mean and categorical variables were presented as counts and percentage of the cohort. A Markov model was developed to analyse the healthcare resource utilisation in different disease states. The multi-state Markov model is a useful way of describing a process in which an individual moves through a series of states in

continuous time (Figure 16)[408–410] . Data includes observations of a continuous-time process at arbitrary times, for example, visits to a hospital to diagnose disease status. The likelihood of being in a certain disease state is calculated in terms of transition probabilities. The study was approved by our Research & Development Department as a service evaluation and no formal ethical approval was required.

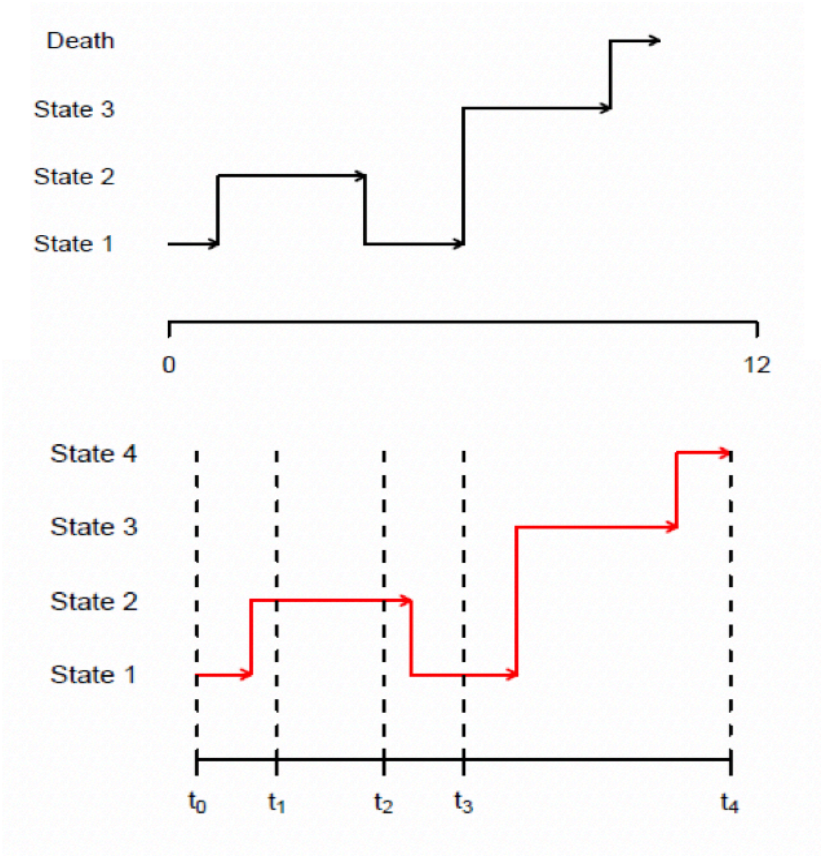


Figure 16: Schematic example of Markov model -
Using a cycle time of 1year (0-12months) and demonstrating transition between disease states at different timepoints (t_0,t_1..t_4).

5.4 Results

Table 14 demonstrates the patient characteristics of the study cohort. In the study, 78 consecutive refractory patients between 2008 and 2017 were included, with a median follow-

up time of 2.6 years encompassing a total number of visits of 1393 and an average of 13 visits per patient.

Table 14: Patient characteristics

Baseline characteristic	(N=78)		
Median Age, years	35-39*		
Gender, male, n (%)	34 (44%)		
Duration CD, years (SD)	17.3 (8.9)		
Montreal Classification	**n (%)**		
	A1 19 (25%)	**B1** 55 (73%)	**L1** 20 (26%)
	A2 52 (68%)	**B2** 11 (14%)	**L2** 14 (18%)
	A3 5 (6.6%)	**B3** 10 (13%)	**L3** 44 (56%)
Presence of proctitis n (%)	19 (24)		
Smoking n (%)	**n (%)**		
Missing	14 (17.9)		
Non-smoker	33 (42.3)		
Past smoker	9 (11.5)		
Current Smoker	22 (28.2)		
Treatment	**n (%)**		
Anti-TNF alone	2 (3%)		
Immunosuppressants	0 (0%)		
Anti-TNF AND Immunosuppressants	76 (97%)		
Neither	0 (0%)		
Seton	**n (%)**		
No	49 (62.8)		
Yes	29 (37.2)		
Previous surgery	**n (%)**		
Yes	47 (60.3)		
No	31 (39.7)		
Other concomitant CD treatments (safety population), n (%)	**n (%)**		
Antibiotics	10 (13%)		
Corticosteroids	11 (14%)		

*median age bracket (range 18-65+)

Table 15: Description of treatments undertaken by study group

Treatment	Number of patients (N=78)	%
Any biologic	65	83.30
Adalimumab	40	61.50
Infliximab	12	15.3
Vedolizumab	13	16.70
Any immunosuppressant	52	66.70
Thiopurines	43	55.10
Methotrexate	11	14.10
Tacrolimus	4	5.10
Any surgery	78	100.00
EUA (+/- other interventions)*	44	56.40
EUA / drainage of abscess or sepsis	20	25.60
EUA / seton insertion	20	25.60
Proctectomy	15	19.20
Defunctioning surgery	15	19.20
Other surgery (VAAFT / LIFT / advancement flap)	5	6.40

*includes core out of tract, fistulectomy, partial lay-open, wound curettage, etc.

The different treatments undergone by the patient cohort are listed in Table 15. The majority of patients were switched to another biologic agent following loss of response to one biologic treatment. Adalimumab was the most common biologic agent (61%) used following loss of response. The majority (52/78, 67%) had additional immunosuppression, most commonly thiopurines. All patients underwent surgical interventions, most commonly examination under anaesthesia (44/78, 56%) and 5/78 (6%) underwent other fistula surgical procedures e.g. VAAFT, LIFT, advancement flap.

Disease state allocation across the study cohort is depicted in Table 16. The majority (58/78, 74%) of patients had spent most time in the "mild" chronic symptomatic fistula disease state with remission being the next most common disease state. The mean sojourn times per patient are depicted in Figure 1, and this demonstrates that a successful proctectomy disease state accounted for the longest period of continuous length of time in any one disease state (mean 4.4 years).

Thirty-six patients (46%) of the patients who were in the remission disease state at any stage during the study period were analysed to assess their disease course following transition into this disease state. Twenty-one patients (21/78, i.e. 27% of entire study population) remained in this disease state for at least 1year; 11 patients had recurrence of their fistula symptoms and transitioned into 'chronic symptomatic fistulas with mild symptoms'. The remaining four patients transitioned to severe symptoms disease state: one remained in this disease state for the rest of the study follow-up; three patients eventually underwent faecal diversion. Two of these latter patients underwent defunctioning surgery (and were both left with residual fistula symptoms); the third patient underwent proctectomy (and had an unhealed perineum at last follow-up, entering the 'unsuccessful proctectomy' disease state). All cases of transition from remission to recurrence of fistula symptoms (i.e. transition to symptomatic disease state) occurred in less than 6 months6months.

Fifteen patients (19%) underwent proctectomy (during the study period). Ten of these patients were in a state of unsuccessful proctectomy (prior to eventual transition to successful proctectomy disease state. A third of the patients with successful proctectomies were still on biologic therapy and 60% on immunomodulators. Review of medical records confirm that these were done for luminal disease. A multi-state Markov model was fitted to the

observational data to estimate transition probabilities between the modelled health states (Table 18). The probability of successful defunctioning was calculated as the ratio of the transition probability from the CSF state with severe symptoms to successful defunctioning over the transition probability from the CSF severe state to defunctioning, both successful or unsuccessful. The resulting probability of successful outcomes post-defunctioning was 0.62. The probability of successful proctectomy was calculated analogously. The ratio of successful proctectomies over the total proctectomies from the CSF severe health state was 0.80.

Figure 18 demonstrates the distribution of health resource utilisation for the study group following the allocation of different disease states. Most patients had biologic treatment regardless of disease state, however this was common in those patients in "chronic symptomatic fistula with severe symptoms" (17/20, 85%), and those in "chronic symptomatic fistula with mild symptoms" (45/58, 78%) and least common in those with successful proctectomy (5/15, 33%). Immunomodulator use had a similar spread across the disease states (ranging from 47-65% of patients in each of the disease states). Examination under anaesthesia (with/without other intervention) was commonest in the group of patients in chronic symptomatic fistula with severe symptoms (11/20, 55%), as well as those with mild symptoms (28/58, 48%),%); and unsuccessful defunctioning surgery disease state (i.e. 7/15, 47%). Hospitalisations were commonest for those with unsuccessful defunctioning surgery (10/15, 67%) and those with chronic symptomatic fistula with severe symptoms (13/20, 65%). Probabilities of all the healthcare resource utilisation for each disease state are depicted in Table 17.

Figure 19 highlights the spread of hospital interactions encountered by the various patients according to different disease states. Gastroenterology outpatient appointments were uniformly common across all disease states, with more than 75% of all patients having regular gastro outpatient follow-up. This was followed by surgical outpatient appointments. IBD and surgical day units had the next most common utilisation amongst the study group (23-55% patients), and were most commonly utilised by patients in the 'chronic symptomatic fistula with severe symptoms' disease state. Fistula related inpatient admissions ranged from 0-27% for surgical admissions and 0-20% for medical admissions and these tended to be in patients with unsuccessful defunctioning surgery, chronic symptomatic fistula with severe symptoms.

Table 16: spread of disease state characteristics across the study group

Disease state	Number of patients	Prevalence (%)	Number of disease state	Average number of disease state per patient	Minimum number of disease state per patient	Maximum number of disease state per patient	Mean time spent in disease state (years)	Median time spent in disease state (years)
Chronic symptomatic fistulas, with mild symptoms	58	74.4	597	7.65	1	50	2.02	1.19
Chronic symptomatic fistulas, with severe symptoms	20	25.6	117	1.5	1	24	0.63	0.36
Remission	36	46.2	189	2.42	1	14	1.83	0.94
Successful defunctioning surgery	13	16.7	98	1.26	1	17	0.92	1.3
Unsuccessful defunctioning surgery	15	19.2	136	1.74	1	20	1.35	1.09
Successful proctectomy	15	19.2	146	1.87	1	34	2.77	1.57
Unsuccessful proctectomy	10	12.8	70	0.9	1	20	1.97	1.44

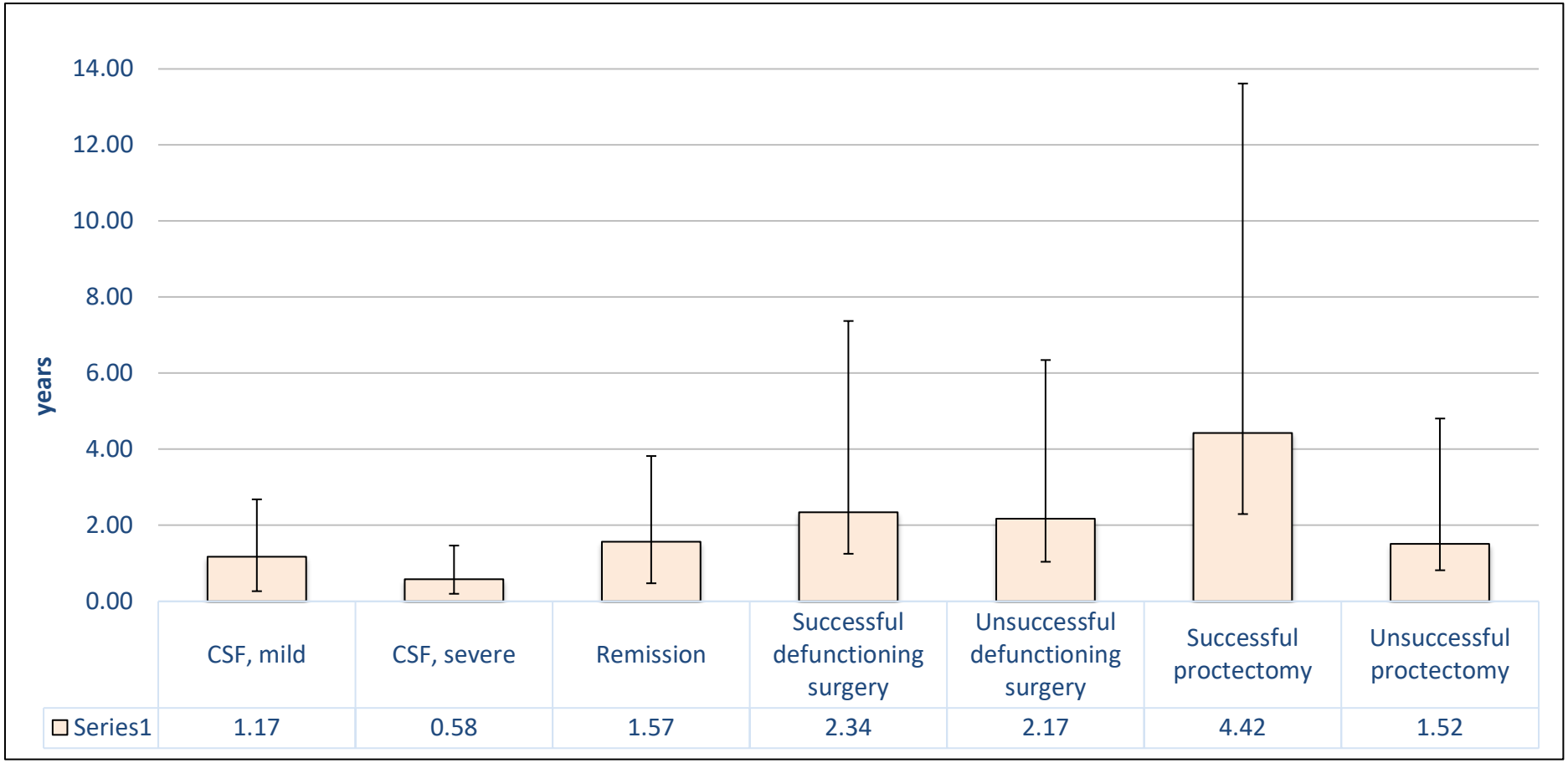
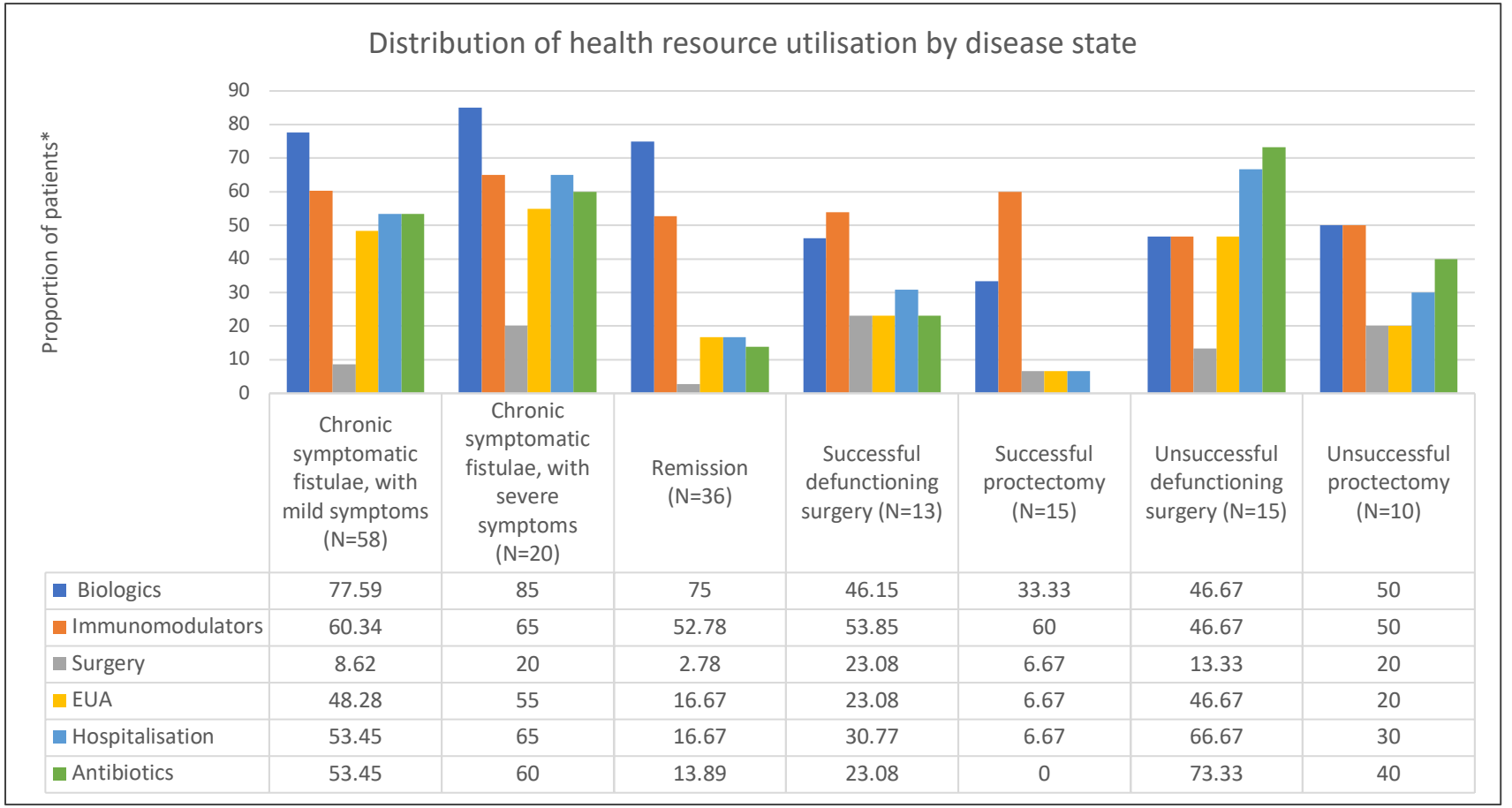
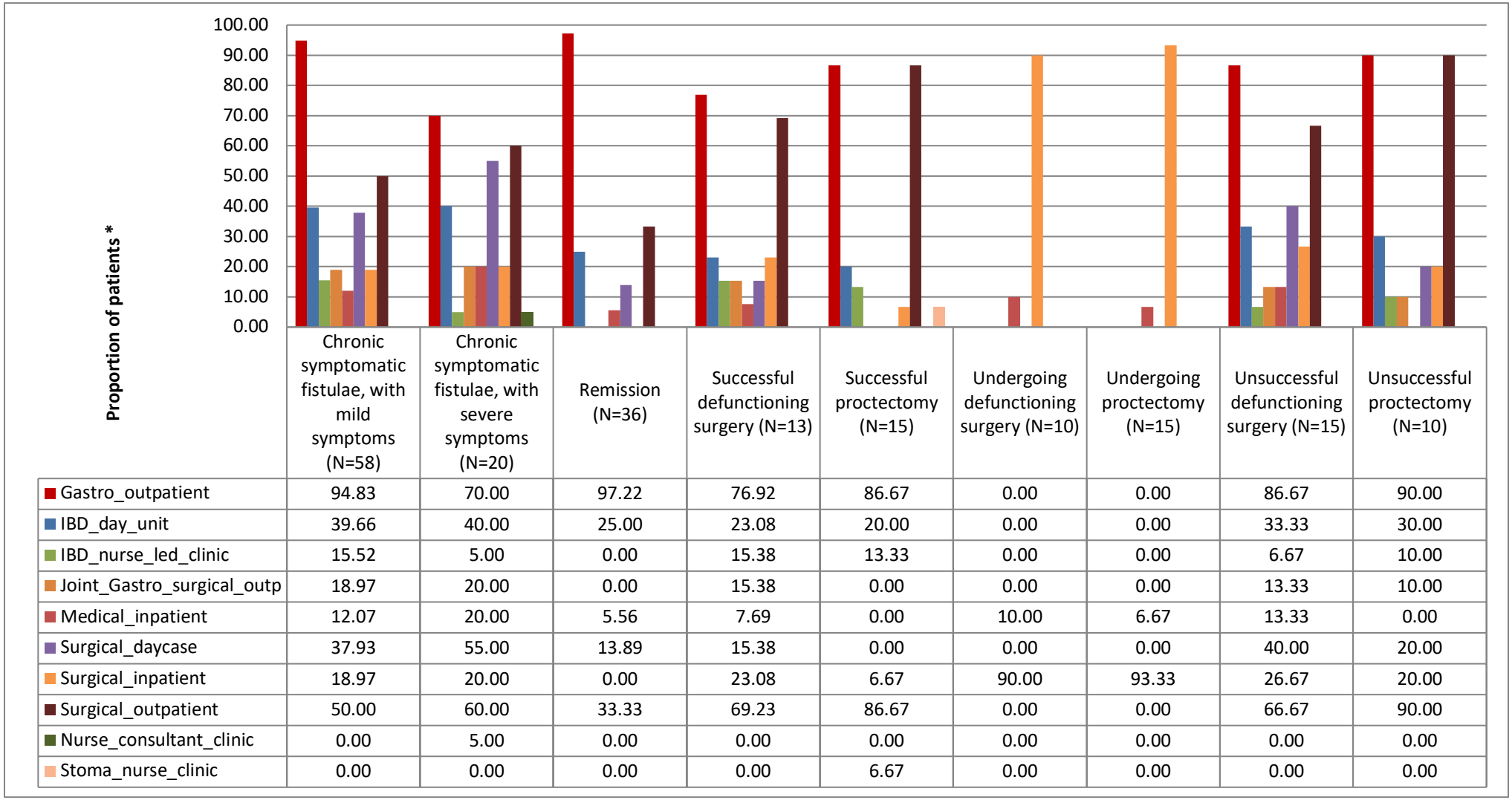


Figure 17: mean sojourn times for disease states



	Chronic symptomatic fistulae, with mild symptoms (N=58)	Chronic symptomatic fistulae, with severe symptoms (N=20)	Remission (N=36)	Successful defunctioning surgery (N=13)	Successful proctectomy (N=15)	Unsuccessful defunctioning surgery (N=15)	Unsuccessful proctectomy (N=10)
Biologics	77.59	85	75	46.15	33.33	46.67	50
Immunomodulators	60.34	65	52.78	53.85	60	46.67	50
Surgery	8.62	20	2.78	23.08	6.67	13.33	20
EUA	48.28	55	16.67	23.08	6.67	46.67	20
Hospitalisation	53.45	65	16.67	30.77	6.67	66.67	30
Antibiotics	53.45	60	13.89	23.08	0	73.33	40

Figure 18: Distribution of health resource utilization by health state
*the denominator corresponds to the number of patients in each health state
** includes definitive surgical procedures, e.g. VAAFT, advancement flap, LIFT etc.
*** with/without other intervetions, as seton insertion



	Chronic symptomatic fistulae, with mild symptoms (N=58)	Chronic symptomatic fistulae, with severe symptoms (N=20)	Remission (N=36)	Successful defunctioning surgery (N=13)	Successful proctectomy (N=15)	Undergoing defunctioning surgery (N=10)	Undergoing proctectomy (N=15)	Unsuccessful defunctioning surgery (N=15)	Unsuccessful proctectomy (N=10)
Gastro_outpatient	94.83	70.00	97.22	76.92	86.67	0.00	0.00	86.67	90.00
IBD_day_unit	39.66	40.00	25.00	23.08	20.00	0.00	0.00	33.33	30.00
IBD_nurse_led_clinic	15.52	5.00	0.00	15.38	13.33	0.00	0.00	6.67	10.00
Joint_Gastro_surgical_outp	18.97	20.00	0.00	15.38	0.00	0.00	0.00	13.33	10.00
Medical_inpatient	12.07	20.00	5.56	7.69	0.00	10.00	6.67	13.33	0.00
Surgical_daycase	37.93	55.00	13.89	15.38	0.00	0.00	0.00	40.00	20.00
Surgical_inpatient	18.97	20.00	0.00	23.08	6.67	90.00	93.33	26.67	20.00
Surgical_outpatient	50.00	60.00	33.33	69.23	86.67	0.00	0.00	66.67	90.00
Nurse_consultant_clinic	0.00	5.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Stoma_nurse_clinic	0.00	0.00	0.00	0.00	6.67	0.00	0.00	0.00	0.00

Figure 19: Distribution of visits by health state

Table 17: Average 'likelihood' (percentages) for each treatment across the disease states

Treatment	Remission	CSF Mild	CSF Severe	Successful Def.	Unsuc. Def.	Successful Proc.	Unsuc. Proc.
Infliximab	14.57%	24.30%	30.86%	11.20%	29.87%	7.65%	27.54%
Adalimumab	49.10%	40.56%	45.86%	26.58%	31.30%	24.79%	31.82%
Dose-escalated infliximab	3.24%	4.69%	5.57%	0.57%	8.57%	0.86%	2.86%
Dose-escalated adalimumab	8.00%	8.89%	3.43%	7.16%	18.10%	0.86%	11.43%
Methotrexate	8.33%	10.34%	5.00%	0.00%	6.67%	13.33%	0.00%
6-MP	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Metronidazole	12.80%	50.32%	59.82%	21.21%	66.07%	1.25%	37.32%
Ciprofloxacin	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Azathioprine	47.22%	52.99%	60.00%	53.84%	40.00%	51.43%	60.00%
Seton	8.33%	23.49%	18.57%	13.18%	13.66%	6.82%	2.86%
Fistulotomy	0.00%	1.72%	5.00%	0.00%	6.67%	0.00%	0.00%
Anal plug	0.00%	14.29%	10.00%	0.00%	0.00%	0.00%	0.00%
Fibrin glue	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rectal flap	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
VAAFT	0.00%	5.17%	10.00%	7.69%	0.00%	0.00%	0.00%
Vedolizumab	9.19%	10.22%	7.57%	8.59%	7.64%	0.43%	4.21%
EUA	11.12%	43.09%	55.00%	6.59%	37.38%	0.00%	24.29%
Biologic	86.11%	91.10%	92.50%	53.85%	63.33%	33.34%	60.00%
Dose escalation	11.11%	13.51%	7.50%	7.69%	16.67%	0.00%	10.00%

*Def., defunctioning; EUA, examination under anaesthesia; Proc., proctectomy; VAAFT-video-assisted anal fistula treatment Unsuc., unsuccessful

Table 18: Markov Multi-state model observed transitions

	to						
from	CSF, mild	CSF, severe	Remission	Successful defunctioning surgery	Unsuccessful defunctioning surgery	Successful proctectomy	Unsuccessful proctectomy
CSF, mild (N=612)	548 (89.5%)	22 (3.6%)	37 (6.1%)	4 (0.7%)	0 (0.0%)	1 (0.2%)	0 (0.0%)
CSF, severe (N=132)	13 (9.9%)	106 (80.3%)	0 (0.0%)	5 (3.8%)	3 (2.3%)	4 (3.0%)	1 (0.8%)
Remission (N=184)	32 (17.4%)	1 (0.5%)	151 (82.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Successful defunctioning surgery (N=94)	0 (0.0%)	0 (0.0%)	0 (0.0%)	87 (92.6%)	7 (7.5%)	0 (0.0%)	0 (0.0%)
Unsuccessful defunctioning surgery (N=144)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	135 (93.8%)	5 (3.5%)	4 (2.8%)
Successful proctectomy (N=135)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	128 (94.8%)	7 (5.2%)
Unsuccessful proctectomy (N=64)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (12.5%)	56 (87.5%)

5.5 Discussion

Tumour necrosis factor-α (TNFα) antagonists are a commonly used therapeutic option in inflammatory bowel diseases, and have changed the treatment landscape in Crohn's perianal fistula management, however, a significant proportion of patients either do not respond or lose response to treatment with time. Patients refractory to biologic treatment pose a management challenge and the considerable burden of disease and management in this cohort of patients, with limited longstanding cure represents an unmet need for alternative treatments (e.g. stem cell therapy) [244,398]. However, there are limited reports[402,411] of disease course and healthcare resource utilisation following failure of anti-TNF therapy, which hampers the ability to perform cost-benefit analysis of new interventions in this cohort. In this single tertiary centre study, the

majority of refractory patients are switched on to alternate anti-TNF therapy and most reside in a mild chronic symptomatic fistula disease state with a minority of patients subsequently transitioning into a severe disease state or remission state (Table 18). The remission rate for our study cohort of 78 patients was 46was46%, however the sustained (1 year1year or more) remission rate was 27%. Some 19% of patients underwent proctectomy. There was considerable healthcare resource utilisation amongst all disease states particularly with biologic therapy, which is the most expensive healthcare resource.

There is a paucity of data providing insight into the durability of Crohn's perianal fistula treatment with infliximab for periods longer than 12 months and even less data for the other anti-TNF agents as well as newer biologic therapies. However, current evidence suggests that response may wane with long-term treatment[326,412]. The overall response rate to a second anti-TNF agent in patients refractory to their first ranges between 50–65%[402,411] and this depends on the definition of response, which is heterogenous in the Crohn's perianal fistula literature. Current estimates from analysis of infliximab studies suggest that approximately 40 % will lose response to infliximab, with an annual risk of loss of response to infliximab calculated to be about 13% per patient-year of treatment[326,412]. Similar assessments have been performed for adalimumab, using dose escalation as a surrogate for loss of response in CD, with an incidence of 24% across randomized trials and observational series[411]. This suggests a variable but relevant proportion of CD patients on long-term anti-TNF treatment lose response. Whether any factors (apart from perhaps anti-drug antibodies) reliably predicts loss of response remains unclear[326]. Current opinion advices therapeutic drug monitoring for determining whether dose intensification, addition of immunomodulators, or switching therapies is most appropriate. Measures can be taken to prevent loss of response including use of concomitant immunomodulators, corticosteroid pre-treatment, early dose optimization, and regularly

scheduled use of the TNFα antagonist. In our study group, 46/78 (60%) refractory patients had been on concomitant immunomodulators and 37/78 (48%) had been on pre-treatment corticosteroid. Prospective clinical trials are needed to determine whether all of these interventions together can substantially lead to a decrease in loss of response[411].

One particularly interesting finding is the rate of success when defunction and proctectomy are performed (62% and 80% respectively). These are valuable data for discussing with patients when considering diversion or ablative surgery, particularly from the point of view of discussing defunctioning as inadequate, in some cases, to bring about the improvement in QoL sought. We can now suggest that that number may be around 40%.

The strengths of this study lie in the utilisation of disease states and healthcare resource utilisations to characterise the burden of disease in a cohort of refractory Crohn's perianal fistula patients. This creates a platform for further work assessing cost-effectiveness of treatment strategies, with the aim of identifying the best treatment strategy for Crohn's perianal fistula. Few studies have addressed this specific area (treatment for refractory patients), although some European studies have reported on health care costs for complex perianal fistula in general, highlighting the main driver being pharmacological therapies, particularly biologic agents, with minimal contributions from surgery/hospitalisations[413,414]. Furthermore a lot of the disease states informing these studies are derived from trial data[413], which don't always mirror real world data as presented in our study. There are some limitations of our study. It was retrospective in terms of the data acquisition as well allocation of disease health states. There were no data collected on adverse effects of biologic therapy and there was a lack of data on antibody to infliximab or infliximab plasma levels, which are known to effect loss of response to the anti-TNF drugs. The absence of prospective QoL data also limits the richness

of the data. An understanding of this and the economic impact of the disease states outside of the healthcare setting, for example days lost to work, the use of sanitary equipment and so on, would create a fuller picture of the true economic cost and symptomatic burden of the different disease states and refractory disease overall.

In summary, our study defines the disease course for patients refractory to anti TNF agents for Crohn's perianal fistulas, using disease states from a vignette study that were reviewed and agreed by an expert consensus team involved in specialist management of Crohn's perianal fistulas. This was done in the context of limited knowledge of real world data on disease course following non/loss of response to biologic therapy and the need for understanding healthcare resource utilisation in these refractory patients, to guide management decisions and inform consideration of novel interventions (e.g. stem cell therapies, novel sphincter sparing treatments, etc) in light of the economic impact of the various disease states..). There is currently no cost-effectiveness evidence on the sequential use of biological treatment in Crohn's perianal fistulas. Health gains and costs of these treatment sequences need to be analysed and compared to identify the most cost-effective treatment strategy, particularly in light of biosimilars[413]. Future directions might involve developing / building a comprehensive registry with a consensus on defined disease states in this challenging condition. This is an often discussed resource that has had previous commercial drives and developed centralised backing with the launch of the National IBD Service Standards in 2009 and the first UK wide registry was launched in 2013. However, improvements are still required particularly with regard to clinician participation and interest, costs of maintaining the registry and data quality assurance[415]. Other future directions include the evaluation of biomarkers of treatment response which would facilitate stratification of patients and prevent prolonged, expensive and futile treatment in those who won't benefit.

Chapter 6. Lack of anti-TNF drugs levels in fistula tissue – a reason for non-response in Crohn's perianal fistula?

6.1 Abstract

Introduction:

Anti-TNF therapy is recommended as treatment for patients with Crohn´s perianal fistulas. However, a significant proportion of patients have a sub-optimal response to anti-TNF therapy. Higher serum levels of anti-TNF agents have been associated with improved outcomes in perianal Crohn's disease. Currently it is unknown whether anti-TNF agent levels can be detected in tissue from fistula tracts themselves and whether this is associated with response.

Aims and Methods:

We undertook a pilot study to develop a method to measure fistula tissue levels of anti-TNF medication (infliximab and adalimumab) using a targeted proteomic technique that employs 'signature peptide detection' following trypsin digestion called ultraperformance liquid chromatography mass spectrometry (UPLC-MS), to quantify a protein. The *targeted UPLC-MS/MS* detection and quantification method implemented was previously validated and MS parameters were optimised to detect specific peptide sequences (signature peptides) from each anti-TNF drug (infliximab / adalimumab) present in fistula tissue samples. Biopsies were obtained from patients with Crohn's disease who underwent examination under anaesthesia for worsening fistula symptoms despite maintenance anti-TNF therapy. Idiopathic (cryptoglandular) tissues were analysed as negative controls and these samples were spiked with anti-TNF drugs as positive controls. The proteins present in fistula samples were extracted

and digested with trypsin to obtain peptide fragments specific to each drug. These were then analysed by UPLC-MS.

Results: Tissue was sampled from the fistula tracts of seven patients with Crohn's perianal disease (5 patients were on adalimumab and 2 patients were on infliximab). Additionally, tissue from the fistula tracts of seven purposively sampled matched (age/gender) patients with idiopathic fistulas were used as controls and anti-TNF drugs were added to these samples as positive controls. The limit of detection (LOD) and linearity range of the method was assessed for each drug in the spiked idiopathic fistula samples. Infliximab and adalimumab had a LOD of 0.004 and 2 μg/ml respectively with linearity demonstrated for both drugs. The anti-TNF drugs, infliximab and adalimumab, were not detected in fistula samples from any of the Crohn's patients despite detection in 'spiked' positive control samples. In addition, to validate the result, samples were concentrated (x10) and still there was no detection of the drugs in the test samples.

Conclusion: The anti-TNF drugs adalimumab and infliximab were not detected in fistula biopsy samples from patients with Crohn´s perianal fistulas with refractory symptoms despite maintenance therapy. This raises the question on the role of tissue penetrance of anti-TNF drugs in response to therapy. Further work is required in a larger number of patients to validate the findings observed and investigate whether any correlation exists between tissue and serum levels of anti-TNF and clinical outcome.

6.2 Introduction

Perianal fistulising Crohn's disease (PFCD) represents a distinct phenotypic manifestation in a third of patient's with Crohn's disease. It follows a chronic course with varying symptoms, including anal pain, purulent discharge and incontinence, and can be associated with high morbidity and an impaired quality of life[416]. This issue assumes particular relevance in younger patients and the associated morbidity of the disease and its treatment modalities can have profound effects on patients. Not only are these effects physical, but they can also affect patients' social lives, educational activities, professional lives and intimate relationships. Perianal fistulising Crohn's disease (PFCD) represents a distinct phenotypic manifestation in a third of patient's with Crohn's disease. It follows a chronic course with varying symptoms, including anal pain, purulent discharge and incontinence, and can be associated with high morbidity and an impaired quality of life[416]. The associated morbidity of the disease and its treatment modalities can have profound effects on patients. Not only are these effects physical, but they can also be far reaching in terms of effect on emotional and social wellbeing[417].

Anti-TNF therapy (e.g. infliximab / adalimumab) is the medical treatment of choice for perianal Crohn's fistulas. However, long term success rates are modest[368,416]. About a third of patients do not respond to anti-TNF therapy (primary non-responders) and almost half of the patients who experience a benefit with these drugs will lose response within the first year (defined as requiring dose change or therapy change)[323,368,411]. This initial response and subsequent loss of response is termed "secondary loss of response" (and the patients are termed secondary non-responders). The reasons why some patients do not respond or lose response after a successful course of therapy is not completely clear but is likely multifactorial and related to metabolism of the drug, the development of antidrug antibodies (immunogenicity),

and possibly other unknown factors[323]. Anti-TNF therapy (e.g. infliximab / adalimumab) is the medical treatment of choice for perianal Crohn's fistulas. However, long term success rates are modest[368,416]. About a third of patients do not respond to anti-TNF therapy (primary non-responders) and almost half of the patients who experience a benefit with these drugs will lose response within the first year (defined as requiring dose change or therapy change)[323,368,411]. This initial response and subsequent loss of response is termed "secondary loss of response" (i.e. secondary non-responders). The reasons why some patients do not respond or lose response after a successful course of therapy is not completely clear but is likely multifactorial and related to metabolism of the drug, the development of antidrug antibodies (immunogenicity), and possibly other unknown factors[323].

Yarur et al. [336] shed some light on potential tissue factors associated with response in luminal Crohn's disease. They reported accurate measurement of tissue levels of anti-TNF drug in luminal Crohn's disease and found that this correlated well with serum levels. They also found that in areas of severe luminal inflammation, the latter acts like a 'sink' for the drug. They suggested this may explain persistent active disease in some patients despite 'therapeutic' drug levels, and further that patients with areas of increased inflammation may need higher dose of drug (anti-TNF), to increase the concentration in those areas of excessive inflammation[336]. More recently the same authors[343], reported higher levels of anti-TNF (infliximab) doses being required for perianal fistula healing/closure than those required for mucosal healing in luminal disease[343,418,419].

If higher serum levels are required to treat anal fistula, and serum levels correlate with tissue levels in luminal disease, but the inflammatory burden might 'subsume' the drug in one area, reducing its concentration and therefore efficacy in another, then might insufficient drug be

reaching the perianal tissues, and can this be measured? No study has assessed fistula tissue levels of anti-TNF, and it is unknown whether this may act as a potential biomarker of treatment response.

Two broad techniques are used to detect and quantify tissue levels of anti-TNF agents: affinity-reagent methods and mass spectrometry (MS). The affinity-reagent methods encompass multiple assays that have been used to measure levels of serum anti-TNF (i.e. monoclonal antibodies)[323]. These include ELISA (enzyme linked immunosorbent assay), HMSA (homogenous mobility shift assay), fluid phase radioimmunoassay and cell-based reporter gene assay[323,420–422]. However, the range and number of proteins quantifiable using affinity-reagent based assays is limited. The other technique increasingly being used is mass spectrometry (MS) based protein identification and quantification (based on signature peptide identification), in a technique known as 'targeted proteomics'[423424]. This technique detects and determines a protein of interest at the peptide level, and uses it as a surrogate analyte following its generation from proteolytic digestion of the protein[425]. It involves the development and use of liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) assays, to detect fragment ion signals from unique signature peptides representing the targeted protein [426,427]. MS techniques have greater precision/accuracy, ability to measure multiple analytes and improved specificity in comparison to the affinity-reagent methods, however, there is not yet a standardised method that allows cross-comparison validation across different studies/laboratories[427–429]. The development of modern mass spectrometry has led to increasing application in protein quantification particularly in view of its wide dynamic range and improved performance[430]. The sensitivity of the platform is particularly attractive when attempting to study fistula tissue levels, as it is envisaged that the drug levels detected are likely to be in lower concentrations than those found in serum.

We adopted a proteomics approach to assess fistula tissue levels of anti-TNF (infliximab and adalimumab) in patients on maintenance anti-TNF therapy for Crohn's perianal fistulas with refractory symptoms. We utilised a platform of liquid chromatography tandem mass spectrometry (LC-MS/MS) in order to attempt detection and quantification of these anti-TNF drugs in fistula tract biopsy samples based on a validated method of quantification of infliximab in human serum[431].

6.3 Materials and Methods:

The study was given ethical approval for conduct by the NHS Health Research Authority (HRA – NRES Committee London – Brent, Ref: 08/H0717/24). Recruitment was via purposive sampling of patients with idiopathic perianal fistulas or perianal fistulas and Crohn's disease requiring examination under anaesthesia for their fistula. Included patients were those on maintenance treatment with infliximab or adalimumab for the primary treatment of Crohn's perianal fistula, as well as those with idiopathic anal fistulas (not on anti-TNF treatment for any disease). Enrolment was done between January of 2016 and December of 2017. All Crohn's anal fistula patients were on maintenance therapy and had completed induction with infliximab (5 mg/kg at weeks 0, 2 and 6) or adalimumab (160 mg at week 0 and 80 mg at week 2). The maintenance dose for infliximab was 5 mg/kg every 8 weeks. For adalimumab maintenance, 40 mg was given every other week. Dosing of the biologics was determined by the treating gastroenterologist. All patients underwent clinically indicated examination under anaesthesia for worsening fistula symptoms despite maintenance anti-TNF therapy.

For all participants included, demographics including age, gender, medical history, and medication use (including concomitant immunosuppression) were ascertained via discussion with patients and review of online medical record on the day of examination under anaesthesia / fistula biopsy. Serum levels are not routinely ordered and were recorded where available. Intraoperatively, full-thickness fistula tract biopsies (fig. 1) were taken from the central aspect of fistula (provided the fistula tract was non-epithelialised). Tissue biopsies were immediately deposited in cryovials and snap frozen with liquid nitrogen and transferred on dry ice then stored at − 80 °C until analysis.

6.3.1 Target proteomic (LC-MS/MS) analysis, signature peptide selection and data acquisition for drug detection / quantification

LC-MS/MS methods were used to analyse the tissue samples. We performed a 'bottom-up' (Figure 20) approach [432] to targeted proteomics whereby the protein is extracted from the bio-sample (serum / fistula tract tissue) and subjected to enzymatic cleavage whereupon resulting peptides (Figure 21) are analysed via mass spectrometry [432]. The workflow for the anti-TNF assay is depicted in Figure 20 and demonstrates the strategy for identification of the specific (unique) peptide relating (in our case) to the anti-TNF: Infliximab (Remicade®) and adalimumab (Humira®).

MS analysis of fistula tract tissue as a biomatrix for detection of anti-TNF has not previously been described in the published literature, and consequently a validated LC–MS/MS analytical assay that was used to detect serum infliximab was replicated, tested and modified in this study[431]. The method has been validated with credibility in assay performance markers, including selectivity, accuracy, precision, carry-over, stability, linearity range, recovery as well as the matrix effect[431]. There were no prior published validated detection/quantification

studies on adalimumab and hence a similar technique/principles was used as for infliximab, with peptide detection (specific to adalimumab) obtained from a manufacturer's application note, by Waters (Waters Corp., Milford, MA, USA) [433]. To confirm ability to detect/quantify infliximab and adalimumab) several LC-MS/MS analytical experiments were performed. Serum and water calibration curves were created using serial dilutions of known concentration of anti-TNF in each fluid to assess the corresponding dose and determine linearity in drug detection. Following this set of experiments, the drug was spiked in a tissue sample from an idiopathic Crohn's anal fistula patient (i.e. not on anti-TNF therapy) in order to confirm drug detection in spiked tissue sample using the LC-MS/MS method. Finally, the method was used to analyse Crohn's anal fistula tract tissue samples from patients on infliximab (two) or adalimumab (five) maintenance therapy. Negative control samples (fistula tissue from idiopathic fistula, i.e. no-anti-TNF treatment) and positive control (idiopathic fistula tissue samples spiked with infliximab / adalimumab) were also analysed for drug detection. A detailed description of sample preparation and workflow for the targeted proteomic analysis is presented in the Supplementary Methods (Appendix 2 – Chapter 14, section 14.1).

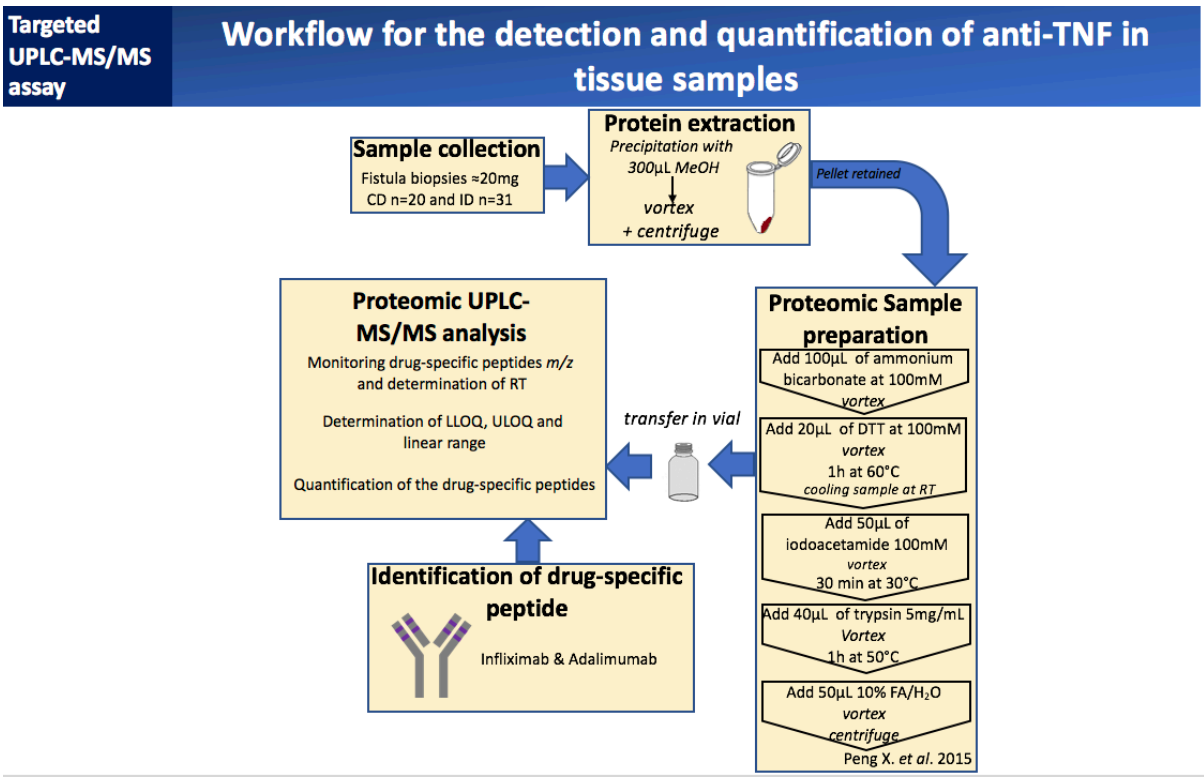


Figure 20: workflow for detection and quantification of anti-TNF (infliximab and adalimumab in fistula tract biopsy samples

*LLOQ – lower limit of quantification, ULOQ – upper limit of quantification, DTT – Dithiothreitol, FA – Formic Acid, RT – retention time m/z – mass to charge ratio H_20 - water

6.3.1.1 *Signature peptide selection*

Antibodies are too heavy (~149 kDa) to be directly quantified using standard LC–MS assays. However, it is possible to detect unique marker peptides specific to the antibodies). There is no known metabolite for monoclonal antibodies[434,435] and as such the assumption made is that all active drugs have the same corresponding peptide sequences. Tryptic peptides (peptides digested by trypsin) used for quantification were initially identified via literature search. These were then assessed during optimisation runs to assess for good chromatographic (UPLC) separation as well as detection on mass spectrometry with regard to ionisation / signal intensity, response to dilution and detection in biological matrix (tissue, via positive controls). Two surrogate peptides for each anti-TNF were selected. F, or infliximab, the peptide selected had previously been confirmed as unique in human serum and had been validated with proven high sensitivity and specificity[431]. For adalimumab, the sequence we chose has previously been

used for adalimumab quantification experiments, described in an application note by Waters (Waters Corp., Milford, MA, USA) [433]. However, in order to verify this, a standard technique of online unique peptide identification was employed. Predicted surrogate peptides were obtained for adalimumab using the silico trypsin digestion [PeptideMass, http://web.expasy.org/peptide_mass/]. These were then compared with the amino acid sequences unique to human proteins using BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi] to target unique peptides specific to adalimumab.

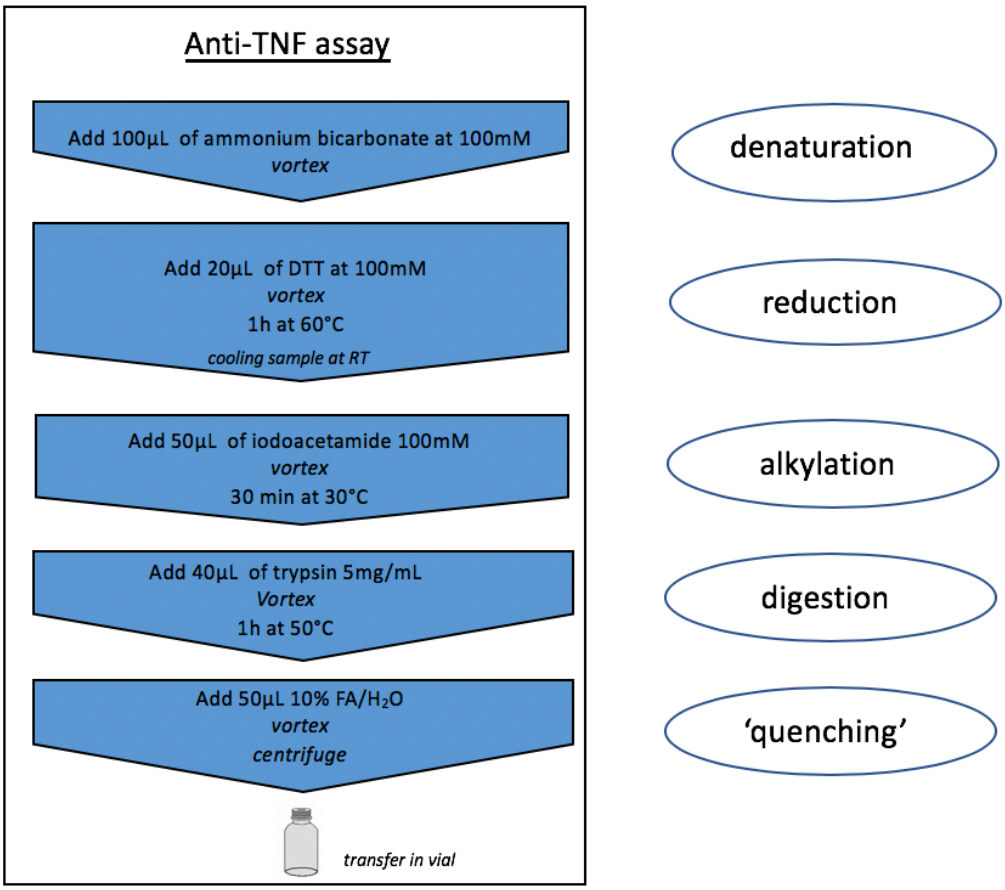


Figure 21: process of protein denaturing and digestion forming signature peptides
These are then introduced to a liquid chromatography (LC) column for separation. Upon eluting from the LC column, peptides are ionized
* DTT – Dithiothreitol, FA – Formic Acid,; H_20 – water

6.3.1.2 *Data acquisition of signature peptides for infliximab / adalimumab*

Data acquisition was performed with MassLynx 4.1 (Waters, Milford, MA). Acquisition was performed on the MRMs transitions specific to each drug (infliximab / adalimumab). In MRM

assays(Figure 22), the first (Q1) and last (Q3) mass analyzers of a triple quadrupole mass spectrometer are used as mass filters to isolate a peptide ion and a corresponding fragment ion. The signal of the fragment ion is then monitored over the chromatographic elution time (Figure 22). The selectivity resulting from the two filtering stages, combined with the high duty cycle, results in quantitative analyses with unmatched sensitivity and specificity. The specific pairs of m/z values associated to the precursor and fragment ions selected are referred to as "transitions" and effectively constitute mass spectrometric assays that allow the identification and quantification of a specific peptide and, by inference, the corresponding protein in a complex protein digest[436,437]. Mass-dependent MRM ion transitions were replicated for infliximab (based on prior published data on infliximab quantification in serum[431] and triple-quadrupole parameters for the adalimumab target peptide were optimized and based on an application note by Waters (Waters Corporation, Milford, MA, USA) for serum quantification[433].

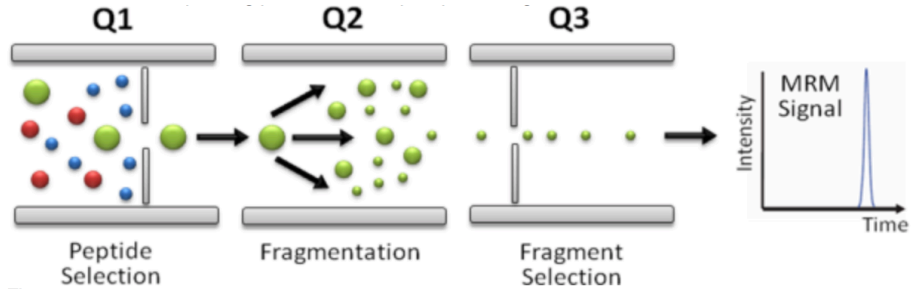


Figure 22: Ion fragmentation and filtering through the quadrupole Q1-3
Adapted from Schmidt et al. 2008 [436].

6.4 Results

Demographic and disease details for the seven Crohn's patients assessed are reported in Table 19. Two patients undergoing infliximab therapy and five patients undergoing adalimumab therapy had their fistula tract biopsies analysed for drug detection and quantification.

Table 19: Characteristics of Crohn's perianal fistula patients

Patient	Sex	Age (years)	Duration of CD (years)	Duration of perianal fistula (years)	Previous fistula surgery (excluding abscess drainage)	Current Stoma	Medication for fistula	Dosing regimen	On concomitant thiopurine (yes/no)	Serum Anti-TNF Drug level (microgram/ml)
1 (CDM04)	Male	22	4	4	Yes	No	Infliximab	5 mg/kg every 8 weeks	Yes	10.0
2 (CDM13)	Male	53	9	5	Yes	Yes	Infliximab	5 mg/kg every 8 weeks	Yes	3.0
3 (CDM03)	Male	39	25	*18*	Yes	Yes	Adalimumab	40 mg every other week	Yes	7.3
4 (CDM05)	Female	24	16	16	Yes	No	Adalimumab	40 mg every other week	No	NA
5 (CDM08)	Female	33	8	3	Yes	No	Adalimumab	40 mg every other week	No	NA
6 (CDM18)	Female	29	4	4	Yes	No	Adalimumab	40 mg every other week	No	NA
7 (CDM20)	Female	27	8	8	Yes	No	Adalimumab	40 mg every other week	No	NA

NA – Not available

Figure 23 demonstrates the confirmation of detection of infliximab specific peptide on triplicate samples of serum and the selectivity was confirmed with the absence of detection of the peptide in the blank control triplicate samples. In the blank samples, no signal was detected that could be confused with the signature peptide ion MRM transitions selected to monitor infliximab concentration [469.6 (3+)→603.8(2+)]. Similar findings were confirmed for adalimumab with transitions at 535.3(2+)→901.44(+).

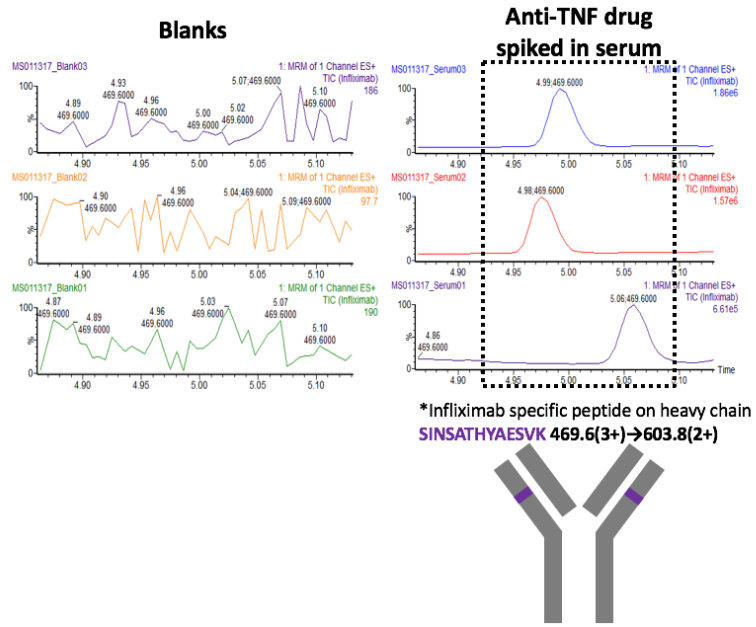


Figure 23: confirmed detection of MRM transition for infliximab specific peptide SINSATHYAESVK

The serial dilutions of infliximab and adalimumab in water were used to determine the calibration curve equation for the quantitation of each drug. The range of linearity was also calculated at 0.004-40 µg/mL for infliximab and 2 – 200 µg/mL for adalimumab (Figure 24).

Analyte	Calibration curve	r^2	Linear range µg/mL
Infliximab	y=22629x-3442.7	0.958	0.004-40
Adalimumab	y=58.2x+755.13	0.979	2-200

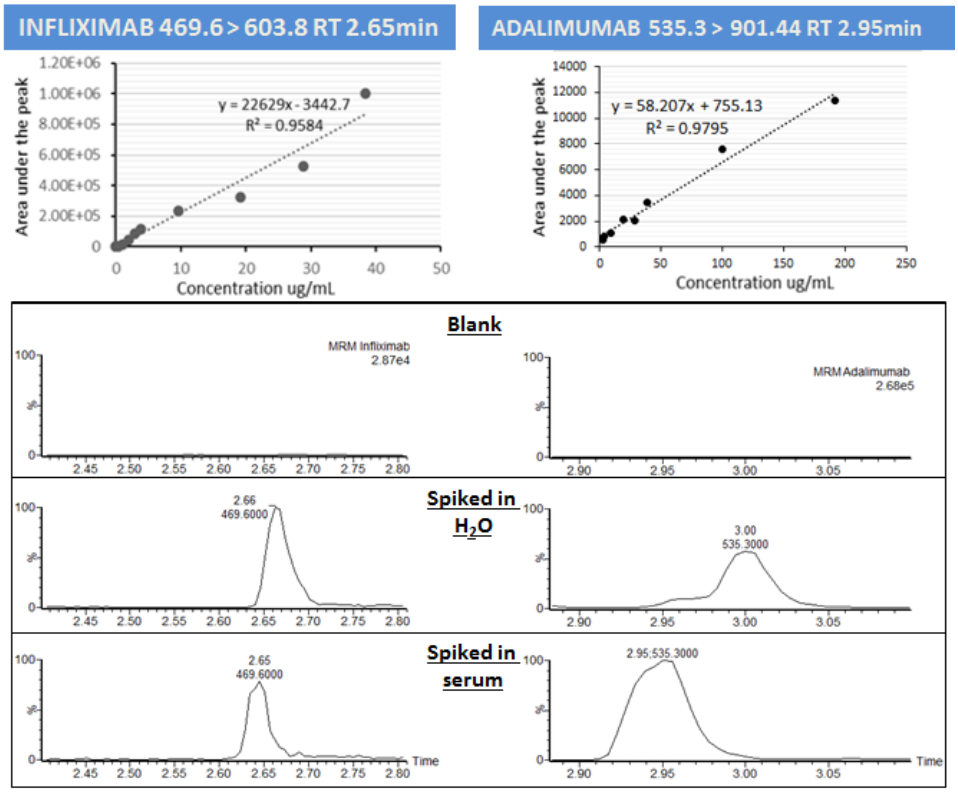


Figure 24: Demonstrates detection Multiple Reaction Monitorings (MRMs) and retention times (RT) for anti-TNF signature surrogate peptides
Results are depicted for infliximab and adalimumab in negative (blank – methanol) and positive (serum / spiked water) sample controls. The linearity is also demonstrated for both drugs.

6.4.1 Findings from Crohn's fistula biopsies

There was complete absence of detection of either infliximab or adalimumab in the fistula tract biopsies of any of these patients. Representative chromatograms are shown in Figure 25 and these are in contrast to the positive controls (fistula tissue spiked with infliximab /adalimumab – Figure 26).

Duplicated idiopathic and fistula samples were analysed. The anti-TNF drugs were not detected in fistula samples. In addition, to validate the result samples were concentrated (x10) and still no detection of the drug was observed in the samples from patients on infliximab or adalimumab therapy.

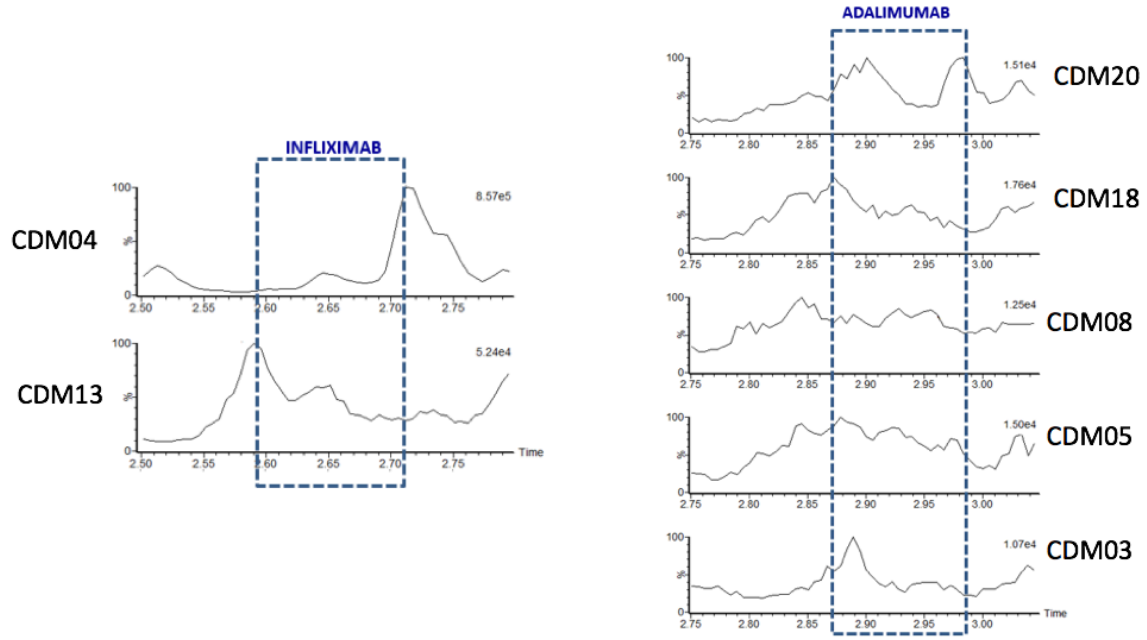


Figure 25: Negative chromatograms (no drug signal) in test Crohn's perianal fistula samples
Negative chromatograms (no drug signal) signifying absence of detection of infliximab in the two tissue samples assessed and absence of adalimumab in all of the five tissue samples assessed.

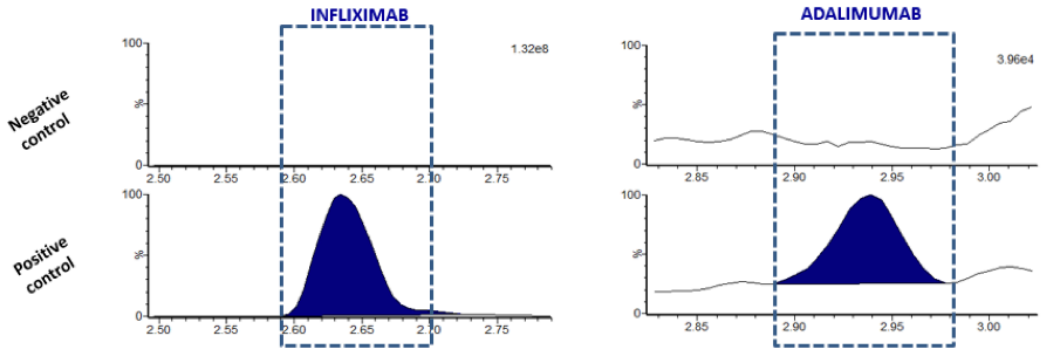


Figure 26: Chromatograms from negative and positive controls
Super-imposed chromatograms from negative controls (idiopathic fistula tissue with no infliximab / adalimumab) and comparative detection of infliximab / adalimumab in positive control (idiopathic fistula tissue spiked with infliximab and adalimumab).

6.5 Discussion

This pilot study demonstrates that infliximab and adalimumab were both feasibly quantifiable using the LC/MS method which was adapted specifically for this study. I have shown that it is possible to detect infliximab and adalimumab in fistula tract tissue using these techniques, although the only positive findings of the drug were when it had been spiked into the tissue immediately before. The linear detection range (LDR) was 0.004 – 40μg/ml for infliximab and 2 – 200 μg/ml for adalimumab. None of the seven patients on anti-TNF for Crohn's anal fistula had any detectable unique peptides for infliximab or adalimumab, consistent with an absence of anti-TNF detection in tissue from their fistula tracts. This inability to detect anti-TNF in the absence of known immunogenicity might mean a genuine absence of the anti-TNF drugs in perianal Crohn's fistula tissue of patients on maintenance treatment. This certainly appears to be the case in the 3 patients (CDM04, CDM13, CDM03), all of whom had had therapeutic serum levels according to recent guidance[438]. Potential reasons for this absence may be due to inadequate penetration of anti-TNF drug into perianal fistula tissues, however, it could also reflect an excessive consumption of anti-TNF agents by perianal fistula tissue.

The principal alternative to this explanation is a failure of the technique or assumptions. It may be, for example, that the peptides detected are only found soon after the drug first reaches the tissues and that they are undetectable weeks later despite the drug being present and detectable via alternative peptide signatures.

The expected levels of anti-TNF agent (or its metabolites) required in the perianal tissue for fistula healing is currently unknown and no published studies have reported on fistula tissue

detection / quantitation of anti-TNF as a biomarker of healing or indeed treatment response. There have been reports that higher serum (trough) levels are associated with favourable fistula response to anti-TNF[439] as well as fistula healing[343] and furthermore local treatments of fistula with injection of anti-TNF (infliximab / adalimumab) have suggested positive 'fistula response'[440]. This suggests there may be a role for local action of anti-TNF, and raises questions as to whether there is positive correlation with serum levels and whether levels of either may be of significance in determining treatment outcome. It is important to note that none of the studies reporting on local therapy reported on tissue levels and the only tissue assessment of anti-TNF in the context of treatment for Crohn's disease was done by Yarur et al[336], in luminal Crohn's disease. They reported detectable levels of anti-TNF in intestinal biopsy tissue and its correlation with endoscopic degree of inflammation[336]. Anti-TNF to TNF ratios were found to be inversely proportional to the degree of inflammation. It is thus possible to hypothesise that similar ratios may serve as a marker of response to therapy in Crohn's anal fistula, particularly as inflammation is thought to be an important factor in their aetiopathogenesis[40,70]. In the context of Crohn's anal fistula treatment, the authors hypothesize that failure or non-response may also be related to inadequate / absent tissue levels of the drug, and this is consistent with the findings discussed above, that higher serum levels are required for perianal than luminal response, that luminal disease often settles but perianal disease persist and that perianal disease is more likely to recur after cessation of treatment than luminal disease. It may simply be the case that anti TNF agents penetrate the perianal tissues less effectively, that levels required for efficacy in perianal tissues are greater than in luminal disease (perhaps because of the inflammatory sump effect described above), or that factors within the perineum degrade the anti TNF agents or their mechanism of action. Any of these explanations would fit with our findings. However, so too would a failure of our technique, as

described above. Future work in this area, underway within the unit, must try to unpick whether these findings are technical or real, and if real, to understand the mechanism of inaction.

The strength of our study lies in the uniqueness of replicating and optimising (using different precipitants / protein digestion conditions) previously validated techniques[218,424,431,441] for anti-TNF detection / quantitation and application in a previously unexplored biomatrix (i.e. perianal fistula tissue) in Crohn's disease. There are, however, several limitations in our study. Firstly, the low number of patient samples tested as a proof of principle of assessment of tissue levels of anti-TNF in patients undergoing therapy for Crohn's anal fistula, although the uniform non-detection of drug in tissues adds some confidence. Also, not all patients had serum levels assessed. Another limitation was the absence of analysis of tissue from normal rectal mucosa close to the fistula internal opening and at a reasonable distance in the anorectum free from inflammation as a comparator. These multiple sites may have given an indication of detection of tissue levels of anti-TNF in these 'control' sites in the same patient, adding greater validity to our findings. Technical aspects of protein quantification may also have been a limitation in our findings, as we have not compared different methods in the detection analysis. The study by Yarur et al.[336] used the homogenous mobility shift assay in their assessment of luminal tissue levels of anti-TNF and had a higher limit of quantitation of infliximab of 1.0 μg/ml (compared to our study findings of 0.004 μg/ml) and similar levels of adalimumab, 1.6 μg/ml (compared to our study findings of 2 μg/ml). It is, however, likely that the levels are not comparable between the studies due to the lack of standardisation of dosing calculations in general and hence, it may be useful to compare immunoaffinity (e.g. ELISA) based techniques, and MS techniques in the detection / quantification in future studies that address tissue levels of anti-TNF assessment. Application of LC-MS in pharmacokinetics and therapeutic drug monitoring is increasing, particularly for monoclonal antibody measurement[424,441] and there

are increasing methods being reported of improving sensitivity without compromising specificity. These include kit-based approaches for facilitating sample digestion and importantly peptide level clean-up and up-front combination of immunoaffinity enrichment approaches with LC-MS techniques to improve accuracy of detection and minimise effects of background proteins in the bio-sample [433,441–443].

A more general limitation is the fact that the outcomes of fistula treatment are less clearly defined and subject to heterogeneity in most fistula studies[444] and the classification of the degree of inflammation in the context of fistulas is less distinguishable than that of luminal Crohn's disease (endoscopically). This makes it difficult to define comparator groups to assess response of inflammation in the fistula relative to tissue levels of drug. We also do not understand the mechanism of action of anti-TNF agents in the perianal tissues. For example, that luminal disease can improve whilst perianal disease persists may not be due to lack of penetration of the agent into perianal tissues at all, and measuring the levels of the same peptides seen in the serum may be based on incorrect assumptions, as discussed above. However, given that the same peptides are identified and associated with inflammation in luminal tissue levels, searching for them in perianal tissue seems reasonable.

In conclusion, this pilot study involved the development of a novel technique for the detection of anti TNF agents in fistula tract tissue, and reports the absence of detection of infliximab / adalimumab in tissue biopsies from fistula tracts of patients with perianal Crohn's fistula on maintenance anti-TNF treatment using an LC-MS/MS methodology. It remains unclear whether anti-TNF levels are likely to be a clinically meaningful biomarker for treatment response in Crohn's anal fistula. Anti-TNF therapies are an important treatment option and assessing response to treatment through laboratory data may improve and personalise patient

care while substantially reducing health-related costs. Further studies with larger numbers and multiple biopsy sites, also correlating serum and tissue levels would aid in improving knowledge as to whether tissue biopsies can help in the determination of treatment response and potentially restrict futile expensive therapy in some patients.

SECTION C - Evaluating novel / minimally invasive surgical treatment strategies in Crohn's perianal fistulas

Chapter 7. Short-term efficacy and safety of three novel sphincter-sparing techniques for anal fistulas: a systematic review

7.1 Abstract

Background:

The surgical treatment of complex anal fistulas, particularly those involving a significant portion of the anal sphincter in which fistulotomy would compromise continence, is challenging. Video-assisted anal fistula treatment (VAAFT), fistula tract laser closure (FiLaC™) and over-the-scope clip (OTSC®) proctology system, are all novel sphincter sparing techniques targeted at healing anal fistulas. In this study, all published articles on these techniques were reviewed to determine efficacy, feasibility and safety.

Methods:

A systematic search of major databases was performed using defined terms. All studies reporting on experience of these techniques were included and outcomes (fistula healing and safety) evaluated.

Results:

Eighteen studies (VAAFT – 12, FiLaC™ – 3, OTSC® – 3) including 1245 patients were analysed. All were case series and outcomes were heterogeneous with follow-up ranging from 6 to 69 months and short-term (<1yr) healing rates of 64–100%. Morbidity was low with only minor complications reported. There was one report of minor incontinence following the first reported study of FiLaC™ and this was treated successfully at 6 months with rubber band

ligation of hypertrophied prolapsed mucosa. There are inconsistencies in the technique in studies of VAAFT and FiLaC^{TM}.

Conclusions:

All three techniques appear to be safe and feasible options in the management of anal fistulas and short-term healing rates are acceptable with no sustained effect on continence. There is however a paucity of robust data with long-term outcomes. These techniques are thus welcome additions, however, their long-term place in the colorectal surgeon's armamentarium, whether diagnostic or therapeutic, remains uncertain.

7.2 Introduction

Anal fistulas have a longstanding place in the history of challenging surgical pathologies [445]. Broadly speaking, most simple fistulas can be laid open with a limited risk of minor continence disturbance.

Whilst fistulotomy will cure a 'high' fistula [446], the increasing concern is the risk of impairment of continence. Furthermore, it is noteworthy that even for simple fistulas, fistulotomy may cause functional disturbance which some patients find unacceptable [445,447]. Thus, to minimize the functional dilemma in fistula surgery with curative intent, several 'sphincter-preserving' techniques have been described which include fibrin glue, anal fistula plug (AFP), anorectal advancement flap (ARAF), and ligation of the intersphincteric tract (LIFT) procedure. These have had initially promising but variable success rates in the published literature. None has been universally accepted as the gold standard surgical approach; one which can offer the success of fistulotomy without the risk of functional deficit.

More recently video-assisted anal fistula treatment (VAAFT) [132], over-the-scope (OTSC®) [448] proctology clip system and fistula tract laser closure, FiLaC™ (using a radial-emitting laser probe) [131], have been added to the surgical armamentarium as new sphincter-preserving techniques.

VAAFT (Figure 27) was first developed by Meinero in 2006[132]. The main features of this technique include the ability to view the fistula from the inside, locate the internal fistula opening and possible secondary tracts or abscess cavities (i.e. diagnostic phase), and the

operative phase which includes destruction of the fistula from the inside using diathermy, cleansing of the fistula tract with irrigation and finally closure of the internal opening.

FiLaC™ (Figure 28) was initially described by Wilhelm in 2011[131], using a novel diode laser source and radial emitting laser probe to obliterate the fistula tract throughout its length from within, whilst using an advancement flap procedure to close the internal opening. Subsequent studies have reported successes without addressing the internal opening.

OTSC was initially described in 2012 by Prosst and Ehni [448]. The technique adopts the OTSC® clip (made from elastic shape memory alloy – Nitinol), which upon application to the internal fistula opening exerts constant compression and theoretical closure.

The aim of this study was to review the efficacy and safety of these novel surgical techniques and to identify their role in anal fistula surgery.

7.3 Materials and methods

7.3.1 Search strategy

A systematic search using MEDLINE and Embase databases was performed from 2006 through to 31st April 2017 according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. We used the following keywords/terms and Medical Subject Headings (MeSH): “fistula”, “fistuloscope”, “video assisted anal fistula treatment”, “Over-the-scope clip”, “over-the-scope proctology clip”, “fistula tract laser closure” “VAAFT”, “OTSC”, “FiLaC”, “laser”, “surgery”.

The studies were supplemented with searches of reference lists and bibliographies of selected articles to ensure that no relevant articles were missed. Two assessors (SA and KS) undertook independent systematic searches and evaluated the abstracts to select the studies for the review.

7.3.2 Inclusion/Exclusion criteria

All available studies published in the English language, on the above techniques were screened for inclusion in this review. Original studies describing a patient population undergoing any of the above three techniques were included. Case reports, conference abstract and review articles were excluded. Data were limited to that for idiopathic / Crohn's related fistula. Rectovaginal fistulas were excluded from the analysis.

7.3.3 Endpoints

Primary: Efficacy of the procedure

Secondary: 1) Complications (safety)

2) Inter-procedure inconsistency/variations of techniques

3) Sub-analysis of patients with Crohn's disease (CD)

7.3.4 Data extraction and statistical analysis

Collected data were expressed in spreadsheet format (using Microsoft Excel, Microsoft, Redmond, WA, USA) and analysed to ascertain any possible conclusions from their collective information. Data collected for each study included: year/country of publication, number of patients, sex distribution, pre-existing CD, operating time, operative success (i.e. healing of

fistula), duration of follow-up, success/healing rates and complications. These were expressed as total (percentage), and in descriptive terms as applicable.

7.4 Results

The literature search revealed 198 citations, from which 21 full text articles were selected and assessed for eligibility. From these, 3 studies were excluded, as they were updated by the relevant authors reporting larger series including the initial reported cohort. A total of 18 studies including 1245 patients (VAAFT n=917, OTSC n=116, FiLaC n=212), were identified and included. All studies were prospective case series (Figure 29).

Table 20 and Table 21 summarize the findings.

7.4.1 Primary endpoint

7.4.1.1 *Efficacy*

VAAFT

Twelve studies reported on VAAFT [191,449–459]. In the study by Schwandner [191] the diagnostic phase of VAAFT was used as an adjunctive therapy to advancement flap repair of complex fistulas in patients with CD. Grolich et al. [455] similarly utilised VAAFT solely for its diagnostic potential in 30 patients, reporting feasibility of fistuloscope assessment in 93% of patients. The internal opening was identified in 67% of patients. It was not specified whether additional findings such as secondary tracts were identified with VAAFT when compared with baseline fistula assessment investigations (fistulography, endosonography, magnetic resonance imaging (MRI)) [455].

Operating time varied from 18–135 minutes. Success rates (i.e. clinical healing) varied from 67% (12/18 patients after an average of 10 months follow-up)[454] to 100% (40/40 at 3 months

follow-up)[451]. Notably, the largest study by Chowbey et al. [459] (involving 416 patients) reported a success rate of 74% (in 99 of 134 patients followed up at 1 year). Meinero et al. [452] (2nd largest study involving 203 patients) reported a 6-month cumulative probability of freedom from fistula estimated at 70% (according to a Kaplan-Meier analysis; 95%CI, 64%–76%).

FiLaC™

Four studies reported on fistula tract laser closure (FiLaC™)[131,460–462]. Two of these were by the same author describing an initial case series of 11 [131] followed by a later report of 5-year experience of 117 patients [462]. The latter study was used in this review of outcomes in FiLaC™ (see table 2).

One study reported on operating times, with a range of 6–35minutes.

Success rates varied between 64% (median follow-up of 25 months) and 82% (median follow-up of 12 months). Healing was defined clinically (no symptoms/signs of recurrence/persistence) and radiologically (no evidence of fistula on endoanal ultrasound).

OTSC

Three studies described the use of the over-the-scope proctology clip system in the treatment of anal fistulas (table 2) [463–465]. Operating time varied from 17 – 66 minutes. Short-term (<1 year) success rates varied between 20% (2/10) and 79% (72/96).

7.4.2 Secondary endpoints

7.4.2.1 *Complications*

VAAFT

One study used a validated stool incontinence questionnaire (Cleveland clinic incontinence score) to assess continence[191], and Kochhar et al. used pre and postoperative anal manometry

as an objective measure of sphincteric function (with no significant difference in mean resting anal / squeeze pressures). There were no reports of deterioration on follow-up questioning of patients across the studies, where mentioned. Across the studies, 52/917 patients (5%) were reported to have suffered complications. Jiang and co-workers report 3 cases of postoperative perianal sepsis that were subsequently treated with cutting setons. They also reported 3 cases of postoperative bleeding (secondary to laceration of rectal mucosa around the internal opening). Meinero and co-workers [452] reported 1 case of scrotal oedema, and Chowbey et al. [459] reported 29 cases of perineal oedema (caused by infiltration of irrigation solution after rupture of the fistula wall). Walega and colleagues reported 1 patient with an anaesthetic complication (delayed discharge due to post puncture syndrome after spinal anaesthesia)[454]. Other complications were 5 cases of postoperative urinary retention, 2 cases of allergy to synthetic cyanoacrylate and 1 patient with delayed discharge (6 days postoperatively) because of a headache related to spinal anaesthesia[452]. Seven patients were readmitted; 5 due to rectal bleeding, and 2 due to bloody discharge from the fistula tract [459].

FiLaC™

There were few complications reported secondary to this procedure. Wilhelm et al. in the initial case series (n = 11)[131] describe 1 case of minor incontinence which was temporary and successfully treated 6 months postoperatively with rubber band ligation of hypertrophied prolapsed mucosa. In the larger series (n = 117) published in 2017, there were no forms of incontinence (solid, liquid stool or gas) reported [462]. Other reported complications of the procedure included temporary pain and anismus in 8 cases (7.5%) and moderate bleeding in 3 cases (2.8%) across the 3 studies.

OTSC

No formal assessment or reporting of postoperative continence was done across the 3 studies. One of the studies [464] reported 3 patients with complications (30%): slight anal discomfort (2/10) and soiling (1/10). The soiling was reported to be related to the OTSC clip, which was removed after approximately 6 months with successful fistula closure. The largest study, incorporating 96 patients, reported on pain scores in 10 of these patients, concluding that this was well controlled with simple analgesia.

7.4.2.2 *Technique variations between studies*

The diagnostic phase of VAAFT was consistently used in all patients, adopting the principles as first described by Meinero and Mori in 2006 [132]. However, there were differences between the studies regarding the operative phase of the procedure, particularly the treatment of the internal opening (IO), as highlighted in Table 20.

Concerning FiLaC, all studies used laser energy for ablation of the fistula tract, adopting similar wavelengths (1470nm), with emitted energy ranging from 12 to 15W. Probe withdrawal speed was different between studies, ranging from approximately 1 to 3mm/sec. The first study on FiLaC by Wilhelm [131] used advancement flaps (both initially and in larger case series) to close the internal opening, whereas subsequent studies had no specific treatment (i.e. other than laser ablation of the tract) for the internal / external openings.

The OTSC technique did not differ between studies.

7.4.2.3 *Crohn's disease*

In the study by Schwandner [191] the diagnostic phase of VAAFT was used as an adjunct to advancement flaps for repair of complex fistulas in patients with CD. In this study, only Crohn's fistulas were assessed.

Overall, a very small minority of patients (46/1245 (4%)) treated had CD-related perianal fistulas. Just under half of these patients (21/46) underwent VAAFT (9 purely diagnostic; 11 in combination with advancement flap), 10/46 had OTSC placement (7 of these 10 had successful fistula closure) and 15 had FiLaC (with primary success rate of 11/15).

7.5 Discussion

The surgical treatment of complex anorectal fistulas remains a problem [445]. Fistulotomy remains the best option when solely addressing the chance of cure. Furthermore, it can even be offered as a treatment modality for high fistulas, albeit in selected cases [446], although a third to a quarter of patients will experience mild leakage of flatus and mucus [446,466]. However, for many patients this remains unacceptable and for some the functional impairment which would follow fistulotomy would be far worse. The goal of curing the disease whilst minimizing the risk of functional impairment has fuelled the development of sphincter-preserving techniques.

The novel therapies assessed in this study promise function-preserving curative surgical therapies for fistula-in-ano. VAAFT provides a minimally invasive technique with the ability to view the fistula from the inside so that all extensions can be identified and eradicated under direct vision using a fistuloscope. A presumed advantage of VAAFT lies in its diagnostic potential with the ability to identify secondary extensions and abscess cavities [191]. Meinero et al. [452] report the ability to characterize true fistulas (where tissue is characteristically red and floating) from the false passages (where the tissue is whitish and not floating), suggesting that fistuloscopy might be more accurate than endosonography and MRI. This diagnostic potential was reported by Schwandner [191] in CD, where additional side tracts not detected preoperatively (clinically or with endosonography) were identified in 64% (7/11) of patients. However, MRI

is often considered the gold standard, for imaging the fistula tracts and perhaps a comparison with MRI would offer a more interesting study.

The operative phase of VAAFT includes fistula destruction (with electrocoagulation), cleansing and closure of the internal opening. This closure is performed by various techniques including suturing, stapling (linear/semi-circular) and advancement flaps. There is currently limited evidence to suggest which closure technique is favourable, with only one, non-randomised study comparing outcomes with stapled versus sutured closure of the internal opening [452]. A statistically significant difference between the two methods in favour of staple closure was found (log-rank test, 6.5; p=0.011).

Our review demonstrates variable success rates with short-term (<1yr) healing rates ranging from 67% [454] to 100% [451]. However, healing was assessed on a clinical basis without radiological assessment and without long- term follow up in most cases. Zarin and colleagues [451] reported 100% success rate at 3 months follow-up, despite stating a mean follow-up period of 6 months. Furthermore, 3/40 patients required re-do surgery, at an unspecified time point, despite the quoted rate of 100% success, suggesting this result should be interpreted with caution. The largest study, by Chowbey et al. [459] (n= 416) reported a success rate of 74% in 99 of 134 patients followed up at 1 year. Meinero et al. [452] (n = 203) reported a 6-month cumulative probability of freedom from fistula estimated at 70% (according to a Kaplan-Meier analysis, 95%CI, 64%–76%).

There were no deaths and morbidity was low, with no significant surgical complications. In particular, there were no reports of incontinence, and 1 study utilised anal manometry in

providing objective evidence of sphincteric function [449], finding no significant difference in resting tone or squeeze increments pre- and post-surgery.

Important considerations for VAAFT include the cost of the equipment and long operating times, although the latter may decrease with increased familiarity with the procedure. Technical aspects also need further evaluation such as progression of the fistuloscope through the fistula tract, which may be hampered by its own rigidity, or by the presence of other tracts with difficult trajectories arising from the primary path [453,467]. There are also concerns regarding iatrogenic false passages (caused by over-aggressive irrigation) and collateral thermal damage by the electrode during ablation, with the possibility of this being a risk for delayed healing and recurrence [467].

FiLaC™ has shown benefit for both simple and complex fistulas (mostly transsphincteric). Closure of the internal opening has been described as an adjunct but 2 of the 3 studies reporting on FiLaC™ have shown success without this extra step [460,468], with the suggestion that it may be unnecessary [469,470]. All 3 studies [131,460,468] used mechanical curettage, followed by introduction of the laser fibre into the fistula tract via the external opening. Curettage cleanses the tract and results in bleeding. These are thought to allow the laser to have maximum effect on tissue, with the suggestion that red blood cells to add to sealing of the tract [471]. The optimum wavelength appears to be 1470nm as this achieves the optimum wattage needed for sealing whist minimising anismus post-operatively. The speed at which the laser emitting fibre is removed was variable between the institutions (1 to 3mm/s). At the slowest speed there was no reported anismus [468]. Success rates after primary treatment were over 64% in our review and Wilhelm and co-workers [462] uniquely reported on secondary success rates following initial FiLaC™ with 28/42 patients achieving healing after repeat treatment. Repeat treatment

consisted of a variety of procedures, including repeat FiLaC, sphincter reconstruction, plug treatment and fistulotomy.

Three studies reporting the use of OTSC were included [463–465]. The technique was initially developed for haemostasis and perforation closure in the gastrointestinal tract following flexible endoscopy [472,473]. Modification of this offers a minimally invasive surgical technique, with no significant damage to the sphincter muscle. The fate of the OTSC is unclear, however, with some patients having the clip left in-situ, spontaneous discharge (with associated fistula persistence [464]) and some patients undergoing operative removal. The data available are conflicting, with Gautier et al. reporting a disappointing experience in comparison to Prosst et al., albeit the former included fewer patients in their study. The included numbers make it difficult to accurately compare outcomes such as fistula healing.

It is not possible to comment on the role of these procedures for fistulating perianal CD, but their minimal morbidity and minimal effect on continence with reports of successful treatment suggest this is an area which should be explored in the future, at least for palliation of symptoms.

Limitations

The present review has several limitations.

Firstly, there is significant heterogeneity in the population studied, fistula morphology and aetiology, with no standardized population. The majority of studies are uncontrolled case series, follow-up times are widely variable and relatively short; high initial success rates (many lacking radiological assessment) may be misleading, recurrences may develop more than a year after surgery [126]. These are common problems in studies of fistula treatment [474], often making

comparison and pooling of data between studies difficult. Adherence to guidelines developed in 2016 for surgical case series reporting [475], as well as the development of a core outcome data set for perianal CD fistulas, should ultimately improve reporting in this field and facilitate meaningful analysis.

The cost of the procedures is an important consideration. All three involve the use of novel medical devices with an associated cost which is not a consideration when performing procedures like fistulotomy, advancement flap or LIFT. No studies assessed cost-effectiveness. This needs to be further investigated.

However, our study has some strengths. We highlighted the discrepancies between techniques with which each of the procedures is performed, with specific emphasis on facets that need to be standardized when planning prospective trials. Most of the series were performed by the same authors or groups, and there is a need to assess the reproducibility and generalisability of the procedures.

Notwithstanding the need for further research, the limited evidence available suggests that VAAFT is safe and feasible with acceptable early healing rates and no reported deterioration in continence. The paucity of studies on FiLaC and OTSC make it difficult to comment accurately on success rates, but early reports are promising, and a lack of continence impairment is reassuring.

Notable advantages of these techniques are their avoidance of sphincter injury, minimal morbidity and the ability to repeat them or perform other surgical techniques following failure. They are a welcome addition to the surgical armamentarium. However, long-term data will need to be appraised to fully understand their role in fistula surgery, since without confirmation of efficacy, they may fall into the same group as fibrin glue and even fistula plugs. Our findings

need to be carefully considered when planning prospective studies on larger samples of patients.

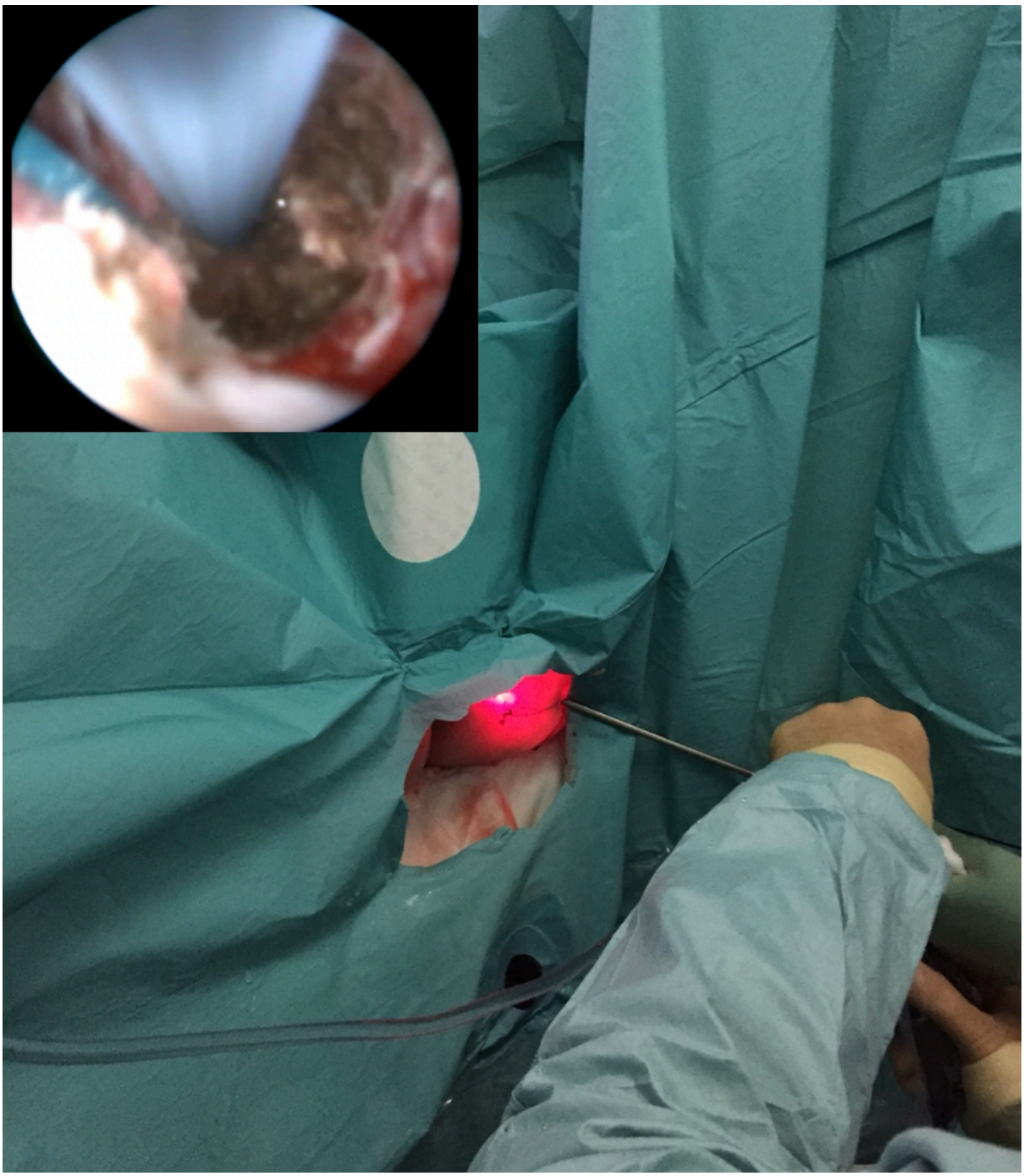


Figure 27: VAAFT procedure – fistula demonstrating fistuloscope in-situ with optical view showing seton as well as electrocautery probe

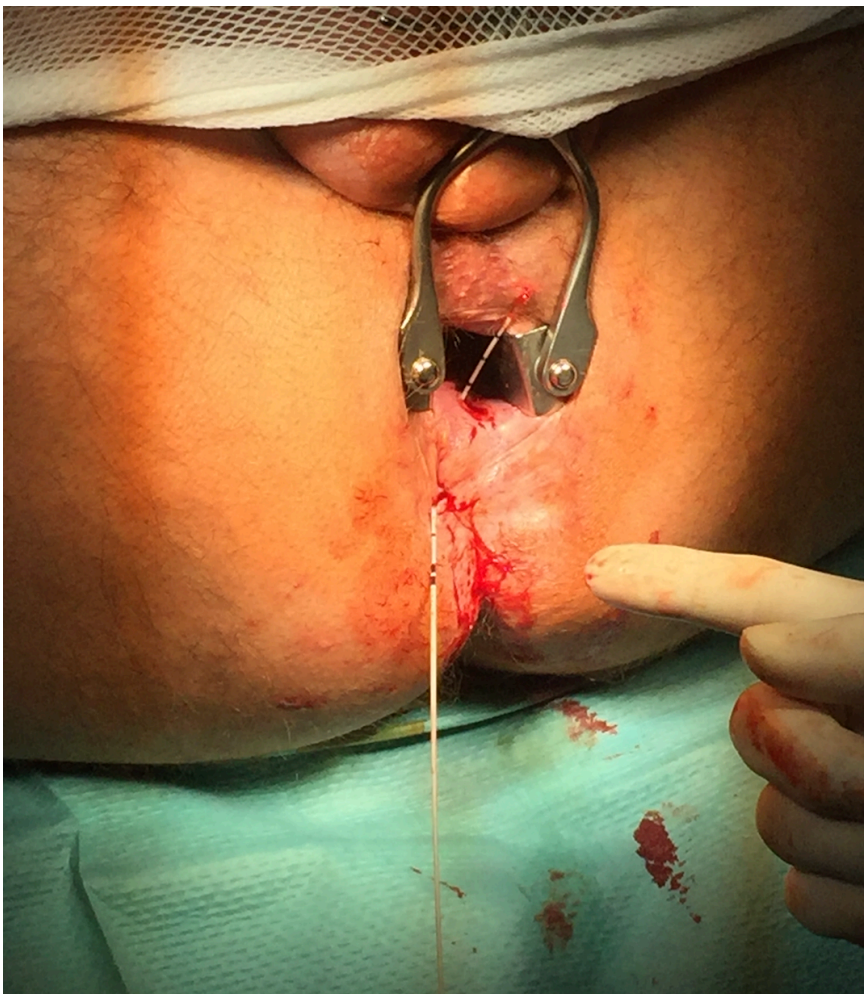


Figure 28: Fistula with Filac™ probe in-situ

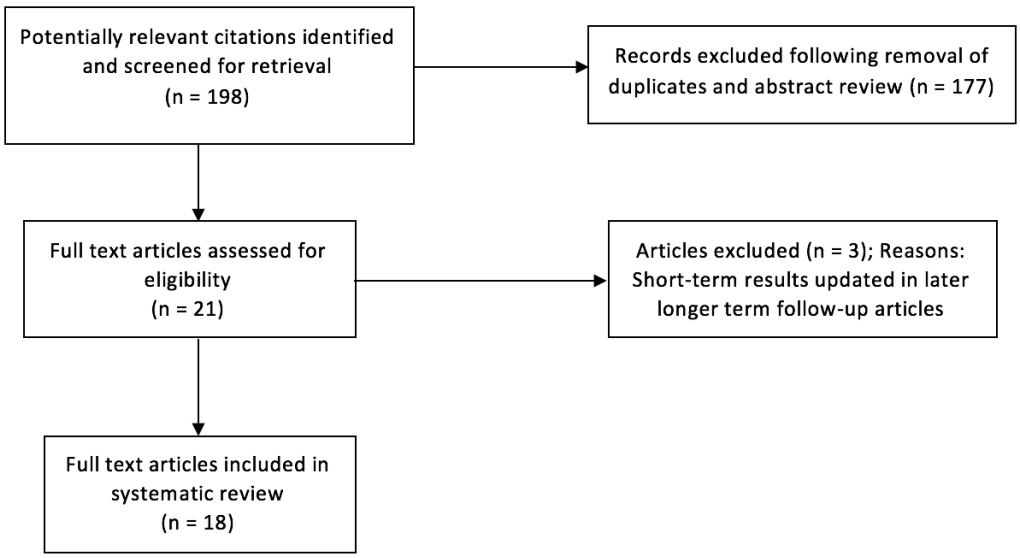


Figure 29: Modified preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram showing selection of articles for review

Table 20: Outcomes for Video-assisted anal fistula treatment (VAAFT).

Author &Publication date	Country	Patients	Age, years	Follow-up, mos.	Closure of internal opening	Crohn's Disease	Operation time, mins	Success
Schwandner 2012[6]	Germany	10	34 (21-51)	8.5 (6-9)	advancement flap	10 (100)	22 (18-42)	9 (81%)
Kochhar 2014[7]	India	82	35	6	sutures or staples	0 (0)	45 (30-90)	69 (84%)
Meinero 2014[8]	Italy	203	42 (21-77)	15 (6-69)	staplers (linear or semi-circular) or advancement flaps	0 (0)	90 (60-120)	74%*
Mendes 2014[9]	Brazil	8	43 (29-66)	5	sutures	0 (0)	31.7 (18-45)	7(88%)
Walega 2014[14]	Poland	18	47	10	mattress sutures / advancement flaps	NR	67 (45 -135)	12(67%)
Grolich 2014[11]	Czech Republic	30	NR	4 (<1-30)	NA	9 (30)	NR	NA
Zarin 2015[17]	Pakistan	40	NR	6	sutures	0	NR	40 (100%)†
Selvarajan 2015[14]	Malaysia	8	42.5	NR	sutures	NR	NR	NR
Chowbey 2015[15]	India	416	NR	NR	linear or semi-circular staplers	NR	50 (22 – 94)	99‡ (73.8)
Pini Prato 2016[16]	Italy	9	9.6 (0.6-15.9)	10 (14-24)	Mucosal advancement flap	1 (11)	38 (25 – 60)	6 (67)
Seow-En. 2016[12]	Singapore	41	44 (18-69)	34 (12-44)	Staplers	0 (0)	NS	29 (71)
Jiang 2017[13]	China	52	48 (19-71)	9	Sutures or staples	1 (0.01)	55 (35-90)	44 (85)
Total		917				21(2)		

Results are n (%) or median (range)
** cumulative probability of ultimate freedom from fistula at 1yr*
† 100% reported healed at 12 weeks (mode of outcome measurement not specified)
‡ 99/134 patients followed up healed at 1yr
Mos: months Mins: Minutes NA not available NR not reported

Table 21: Outcomes for fistula tract laser closure (FiLaC™) and over-the-scope clip (OTSC®) proctology system

OTSC®

Author &Publication date	Country	Patients	Age, years	Follow-up, mos	Crohn's Disease	Operation time, mins	Success
Prosst 2016[24]	Germany	96	50 (20 – 80)	6	NS	32 (17 – 66)	72 (79%)
Mennigen 2015[23]	Germany	10	41 (26 – 69)	7 (5 – 17)	6	41 (24 – 64)	7 (70%)
Gautier 2015[22]	France	10*	43 (24 – 86)	5 (1 – 13)	4(40)	25 (15 – 35)	2 (20%)
Total		116			10(9)		

FILAC™

Author &Publication date	Country	Patients	Age, years	Follow-up, mos	Crohn's Disease	Operation time, mins	Success
Giamundo 2015[28]	Italy	45	46 (18-78)	30 (6-46)	2 (4)	20 (6-35)	32 (71)
Ozturk 2014[18]	Turkey	50	41 (23-83)	12 (2 – 18)	0 (0)	NR	41 (82)
Wilhelm 2017[21]	Germany	117	46 (17-82)	25.4 (6-60)	13 (11.1)	NR	75 (64)
Total		212			15 (7)		

Results are n (%) or median (range)
**excluding rectovaginal fistulas*
NS- not specified (study included 11 fistulas – 8 Crohn's disease, 3 Ulcerative colitis; however unclear whether multiple fistulas in same patient were counted as separate)
Mos: months Mins: Minutes NR not reported

Chapter 8. Review of local injection of anti-TNF for perianal fistulising Crohn's disease.

8.1 Abstract

Background:

Perianal fistulising Crohn's disease (PFCD) affects a third of Crohn's disease patients and represents a disabling phenotype with poor outcome. The anti-tumour necrosis factor alpha (TNF) therapies have been shown to maintain clinical remission in a third of patients after 1 year of treatment. Maintenance therapy with systematic administration schedule confers greatest benefit, but exposes patients to risks/side effects of continued systemic use and led to consideration of local drug delivery (first described in 2000). In this review, we analyse all published articles on local anti-TNF therapy in the treatment of Crohn's perianal fistulas.

Methods:

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were used to systematically search Medline and Embase using the medical subject headings "fistula", "anus", "Crohn disease", "infliximab", "adalimumab". This was combined with free text searches, e.g. 'local injection', 'Crohn's perianal disease'. Studies/abstracts describing local injection treatment with anti-TNF were included in this review.

Results:

Six pilot studies including a total of 92 patients were included in this review. Outcomes reported were mostly clinical and included 'complete/partial response' to therapy and short-

term results varied between 40-100%. There were no significant adverse events and the local injections were well tolerated.

Conclusions:

There is paucity of data assessing this treatment modality. Local anti-TNF therapy appears safe, but outcome reporting is heterogeneous, subjective, and long-term data are unavailable. Our review suggests a potential role may be in those in whom systemic treatment is contraindicated and calls for standardised reporting of outcomes in this field to enable better data interpretation.

8.2 Introduction

Perianal fistulising Crohn's disease represents a particularly disabling phenotype of Crohn's disease (CD) with poor outcomes. Incidence rises with increased duration of Crohn's disease and reports of lifetime risk can be up to 40%[15]. It represents a distinct subset of Crohn's disease as reflected in the Montreal classification of inflammatory bowel disease (IBD)[100] and often signifies an aggressive form of Crohn's disease. Treatment of this condition has historically proved frustrating, often following a chronic and relapsing course, with up to 40% of patients previously undergoing eventual proctectomy[205]. The advent of medical therapies, particularly biological therapy, heralded a positive change in the burden of disability associated with this condition. Anti-TNF therapy (i.e. infliximab, adalimumab) has been shown to maintain clinical remission in approximately a third of patients after 1 year of treatment[122]. Maintenance therapy with systematic administration schedules (rather than episodic use) of anti-TNF confers greatest benefit[140,476]. However, this in turn exposes patients to the risks and side effects associated with continued use, including auto-antibody formation, infusion reactions, infections and malignancies [477]. This has led to consideration of local drug delivery, which was first described in 2000[275]. In this review, we analyse all published articles on local anti-TNF therapy in the treatment of perianal fistulising CD.

8.3 Methods

All articles/abstracts in the English literature reporting the use of local injection of anti-TNF for the treatment of perianal fistula in patients with Crohn's disease were considered. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines

were used to systematically search Medline and Embase (between January 2000 – December 2016) using the medical subject headings "fistula", "anus", "Crohn disease", "infliximab", "adalimumab". This was combined with free text searches, e.g. 'local injection', 'Crohn's perianal disease' and cross-references. Studies/abstracts describing local injection treatment with anti-TNFα were considered in this review. Retrieved citations and abstracts were reviewed by two independent reviewers (SOA and KS) and all relevant articles were selected. Any discrepancies in article selection were discussed and a final consensus was agreed. All relevant articles/abstracts describing the patient population with a sample size ≥5 patients were included. Data from the articles were expressed in spreadsheet format (using Microsoft Excel, Microsoft, Redmond, WA) and analysed to ascertain conclusions (where possible) from their collective information. Data extracted included participant numbers, age range, gender distribution, type / dose / dosing regimen of anti-TNF agent, duration of follow-up, and endpoints, including complications. Quantitative data analysis was not possible due to methodological differences amongst the studies. Data were pooled without formal statistical analysis and meta-analysis due to study heterogeneity, small patient numbers and lack of comparative studies.

8.4 Results

Six studies (four original articles, two cohort study abstracts) were included in this review[478–483] (Figure 30). A total of 92 patients were evaluated and the demographics are demonstrated in Table 22. All studies were pilot/prospective cohort studies. In all studies, patients largely continued concomitant therapy (i.e. steroids, 6-mercaptopurine, mesalazine, azathioprine). Tonelli et al.[482] stated that in their study no patient received therapy with systemic immunosuppressive drugs. In the remaining five studies[478–481,483], some included patients on

concurrent systemic anti-TNF therapy[479], whereas the rest did not explicitly report this. Four studies[478–481] assessed infliximab as the local anti-TNF agent used, whilst the remaining two[482,483] assessed adalimumab. Technique of injection, where specified, was similar between studies with examination under anaesthesia, curettage[482] of the fistula tract (in one case fistulectomy[479]) and injection of anti-TNF agent along the fistula tracts and circumferentially around the external and/or internal openings. Doses given varied between 15-25 mg infliximab and 20-40mg adalimumab. Dosing intervals varied between 2 – 6 weeks. All treatment regimens employed by the varying studies required at least two sessions of treatment (i.e. injections at the varying time intervals, see Table 23). Follow-up ranged from 1 – 43 months. Outcome measures were mostly clinical with primary end points being complete or partial healing of fistula. Some studies used radiological techniques (ultrasonography / MRI), in addition to clinical findings[479,482]. Success rates, as defined by the studies, were signified by complete / partial response to anti-TNF treatment. This was ascertained by clinical examination, to assess for discharge, with complete response signifying absence of discharge / clinical healing. The study with the longest follow-up, demonstrated response rates of 62.5% (5/8 patients) with complete healing (clinical assessment) at median follow-up of 35 months[479]. The response rates in the rest of the studies revealed a partial/complete response varying from 40 – 100% (Table 24). Morbidity was low with the procedure. Reports of minor symptoms of local irritation/burning/heaviness were largely self-limiting. Poggioli and colleagues reported 3 adverse events in their study of 15 patients – 1 case of pre-existing rectal stenosis worsened after treatment, 1 case of new recto-urethral fistula requiring surgery, 1 case of poor sphincter function after treatment. Alessandroni et al.[479] reported a delayed hypersensitivity reaction in a patient who was treated with local infliximab, but then subsequently had to abandon treatment and go on to intravenous infliximab due to relapse of intestinal symptoms. The patient

developed a delayed hypersensitivity reaction after first infusion and was subsequently lost to follow-up.

8.5 Discussion

8.5.1 Local anti-TNF therapy as a potential therapeutic option

The introduction of anti-TNFα heralded a significant addition to treatment of perianal Crohn's fistulas. Initial remission rates have been reported as up to 55% in the literature, with maintenance treatment resulting in continued remission in about a third of these patients at 1 year[120,140,151,152,484]. Infliximab was the first of the anti-TNF therapies to have demonstrated benefit. Fistula response in the ACCENT 2 trial was prolonged by maintenance intravenous infusion every 8 weeks[120,485]. This treatment strategy has since been accepted into guidelines in managing fistulising perianal Crohn's disease[119]. However, as with all immunomodulators, there are risks of adverse events with continued use (e.g. infusion reactions, neurological events, infections). These concerns led to the proposal of local injection of anti-TNF as an alternative to systemic infusion. Theoretical advantages include more efficient delivery with direct diffusion/interstitial fluid movement of antibody to target site, preventing the need for high systemic concentrations[486]. Lichtiger initially described the technique in a small case series with injection into the fistula tract and circumferentially around tract (subcutaneously). They reported partial clinical response in 78% (7/9patients) and complete closure in 44% (4/9patients) and no significant adverse events. However, follow-up period was only 1 month, which makes the actual efficacy / healing rates difficult to assess in this study. In our review, three other case series assessed local infliximab injection[479–481]. They used a modified version of the technique (described by Lichtiger) with injection around the internal opening, as well as the fistula tract/external opening, this was combined with debridement / fistulectomy of the

tract. Partial / complete response was demonstrated in approximately 62.5 – 73% at ≥1year. Similarly, no significant adverse events were reported. Dosing (15-25mg) and intervals (4-6weeks) were similar.

Two studies have been reported in the literature regarding adalimumab use. Tonelli and colleagues[482] reported a 100% partial/complete response in 12 patients who had 20mg twice weekly after median follow-up of 18 months. Some 75% of these demonstrated complete closure. Laureti et al.[483] who followed on from their experience with infliximab[481], reported 40% complete closure (13/33 patients) at 11 months after a median of 9 treatments with 40mg adalimumab.

8.5.2 Adverse Events / Limitations of local anti-TNF therapy

In general, the studies on local anti-TNF injections were free of significant adverse events. However, it is important to note the report of delayed hypersensitivity reaction in a patient undergoing intravenous infliximab following previous local therapy. The variable dosing intervals may indeed theoretically provoke such a response and this may be a significant concern with a non-standardised dosing regimen. It is possible that the local injections may stimulate formation of antibodies which, in turn, may render the patient sensitised and intolerant to future systemic treatment[487]. The complications reported by Poggioli et al. give cause for concern. They reported 1 case of pre-existing rectal stenosis worsened after treatment, 1 case of new recto-urethral fistula requiring surgery and 1 case of poor sphincter function after treatment. The exact continence impairment in the latter is not clear and might be most, but none should be expected. Similarly, the worsening of rectal stenosis might represent disease progression rather than the effect of the intervention, but this cannot be disregarded. The new rectourethral fistula, which carries serious morbidity and the need for a major procedure perhaps including a defunctioning stoma and coloanal pull through procedure, is the most

major concern. It is conceivable that this fistula was created as a result of the intervention and would represent a real worsening of disease. These complications should be at the forefront of investigators' minds in future studies, so that such occurrences are detected even if subtle.

A major limitation in the studies we reviewed was the nature of outcome reporting. There is significant heterogeneity of outcome reporting, which represents a widespread issue across the Crohn's fistula literature. The most widely used instrument for assessing treatment outcomes in perianal Crohn's clinical trials is the Fistula Drainage Assessment[120]. Fistulas are classified as open (when purulent material can be expelled with gentle pressure) or closed. A fistula should remain closed for 2 consecutive visits (at least 4 weeks apart) to be considered closed (complete healing) according to the Fistula Drainage Assessment (FDA). If half of all external openings are closed, the patient has responded (partial healing). Other studies report fistula drainage 'semi-quantitatively' based on frequency/quantity of dressing/pad changes. In our review, most studies reported clinical assessment of healing, and classified this as complete or partial (definitions of these were heterogeneous, not necessarily sticking closely to the definitions in the FDA). These clinical outcome measures are subjective and don't account for temporal changes in fistula drainage and may be subject to recall bias. Clinical outcome reporting is also exacerbated by the fact that clinical healing is not always readily achievable and does not always correlate with radiological healing even when it does occur. In fact, MRI confirmation of deep healing has been shown to occur a median of 12 months after closure of the external opening(s)[122,383]. Two studies[479,482] in our review, used radiological outcomes (MRI / endo-anal ultrasound) in addition to clinical assessment. In the study by Alessandroni et al.[479], all patients underwent MRI at 1 year follow-up to check complete resolution of fistula / healing, as well as to assess the fistula anatomy, if recurrence was suspected. Interestingly, the MRI was diagnostic of an intersphincteric fistula in a patient that had been clinically deemed to have complete resolution after treatment. Otherwise the MRI and clinical findings

correlated. Tonelli et al.[482] used MRI/USS examination to confirm complete closure of fistula tract and again imaging demonstrated persistence of a fistula in a patient that was deemed to have clinically healed. This required further treatment. Another limitation was the inability to fully stratify according to type of fistula in order to correlate this with response. Table 22 demonstrates that most fistulas were transsphincteric, and the small numbers of the rest (e.g. intersphincteric / suprasphincteric) make it difficult to accurately compare this.

8.5.3 Conclusion

The data available on this technique are limited, outcomes are heterogeneous, and this make interpretation difficult. There does seem to be a positive response towards fistula healing, however, this is difficult to quantify statistically, given the scant evidence available and follow up was often very short. Other limitations include the lack of comparison with placebo and furthermore, the heterogeneity and paucity of long-term data with reproducible outcomes make it difficult to determine duration of remission / recurrence rates. The ideal dosing regimen and intervals remain unclear, as well as the risk of antibody formation and thus hypersensitivity with future treatments. There may however be scope for treatment with local injection of anti-TNF in a subset of patients who are either intolerant to, or in whom systemic therapy is contraindicated. Furthermore, lessons might be learnt for the Admire CD data on mesenchymal stem cell use, in which thorough curettage and closure of the internal opening were adjuncts to stem cell injection which, like local infliximab, might be acting to reduce local inflammation and promote wound repair, and might therefore benefit from the same additional adjuncts which remove epithelialisation and disconnect the tract form the gut.

Table 22: Demographics

Study	Design	Numbers	Age in yrs. Median (range)	Male: Female Ratio	Type of fistula	Type of LA treatment
Lichtiger S. 2001 (USA)	Pilot	9	NS	NS	NS	Infliximab
Poggioli et al. 2005 (Ita)	Pilot	15	29.7	12:3	12: High TS 2: High IS 1: SS	Infliximab
Asteria et al. 2006 (Ita)	Pilot	11	38.9 (28 – 44)	4:7	7: Low TS 1: Low IS 3: Low AV / TS	Infliximab
Alessandroni et al. 2011 (Ita)	Prospective cohort / Pilot	12	40* (18 – 52)	8:4	5: High TS 5: TS 2: IS	Infliximab
Laureti et al. 2012 (Ita)	Pilot	33	NS	NS	NS	Adalimumab
Tonelli et al. 2012 (Ita)	Pilot / Uncontrolled study	12	43.5 (27 – 59)	3:9	7: TS 3: AV 2: 'Complex'	Adalimumab
		92				

*NS – Not specified, TS – transsphincteric, IS – intersphincteric, SS – suprasphincteric, AV – anovaginal

Table 23: Treatment regimen

Study	Numbers	Median follow-up in months (range)	Dose (mg)	No. of Treatments	Dosing Interval (wks.)	Type of LA treatment	Mode of injection
Lichtiger S. 2001 (USA)	9	1	20	3	1 & 2	Infliximab	Circumferential + Intrafistula
Poggioli et al. 2005 (Ita)	15	18.2 (3 – 30)	15 – 21	≥6	4	Infliximab	(Fistulectomy +) Circumferential (IO/EO) + intrafistula
Asteria et al. 2006 (Ita)	11	10.5 (7 – 18)	20	≥3	4	Infliximab	Circumferential (IO/EO) + intrafistula
Alessandroni et al. 2011 (Ita)	12	35* (19 – 43)	20 – 25	≥2	4 – 6	Infliximab	Circumferential (IO/EO) + intrafistula
Laureti et al. 2012 (Ita)	33	11* (7 – 14)	40	≥2	2	Adalimumab	Submucosal around I.O.
Tonelli et al. 2012 (Ita)	12	17.5 (5 – 30)	20	≥4	2	Adalimumab	Circumferential (IO/EO) + intrafistula

*median follow-up, IO – internal opening, EO – external opening, LA – local anaesthetic

Table 24: Outcomes

Study	Numbers	Median follow-up in months (range)	Outcomes
Lichtiger S. 2001 (USA)	9	1	44% (4/9) demonstrated complete and 33% (3/9) partial response
Poggioli et al. 2005 (Ita)	15	18.2 (3 – 30)	67% (10/15) demonstrated complete response
Asteria et al. 2006 (Ita)	11	10.5 (7 – 18)	36% (4/11) demonstrated complete and 36% (4/11) partial response
Alessandroni et al. 2011 (Ita)	12	35 (19 – 43)	62.5% (5/8) demonstrated complete response
Laureti et al. 2012 (Ita)	33	11* (7 – 14)	40% demonstrated complete response
Tonelli et al. 2012 (Ita)	12	17.5 (5 – 30)	75% (9/12) demonstrated complete and 25%(3/12) partial response

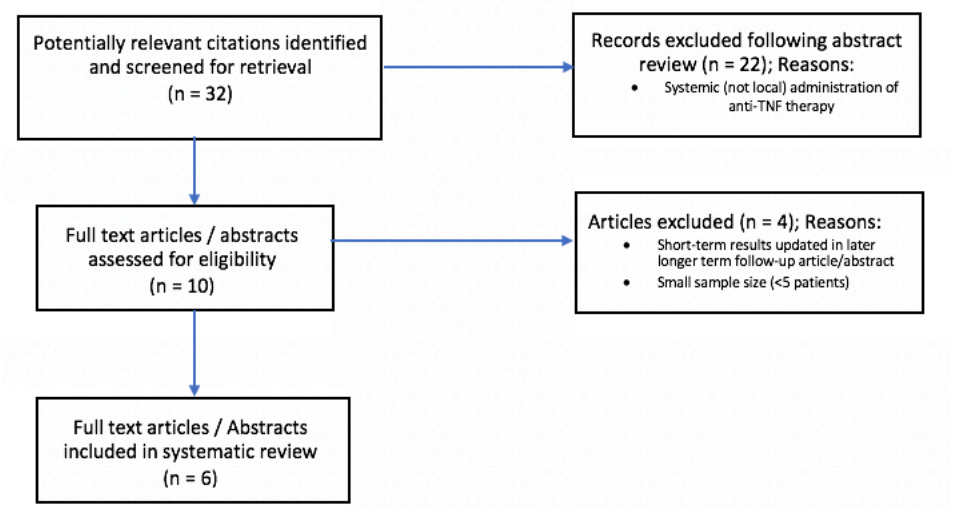


Figure 30: Flow diagram of search strategy

Chapter 9. Video-assisted anal fistula treatment (VAAFT) in patients with Crohn's perianal fistulas – patient reported outcomes of symptomatic treatment

9.1 Abstract

Introduction:

A third of patients with Crohn's disease develop perianal fistulas. These are associated with a significant burden of symptoms and negative impact on quality of life. This study reports the use of VAAFT as a means of symptom improvement with minimally invasive access to the fistula tract, facilitating diagnosis/ drainage of deep/complex secondary extensions with cauterization of inflammatory tissue.

Methods:

Consecutive patients with complex Crohn's fistula undergoing a VAAFT for symptomatic control were included. They were identified from a prospectively maintained database, which was interrogated from June 2015-November 2017. Patients underwent diagnostic fistuloscopy and fulguration of tracts/secondary extensions. Setons were sited/replaced after the procedure to maintain postoperative drainage. Primary endpoint was completion of the "Measure your medical outcome profile" (MYMOP2) quality of life (QoL) questionnaire at 6 weeks postoperatively. Secondary outcome measures were a decisional regret scale (DRS), post-operative complications and the thirty-day re-operation rate.

Results:

Twenty-five patients underwent the procedure during the study period and 21/25 (84%) completed MYMOP2 QoL data demonstrating a statistically significant improvement in both pain and discharge scores. Some 81% of patients who completed the decisional regret scale agreed/strongly agreed that the procedure was the right decision and no patient regretted undergoing the procedure. There was 1 reoperation but otherwise no complications.

Conclusions:

This study demonstrates feasibility, safety and importantly an improvement in patient reported outcomes in a series of patients undergoing VAAFT for complex Crohn's anal fistula. VAAFT reduced the main symptoms (pain and discharge) in patients with complex refractory anal fistulas in this unblinded study.

9.2 Introduction

Perianal fistula occur in approximately a third of all patients with Crohn's Disease (CD) and represent a distinct and aggressive phenotype[488,489]. Complex Crohn's perianal fistulas, can involve a significant portion of the anal sphincter muscle, often have multiple secondary tracts / openings and can be associated with other manifestations of perianal Crohn's disease[107]. Treatment in this context is challenging with limited medical and surgical options for sustained cure.

The introduction of anti-TNF agents (e.g. infliximab and adalimumab) promised improvement to treatment with clinical response rates of up to 68% and reported complete healing rates of 55% in the short-term[120]. Recent guidelines[119] in the treatment of perianal fistulising Crohn's disease, recommend anti-TNF agents as the current gold standard, with antibiotics and immunosuppressant agents offering a role as adjunctive treatments. Despite best medical treatment a significant number of patients either never achieve response or subsequently lose response to biologic treatments. Only a third of patients with Crohn's perianal fistula which closed on induction remain in remission on long term maintenance treatment, and this number reduces further with time[122].

Surgical treatment options are limited due to the anatomical complexity limiting the suitable sphincter sparing options[67,203]. Surgical procedures aim to heal fistulas but these too have disappointing results with frequent recurrences. Each recurrence or operation can lead to tissue destruction, distorting anatomy and making subsequent procedures more challenging. In about

12-20%, proctectomy may ultimately be required[490]. Combined surgical and medical therapies offer improved outcomes, yet robust, sustained healing remains an elusive goal[205].

Video-assisted anal fistula treatment (VAAFT) is a sphincter sparing minimally invasive technique[132], comprising diagnostic and operative phases. The diagnostic phase involves viewing the fistula from the inside using a fistuloscope, and identifying all secondary extensions under direct vision. As described by the original authors[132], the fistuloscope allows detection of true fistulas as distinct from false passages and facilitates intraoperative delineation of complex fistula anatomy. The operative phase employs cautery for tract ablation with various options (suture / staple closure or advancement flap) to close the internal opening. Several studies have reported 'success' in cryptoglandular fistulas, albeit with heterogeneous outcomes. Data on use in Crohn's perianal fistula are sparse, with few studies evaluating this procedure. The aim, in studies published to date, is to achieve closure of the fistula.

Qualitative studies reveal that with refractory disease, not only is a patient's physical state affected, but also their emotional wellbeing, social life, educational activities, professional lives and intimate relationships[491]. In the face of this impact on quality of life, there is a need to address symptom burden in those cases where definitive cure is not always achievable. Whilst robust healing of Crohn's anal fistula with medical or surgical treatment is rare, we noticed that patients gained symptomatic benefit from VAAFT procedures, even when healing did not occur (which was usually the case). Many patients are managed in the long term on biologic agents which control their symptoms incompletely. We hypothesised that VAAFT (performed with the intent of symptom amelioration rather than fistula closure) might further improve symptoms from Crohn's anal fistula.

In this study, we analyse the use of VAAFT as a minimally invasive technique to access the fistula track and facilitate drainage of secondary extensions with cauterization of excessive inflammatory tissue with the intent of symptom amelioration. We sought to assess the role of VAAFT as a symptomatic treatment in perianal Crohn's fistula using patient reported outcome measures as our primary endpoint, and secondary outcome measures of complications and thirty-day re-operation.

9.3 Methods:

We performed an analysis from a prospectively maintained database of consecutive Crohn's fistula patients undergoing VAAFT between June 2015 and November 2017. All patients had persistent fistula symptoms in the context of multiple previous procedures, previous / current use of biologic therapy, and complex fistulas according to the American Gastroenterological Association (AGA) classification[107], i.e. high (high intersphincteric / high trans-sphincteric or extrasphincteric or suprasphincteric origin of the fistula tract), +/- multiple external openings, +/- collection, +/- rectovaginal fistula / anorectal stricture / active rectal disease at endoscopy.

9.3.1 Operative procedure

Patients were placed in the lithotomy position under general anaesthesia. The fistuloscope was used to survey the fistula tract network from the external opening(s) to the internal opening(s). The technique as described by Meinero[132] was used with the exception of the treatment of the internal opening which was not closed, as fistula closure was not sought. All fragments of the whitish material adhering to the fistula wall were cauterised with careful observation for secondary extensions any abscess cavities or any possible fistula tract. Continuing under direct vision, any inflammatory or necrotic material was removed with endo-brush and continuous

jet irrigation (glycine-mannitol 1%) ensuring all waste exits via the internal or external openings. Setons were changed or sited to facilitate postoperative drainage.

9.3.2 Patient reported outcome measures:

9.3.2.1 *Quality of life questionnaire ("Measure your medical outcome profile" MYMOP)*

Patients completed quality of life questionnaire using a generic "(MYMOP2) quality of life questionnaire. MYMOP was developed by Paterson and initially published in 1996 [6], a revised version including items on medication was validated in 1999 [7,8]. Since then, the MYMOP has been used in several studies and has proven to be a sensitive measure of within-person change over time [8,9]. Patients rated their two most important symptoms on a 1-6 Likert scale both preoperatively and postoperatively (at six weeks follow up).

Pre-and postoperative MYMOP2 scores as well as decisional regret scores were then collated and expressed in spreadsheet format (using Microsoft Excel, Microsoft, Redmond, WA), and analysed. Any difference between their chosen MYMOP2 scores pre-and postoperatively were determined, as this difference represents the magnitude of the effect on symptoms. Secondary outcome measures were 30-day re-operation rate, to identify a tendency to provoke abscess formation, and any complications recorded on review of electronic records.

9.3.2.2 *Decisional Regret Scale*

Decision regret has been associated with lower satisfaction with medical decision making and quality of life, poorer health outcomes, and negative experiences with the health care system[492], thus making it a potential indicator for assessing the quality of health decisions[492–494]. The validated Decisional Regret Scale (DRS)[493] was used to measure distress or remorse after the decision to have surgery. The scale uses a five-item self-reported Likert Scale (1, 'strongly

agree'; 2, 'agree'; 3, 'neither agree nor disagree'; 4, 'disagree'; and 5, 'strongly disagree'). Patients completed the decisional regret questionnaires at the six-week follow-up.

9.4 Results

A total of 25 patients underwent the VAAFT procedure for symptom improvement of Crohn's anal fistulas between June 2015 and November 2017. The median age was 32 (range 17 – 64) years. The majority (23/25, 92%) were on biologic medication with concurrent azathioprine (92%), with 32% (8/25) having been on two or more previous courses of biologic treatment (Table 25). All operations where done as day case procedures with no overnight stays.

The procedure was completed as planned in 24/25 patients. In one patient, the planned VAAFT procedure was abandoned for routine examination under anaesthesia, due to the inability to intubate the narrow fistula tract with the fistuloscope.

Patient reported outcome measures were collected for 21/24 patients who underwent the procedure as planned, and completed MYMOP scores pre-and postoperatively (at 6 weeks) as well as decisional regret scale postoperatively. Three patients (3/24) missed their follow-up appointments and had no recorded postoperative scores. All patients who completed questionnaires chose pain and discharge as their two most important symptoms for the MYMOP2 score.

Figure 31 demonstrates the pre-and postoperative MYMOP pain scores. Median preoperative pain score was 4 (range 1-6), and this result decreased to a median postoperative pain score of

1 (range 0-4). This difference between these paired groups was statistically significant on Wilcoxon signed rank testing ($p<0.001$).

Figure 32 demonstrates the pre-and postoperative MYMOP discharge scores. Median discharge score was 4 (range 1-6), and this decreased to a median postoperative discharge score of 1 (range 0-5). This difference between these paired groups was also statistically significant on Wilcoxon signed rank testing ($p<0.001$).

The results of completed decisional regret scales are shown in Table 27. Eighty-one percent of patients who underwent the VAAFT procedure agreed or strongly agreed that it was the right decision and 71% agreed or strongly agreed that they would make the decision to undergo the procedure again, in the same situation. All patients disagreed or strongly disagreed with the statement that they regretted undergoing the procedure, and 95% disagreed or strongly disagreed that the choice did them harm.

There was one return to theatre at two weeks postoperatively for an examination under anaesthesia (EUA) due to clinical suggestion of an abscess. No abscess or collections were identified at EUA in this patient. There were no reported complications in the remaining 20 patients.

9.5 Discussion

In this study, we used patient reported outcome measures to determine the benefit produced by VAAFT with the intent of symptom improvement for Crohn's perianal fistula. No attempt was

made to close the fistula, the internal opening was left open and a seton was left in situ. In these patients with refractory fistula, medical treatment and seton drainage were added to the VAAFT technique, in an attempt to reduce symptoms but not to affect fistula closure.

The increasingly recognised importance of improving quality of life in patients with perianal Crohn's disease is further highlighted in such studies as the PISA trial, which used quality of life as a principal outcome measure[495]. Techniques to improve quality of life in patients with refractory perianal Crohn's disease, remain crucial where remission is not always achievable. Our study demonstrated that for complex fistulas, VAAFT was associated with a significant improvement in pain and discharge, measured using MYMOP2 six week postoperatively. Fistuloscopy was feasible in 24/25 patients. In total, 81% of patients felt that undergoing VAAFT was the right decision for them and no patient regretted it. One patient returned to theatre due to increased pain and swelling, however, no abscess was found. No other complications were observed.

An important factor in assessing the role of this technique may derive from an improved understanding of the underlying mechanism of action, and how this addresses the pathogenesis of Crohn's anal fistula. VAAFT and Fistula tract Laser Closure (FiLaC™) are novel therapies for definitive sphincter sparing surgical treatment of anal fistulas, which have largely been evaluated in cryptoglandular fistula[496]. The rationale behind these minimally invasive procedures, which probe the fistula tract via the external opening, is to ablate the tract either by electrocautery (VAAFT) or laser energy (FiLaC™), damaging the lining of the tract and leaving a healthier wound behind, allowing potential tissue repair by the macrophages and fibroblasts recruited from the surrounding healthy connective tissue[497]. They have the benefit of causing minimal damage to surrounding tissues, which in turn allows the opportunity for

repeat procedures. The exact mechanism of action remains unknown, with no studies assessing the changes on a cellular level or in cytokine milieu, before and after treatment.

There are very few studies assessing the VAAFT procedure in patients with Crohn's anal fistula. A recent review article on VAAFT as well as other novel sphincter sparing techniques reported a total of 917 patients undergoing VAAFT across 12 studies[496]. Of these 917 patients, 21 (2%) underwent VAAFT for Crohn's anal fistula[191,450,455,457,496]. The studies reported varied treatment of the internal opening, with advancement flap in 11/21[191,450]; no closure in 9/21 (VAAFT used purely for diagnosis and evaluating anatomy)[455]; and suture/staple in 1/21[457]. Success was assessed clinically and varied across the studies and was seen in 12/21 at maximum follow-up of 9 months. Conclusions are difficult to draw in view of the heterogeneity in the procedure and outcome measurement, and the overall paucity of data. The largest Crohn's series in the review was by Schwandner et al. [191], reporting on 11 patients who had VAAFT in combination with advancement flap repair of complex fistulas in patients with Crohn's disease with an 81% success rate(9/11 patients). 'Success' was defined clinically as closure of internal/external openings, absence of fistula drainage / abscess formation; and this was assessed on a 3-monthly basis with maximum follow-up of 9 months. The VAAFT element was employed for diagnosis of secondary extensions with disruption of these with electrocautery or brushing. There was a diagnostic benefit noted in 64% (7/11) of patients in whom additional side tracts, not seen with preoperative clinical/endosonographic evaluation, were detected[191,496].

To our knowledge, no other studies to date have employed patient reported outcomes as the primary outcome following intervention on Crohn's anal fistula. Sahnan et al.[498], highlighted an important problem with studies reporting on interventions in Crohn's with regard to

heterogeneity in outcome reporting[498]. This heterogeneity significantly affects robust data synthesis across studies, making it difficult to determine the role and value of interventions in perianal Crohn's disease. A Core Outcome Set for this disease has recently been published by a national collaborative group, and the importance of PROMs were emphasised in this process, and in the resulting COS[310].

There are some limitations to the present study. This was a case series to determine feasibility and identify if any beneficial effect was produced, there was no comparative control group, and hence, it is not possible to exclude the placebo effect of having undergone a general anaesthetic and surgery. A sham surgery randomised controlled trial, with optimised medical management and EUA as standard in both arms, is required to answer that question. The duration of the symptomatic effect was also not ascertained in this study and can also only be determined in a placebo controlled trial. Anecdotally, symptoms (pain and discharge) appeared to slowly deteriorate after approximately two months to return to their pre-operative level. Some of the patients in this study had multiple procedures (Table 26). This was on a selective basis according to patient request following symptomatic benefit. Future studies need to assess the benefit and timing of repeated VAAFT procedures within a programme of treatment which includes optimised medical management, and whether this maintains an improvement in symptoms on each occasion. Clearly, a cost effectiveness analysis would be required to assess the health economic benefits of this approach. The various aspects of this optimised package of care aiming to produce symptom improvement and will develop and evolve in the coming years. Furthermore, closure of the internal opening (whether by advancement flap, suture or staple device) would enable assessment of the curative efficacy of VAAFT for Crohn's fistulas. VAAFT also has the potential to deliver medication to the fistula tract[440]. This application has

great potential in view of increasing evidence of injectable treatments such as stem cells, platelet rich plasma, fat[244,246,499] and also for local injection of drugs.

9.5.1 Conclusions

This study introduces the concept of symptom improvement as a clinical endpoint in its own right, in a study of a novel, palliative use for a new surgical technique in Crohn's anal fistula. Although there are outstanding questions to be addressed, including mechanism of action, efficacy when measured against placebo in a blinded study, duration of effect, and cost effectiveness, our study suggests that VAAFT may offer symptomatic improvement for some patients with Crohn's perianal fistula. Given the current low rates of fistula healing in response to any technique, it is imperative that the symptom burden associated with this challenging condition is addressed primarily. The need for a disease specific patient reported quality of life based outcome measure is highlighted once again.

Table 25: Patient and fistula characteristics

Patient Demographics	
Sex (Male: Female)	11:14
Age – median (range)	32 (17 – 64)
Median duration of CD diagnosis	5.5yrs (0 – 24)
Median duration of fistula(s)	5yrs (0 – 19)
Number of patients on biologic medication	23/25 (92%)
Number of patients with ≥ 2 previous courses biologic medication	8/25 (32%)
Presence of proctitis	6/25 (24%)
Previous surgery for fistula (including EUA)	25/25 (100%)
• Median number of previous surgeries	4 (2 – 12)
Fistula complexity	
• High fistula IO	12/25 (48%)
• Presence of horseshoeing	10/25 (40%)
• ≥1 secondary extension	25/25 (100%)
• multiple IO / fistulas	9/25 (36%)
• >1 EO	11/25 (44%)
• concomitant perianal disease (stricture/ulceration)	5/25 (20%)

Table 26: Follow-up data at end of study period

Follow-up data for patients undergoing VAAFT (n=24/25)	
Duration of follow-up	13months (4 – 27)
Number of patients who underwent repeat VAAFT procedures	9
• Median number (and range) of repeat VAAFT procedures	1 (0 – 3)

Table 27: Decision Regret Scores for patients postoperatively

Decision Regret Scale items	Strongly agree	Agree	Neither agree nor disagree	Disagree	Strongly disagree
1. it was the right decision	11/21	6/21	4/21	0/21	0/21
2. I regret the choice that was made	0/21	0/21	0/21	8/21	13/21
3. I would make the same choice if I had to do it over again	9/21	6/21	6/21	0/21	0/21
4. The choice did me a lot of harm	0/21	0/21	1/21	4/21	16/21
5. The decision was a wise one	10/21	5/21	6/21	0/21	0/21

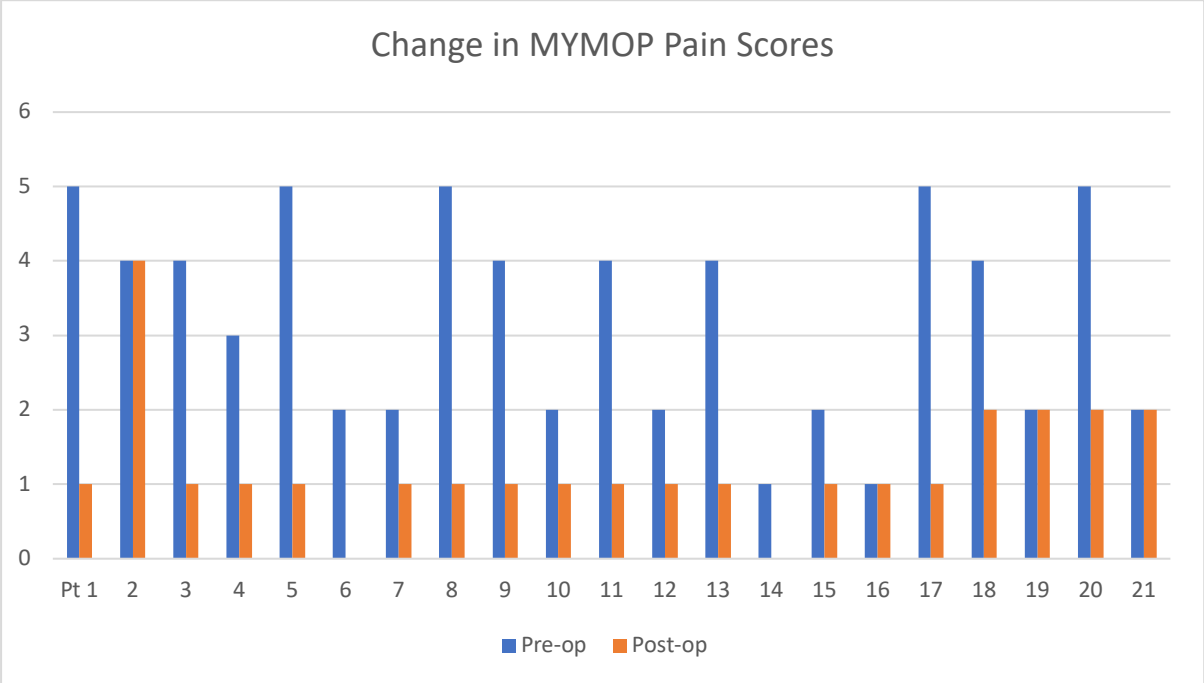


Figure 31: Demonstrates the pre-and postoperative MYMOP pain scores

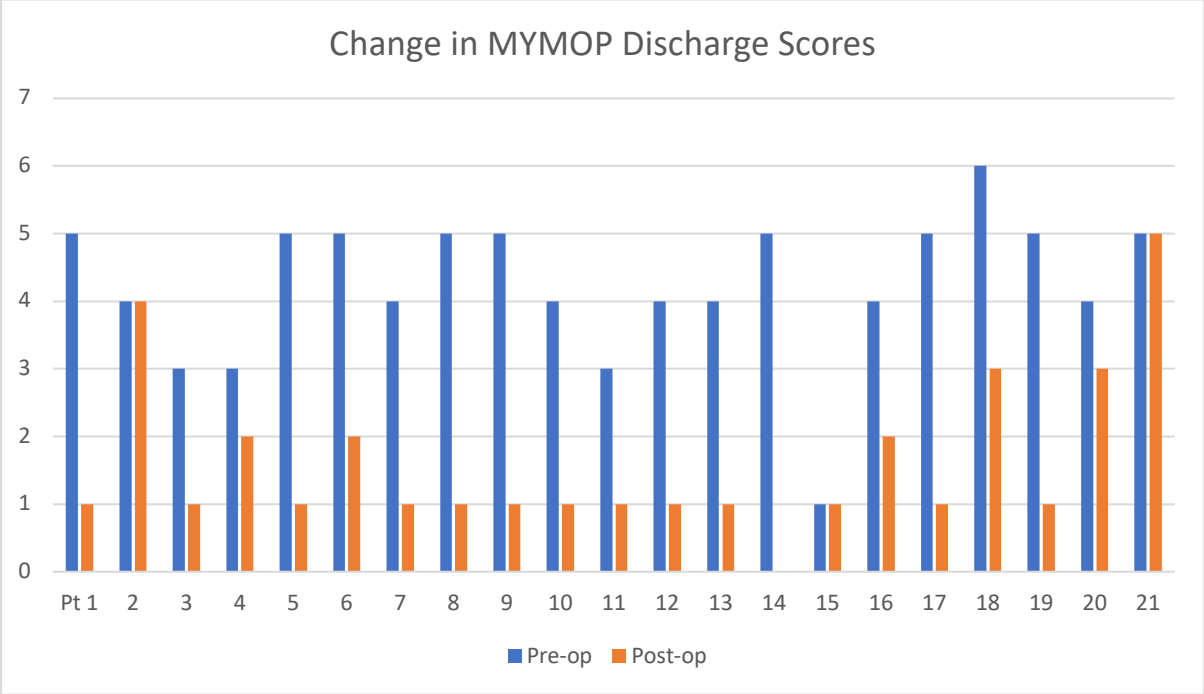


Figure 32: Demonstrates the pre-and postoperative MYMOP discharge scores

Section D - Development of a patient reported outcome measure for Crohn's perianal fistula

Chapter 10. Burden of disease and adaptation to life in patients with Crohn's perianal fistula – a qualitative exploration

10.1 Abstract

Background:

Perianal fistulas are a challenging manifestation of Crohn's disease. Best medical and surgical therapy results in only about a third of patients remaining in remission at one year on maintenance treatment and sustained healing is often elusive. There is little published data on patient perspective of living with the condition or coping strategies in the face of non-curative/non-definitive treatment. We aimed to understand the experience of living with perianal fistula(s) and their impact on quality of life and routine functioning.

Methods:

This exploratory qualitative study used purposive sampling to recruit participants with current / previous diagnosis of Crohn's anal fistulas, from national IBD / bowel disease charities. Unstructured individual face-to-face interviews were audio recorded, transcribed and analysed thematically. Early themes were reviewed by the study team including patient advocates, clinicians and qualitative researchers.

Results:

Twelve interviews were conducted, achieving apparent data saturation. Three broad themes were uncovered: *Burden of symptoms*; *Burden of treatment*; and *Impact on emotional, physical*

and social well-being. Each included several sub-themes, with considerable interplay between these. The impact of perianal fistula(s) on patients with CD is intense and wide reaching, negatively affecting intimate, close and social relationships. Fistulas cause losses in life and work-related opportunities, and treatments can be difficult to tolerate.

Conclusion:

Perianal Crohn's fistulas exert a heavy negative physical and emotional impact on patients. These findings will inform development of a patient reported outcome measure to assess treatment effectiveness and quality of life for patients living with this challenging condition.

Burden of disease and adaptation to life in patients with Crohn's perianal fistula – a qualitative exploration

'I think it just affects everything. It affects what I wear, it affects what I do, it affects my marriage, it affects everything (P2).

10.2 Introduction

Perianal Crohn's fistula(s) affect a third of patients with Crohn's disease (CD). Fistulas often represent an aggressive phenotype of CD [488,489], and follow a chronic course with symptoms including anal pain and purulent discharge. Despite best medical treatment a significant number of patients either never achieve response or subsequently lose response to biologic treatments. Only a third of patients with Crohn's perianal fistulas which close on induction, remain in remission at one year on maintenance treatment, and this number falls further with time [122]. Surgical options include drainage and seton insertion (which minimises sepsis) and definitive options (e.g. fistula plug/glues, advancement flap, LIFT – ligation of intersphincteric tract and more novel sphincter-sparing techniques). These, however, tend to fare little better than medical therapy alone and whilst combined surgical and medical therapy offers improved benefits, cure often remains elusive. Most patients experience recurrence or persistence and a relapsing-remitting course of fistula activity, which is likely to severely impair quality of life.

The only current validated clinical assessment tool designed to assess Crohn's perianal fistula activity is the Perianal Disease Activity Index (PDAI)[123]. It assesses pain, restriction of activities, restriction of sexual activities and perianal disease severity (discharge, disease type and induration). Items are scored on a 0 (no problem) to 4 (severe problem) Likert scale [123]. A

core weakness of the PDAI is the lack of patient involvement during development, and hence it only reflects what clinicians view as important. Consequently, it does not assess the global quality of life impact on patients, and its relevance to what patients consider to represent successful treatment of fistulas is unknown. There is little evidence available on patients' experiences of perianal fistulas in CD, but one early exploratory study interviewed patients with either CD or idiopathic perianal fistulas, and highlighted the extensive impact of these fistulas beyond pain and restriction of sexual activity [417]. The purpose of this investigation was to obtain detailed information from patients with Crohn's disease, describing the experience of living with perianal fistula(s) and the impact it has on their quality of life and routine functioning.

10.3 Materials and Methods

As part of a larger project to develop a new Crohn's fistula patient-reported outcome measure (PROM), we conducted an exploratory qualitative study to understand the experiences of those living with CD-related perianal fistulas [500–503]. Established qualitative techniques [502] such as purposive sampling, small sample sizes, narrative methods of data collection, and thematic analysis of data were employed. The research question was: What are patients' experiences of living with perianal fistulas related to Crohn's disease?

10.3.1 Recruitment / sampling

Using purposive sampling, community-dwelling individuals with experience of CD-related perianal fistulas were recruited from the membership of collaborating specialist charities in the United Kingdom (Crohn's & Colitis UK; ForCrohns; Bowel Disease Research Foundation). Advertisements were released online and via social media by these charities.

10.3.2 Inclusion / Exclusion Criteria

Inclusion criteria were: aged >16, living in the UK, self-reported diagnosis of Crohn's disease with current experience of perianal fistula; ability to speak, understand, read and write in English language; ability to give informed consent. Patients without a diagnosis of Crohn's disease were excluded.

10.3.3 Data collection methods

Individual unstructured face to face, video-calling or telephone interviews were conducted, according to participant preference. Following introductory procedures (consent, collecting demographic and disease classification details), the interviewer prompted each participant to '*Tell me what life is like for you with a perianal fistula*.' Follow-up questions and prompts were guided by the unfolding dialogue, thus enabling the participant to freely address their own fistula-related concerns. The aim of the interview was to secure an in-depth understanding of the experience of living with Crohn's perianal fistula, including the impact of fistula diagnosis and subsequent surgical and / or medical treatments. Interviews were recorded on a digital audio device and transcribed and anonymised by an independent professional.

10.3.4 Data analysis

All anonymised transcripts were returned to the study team (SA, LD, CN, PT, KS, TW, AH, NY and a patient/public involvement group – see below) for thematic analysis. Analysis was guided by the analytical hierarchy described by Spencer, Ritchie and O'Connor [504]; this involves several stages through which researchers individually identify themes and concepts within the data, then collaborate to agree themes, with analysis becoming increasingly interpretive as it progresses.

Each team member independently read the transcripts assigned to them, identified potential early themes and allocated provisional codes (labels). Each transcript was initially analysed by at least two team members. These early analyses were then submitted to a core group (LD, CN, SA, TW, PT) who subsequently discussed, refined codes, and agreed final themes.

10.3.5 Ethical considerations

The study was approved by a university ethics committee (17/LO/1563). Informed consent was collected immediately prior to data collection. Interviews were conducted by two researchers, SA (a clinician) and LD (an experienced qualitative researcher).

10.3.6 Patient and Public involvement (PPI) team

Four members of Crohn's and Colitis UK (CCUK), including an experienced PPI advocate were recruited to help with study design and data analysis. All had Crohn's disease and previous or current experience of living with a perianal fistula. The PPI team helped with analysis and contributed to discussion, development and finalising of emerging themes.

10.4 Results

Fourteen people with current or previous Crohn's anal fistula participated (11 female, three male, aged 16-52). During interview, one (52-year-old, female) was found not to have a perianal fistula (but a previous abscess), and another (16-year-old female) had an entero-enteric fistula. Their data were not used in the analysis. Demographics for the 12 eligible participants included in the analysis are shown in Table 28. Participants provided very detailed and highly descriptive interviews; they frequently suggested that being able to divulge details about their life with Crohn's anal fistula was very therapeutic. Following introduction of the trigger

question, interviews were largely patient-led, with open questions and few interruptions from the interviewer. Median length of interview was 43mins (range 18 – 145mins). Apparent data saturation was achieved after 10 interviews and confirmed in two subsequent interviews. During analysis, three themes, each with several sub-themes emerged from the transcripts, characterising the experience of living with Crohn's anal fistula (Figure 33):

- Burden of symptoms
- Burden of treatment
- Impact on emotional, physical and social well-being.

Verbatim excerpts from interviews illustrate each theme and sub-theme. Participants are represented by a Study ID number *('PXX').*

10.4.1 Theme 1: Burden of symptoms

This theme encompassed several key sub-themes, which were:

- Fistula-related pain
- Fistula-related discharge
- Restricted mobility due to the fistula
- Fistula-related fatigue

The predominant fistula-related symptoms experienced and expressed by every participant were pain and discharge, although the resulting perceived impact varied between participants.

Table 28: Demographic details for participants taking part in the unstructured face to face interviews

Participant	Sex	Age	Duration of CD (years)	Duration of perianal fistula (years)	Previous fistula surgery (excluding abscess drainage)	Current Stoma	Medication for fistula
1	Male	26	13	4	Yes	Yes	Nil
2	Female	26	4	1	Yes	No	Antibiotics – cyclical
3	Female	34	14	5	Yes	Yes	Nil
4	Male	23	5	3	Yes	No	Anti-TNF
5	Male	23	2	2	Yes	No	Nil
6	Female	16	2	0.5	no	No	Nil
7	Female	39	5	5	Yes	No	Anti-TNF
8	Female	52	9	7	Yes	No	Anti-TNF
9	Female	37	6	2	Yes	No	AZT
10	Female	35	15	1	Yes	No	Anti-TNF + AZT
11	Female	43	19	14	Yes	No	Anti-TNF + AZT
12	Female	20	3	3	Yes	No	Anti-TNF + AZT

AZT – Azathioprine, anti-TNF – biologic medication, specifically anti-TNF alpha drugs (infliximab / adalimumab)

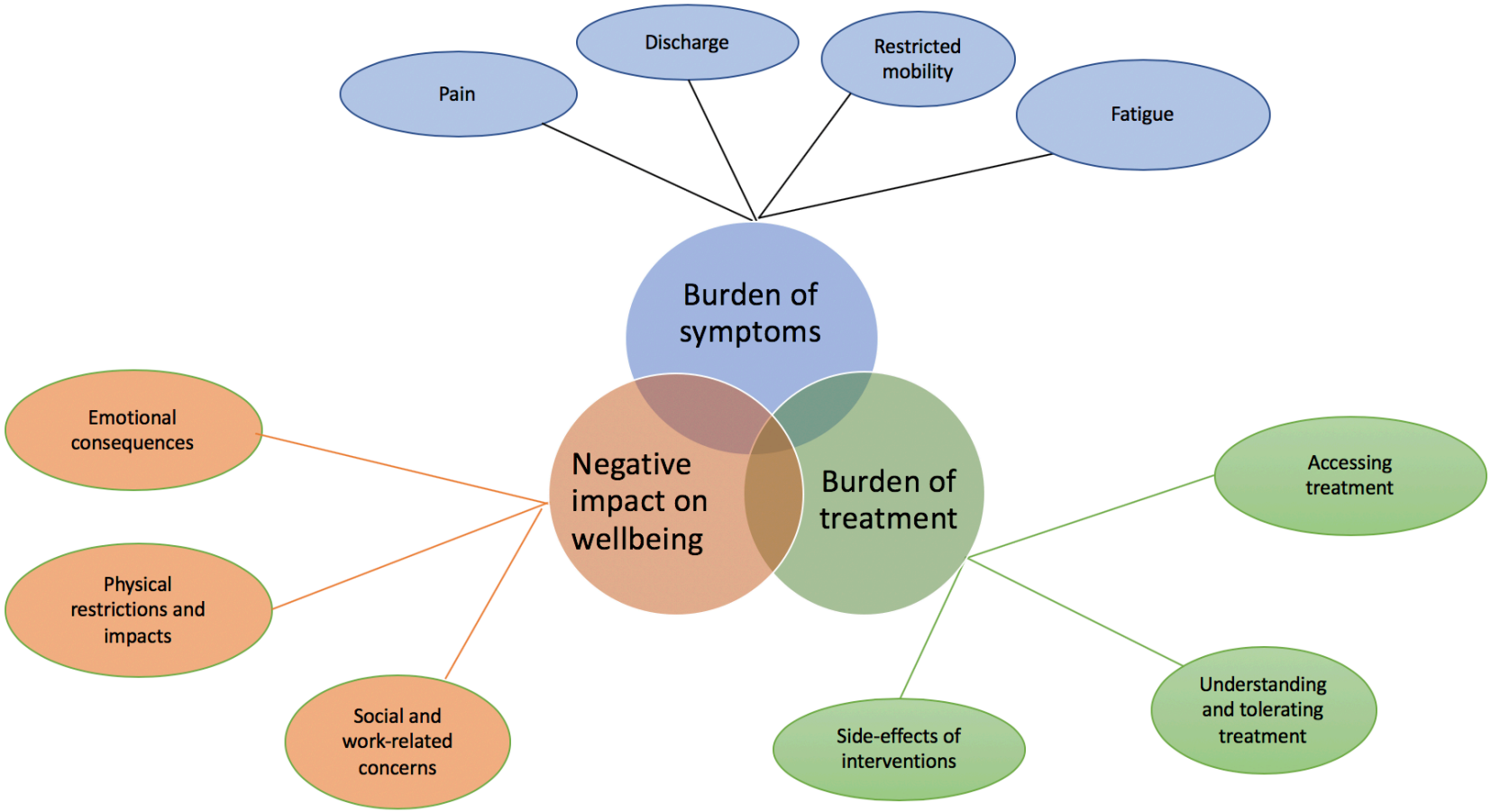


Figure 33: Thematic depiction of the experiences of living with Crohn's anal fistula
Themes are located in the central circles with the subthemes in the peripheries

10.4.1.1 *Fistula-related pain*

Fistula-related pain was a prominent and significant source of impaired wellbeing, and participants described different pain sources. Pain was usually associated with a deterioration in health state, often coinciding with initial diagnosis of a fistula, the development of an abscess, or a flare up of fistula inflammation/infection. Pain was experienced as intense or sharp, and varied in frequency / occurrence with the number of local fistula complications (e.g. abscess, infection). Participants also described a more constant background discomfort associated with the mere presence of a fistula:

'*I know I haven't had a day without pain for nearly four years… Pain contributes a lot to my life...' (P7).*

Overall, pain from the fistula had knock-on effects, which included needing powerful analgesia:

'there's no way that you could get past [the pain]. Because I couldn't sit down or I was in like such bad pain that I was on Oramorph [oral morphine] and then you've probably got to be a special kind of, of intolerant or super tolerant person, I don't know how you put it, to like to be able to function as normal when you're on Oramorph' (P1)

Pain further impacts on daily functioning, as discussed in subsection (ii), below.

10.4.1.2 *Fistula-related discharge*

All participants described the burden caused by fistula discharge. There were concerns about the quantity and smell of fistula discharge

'Quite frequently ... probably once a day, or once every couple of days or whatever I have to sit on the toilet and release a load of discharge' (P1).

Whether described as leakage, seepage or discharge, the output from fistulas caused embarrassment to participants, amid concerns about odour and visible leakage, effect on surrounding skin and clothing (discussed in Theme 3, subsection(i)). Persistent discharge associated with setons left some with the feeling of incontinence and increased the need for some degree of barrier protection (e.g. absorbent pads or gauze in their underwear) against skin irritation.

'...there was this constant stuff coming out, and having to wear sanitary towels to try and make sure it didn't leak through clothing ... it was not an easy thing to try and manage' (P7)

10.4.1.3 *Restricted mobility due to fistula*

Participants reported an overall impairment in mobility associated with fistula(s).

Factors contributing to this include the anatomical location, for instance - involving musculature and limiting range of mobility. Position- related pain or irritation around the fistulas was also brought on by sitting down/certain stances/walking/running:

'On a day-to-day basis ... I can't really tolerate more than fifteen minutes sitting down' (P11).

Similarly, irritation from setons in the perineum was exacerbated by certain sitting / standing positions or mobilising:

'Because [the setons] are in my bum, it affects the muscles around my bum and it affects the way I can sit down' (P4)
'I don't know whether it was the position of them, but I struggled to walk for much, for any length of time'... (P7).

Scarring or inflammation in the tissues around the perineum were also reported to result in loss of ability to move in their premorbid manners and in some cases resulted in an antalgic gait.

Discomfort caused by irritation from pads or gauze used to absorb leakage, played a role in restricting freedom of movement and patients often reported the need to constantly change positions as prolonged time in any particular position frequently resulted in discomfort.

10.4.1.4 *Fistula-related fatigue*

Participants often reported significant fatigue attributed to their constant experience of fistula pain. This pain-related fatigue was sometimes exacerbated by lethargy associated with use of analgesics. In these instances, patients reported being trapped in a cycle of pain - analgesia - fatigue - lethargy. Whilst a feeling of lethargy was attributed to underlying Crohn's disease in some cases, it is important to note that some patients reported this being associated specifically with the anal fistula and not CD:

'There's like a lethargy which is resultant of the fistulas and also the Crohn's, but I actually think that an awful lot of my lethargy came from the pain from the fistulas and also from, for example, not being able to get back to sleep because you are in pain because of the fistulas' (P1).

There were reports of feeling exhausted due to the rigours of managing the mental and physical requirements of dealing with challenging fistulas. Some participants reported sleep disturbances due to waking up to change dressings or because of abrupt pain secondary to a seton knot catching within the fistula tract or on clothing material. Others described a general 'wearing down' due to the burden of symptoms and treatment:

'You're done in. Yes, it just wipes you mentally, which mentally makes you just want to lie in bed. There's your physical side gone; you just don't want to do anything' (P12).

Furthermore, participants described the impact of fatigue on social and preferred activities, with their horizons becoming narrowed as fatigue related to fistula pain prevented them from pursuing hobbies and interests.

10.4.2 Theme 2: Burden of disease treatment

Treatment of perianal Crohn's fistulas is often multidisciplinary with long-term use of immunosuppressive or immunomodulating medications and surgical interventions for drainage or an attempt at definitive treatment. Often these therapies fail, and participants report undergoing a cycle of repeated burdensome treatments with elusive long-term cure. Under this theme, the key sub-themes were:

- Accessing treatment
- Understanding and tolerating treatment goals and processes
- Side effects of medical and surgical interventions

10.4.2.1 *Accessing treatment*

Participants often described experiences of needing frequent access to treatment; multiple interactions with healthcare services were varied and included positive and negative perspectives. There was often a distinction between the experience of access to general and emergency services compared to specialist clinicians. When emergency care was required, participants often did not have access to tertiary care healthcare professionals (HCPs) with expert interest in Crohn's anal fistula:

'I have a problem sometimes if I go into hospital for an emergency operation, because the surgeon I [usually] see doesn't work at the hospital with an A&E. He's never there. (P4).

Participants reported some negative experiences when interacting with HCPs with poor understanding of the condition, its presentation, or acute exacerbations. Some participants reported receiving conflicting advice during management which led to loss of faith in the treating practitioner. Early identification of a developing or worsening abscess or fistula is essential to ensure timely, targeted intervention and to rescue and preserve deep tissues.

However, participants reported that expert opinion was often lacking amongst receiving Accident & Emergency staff:

'so, you go to A&E and what I tend to have found is that the majority of doctors won't have necessarily seen [Crohn's abscesses or fistulas] before and so, they don't necessarily, they don't necessarily know whether something is gearing up' (P1).

In this space between the expert knowledge of the patient, and the non-specialist knowledge of the frontline staff, the patient's condition can deteriorate. The challenges of self-presenting in emergency departments and difficulties accessing specialist opinions either at diagnosis, or at moments of exacerbation was a source of stress for participants, particularly if referral between hospital departments was either not expedited or there was a prolonged delay from time of referral from primary care:

'My GP has told me many times, "There's nothing that I can do to help you, you need to phone the hospital." And then hospital appointments are so far, you know, few and far between….' (P3).

Delay in diagnosis, misdiagnosis, or lack of capacity to provide required specialist care could result in anxiety and loss of confidence in the healthcare service. Prompt access was often viewed positively, and positive established relationships with HCPs was linked to greater confidence in the care received.

10.4.2.2 *Understanding and tolerating treatment goals*

Participants reported that healthcare professionals often did not explain goals and methods of treatment coherently, or provide realistic expectations about setons and pain following surgery, or the extent of surgery:

'It was a male doctor, a male colorectal surgeon that did it. And I didn't realise that that operation was going to be more towards the vagina. And I had felt like I had just been butchered. That's how I felt. It was so painful and I just remember thinking, "What have they done to me, what have they done to me?" And I never went back again. It was so painful' (P3).

For this participant, the poor preparation and explanation of her surgery resulted in her withdrawing from health care support altogether. For others, mixed messages lead to confusion and difficulty knowing who to trust:

'It's just I have different teams telling me all different things. I have [one hospital] telling me 'You need a [stoma] bag'. I have my surgeon telling me I need more setons in. I have my consultant at [another hospital] saying 'You won't need a bag'. It's just, I don't have one opinion, I have four different and I don't know who to trust. I don't know who to say, "I'll believe, I put my life in your hands and know that you're going to make me better and do the best for me." I just don't feel like I have that with anyone' (P12).

Consequently, participants often sought information from alternate sources, such as online searches and support groups to better understand the best treatment for them.

Others described more positive experiences, including developing rapport and trust with the surgeon who had performed several operations on them, and doctors following up when concerned about an individual's wellbeing:

'My doctor physically phoned me just to say, "Oh I was a bit concerned with you in clinic yesterday, how are you doing, is that alright, do we need to see you sooner than your next appointment that we booked?"' (P5).

Understanding and having realistic expectations about interventions and outcomes was found to be important in helping patients cope with the possible relentlessness of CD-related fistulas. Several participants reported that the repeated treatments and the cycle of flare-ups gave them a sense of being on a constant treadmill of appointments, medication and surgical intervention.

Some participants' experiences revealed the perspective of fistula treatment being often onerous and seemingly futile. In particular, those with longstanding fistulas and multiple previous interventions seemed resigned to the lack of definitive cure and anticipated a future with repeated fistula relapses and remissions:

'It makes me feel a bit hopeless, because I feel like there's no hope of it getting fixed, ever' (P9).

Participants also expressed frustration that HCPs themselves do not necessarily understand the impact of the very treatments they propose:

'I am probably one hundred percent confident that no surgeon that has ever put a seton in, has had a seton themselves.' (P1).

Failing to explain treatments adequately or showing lack of insight and empathy in respect of the impact and relentlessness of treatments, can be detrimental for some patients.

10.4.2.3 *Side effects of medical and surgical interventions*

Alongside dealing with the symptoms of and treatments for fistulas, participants revealed the burden of coping with the side effects of medical interventions. Concerns included systemic symptoms such as gastrointestinal disturbances, minor infections, rashes and headaches, and the potential risks of medication:

'It has taken me fourteen years to accept medication. It has. There's this thing in my mind where I think to myself, "Well, yes I know I've got the fistulas, but I cope. I work, I do this, I do that. Do I really want to take medication that's going to increase my risk of cancer and it's going to give me headaches and make me feel more fatigued? And, you know, I could suffer with anaphylactic shock." And sometimes I see that the medicine will restrict my quality of life more. And I really worry about that, because nobody really knows until you take it.' (P3).

The need for medications resulted in varying degrees of loss of independence, particularly for participants who were on infused medication requiring hospital visits for administrations.

The impact of surgery brought mixed responses from participants. Many viewed surgery as a positive necessity when, for example, they had developed an abscess requiring drainage. There were many strong negative sentiments about seton use, and some participants felt the presence of the seton was worse than having the fistula itself:

'Every time that I've had one, the seton has caused a lot more discomfort than the abscess has given me in the first place…on two different occasions I've had such agony from the setons, I've had to cut the seton out myself' (P1).

'My setons were placed a year ago. They're the most uncomfortablest [sic] things I think I've ever experienced … they're as thick as a straw and they're knotted about eight times. You can imagine the thickness. The pain that they cause. They cause rubbing, they cause bleeding … they've caused little scars between my vagina and my bottom because they're that thick, they're literally … making you bleed throughout the day' (P12).

The type of seton material used and method of securing it in place (degree of tightness and number of knots) varies between surgeons and can vary from silastic setons (bulkier) with multiple knots (or knotless), to suture material (finer in comparison) with smaller knots, with some newer setons having no knots at all. Participants reported a preference for suture material (which caused less discomfort), but the sporadic sensation of seton knots in the fistula caused pain, which often led to a degree of apprehension about mobility, sitting down or making sudden movements:

'I don't know whether it was the position of them, but I struggled to walk for any length of time'... (P7). 'I used to have the bigger seton which I hated because it gave so much pain and now, I have the stitch seton which is better, but still uncomfortable because of all the knots... my latest seton, I think it's too long' (P9).

Participants also reported difficult experiences secondary to pain or discomfort when recovering from surgery, particularly following drainage or lay-open procedures. This post-operative period was often described as challenging particularly if there were large wounds after surgery requiring regular management:

'Nobody really prepares you again for these operations. They tell you what it is and they didn't really kind of explain every time, they didn't explain the pain that I would feel' (P3).

Furthermore, residual scarring and hardening of tissues around the buttock area from previous, often repeated, surgery also caused some negative experiences emotionally and physically:

'I think the scarring around the fistulas creates this – not really a fear, but it just sets you in the mindset of if I push myself too far, it's going to rip, something is going to happen. And you can feel that' (P3).

Participants who were living with chronic fistulas present for 12 months or more, often expressed fear and uncertainty about possible future treatments. The formation of a temporary or permanent stoma, diverting faecal flow away from the fistula(s) is sometimes a necessary therapy when perianal disease becomes unmanageable. Proctectomy with removal of the rectum and anus may also be required. Some participants expressed an apprehension about needing a stoma in the future:

'They have been talking about a stoma, so then you're thinking, you know, is that something that I've got to consider…' (P9).

Those who had undergone proctectomy and permanent stoma formation experienced this in the context of aggressive debilitating disease which had impacted negatively on quality of life. They had desired a more permanent resolution to their symptom burden due to the overwhelming negative experience of their fistula-related problems:

'the fistulas got really bad to the point where I needed the stoma' (P3).

Participants for whom the proctectomy proved curative for their fistula described positive outcomes, with relief being a common thread, as well as a sense of 'having their life back'. They did however express that the decision-making was complicated as they perceived a lot of stigma related to the stoma.

10.4.3 Theme 3: Negative Impact on Wellbeing

Several issues including the mere presence of a fistula and the symptoms related to it, and having to undergo fistula treatment, had various, often negative impacts on participant's sense of wellbeing. The key sub-themes that emerged were:

- Emotional consequences
- Physical restrictions and impacts
- Social and work-related concerns

10.4.3.1 *Emotional consequences*

Participants described wide-ranging emotional consequences of living with CD-related perianal fistulas, including impact on mental health, body image and self-confidence.

Mental health effects included worry, anxiety, feeling low or depressed, and mental exhaustion. Feelings of embarrassment often arose due to the fistula/seton and its location in an intimate area, leading to feelings of 'not being normal'. Some participants described significant changes in personality and outlook and two participants described periods of significantly low mood and suicidal ideation driven by the burden of living with a fistula - both sought therapy and had or could overcome these periods:

'The mental side of it, for me, is massive. It's probably the hardest part for me to deal with. But I'm trying to deal with it every day. So that's why it gets so hard.' (P4).

Some participants felt more optimistic during periods of reduced symptom burden and decreased need for treatments, describing hopefulness, positive outlook, and acceptance. Positive interactions with health specialists, and strong support networks and relationships as well as online support groups often contributed to positive emotions:

'There's a fantastic closed group for fistula, American-based, but I think there's about a thousand people in it, roughly and it's amazing. There are people there who are just patients themselves but are very, very knowledgeable. And you will always get an answer for something or a suggestion or even if it's just, "I understand."' (P11).

However, in some cases a necessitated increased reliance on others during periods of increased symptom burden led some to feelings of guilt and other negative emotions including feelings of depression.

As well as impacting on mental health, living with a fistula was also potentially detrimental to some individual's self-confidence and body image. Participants described that the presence of the fistula in an intimate part of the body, with the often-associated (and sometimes faecal) discharge, resulted in feelings of altered body image:

'[The fistula] does attack your self-confidence a little bit … I don't really want to see it – I know what's happened to me, I don't want to see it, I don't want to deal with it. Everyone else has got to dress me anyway, and do the dressings, and just tell me whether it's good or bad. And I've kind of kept with that. And it does make me upset. I have seen it once, and I don't like the appearance of it.' (P2).

Scarring arising from surgical procedures also contributed to the sense of altered body image, while the presence of setons (particularly the bulkier silastic setons) contributed to participants feeling 'abnormal'. These changes, combined with the need for pads/gauze or larger underwear

for ‘hiding’ the changes, as well as restricted clothing options for some, led to feelings of reduced self-confidence and negative perceived body image:

‘You just want to look normal down there… I think because I have [the fistula] and it’s made me feel ugly, then it makes me start obsessing about other areas of my appearance’ (P9).

Many participants explained that they could not wear tight clothing, as this increased the discomfort they experienced from their fistula, and women in particular reported feeling limited in the choice of outfits available to them, further impacting on their sense of self, confidence and self-esteem:

‘You have to pick what clothing you wear – leggings can start infection, because they’re tight. Then, you’ve got to wear everything loose, which is a nightmare’ (P2).

There was considerable concern about risk of clothing being stained and the need to choose clothing colours specifically to mask any unpredictable discharge:

‘My knickers are constantly ruined because it’s, my, the leakage is, is going through my knickers or my brother’s boxers, if I wear his. Towels. I feel like towels just aren’t clean, because I’m constantly - it’s like yellow and green infection all over your belongings. I mean dark clothes are a definite.’ (P12).

Scarring, altered body image and low confidence could also have consequences for intimate relationships, with some participants volunteering emotional reasons explaining why they avoided sexual intercourse altogether:

‘I think you always have that anxiousness in your head, thinking, “Oh no, he’s going to, he’s just going to think I’m absolutely disgusting.’ (P12).

Participants reported high levels of anxiety around sexual intimacy, often related to the fear of leakage or unpleasant odour. This had a negative influence on emotional wellbeing, with restricted sexual freedom and physical pleasure often decreasing sexual desire.

'The main problem is, for me, is the embarrassment if there was discharge. It's happened before and it's just like, it's just put me to a point where I just say, "I'm so sorry," and I just literally like just sat on the edge of the bed and just cried.' (P4).

For many participants, from newly-weds through to those who had been married for many years, the lack of physical intimacy in their relationship was experienced as a huge loss.

10.4.3.2 *Physical restrictions and impacts*

In addition to the emotional impact, fistulas caused, they resulted in physical restrictions in activities of daily living and intimate activities, as well as impacting on cleanliness and hygiene. Restriction of simple daily functions like sitting down had knock on effects on wide-ranging activities of daily living. Walking and running were also sometimes affected in some participants leading to aversion and coping strategies, included limping, and avoiding walking long distances or running activities that would trigger discomfort, with negative impact on regular exercise and sporting activities, and certain activities (e.g. cycling, swimming) becoming impractical for some.

'Well you learn to walk differently, you learn to sit differently, you learn to stand up differently'. (P3).

'I felt like I couldn't [go and] swim. Whether I could or not, it didn't feel hygienic to swim with the fistula because especially if it's still leaking, it just doesn't feel right' (P7).

'Driving was one thing that I really found difficult to begin with, just speed bumps, holes, you know, things like that' (P5).

Fistulas were understood to negatively alter intimate sexual experiences, as participants described physical restrictions limiting freedom of movement and enjoyment, as well as pain from the fistula being exacerbated with intercourse:

'We don't do sex anymore…it's like l am a born-again virgin, in a sense, that's how I feel, because the pain, it just hurts' (P11).

Setons also restricted freedom of movement during sexual activity:

'With the seton again, you feel a bit embarrassed, especially if it's a seton that hangs low or moves or anything. You know, you just can't settle or enjoy' (P3).

Participants often expressed a significant change in their daily physical routines to compensate for the physical restrictions, and also reported an increased level of 'self-maintenance' required because of the fistula. This was reflected in participants' experiences of maintaining self-hygiene and cleanliness, leading to altered hygiene habits. Behavioural changes included frequent shower use and carrying spare underwear and/or a full change of clothing in case of accidents. This often led to prolonged time spent in the bathroom, increased toilet usage and in some cases almost 'ritualistic' toilet use brought on by having multiple fistulas:

'I find a bit of a Catch 22 with it is, everyone tells you to keep it clean, keep it clean, keep it clean. But the area that it's in and the fact that it's draining pus..., that's not an easy thing to do.' (P5).

Increased toilet usage was sometimes described as necessary to avoid soiling accidents and public embarrassment. Some participants reported difficulty in maintaining a clean genital area, a struggle made worse by increased fistula discharge or multiple setons:

'Trying to use toilet roll when you've got this mess of abscess and seton is just a nightmare, because it just all gets caught up' (P7).

10.4.3.3 *Social and work-related concerns*

The presence of perianal fistula(s) was often described as adding a layer of complexity to managing and living with Crohn's disease. The social and work-related concerns arising from having a fistula were revealed in participants' descriptions of the effects on employment and finances, independence, social interactions, relationships, and development of coping strategies to deal with these.

Participants described having to take time off work/education usually due to fistula-related surgery, treatment or flare. Whilst participants struggled to quantify the losses, most spoke of a sense of loss of work options or potential, and concerns about job stability:

'I've been off sick since February 2015. 'For the last few years prior to that, the holidays when you get four, five weeks' holiday in a year, I was taking them in a sense as sick days, because I didn't want to lose my job because I'm scared of who's going to employ me with my sick record' (P11).

Participants felt they had restricted options for the types of jobs they could manage, for example needing to avoid those involving prolonged sitting (or jobs involving driving). Some participants changed their occupation to avoid exacerbating fistula pain and discomfort, Similarly, younger patients reported problems with prolonged sitting in school:

'It was just totally impossible for me to, to be able to sit for three hours to do an exam, so, I remember doing the exams while I was kneeling' (P1).

There were also reports of workplace modifications, such as adjusted seating to suit a participant's specific needs. For those participants who were working, many felt supported by their workplace:

'I ended up missing quite a lot of work with the abscess and the fistula combined… I was so lucky, I had a very, very understanding boss, who was just brilliant ...' (P10).

Managing the fistula had financial implications, not least due to the increased use of pads, gauze, and underwear to maintain cleanliness:

'It's unbelievable. I've probably got through a box; how many boxes a week? Three, four boxes a week of pads. I'm constantly buying them' (P12).

Participants also described loss of earnings related to the fistula due to the inability to work, or perceived reduction in career potential due to needing substantial time off work. Some participants also reported an increased dependency on friends and family for financial support as well as practical support which in some case led to a sense of loss of independence:

'I haven't had any salary since January. And so, I quite rely on family to get me by, and also, my partner's income. It's also affected, affecting even looking forwards around the type of job that I get, because I'm wary of getting a full-time job [in case I] fall back into this like boom and bust way of managing my healthcare' (P1).

The uncertainty of fistula-related exacerbations had no respect for important periods of participants working / education lives, or indeed other personal milestones. Participants' sense of 'self', arising from being independent, in control, and having choices in life, was negatively affected during periods of exacerbation and rescue treatments. The fistula was understood as robbing them of potential or ability to fulfil their dreams:

'[The fistula] has a mind of its own and I'm along for the ride' (P2).

Living with a fistula reduced social opportunity and some participants described avoidance of social situations due to a need for 'toilet-mapping', which felt restrictive. The presence of discharging fistula(s) raised the constant need for access to a suitable toilet, causing participants to always frequently look for facilities in new environments. This sometimes restricted options for going out, with participants preferring venues where the toilet facilities were easily accessible and clean.

'So, I wouldn't go there if there's no toilet' (P2).

'I still went out with, with my daughter, but we often just used to go to cafés or whatever, where I knew there was toilets' (P7).

Participants who experienced frequent exacerbation of fistula symptoms tended to reduce social interactions to avoid either the physical requirements (e.g. frequent toilet use, prolonged sitting) or the degree of pre-planning that would precede being able to attend. Others cancelled plans due to low mood or fatigue and decreased desire for social engagements, and in some cases, due to concern about stigmatising attitudes from others. Participants sometimes selectively limited disclosure of their illness as an attempt to avoid potential stigma:

'I don't really go out. Since having perianal disease, your social life as a twenty-year-old completely disappears. You can't do anything because no one understands.' (P12).

Participants reported varied experience of relationships secondary to having a fistula and these were both positive and negative. Some participants experienced increased closeness to family members or a close circle in whom they confided and who provided a support network subsequent to their diagnosis of Crohn's anal fistula:

'*I genuinely do think that I probably am lucky to have a partner that is, not just understanding, has time to listen, but also probably understands it more than me*.' (P1).

A recurrent theme was apprehension about starting new relationships, particularly intimate ones, and these feelings often coincided with periods of fistula exacerbation or need for recurrent rescue treatments:

'*It affects your relationships with your friends. And family as well. Because it's hidden as well, I find that I'm always pretending, you know, "I'm fine" is the two famous words. And just keep on smiling, even though if you feel really dire at times. Because people can't see what's going on inside, they just assume that everything is fine*' (P11).

To overcome these many negative fistula-related impacts, some participants developed coping strategies. These included seeking further information and understanding, as well as seeking support via family, or through groups and individuals with similar diagnosis, particularly online support groups:

'Just to touch base with someone who's been through a similar thing is always good for me to do' (P5).

Career, work and personal aspirations were often modified due to symptoms and frequency of interventions. Discussion with work colleagues (particularly those in senior positions) sometimes resulted in positive modifications of work environments which helped participants. Prompt access to, and faith in, expert HCPs as well as successful interventions – including fine thread setons and medication which reduced fistula leakage - were described as positive experiences which enabled participants to cope and live better with the fistula:

'I have had huge support from, particularly, my husband and from all the medical professions that I've been dealing with' (P8).

In contrast, some participants described avoidance of coping mechanisms and negative emotions, and absence of a support network. Typically, these participants described some

degree of withdrawal from society, with an inevitable negative impact on social interactions and development of new relationships. There were also reports of withdrawal and 'distancing' from established relationships with a pretence of coping well:

'*I basically try and make it so that no one thinks I'm having any issues, because to me, that's the easiest way to deal with it, because then less people ask more questions*.' (P4).

10.5Discussion

This study reports the results of the first in-depth qualitative exploration into experiences of living with Crohn's anal fistula. The rich data obtained provide a deeper understanding of the potential challenges facing patients. The experiences of patients with Crohn's anal fistula are far-reaching and extend far beyond the fistula-related symptoms. Our results demonstrate that these experiences can be broadly divided into three categories: burden of symptoms, burden of treatment and impact on physical, emotional and social aspects of wellbeing. There is considerable interplay between these themes and the extent to which they were experienced varied from participant to participant [Figure 33].

Providing accurate, in-depth and targeted information for patients at the point of fistula diagnosis is likely to be beneficial. Health care professionals could use Crohn's anal fistula information leaflets, and visual representation of the patient's fistula using novel imaging techniques which have been recently been described (e.g. 3D MRI and 3D printing of fistulas) [505,506] to achieve this. Patients' source of information and the psychological response to the diagnosis has a bearing on how they perceive their fistulas. Trusted healthcare professionals, support groups and online sources often help with building positive experience.

Following diagnosis, the nature of the symptoms and subsequent disease course or treatment that a patient receives, determines the experience that the patient subsequently has. Those with mild and less troublesome symptoms or who achieve early healing, can be anticipated to have a reasonably limited deterioration in their quality of life secondary to the fistulas. However, this study demonstrates that those with more chronic fistulas or significant symptoms, have a very different experience which is often restrictive and negative in nature. Areas affected can vary from restriction in daily activities, to decreased career and financial potential as well as significant restriction of factors that contribute to emotional, physical and social wellbeing. The unintended detrimental effects of setons may be reduced through thoughtful use of finer sized seton materials (with fewer knots) that are available to surgeons.

Chronic illness adaptation requires successful negotiation to a new identity [507] and this can sometimes be challenging in Crohn's disease due to unpredictable disease flares [508]. Our findings demonstrate that the impact of Crohn's disease anal fistulas on patients extends far beyond the limited clinical, physical and sexual activity focus of the PDAI[123]. There is no available tool which assesses clinical markers of treatment effectiveness as well as the global quality of life impact on patients, developed to current standards and with patient input. There is a need for a patient reported outcome measure (PROM) that encompasses the key aspects covering the range of patient experience as demonstrated in this study.

Strengths and Limitations

Individual, followed by team analysis and agreement, enhanced credibility of findings by ensuring data analysis and subsequent findings were not influenced by a lone researcher, but are a consensus of the study team. The study benefitted from robust input from a patient and public involvement group who assisted in reviewing anonymised transcripts and developing

and refining early themes. A multidisciplinary steering group allowed for crucial stakeholder perspectives to be included in the analysis and review of emerging themes. Limitations included inability to transfer findings to the elderly population of patients with CD fistulas, since the oldest study participant was 52 years old. A limitation of the study was the inability to transfer findings to the elderly population of patients with CD fistulas, since the oldest study participant was 52 years old.

Conclusion

The impact of perianal fistula(s) on patients with CD is intense, leading to reductions in life opportunities and negatively affecting intimate, close and social relationships. Capturing patient experience that extends beyond disease-related symptoms improves understanding and gives insight into the overall impact of Crohn's anal fistulas. Currently, no patient reported outcome measures are available for use in routine practice in Crohn's fistula management and as a result, patient outcomes (other than disease-related symptoms of pain and discharge) are rarely captured [509]. The patient experience is invaluable in the development of a PROM for this condition which would benefit in monitoring and improving the care for individual patients [310,510–512]. Potential areas of use include routine practice, monitoring treatment efficacy, clinical trials, and telemedicine systems[500,511,513]. Work is currently underway to develop a Crohn's Anal Fistula Quality of Life tool, using the information gathered in this study[514].

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Chapter 11. Development and validation of a patient reported outcome measure for Crohn's perianal fistula – Crohn's Anal Fistula Quality of Life questionnaire (CAF-QoL)

11.1 Abstract

Introduction:

Crohn's perianal fistulas are challenging for clinicians and patients. Many do not respond to available treatments, and despite recommendations by a recent global consensus, there are currently no specific patient-derived quality of life (QoL) tools to measure outcomes including response to treatment, in patients with Crohn's perianal fistulas. Exploratory qualitative work has demonstrated that Crohn's perianal fistulas reduce QoL far beyond restricting daily and sexual activities, and a patient-centred, patient derived tool is needed. We present a patient reported outcomes measure (PROM) for this complicated disease phenotype.

Methods:

A 35 item draft questionnaire was generated using information from: a) unstructured qualitative patient interviews, exploring the experience of living with Crohn's perianal fistulas; b) a consensus exercise analysing outcomes from a systematic review of studies assessing medical, surgical and combined (medical/surgical) treatment of Crohn's perianal fistula c) a patient and public involvement meeting. Psychometric properties were assessed including construct validity (by comparison with the Hospital Anxiety and Depression Scale, HADS and the United

Kingdom version of the Inflammatory Bowel Disease Questionnaire, UK-IBDQ), and reliability and responsiveness assessed by test-retest analysis.

Results:

Data from 211 patients contributed to the development of the questionnaire. Cronbach's alpha was 0.88, and item-total correlations were very good. Analysis aided reduction of the questionnaire to 26 items that demonstrated good internal consistency, good stability (intra-class correlation 0.98) and good construct validity with positive correlation with the UK IBDQ and HADS.

Conclusion:

A disease specific PROM to assess clinical outcome (i.e. QoL) as baseline and following interventions in patients with Crohn's anal fistula – the CAF-QoL – is ready for use. Translation and cross-cultural validation will aid wider international dissemination.

11.2 Introduction

Crohn's disease (CD), affects approximately 145 people per 100 000 population in the UK, and between 4 and 250 people per 100 000 population worldwide[515]. Perianal fistulas occur in a third of all CD patients[489]. They represent a distinct and aggressive phenotype of Crohn's disease[488,489] and follow a chronic course with symptoms including anal pain and purulent discharge, often leading to a severely impaired quality of life. The associated morbidity of the disease and its treatment can have profound effects not only on patients' physical wellbeing, but also their emotional wellbeing, social life, educational activities, professional lives and intimate relationships.

Crohn's perianal fistulas are challenging to treat not least because they are often complex in nature and refractory to conventional medical treatment strategies such as antibiotics and immunomodulators[67,203]. The introduction of anti-TNF agents promised improvement in treatment with clinical response rates of up to 68% and complete healing rates of 55% reported in the short-term[120], but true healing remains elusive[377,516]. In 2014 Gecse and colleagues [119], representing an expert global consensus group on perianal Crohn's disease from ECCO, published guidelines on the treatment of perianal fistulising Crohn's disease. They recommend anti-TNF agents as the current gold standard, with antibiotics and immunosuppressant agents offering a role as adjunctive treatments. It is important to note, however, that despite best medical treatment a significant number of patients either never achieve response or subsequently lose response to biologic treatments. Only a third of patients with Crohn's perianal fistulas which closed on induction remain in 'remission' at 1 year on maintenance treatment and this number falls further with time[122]. Surgical treatments fare little better and

despite benefits from a multidisciplinary approach, sustained cure is often an elusive goal and most patients experience recurrence or persistence.

In clinical trials, success is usually measured by clinical assessment of the fistula tracks and whether they close in response to treatment. This may rarely be accompanied by radiological assessment to confirm 'healing'. This is appropriate in trials of treatment with 'curative intent', but given that most patients do not achieve sustained closure of their fistula, there is a need to measure any benefit produced by treatments in those whose fistulas do not 'heal'. Further, the additional impacts of any intervention may have an impact on quality of life even if successful 'healing' occurs. For example, continence impairment might occur after a 'curative' fistulotomy or advancement flap repair. There are also interventions which are not designed to heal fistula and are instead performed (and studied) with palliative intent. QoL would represent the primary outcome in such studies.

The only current measure designed to assess Crohn's perianal fistula activity and its impact on quality of life is the Perianal Disease Activity Index (PDAI). It is a validated clinical assessment tool that aims to measure disease activity in patients with Crohn's perianal fistulas. It assesses pain, restriction of activities, restriction of sexual activities) and perianal disease severity (discharge, disease type and induration). Items are scored on a 0 (no problem) to 4 (severe problem) Likert scale[123]. Although the PDAI reaches 87% accuracy when compared with clinical assessment[517] and addresses fistula classification and activity, correlations between it and other measurement techniques can be weak[518]. A further weakness is the lack of patient involvement in the development of the PDAI; reliance on clinician-only input means that the short index addresses aspects which reflect clinical importance, or quality of life issues which physicians believe will be important to patients, but do not assess the global quality of

life impact on patients, and its relevance to what patients themselves consider to represent success or important features of QoL is unknown. Recent guidelines recommend that improved instruments are needed to score perianal CD fistula activity and impact on quality of life[119]. In particular, a tool which assesses the impact on a patient's quality of life due to their fistula and which detects a change to their quality of life after treatment in a meaningful way, is required. Early exploratory qualitative work demonstrates that the impact of CD perianal fistulas on quality of life reaches much further than restricting daily and sexual activities[417,519] and a patient-centred, patient-derived tool is needed. We have developed a new quality of life assessment scale for CD patients with perianal fistulas, using published methodologies, and ensuring patients are involved in all phases of questionnaire creation.

11.3 Methods

The study was a three-phase mixed methods design utilising an exploratory instrument development model[520,521] to support the instrument development process as evidenced by Rothrock et al[522]. The first phase uses an exploratory qualitative approach, recommended for investigation of novel subjects about which little is known[500]. The second phase involves a process of cognitive interviews to refine items on the questionnaire and the third phase involved initial testing of the questionnaire by participants with Crohn's perianal fistula. Members of the study steering group included all stakeholders for this condition, consisting of a tertiary centre colorectal surgeon specialising in pCf, an IBD gastroenterologist, specialist nurses, and patient representatives. The latter constituted our patient and public involvement team and consisted of four members of Crohn's and Colitis UK (CCUK), including an experienced PPI advocate, who were recruited to help with study design and data analysis. All had Crohn's disease and previous or current experience of living with a perianal fistula. The PPI team helped

with analysis and contributed to discussion and development of the preliminary items for the draft questionnaire.

11.3.1 Recruitment and Sampling

Using purposive sampling, we recruited from the membership of our collaborating organisations (Crohn's and Colitis UK, ForCrohns, UK IBD BioResource) as well as via outpatient clinics in St Mark's Hospital, aiming to recruit equal numbers of men and women, with a range of ages and from a variety of ethnic groups. A preliminary recruitment message was disseminated via collaborating organisations on our behalf, in order to register their interest in any or all phases of the study. Potential recruits from outpatient clinics where approached and study details were provided with attached invitation to register their interest.

Selection of participants

Inclusion criteria: over 16 years (no upper age limit); living in the UK; self-reported diagnosis of Crohn's disease with perianal fistula; ability to speak and understand English (Phases 1 & 2); ability to read, understand and write English (Phase 3); ability to give informed consent.

Exclusion criteria: Patients with resolved (i.e. absence of current) fistulas were excluded from Phase 3.

11.3.2 Ethical considerations

The study was approved by the Health Research Authority (HRA) for NHS England (Research and ethics committee reference: 17/LO/1563), enabling the study to recruit from NHS and community populations. Informed consent was collected immediately prior to data collection.

Written informed consent was secured from Phase one, whereby invited recruits were sent copies of written consent forms and completed an expression of interest form to indicate their preferences for which phase they wanted to partake in. Some patients were recruited form the IBD bioresource and identifying and contacting them was undertaken on the basis of the Bioresource's own consent. Patients then completed the CAF-QoL study specific consent process if they agreed to take part.

11.3.3 Data Collection / Generation of a draft questionnaire

11.3.3.1 *Phase 1*

a) We conducted individual unstructured interviews either face to face or by telephone / Skype interviews, according to participant preference; exploring the experience of living with CD-related perianal fistulas and the impact on the individual of the disease and the necessary surgical and / or medical treatments on the individual. The aim was to secure an in-depth understanding of this complex experience. Interviews were recorded on a digital audio device and transcribed by an independent professional. Following analysis a long list of items for inclusion in the draft PROM was created[519].

b) To enhance the face and content validity of the questionnaire, we also included data from a systematic review of outcomes and consensus exercise[310] undertaken to develop a Core Outcome Set setting the most appropriate outcome measures to be used in assessing and managing patients with Crohn's perianal fistulas[310]. This included more than 80 stakeholders from across the UK[310]. The consensus exercise also sampled 160 clinicians from across the UK (including surgeons, gastroenterologists, radiologists and IBD nurses). Patients were the primary stakeholders in the exercise, and guided inclusion of quality of life outcomes and some clinical outcomes of relevance to patients, and clinician stakeholders identified clinical and

radiological outcomes thought to be important in management and assessment of success. Clinicians also commented and voted on quality of life outcomes but patients had primacy. The resulting COS and the discussions with that large patient cohort during the COS development were factored into the initial draft PROM[310]. We also included a background section (including 16 questions) collating demographic data and disease details (including duration, number of fistulas, medication and surgical history).

11.3.3.2 *Phase 2*

Participants with CD-related perianal fistulas then provided further content validation via the more formal process of cognitive interviews. Cognitive interviews enable researchers to refine the questionnaire using think-aloud techniques and verbal probing. Patients were interviewed as they completed the draft QoL PROM. Participants were asked to read each item aloud and tell the researcher all the thoughts that came to mind when hearing the item. Relevant probes were used to clarify and explore issues that were raised. Participants gave free text qualitative feedback on aspects of fistula-related QoL not covered in the draft PROM. Interviews were recorded on a digital audio device, and transcribed by a professional transcriber. The data were analysed by the research team and used to modify the questionnaire, adding or removing areas not covered or not needed, and responding to suggestions for modifications such as language, form and structure to avoid misinterpretation of items. Amendments were discussed within the study management team including the patient representatives. Four further rounds of cognitive interviews, analysis and amendment took place until no further adjustments were required.

11.3.3.3 *Item reduction of the draft questionnaire*

Item reduction occurred in two stages – after the Phase 2 cognitive interviews were complete, and following statistical analysis. Following each of four rounds of cognitive interviews, there was a review of items following patient feedback to ensure any suggestions regarding wording / understanding were modified by consensus and further interviews were used to confirm acceptability until no further suggestions were received.

11.3.4 Initial testing of the draft (CAF-QoL) questionnaire

11.3.4.1 *Phase 3*

Participants with CD and perianal fistulas were sent the draft QoL PROM by post or online (following consultation with patients at a public and patient involvement day where the study design was presented for critical feedback). The participants were asked to complete the same draft PROM questionnaire two weeks later so that test-retest reliability can be ascertained. At the initial administration of the questionnaire, participants were also be asked to complete a demographic details form, and the validated UK-IBDQ[523] . We also collected disease activity information at each administration of the questionnaire, using a modified version of the Harvey-Bradshaw Crohn's Disease Activity Index[524,525]. This allowed stratification to those whose disease activity level changes from the test-retest analysis, as alterations in disease activity may affect fistula-related QoL and allowed a sub-analysis for testing sensitivity to change. Data were input into a study-specific spreadsheet by a third party independent data input operative prior to statistical analysis

11.3.4.2 *Testing of psychometric properties*

Validity

Content Validity. This assesses the applicability, relevance, and clarity of question items in order to maximise the accurate completion of the questionnaire. This was assured through the involvement of patients (who serve as the crucial stakeholders in PROM development) being involved in the consensus exercise prioritising the need the for the PROM, as well as being an integral part of the gathering of information (qualitative exploration of self-reported experience of living with Crohn's perianal fistulas). Further content validity was, was explored by evaluating the levels of missing data per item in phase 3. The overall response rates was analysed to indicate the feasibility of the questionnaire for self-completion.

Construct Validity. Construct validity assesses the consistency of the scale with other instruments known to assess similar attributes with good validity and reliability.

It is commonly assessed by Pearson's correlation coefficient (r). Two outcome measures were used; the UK-IBDQ [523]and the Hospital Anxiety Distress Scale (HADS)[207], both validated PROMs to evaluate the relationships between patients' reports as they both cover some similar concepts.

Reliability

Test-retest reliability (reproducibility or stability) assesses the consistency between successive applications of CAF-QoL[526,527]. Responses from participants completing the phase 3 test-retest in whom the IBDQ, HADS and anchor questions (i.e. ascertaining self-reported global rating) indicated stable disease were used to assess reliability as is normal practice[523,528,529]. The anchor questions were a general rating of fistula condition (on a Likert scale of 0-10) as well as whether the patient had had any flare up of their fistula (requiring medication, operation or seton) since they first completed the questionnaire. Those who reported no changes (absence of flare up,

≤1 point difference on Likert scale and stable overall IBD disease activity) were included in the reproducibility analysis. For these patients, the first and second set of responses should be consistent. The differences between the test and retest responses was appraised using weighted statistics. The reproducibility of the scores for stable patients was assessed using the intraclass correlation (ICC) coefficient. The ICC involves dividing the total variation in the CAF-QoL scores into the variation between patients, and the variation within patients (due to repeat measurements of the same patient). The ICC is the proportion of total variation that is between patients. If the agreement between test and retest scores is high, the within-patient variation should be small and thus the ICC value should be close to 1. An intraclass correlation between the first and second set of questionnaires should exceed 0.75 for good reproducibility[526].

Agreement between the test and retest questionnaires was assessed using the weighted kappa method due to the categorical nature of outcomes. These methods measure the agreement between repeat responses over and above that which would be expected due to chance. The kappa method gives more weight to closer disagreements (e.g. by only one category) than larger disagreements (e.g. by 4 categories). Kappa is measured on a scale ranging up to a maximum agreement of 1, and a suggested interpretation of the values is given in Table 29. The kappa values were calculated along with a corresponding confidence interval indicating the uncertainty in the values. The analysis was restricted to patients with stable disease only between timepoints.

Table 29: Interpretation of Kappa values

Value of Kappa	Strength of agreement
< 0.20	Poor
0.21 – 0.40	Fair
0.41 – 0.60	Moderate
0.61 – 0.80	Good
0.81 – 1.00	Very Good

Agreement in the total CAF-QoL scores between the test and retest datasets calculations were restricted only to patients with stable disease. Two methods were used to examine agreement, the Bland-Altman method and the intra-class correlation (ICC) method. The Bland-Altman limits of agreement method quantifies, in the units of the score, the size of differences between test/retest values that are likely to occur. The measure is obtained by first calculating the difference between the test and retest scores for each patient. The 95% limits of agreement (within which 95% of all differences between values should occur) were then calculated using Mean difference ± 1.96*(standard deviation of differences). The final analyses compared the average change in CAF-QoL values between test and retest measurements in different subgroups of patients. The change in CAF-QoL scores between the two questionnaires was found to be normally distributed, and so the paired t-test was used for the analyses.

Sensitivity to Change

Sensitivity to change (or responsiveness), is the ability of the CAF-QoL to detect change between successive applications, if change has occurred. In contrast to reproducibility, we assessed responsiveness in retested patients who reported a change in their fistula status (measured on a Likert scale of 0-10). Comparisons between baseline and retest scores were assessed using mean change between questionnaires, with a corresponding confidence interval, and also the p-values indicating the significance of the changes.

11.3.4.3 *Data Analysis*

Qualitative analysis

All audio files were transcribed professionally as each phase was completed, and anonymised transcripts returned to the study team (including PPI group) for analysis. Each team member

independently identified themes, before meeting to collaborate and agree final themes. The analytical hierarchy described by Spencer, Ritchie and O'Connor[504] was followed to support thematic analysis. The hierarchy involves several stages through which team members individually identify themes and concepts within the data, then collaborate to agree themes and to synthesise these into items for inclusion in the draft PROM. Individual, followed by team analysis and agreement, enhances credibility by ensuring data analysis and subsequent findings are not influenced by a lone researcher, but are a consensus of the study team. Findings from Phase 1a were combined with results from the Core Outcome Sets exercise in Phase 1b[310] to create a long list of items for inclusion in the draft PROM.

Statistical analysis

We conducted several stages of psychometric testing of the draft PROM – some of which have already been described. Test-retest stability was explored, as were rates of response and of missing items. Factor analysis (FA) with Principal Axis Factoring (PAF) extraction and varimax rotation were used to determine individual dimensions or subscales of anal fistula-related quality of life and to refine the number of items[530]. The internal reliability of any subscales was determined using Cronbach's alpha (this value should exceed 0.7 for good consistency)[531]. The strength of association between individual questions was also assessed using Pearson's correlation coefficient to identify questions that were strongly correlated. The biggest area concern was for questions that are very strongly correlated, and would be almost duplicating the information. Any of these were subsequently reviewed (by the steering group) with a view to achieving consensus in excluding those questions agreed to be duplication of information.

11.4 Results

A total of 211 participants with Crohn's perianal fistula were involved in the process across all three phases of the study (Table 30). The majority of participants (~60%) had IBD for longer than 10 years and >80% had a duration Crohn's perianal fistulas of at least one year. Fewer than 15% of participants had a stoma (temporary or permanent).

Table 30: Participant characteristics

Variable	Category	Number	Percentage
Gender	Female	122	57.8%
	Male	88	41.7%
	Transgender	1	0.5%
Age (*)	-	42.9 ± 12.7	
Duration of Crohn's Disease	< 1 year	7	3.3%
	1 – 4 years	31	14.7%
	5 – 9 years	47	22.2%
	10 – 14 years	34	16.1%
	15+ years	92	43.6%
Duration of perianal fistula	< 1 year	18	8.5%
	1 – 4 years	67	31.8%
	5 – 9 years	61	28.9%
	10 – 14 years	25	11.8%
	15+ years	40	18.9%
Medication for perianal fistula	No	107	50.1%
	Yes	104	49.2%
Previous surgery for perianal fistula	No	61	28.9%
	Yes	150	71.1%
Previous seton	Yes	131	62.1%
	No	59	27.9%
	Unsure	21	10.0%
Stoma due to perianal fistula	Yes	31	14.7%
	No	180	85.3%

(*) Mean ± standard deviation reported

11.4.1 Phase 1

Twelve interviews were conducted, achieving apparent data saturation. Median length of interview was 43mins (range 18 – 145mins). Three broad themes were uncovered: *Burden of symptoms*; *Burden of treatment*; and *Impact on emotional, physical and social well-being*. Each included several sub-themes, with considerable interplay between these[519]. In summary, the impact of perianal fistula(s) on patients with CD was intense and wide reaching, negatively affecting intimate, close and social relationships and causing losses in life and work-related opportunities[514]. The 'long list' of issues of importance / concern to patients derived from these interviews was combined with a list of outcomes incorporated from a pre-existing multidisciplinary systematic review / consensus exercise assessing outcomes in patients undergoing interventions for pCf[310]. The list was collated and the number of items refined at a further team meeting by eliminating repetition and overlap. From the original long list of 45 items, 35 items were retained under the three domains (A-C) mirroring the broad themes described above. The list was reviewed by the study team and converted into questions (Domain A – relating to symptoms) and statements (Domain B – relating to treatment; and C – relating to quality of life impact). The team also agreed to adopt Likert scales measuring frequency (0-4) in Domain A, and degree of agreement (Likert scales 0-4) in domains B and C. The questions and statements were phrased to ensure that positive answers were always at the same end of the Likert scale.

11.4.2 Phase 2

At this stage the draft CAF-QoL questionnaire is demonstrated in Figure 34. This questionnaire was put through four rounds of cognitive interviews involving a total of 15 patients with several changes and modifications following each round until no subsequent

changes were recommended. This draft was then taken forward for test-retest analysis (Phase 3).

Crohn's Anal Fistula Quality of Life Scale (CAFQoL)

*Please consider how your anal fistula has affected your life over the last **6-8 weeks**.*
If you are unsure how to answer any question, please just give the best answer you can. Do not spend too much time thinking about your answer as your first thoughts are likely to be most accurate.

	Domain A *This section is about symptoms from your fistula* PLEASE CIRCLE ONE NUMBER FOR EACH QUESTION THAT COMES CLOSEST TO REFLECTING YOUR OPINION. IF YOU HAVE MORE THAN ONE FISTULA, TELL US ABOUT YOUR **WORST** CURRENT FISTULA	Never	Occasionally	One/ few times a week	Daily	Several times a day
17	Do you get sharp, intense pain around the anus because of your fistula(s)?	0	1	2	3	4
18	Do you get a dull / background discomfort around the anus because of your fistula(s)	0	1	2	3	4
19	Do you take painkillers for pain or discomfort related to your fistula(s)?	0	1	2	3	4
20	Do you get any type of discharge from your fistula(s)?	0	1	2	3	4
21	Do you need to use pads or gauze for the discharge from your fistula(s)?	0	1	2	3	4
22	Do you get sore skin around your fistula(s) because of discharge?	0	1	2	3	4
23	Is sitting, standing or walking restricted by your fistula(s)?	0	1	2	3	4
24	Do you get urinary tract (bladder) infections that you believe are caused by your fistula(s)?	0	1	2	3	4

	Domain B *This section is about the effects of your current fistula treatment (medication, operations, seton)* PLEASE CIRCLE ONE NUMBER FOR EACH QUESTION THAT COMES CLOSEST TO REFLECTING YOUR OPINION	Strongly Disagree	Disagree	Unsure	Agree	Strongly agree	
25	I am bothered by the side effects from the medication I take for my fistula	0	1	2	3	4	no meds
26	My seton (stitch/string/loop) causes me pain/discomfort / irritation	0	1	2	3	4	no seton
27	I feel that my seton is not helpful	0	1	2	3	4	no seton
28	I don't understand the treatment I am receiving for my fistula	0	1	2	3	4	
29	Understanding the treatment I get for my fistula is important to me	0	1	2	3	4	
30	I am not sure that my current fistula treatment is right for me	0	1	2	3	4	
31	Anything else about your symptoms or the effects of your current treatment you want to say:						

	Domain C: This section is about how your fistula impacts upon your quality of life over the last **6-8 weeks** PLEASE CIRCLE ONE NUMBER FOR EACH QUESTION THAT COMES CLOSEST TO REFLECTING YOUR OPINION	Strongly Disagree / Disagree / Unsure / Agree / Strongly agree
32	My sleep is disturbed because of my fistula	0 1 2 3 4
33	I avoid getting physically close to another person (hugging, sitting next to each other etc.) because of my fistula	0 1 2 3 4
34	I do, or would avoid getting into new relationships because of my fistula	0 1 2 3 4
35	My sexual activity is (or would be) restricted because of my fistula	0 1 2 3 4
36	My socialising (meeting friends/going to parties, other social events) is restricted because of my fistula	0 1 2 3 4
37	My exercise / activities (e.g. swimming, cycling, running) that I would like to do are restricted because of my fistula	0 1 2 3 4
38	My travelling (driving, taking the train/plane etc.) is restricted because of my fistula	0 1 2 3 4
39	Having a fistula causes me embarrassment / shame	0 1 2 3 4
40	I am concerned that others may find out that I have a fistula	0 1 2 3 4
41	My ability to work or study is restricted because of my fistula	0 1 2 3 4
42	My ability to advance in my education / career is limited because of my fistula	0 1 2 3 4
43	I have lost out financially because of my fistula	0 1 2 3 4
44	Because of my fistula, I worry about finding or needing the toilet ('toilet mapping') when I am away from home	0 1 2 3 4
45	Because of my fistula, I only go to places where I know there's a clean toilet and washing facilities	0 1 2 3 4
46	Because of my fistula, I have to take spare underwear and wipes with me when I go out	0 1 2 3 4
47	It is hard for me to keep myself feeling clean because of my fistula	0 1 2 3 4
48	I am concerned that other people may be able to smell the discharge from my fistula	0 1 2 3 4
49	I feel anxious or depressed, down or hopeless because of my fistula	0 1 2 3 4
50	I worry that my fistula will never be cured	0 1 2 3 4
51	I worry I might one day need to have a stoma because of my fistula	0 1 2 3 4 Tick if you have a stoma ☐
52	I worry about my temporary stoma becoming permanent because of my fistula	0 1 2 3 4 Tick if any below apply: ☐ I don't have a temporary stoma ☐ I have a permanent stoma

Figure 34: Initial Draft CAF-QoL prior to test-retest analyses.

11.4.3 Phase 3

(Additional data analysis from this phase is presented in supplementary results file-*section* 15.1)

11.4.3.1 *Completeness of the data*

The 'test' data consisted of data from 184 patients. Of these, 3 patients did not complete any of the questions from Q17 onwards, which related to the new CAF-QoL scale. Therefore, these were excluded completely from the analysis, leaving 181 patients for analysis. The completeness of the data was examined, and the information is presented in supplementary Table 42. The figures are the number and percentage of missing data values, and also the number of items where the question was not applicable. The final column gives the combined total of missing and not applicable responses. The data suggested that there was very little in the way of missing data. Two patients had missing values for all questions from question 32 onwards (Figure 34). This suggests they gave up on the questionnaire completely at this stage, rather than any specific issues with the questions. A number of questions had 'not applicable' responses. These were the questions relating to presence of seton / stoma or side effects of medication. The 'not applicable' occurred in over half of patients for questions 27, 28 and particularly question 53 where over 80% of responses were 'not applicable'.

11.4.3.2 *Data spread*

Each item consisted of five response categories. Summaries of the spread of responses were produced for questions relating to frequency of symptoms, i.e. Domain A (supplementary

Table 43). This showed reasonable spread of responses throughout the five outcome categories. Summaries of the spread of responses were produced for questions that had an 'agree/disagree' response scale (Domains B and C). A summary of the responses for these questions are given in supplementary Table 44. The responses suggested there was a reasonable spread of values throughout the five response categories for the majority of the items.

11.4.3.3 *Test-retest agreement (reliability)*

The next set of analyses examined the agreement between the test and retest questionnaires. The weighted kappa method was used to determine the level of agreement, and the results are summarised in supplementary Table 45. The figures are the calculated kappa values, along with corresponding confidence intervals. The results suggested that for the majority of the items there was good agreement between the test and retest results, and in some cases very good agreement. None of the items displayed 'poor/fair' agreement between the two sets of measurements. However, for some items the agreement was only 'moderate' (0.4-0.6).

11.4.3.4 *Associations between questions*

Every pair of individual questions was correlated against each other, using a Pearson correlation coefficient. Supplementary Table 46 shows the questions where the correlation between variables was 0.75 or higher. The results demonstrated that there were no 'perfect' correlations between variables, with no correlations over 0.9. Therefore, no questions were perfectly duplicating information. However, there were a number of questions where the questions were fairly strongly correlated, and thus were giving broadly similar information. These findings were all for questions that were adjacent to each other in the questionnaire,

namely Q52 and Q53, Q42 and Q43, Q35 and Q36, and Q20 and Q21. There were also fairly strong correlations between Q26 and several other questions. Although, it is noted that these results are based on a smaller number of observations (n = 28)**see section on data limitations (section 11.5).*

11.4.4 Item reduction after first set of statistical analysis

Following initial statistical analysis, the team undertook further questionnaire review and items that yielded contention based on analysis were discussed and consensus achieved. This resulted in 9 questions reduced from the draft questionnaire. The updated questionnaire is demonstrated in Figure 35

- From Domain A - excluded **questions 19, 21 and 24**
- From Domain B - excluded **questions 28, 29 30 - 31**
- From Domain C- excluded **question 35, 43**

The reductions were made by consensus of the steering group and reasons included overlapping themes, as well as poor performance on initial screening statistics as described above. The final version of CAF-QoL is in Figure 35 below.

Crohn's Anal Fistula Quality of Life Scale (CAFQoL)

*Please consider how your anal fistula has affected your life over the last **6-8 weeks**.*
If you are unsure how to answer any question, please just give the best answer you can. Do not spend too much time thinking about your answer as your first thoughts are likely to be most accurate.

	Domain A *This section is about symptoms from your fistula* PLEASE CIRCLE ONE NUMBER FOR EACH QUESTION THAT COMES CLOSEST TO REFLECTING YOUR OPINION. IF YOU HAVE MORE THAN ONE FISTULA, TELL US ABOUT YOUR **WORST** CURRENT FISTULA	Never	Occasionally	One/ few times a week	Daily	Several times a day
17	Do you get sharp, intense pain around the anus because of your fistula(s)?	0	1	2	3	4
18	Do you get a dull / background discomfort around the anus because of your fistula(s)	0	1	2	3	4
20	Do you get any type of discharge from your fistula(s)?	0	1	2	3	4
22	Do you get sore skin around your fistula(s) because of discharge?	0	1	2	3	4
23	Is sitting, standing or walking restricted by your fistula(s)?	0	1	2	3	4

	Domain B *This section is about the effects of your current fistula treatment (medication, operations, seton)* PLEASE CIRCLE ONE NUMBER FOR EACH QUESTION THAT COMES CLOSEST TO REFLECTING YOUR OPINION	Strongly Disagree	Disagree	Unsure	Agree	Strongly agree	
25	I am bothered by the side effects from the medication I take for my fistula	0	1	2	3	4	no meds
26	I am bothered by the side effects of surgery I had for my fistula (e.g. scarring, appearance, wound problems)	0	1	2	3	4	no surgery
27	My seton (stitch/string/loop) causes me pain/discomfort/irritation	0	1	2	3	4	no seton
32	Anything else about your symptoms or the effects of your current treatment you want to say:						

	Domain C: This section is about how your fistula impacts upon your quality of life over the last **6-8 weeks** PLEASE CIRCLE ONE NUMBER FOR EACH QUESTION THAT COMES CLOSEST TO REFLECTING YOUR OPINION	Strongly Disagree / Disagree / Unsure / Agree / Strongly agree
33	My sleep is disturbed because of my fistula	0 1 2 3 4
34	I avoid getting physically close to another person (hugging, sitting next to each other etc.) because of my fistula	0 1 2 3 4
36	My sexual activity is (or would be) restricted because of my fistula	0 1 2 3 4
37	My socialising (meeting friends/going to parties, other social events) is restricted because of my fistula	0 1 2 3 4
38	My exercise / activities (e.g. swimming, cycling, running) that I would like to do are restricted because of my fistula	0 1 2 3 4
39	My travelling (driving, taking the train/plane etc.) is restricted because of my fistula	0 1 2 3 4
40	Having a fistula causes me embarrassment / shame	0 1 2 3 4
41	I am concerned that others may find out that I have a fistula	0 1 2 3 4
42	My ability to work or study is restricted because of my fistula	0 1 2 3 4
44	I have lost out financially because of my fistula	0 1 2 3 4
45	Because of my fistula, I worry about finding or needing the toilet ('toilet mapping') when I am away from home	0 1 2 3 4
46	Because of my fistula, I only go to places where I know there's a clean toilet and washing facilities	0 1 2 3 4
47	Because of my fistula, I have to take spare underwear and wipes with me when I go out	0 1 2 3 4
48	It is hard for me to keep myself feeling clean because of my fistula	0 1 2 3 4
49	I am concerned that other people may be able to smell the discharge from my fistula	0 1 2 3 4
50	I feel anxious or depressed, down or hopeless because of my fistula	0 1 2 3 4
51	I worry that my fistula will never be cured	0 1 2 3 4
52	I worry I might one day need to have a stoma because of my fistula	0 1 2 3 4 Tick if you have a stoma ☐
53	I worry about my temporary stoma becoming permanent because of my fistula	0 1 2 3 4 Tick if any below apply: ☐ I don't have a temporary stoma ☐ I have a permanent stoma

Figure 35: CAF-QoL taken forward for further statistical / psychometric evaluation (following item reduction).

11.4.5 Further statistical analysis

Following preliminary analysis, the original questionnaire items were reduced to only those to be included in the final score. Each individual item was scored from 0 to 4, and the total score was calculated by summing the scores from the individual items together. The first set of analyses summarised the scores for the patient group as a whole. The next analyses examined psychometric properties of the calculated score. Cronbach's alpha was used to examine the internal consistency of the score. Additionally, factor analysis was used to examine how the individual items were associated with each other, and whether the score could be subdivided into subscales.

11.4.5.1 *Score summaries*

The CAF-QoL score consisted of 28 items, 27 of which were scored from 0 to 4 (the other is a free-text question). This gives a theoretical range of scores from 0 to 108. The questions are scored in such a way that higher scores indicate worsening quality of life. The score was able to be calculated for 179 patients. The observed range of values was from 0 to 95. The mean score was 42.0, with a standard deviation of 26.0. A graphical illustration of the scores is shown in Figure 36.

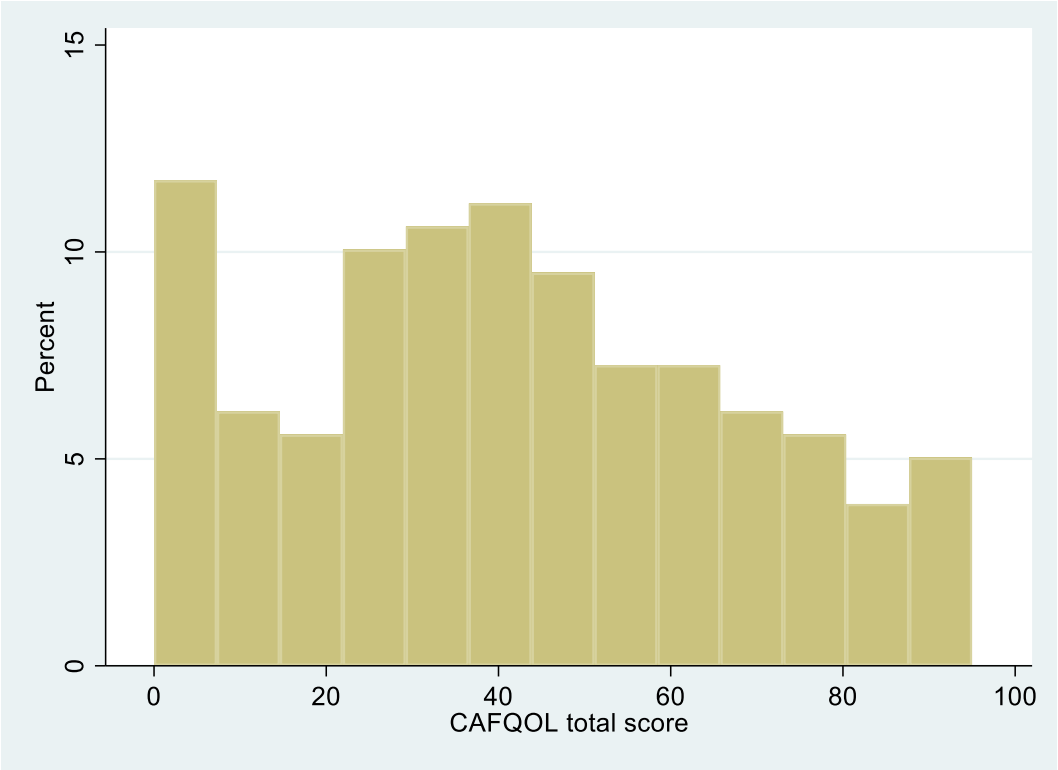

Figure 36: Histogram of calculated total CAF-QoL scores

11.4.5.2 *Psychometric properties of the score*

Internal consistency and testing for any sub-scales within the CAF-QoL

Cronbach's alpha calculated value was 0.88, suggesting a good degree of internal consistency amongst the individual items. Also performed was a factor analysis to analyse if the score naturally broke down into different subscales. Results were depicted via scree plot (supplementary Figure 40), which demonstrates the sizes of 'eigenvalues' attributed to individual components. The plot shows that the natural change in slope direction was after the first component. This suggests that only one factor is worthy of interpretation, i.e. the items on the questionnaire do not subdivide into sub-scales on the basis of participants responses. A

higher eigenvalue would imply that a greater amount of variability is associated with that component. Results for the first five components are listed in supplementary Table). The factor loadings for the one significant component involved the majority of questions (supplementary Table 48), with no natural subdivision into sub-scales. Therefore, it can be interpreted that CAF-QoL gives primarily an overall measure of the components as a whole with no evidence of any subdivision into subscales.

Agreement between test and retest scores

Sixty nine patients met the criteria for disease stability (as defined by: $\leq$1 point difference between test/retest scores on global self-rating of fistula status, and stable disease on HBDAI and no fistula flare between test/retest completion. The mean difference between the retest and test measurements (calculated as retest minus test) was -0.6 with a standard deviation of 6.0. The 95% Bland-Altman limits[532–534] were calculated as from -12.7 to +11.6. These results demonstrated that the majority of all test/retest measurements were within around 12 units of each other. This demonstrates good agreement when set against the range of theoretical scores, (0 to 108). A graphical illustration of the differences between the test and retest scores is shown in Figure 37. The agreement between test and retest scores was also examined using intra-class correlation (ICC) method. This method gave an ICC value of 0.98 (95% confidence interval: 0.96 to 0.99). This high value demonstrates very good agreement between the test and retest scores.

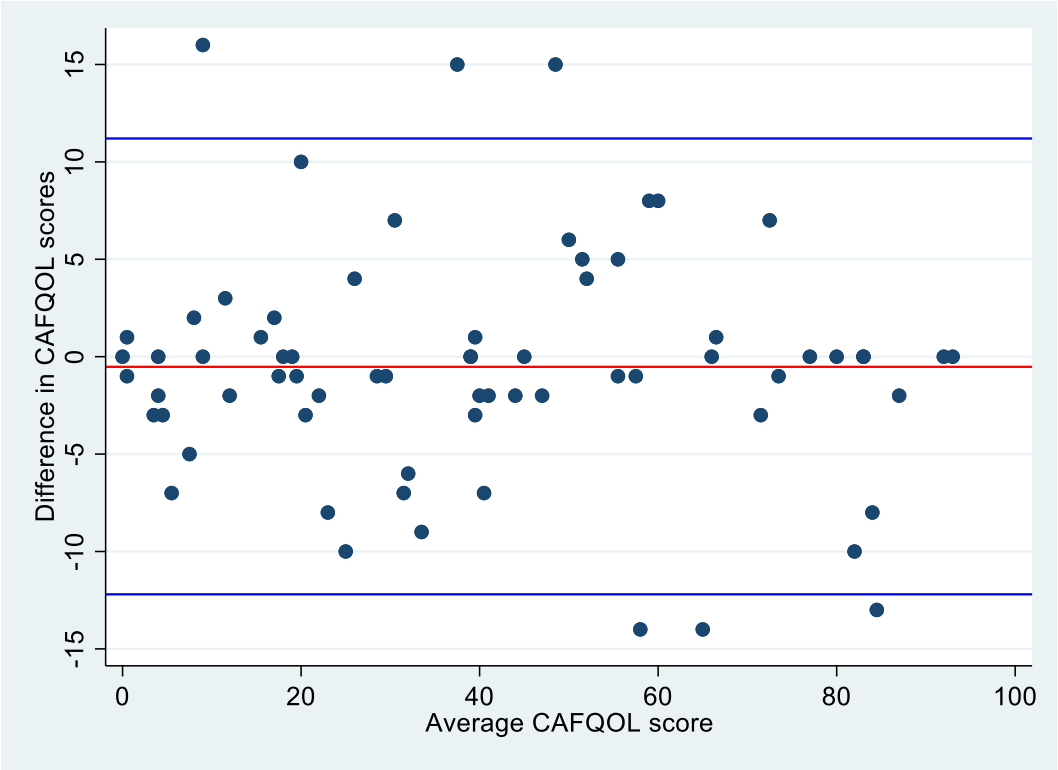

Figure 37: Bland-Altman plot showing agreement between repeat CAF-QoL scores
The red line represents the mean difference between the test and retest scores, whilst the blue lines show the Bland-Altman limits.

11.4.5.3 *Association with other health related measures*

Assessment of the agreement between the total CAF-QoL scores and other health related measures (UK-IBDQ / HADS) is demonstrated by way of the Pearson correlation results, which are summarised in Table 31.

Table 31: Association between CAF-QoL scores and other health scores

Variable	n	Correlation coefficient	P-value
HADS anxiety	174	0.52	<0.001
HADS depression	174	0.53	<0.001
IBD-Q	176	0.58	<0.001

The results demonstrated significant positive correlations between the total CAF-QoL score and each of the other health-related measures. Higher CAF-QoL scores were associated with higher levels of anxiety and depression, and also higher IBD-Q scores.

11.4.5.4 *Changes between test and retest scores*

In order to examine the change in CAF-QoL total score between test and retest in specific groups of patients, three groups were examined, relating to patients with 'stable', 'improving' and 'worsening' global fistula rating. The. The results are summarised in Table 32. The first figures shown are the number of patients in the analysis, the mean and standard deviation at each time. Also shown are the mean change in scores between questionnaires, with a corresponding confidence interval, and also the p-values indicating the significance of the changes.

Table 32: Changes in CAF-QoL score from test to retest

Patient group	n	Test Mean ± SD	Retest Mean ± SD	Change Mean (95% CI)	P-value
Stable (*)	69	41.5 ± 28.4	40.9 ± 27.8	-0.6 (-2.0, 0.9)	0.46
Improved (**)	12	52.2 ± 20.7	48.2 ± 22.3	-4.0 (-7.0, -1.0)	**0.01**
Worsening (***)	9	30.1 ± 17.3	37.7 ± 21.5	7.6 (1.6, 13.5)	**0.02**

(*) Defined as Q6 test/retest scores of ± 1 point, no fistula flare up, and same test/retest Harvey-Bradshaw criteria
(**) Defined as global fistula rating on *retest* scores of ≥ 3 units **lower** than *test* score
(***) Defined as global fistula rating on *retest* scores of ≥ 3 units **higher** than *test* score

The results suggested that in patients with stable disease there was no significant change in CAF-QoL scores between the two timepoints. CAF-QoL scores decreased significantly at the retest timepoint in those with improved disease, by an average of 4 units. Conversely in those with worsening global fistula rating scores increased significantly between timepoints, by a mean of 7 units.

11.5Discussion

The aim of this study was to develop a reliable and valid disease specific PROM for Crohn's perianal fistula. We have produced a set of 28 questions, the Crohn's Anal Fistula Quality of Life Questionnaire (CAF-QoL). On initial testing, the CAF-QoL has been found to have good face and content validity and good reliability and acceptability, scoring it well in terms of criteria for judging PROMs. These findings allow its recommendation for use in interventional studies and prospective studies assessing patients with Crohn's perianal fistula. The robustness of the questionnaire is confirmed through the rigorous developmental process, with participants with Crohn's perianal fistula contributing throughout, not only as participants but also on the study management committee, ensuring that the language is straightforward, using lay terms that increase readability and enable accurate interpretation, and that the content is relevant to patients. Assessing the quality of life in patients with IBD is an important component of medical and surgical management and clinical decision-making, and the last decade has seen a rapid increase in the number of measures to assess the QoL in patients with IBD[523]. However due to the broad range of phenotypes encompassed in IBD, a single tool cannot capture the specific patient burdens associated with all the varying manifestations. There was previously no validated outcome measure that captures the patients' evaluation of the effect of interventions for Crohn's perianal fistulas on their wellbeing. This has taken even more importance in the face of elusive cure / sustained fistula closure, and is equally valuable as a secondary outcome measure for determining the impact of interventions in studies with curative intent, and as a primary outcome in studies of palliative interventions.

There is limited evidence in the literature on quality of life in patients with anal fistula. One study examining the QoL of patients with benign anorectal disorders used a validated gastrointestinal QoL index (GIQLI) on patients who presented to the outpatient clinic. Patients with incontinence were reported to have a reduced QoL over time compared to age-matched controls. Anal fistula patients' QoL was not significantly different from controls as long as they remained continent. Adaptation to the fistula and its symptoms over time was one of the possible explanations for the stability in QoL, although this was based on the authors' interpretation rather than patients' views. This study suggested that patients favoured continence over cure of the fistula[535]. Another study assessing patient satisfaction after fistula surgery invited 624 patients to take part, with 375 returning the questionnaires. Patients were mostly dissatisfied if they suffered with recurrence of their fistula or incontinence (61% and 24% dissatisfaction rates)[536]. These two studies give different weights to concerns regarding cure and continence from the patients' and surgeons' points of view, but nonetheless highlight the fact that patients have concerns with regards to anal fistulas and the treatment options. An Australian study by Wong et al.[537] compared surgeon and patient preferences for surgical operations for idiopathic anal fistulas. The different cure and incontinence rates for each procedure were quoted for the patients to consider and their preferences explored. Patients and colorectal surgeons self-completed a questionnaire. Surgeons were asked about their treatment preferences for high anal fistulas, and their opinion of how much of an incontinence risk patients were willing to take to be rid of the fistula. Patients were asked to nominate up to 5 QoL aspects they would consider when thinking about anal fistula treatments and rate each on a scale of 1-10. Seventy-five surgeons (56% of those approached) and 28 patients (14%) replied to the postal questionnaire. A clear mismatch was reported between what the surgeons and patients felt were important QoL issues when considering anal fistula surgery. In order of priority, surgeons nominated continence, leakage, pain, cure and sepsis as the five most

important QoL factors. Patients' nominated independent activity in good health, pain, continence, psychological health and leakage as their five most important factors. Ninety-one percent of the surgeons and 25% of the patients nominated continence as an important QoL issue[537]. The study demonstrated the potential assumptions made by clinicians with regards to the impact of fistula surgery on patients' QoL. More recently, Ferrer-Marquez et al.[538] developed a Quality of Life in Patients with Anal Fistula Questionnaire, demonstrating it to be a valid, reliable, and concise tool, however, major limitations of this study were the use of a small sample of Spanish-speaking patients, and crucially, not including patients in the initial development of the questionnaire, and developing the scoring system using a summation method. There is no objective patient-centred disease scoring tool for Crohn's perianal fistula and this results in the inability to adequately quantify the effects on psychosocial wellbeing and has led to calls to address the unmet need for a disease specific PROM[395].

Study Strengths and limitations

The strength of the study lies in its design, incorporating a literature review process, consensus exercise and patient focus groups through to individual patient interviews[500] (unstructured and cognitive) to inform the item generation for the CAF-QoL questionnaire. Recruitment of participants occurred via nationwide charities (ForCrohns, CCUK, UK-IBD-BioResource) as well as via hospital recruitment, and via social media. This ensured a rich and broad sample of those with experience of Crohn's perianal fistulas. The involvement of patients in every step of the process both as participants and in the steering group ensured that the patient voice was central in every stage of the process , facilitating creation of a true PROM. This is often lacking in PROM development, as a recent systematic review demonstrated, reporting a

considerable variability in level of involvement.[510]. Another strength was the diversity in the study management group with stakeholder leads (gastroenterologist, colorectal surgeon, specialist nurses, qualitative researchers and patient representatives) all ensuring that patient input was always prioritised. Limitations include the fact we did not collect data on those patients with other manifestations of perianal Crohn's disease aside from Crohn's fistula and did not carry out separate analysis for patients with temporary defunctioning stoma versus proctectomy, taking the view that such patients might well have different priorities, goals and symptoms, and might have contributed to a score which was useful for neither group.

There were some assumptions / limitations that were found during the analysis process. These need to be taken into account when interpreting the analysis results. The main data limitations were as follows:

'Not applicable' CAF-QoL questions

Some CAF-QoL questions were applicable only to specific groups of patients, for example those on medication or those with a temporary stoma. Patients to whom the question did not apply were given a score of zero, the best possible outcome (because they were less likely to have had any medication/stoma related impairment in QoL) .

Missing data for CAF-QoL *Q26- 'I am bothered by the side effects of surgery…'*

Due to an administrative error, the majority of patients were not asked to provide a response to one question on and so analysis of the strengths of this question was based on fewer completions. However, as it scored well, there was consensus to keep the question due to it surgery being deemed a potential important effector on quality of life in perianal Crohn's fistula patients.

Incorrect response for HADS depression score

For one question on the HADS scale, the majority of patients were not given the correct response categories to choose from due to a typographical error. This made very little difference to overall scores.

Not applicable IBD-Q questions

Two items on the IBD-Q score had possible responses of 'does not apply to me'. Patients with these responses were scored as 1, the 'best' possible score as it was presumed the response suggested little or no impact on their QoL. It is noted that this applied to a relatively small number of patients.

Sensitivity to change calculation

Responsiveness and stability calculations are limited by the fact that there is no true external gold standard to define a change in disease states and control so we performed analyses utilising self-reported global ratings to define a change in health state between test-retest analyses, which is the standard approach in this situation but remains suboptimal[523,528,529]. It is inevitably the case with the first disease specific QoL score produced in a given area.

SUMMARY AND FUTURE DIRECTION

The CAF-QoL is the first disease specific QoL score questionnaire in Crohn's perianal fistula. It has been developed with a truly patient-centred methodology It can be completed face to face with the clinician present, or independently by the patient. As such, it can create a communication bridge and promote discussion and assessment of patient needs. It also

identifies changing concerns and priorities that arise for patients between remission and relapse, information that can guide individualised patient advice and support. It can also be used to assess interventions and define disease impact as potential decision aid for those who warrant proctectomy for its effect on their quality of life. Further work is required to determine whether the new CAF-QoL correlates with the PDAI, which incorporates several clinical components, to determine whether the two scoring tools correlate. It is also necessary to determine CAF-QoL's relationship with diagnosis and fistula activity, to assess its ability to detect sensitivity to change (using defined clinical indices). This however is hampered, because the cut-off for clinically significant response has never been determined for PDAI despite its considerable use in clinical trials[395]. A comprehensive classification for Crohn's perianal fistulas, which integrates all elements that are important for medical and surgical management is required and may need to incorporate a combination of diagnostics (endoscopy / MRI / endoanal ultrasound) and examination under anaesthesia to ensure robustness. Furthermore, the CAF-QoL will need translation into different languages and cross cultural validation for international use. Its use in interventional studies and clinical practice will also lead to further data collation and analysis with a view to shortening the questionnaire and ultimately improving the experience for patient completion and uptake.

Chapter 12. Thesis Discussion

12.1 Main findings of the Thesis.

This thesis describes several research studies directed towards improving our understanding of disease aetiopathogenesis and optimising the management strategy of Crohn's perianal fistulas. We used metabonomics as a new tool of exploring fistula aetiopathogenesis with a view to improving our understanding of Crohn's perianal fistula and its relationship with the more common idiopathic (cryptoglandular) fistula. Using real world data, we explored the burden and disease course facing a cohort of Crohn's perianal fistula patients on current internationally recommended medical treatment and assessed the response and economic burden in the event of failure to respond, as patients moved through a number of disease states. We evaluated novel surgical techniques and their outcomes and introduced a patient centred evaluation and the concept of symptom palliation using one of these surgical techniques in the treatment of Crohn's perianal fistula. Finally, this thesis also describes the development of the first disease specific, patient derived PROM for Crohn's perianal fistula. Specific sections, and themes which emerged from the thesis are discussed below.

12.2 Section A – Novel approach to fistula aetiopathogenesis

Our study identified a fistula tissue metabonome (metabolic phenotype) that is differentially expressed in Crohn's perianal fistulas when compared to idiopathic perianal fistulas. Forty-one differentially expressed lipid/amino acid metabolites were identified, distinguishing

Crohn's and idiopathic fistulas. This is the first time such a difference has been described on a molecular level, and further studies are required to corroborate these findings. Pathway mapping for these metabolites was performed and identified pathways in amino acid, lipid and fatty acid metabolism involved in various roles including cell signalling, inflammation, and membrane stability and integrity amongst others. Some of the differentiating metabolites in our study have also been reported in a recent study describing an EMT-generated metabolome, which further corroborates the role of EMT in fistula aetiopathogenesis. Current knowledge and clinical translation of these pathways in fistula aetiopathogenesis theories remain rudimentary and studies with correlation of data across various platforms are lacking. There is a need for integration of multi-omics platforms (metabonomic, genomic, transcriptomic, microbiome data) in order to make further in-roads in our understanding of pathogenesis[234].

Some progress has been made more broadly in IBD, for example metabolic phenotyping has been carried out on urine, plasma and faecal samples both to characterize the metabolic consequences of IBD and to identify disease-induced changes in the metabolites deriving from the gut microbiota[539,540]. Marchesi and colleagues recorded the first IBD metabolomics publication, examining the fecal metabolome (via fecal extracts) of CD, UC, and healthy controls. The investigators found marked differences in the fecal metabolomes (particularly short chain fatty acids butyrate and acetate) of IBD patients compared with those of healthy controls[214]. The attributed the influence of the gut microbiota to the cause of this difference due to reduction in *Clostridium coccoides* and *C. leptum* as these bacteria groups (mainly responsible for production of short chain fatty acids) were also noted to be reduced in IBD patients compared with healthy controls[214].

Our study applies a novel tool to provide information on the metabolic state of perianal fistula tissues and correlates this with the host phenotype. There was, however, a lack of correlation with microbiome data. Capture of such data as well as metabolomic characterization could uncover host microbe interaction, which is key for understanding their actual roles in health and disease. Aside from providing novel insights into disease, biological profiling (for example metabolic phenotyping) has the potential to improve detection of diagnostic biomarkers of disease, which combined with improved clinical diagnostic criteria can enhance the characterization of Crohn's perianal fistula. The creation of enhanced diagnostic biomarker profiles at each stage of the patient journey through intervention and linking this to outcomes, could lead to prospective or prognostic analysis of patient outcomes and better treatment stratification[540]

12.3 Section B - Natural history of Crohn's perianal fistulas on biologic treatment

The next section of this thesis involved studies in the medical treatment of Crohn's perianal fistula. We reviewed the use of biologic therapy in the treatment of Crohn's disease and the few prospective trials leading to its recommendation as first line therapy for patients with Crohn's perianal fistula. Response to medication is moderate with a significant proportion failing to achieve cure or sustained fistula closure and similarly a significant proportion of patients do not respond at all or lose response to therapy. There are limited data on the long term outcomes of patients with Crohn's perianal fistula on biologic treatment outside the

context of trials and there is lack of real world data on the disease burden and the clinical course in the face of long term therapy and failure to achieve sustained fistula closure and healing.

We undertook a retrospective study assessing real world data from our institution, with one of the largest patient cohorts with Crohn's perianal fistula undergoing treatment with biologic (anti-TNF) agents with a median follow up of approximately 7 years. We found that during the study period, 37/202 (18%) patients had radiological fistula healing[377]. The proctectomy rate for our study cohort was 10%. Due to its retrospective nature, data on clinical remission and relapse were not reliably recorded in this study. We have therefore not presented these data. Further, the lack of a high quality disease specific quality of life tool during the study period prevented robust measurement of subjective outcomes from the patient's point of view. We therefore chose to focus on robust, objective outcome measurements and radiological healing and proctectomy were chosen for this purpose. Radiological healing is the most robust method of determining deep tissue healing of the fistula, the stated objective of anti-TNF agents. Proctectomy is a consistent outcome measure in studies of pCf over the years and identifying a proctectomy rate in the biologic era is a useful marker of the overall efficacy of anti TNF agents. The problem with proctectomy as an outcome measure, in common with measuring pouch excision/defunctioning for pouch failure, for example, is that a decision by surgeon and patient is required for the outcome to be reached, rather than a defined change in disease state or symptom profile. Some patients meet a reasonable threshold for proctectomy but decline it, preferring to live with difficult symptoms, whereas others might undergo the procedure with a much lower burden of symptoms because of a perceived quality of life improvement which will be achieved afterwards. Historically proctectomy represented final failure of all other options whereas newer thinking (mostly coming to the fore after the study

period) suggests that proctectomy should be an option earlier in the disease course, rather than representing final failure.

A majority of patients (75%) underwent surgical procedures with 13% undergoing more than one procedure per year on average. The majority of these procedures where for drainage of abscess/collection +/- seton management. A third of patients lost response to treatment with 13% being primary non-responders. This study demonstrated limited long term healing in Crohn's perianal fistulas. This means that patients are on long term immunosuppressive medication and still require multiple surgical interventions in the context of relapse and remission, or chronic symptomatic disease. The study also demonstrated a significant number of patients who require cessation of treatment due to toxicity or as a result of failure/loss of response to anti-TNF therapy. Combined, this figure represented 158/218 patients (72%) and hence a proportion of patients who are refractory to treatment and for whom the options of treatment are limited to a switch in therapy or consideration of more extensive surgery. This fits with the view of many IBD specialists who feel that the ever expanding list of biologic agents represents a buffet through which patients progress until they run out of options, rather than a menu from which the ideal course can be chosen. Historically, a high proportion of patients underwent proctectomy for disabling disease (up to 40%[13,205]). This is particularly the case when the comparison is with older cohorts in the pre-biologic era[13]. Although our cohort proctectomy rate of 10% is on the lower end of quoted rates[9,13,374], it remains unclear whether this is truly an effect of improvements in medical therapy / multidisciplinary management over recent decades.

In order to further understand the disease burden in patients refractory to biologic treatment (in the context of elusive fistula healing), we assigned disease states to patients with Crohn's

perianal fistula refractory to at least one anti-TNF treatment, on the basis of clinical documentation of fistula status. This allowed exploration of the disease course and healthcare resource utilisation in this group of patients. Our single centre study demonstrated that a majority of patients with refractory disease are switched on to alternate anti-TNF therapy and most reside in a mild chronic symptomatic fistula disease state with a minority of patients subsequently transitioning into a severe disease state or remission state. The overall remission rate (of any duration) for our study cohort of 78 patients was 46%, however the sustained (1 year or more) remission rate was 27%. Some 19% of the total cohort underwent proctectomy. There was considerable healthcare resource utilisation amongst all disease states particularly with biologic therapy, which is the most expensive healthcare resource. Those patients refractory to treatment, represent a group with an urgent need for better treatment options in order to avoid proctectomy.

Loss of response to treatment remains an area that is still poorly understood particularly with regard to routinely predicting those patients who will develop this. The ability to detect this patient group would potentially avoid the burden of unnecessary futile treatments and allow their stratification towards novel therapeutic options, even if it be in the context of research settings initially[164,170,244,246,499,541–545]. The subsequent chapter explored a novel analytical technique (proteomics) to evaluate a potential biomarker of treatment response in patients undergoing anti-TNF therapy for Crohn's perianal fistula. This pilot study demonstrated that it is possible to detect and quantify infliximab and adalimumab in fistula tissue using the LC/MS method and detection of signature peptides for both drugs. None of the seven patients on anti-TNF for Crohn's anal fistula had any detectable unique peptides for infliximab or adalimumab, consistent with an absence of anti-TNF detection in tissue from their fistula tracts. This inability to detect anti-TNF in the absence of known immunogenicity suggests a genuine

absence of the anti-TNF drugs in perianal Crohn's fistula tissue of patients on maintenance treatment. Potential reasons for this may be due to inadequate penetration of anti-TNF drug into perianal fistula tissues, however, it could also reflect an excessive consumption of anti-TNF agents by perianal fistula tissue. It also remains possible that there is a technical experimental reason for the negative findings.

This study introduces an interesting concept in the role of fistula tissue levels and their relevance in the pharmacokinetics of biologic therapy. Whilst our study suggests there is an absence of drug in the Crohn's perianal fistula tissue in our cohort of patients on maintenance therapy, it does not answer the questions on significance. Studies suggesting higher levels of drug correlating with better response allude to our results perhaps being relevant in the context of failure to respond. All patients in our study underwent EUAs suggesting symptom burden despite maintenance therapy and it may well prove the case that tissue levels correlate with clinical response. However, further work is required to evaluate this phenomenon.

12.4 Section C – Evaluating novel / minimally invasive surgical treatment strategies in Crohn's perianal fistulas

Section B's chapters on disease course in Crohn's perianal fistula patients on biologic treatment revealed limited use of definitive surgical reparative techniques even in those patients without proctitis. This suggests only a small proportion of patients with Crohn's perianal fistula

undergo attempted definitive reparative surgery. Reasons may include the heightened reluctance to endanger the sphincter complex which may accompany repeated failed attempts. This section evaluated minimally invasive therapies targeted at fistula cure with minimal continence impact. We reviewed local injection of anti-TNF into the fistula (and/or peri-fistula) tracts, demonstrating that it seems to have a positive response towards fistula healing, however, this is difficult to quantify statistically, given the scant evidence available. The ideal dosing regimen and intervals remain unclear, as well as the risk of antibody formation and thus hypersensitivity with future treatments. There may however be scope for treatment with local injection of anti-TNF in a subset of patients who are either intolerant to systemic therapy, or in whom it is contraindicated.

Several novel sphincter sparing techniques targeting cure of fistula without continence impairment, have been described in the surgical literature. We reviewed the use and application of a few of these in Crohn's perianal fistula, demonstrating that only a very small minority of patients in the reviewed cohort (46/1245 (4%)) had CD-related perianal fistulas. Just under half of these patients (21/46) underwent VAAFT (9 purely diagnostic; 11 in combination with advancement flap), 10/46 had OTSC placement (7 of these 10 had successful fistula closure) and 15 had FiLaC (with primary success rate of 11/15). It is not possible at present to comment on the efficacy for Crohn's perianal fistula, but their minimal morbidity and minimal effect on continence with reports of successful treatment represent an exciting area of surgical interest for exploration. We did however highlight the discrepancies between techniques with which each of the procedures is performed, with specific emphasis on facets that need to be standardized when planning prospective trials. Most of the series were performed by the same authors or groups, and there is a need to assess the reproducibility and generalisability of the procedures and the rates of fistula healing.

We adopted a patient centred approach at evaluating one of these novel sphincter sparing strategies for symptomatic (rather than attempted reparative) benefit in patients with refractory symptoms despite medical therapy. The strength of this evaluation was its primary use of outcomes targeted at and relevant to patients; the primary outcome measure was a patient completed "Measure your medical outcome profile" (MYMOP2) quality of life (QoL) questionnaire at 6 weeks postoperatively, with secondary outcome measures being a decisional regret scale (DRS), post-operative complications and the thirty-day re-operation rate. The study also introduced the concept of symptom amelioration / palliation as a treatment intent in the context of refractory disease. We used a modified operative strategy of VAAFT in which no attempt was made to close the fistula, the internal opening was left open and a seton was left in situ. In these patients with refractory fistula, medical treatment and seton drainage continued in addition to the VAAFT technique, in an attempt to reduce symptoms but not to affect fistula closure. Our (unblinded) study demonstrated that for complex fistulas, VAAFT was associated with a significant improvement in pain and discharge, measured using MYMOP2 six weeks postoperatively. Fistuloscopy was feasible in 24/25 patients. In total, 81% of patients felt that undergoing VAAFT was the right decision for them and no patient regretted it. One patient returned to theatre due to increased pain and swelling, however, no abscess was found. No other complications were observed. Given the current low rates of fistula healing in response to any technique, it is imperative that the symptom burden associated with this challenging condition is addressed primarily (especially in those refractory to first line interventions). Better patient reported outcome measures are required for this, particularly those that relate specifically to Crohn's and this was the focus of the next section.

12.5 Section D – Development of a patient reported outcome measure for Crohn's perianal fistula

All through this thesis, it has been clear that the outcome of sustained fistula closure or healing has been a challenging one, both to achieve on treatment and to define clinically because of the high rates of failure, recurrence or symptom relapse. In this context, assessment of impact of interventions on quality of life is increasingly important. Section D centred around developing a useful outcome measure for assessment of interventional studies and clinical decision making that was relevant to patients in this context of elusive cure. The chapters centred around developing a patient reported outcome measure for better outcome assessment in Crohn's perianal fistula management. We undertook a qualitative exploratory study and used this information in combination with a nationwide consensus exercise and patient focus groups to inform the initial items that would contribute to this study. Over several rigorous processes, this ultimately led to an initial draft CAF-QoL questionnaire, consisting of 35 items under three domains (A, B, C): *Burden of symptoms*; *Burden of treatment*; and *Impact on emotional, physical and social well-being*.

The initial questionnaire was subject to a process of refinement by cognitive interviews to adjust wording and applicability to the target crucial stakeholders (patients with Crohn's perianal fistulas) as well as testing in order to further reduce the items on the questionnaire and assess psychometric properties in interventional / prospective studies evaluating new treatments[444]. This resulted in a final draft of 26 questions, which we present as the Crohn's Anal Fistula Quality of Life Questionnaire (CAF-QoL) for use and dissemination as per the

COS. The CAF-QoL was found to have good face and content validity and good reliability and acceptability, scoring it well in terms of criteria for judging PROMs. These findings allow its recommendation for use in interventional studies and prospective studies assessing patients with Crohn's perianal fistula. The robustness of the questionnaire is confirmed through the rigorous development process, with participants (including a PPI steering group) with Crohn's perianal fistula contributing throughout, ensuring that the language is straightforward, using lay terms that increase readability and enable accurate interpretation. We developed the first Crohn's perianal fistula PROM and recommend its use along with the COS in interventional / prospective studies evaluating new treatments.

12.6 Thesis Limitations

There are several limitations to this thesis. Some of these are borne out of the complexity of the condition and different phenotypes that are often coexistent with Crohn's fistulas (e.g. ulceration, fissures and strictures/stenosis) which add to the difficulty in optimising strategy in view of the nuanced treatment of each of the separate conditions and uncertainty as to the effect of treatment on coexisting manifestations.

In studying aetiopathogenesis, only a handful of studies[40,78,82,93,212] have attempted to compare the characteristics of Crohn's versus idiopathic fistulas. Whilst this thesis does this in section A, it is based on the assumption that there is a difference between Crohn's and idiopathic fistulas at a molecular/cellular level. Studies much like this have highlighted some differences between the groups. However, many of the fistula tracts in the present study were longstanding. This is due to the particular patient cohort referred to our institution, many of whom have complex recurrent fistulas. Given the median duration of fistula tracts studied, our data may be

relevant to persistence of established fistula tracts rather than to the initial insult leading to fistula formation, which may well be driven by initial bacterial infection (as may occasional flare-ups)[78]. Furthermore, the numbers used in our study (much like others in the literature) is small, especially when considering the capacity of the novel molecular platforms for high-throughput data. These small numbers suffer from additional limitations including heterogenous patients (in terms of their luminal and perianal CD, fistula duration and classification) and mix of medical therapies at the time of sampling[70]. The main study site for recruitment was a tertiary referral centre and this can lead to a selection bias on the data, given that the patients sampled may not be entirely representative of the spectrum of Crohn's perianal fistulas encountered in general.

Similarly, Section B limitations pertain to the single centre nature of the data chapters as well as their retrospective nature. Furthermore, the lack of effective classification limits research by combining groups with potentially different aetiological aspects, at different points in their natural history or with different factors provoking persistence such that the benefit produced in one sub-group of patients is obscured by inevitable failure in another[70]. An effective classification system, which integrates all elements that are important for medical and surgical management, remains an unmet need[395]. Another aspect limiting our study is the lack of a robust, validated outcome measure of sustained fistula closure or response to treatment. The definition of radiological healing is controversial and whilst MRI is the gold standard for identifying perianal fistula morphology, it is not validated to evaluate response to treatment[377,395]. In this thesis we utilised a radiological healing definition that has previously been published by our group (absence of high-signal tracks on fat saturated T2 sequences). If fistula tracts remained visible (not healed), paired examinations were rated as ' improved, ' ' unchanged, ' or ' worse[383]. Also, the implication of collections and their size and relation to

fistula remission remain controversial[244], and recently an interventional study of Crohn's perianal fistula included collections measuring less than 2cm in at least two dimensions in their radiological remission definition. A further limitation of this study was the absence of prospectively collected clinical healing data and patient reported QoL data, neither of which was sufficiently robust in this retrospective study to draws conclusions from, so we have not presented these data.

The limitations on the proteomic technique of tissue drug level quantification are detailed in the corresponding chapter, however, the relatively small numbers of patients assessed and the lack of clearly defined strategies as to loss of response criteria, immunogenicity testing (anti-drug antibody levels) and comparison with serum and local luminal mucosal drug levels make the findings of our pilot study exciting, but rudimentary and in need of further work to corroborate our findings. Nevertheless, the initial development of the technique remains an important step towards this goal. We also do not understand the mechanism of action of anti-TNF agents in the perianal tissues. For example, that luminal disease can improve whilst perianal disease persists may not be due to lack of penetration of the agent into perianal tissues at all and measuring the levels of the same peptides seen in the serum may be based on incorrect assumptions. However, given that the same peptides are identified and associated with inflammation in luminal tissue levels, searching for them in perianal tissue seems reasonable.

The studies reported in section C suffer from the aforementioned limitation of heterogeneity in the population studied, fistula morphology and aetiology, with no standardized population. The majority of studies of novel surgical interventions are uncontrolled case series, follow-up times are widely variable and relatively short. High initial success rates (many lacking radiological assessment) may be misleading and recurrences may develop more than a year after surgery

[126]. There was significant heterogeneity of outcome reporting, which represents a widespread issue in assessing outcome of interventions in the perianal Crohn's fistula literature. Most studies reported clinical assessment of healing and classified this as complete or partial (definitions of these were heterogeneous). These clinical outcome measures are subjective and don't account for temporal changes in fistula drainage and may be subject to recall bias. Clinical outcome reporting is also exacerbated by the fact that clinical healing is not always readily achievable and does not always correlate with radiological healing. In fact, MRI confirmation of deep healing has been shown to occur a median of 12 months after closure of the external openings[122,383]. These are common problems in studies of fistula treatment [474], often making comparison and pooling of data between studies difficult. In our study evaluating a novel therapy for primary symptom amelioration, we used a generic PROM, which was not validated for use specifically in Crohn's perianal fistulas and thus is limited in its long term use in this condition. A better disease specific PROM is required. Adherence to guidelines developed in 2016 for surgical case series reporting [475], as well as the development of a core outcome data set for perianal CD fistulas[444], should ultimately improve reporting in this field and facilitate meaningful analysis.

Section D had some limitations in the development of the CAF-QoL questionnaire. During the experience interviews, it should be noted that the group who participated were self- selecting and therefore the experiences of those who did not participate may differ. In addition, only English speaking patients were recruited so the views of non-English speaking patients were not included and may differ. Cultural differences between English speakers and some groups of patients who do not speak English, might reduce generalisability outside the English speaking world. Other limitations include the fact that we did not collect data on patients with other manifestations of perianal Crohn's disease aside from Crohn's fistula and did not carry

out separate analysis for patients with temporary defunctioning stoma or proctectomy. We took the view that this was outside the scope of the current study, which focussed on developing a QoL score specific to patients with fistula before and after intervention, and the symptoms and burden attributed to the fistula itself, rather than focussing on the smaller group of patients who have had faecal diversion or proctectomy. Specific limitations pertaining to questionnaire testing are detailed in the corresponding chapter.

12.7 Future direction

This thesis has identified several areas that would benefit from further work in order to drive towards our goal of developing the optimal management strategy in Crohn's perianal fistula. Work on aetiopathogenesis remains crucial to further our approach to treatment in this challenging condition. Section A demonstrates a new foray into molecular techniques for clinicians with the new tools available and the onus is on using these appropriately in order that we obtain useful answers. To this end, the ENiGMA working group is an alliance of dedicated physicians (expert gastroenterologists), specialist colorectal surgeons, trainees and patient representatives, that responded to the call to optimise the strategy for managing Crohn's anal fistulas. The group has a schedule of several workstreams and hopes to incorporate Crohn's fistula mechanistic work into a more structured approach to assessment. There is a need for defined patient cohorts when assessing aetiopathogenesis, and there is ongoing work on developing a new classification system that incorporates a combination of diagnostics comprising endoscopy, magnetic resonance imaging [MRI] and/or endoanal ultrasound [EUS], and examination under anaesthesia [EUA]. Cohorts addressed should be carefully phenotyped and should include an 'inception cohort' of new diagnosis of pCf patients as well as potentially other fistula phenotypes (i.e. (e.g. pouch fistulas, rectovaginal fistulas and

idiopathic/cryptoglandular fistulas). An important resource would be the establishment of a biobank of longitudinal bio-samples (serum, stool, urine, faces, rectal/fistula biopsy tissue) taken from patients at presentation, endoscopy, surgery and through their course of treatment and stored for later downstream investigations. A grant has since been secured by our study group to lead this aspect and establish a biobank of patients from which downstream high-throughput studies can be generated with the numbers that would allow for validation of meaningful findings. There is also a need to correlate the data across various multi-omic platforms, i.e. genomic / proteomic / metabonomic and microbiome. An integration of the chemical data with conventional clinico-radiological data would enable better disease stratification, notwithstanding the greater goal of understanding the complex microbial and metabolic interactions that impact and modulate this phenotypic manifestation. By assessing this cohort longitudinally, we hope to develop diagnostic and therapeutic biomarkers of disease which can add guidance in the personalization/stratification of patient treatments into efficacious treatment and minimise the morbidity / economic cost of futile treatments.

Improved classification systems and better capture of disease course and response to therapy via robust prospectively maintained databases would help in evaluation of real world outcomes of Crohn's perianal fistula patients. Tertiary centres may help in this process, due to more infrastructure for patient support and assessment and often better facilities and collating data on patient pathways. Furthermore, St Mark's hospital has in the last couple of years adopted an algorithm for management of Crohn's perianal fistulas, which includes schedules for regular MRI, which would aid in ensuring patients have similar management strategies allowing for better real world evaluation of interventions. Thus the collation and interrogation of a prospectively maintained database on Crohn's and idiopathic perianal fistulas with carefully phenotyped and classified cohorts detailing interventions, outcomes and time frames would be

a huge benefit in informing the literature on real world outcomes albeit from a tertiary centre perspective[546–548].

There also needs to be better evaluation of response to treatment with regard to disease indices and drug levels. Despite the paradigm shift seen over the last two decades with anti-TNF treatment in Crohn's disease, the response that an individual patient will have to a specific anti-TNF and dose is difficult to predict when compared with conventional (non-biologic) therapies. Research into the role of therapeutic drug monitoring and necessary parameters as well as immunogenicity are still ongoing with a need for consensus on definitions of the criteria for response failures to biologic therapy[402342]. It also remains unclear whether anti-TNF levels are likely to be a clinically meaningful biomarker for treatment response in Crohn's anal fistula. Small case series suggest higher trough levels associate with better response, but this is yet to be widely accepted. Biologic therapies are clearly an important treatment option and assessing response to treatment through laboratory data may improve and personalise patient care while substantially reducing health-related costs.

Our study on tissue drug levels as a potential biomarker requires larger numbers and multiple biopsy sites to aid in improving knowledge as to whether tissue biopsies can help in the determination of treatment. We also do not understand the mechanism of action of anti-TNF agents in the perianal tissues yet. Further work examining serum and luminal mucosal levels of anti TNF drugs at the same time as fistula samples would help answer several questions about the lack of drug seen in the 7 samples analysed so far. This would help in the road to identification of a predictor of those patients that are likely to go on to achieve response and save prolonged expensive and futile treatment in those who won't.

This thesis also brought forward the concept of symptom amelioration as a treatment intent in patients with refractory fistulas, to be obtained using the novel minimal invasive technique, VAAFT. There is a need for evaluating whether this has a genuine benefit above a placebo effect. It's assessment against sham surgery and the use of the CAF-QoL to assess outcome would enable better judgement of this. Long term data on the other novel surgical techniques reviewed (FiLaC, VAAFT, OTSC) are required and proper evaluation as to their role in Crohn's perianal fistula surgery, and patient benefit ought to be better characterised. These techniques demonstrate a shift towards 'sphincter preserving surgery' which have an obvious appeal in patients with Crohn's perianal fistula due to the need to preserve sphincter function in patients with a destructive disease process and the potential for a loose stool in years to come, and also to avoid problems with defective wound healing. However, their ability to singularly cure Crohn's perianal fistula remains to be seen. Future studies should assess their role in relation to certain fistula subtypes, where cure may well be achievable with minimal sphincter impairment. Furthermore it is imperative that the reporting of studies adopt a recent Crohn's perianal core outcome set[444] which would aid better evaluation and data summation across the various interventional studies.

The PROM developed in this thesis, CAF-QoL, is planned for dissemination and widespread use in Crohn's perianal fistula clinical practice. Future work into its role in assessing QoL impact and health care evaluation in interventional studies for Crohn's perianal fistula needs to be evaluated. There is also the potential of its use to aid the consent/ decision-making process towards proctectomy for Crohn's perianal fistulas in patients that are refractory. Other avenues for further development and wider dissemination include its cross-cultural validation and translation for use in non-English speaking regions.

Chapter 13. Appendix 1 – Supplementary material for Chapter 2 - Differences in amino acid and lipid metabolism distinguish Crohn's from idiopathic perianal fistulas by tissue metabonomic profiling and may offer clues to underlying pathogenesis)

13.1 Supplementary Material and Methods

13.1.1 Metabonomic profiling:

Two analytical platforms were attempted to achieve broad metabolome coverage; a chromatographic tool, hydrophilic interaction liquid chromatography/mass spectrometry (HILIC-MS) [220], was used to generate polar metabolites, and lipids were generated using ultra-high performance liquid chromatography/mass spectrometry (UPLC-MS) profiling[218,221]. Profiling analyses of metabolites was performed on fistula tract biopsies from Crohn's and idiopathic patients. A detailed description of sample preparation is presented in the Supplementary Methods. A schematic illustration of the tissue sampling strategy is given in Figure 38.

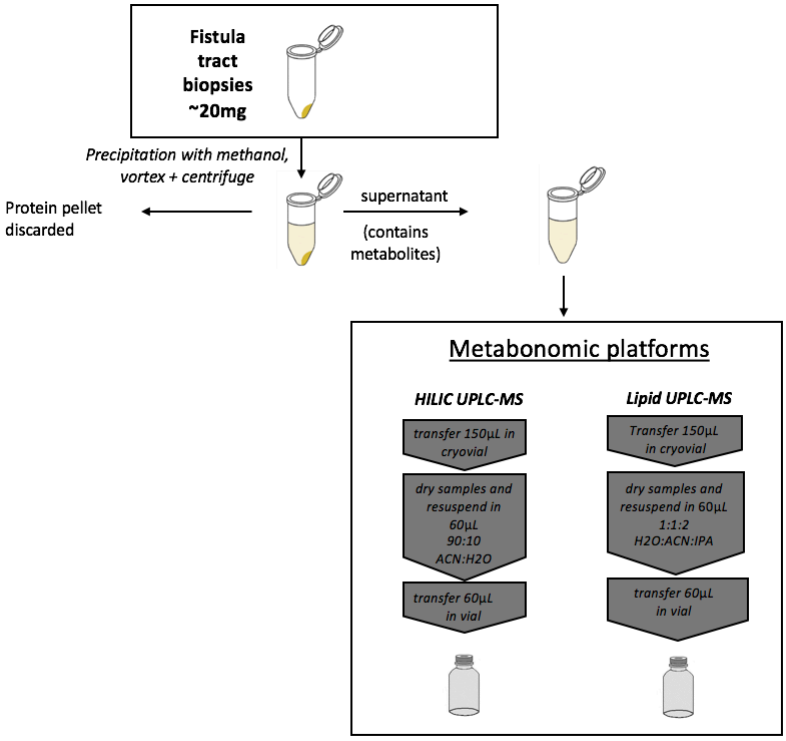

Figure 38: Overview of sample preparation for metabonomic profiling assays (HILIC UPLC-MS and Lipid UPLC-MS)

13.1.1.1 *Chemical materials:*

Solvents and solvent additives for UPLC-MS profiling. Organic solvents (HPLC grade) used for sample preparation were obtained from Sigma-Aldrich (Dorset, UK). Mobile phases were prepared with grade LC-MS solvents and modifiers from Sigma-Aldrich (Dorset, UK).

13.1.1.2 *sample preparation / metabolite extraction for UPLC profiling (HILIC / lipid UPLC profiling)*

An overview of the sample preparation is demonstrated in Figure 38. Prior to analysis, fistula tract biopsies were cut and weighed, with target sample specimen of approximately 20mg (confirmed on previous practice runs for satisfactory analysis). Tissue lysis and metabolite extraction was performed using a bead beater (Bertin Technologies) following previous

freezing with liquid nitrogen upon collection. Tissue samples (range of weighted samples 10.3 – 23.1mg) were loaded into shatter resistant bead beating tubes ("VWR® Reinforced 2ml Bead Mill Tubes", VWR international, UK), each with six zircon beads. A pre-chilled *methanol (MeOH) solution* of 300microliters was added to the tissue samples. This was done to precipitate the proteins so the supernatant could be analysed for metabolites. The bead beater was set to vibrate at 6500 Hz for 60 seconds and a 30second break followed by two further 60second cycles. Samples (for lipid profiling) were vortexed and centrifuged (Eppendorf, Centrifuge 5417R, Germany) at 14,000*g* for 20 min at -4°C. For each sample, the supernatant fraction was Total Recovery vials with low volume inserts (Waters Corp, USA) and stored at -80°C awaiting UPLC-MS analysis. The supernatant of samples prepared for the polar metabolite (HILIC) assay was evaporated at room temperature in a vacuum concentrator (Eppendorf Concentrator Plus) and reconstituted in 60µL of solvent mixture with acetonitrile/H_2O (90:10) and centrifuged for 20 min at 14000g,4 °C and then transferred into Total Recovery vials with low volume inserts (Waters Corp, USA). For lipid profiling assay, samples were was evaporated at room temperature in a vacuum concentrator (Eppendorf Concentrator Plus) and reconstituted in 60µL of solvent mixture with of water /acetonitrile / isopropranolol at a ratio of (1:1:2).The supernatant of samples prepared for the polar metabolite (HILIC) assay was evaporated at room temperature in a vacuum concentrator (Eppendorf Concentrator Plus) and reconstituted in 60µL of solvent mixture with acetonitrile/H_2O (90:10) and centrifuged for 20 min at 14000g,4 °C and then transferred into Total Recovery vials with low volume inserts (Waters Corp, USA).

Quality Control (QC) samples were prepared from pooled biopsy samples (10µL from each sample, pooled in a Total recovery vial). These samples were analysed using reversed phase-UPLC-MS lipid profiling, and HILIC UPLC-MS profiling[218]. A standard QC strategy[549] was used for both the lipid UPLC-MS and HILIC-MS analysis, whereby injected several times

before initiating the mass spectrometry run, and then injected every 10 samples throughout the analytical run and at the end of the run for assessment of instrument stability and analyte run. Following QC and sample extraction and preparation, these were stored at -80°C awaiting UPLC-MS analysis.

13.1.1.3 *Analysis of metabolite extracts via UPLC-MS profiling method (HILIC-MS / lipid profiling)* [550]

For the HILIC-MS[220], the chromatographic analysis (UPLC) was performed using an Acquity UPLC system (Waters Ltd, Elstree, UK) coupled to a mass spectrometer(MS), XEVO G2 QTof (Waters MS Technologies, UK) with an electrospray in positive ion mode. For the UPLC, separation was achieved using gradient elution (Table 33) with the mobile phase compositions of 20 mM ammonium formate (LC-MS grade, Fluka, USA) in water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The initial condition was 0.6 mL/min flow rate of 99% A for 10 min followed by 4 min of washing step and 1 min to return to initial conditions. 10 µL of the samples were injected onto a 2.1 × 150 mm Acquity BEH HILIC column (Waters Corp., Milford, MA, USA) thermostatted at 40 °C. The MS parameters implemented were as followed; capillary Voltage 1.5kV, cone voltages at 20 V, desolvation temperature at 600 °C, and source temperature at 120 °C. The cone gas flow rate was 150 L/h, and desolvation gas flow rate at 1000 L/h (see Table 35)). Acquisition was performed from *m/z* 50 to 1200. Leucine enkephalin was continuously injected to perform mass correction.

For the Lipid profiling UPLC-MS[218,221], the same instruments as above, were used for UPLC and MS. The UPLC separation was achieved using gradient elution highlighted in Table 34- Mobile phase A consisted of acetonitrile / water (60:40) with 10mM of ammonium fomate and 0.1% formic acid; mobile phase B consisted of isopropranolol / acetonitrile (90:10) with 10mM of ammonium fomate and 0.1% formic acid. The initial condition was 0.4 mL/min flow rate of 60% A for 10 min followed by 4 min of washing step and 1 min to return to initial conditions.

10 µL of the samples were injected onto 2.1 × 100 mm Acquity UPLC CSH C18 column (Waters Corp, USA) was thermostatted at 40 °C. MS parameters are highlighted in Table 35.

Table 33: Gradient profile used in the separation of metabolites in the HILIC assay

	Time(min)	Flow rate µL /min	%A	%B	Curve
1	Initial	0.6	5	95	Initial
2	0.1	0.6	5	95	6
3	4.6	0.6	20	80	6
4	5.5	0.6	50	50	6
5	7	0.6	50	50	6
6	7.1	0.605	5	95	6
7	7.2	0.61	5	95	6
8	7.3	0.62	5	95	6
9	7.4	0.65	5	95	6
10	7.5	0.7	5	95	6
11	7.6	0.8	5	95	6
12	7.7	0.9	5	95	6
13	7.8	1	5	95	6
14	14.5	1	5	95	6
15	14.65	0.6	5	95	6

Table 34: Chromatograph gradient used for lipid profiling

	Time(min)	Flow rate µL/min	%A	%B	Curve
1	Initial	0.4	60	40	Initial
2	2	0.4	57	43	6
3	2.1	0.4	50	50	1
4	12	0.4	46	54	6
5	12.1	0.4	30	70	1
6	18	0.4	1	99	6
7	18.1	0.4	60	40	1
8	20	0.4	60	40	

Table 35: Settings for MS conditions

	HILIC-MS	Lipid UPLC-MS
MS conditions	ESI (electrospray ionisation)	ESI (electrospray ionisation)
Capillary voltage	1.5kV	1kV
Cone voltage	20V	30V
Desolvation temperature	600ºC	550ºC
Source temperature	120 ºC	120 ºC
Cone gas flow rate	150L/hr	150L/hr
Desolvation gas flow rate	1000L/hr	900L/hr

13.1.1.4 *Data pre-processing using XCMS*

The UPLC-MS raw data were converted to netCDF format using MassLynx™ software (Waters Corporation, Milford, USA). Data extraction and processing was implemented using the XCMS package in R programming software, with peak settings highlighted below Table 36). An in-house script was used incorporating various programs and algorithm combination in order process the data. Peak detection was implemented using centWave (algorithm for chromatographic peak detection for high resolution MS data in centroid mode) [223] with application of various filters (including, ppm, signal-to-noise threshold, number of scans, peak width and signal intensity). Peaks were grouped across the samples according to mass to charge (m/z) ratios for given retention times. They were also screened to ensure a predefined minimum of required fraction of samples in which peaks for the peak group were identified (minimum fraction filter).

The dataset was normalized using the median fold change method [551] to total spectral area. Coefficient variation (defined by the standard deviation divided by the mean of features intensities) were calculated across QC samples injected through the run, and ensured reproducibility[552].

Table 36: Settings setup for pre-processing steps on XCMS program package of "R".

	Function	Settings (HILIC)	Settings (lipid)
Peak detection	Method	centWave	centWave
	Peak width	10 to 30 sec	10 to 50 sec
	Ppm	10	40
	Signal to noise threshold	10	10
Peak alignment / Grouping	Method 1	group	group
	m/z	0.06 Dalton	0.03dalton
	RT error	8 seconds	6seconds
	Method 2	retcor	retcor
	Method 3	peakgroups	peakgroups
Peak Filtering	Minfrac	0.2	0.2
	CV	30%	30%
Normalisation	Method	Median fold change	Median fold change

13.1.1.5 *Univariate and multivariate and statistical data analysis*

Multivariate data analysis was performed using the SIMCA package (v.13.0.2, Umetrics, Umeå, Sweden). Linear-projection methods, such as principal-components analysis (PCA) and partial-least-squares discriminant analysis (PLS-DA), were used to map samples on the basis of their biochemical similarity and to extract patterns of metabolites that relate to a particular phenotype[553] (in our case Crohn's disease anal fistulas vs idiopathic fistula). Multivariate Pareto scaled data were modelled using (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The OPLS-DA parameters (R^2X, R^2Y, Q^2Y and cross validated CV ANOVA p-value) aimed to assess the inherent metabolic variation and robustness of each model. Markers were identified by selection from a coefficient correlation plot using the SIMCA package and these were defined as markers from the statistically significant OPLS-DA model that served as predictive of either CD or idiopathic fistulas.

Univariate analysis, using the student two-tailed t-test was applied to the main discriminatory features (i.e. metabolite markers / intensity of peaks) of each model (Office Excel 2007) to assess for significant difference between sample groups (CD vs. idiopathic). The p-values associated with the both HILIC and Lipid profiling models were adjusted for multiple testing using the method of Benjamini and Hochberg[225] to control the false detection rate at 0.05 level. A Python script was developed to plot the receiver operating characteristic (ROC) and calculate the area under the curve (AUC) with confusion matrix parameters (accuracy, sensitivity and specificity) of each marker [226].

13.1.1.6 *Metabolite assignment*

Markers list was retrieved from significant OPLS-DA model, and inspection of spectra was performed for each marker feature in order to select the base peak (i.e. the peak from the most abundant and often most stable ion). Metabolite identification by MS was conducted by matching accurate *m/z* measurements of detected chromatographic peaks to theoretical values from in-house databases and on-line databases such as the human metabolite database (HMDB, http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/ligand.html), and METLIN (http://metlin.scripps.edu/) , LIPID MAPS (http://www.lipidmaps.org/tools/index.html). For the HILIC data, spearman correlations were applied to identify ions (e.g. [M+H]+, [M+Na]+, [M+NH_4]) that were adducts in the spectra and hence related to the same marker. For the lipid profiling data, ions that were adducts included [M+K]+, [M+H-H2O]+, in addition to those seen in the HILIC data. Tandem MS fragmentation patterns were obtained for further structural elucidation and confirmed with an authentic standards matching for retention time and *m/z*.

13.2 Supplementary Results

13.2.1 Differences in metabolic profiles observed between CD fistula patients and idiopathic fistula patients using UPLC-MS approaches

Table 37 demonstrates the feature identification process, from the initial raw data with over 1800 features (i.e. ionised mass: charge ratio, m/z, at specific retention time, RT) on each assay. These were processed as described in the methods section (data pre-processing) in order to characterise statistically significant metabolites distinguishing Crohn's and idiopathic fistula tract biopsies, i.e. n = 22 (HILIC) and n = 19 (lipid profiling).

Table 37: Process of feature identification to assigned markers

	Number (n=…) – HILIC	Number (n=…) - LIPID
Raw norm features	1802	3334
Raw norm CV features (coefficient variation)	1707	3099
Predictive features	1783	1752
Mono-isotopic peaks	106	598
Base peaks	81	564
Assigned base peak	38	274
Assigned markers (statistically significant on student t-test)	22	19
Crohn's fistula tissue predictive markers	12	18
Idiopathic fistula tissue predictive markers	10	0

13.2.2 Metabolic predictors of fistula type using UPLC-MS approaches to fistula tract biopsy

Receiver operating characteristic (ROC) curve analysis was applied to provide a measure of utility of the markers in distinguishing between Crohn's and idiopathic fistula biopsy samples. Mean ROC statistics were done for individual metabolites, and this was highest for Decanoyl L-Carnitine being predictive of Idiopathic fistula (AUC = 0.87 +/- 0.15), and Dimethylarginine was highest (AUC = 0.83 +/- 0.17) for prediction of Crohn's fistula. Highest mean ROC statistics for prediction of Crohn's fistula were Arginine (AUC= 0.78 +/-0.18) Methionine (AUC= 0.77 +/-0.20), Lysine (AUC=0.75 +/- 0.20). Lowest mean ROC statistics for prediction of Crohn's fistula were AUC = 0.67 +/- 0.20 (Proline), AUC (area under the curve) = 0.65 +/- 0.22 (Palmitoyl L-Carnitine), AUC = 0.64 +/-0.22 (Phosphocholine), AUC = 0.39 +/- 0.24 (Guanine). Combined analysis ROC curve for all predictive metabolites for either Crohn's or idiopathic fistula revealed an AUC of 0.72 +/- 0.21 and is depicted in (supplementary Figure 39). This was based on combination of metabolites with the highest AUC values (i.e. excluding Palmitoyl carnitine, Phosphocholine, Proline, Phenylalanine, Guanine).

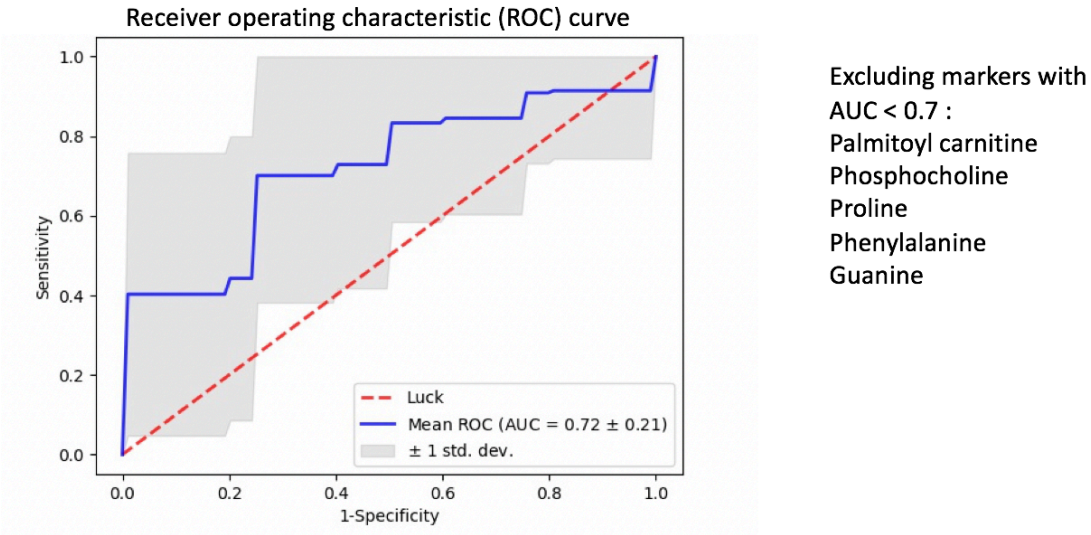

Figure 39: Combined ROC for significant marker features predictive of Crohn's fistula / Idiopathic fistula extracted from the OPLS-DA model

Table 38: list of distinguishing marker metabolites on HILIC assay between CD / Idiopathic fistulas from the OPLS-DA model in Figure 2

Marker metabollite	Ions	m/z	RT	Trend in CD fistula	Class of metabolite	p-value (from student t-test)	corrected p-value (Benjaminin-Hochberg)	Spearman Correlation
Arginine	[M+H]+	175.119	5.98	↑	Amino acid	0.0090	0.008	0.407
Threonine	[M+H]+	120.066	5.02	↑	Amino acid	0.0410	0.027	0.332
Tyrosine	[M+H-NH3]+	165.055	4.16	↑	Amino acid	0.0126	0.015	0.371
Tyrosine	[M+H–CO2H]	136.076	4.16	↑	Amino acid	0.0126	0.015	0.371
Tyrosine	[M+H]+	182.081	4.16	↑	Amino acid	0.0126	0.015	0.371
Lysine	[M+H-NH2]+	130.086	6.01	↑	Amino acid	0.0112	0.007	0.419
Lysine	[M+H]+	147.113	6.01	↑	Amino acid	0.0112	0.007	0.419
Citrulline	[M+H]+	176.103	5.68	↑	Amino acid	0.0219	0.013	0.384
Proline	[M+2Na-H]1+	160.035	4.51	↑	Amino acid	0.0200	0.021	0.348
Methionine	[M+H-NH2]+	133.032	4.3	↑	Amino acid	0.0026	0.004	0.455
Methionine	[M+H]+	150.058	4.3	↑	Amino acid	0.0026	0.004	0.455
Methionine	[M+2Na-H]+	194.022	4.29	↑	Amino acid	0.0026	0.004	0.455
Phenylalanine	[M+2Na-H]+	210.05	4.08	↑	AMIno acid	0.0440	0.065	0.277
Homoarginine	[M+H]+	189.16	6.23	↑	amino acid	0.0112	0.007	0.419
Dimethylarginine	[M+H]+	203.15	6.05	↑	amino acid	0.0105	0.000	0.568
Guanine	[M+K]+	190.013	2.16	↑	Nucleoside	0.5380	0.949	0.010
Guanine	[M+Na]+	174.039	2.16	↑	Nucleoside	0.5380	0.949	0.010
Guanine	[M+2Na-H]+	196.021	2.16	↑	Nucleoside	0.5380	0.949	0.010
Guanine	[M+H]+	152.057	2.15	↑	Nucleoside	0.5380	0.949	0.010
O acetyl L-carnitine	[M+H-NH3]+	187.108	5.14	↑	amino acid	0.0138	0.008	0.407
Octanoyl L-Carnitine	[M+H]+	288.217	4.17	↑	amino acid	0.0191	0.018	0.358
Octanoyl L-Carnitine	[M+Na]+	310.201	4.18	↑	amino acid	0.0191	0.018	0.358
N-Acetyllysine	[M+H]+	189.123	5.58	↑	amino acid	0.0175	0.015	0.374
N-Acetyllysine	[M+Na]+	211.105	5.58	↑	amino acid	0.0175	0.015	0.374
Creatinine	[2M+H]+	227.125	2.51	↓	UNCLASSIFIED	2.49E-05	0.001	-0.536
Creatinine	[M+H]+	114.066	2.51	↓	UNCLASSIFIED	2.49E-05	0.001	-0.536
Adenosine	[M+H]+	268.104	1.74	↓	Nucleoside	4.17E-04	0.002	-0.490
Phosphocholine PC 34:2	[M+Na]+	780.552	3.95	↓	lipid	0.0647	0.106	-0.242
Monoacylglycerol 18:3	[M+NH4]+	370.295	3.82	↓	lipid	0.0065	0.003	-0.468
Hexanoyl L-Carnitine	[M+H]+	260.186	4.37	↓	amino acid	1.48E-04	0.000	-0.552
S-methyl-5-thioadenosine	[M+H]+	298.097	1.29	↓	Nucleoside	0.0190	0.006	-0.439
Decanoyl L-Carnitine	[M+H]+	316.248	4.04	↓	amino acid	1.30E-05	0.000	-0.649
Palmitoyl L-Carnitine	[M+H]+	400.342	3.76	↓	amino acid	0.1500	0.047	-0.300

Univariate statistical analysis calculated using the Two-tailed Student's t-test with significance set at $p<0.05$. Benjamini-Hochberg statistical adjustment was applied confirming significance of all values except guanine, phenylalanine, phosphocholine.

*m/z (mass-to-charge ratio); RT – retention time; ESI+ (electrospray ion positive mode)

Table 39: list of distinguishing marker metabolites on Lipid profiling assay fistulas from the OPLS-DA model in Error! Reference source not found.

Univariate statistical analysis calculated using the Two-tailed Student's t-test with significance set at $p<0.05$. Benjamini-Hochberg statistical adjustment was applied confirming significance of 2 metabolite (HexCer (d18:1/23:0), Diglyceride DG 38:5.

*m/z (mass-to-charge ratio); RT – retention time; ESI+ (electrospray ionisation- positive mode)

Chapter 14. Appendix 2 – Supplementary material for Chapter 7 (Lack of anti-TNF drugs levels in fistula tissue – a reason for non-response in Crohn's perianal fistula?)

14.1 Supplementary Materials and Methods:

14.1.1 Chemical materials:

Infliximab (Remicade®) and adalimumab (Humira®) were obtained from Northwick Park Hospital pharmacy department and stored according to manufacturer instructions. Infliximab (Remicade; Centocor, Horsham, PA) was supplied as a sterile, white, lyophilized powder for intravenous infusion. Following reconstitution with 10mL of Sterile Water for injection, USP, the resulting pH is approximately 7.2. Each single-use vial contains 100mg infliximab, 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate, and 6.1 mg dibasic sodium phosphate. No preservatives are present. Concentration of infliximab following reconstitution (as for injection) 10mg / ml, was reconstituted and stored at -80°C until use. Adalimumab (Humira; Abbott Laboratories, Abbott Park, IL) was supplied as a sterile solution (of drug dissolved in water) with concentration of 40mg in 0.4ml i.e. 100mg / ml.

Organic solvents (HPLC grade) used for proteomic sample preparation and were obtained from Sigma-Aldrich (Dorset, UK). Other components, ammonium bicarbonate, Dithiothreitol (DTT), iodoacetamide (IDT), were all obtained from Sigma-Aldrich (Dorset). Trypsin was obtained from Promega (WI, USA). LC-MS grade solvents were used to prepare all mobile

phases, i.e., acetonitrile, formic acid and water from Sigma-Aldrich (Dorset, UK). [431]

14.1.2 Sample preparation (Figure 21):

14.1.2.1 *Precipitation with methanol (Protein extraction from bio-sample – serum/tissue)*

Protein extraction using methanol requires different preparation for liquid (e.g. serum) and solid (i.e. fistula tract biopsy tissue) bio-samples. In order to test detection in liquid biofluids, infliximab and adalimumab were each spiked in both serum and water respectively. For purposes of quantitation, serial dilutions were aliquoted (see below). Precipitation of all samples was performed by addition of pre-chilled methanol solution (300 µL) to the sample. Samples were vortexed and centrifuged at 14,000g for 20 min at -4°C. The supernatant was discarded, leaving the resultant protein pellet sediment for further sample preparation (protein denaturation and digestion).

For both adalimumab and infliximab calibration curves, an initial concentration of 500 µg mL−1 samples was made from pipetting the drug into human serum, and then different concentration levels of samples (100, 75, 50, 50, 25, 10, 7.5, 5, 2.5, 1, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.01 µg mL−1) were prepared from the 500 µg mL−1 samples by serial dilutions with human serum. An equivalent dilution series was done for both drugs using water as the 'diluent', this was in order to have a *'quality control'* samples for a range of concentrations that were used as a calibration standard against the test fistula tract tissue biopsy samples. Each calibration sample was precipitated in the same way as for methanol with the supernatant aliquoted and discarded leaving just the drug protein (infliximab / adalimumab monoclonal antibody) in the cryovial for protein digestion and analysis.

For protein extraction from fistula tract tissue biopsies, these were cut and weighed, with target sample specimen of approximately 20mg. Tissue lysis and metabolite extraction was

performed using a bead beater (Bertin Technologies) following previous freezing with liquid nitrogen upon collection. Tissue samples (range of weighted samples 10.3 – 23.1mg) were loaded into shatter resistant bead beating tubes ("VWR® Reinforced 2ml Bead Mill Tubes", VWR international, UK), each with six zircon beads. A pre-chilled *methanol solution*, of 300 µL was added to each tissue sample (resulting in tissue precipitation). The bead beater was set to vibrate at 6500 Hz for 60 seconds and a 30second break followed by two further 60second cycles. Samples were kept on dry ice in between cycles and following bead beating were subsequently vortexed and centrifuged (Eppendorf, Centrifuge 5417R, Germany) at 14,000*g* for 20 min at 4°C. The resultant supernatant was aliquoted off and discarded, leaving the protein pellet sediment for analysis following sample preparation.

NB> For the positive control tissue samples, these were spiked using 20 µL of infliximab (10mg / ml) infliximab or 20 µL of adalimumab (100mg / ml) prior to protein extraction.

14.1.2.2 *Protein digestion (Figure 21)*

The precipitation of spiked serum / water and fistula tract tissue sample was followed by digestion of the precipitated protein sediment via sequential stages of processing to extract the required peptide (signature peptide). These were then introduced to a liquid chromatography column for separation. Upon eluting from the LC column, peptides are ionized and analysed by the mass spectrometer.

The processes involved were denaturation, reduction of di-sulphite bonds, (Dithiothreitol, DTT) Alkylation (iodoacetamide, IDT), digestion by trypsin. The protein pellets in aliquots (following tissue extraction) are resuspended in 50µL of ammonium bicarbonate (later optimised to 100µL) for denaturation of the proteins by breaking of the hydrogen bonds and also persistent disulphide bonds are reduced with dithiothreitol (10 µL of 100mM, optimised

to 20 µL). The samples were vortexed and incubated at 60 ºC for 60minutes. They are subsequently cooled at room temperature for about 30minutes in preparation for the next step, alkylation with 25 µL of 100 mM iodoacetamide (optimised to 50 µL) which prevents refolding of proteins (i.e. reforming of the disulphide bonds). The process involved storing the samples in the dark at 30 ºC for 30minutes. The final step is the digestion of the resultant proteins into the specific peptides that can be analysed by mass spectrometry. This process involves addition of 25 µL (optimised to 40 µL) of 5mg / ml Trypsin (Promega, WI, USA). The samples are incubated for 60mins at 50 ºC. This process of digestion by trypsin is finally aborted / 'quenched' with 25 µL (optimised to 50 µL) of *10% Formic acid /water solution (1:1*). The samples are vortexed and centrifuged (Eppendorf, Centrifuge 5417R, Germany) at 14,000*g* for 20 min at 4°C. The supernatant is transferred into mass spec vials with inserts (50 µL in each vial), which were placed in the autosampler at 4°C ready for liquid chromatographic separation and elution and ionisation in the mass spectrometer. The final concentration levels of infliximab / adalimumab in each calibration sample was calculated by accounting for various volumes of solvents described above (these were 192.31, 38.46, 28.85, 19.23, 9.62, 3.85, 2.89, 1.92, 0.96, 0.38, 0.29, 0.19, 0.097, 0.038, 0.029, 0.019, 0.0096, 0.0038 µg mL−1).

14.1.3 Signature peptide selection

The process of trypsin denaturation described above was specific to infliximab and adalimumab, and a necessary step for detection/quantification as antibodies are too heavy (~149 kDa) to be directly quantified using standard LC–MS assays. However, it is possible to detect unique marker peptides specific to the antibodies. Therefore, signature peptide selection is of paramount importance for the quantification of the mononuclear antibodies (i.e. infliximab and adalimumab). Tryptic peptides (i.e. peptides digested by trypsin) used for quantification were initially identified via literature search. These were then assessed during optimisation

runs to assess for good chromatographic (UPLC) separation as well as detection on mass spectrometry with regard to ionisation / signal intensity, response to dilution, detection in biological matrix (i.e. tissue, via positive controls). The two surrogate peptides for each anti-TNF are described below:

- "SINSATHYAESVK" for infliximab- is located in the complementarity determining region (CDR) of the heavy-chain variable region (VH) of infliximab. It was confirmed as unique in human serum and had been previously validated for showed high sensitivity and specificity[431]. Reported MRM transition for this peptide was 469.6→603.8[431], however this was also confirmed on MS assay optimisation – m/z values (i.e. 'transitions') of precursor and fragment ions following ion fragmentation obtained following application of high collision energy (in quadrupole 2) in the MS.

- "APYTFGQGTK" for adalimumab – is located in the light chain of adalimumab and being a fully humanized monoclonal antibody, it can be difficult to quantify in human serum/plasma, as it shares very close sequence homology with endogenous human IgG. This severely restricts the tryptic peptide options available for adalimumab's quantification. The sequence we chose has previously been used for adalimumab quantification experiments, described in an application note by Waters (Waters Corp., Milford, MA, USA) [433], however in order to verify this, a standard technique of online unique peptide identification was employed. Predicted surrogate peptides were obtained for adalimumab using the silico trypsin digestion [PeptideMass, http://web.expasy.org/peptide_mass/]. These were then compared with the amino acid sequences

unique to human proteins using BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi] to target unique peptides specific to the adalimumab. Following unique signature peptide identification, development and optimization of the MRM method was experimentally determined using a Skyline/MassLynx workflow performed on the Xevo TQ-XS Tandem Quadrupole MS using a tryptic digest of adalimumab /infliximab in serum and water. This corroborated the application note finding, with MRM transition 535.27>901.44.

14.1.4 Analysis of tissue extract using UPLC-MS/MS (LC–MS Setup for Signature Peptide Identification Procedure and Quantitative Analysis)

The chromatographic analysis (UPLC) was performed using an Acquity UPLC system (Waters Ltd, Elstree, UK) coupled to triple quadrupole mass spectrometer(MS), XEVO TQ-S (Waters MS Technologies, UK) with an electrospray in positive ion mode (with multiple reaction monitoring, MRM – discussed later). For the UPLC, separation was achieved using gradient elution (Table 40) with the mobile phases composition of 0.1% formic acid in water (A) and acetonitrile with 0.1% formic acid (B). The initial condition was 0.3 mL/min flow rate of 100% A for 6.5 min, gradient was then continued to return to initial conditions at 7.1minute, with subsequent equilibration. 10 µL of the samples were injected onto a 2.1 × 150 mm Acquity UPLC® BEH C18 column (Waters Corp., Milford, MA, USA) thermostatted at 60 °C. The flow rate was 0.3 mL min−1, and the auto-sampler temperature was set at 4 °C. The MS parameters implemented are displayed in the (Table 41). Standardised MS parameters

implemented were capillary Voltage 1.5kV, cone voltages at 20 V, desolvation temperature at 600 °C, and source temperature at 120 °C. The cone gas flow rate was 150 L/h, and desolvation gas flow rate at 1000 L/h (Table 41). Collision energy was optimised between 5 – 20kV in order to ascertain the energy required for peak intensity for parent ion generation for infliximab / adalimumab.

Table 40: Gradient profile used for UPLC separation

	Time (min)	Flow rate mL/min	%A	%B	Curve
1	Initial	0.3	100	0.0	Initial
2	6.5	0.3	40.0	60.0	6
3	7.0	0.3	0.0	100.0	6
4	7.10	0.3	100.0	0.0	6
5	7.2	0.3	100.0	0.0	6

Table 1 – Gradient profile used for UPLC separation

Table 41: Settings for MS conditions.

MS conditions	ESI (electrospray ionisation)
Capillary voltage	1.5kV
Cone voltage	20V
Collision Energy	Q1- 10 kV Q2 - 20kV (infliximab)
Desolvation temperature	600ºC
Source temperature	120 ºC
Auto-sampler temperature	4 ºC
Cone gas flow rate	150L/hr
Desolvation gas flow rate	1000L/hr

Table 2 - Settings for MS conditions.

Certain techniques were performed as quality controls for the mass spectrometer sample analysis runs, these include:

- the autosampler being kept at a temperature of 4 °C in preparation for sample injection into the mass spectrometer.

- Test runs were performed, before analysis of tissue samples to ensure detection and quantification of the drugs in serum / water.
- A quality control run strategy for the tissue analysis run was performed in tandem with the tissue samples being analysed. This involved sequentially analysing serial dilutions of drug (both infliximab and adalimumab) in water, in the same experiment run as the test samples (positive / negative control tissue samples and fistula tract biopsy samples). This was done to ensure a positive control and calibration curve for quantitation. The ordering of the run cycle was set up so that the order started with injections of a few blank samples (methanol) so as to first exclude contamination, next in the run cycle, was injection of positive controls (adalimumab) spiked in tissue to test the detection in the specific biological matrix). This was followed by sequential injection of patient biopsy samples and then a re-run of the calibration samples (anti-TNF in water) followed by blank samples after highest concentration samples to ensure no ‘carry-over’ or contamination of samples.

Chapter 15. Appendix 3 – Supplementary material for Chapter 11; Development and validation of a patient reported outcome measure for Crohn's perianal fistula – Crohn's Anal Fistula Quality of Life questionnaire (CAF-QoL)

15.1 Supplementary Results section

15.1.1 Completeness of the data

The 'test' data consisted of data from 184 patients. Of these, 3 patients did not complete any of the questions from Q17 onwards, which related to the new CAF-QoL scale. Therefore, these were excluded completely from the analysis, leaving 181 patients for analysis. The completeness of the data was examined, and the information is presented in Table 42. The figures are the number and percentage of missing data values, and also the number of items where the question was not applicable. The final column gives the combined total of missing and not applicable responses. The data suggested that there was very little in the way of missing data. Two patients had missing values for all questions from question 32 onwards. This suggests they gave up on the questionnaire completely at this stage, rather than any specific issues with the questions. A number of questions had 'not applicable' responses. The 'not applicable' occurred in over half of patients for questions 27, 28 and particularly question 53

where over 80% of responses were ‘not applicable’. Given that these questions were not relevant to over half of patients, this suggests that these may be less useful or applicable to include as part of a scoring system.

Table 42: Data completeness

Question number	Not applicable N (%)	Missing data N (%)	N/A + Missing N (%)
17	-	0 (0%)	0 (0%)
18	-	0 (0%)	0 (0%)
19	-	0 (0%)	0 (0%)
20	-	0 (0%)	0 (0%)
21	-	0 (0%)	0 (0%)
22	-	0 (0%)	0 (0%)
23	-	0 (0%)	0 (0%)
24	-	0 (0%)	0 (0%)
25	64 (35%)	0 (0%)	64 (35%)
26 (*)	5 (18%)	0 (0%)	5 (18%)
27	118 (65%)	0 (0%)	118 (65%)
28	119 (66%)	0 (0%)	119 (66%)
29	-	0 (0%)	0 (0%)
30	-	0 (0%)	0 (0%)
31	-	0 (0%)	0 (0%)
33	-	2 (1%)	2 (1%)
34	-	2 (1%)	2 (1%)
35	-	2 (1%)	2 (1%)
36	-	2 (1%)	2 (1%)
37	-	2 (1%)	2 (1%)
38	-	2 (1%)	2 (1%)
39	-	2 (1%)	2 (1%)
40	-	2 (1%)	2 (1%)
41	-	2 (1%)	2 (1%)
42	-	2 (1%)	2 (1%)
43	-	2 (1%)	2 (1%)
44	-	2 (1%)	2 (1%)
45	-	2 (1%)	2 (1%)
46	-	2 (1%)	2 (1%)
47	-	2 (1%)	2 (1%)
48	-	2 (1%)	2 (1%)
49	-	2 (1%)	2 (1%)
50	-	2 (1%)	2 (1%)
51	-	2 (1%)	2 (1%)
52	30 (17%)	2 (1%)	32 (18%)
53	151 (83%)	2 (1%)	153 (85%)

(*) Results based a smaller number of patients compared to other questions (n=28)

15.1.2 Data spread

Each item consisted of five response categories. Summaries of the spread of responses were produced for questions relating to frequency of symptoms, i.e. Domain A (Table 43)

Table 43: Descriptive summary of frequency questions (Domain A)

Question number	Never N (%)	Occasion. N (%)	Few/wk. N (%)	Daily N (%)	Several N (%)	SD
17	77 (43%)	61 (34%)	13 (7%)	19 (11%)	11 (6%)	1.2
18	53 (29%)	67 (37%)	17 (9%)	25 (14%)	19 (11%)	1.3
19	115 (64%)	40 (22%)	10 (6%)	10 (6%)	6 (3%)	1.0
20	47 (26%)	39 (22%)	16 (9%)	33 (18%)	46 (25%)	1.6
21	73 (40%)	26 (14%)	7 (4%)	29 (16%)	46 (25%)	1.7
22	74 (41%)	51 (28%)	21 (12%)	19 (11%)	16 (9%)	1.3
23	81 (45%)	55 (30%)	12 (7%)	14 (8%)	19 (11%)	1.3
24	155 (86%)	23 (13%)	2 (1%)	0 (0%)	1 (1%)	0.5

The first figures are the number and percentage of patients in each category. The final column gives the standard deviation of the measurements, another measure of the spread of the responses.

Table 43 demonstrates reasonable spread of responses throughout the five outcome categories. The exception was for question 24. The responses were grouped toward the lower end of the scale, and the standard deviation for this question was noticeably lower than for the other questions. There was also less spread of responses for question 19, but this was not so pronounced as for question 24(Figure 34).

Summaries of the spread of responses were produced for questions that had a agree/disagree response scale (Domain B & C). A summary of the responses for these questions are given in Table 44.

Table 44: Descriptive summary of agree/disagree questions (Domain B and C).

Question number	Str Disagree N (%)	Disagree N (%)	Unsure N (%)	Agree N (%)	Str Agree N (%)	SD
25	25 (21%)	31 (27%)	20 (17%)	18 (15%)	23 (20%)	1.4
26 (*)	4 (17%)	7 (30%)	3 (13%)	5 (22%)	4 (17%)	1.4
27	15 (24%)	3 (5%)	4 (6%)	19 (30%)	22 (35%)	1.6
28	15 (24%)	24 (39%)	16 (26%)	5 (8%)	2 (3%)	1.0
29	83 (46%)	52 (29%)	24 (13%)	16 (9%)	6 (3%)	1.2
30	2 (1%)	1 (1%)	17 (9%)	63 (35%)	98 (54%)	0.8
31	46 (25%)	46 (25%)	63 (35%)	16 (9%)	10 (6%)	1.1
33	69 (39%)	50 (28%)	15 (8%)	38 (21%)	7 (4%)	1.3
34	72 (40%)	45 (25%)	9 (5%)	38 (21%)	15 (8%)	1.4
35	54 (30%)	21 (12%)	26 (15%)	26 (15%)	39 (22%)	1.6
36	40 (22%)	21 (12%)	25 (14%)	44 (25%)	49 (27%)	1.5
37	63 (35%)	53 (30%)	17 (10%)	30 (17%)	16 (9%)	1.3
38	47 (26%)	25 (14%)	15 (8%)	53 (30%)	39 (22%)	1.5
39	60 (34%)	55 (31%)	16 (9%)	28 (16%)	20 (11%)	1.4
40	38 (21%)	32 (18%)	12 (7%)	63 (35%)	34 (19%)	1.5
41	53 (30%)	49 (27%)	15 (8%)	41 (23%)	21 (12%)	1.4
42	61 (34%)	49 (27%)	19 (11%)	30 (17%)	20 (11%)	1.4
43	64 (36%)	48 (27%)	25 (14%)	25 (14%)	21 (12%)	1.4
44	65 (36%)	38 (20%)	13 (7%)	33 (18%)	30 (17%)	1.5
45	44 (25%)	36 (20%)	14 (8%)	50 (28%)	35 (20%)	1.5
46	41 (23%)	45 (25%)	20 (11%)	41 (23%)	32 (18%)	1.5
47	38 (21%)	28 (16%)	6 (3%)	62 (35%)	45 (25%)	1.5
48	35 (20%)	41 (23%)	11 (6%)	55 (31%)	37 (21%)	1.5
49	42 (23%)	36 (20%)	19 (11%)	44 (25%)	38 (21%)	1.5
50	47 (26%)	43 (24%)	21 (12%)	47 (26%)	21 (12%)	1.4
51	23 (13%)	20 (11%)	36 (20%)	41 (23%)	59 (33%)	1.4
52	20 (13%)	22 (15%)	35 (23%)	34 (23%)	38 (26%)	1.4
53	11 (39%)	2 (7%)	6 (21%)	3 (11%)	6 (21%)	1.6

(*) Results based a smaller number of patients compared to other questions (n=28 valid responses)

The responses suggested there was a reasonable spread of values throughout the five response categories for the majority of the items. The lowest spread was for question 30, where the responses were grouped in the upper categories, with few items in the lower categories. The standard deviation was the lowest for this question.

15.1.3 Intra-observer agreement

The next set of analyses examined the intra-observer agreement, the agreement between the test and retest questionnaires. The weighted kappa method (Table 29) was used to determine the level of agreement, and the results are summarised in Table 45. The figures are the calculated kappa values, along with corresponding confidence intervals. (kappa methods were used to calculate the agreement between the two sets of measurements (test-retest), due to the categorical nature of outcomes. These methods measure the agreement between repeat responses over and above that which would be expected due to chance. Due to the ordinal nature of the responses, the weighted kappa method was used. This gives more weight to closer disagreements (e.g. by only one category) than larger disagreements (e.g. by 4 categories). Kappa is measured on a scale ranging up to a maximum agreement of 1, and a suggested interpretation of the values is given in the table below. The kappa values were calculated along with a corresponding confidence interval indicating the uncertainty in the values. The analysis was restricted to patients with stable disease only between timepoints.

Table 45: Agreement between test and retest questionnaires

Question number	n	Kappa (95% CI)	Kappa Interpretation
17	70	0.76 (0.60, 0.92)	Good
18	70	0.80 (0.64, 0.97)	Good / Very Good
19	70	0.72 (0.56, 0.89)	Good
20	70	0.87 (0.69, 1.00)	Very Good
21	70	0.88 (0.69, 1.00)	Very Good
22	70	0.74 (0.57, 0.90)	Good
23	70	0.76 (0.59, 0.92)	Good
24	70	0.70 (0.48, 0.91)	Good
25	47	0.68 (0.48, 0.87)	Good
26	16	0.96 (0.62, 1.00)	Very Good
27	24	0.72 (0.43, 1.00)	Good
28	24	0.51 (0.26, 0.77)	Moderate
29	70	0.59 (0.42, 0.75)	Moderate / Good
30	70	0.56 (0.40, 0.72)	Moderate
31	70	0.57 (0.41, 0.73)	Moderate
33	69	0.78 (0.60, 0.96)	Good / Very Good
34	69	0.78 (0.60, 0.96)	Good / Very Good
35	69	0.82 (0.65, 0.99)	Good / Very Good
36	69	0.70 (0.54, 0.88)	Good
37	69	0.81 (0.64, 0.98)	Good / Very Good
38	69	0.75 (0.57, 0.93)	Good
39	69	0.74 (0.57, 0.92)	Good
40	69	0.69 (0.52, 0.86)	Good
41	69	0.75 (0.58, 0.92)	Good
42	69	0.80 (0.63, 0.97)	Good / Very Good
43	69	0.77 (0.60, 0.94)	Good
44	69	0.80 (0.62, 0.98)	Good / Very Good
45	69	0.75 (0.57, 0.93)	Good
46	69	0.78 (0.61, 0.96)	Good / Very Good
47	69	0.79 (0.63, 0.96)	Good / Very Good
48	69	0.81 (0.64, 0.99)	Good / Very Good
49	69	0.83 (0.66, 1.00)	Very Good
50	69	0.77 (0.59, 0.94)	Good
51	69	0.73 (0.57, 0.89)	Good
52	58	0.69 (0.52, 0.86)	Good
53	7	0.49 (0.04, 0.94)	Moderate

(*) Results based a smaller number of patients compared to other questions (n=28 valid responses)

The results suggested that for the majority of the items there was good agreement between the test and retest results, and in some cases very good agreement. None of the items displayed poor agreement between the two sets of measurements. However, for some items the agreement was only ‘moderate’. This was the case for question 28, 30, 31 and 53, 29.

15.1.4 Associations between questions

The final set of analyses examined the strength of association between the individual questions. Every pair of individual questions was correlated against each other. The biggest area concern would be for questions that are very strongly correlated and would be almost duplicating the information. Therefore, attention is focussed on these findings. Table 46shows the questions where the correlation between variables was 0.75 or higher. The table is split into two sections. The first relates to associations between questions where the full dataset was collected, which the second part relates only to associations with Q26, which was data collected on a smaller number of patients. Within each section, the table is ordered so that the highest correlations are shown first, which the correlations descending in size order.

Table 46: Largest associations between questions

Question 1	Question 2	Correlation Coefficient
42	43	0.88
52	53	0.85
35	36	0.84
26	31	0.84
26	17	0.84
26	42	0.84
26	19	0.83
26	45	0.83
20	21	0.83
21	53	0.83
45	46	0.80
48	49	0.79
33	53	0.81
27	35	0.80
43	44	0.79
20	22	0.79
51	53	0.79
37	39	0.79
37	42	0.78
37	43	0.78
39	42	0.78
43	44	0.77
20	22	0.76
39	43	0.76
40	41	0.76
34	37	0.75
33	53	0.75
35	50	0.75
38	48	0.75

The results suggest that there were no 'perfect' correlations between variables, with no correlations over 0.9. Therefore, no questions were perfectly duplicating information. However, there were a number of questions where the questions were fairly strongly correlated, and thus were giving broadly similar information. These findings were all for questions that were adjacent to each other in the questionnaire, namely Q52 and Q53, Q42 and Q43, Q35 and Q36, and Q20 and Q21. There were also fairly strong correlations between Q26 and several other questions. Although, it is noted that these results are based on a smaller number of observations (n = 28).

15.1.5 Further statistical analysis

15.1.5.1 *Internal consistency and components*

Cronbach's alpha was calculated to measure the internal consistency of the score. The calculated value was 0.88, suggesting a good degree of internal consistency amongst the individual items. Also performed was a factor analysis to analyse if the score naturally broke down into different components. A summary of the first stage of the process is shown in Table 47. This reports the eigenvalues attributed to individually components, and also the percentage of the total variability in the scores attributed to each component. A higher eigenvalue would imply that a greater amount of variability is associated with that component. Only results for the first five components are listed.

Table 47: Summary of factor analysis

Component	Eigenvalue	% of total
1	12.8	49.2
2	1.8	6.9
3	1.5	5.7
4	1.1	4.1
5	1.0	3.9

The table suggests that the first component is much more important than the other components. This accounts for almost half of the variability in the data, with all other components explained 7% or less variation. The sizes of the eigenvalues are also illustrated in a scree plot, which is shown in Figure 40.

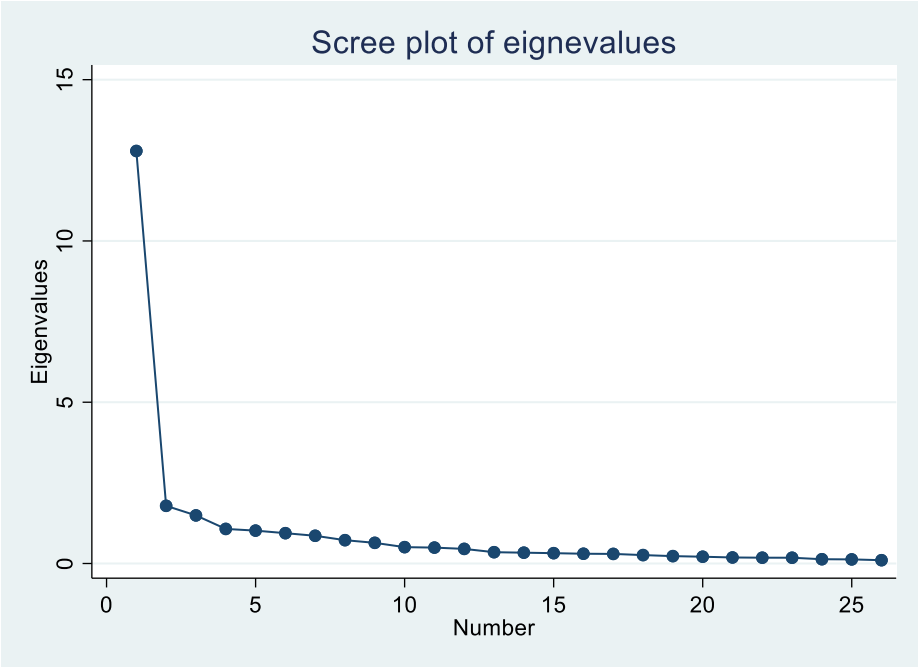

Figure 40: Scree plot of eigenvalues from factor analysis

The plot shows that the natural change in slope direction was after the first component. This suggests further evidence that just one factor is worthy of interpretation, i.e. the items on the questionnaire do not subdivide into sub-scales on the basis of participants responses.

A summary of the factor loadings for the one important component is presented in Table 48. For ease of interpretation, only factor loadings of 0.4 or higher are shown.

Table 48: Factor loadings from the factor analysis

Question number	Component 1 factor loading
17	-
18	-
20	-
22	-
23	0.47
25	-
26	-
27	-
33	0.56
34	0.53
36	-
37	0.64
38	0.51
39	0.71
40	-
41	-
42	0.72
44	0.72
45	0.69
46	0.68
47	0.54
48	0.41
49	0.41
50	0.44
51	-
52	-
53	0.43

The loadings suggest that a large proportion of questions contribute towards this first component. Therefore, it can be interpreted primarily as an overall measure of the components as a whole with no evidence of any subdivision into subscales.

References:

1. Soliman F, Sturgeon G, Hargest R. Revisiting an ancient treatment for transphincteric fistula-in-ano 'There is nothing new under the sun' Ecclesiastes 1v9. *J. R. Soc. Med.* 2015;108:482–489.
2. Cosman BC. All's Well That Ends Well: Shakespeare's treatment of anal fistula. *Dis. Colon Rectum*. 1998;41:914–24.
3. Fry RD. History of anal fistulas. *Semin. Colon Rectal Surg.* 2014;25:173–175.
4. Zanotti C, Martinez-Puente C, Pascual I, et al. An assessment of the incidence of fistula-in-ano in four countries of the European Union. *Int. J. Colorectal Dis.* 2007;22:1459–62.
5. Census 2011. UK Census.
6. Sahnan K, Askari A, Adegbola SO, et al. Natural history of anorectal sepsis. *Br. J. Surg.* 2017;104:1857–1865.
7. CROHN BB, GINZBURG L, OPPENHEIMER GD. Regional ileitis; a pathologic and clinical entity. *Am. J. Med.* 1952;13:583–90.
8. Penner A, Crohn BB. PERIANAL FISTULAE AS A COMPLICATION OF REGIONAL ILEITIS. *Ann. Surg.* 1938;108:867–73.
9. Schwartz DA, Loftus E V., Tremaine WJ, et al. The natural history of fistulizing Crohn's disease in Olmsted County, Minnesota. *Gastroenterology*. 2002;122:875–880.
10. Castaño-Milla C, Chaparro M, Saro C, et al. Effectiveness of Adalimumab in Perianal Fistulas in Crohn's Disease Patients Naive to Anti-TNF Therapy. *J. Clin. Gastroenterol.* 2015;49:34–40.
11. Cosnes J, Cattan S, Blain A, et al. Long-term evolution of disease behavior of Crohn's disease. *Inflamm. Bowel Dis.* 2002;8:244–50.
12. Karban A, Itay M, Davidovich O, et al. Risk Factors for Perianal Crohn's Disease: The Role of Genotype, Phenotype, and Ethnicity. *Am. J. Gastroenterol.* 2007;102:1702–1708.
13. Hellers G, Bergstrand O, Ewerth S, et al. Occurrence and outcome after primary treatment of anal fistulae in Crohn's disease. *Gut*. 1980;21:525–7.
14. Tang LY, Rawsthorne P, Bernstein CN. Are perineal and luminal fistulas associated in Crohn's disease? A population-based study. *Clin. Gastroenterol. Hepatol.* 2006;4:1130–4.
15. Ingle SB, Loftus E V. The natural history of perianal Crohn's disease. *Dig. Liver Dis.* 2007;39:963–969.
16. Adegbola SO, Sahnan K, Tozer PJ, et al. Management of Perianal Crohn's Disease in the Biologic Era. In: *Coloproctology*. Cham: Springer International Publishing; 2017:1–27.
17. Read DR, Abcarian H. A prospective survey of 474 patients with anorectal abscess. *Dis. Colon Rectum*. 1979;22:566–568.
18. Ramanujam PS, Prasad ML, Abcarian H, et al. Perianal abscesses and fistulas - A study of 1023 patients. *Dis. Colon Rectum*. 1984;27:593–597.
19. Vasilevsky CA, Gordon PH. The incidence of recurrent abscesses or fistula-in-ano following anorectal suppuration. *Dis. Colon Rectum*. 1984;27:126–130.
20. Henrichsen S, Christiansen J. Incidence of fistula-in-ano complicating anorectal sepsis: a prospective study. *Br. J. Surg.* 1986;73:371–372.
21. Nelson R. Anorectal abscess fistula: What do we know? *Surg. Clin. North Am.* 2002;82:1139–1151.
22. Whiteford M. Perianal Abscess/Fistula Disease. *Clin. Colon Rectal Surg.* 2007;20:102–

109.
23. Abcarian H. Anorectal Infection : Abscess – Fistula. 2011;1:14–21.
24. Narayanan A, Sundararaman S, Varadhan L, et al. The value of microbiological analysis of pus swabs in perianal abscess. Have they stood the test of time and antibiotic usage? *Int. Surg. J.* 2015;2:175.
25. Grace RH, Harper IA, Thompson RG. Anorectal sepsis: microbiology in relation to fistula-in-ano. *Br. J. Surg.* 1982;69:401–3.
26. Sahnan K, Adegbola SO, Tozer PJ, et al. Perianal abscess. *BMJ*. 2017;356:j475.
27. Sainio P. Fistula-in-ano in a defined population. Incidence and epidemiological aspects. *Ann. Chir. Gynaecol.* 1984;73:219–24.
28. Wolff BG, Culp CE, Beart Jr. RW, et al. Anorectal Crohn's disease. A long-term perspective. *Dis Colon Rectum*. 1985;28:709–711.
29. Eglinton TW, Barclay ML, Gearry RB, et al. The spectrum of perianal Crohn's disease in a population-based cohort. *Dis. Colon Rectum*. 2012;55:773–7.
30. Vavricka SR, Spigaglia SM, Rogler G, et al. Systematic evaluation of risk factors for diagnostic delay in inflammatory bowel disease. *Inflamm. Bowel Dis.* 2012;18:496–505.
31. Schoepfer AM, Dehlavi M-A, Fournier N, et al. Diagnostic Delay in Crohn's Disease Is Associated With a Complicated Disease Course and Increased Operation Rate. *Am. J. Gastroenterol.* 2013;108.
32. D'Haens G, Baert F, Assche G van, et al. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet*. 2008;371:660–667.
33. McColl I. The comparative anatomy and pathology of anal glands. Arris and Gale lecture delivered at the Royal College of Surgeons of England on 25th February 1965. *Ann. R. Coll. Surg. Engl.* 1967;40:36–67.
34. Ghahramani L, Reza Minaie M, Arasteh P, et al. Antibiotic therapy for prevention of fistula in-ano after incision and drainage of simple perianal abscess: A randomized single blind clinical trial. 2017.
35. Lunniss PJ, Jenkins PJ, Besser GM, et al. Gender differences in incidence of idiopathic fistula-in-ano are not explained by circulating sex hormones. *Int. J. Colorectal Dis.* 1995;10:25–8.
36. Sahnan K, Askari A, Adegbola SO, et al. Persistent Fistula After Anorectal Abscess Drainage. *Dis. Colon Rectum*. 2019;62:327–332.
37. Hamadani A, Haigh PI, Liu ILA, et al. Who is at risk for developing chronic anal fistula or recurrent anal sepsis after initial perianal abscess? *Dis. Colon Rectum*. 2009;52:217–221.
38. Wang D, Yang G, Qiu J, et al. Risk factors for anal fistula: a case–control study. *Tech. Coloproctol.* 2014;18:635–639.
39. Sugrue J, Johan Nordenstam B, Herand Abcarian B, et al. Pathogenesis and persistence of cryptoglandular anal fistula: a systematic review. *Tech. Coloproctol.* 21.
40. Tozer PJ, Whelan K, Phillips RKS, et al. Etiology of perianal Crohn's disease: Role of genetic, microbiological, and immunological factors. *Inflamm. Bowel Dis.* 2009;15:1591–1598.
41. EISENHAMMER S. The internal anal sphincter and the anorectal abscess. *Surg. Gynecol. Obstet.* 1956;103:501–6.
42. Parks A. A classification of fisula in ano. *Br. J. Surg.* 1976;63:1–12.
43. Seow-Choen F, Ho JM. Histoanatomy of anal glands. *Dis. Colon Rectum*. 1994;37:1215–

8.
44. PARKS AG. Pathogenesis and treatment of fistuila-in-ano. *Br. Med. J.* 1961;1:463–9.
45. Eykyn SJ, Grace RH. The relevance of microbiology in the management of anorectal sepsis. *Ann. R. Coll. Surg. Engl.* 1986;68:237–9.
46. Toyonaga T, Matsushima M, Tanaka Y, et al. Microbiological analysis and endoanal ultrasonography for diagnosis of anal fistula in acute anorectal sepsis. *Int. J. Colorectal Dis.* 2007;22:209–13.
47. Lunniss PJ, Phillips RK. Surgical assessment of acute anorectal sepsis is a better predictor of fistula than microbiological analysis. *Br. J. Surg.* 1994;81:368–9.
48. Whitehead SM, Leach RD, Eykyn SJ, et al. The aetiology of perirectal sepsis. *Br. J. Surg.* 1982;69:166–8.
49. Gosselink MP, Onkelen RS van, Schouten WR. The cryptoglandular theory revisited. *Color. Dis.* 2015;17:1041–1043.
50. Oliver I, Lacueva FJ, Pérez Vicente F, et al. Randomized clinical trial comparing simple drainage of anorectal abscess with and without fistula track treatment. *Int. J. Colorectal Dis.* 2003;18:107–10.
51. Raghavaiah N V. Anal fistula in India. *Int. Surg.* 1976;61:243–5.
52. Hyman N. Anorectal abscess and fistula. *Prim. Care*. 1999;26:69–80.
53. Marcus RH, Stine RJ, Cohen MA. Perirectal abscess. *Ann. Emerg. Med.* 1995;25:597–603.
54. Turra G, Gherardi GM, Mangiarotti S, et al. [Recurrent anorectal abscesses]. *Chir. Ital.* 1984;36:266–71.
55. Fucini C. One stage treatment of anal abscesses and fistulas. A clinical appraisal on the basis of two different classifications. *Int. J. Colorectal Dis.* 1991;6:12–6.
56. Ho YH, Tan M, Chui CH, et al. Randomized controlled trial of primary fistulotomy with drainage alone for perianal abscesses. *Dis. Colon Rectum*. 1997;40:1435–8.
57. Tang CL, Chew SP, Seow-Choen F. Prospective randomized trial of drainage alone vs. drainage and fistulotomy for acute perianal abscesses with proven internal opening. *Dis. Colon Rectum*. 1996;39:1415–7.
58. Schouten WR, Vroonhoven TJ van. Treatment of anorectal abscess with or without primary fistulectomy. Results of a prospective randomized trial. *Dis. Colon Rectum*. 1991;34:60–3.
59. Hughes LE. Surgical pathology and management of anorectal Crohn ' s disease '. *J. R. Soc. Med.* 1978;71:644–651.
60. Armuzzi A, Ahmad T, Ling K-L, et al. Genotype-phenotype analysis of the Crohn's disease susceptibility haplotype on chromosome 5q31. *Gut*. 2003;52:1133–9.
61. Vermeire S, Pierik M, Hlavaty T, et al. Association of organic cation transporter risk haplotype with perianal penetrating Crohn's disease but not with susceptibility to IBD. *Gastroenterology*. 2005;129:1845–53.
62. Latiano A, Palmieri O, Cucchiara S, et al. Polymorphism of the IRGM gene might predispose to fistulizing behavior in Crohn's disease. *Am. J. Gastroenterol.* 2009;104:110–6.
63. Kanaan Z, Ahmad S, Bilchuk N, et al. Perianal Crohn's Disease: Predictive Factors and Genotype-Phenotype Correlations. *Dig. Surg.* 2012;29:107–114.
64. Grant DR, Cohen Z, McLeod RS. Loop ileostomy for anorectal Crohn's disease. *Can. J. Surg.* 1986;29:32–5.
65. Yamamoto T, Allan RN, Keighley MR. Effect of fecal diversion alone on perianal Crohn's

disease. *World J. Surg.* 2000;24:1253–1258.
66. Stringer EE, Nicholson TJ, Armstrong D. Efficacy of topical metronidazole (10 percent) in the treatment of anorectal Crohn's disease. *Dis. Colon Rectum*. 2005;48:970–4.
67. Thia KT, Mahadevan U, Feagan BG, et al. Ciprofloxacin or metronidazole for the treatment of perianal fistulas in patients with Crohn's disease: A randomized, double-blind, placebo-controlled pilot study. *Inflamm. Bowel Dis.* 2009;15:17–24.
68. Bernstein LH, Frank MS, Brandt LJ, et al. Healing of perineal Crohn's disease with metronidazole. *Gastroenterology*. 1980;79:599.
69. Brandt LJ, Bernstein LH, Boley SJ, et al. Metronidazole therapy for perineal Crohn's disease: a follow-up study. *Gastroenterology*. 1982;83:383–7.
70. Tozer PJ, Lung P, Lobo AJ, et al. Review article: pathogenesis of Crohn's perianal fistula-understanding factors impacting on success and failure of treatment strategies. *Aliment. Pharmacol. Ther.* 2018;48:260–269.
71. Torres J, Mehandru S, Colombel J-F, et al. Crohn's disease. *Lancet*. 2017;389:1741–1755.
72. Rieder F, Karrasch T, Ben-Horin S, et al. Results of the 2nd Scientific Workshop of the ECCO (III): Basic mechanisms of intestinal healing. *J. Crohn's Colitis*. 2012;6:373–385.
73. Scharl M, Rogler G, Scharl M, et al. Pathophysiology of fistula formation in Crohn ' s disease. 2014;5:205–212.
74. Scharl M, Rogler G, Biedermann L. Fistulizing Crohn's Disease. *Nat. Publ. Gr.* 2017;8.
75. San Ildefonso Pereira A de, Maruri Chimeno I, Facal Alvarez C, et al. Bacteriology of anal fistulae. *Rev. española enfermedades Dig. organo Of. la Soc. Española Patol. Dig.* 2002;94:533–6.
76. Seow-Choen F, Hay AJ, Heard S, et al. Bacteriology of anal fistulae. *Br. J. Surg.* 1992;79:27–8.
77. Lunniss PJ, Faris B, Rees HC, et al. Histological and microbiological assessment of the role of microorganisms in chronic anal fistula. *Br. J. Surg.* 1993;80:1072.
78. Tozer PJ, Rayment N, Hart AL, et al. What role do bacteria play in persisting fistula formation in idiopathic and Crohn's anal fistula? *Color. Dis.* 2015;17:235–241.
79. Laman JD, Schoneveld AH, Moll FL, et al. Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. *Am. J. Cardiol.* 2002;90:119–23.
80. Inohara N, Ogura Y, Fontalba A, et al. Host Recognition of Bacterial Muramyl Dipeptide Mediated through NOD2. *J. Biol. Chem.* 2003;278:5509–5512.
81. Onkelen RS van, Mitalas LE, Gosselink MP, et al. Assessment of microbiota and peptidoglycan in perianal fistulas. *Diagn. Microbiol. Infect. Dis.* 2013;75:50–54.
82. Bataille F, Klebl F, Rummele P, et al. Morphological characterisation of Crohn's disease fistulae. *Gut*. 2004;53:1314–1321.
83. Siegmund B, Feakins RM, Bamias G, et al. Results of the Fifth Scientific Workshop of the ECCO (II): Pathophysiology of Perianal Fistulizing Disease. *J. Crohn's Colitis*. 2016:377–386.
84. Zoeten EF de, Pasternak BA, Mattei P, et al. Diagnosis and Treatment of Perianal Crohn Disease. *J. Pediatr. Gastroenterol. Nutr.* 2013;57:401–412.
85. Koperen PJ van, Kate FJW ten, Bemelman WA, et al. Histological identification of epithelium in perianal fistulae: a prospective study. *Colorectal Dis.* 2010;12:891–5.
86. Mitalas LE, Onkelen RS van, Monkhorst K, et al. Identification of epithelialization in high transsphincteric fistulas. *Tech. Coloproctol.* 2012;16:113–7.

87. Scharl M, Weber A, Fürst A, et al. Potential role for SNAIL family transcription factors in the etiology of Crohn's disease-associated fistulae. *Inflamm. Bowel Dis.* 2011;17:1907–1916.
88. Scharl M, Frei S, Pesch T, et al. Interleukin-13 and transforming growth factor β synergise in the pathogenesis of human intestinal fistulae. *Gut*. 2013;62:63–72.
89. Ruffolo C, Scarpa M, Faggian D, et al. Cytokine Network in Rectal Mucosa in Perianal Crohn ' s Disease : Relations with Inflammatory Parameters and Need for Surgery. 2008;14:1406–1412.
90. Kiehne K, Fincke A, Brunke G, et al. Antimicrobial peptides in chronic anal fistula epithelium. *Scand J Gastroenterol*. 2007;42:1063–1069.
91. Ratto C, Litta F, Lucchetti D, et al. Immunopathological characterization of cryptoglandular anal fistula: a pilot study investigating its pathogenesis. *Color. Dis.* 2016;18:O436–O444.
92. Onkelen RS van, Gosselink MP, Meurs M van, et al. Pro-inflammatory cytokines in cryptoglandular anal fistulas. *Tech. Coloproctol.* 2016;20:619–25.
93. Bataille F, Rohrmeier C, Bates R, et al. Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease. *Inflamm. Bowel Dis.* 2008;14:1514–27.
94. Kirkegaard T, Hansen A, Brynskov J. Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. *Gut*. 2004;53:701–709.
95. Lampe B von, Barthel B, Coupland SE, et al. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut*. 2000;47:63–73.
96. Clutterbuck AL, Asplin KE, Harris P, et al. Targeting matrix metalloproteinases in inflammatory conditions. *Curr. Drug Targets*. 2009;10:1245–54.
97. Beaugerie L, Seksik P, Nion-Larmurier I, et al. Predictors of Crohn's disease. *Gastroenterology*. 2006;130:650–6.
98. Bernell O, Lapidus a, Hellers G. Risk factors for surgery and postoperative recurrence in Crohn's disease. *Ann. Surg.* 2000;231:38–45.
99. Whelan G, Farmer RG, Fazio VW, et al. Recurrence after surgery in Crohn's disease. Relationship to location of disease (clinical pattern) and surgical indication. *Gastroenterology*. 1985;88:1826–33.
100. Satsangi J, Silverberg MS, Vermeire S, et al. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut*. 2006;55:749–53.
101. Mahadev S, Young JM, Selby W, et al. Quality of life in perianal Crohn's disease: what do patients consider important? *Dis. Colon Rectum*. 2011;54:579–85.
102. Haggett PJ, Moore NR, Shearman JD, et al. Pelvic and perineal complications of Crohn's disease: assessment using magnetic resonance imaging. *Gut*. 1995;36:407–10.
103. Parks AG, Thomson JP. Intersphincteric abscess. *Br. Med. J.* 1973;2:537–9.
104. Viganò C, Losco A, Caprioli F, et al. Incidence and clinical outcomes of intersphincteric abscesses diagnosed by anal ultrasonography in patients with crohn's disease. *Inflamm. Bowel Dis.* 2011;17:2102–2108.
105. Hanley PH. Reflections on anorectal abscess fistula: 1984. *Dis. Colon Rectum*. 1985;28:528–33.
106. Parks AG, Gordon PH, Hardcastle JD. A classification of fistula-in-ano. *Br. J. Surg.* 1976;63:1–12.

107. Sandborn WJ, Fazio VW, Feagan BG, et al. AGA technical review on perianal Crohn's disease. *Gastroenterology*. 2003;125:1508–30.
108. Seow-Choen, Phillips RK. Insights gained from the management of problematical anal fistulae at St. Mark's Hospital, 1984-88. *Br. J. Surg.* 1991;78:539–41.
109. Halligan S, Buchanan G. MR imaging of fistula-in-ano. *Eur. J. Radiol.* 2003;47:98–107.
110. Tozer PJ, Burling D, Gupta A, et al. Review article: medical, surgical and radiological management of perianal Crohn's fistulas. *Aliment. Pharmacol. Ther.* 2011;33:5–22.
111. Siddiqui MRS, Ashrafian H, Tozer P, et al. A diagnostic accuracy meta-analysis of endoanal ultrasound and MRI for perianal fistula assessment. *Dis. Colon Rectum*. 2012;55:576–85.
112. Barker PG, Lunniss PJ, Armstrong P, et al. Magnetic resonance imaging of fistula-in-ano: technique, interpretation and accuracy. *Clin. Radiol.* 1994;49:7–13.
113. deSouza NM, Williams AD, Gilderdale DJ. High-resolution magnetic resonance imaging of the anal sphincter using a dedicated endoanal receiver coil. *Eur. Radiol.* 1999;9:436–43.
114. Spencer JA, Chapple K, Wilson D, et al. Outcome after surgery for perianal fistula: predictive value of MR imaging. *AJR. Am. J. Roentgenol.* 1998;171:403–6.
115. Schwartz DA, Wiersema MJ, Dudiak KM, et al. A comparison of endoscopic ultrasound, magnetic resonance imaging, and exam under anesthesia for evaluation of Crohn's perianal fistulas. *Gastroenterology*. 2001;121:1064–72.
116. Skalej M, Makowiec F, Weinlich M, et al. [Magnetic resonance imaging in perianal Crohn's disease]. *Dtsch. Med. Wochenschr.* 1993;118:1791–6.
117. Koelbel G, Schmiedl U, Majer MC, et al. Diagnosis of fistulae and sinus tracts in patients with Crohn disease: value of MR imaging. *AJR. Am. J. Roentgenol.* 1989;152:999–1003.
118. Scholefield JH, Berry DP, Armitage NC, et al. Magnetic resonance imaging in the management of fistula in ano. *Int. J. Colorectal Dis.* 1997;12:276–9.
119. Gecse KB, Bemelman W, Kamm MA, et al. A global consensus on the classification, diagnosis and multidisciplinary treatment of perianal fistulising Crohn's disease. *Gut*. 2014;63:1381–92.
120. Present DH, Rutgeerts P, Targan S, et al. Infliximab for the treatment of fistulas in patients with Crohn's disease. *N. Engl. J. Med.* 1999;340:1398–405.
121. Horsthuis K, Ziech MLW, Bipat S, et al. Evaluation of an MRI-based score of disease activity in perianal fistulizing Crohn's disease. *Clin. Imaging*. 35:360–5.
122. Tozer P, Ng SC, Siddiqui MR, et al. Long-term MRI-guided combined anti-TNF-α and thiopurine therapy for Crohn's perianal fistulas. *Inflamm. Bowel Dis.* 2012;18:1825–34.
123. Irvine EJ. Usual therapy improves perianal Crohn's disease as measured by a new disease activity index. McMaster IBD Study Group. *J. Clin. Gastroenterol.* 1995;20:27–32.
124. Göttgens KWA, Smeets RR, Stassen LPS, et al. Systematic review and meta-analysis of surgical interventions for high cryptoglandular perianal fistula. *Int. J. Colorectal Dis.* 2015;30:583–93.
125. Williams G, Williams A, Tozer P, et al. The treatment of anal fistula : second ACPGBI Position Statement – 2018. 2018;20:5–31.
126. Hagen SJ van der, Baeten CG, Soeters PB, et al. Long-term outcome following mucosal advancement flap for high perianal fistulas and fistulotomy for low perianal fistulas: Recurrent perianal fistulas: Failure of treatment or recurrent patient disease? *Int. J. Colorectal Dis.* 2006;21:784–790.

127. Soltani A, Kaiser AM. Endorectal Advancement Flap for Cryptoglandular or Crohn's Fistula-in-Ano. *Dis. Colon Rectum*. 2010;53:486–495.
128. Murugesan J, Mor I, Fulham S, et al. Systematic review of efficacy of LIFT procedure in crpytoglandular fistula-in-ano. *J. Coloproctology*. 2014;34:109–119.
129. Rojanasakul A. LIFT procedure: a simplified technique for fistula-in-ano. *Tech. Coloproctol.* 2009;13:237–240.
130. Hong KD, Kang S, Kalaskar S, et al. Ligation of intersphincteric fistula tract (LIFT) to treat anal fistula: systematic review and meta-analysis. *Tech. Coloproctol.* 2014;18:685–691.
131. Wilhelm A. A new technique for sphincter-preserving anal fistula repair using a novel radial emitting laser probe. *Tech. Coloproctol.* 2011;15:445–449.
132. Meinero P, Mori L. Video-assisted anal fistula treatment (VAAFT): a novel sphincter-saving procedure for treating complex anal fistulas. *Tech. Coloproctol.* 2011;15:417–422.
133. Dubois A, Carrier G, Pereira B, et al. Therapeutic management of complex anal fistulas by installing a nitinol closure clip: study protocol of a multicentric randomised controlled trial-FISCLOSE. *BMJ Open*. 2015;5:e009884.
134. Bell SJ, Williams AB, Wiesel P, et al. The clinical course of fistulating Crohn's disease. *Aliment. Pharmacol. Ther.* 2003;17:1145–51.
135. West RL, Woude CJ van der, Hansen BE, et al. Clinical and endosonographic effect of ciprofloxacin on the treatment of perianal fistulae in Crohn's disease with infliximab: a double-blind placebo-controlled study. *Aliment. Pharmacol. Ther.* 2004;20:1329–36.
136. Dewint P, Hansen BE, Verhey E, et al. Adalimumab combined with ciprofloxacin is superior to adalimumab monotherapy in perianal fistula closure in Crohn's disease: a randomised, double-blind, placebo controlled trial (ADAFI). *Gut*. 2014;63:292–299.
137. Freire P, Portela F, Donato MM, et al. CARD15 mutations and perianal fistulating Crohn's disease: correlation and predictive value of antibiotic response. *Dig. Dis. Sci.* 2011;56:853–9.
138. Jones JL, Kaplan GG, Peyrin-Biroulet L, et al. Effects of Concomitant Immunomodulator Therapy on Efficacy and Safety of Anti-Tumor Necrosis Factor Therapy for Crohn's Disease: A Meta-analysis of Placebo-controlled Trials. *Clin. Gastroenterol. Hepatol.* 2015;13:2233-2240.e2.
139. Ford AC, Sandborn WJ, Khan KJ, et al. Efficacy of Biological Therapies in Inflammatory Bowel Disease: Systematic Review and Meta-Analysis. *Am. J. Gastroenterol.* 2011;106:644–659.
140. Sands BE, Anderson FH, Bernstein CN, et al. Infliximab maintenance therapy for fistulizing Crohn's disease. *N. Engl. J. Med.* 2004;350:876–885.
141. Lichtenstein GR, Yan S, Bala M, et al. Infliximab maintenance treatment reduces hospitalizations, surgeries, and procedures in fistulizing Crohn's disease. *Gastroenterology*. 2005;128:862–869.
142. Ng SC, Plamondon S, Gupta A, et al. Prospective assessment of the effect on quality of life of anti-tumour necrosis factor therapy for perineal Crohn's fistulas. *Aliment. Pharmacol. Ther.* 2009;30:757–66.
143. Regueiro M, Mardini H. Treatment of perianal fistulizing Crohn's disease with infliximab alone or as an adjunct to exam under anesthesia with seton placement. *Inflamm. Bowel Dis.* 2003;9:98–103.
144. Kotze PG, Albuquerque IC de, Luz Moreira A da, et al. Perianal complete remission with combined therapy (seton placement and anti-TNF agents) in Crohn's disease: a

Brazilian multicenter observational study. *Arq. Gastroenterol.* 51:284–9.
145. Hagen SJ van der, Baeten CG, Soeters PB, et al. Anti-TNF-alpha (infliximab) used as induction treatment in case of active proctitis in a multistep strategy followed by definitive surgery of complex anal fistulas in Crohn's disease: a preliminary report. *Dis. Colon Rectum*. 2005;48:758–67.
146. Roumeguère P, Bouchard D, Pigot F, et al. Combined approach with infliximab, surgery, and methotrexate in severe fistulizing anoperineal Crohn's disease: results from a prospective study. *Inflamm. Bowel Dis.* 2011;17:69–76.
147. Higashi D, Futami K, Egawa Y, et al. Infliximab treatment for anal fistula in patients with Crohn's disease. *Anticancer Res.* 2009;29:927–33.
148. Louis E, Mary J-Y, Vernier-Massouille G, et al. Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab therapy is stopped. *Gastroenterology*. 2012;142:63-70.e5.
149. Yanai H, Lichtenstein L, Assa A, et al. Levels of drug and antidrug antibodies are associated with outcome of interventions after loss of response to infliximab or adalimumab. *Clin. Gastroenterol. Hepatol.* 2015;13:522-530.e2.
150. Assche G Van, Vermeire S, Ballet V, et al. Switch to adalimumab in patients with Crohn's disease controlled by maintenance infliximab: prospective randomised SWITCH trial. *Gut*. 2012;61:229–34.
151. Colombel J-F, Sandborn WJ, Rutgeerts P, et al. Adalimumab for Maintenance of Clinical Response and Remission in Patients With Crohn's Disease: The CHARM Trial. *Gastroenterology*. 2007;132:52–65.
152. Colombel JF, Schwartz DA, Sandborn WJ, et al. Adalimumab for the treatment of fistulas in patients with Crohn's disease. *Gut*. 2009;58:940–948.
153. Sandborn WJ, Rutgeerts P, Enns R, et al. Adalimumab induction therapy for Crohn disease previously treated with infliximab: A randomized trial. *Ann. Intern. Med.* 2007;146:829–838.
154. Lichtiger S, Binion DG, Wolf DC, et al. The CHOICE trial: adalimumab demonstrates safety, fistula healing, improved quality of life and increased work productivity in patients with Crohn's disease who failed prior infliximab therapy. *Aliment. Pharmacol. Ther.* 2010;32:1228–1239.
155. Echarri A, Castro J, Barreiro M, et al. Evaluation of adalimumab therapy in multidisciplinary strategy for perianal Crohn's disease patients with infliximab failure. *J. Crohn's Colitis*. 2010;4:654–660.
156. Schreiber S, Lawrance IC, Thomsen OØ, et al. Randomised clinical trial: certolizumab pegol for fistulas in Crohn's disease - subgroup results from a placebo-controlled study. *Aliment. Pharmacol. Ther.* 2011;33:185–93.
157. White RA, Eisenstat TE, Rubin RJ, et al. Seton management of complex anorectal fistulas in patients with Crohn's disease. *Dis. Colon Rectum*. 1990;33:587–9.
158. Williams JG, Rothenberger DA, Nemer FD, et al. Fistula-in-ano in Crohn's disease. Results of aggressive surgical treatment. *Dis. Colon Rectum*. 1991;34:378–84.
159. Zoeten EF de, Pasternak BA, Mattei P, et al. Diagnosis and treatment of perianal Crohn disease: NASPGHAN clinical report and consensus statement. *J. Pediatr. Gastroenterol. Nutr.* 2013;57:401–12.
160. Hyder SA, Travis SPL, Jewell DP, et al. Fistulating anal Crohn's disease: results of combined surgical and infliximab treatment. *Dis. Colon Rectum*. 2006;49:1837–41.
161. Tanaka S, Matsuo K, Sasaki T, et al. Clinical advantages of combined seton placement

and infliximab maintenance therapy for perianal fistulizing Crohn's disease: when and how were the seton drains removed? *Hepatogastroenterology.* 57:3–7.
162. Vitton V, Gasmi M, Barthet M, et al. Long-term healing of Crohn's anal fistulas with fibrin glue injection. *Aliment. Pharmacol. Ther.* 2005;21:1453–7.
163. O'Riordan JM, Datta I, Johnston C, et al. A systematic review of the anal fistula plug for patients with Crohn's and non-Crohn's related fistula-in-ano. *Dis. Colon Rectum*. 2012;55:351–8.
164. Garcia-Olmo D, Herreros D, Pascual I, et al. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Dis. Colon Rectum*. 2009;52:79–86.
165. McClane SJ, Rombeau JL. Anorectal Crohn's disease. *Surg. Clin. North Am.* 2001;81:169–83, ix.
166. Makowiec F, Jehle EC, Becker HD, et al. Clinical course after transanal advancement flap repair of perianal fistula in patients with Crohn's disease. *Br. J. Surg.* 1995;82:603–6.
167. Gingold DS, Murrell ZA, Fleshner PR. A prospective evaluation of the ligation of the intersphincteric tract procedure for complex anal fistula in patients with Crohn's disease. *Ann. Surg.* 2014;260:1057–61.
168. Kamiński JP, Zaghiyan K, Fleshner P. Increasing experience of ligation of the intersphincteric fistula tract for patients with Crohn's disease: what have we learned? *Color. Dis.* 2017;19:750–755.
169. la Portilla F de, Alba F, García-Olmo D, et al. Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. *Int. J. Colorectal Dis.* 2013;28:313–23.
170. Lee WY, Park KJ, Cho YB, et al. Autologous adipose tissue-derived stem cells treatment demonstrated favorable and sustainable therapeutic effect for Crohn's fistula. *Stem Cells*. 2013;31:2575–81.
171. Cho YB, Park KJ, Yoon SN, et al. Long-term results of adipose-derived stem cell therapy for the treatment of Crohn's fistula. *Stem Cells Transl. Med.* 2015;4:532–7.
172. Ciccocioppo R, Gallia A, Sgarella A, et al. Long-Term Follow-Up of Crohn Disease Fistulas After Local Injections of Bone MarroweDerived Mesenchymal Stem Cells. 2015;90:747–755.
173. Singh S, Ding NS, Mathis KL, et al. Systematic review with meta-analysis: faecal diversion for management of perianal Crohn's disease. *Aliment. Pharmacol. Ther.* 2015;42:783–92.
174. Yamamoto T, Bain IM, Allan RN, et al. Persistent perineal sinus after proctocolectomy for Crohn's disease. *Dis. Colon Rectum*. 1999;42:96–101.
175. Radcliffe AG, Ritchie JK, Hawley PR, et al. Anovaginal and rectovaginal fistulas in Crohn's disease. *Dis. Colon Rectum*. 1988;31:94–9.
176. Frizelle FA, Santoro GA, Pemberton JH. The management of perianal Crohn's disease. *Int. J. Colorectal Dis.* 1996;11:227–37.
177. Gyde SN, Prior P, Macartney JC, et al. Malignancy in Crohn's disease. *Gut*. 1980;21:1024–9.
178. Givel JC, Hawker P, Allan RN, et al. Enterovaginal fistulas associated with Crohn's disease. *Surg. Gynecol. Obstet.* 1982;155:494–6.
179. Valente MA. Contemporary surgical management of rectovaginal fistula in Crohn's disease. *World J. Gastrointest. Pathophysiol.* 2014;5:487.

180. Sands BE, Blank MA, Patel K, et al. Long-term treatment of rectovaginal fistulas in Crohn's disease: response to infliximab in the ACCENT II Study. *Clin. Gastroenterol. Hepatol.* 2004;2:912–20.
181. Songne K, Scotté M, Lubrano J, et al. Treatment of anovaginal or rectovaginal fistulas with modified Martius graft. *Colorectal Dis.* 2007;9:653–6.
182. Andreani SM, Dang HH, Grondona P, et al. Rectovaginal fistula in Crohn's disease. *Dis. Colon Rectum*. 2007;50:2215–22.
183. O'Leary DP, Milroy CE, Durdey P. Definitive repair of anovaginal fistula in Crohn's disease. *Ann. R. Coll. Surg. Engl.* 1998;80:250–2.
184. Hull TL, Fazio VW. Surgical approaches to low anovaginal fistula in Crohn's disease. *Am. J. Surg.* 1997;173:95–8.
185. Hesterberg R, Schmidt WU, Müller F, et al. Treatment of anovaginal fistulas with an anocutaneous flap in patients with Crohn's disease. *Int. J. Colorectal Dis.* 1993;8:51–4.
186. Robertson WG, Mangione JS. Cutaneous advancement flap closure: alternative method for treatment of complicated anal fistulas. *Dis. Colon Rectum*. 1998;41:884–6; discussion 886-7.
187. Simmang CL, Lacey SW, Huber PJ. Rectal sleeve advancement: repair of rectovaginal fistula associated with anorectal stricture in Crohn's disease. *Dis. Colon Rectum*. 1998;41:787–9.
188. Lee PY, Fazio VW, Church JM, et al. Vaginal fistula following restorative proctocolectomy. *Dis. Colon Rectum*. 1997;40:752–9.
189. Hatch Q, Champagne BJ, Maykel JA, et al. The impact of pregnancy on surgical Crohn disease: an analysis of the Nationwide Inpatient Sample. *J. Surg. Res.* 2014;190:41–6.
190. Caprilli R, Gassull MA, Escher JC, et al. European evidence based consensus on the diagnosis and management of Crohn's disease: special situations. *Gut*. 2006;55 Suppl 1:i36-58.
191. Schwandner O. Video-assisted anal fistula treatment (VAAFT) combined with advancement flap repair in Crohn's disease. *Tech. Coloproctol.* 2013;17:221–225.
192. Bourikas LA, Koutroubakis IE. Anti-TNF and fistulizing perianal Crohn's disease: use in clinical practice. *Curr. Drug Targets*. 2010;11:187–97.
193. Karmiris K, Bielen D, Vanbeckevoort D, et al. Long-Term Monitoring of Infliximab Therapy for Perianal Fistulizing Crohn's Disease by Using Magnetic Resonance Imaging. *Clin. Gastroenterol. Hepatol.* 2011;9:130-136.e1.
194. Bode M, Eder S, Schürmann G. [Perianal fistulas in Crohn's disease--biologicals and surgery: is it worthwhile?]. *Z. Gastroenterol.* 2008;46:1376–83.
195. El-Gazzaz G, Hull T, Church JM. Biological immunomodulators improve the healing rate in surgically treated perianal Crohn's fistulas. *Colorectal Dis.* 2012;14:1217–23.
196. Levien DH, Surrell J, Mazier WP. Surgical treatment of anorectal fistula in patients with Crohn's disease. *Surg. Gynecol. Obstet.* 1989;169:133–6.
197. Adegbola SO, Pisani A, Sahnan K, et al. Medical and surgical management of perianal Crohn's disease. *Ann. Gastroenterol.* 2018;31:129–139.
198. Sandborn WJ, Fazio VW, Feagan BG, et al. AGA Technical Review on Perianal Crohn's Disease. *Gastroenterology*. 2003;125:1508–1530.
199. Hughes LE. Clinical classification of perianal Crohn's disease. *Dis. Colon Rectum*. 1992;35:928–32.
200. Pikarsky AJ, Gervaz P, Wexner SD. Perianal Crohn disease: a new scoring system to evaluate and predict outcome of surgical intervention. *Arch. Surg.* 2002;137:774–7;

discussion 778.
201. Safar B, Sands D. Perianal Crohn's disease. *Clin. Colon Rectal Surg.* 2007;20:282–93.
202. Jorge JM, Wexner SD. Etiology and management of fecal incontinence. *Dis. Colon Rectum*. 1993;36:77–97.
203. Present DH, Korelitz BI, Wisch N, et al. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. *N. Engl. J. Med.* 1980;302:981–7.
204. Allan A, Linares L, Spooner HA, et al. Clinical index to quantitate symptoms of perianal Crohn's disease. *Dis. Colon Rectum*. 1992;35:656–61.
205. Yassin NA, Askari A, Warusavitarne J, et al. Systematic review: the combined surgical and medical treatment of fistulising perianal Crohn's disease. *Aliment. Pharmacol. Ther.* 2014;40:741–9.
206. Gu J, Valente MA, Remzi FH, et al. Factors affecting the fate of faecal diversion in patients with perianal Crohn's disease. *Color. Dis.* 2015;17:66–72.
207. Régimbeau JM, Panis Y, Cazaban L, et al. Long-term results of faecal diversion for refractory perianal Crohn's disease. *Color. Dis.* 2001;3:232–237.
208. Hong MK, Craig Lynch A, Bell S, et al. Faecal diversion in the management of perianal Crohn's disease. *Color. Dis*. 2011;13:171–176.
209. Makowiec F, Jehle EC, Starlinger M. Clinical course of perianal fistulas in Crohn's disease. *Gut*. 1995;37:696–701.
210. Panés J, Rimola J. Perianal fistulizing Crohn's disease: pathogenesis, diagnosis and therapy. *Nat. Rev. Gastroenterol. Hepatol.* 2017.
211. Hadzhiev BD, Murdjeva MA. Immunopathological Characteristics of Cryptoglandular and Crohn's Anorectal Fistulas. *Folia Med. (Plovdiv).* 2010;52:5–12.
212. Yassin NA, Mallapa S, Sarker S, et al. THE PRESENCE OF ENDOTOXINS IN CROHN'S AND IDIOPATHIC PERIANAL FISTULA TRACKS. *United Eur. Gastroenterol. J.* 2013:A506.
213. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica*. 1999;29:1181–1189.
214. Lin HM, Helsby NA, Rowan DD, et al. Using metabolomic analysis to understand inflammatory bowel diseases. *Inflamm. Bowel Dis.* 2011;17:1021–1029.
215. Titz B, Gadaleta R, Sasso G Lo, et al. Proteomics and Lipidomics in Inflammatory Bowel Disease Research: From Mechanistic Insights to Biomarker Identification. *Int. J. Mol. Sci.* 2018;19:2775.
216. Fiehn O, Kim J. Metabolomics Insights Into Pathophysiological Mechanisms of Interstitial Cystitis. *Int. Neurourol. J.* 2014;18:106.
217. Wilkins JM, Trushina E. Application of Metabolomics in Alzheimer's Disease. *Front. Neurol.* 2018;8:1.
218. Vorkas PA, Isaac G, Anwar MA, et al. Untargeted UPLC-MS profiling pipeline to expand tissue metabolome coverage: Application to cardiovascular disease. *Anal. Chem.* 2015;87:4184–4193.
219. Holmes E, Wijeyesekera A, Taylor-Robinson SD, et al. The promise of metabolic phenotyping in gastroenterology and hepatology. *Nat. Rev. Gastroenterol. Hepatol.* 2015;12:458–471.
220. Lewis MR, Pearce JTM, Spagou K, et al. Development and Application of Ultra-Performance Liquid Chromatography-TOF MS for Precision Large Scale Urinary Metabolic Phenotyping. *Anal. Chem.* 2016;88:9004–9013.

221. Isaac G, Mcdonald S, Astarita G. *Lipid Separation using UPLC with Charged Surface Hybrid Technology*.
222. Smith CA, Want EJ, O'maille G, et al. XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. 2006.
223. Tautenhahn R, Bottcher C, Neumann S. Highly sensitive feature detection for high resolution LC/MS. *BMC Bioinformatics*. 2008;9:504.
224. Eriksson L, Trygg J, Wold S. CV-ANOVA for significance testing of PLS and OPLS® models. *J. Chemom.* 2008;22:594–600.
225. Benjamini Y, Hochberg Y. *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing*. 1995.
226. Patti GJ, Tautenhahn R, Siuzdak G. Meta-analysis of untargeted metabolomic data from multiple profiling experiments. *Nat. Protoc.* 2012;7:508–516.
227. Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*. 2007;3:211–221.
228. Lindgren F, Hansen B, Karcher W. Model Validation By Permutation Tests : *J. Chemom.* 1996;10:521–532.
229. Scoville EA, Allaman MM, Brown CT, et al. Alterations in lipid, amino acid, and energy metabolism distinguish Crohn's disease from ulcerative colitis and control subjects by serum metabolomic profiling. *Metabolomics*. 2018;14:17.
230. Hisamatsu T, Okamoto S, Hashimoto M, et al. Novel, Objective, Multivariate Biomarkers Composed of Plasma Amino Acid Profiles for the Diagnosis and Assessment of Inflammatory Bowel Disease Niess J-H, ed. *PLoS One*. 2012;7:e31131.
231. Brosnan JT. Interorgan Amino Acid Transport and its Regulation. *J. Nutr.* 2003;133:2068S-2072S.
232. Kalluri R, Weinberg RA. Review series The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* 2009;119.
233. Bhowmik SK, Ramirez-Peña E, Arnold JM, et al. EMT-induced metabolite signature identifies poor clinical outcome. *Oncotarget*. 2015;6:42651–60.
234. Blum BC, Mousavi F, Emili A. Single-platform 'multi-omic' profiling: unified mass spectrometry and computational workflows for integrative proteomics–metabolomics analysis. *Mol. Omi.* 2018;14:307–319.
235. Kumar N, Hoque MA, Shahjaman M, et al. Metabolomic Biomarker Identification in Presence of Outliers and Missing Values. *Biomed Res. Int.* 2017;2017:2437608.
236. Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. *Nat. Cell Biol.* 2016;18:246–254.
237. Young M, Reed KR. Organoids as a Model for Colorectal Cancer. *Curr. Colorectal Cancer Rep.* 2016.
238. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. 2011;141:1762–1772.
239. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. 2009;459:262–265.
240. Dye BR, Hill DR, Ferguson MA, et al. In vitro generation of human pluripotent stem cell derived lung organoids.
241. Boj SF, Hwang C-I, Baker LA, et al. Model organoids provide new research opportunities

for ductal pancreatic cancer. *Mol. Cell. Oncol.* 2016;3:e1014757.
242. Xu AT, Tong JL, Ran ZH. Organoids derived from digestive tract, liver, and pancreas. *J. Dig. Dis.* 2016;17:3–10.
243. Olayanju A, Jones L, Greco K, et al. Application of porcine gastrointestinal organoid units as a potential in vitro tool for drug discovery and development. 2018.
244. Panés J, García-Olmo D, Assche G Van, et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet*. 2016;388:1281–1290.
245. Molendijk I, Bonsing BA, Roelofs H, et al. Allogeneic Bone Marrow–Derived Mesenchymal Stromal Cells Promote Healing of Refractory Perianal Fistulas in Patients With Crohn's Disease. *Gastroenterology*. 2015;149:918-927.e6.
246. Norderval S, Lundby · L, Hougaard · H, et al. Efficacy of autologous fat graft injection in the treatment of anovaginal fistulas. *Tech. Coloproctol.* 1.
247. de L, Weum S, Norderval S. Novel treatment for recalcitrant rectovaginal fistulas: fat injection. *Int. Urogynecol. J.* 2015;26:139–144.
248. García-Gómez I, Elvira G, Zapata AG, et al. Expert Opinion on Biological Therapy Mesenchymal stem cells: biological properties and clinical applications. 2010.
249. Salgado AJBOG, Reis RLG, Sousa NJC, et al. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Curr. Stem Cell Res. Ther.* 2010;5:103–10.
250. Mcnairn AJ, Guasch G. Epithelial transition zones: Merging microenvironments, niches, and cellular transformation. *Eur. J. Dermatology*. 2011;21:21–28.
251. Tanaka E, Noguchi T, Nagai K, et al. Morphology of the epithelium of the lower rectum and the anal canal in the adult human. *Med. Mol. Morphol.* 2012;45:72–79.
252. Xinaris C, Brizi V, Remuzzi G. E-Mail Organoid Models and Applications in Biomedical Research. *Nephron*. 2015;130:191–199.
253. Molodecky NA, Soon IS, Rabi DM, et al. Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review. *Gastroenterology*. 2012;142:46-54.e42.
254. Gasche C. Genotypes and phenotypes in Crohn's disease: do they help in clinical management? *Gut*. 2005;54:162–167.
255. Sartor RB. Mechanisms of Disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat. Clin. Pract. Gastroenterol. Hepatol.* 2006;3:390–407.
256. Targan SR, Hanauer SB, Deventer SJ van, et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N. Engl. J. Med.* 1997;337:1029–35.
257. Hart AL, Ng SC. Crohn's disease. *Medicine (Baltimore).* 2015;43:282–290.
258. Ghazi LJ, Patil SA, Rustgi A, et al. Step up versus early biologic therapy for Crohn's disease in clinical practice. *Inflamm. Bowel Dis.* 2013;19:1397–1403.
259. Gomollón F, Dignass A, Annese V, et al. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *J. Crohn's Colitis*. 2017;11:3–25.
260. Marchetti M, Liberato NL, Sabatino A Di, et al. Cost-effectiveness analysis of top-down versus step-up strategies in patients with newly diagnosed active luminal Crohn's disease. *Eur. J. Heal. Econ.* 2013;14:853–861.
261. Rogler G. Top-down or step-up treatment in Crohn's disease? *Dig. Dis.* 2013;31:83–90.
262. Tracey D, Klareskog L, Sasso EH, et al. Tumor necrosis factor antagonist mechanisms of

action: a comprehensive review. *Pharmacol. Ther.* 2008;117:244–79.
263. Eissner G, Kirchner S, Lindner H, et al. Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *J. Immunol.* 2000;164:6193–8.
264. Horiuchi T, Mitoma H, Harashima S -i., et al. Transmembrane TNF- : structure, function and interaction with anti-TNF agents. *Rheumatology*. 2010;49:1215–1228.
265. Mitoma H, Horiuchi T, Tsukamoto H, et al. Molecular mechanisms of action of anti-TNF-α agents – Comparison among therapeutic TNF-α antagonists. *Cytokine*. 2018;101:56–63.
266. Deventer SJ Van. Tumour necrosis factor and Crohn's disease. *Gut*. 1997;40:443–8.
267. Vandenabeele P, Declercq W, Beyaert R, et al. Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol.* 1995;5:392–9.
268. Begue B, Wajant H, Bambou J, et al. Implication of TNF-Related Apoptosis-Inducing Ligand in Inflammatory Intestinal Epithelial Lesions. *Gastroenterology*. 2006;130:1962–1974.
269. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho J-K. Role of cytokines in inflammatory bowel disease. *World J. Gastroenterol.* 2008;14:4280–8.
270. Paleolog EM, Delasalle SA, Buurman WA, et al. Functional activities of receptors for tumor necrosis factor-alpha on human vascular endothelial cells. *Blood*. 1994;84:2578–90.
271. Kuijpers TW, Hakkert BC, Hart MH, et al. Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: a role for platelet-activating factor and IL-8. *J. Cell Biol.* 1992;117:565–72.
272. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood*. 1994;84:2068–101.
273. Kindler V, Sappino AP, Grau GE, et al. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 1989;56:731–740.
274. Myatt N, Coghill G, Morrison K, et al. Detection of tumour necrosis factor alpha in sarcoidosis and tuberculosis granulomas using in situ hybridisation. *J. Clin. Pathol.* 1994;47:423–6.
275. Poggioli G, Laureti S, Campieri M, et al. Infliximab in the treatment of Crohn's disease. *Ther. Clin. Risk Manag.* 2007;3:301–8.
276. Murch SH, Braegger CP, Walker-Smith JA, et al. Location of tumour necrosis factor a by immunohistochemistry in chronic inflammatory bowel disease. *Gut*. 1993;34:1705–1709.
277. Yapali S, Hamzaoglu HO. Anti-TNF treatment in inflammatory bowel disease. *Ann. Gastroenterol.* 2007;20:48–53.
278. Avdagić N, Babić N, Šeremet M, et al. Tumor necrosis factor-alpha serum level in assessment of disease activity in inflammatory bowel diseases. *Med. Glas. (Zenica).* 2013;10:211–6.
279. Braegger CP, Nicholls S, Murch SH, et al. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet (London, England)*. 1992;339:89–91.
280. Murch SH, Lamkin VA, Savage MO, et al. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut*. 1991;32:913–7.
281. Assche G Van, Rutgeerts P. Anti-TNF agents in Crohn's disease. *Expert Opin. Investig. Drugs*. 2000;9:103–111.
282. Mullin JM, Snock K V. Effect of Tumor Necrosis Factor on Epithelial Tight Junctions and

Transepithelial Permeability. *Cancer Res.* 1990;50:2172–2176.
283. Rampart M, Smet W de, Fiers W, et al. Inflammatory properties of recombinant TNF in rabbit skin in vivo. *J.Exp.Med.* 1989;169:2227–2232.
284. Amiri P, Locksley RM, Parslow TG, et al. Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. *Nature*. 1992;356:604–7.
285. Derkx B, Taminiau J, Radema S, et al. Tumour-necrosis-factor antibody treatment in Crohn's disease. *Lancet*. 1993;342:173–174.
286. Dullemen HM Van, Deventer SJH Van, Hommes DW, et al. Treatment of Crohn's Disease With Anti-Tumor Necrosis Factor Chimeric Monoclonal Antibody (cA2). *Gastroenterology*. 1995;109:129–135.
287. Berns M, Hommes DW. Anti-TNF-α therapies for the treatment of Crohn's disease: the past, present and future. *Expert Opin. Investig. Drugs*. 2016;25:129–143.
288. Eissner G, Kolch W, Scheurich P. Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. *Cytokine Growth Factor Rev.* 2004;15:353–366.
289. Harashima S, Horiuchi T, Hatta N, et al. Outside-to-inside signal through the membrane TNF-alpha induces E-selectin (CD62E) expression on activated human CD4+ T cells. *J. Immunol.* 2001;166:130–6.
290. Hove T ten, Montfrans C van, Peppelenbosch MP, et al. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut*. 2002;50:206–11.
291. Brande JMH Van Den, Koehler TC, Zelinkova Z, et al. Prediction of antitumour necrosis factor clinical efficacy by real-time visualisation of apoptosis in patients with Crohn's disease. *Gut*. 2007;56:509–517.
292. Akobeng AK, Zachos M. Tumor necrosis factor-alpha antibody for induction of remission in Crohn's disease. *Cochrane Database Syst. Rev.* 2003:10–13.
293. D'Haens G, Deventer S Van, Hogezand R Van, et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A european multicenter trial. *Gastroenterology*. 1999;116:1029–1034.
294. Baert FJ, D'Haens GR, Peeters M, et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology*. 1999;116:22–8.
295. Hanauer SB, Feagan BG, Lichtenstein GR, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet (London, England)*. 2002;359:1541–9.
296. Sands BE, Blank MA, Patel K, et al. Long-term treatment of rectovaginal fistulas in Crohn's disease: Response to infliximab in the ACCENT II study. *Clin. Gastroenterol. Hepatol.* 2004;2:912–920.
297. Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, Azathioprine, or Combination Therapy for Crohn's Disease. *N. Engl. J. Med.* 2010;362:1383–1395.
298. Hanauer SB, Sandborn WJ, Rutgeerts P, et al. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: The CLASSIC-I trial. *Gastroenterology*. 2006;130:323–332.
299. Sandborn WJ, Hanauer SB, Rutgeerts P, et al. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut*. 2007;56:1232–1239.
300. Schreiber S, Rutgeerts P, Fedorak RN, et al. A Randomized, Placebo-Controlled Trial of Certolizumab Pegol (CDP870) for Treatment of Crohn's Disease. *Gastroenterology*. 2005;129:807–818.

301. Sandborn WJ, Feagan BG, Stoinov S, et al. Certolizumab pegol for the treatment of Crohn's disease. *N. Engl. J. Med.* 2007;357:228–38.
302. Sandborn WJ, Schreiber S, Feagan BG, et al. Certolizumab Pegol for Active Crohn's Disease: A Placebo-Controlled, Randomized Trial. *Clin. Gastroenterol. Hepatol.* 2011;9:670-678.e3.
303. Schreiber S, Khaliq-Kareemi M, Lawrance IC, et al. Maintenance therapy with certolizumab pegol for Crohn's disease. *N. Engl. J. Med.* 2007;357:239–50.
304. Dassopoulos T, Sultan S, Falck–Ytter YT, et al. American Gastroenterological Association Institute Technical Review on the Use of Thiopurines, Methotrexate, and Anti–TNF-α Biologic Drugs for the Induction and Maintenance of Remission in Inflammatory Crohn's Disease. *Gastroenterology*. 2013;145:1464-1478.e5.
305. Rutgeerts P, Assche G Van, Sandborn WJ, et al. Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology*. 2012;142:1102-1111.e2.
306. Oikonomopoulos A, Deen WK van, Hommes DW. Anti-TNF antibodies in inflammatory bowel disease: do we finally know how it works? *Curr. Drug Targets*. 2013;14:1421–32.
307. Nesbitt A, Fossati G, Bergin M, et al. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. *Inflamm. Bowel Dis.* 2007;13:1323–32.
308. Rutgeerts P, Schreiber S, Feagan B, et al. Certolizumab pegol, a monthly subcutaneously administered Fc-free anti-TNFα, improves health-related quality of life in patients with moderate to severe Crohn's disease. *Int J Color. Dis*. 2008;23:289–296.
309. Lichtenstein GR, Thomsen OØ, Schreiber S, et al. Continuous Therapy With Certolizumab Pegol Maintains Remission of Patients With Crohn's Disease for up to 18 Months. *Clin. Gastroenterol. Hepatol.* 2010;8:600–609.
310. Sahnan K, Tozer PJ, Adegbola SO, et al. Developing a core outcome set for fistulising perianal Crohn's disease. *Gut*. 2019;68:226–238.
311. Patil SA, Rustgi A, Langenberg P, et al. Comparative effectiveness of anti-TNF agents for Crohn's disease in a tertiary referral IBD practice. *Dig. Dis. Sci.* 2013;58:209–15.
312. Kestens C, Oijen MGH van, Mulder CLJ, et al. Adalimumab and Infliximab Are Equally Effective for Crohn's Disease in Patients Not Previously Treated With Anti-Tumor Necrosis Factor-alpha Agents. *Clin. Gastroenterol. Hepatol.* 2013;11:826–831.
313. Osterman MT, Haynes K, Delzell E, et al. Comparative Effectiveness of Infliximab and Adalimumab for Crohn's Disease Critical revision of the manuscript for important intellectual content Statistical analysis Obtained funding. *Clin Gastroenterol Hepatol*. 2014;12:811–817.
314. Stidham RW, Lee TCH, Higgins PDR, et al. Systematic review with network meta-analysis: the efficacy of anti-TNF agents for the treatment of Crohn's disease. *Aliment. Pharmacol. Ther.* 2014;39:1349–1362.
315. Fiorino G, Fazio M, Danese S. Biosimilar therapies in inflammatory bowel disease: should we care about patient profile? *Expert Rev. Clin. Immunol.* 2016;12:361–3.
316. Papamichael K, Stappen T Van, Jairath V, et al. Review article: Pharmacological aspects of anti-TNF biosimilars in inflammatory bowel diseases. *Aliment. Pharmacol. Ther.* 2015;42:1158–1169.
317. Danese S, Bonovas S, Peyrin-Biroulet L. Biosimilars in IBD: from theory to practice. *Nat. Rev. Gastroenterol. Hepatol.* 2017;14:22–31.
318. Murphy C, Sugrue K, Mohamad G, et al. Biosimilar but not the same. *J Crohn's Colitis*.

2015;9(suppl 1):S224–S225.
319. Torres J, Boyapati RK, Kennedy NA, et al. Systematic Review of Effects of Withdrawal of Immunomodulators or Biologic Agents From Patients With Inflammatory Bowel Disease. *Gastroenterology*. 2015;149:1716–30.
320. Cohen BL, Sachar DB. Update on anti-tumor necrosis factor agents and other new drugs for inflammatory bowel disease. *BMJ*. 2017;357:j2505.
321. Danese S, Fiorino G, Raine T, et al. ECCO Position Statement on the Use of Biosimilars for Inflammatory Bowel Disease—An Update. *J. Crohn's Colitis*. 2017:26–34.
322. Gecse KB, Lovász BD, Farkas K, et al. Efficacy and Safety of the Biosimilar Infliximab CT-P13 Treatment in Inflammatory Bowel Diseases: A Prospective, Multicentre, Nationwide Cohort. *J. Crohn's Colitis*. 2016;10:133–140.
323. Yarur AJ, Rubin DT. Therapeutic Drug Monitoring of Anti-tumor Necrosis Factor Agents in Patients with Inflammatory Bowel Diseases. *Inflamm. Bowel Dis.* 2015;21:1709–1718.
324. Sandborn WJ, Abreu MT, D'Haens G, et al. Certolizumab Pegol in Patients With Moderate to Severe Crohn's Disease and Secondary Failure to Infliximab. *Clin. Gastroenterol. Hepatol.* 2010;8:688-695.e2.
325. Billioud V, Sandborn WJ, Peyrin-Biroulet L. Loss of Response and Need for Adalimumab Dose Intensification in Crohn's Disease: A Systematic Review. *Am. J. Gastroenterol.* 2011;106:674–684.
326. Gisbert JP, Panés J. Loss of Response and Requirement of Infliximab Dose Intensification in Crohn's Disease: A Review. *Am. J. Gastroenterol.* 2009;104:760–767.
327. Steenholdt C, Palarasah Y, Bendtzen K, et al. Pre-existing IgG antibodies cross-reacting with the Fab region of infliximab predict efficacy and safety of infliximab therapy in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 2013;37:1172–1183.
328. Casteele N Vande, Gils A, Singh S, et al. Antibody Response to Infliximab and its Impact on Pharmacokinetics can be Transient. *Am. J. Gastroenterol.* 2013;108:962–971.
329. Gisbert JP, Marín AC, McNicholl AG, et al. Systematic review with meta-analysis: the efficacy of a second anti-TNF in patients with inflammatory bowel disease whose previous anti-TNF treatment has failed. *Aliment. Pharmacol. Ther.* 2015;41:613–623.
330. Mahadevan U, Cucchiara S, Hyams JS, et al. The London Position Statement of the World Congress of Gastroenterology on Biological Therapy for IBD With the European Crohn's and Colitis Organisation: Pregnancy and Pediatrics. *Am. J. Gastroenterol.* 2011;106:214–223.
331. Billiet T, Rutgeerts P, Ferrante M, et al. Targeting TNF-α for the treatment of inflammatory bowel disease. *Expert Opin. Biol. Ther.* 2014;14:75–101.
332. Maser EA, Villela R, Silverberg MS, et al. Association of Trough Serum Infliximab to Clinical Outcome After Scheduled Maintenance Treatment for Crohn's Disease. *Clin. Gastroenterol. Hepatol.* 2006;4:1248–1254.
333. Schnitzler F, Fidder H, Ferrante M, et al. Long-term outcome of treatment with infliximab in 614 patients with Crohn's disease: results from a single-centre cohort. *Gut*. 2009;58:492–500.
334. Casteele N Vande, Gils A. Pharmacokinetics of anti-TNF monoclonal antibodies in inflammatory bowel disease: Adding value to current practice. *J. Clin. Pharmacol.* 2015;55:S39–S50.
335. Colombel J-F, Feagan BG, Sandborn WJ, et al. Therapeutic Drug Monitoring of Biologics for Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* 2012;18:349–358.

336. Yarur AJ, Jain A, Sussman DA, et al. The association of tissue anti-TNF drug levels with serological and endoscopic disease activity in inflammatory bowel disease: the ATLAS study. *Gut*. 2016;65:249–55.
337. Diane R. Mould BG. Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies. *Biodrugs*. 2010;24:23–39.
338. Casteele N Vande, Ferrante M, Assche G Van, et al. Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease. *Gastroenterology*. 2015;148:1320-1329.e3.
339. D'Haens G, Vermeire S, Lambrecht G, et al. Increasing Infliximab Dose Based on Symptoms, Biomarkers, and Serum Drug Concentrations Does Not Increase Clinical, Endoscopic, and Corticosteroid-Free Remission in Patients With Active Luminal Crohn's Disease. *Gastroenterology*. 2018;154:1343-1351.e1.
340. Feuerstein JD, Nguyen GC, Kupfer SS, et al. American Gastroenterological Association Institute Guideline on Therapeutic Drug Monitoring in Inflammatory Bowel Disease. *Gastroenterology*. 2017;153:827–834.
341. Lichtenstein GR, Loftus E V, Isaacs KL, et al. ACG Clinical Guideline: Management of Crohn's Disease in Adults. *Am. J. Gastroenterol.* 2018;113:481–517.
342. Casteele N Vande, Herfarth H, Katz J, et al. American Gastroenterological Association Institute Technical Review on the Role of Therapeutic Drug Monitoring in the Management of Inflammatory Bowel Diseases. *Gastroenterology*. 2017;153:835-857.e6.
343. Yarur AJ, Kanagala V, Stein DJ, et al. Higher infliximab trough levels are associated with perianal fistula healing in patients with Crohn's disease. *Aliment. Pharmacol. Ther.* 2017;45:933–940.
344. Torres J, Cravo M, Colombel J-F. Anti-TNF Withdrawal in Inflammatory Bowel Disease. *GE Port. J. Gastroenterol.* 2016;23:153–161.
345. Papamichael K, Vermeire S. Withdrawal of anti-tumour necrosis factor α therapy in inflammatory bowel disease. *World J Gastroenterol Gastroenterol*. 2015;21:4773–4778.
346. Brooks AJ, Sebastian S, Cross SS, et al. Outcome of elective withdrawal of anti-tumour necrosis factor-α therapy in patients with Crohn's disease in established remission. *J. Crohn's Colitis*. 2014;11:1456–1462.
347. Steenholdt C, Molazahi A, Ainsworth MA, et al. Outcome after discontinuation of infliximab in patients with inflammatory bowel disease in clinical remission: an observational Danish single center study. *Scand. J. Gastroenterol.* 2012;47:518–527.
348. Dai C, Liu W-X, Jiang M, et al. Mucosal Healing Did Not Predict Sustained Clinical Remission in Patients with IBD after Discontinuation of One-Year Infliximab Therapy. *PLoS One*. 2014;9.
349. Waugh AWG, Garg S, Matic K, et al. Maintenance of clinical benefit in Crohn's disease patients after discontinuation of infliximab: long-term follow-up of a single centre cohort. *Aliment. Pharmacol. Ther.* 2010;32:1129–1134.
350. Molnár T, Farkas K, Miheller P, et al. Is the efficacy of successful infliximab induction therapy maintained for one year lasting without retreatment in different behavior types of Crohn's disease? *J. Crohn's Colitis*. 2008;2:322–326.
351. Bau M, Zacharias P, Ribeiro DA, et al. SAFETY PROFILE OF ANTI-TNF THERAPY IN CROHN'S DISEASE MANAGEMENT: A BRAZILIAN SINGLE-CENTER DIRECT RETROSPECTIVE COMPARISON BETWEEN INFLIXIMAB AND ADALIMUMAB. *Arq.*

Gastroenterol. 2017;54:328–332.
352. Feuerstein JD, Cheifetz AS. Miscellaneous Adverse Events with Biologic Agents (Excludes Infection and Malignancy). *Gastroenterol. Clin. North Am.* 2014;43:543–563.
353. Vermeire S, Assche G Van, Rutgeerts P. Serum sickness, encephalitis and other complications of anti-cytokine therapy. *Best Pract. Res. Clin. Gastroenterol.* 2009;23:101–112.
354. Miehsler W, Novacek G, Wenzl H, et al. A decade of infliximab: The Austrian evidence based consensus on the safe use of infliximab in inflammatory bowel disease. *J. Crohn's Colitis*. 2010;4:221–256.
355. Casteele N Vande, Breynaert C, Vermeire S, et al. Incidence of acute severe infusion reactions to infliximab depends on definition used rather than assay. *Aliment. Pharmacol. Ther.* 2011;34:401–403.
356. O'Meara S, Nanda KS, Moss AC. Antibodies to Infliximab and Risk of Infusion Reactions in Patients With Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* 2014;20:1–6.
357. Rahier JF, Magro F, Abreu C, et al. Second European evidence-based consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease. *J. Crohn's Colitis*. 2014;8:443–468.
358. Lichtenstein GR, Feagan BG, Cohen RD, et al. Serious Infections and Mortality in Association With Therapies for Crohn's Disease: TREAT Registry. *Clin. Gastroenterol. Hepatol.* 2006;4:621–630.
359. Colombel J-F, Sandborn WJ, Panaccione R, et al. Adalimumab safety in global clinical trials of patients with Crohn's disease. *Inflamm. Bowel Dis.* 2009;15:1308–1319.
360. Pagnini C, Arseneau KO, Cominelli F. Safety considerations when using anti-TNFα therapy to treat Crohn's disease. *Expert Opin. Drug Saf.* 2015;14:31–44.
361. Keane J, Gershon S, Wise RP, et al. Tuberculosis Associated with Infliximab, a Tumor Necrosis Factor α–Neutralizing Agent. *N. Engl. J. Med.* 2001;345:1098–1104.
362. Bongartz T, Sutton AJ, Sweeting MJ, et al. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: Systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *J. Am. Med. Assoc.* 2006;295:2275–2285.
363. Hansen RA, Gartlehner G, Powell GE, et al. Serious Adverse Events With Infliximab: Analysis of Spontaneously Reported Adverse Events. *Clin. Gastroenterol. Hepatol.* 2007;5:729-735.e1.
364. Nyboe Andersen N, Pasternak B, Basit S, et al. Association between tumor necrosis factor-α antagonists and risk of cancer in patients with inflammatory bowel disease. *JAMA*. 2014;311:2406–13.
365. Long MD, Martin CF, Pipkin CA, et al. Risk of Melanoma and Nonmelanoma Skin Cancer Among Patients With Inflammatory Bowel Disease. *Gastroenterology*. 2012;143:390-399.e1.
366. Beaugerie L, Itzkowitz SH. Cancers complicating inflammatory bowel disease. *N. Engl. J. Med.* 2015;372:1441–52.
367. Singh S, Nagpal SJS, Murad MH, et al. Inflammatory Bowel Disease Is Associated With an Increased Risk of Melanoma: A Systematic Review and Meta-analysis. *Clin. Gastroenterol. Hepatol.* 2014;12:210–218.
368. Peyrin-Biroulet L, Deltenre P, Suray N de, et al. Efficacy and Safety of Tumor Necrosis Factor Antagonists in Crohn's Disease: Meta-Analysis of Placebo-Controlled Trials. *Clin. Gastroenterol. Hepatol.* 2008;6:644–653.

369. Fidder H, Schnitzler F, Ferrante M, et al. Long-term safety of infliximab for the treatment of inflammatory bowel disease: A single-centre cohort study. *Gut*. 2009;58:501–508.
370. Biancone L, Orlando A, Kohn A, et al. Infliximab and newly diagnosed neoplasia in Crohn's disease: A multicentre matched pair study. *Gut*. 2006;55:228–233.
371. Ferrante M, Vermeire S, Rutgeerts PJ. Drug safety evaluation of certolizumab pegol. *Expert Opin. Drug Saf.* 2014;13:255–266.
372. Kane S V, Acquah LA. Placental Transport of Immunoglobulins: A Clinical Review for Gastroenterologists Who Prescribe Therapeutic Monoclonal Antibodies to Women During Conception and Pregnancy. *Am. J. Gastroenterol.* 2009;104:228–233.
373. Kamm MA, Ng SC. Perianal Fistulizing Crohn's Disease: A Call to Action. *Clin. Gastroenterol. Hepatol.* 2008;6:7–10.
374. Park SH, Aniwan S, Harmsen ¶ W Scott, et al. Update on the Natural Course of Fistulizing Perianal Crohn's Disease in a Population-Based Cohort. *Inflamm Bowel Dis* •. 2019;25.
375. Groof EJ de, Sahami S, Lucas C, et al. Treatment of perianal fistula in Crohn's disease: a systematic review and meta-analysis comparing seton drainage and anti-tumour necrosis factor treatment. *Color. Dis.* 2016;18:667–675.
376. Kotze PG, Shen B, Lightner A, et al. Modern management of perianal fistulas in Crohn's disease: future directions. *Gut*. 2018;67:1181–1194.
377. Mak WY, Lung PFC, Hart A. In Patients With Perianal Crohn's Fistulas, What Are the Outcomes When 'Radiological Healing' Is Achieved? Does Radiological Healing of Perianal Crohn's Fistulas Herald the Time Point for Stopping a Biologic? *J. Crohn's Colitis*. 2017;11:1506–1506.
378. Uchino M, Ikeuchi H, Bando T, et al. Long-term efficacy of infliximab maintenance therapy for perianal Crohn's disease. *World J Gastroenterol*. 2011;17:1174–1179.
379. Bouguen G, Siproudhis L, Gizard E, et al. Long-term Outcome of Perianal Fistulizing Crohn's Disease Treated With Infliximab. *Clin. Gastroenterol. Hepatol.* 2013;11:975-981.e4.
380. Antakia R, Shorthouse AJ, Robinson K, et al. Combined modality treatment for complex fistulating perianal Crohn's disease. *Color. Dis.* 2013;15:210–216.
381. Tougeron Guillaume Savoye AE Céline Savoye-Collet AE Edith Koning AE Francis Michot AE Eric Lerebours DA. Predicting Factors of Fistula Healing and Clinical Remission After Infliximab-Based Combined Therapy for Perianal Fistulizing Crohn's Disease.
382. Hotokezaka M, Ikeda T, Uchiyama S, et al. Results of Seton Drainage and Infliximab Infusion for Complex Anal Crohn's Disease. *Hepatogastroenterology*. 2011;58:1189–1192.
383. Ng SC, Plamondon S, Gupta A, et al. Prospective evaluation of anti-tumor necrosis factor therapy guided by magnetic resonance imaging for Crohn's perineal fistulas. *Am. J. Gastroenterol.* 2009;104:2973–2986.
384. Cosnes J, Nion-Larmurier I, Beaugerie L, et al. Impact of the increasing use of immunosuppressants in Crohn's disease on the need for intestinal surgery. *Gut*. 2005;54:237–241.
385. Wolters FL, Russel MGVM, Stockbrugger RW. Has disease outcome in Crohn's disease changed during the last four decades? *Aliment. Pharmacol. Ther.* 2004;20:483–496.
386. Boyapati RK, Ho GT, Satsangi J. Top-down in the Long Term in Crohn's Disease. *J. Crohn's Colitis*. 2018:513–514.

387. Tsui JJ, Huynh HQ. Is top-down therapy a more effective alternative to conventional step-up therapy for Crohn's disease? *Ann Gastroenterol*. 2018;31:413–424.
388. Sauk J, Nguyen D, Yajnik V, et al. Natural history of perianal crohn's disease after fecal diversion. *Inflamm. Bowel Dis.* 2014;20:2260–2265.
389. Panchal H, Wagner M, Chatterji M, et al. Earlier Anti-Tumor Necrosis Factor Therapy of Crohn's Disease Correlates with Slower Progression of Bowel Damage. *Dig. Dis. Sci.* 1:3.
390. Khanna R, Bressler B, Levesque BG, et al. Early combined immunosuppression for the management of Crohn's disease (REACT): a cluster randomised controlled trial. *Lancet*. 2015;386:1825–1834.
391. Rungoe C, Langholz E, Andersson M, et al. Changes in medical treatment and surgery rates in inflammatory bowel disease: a nationwide cohort study 1979-2011. *Gut*. 2014;63:1607–16.
392. Kotze PG, Magro DO, Augusto C, et al. Long Time from Diagnosis to Surgery May Increase Postoperative Complication Rates in Elective CD Intestinal Resections : An Observational Study. 2018;2018.
393. Iesalnieks I, Kilger A, Glass H, et al. Perforating Crohn's ileitis: delay of surgery is associated with inferior postoperative outcome. *Inflamm. Bowel Dis.* 2010;16:2125–30.
394. Lee MJ, Freer C, Adegbola S, et al. Patients with perianal Crohn's fistulas experience delays in accessing anti-TNF therapy due to slow recognition, diagnosis and integration of specialist services: Lessons learned from three referral centres. *Color. Dis.* 2018;20:1–7.
395. Gecse KB, Sebastian S, Hertogh G de, et al. Results of the Fifth Scientific Workshop of the ECCO [II]: Clinical Aspects of Perianal Fistulising Crohn's Disease—the Unmet Needs: Table 1. *J. Crohn's Colitis*. 2016:jjw039.
396. Gaertner B, Decanini A, Mellgren A, et al. Does infliximab infusion impact results of operative treatment for Crohn's perianal fistulas? *Dis. Colon Rectum*. 2007;50:1754.
397. Galandiuk S, Kimberling J, Al-Mishlab TG, et al. Perianal Crohn disease: predictors of need for permanent diversion. *Ann. Surg.* 2005;241:796–801; discussion 801-2.
398. Lightner AL, Faubion WA. Mesenchymal Stem Cell Injections for the Treatment of Perianal Crohn's Disease: What We Have Accomplished and What We Still Need to Do. *J. Crohn's Colitis*. 2017;11:1267–1276.
399. Vollebregt PF, Bodegraven AA van, Markus-de Kwaadsteniet TML, et al. Impacts of perianal disease and faecal incontinence on quality of life and employment in 1092 patients with inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 2018;47:1253–1260.
400. Molendijk I, Bonsing BA, Roelofs H, et al. Allogeneic Bone MarrowLDerived Mesenchymal Stromal Cells Promote Healing of Refractory Perianal Fistulas in Patients With Crohn's Disease. *Gastroenterology*. 2015;149:918-927.e6.
401. Sprakes MB, Ford AC, Warren L, et al. Efficacy, tolerability, and predictors of response to infliximab therapy for Crohn's disease: A large single centre experience. *J. Crohn's Colitis*. 2012;6:143–153.
402. Allez M, Karmiris K, Louis E, et al. Report of the ECCO pathogenesis workshop on anti-TNF therapy failures in inflammatory bowel diseases: Definitions, frequency and pharmacological aspects. *J. Crohn's Colitis*. 2010;4:355–366.
403. Ben-Horin S, Chowers Y. Review article: loss of response to anti-TNF treatments in Crohn's disease. *Aliment. Pharmacol. Ther.* 2011;33:987–995.

404. Ben-Horin S. Loss of Response to Anti-Tumor Necrosis Factors: What Is the Next Step? *Dig. Dis.* 2014;32:384–388.
405. Longworth L, Fountain D, Singh J, et al. Elicitation of Health-Related Utility in Perianal Fistula in Crohn's Disease. *Patient - Patient-Centered Outcomes Res.* 2019;12:339–348.
406. Arseneau KO, Cohn SM, Cominelli F, et al. Cost-Utility of Initial Medical Management for Crohn's Disease Perianal Fistulae. 2001.
407. Tosh JC, Longworth LJ, George E. Outcomes Assessment Utility values in National Institute for Health and Clinical Excellence (NICE) Technology Appraisals. 2011.
408. Andeweg CS, Groenewoud J, Wilt GJ van der, et al. A Markov Decision Model to Guide Treatment of Recurrent Colonic Diverticulitis. *Clin. Gastroenterol. Hepatol.* 2016;14:87-95.e2.
409. Rencz F, Gulácsi L, Péntek M, et al. Cost-utility of biological treatment sequences for luminal Crohn's disease in Europe. *Expert Rev. Pharmacoecon. Outcomes Res.* 2017;17:597–606.
410. Sonnenberg FA, Beck JR. Markov Models in Medical Decision Making. *Med. Decis. Mak.* 1993;13:322–338.
411. Roda G, Jharap B, Neeraj N, et al. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin. Transl. Gastroenterol.* 2016;7.
412. M. C, J. P, V. G, et al. Long-term durability of infliximab treatment in Crohn's disease and efficacy of dose 'escalation' in patients losing response. *J. Clin. Gastroenterol.* 2011;45:113–118.
413. Baji P, Gulácsi L, Brodszky V, et al. Cost-effectiveness of biological treatment sequences for fistulising Crohn's disease across Europe. *United Eur. Gastroenterol. J.* 2018;6:310–321.
414. Chaparro M, Zanotti C, Burgueño P, et al. Health care costs of complex perianal fistula in Crohn's disease. *Dig. Dis. Sci.* 2013;58:3400–3406.
415. Alrubaiy L, Rikaby I, Hutchings HA, et al. Can the inflammatory bowel disease biologics registry lead to improved quality of care? *J. Taibah Univ. Med. Sci.* 2015;10:373–375.
416. Sica GS, Carlo S Di, Tema G, et al. Treatment of peri-anal fistula in Crohn's disease. *World J. Gastroenterol.* 2014;20:13205–10.
417. Yassin N, Dibley L, Nachiappan S, et al. PTU-201 Living with anal fistulae. *Gut*. 2015;64:A151.2-A151.
418. Yarur AJ, Kubiliun MJ, Czul F, et al. Concentrations of 6-Thioguanine Nucleotide Correlate With Trough Levels of Infliximab in Patients With Inflammatory Bowel Disease on Combination Therapy. *Clin. Gastroenterol. Hepatol.* 2015;13:1118-1124.e3.
419. Ungar B, Levy I, Yavne Y, et al. Optimizing Anti-TNF-α Therapy: Serum Levels of Infliximab and Adalimumab Are Associated With Mucosal Healing in Patients With Inflammatory Bowel Diseases. 2016.
420. Lallemand C, Kavrochorianou N, Steenholdt C, et al. Reporter gene assay for the quantification of the activity and neutralizing antibody response to TNFα antagonists. 2011.
421. Wang S-L, Ohrmund L, Hauenstein S, et al. Development and validation of a homogeneous mobility shift assay for the measurement of infliximab and antibodies-to-infliximab levels in patient serum. *J. Immunol. Methods*. 2012;382:177–88.
422. Steenholdt C, Ainsworth MA, Tovey M, et al. Comparison of Techniques for Monitoring Infliximab and Antibodies Against Infliximab in Crohn's Disease. *Ther. Drug Monit.* 2013;35:530–538.

423. Ebhardt HA, Root A, Sander C, et al. Applications of targeted proteomics in systems biology and translational medicine. *Proteomics*. 2015;15:3193–3208.
424. Ladwig PM, Barnidge DR, Willrich MA V. Mass Spectrometry Approaches for Identification and Quantitation of Therapeutic Monoclonal Antibodies in the Clinical Laboratory Papasian CJ, ed. *Clin. Vaccine Immunol.* 2017;24.
425. Yu Y, Xu J, Liu Y, et al. Quantification of human serum transferrin using liquid chromatography-tandem mass spectrometry based targeted proteomics. *J. Chromatogr. B*. 2012;902:10–15.
426. Yang T, Chen F, Xu F, et al. A liquid chromatography-tandem mass spectrometry-based targeted proteomics assay for monitoring P-glycoprotein levels in human breast tissue. *Clin. Chim. Acta*. 2014;436:283–289.
427. Doerr A. Mass spectrometry-based targeted proteomics. *Nat. Methods*. 2013;10:23.
428. Yang T, Xu F, Xu J, et al. Comparison of liquid chromatography-tandem mass spectrometry-based targeted proteomics and conventional analytical methods for the determination of P-glycoprotein in human breast cancer cells. *J. Chromatogr. B*. 2013;936:18–24.
429. Schmitz EE. Development and evaluation of protein quantitation assays for use in health care. 2018.
430. Liu L, Xu Q, Hao S, et al. A Quasi-direct LC-MS / MS-based Targeted Proteomics Approach for miRNA Quantification via a Covalently Immobilized DNA- peptide Probe. *Sci. Rep.* 2017:1–12.
431. Peng X, Liu B, Li Y, et al. Development and Validation of LC–MS/MS Method for the Quantitation of Infliximab in Human Serum. *Chromatographia*. 2015;78:521–531.
432. Karpievitch Y V, Polpitiya AD, Anderson GA, et al. Liquid chromatography mass spectrometry-based proteomics: Biological and technological aspects. *Ann. Appl. Stat.* 2010;4:1797–1823.
433. Dunning C, Lame M, Calciano S, et al. *Accurate and Sensitive LC-MS/MS Quantification of Adalimumab in Serum/Plasma: Impact of Sample Preparation on Method Performance*. 2017.
434. Ryman JT, Meibohm B. Pharmacokinetics of Monoclonal Antibodies. *CPT Pharmacometrics Syst. Pharmacol*. 2017;6:576–588.
435. Keizer RJ, Huitema ADR, Schellens JHM, et al. Clinical Pharmacokinetics of Therapeutic Monoclonal Antibodies. *Clin. Pharmacokinet.* 2010;49:493–507.
436. Schmidt A, Picotti P, Ruedi Aebersold U. Proteomanalyse und Systembiologie. *Biospektrum*. 2008;14:44–46.
437. Stahl-Zeng J, Lange V, Ossola R, et al. High Sensitivity Detection of Plasma Proteins by Multiple Reaction Monitoring of N -Glycosites. *Mol. Cell. Proteomics*. 2007;6:1809–1817.
438. Hendy P, Hart A, Irving P. Anti-TNF drug and antidrug antibody level monitoring in IBD: a practical guide.
439. Davidov Y, Ungar B, Bar-Yoseph H, et al. Association of Induction Infliximab Levels With Clinical Response in Perianal Crohn's Disease. *J. Crohn's Colitis*. 2016:jjw182.
440. Adegbola SO, Sahnan K, Tozer PJ, et al. Review of local injection of anti-TNF for perianal fistulising Crohn's disease. *Int. J. Colorectal Dis.* 2017;32:1539–1544.
441. Willrich MA V, Murray DL, Barnidge DR, et al. Quantitation of infliximab using clonotypic peptides and selective reaction monitoring by LC-MS/MS. *Int. Immunopharmacol.* 2015;28:513–520.

442. Ackermann BL, Berna MJ. Coupling immunoaffinity techniques with MS for quantitative analysis of low-abundance protein biomarkers. *Expert Rev. Proteomics*. 2007;4:175–186.
443. Jourdil J-F, Lebert D, Gautier-Veyret E, et al. Infliximab quantitation in human plasma by liquid chromatography-tandem mass spectrometry: towards a standardization of the methods?
444. Sahnan K, Tozer PJ, Adegbola SO, et al. Developing a core outcome set for fistulising perianal Crohn's disease. *Gut*. 2019;68:226–238.
445. Dudukgian H. Why do we have so much trouble treating anal fistula? *World J. Gastroenterol.* 2011;17:3292.
446. Atkin GK, Martins J, Tozer P, et al. For many high anal fistulas, lay open is still a good option. *Tech. Coloproctol.* 2011;15:143–50.
447. Garcia-Aguilar J, Belmonte C, Wong WD, et al. Anal fistula surgery. Factors associated with recurrence and incontinence. *Dis. Colon Rectum*. 1996;39:723–9.
448. Prosst RL, Ehni W. The OTSC® Proctology clip system for anorectal fistula closure: the 'Anal Fistula Claw': Case report. *Minim. Invasive Ther. Allied Technol.* 2012;21:307–312.
449. Kochhar G, Saha S, Andley M, et al. Video-assisted anal fistula treatment. *JSLS J. Soc. Laparoendosc. Surg.* 2014;18:354–359.
450. Pini Prato A, Zanaboni C, Mosconi M, et al. Preliminary results of video-assisted anal fistula treatment (VAAFT) in children. *Tech. Coloproctol.* 2016;20:279–285.
451. Zarin M, Imran khan M, Ahmad M, et al. VAAFT: Video assisted Anal fistula treatment; Bringing revolution in Fistula treatment. *Pakistan J. Med. Sci.* 2015;31:716–721.
452. Meinero P, Mori L, Gasloli G. Video-Assisted Anal Fistula Treatment. *Dis. Colon Rectum*. 2014;57:354–359.
453. Mendes CRS, Ferreira LS de M, Sapucaia RA, et al. Video-assisted anal fistula treatment: technical considerations and preliminary results of the first Brazilian experience. *Arq. Bras. Cir. Dig.* 2014;27:77–81.
454. Wałęga P, Romaniszyn M, Nowak W. VAAFT: a new minimally invasive method in the diagnostics and treatment of anal fistulas--initial results. *Pol. Przegl. Chir.* 2014;86:7–10.
455. Grolich T, Skricka T, Robek O, et al. Role of video assisted anal fistula treatment in our management of fistula-in-ano. *Acta Chir. Iugosl.* 2014;61:83–85.
456. Seow-En I, Seow-Choen F, Koh PK. An experience with video-assisted anal fistula treatment (VAAFT) with new insights into the treatment of anal fistulae. *Tech. Coloproctol.* 2016;20:389–393.
457. Jiang H, Liu H, Li Z, et al. Video-Assisted Anal Fistula Treatment (VAAFT) for Complex Anal Fistula: A Preliminary Evaluation in China. *Med. Sci. Monit.* 2017;23:2065–2071.
458. Selvarajan A. Video-Assisted Anal Fistula Treatment (VAAFT): Johor Bahru's Humble Beginnings. *Int. J. Integr. Med. Sci.* 2015;2:175–177.
459. Chowbey PK, Khullar R, Sharma A, et al. Minimally Invasive Anal Fistula Treatment (MAFT)—An Appraisal of Early Results in 416 Patients. *Indian J. Surg.* 2015;77:716–721.
460. Öztürk E, Gülcü B. Laser Ablation of Fistula Tract. *Dis. Colon Rectum*. 2014;57:360–364.
461. Giamundo P, Geraci M, Tibaldi L, et al. Closure of fistula-in-ano with laser--FiLaC™: an effective novel sphincter-saving procedure for complex disease. *Colorectal Dis.* 2014;16:110–5.
462. Wilhelm A, Fiebig A, Krawczak M. Five years of experience with the FiLaC™ laser for fistula-in-ano management: long-term follow-up from a single institution. *Tech.*

Coloproctol. 2017.
463. Gautier M, Godeberge P, Ganansia R, et al. Easy clip to treat anal fistula tracts: a word of caution. *Int. J. Colorectal Dis.* 2015;30:621–624.
464. Mennigen R, Laukötter M, Senninger N, et al. The OTSC® proctology clip system for the closure of refractory anal fistulas. *Tech. Coloproctol.* 2015;19:241–246.
465. Prosst RL, Joos AK. Short-term outcomes of a novel endoscopic clipping device for closure of the internal opening in 100 anorectal fistulas. *Tech. Coloproctol.* 2016;20:753–758.
466. Tozer P, Sala S, Cianci V, et al. Fistulotomy in the Tertiary Setting can Achieve High Rates of Fistula Cure with an Acceptable Risk of Deterioration in Continence. *J. Gastrointest. Surg.* 2013;17:1960–1965.
467. Chivate SD. Comment on Meinero and Mori: Video-assisted anal fistula treatment (VAAFT): a novel sphincter-saving procedure to repair complex anal fistulas. *Tech. Coloproctol.* 2012;16:465–470.
468. Giamundo P, Esercizio L, Geraci M, et al. Fistula-tract Laser Closure (FiLaCTM): long-term results and new operative strategies. *Tech. Coloproctol.* 2015;19:449–453.
469. Stijns J, Wasowicz DK, Zimmerman DDE. Does laser fistuloplasty (FiLaC™) offer any benefit over surgical closure of the internal orifice? *Tech. Coloproctol.* 2017.
470. Wilhelm A. Anal fistula surgery: what are the patients expecting from the surgeon? *Tech. Coloproctol.* 2017;21:491–492.
471. Akbari A. The role of red blood cells in wound healing. 2011.
472. Kirschniak A, Kratt T, Stüker D, et al. A new endoscopic over-the-scope clip system for treatment of lesions and bleeding in the GI tract: first clinical experiences. *Gastrointest. Endosc.* 2007;66:162–7.
473. Mennigen R, Senninger N, Laukoetter MG. Novel treatment options for perforations of the upper gastrointestinal tract: Endoscopic vacuum therapy and over-the-scope clips. *World J. Gastroenterol.* 2014;20:7767–7776.
474. Kontovounisios C, Tekkis P, Tan E, et al. Adoption and success rates of perineal procedures for fistula-in-ano: a systematic review. *Color. Dis.* 2016;18:441–458.
475. Agha RA, Fowler AJ, Rajmohan S, et al. Preferred reporting of case series in surgery; the PROCESS guidelines. *Int. J. Surg.* 2016;36:319–323.
476. Griggs L, Schwartz D a. Medical options for treating perianal Crohn's disease. *Dig. Liver Dis.* 2007;39:979–987.
477. Condino G, Calabrese E, Zorzi F, et al. Anti-TNF-alpha treatments and obstructive symptoms in Crohn's disease: a prospective study. *Dig. Liver Dis.* 2013;45:258–62.
478. Lichtiger S. Healing of perianal fistulae by local injection of antibody to TNF. *Gastroenterology*. 2001;120:A621.
479. Alessandroni L, Kohn A, Cosintino R, et al. Local injection of infliximab in severe fistulating perianal Crohn's disease: an open uncontrolled study. *Tech. Coloproctol.* 2011;15:407–12.
480. Asteria CR, Ficari F, Bagnoli S, et al. Treatment of perianal fistulas in Crohn's disease by local injection of antibody to TNF-α accounts for a favourable clinical response in selected cases: A pilot study. *Scand. J. Gastroenterol.* 2006;41:1064–1072.
481. Poggioli G, Laureti S, Pierangeli F, et al. Local injection of Infliximab for the treatment of perianal Crohn's disease. *Dis. Colon Rectum*. 2005;48:768–74.
482. Tonelli F, Giudici F, Asteria CR. Effectiveness and Safety of Local Adalimumab Injection in Patients With Fistulizing Perianal Crohn's Disease. *Dis. Colon Rectum*. 2012;55:870–

875.
483. Laureti S, Coscia M, Gentilini L, et al. P393 Combination of surgical therapy and local injections of adalimumab in treatment of complex perianal Crohn's disease. *J. Crohn's Colitis*. 2012;6:S166.
484. Tozer P, Borowski DW, Gupta A, et al. Managing perianal Crohn's fistula in the anti-TNFα era. *Tech. Coloproctol.* 2015;19:673–8.
485. Sandborn WJ, Hanauer SB. Infliximab in the treatment of Crohn's disease: a user's guide for clinicians. *Am. J. Gastroenterol.* 2002;97:2962–72.
486. Jones RG a, Martino A. Targeted localized use of therapeutic antibodies: a review of non-systemic, topical and oral applications. *Crit. Rev. Biotechnol.* 2015;00:1–15.
487. Hendel J, Karstensen JG, Vilmann P. Serial intralesional injections of infliximab in small bowel Crohn's strictures are feasible and might lower inflammation. *United Eur. Gastroenterol. J.* 2014;2:406–412.
488. Economou M, Zambeli E, Michopoulos S. Incidence and prevalence of Crohn ' s disease and its etiological influences. 2009:158–167.
489. Ardizzone S, Porro GB. Perianal Crohn's disease: overview. *Dig. Liver Dis.* 2007;39:957–8.
490. Singh B, McC Mortensen NJ, Jewell DP, et al. Perianal Crohn's disease. *Br. J. Surg.* 2004;91:801–14.
491. Adegbola SO, Dibley L, Sahnan K, et al. A qualitative exploration into experiences of living with Crohn's anal fistula. In: *European Crohn's & Colitis Organisation*.; 2018:ABSTRACT NUMBER: A-1881.
492. Becerra Pérez MM, Menear M, Brehaut JC, et al. Extent and Predictors of Decision Regret about Health Care Decisions. *Med. Decis. Mak.* 2016;36:777–790.
493. Brehaut JC, O'Connor AM, Wood TJ, et al. Validation of a Decision Regret Scale. *Med. Decis. Mak.* 2003;23:281–292.
494. Davison BJ, So AI, Goldenberg SL. Quality of life, sexual function and decisional regret at 1 year after surgical treatment for localized prostate cancer. *BJU Int.* 2007;100:780–785.
495. Groof EJ de, Buskens CJ, Ponsioen CY, et al. Multimodal treatment of perianal fistulas in Crohn's disease: seton versus anti-TNF versus advancement plasty (PISA): study protocol for a randomized controlled trial. *Trials*. 2015;16:366.
496. Adegbola SO, Sahnan K, Pellino G, et al. Short-term efficacy and safety of three novel sphincter-sparing techniques for anal fistulae: a systematic review. *Tech. Coloproctol.* 2017;21:775–782.
497. Altomare DF. Anal fistula closure with FiLaC: new hope or the same old story? *Tech. Coloproctol.* 2015;19:441–442.
498. Sahnan K, Adegbola SO, Tozer PJ, et al. P245 A systematic review of outcomes reported in studies on fistulising perianal Crohn's disease. *J. Crohn's Colitis*. 2017;11:S202–S202.
499. Moreno-Serrano A, García-Díaz JJ, Ferrer-Márquez M, et al. Using autologous platelet-rich plasma for the treatment of complex fistulas. *Rev esp enfeRm dig*. 2016;108:123–128.
500. Brédart A, Marrel A, Abetz-Webb L, et al. Interviewing to develop Patient-Reported Outcome (PRO) measures for clinical research: eliciting patients' experience. *Health Qual. Life Outcomes*. 2014;12:1–10.
501. Tong A, Sainsbury P, Craig J. Consolidated criteria for reporting qualitative research (COREQ): a 32-item checklist for interviews and focus groups. *Int. J. Qual. Heal. Care*.

2007;19:349–357.
502. Kim H, Sefcik JS, Bradway C. Characteristics of Qualitative Descriptive Studies: A Systematic Review. *Res. Nurs. Heal.* 2017;40:23–42.
503. Stebbins R. *Exploratory Research in the Social Sciences*. 2455 Teller Road, Thousand Oaks California 91320 United States of America: SAGE Publications, Inc.; 2001.
504. Ritchie J, Spencer L, O' Connor W. 'Carrying Out Qualitative Analysis' IN: Ritchie, J. and Lewis, J. - Qualitative research practice: a guide for social science students and researchers. In: Ritchie J, Lewis J, eds. *Qualitative research practice: a guide for social science students and researchers*. London: SAGE; 2003:219–262.
505. Sahnan K, Adegbola SO, Tozer PJ, et al. Improving the Understanding of Perianal Crohn Fistula Through 3D Modeling. *Ann. Surg.* 2018;267:e105–e107.
506. Sahnan K, Adegbola SO, Tozer PJ, et al. Innovation in the imaging perianal fistula: a step towards personalised medicine. *Therap. Adv. Gastroenterol.* 2018;11:1756284818775060.
507. Dibley L, Norton C, Whitehead E. The experience of stigma in inflammatory bowel disease: An interpretive (hermeneutic) phenomenological study. *J. Adv. Nurs.* 2018;74:838–851.
508. Stjernman H, Tysk C, Almer S, et al. Worries and concerns in a large unselected cohort of patients with Crohn's disease. *Scand. J. Gastroenterol.* 2010;45:696–706.
509. Kim AH, Roberts C, Feagan BG, et al. Developing a Standard Set of Patient-Centred Outcomes for Inflammatory Bowel Disease – an International, Cross-disciplinary Consensus. *J. Crohn's Colitis*. 2017:408–418.
510. Wiering B, Boer D de, Delnoij D. Patient involvement in the development of patient-reported outcome measures: a scoping review. *Heal. Expect.* 2017;20:11–23.
511. Black N. Patient reported outcome measures could help transform healthcare. *BMJ*. 2013;346:f167.
512. Devlin NJ, Parkin D, Browne J. Patient-reported outcome measures in the NHS: new methods for analysing and reporting EQ-5D data. *Health Econ.* 2010;19:886–905.
513. Jong MJ de, Huibregtse R, Masclee AAM, et al. Patient-Reported Outcome Measures for Use in Clinical Trials and Clinical Practice in Inflammatory Bowel Diseases - A Systematic Review. *Clin. Gastroenterol. Hepatol.* 2017.
514. Adegbola S, Dibley L, Sahnan K, et al. N001 Developing a quality of life score for Crohn's anal fistula (CAF-QoL). *J. Crohn's Colitis*. 2018;12:S568–S568.
515. Anon. NICE. Clinical Knowledge Summary-Crohn's Disease. *Ref Type Online Source*. 2017:0–7.
516. Mak WY, Adegbola S, Sahnan K, et al. *P319 Long-term outcomes after anti-TNF therapy withdrawal in patients with radiologically healed perianal Crohn's fistulas*. 2014.
517. Losco A, Viganò C, Conte D, et al. Assessing the activity of perianal Crohn's disease: comparison of clinical indices and computer-assisted anal ultrasound. *Inflamm. Bowel Dis.* 2009;15:742–9.
518. Horsthuis K, Ziech MLW, Bipat S, et al. Evaluation of an MRI-based score of disease activity in perianal fistulizing Crohn's disease. 2011.
519. Adegbola S, Dibley L, Sahnan K, et al. N002 A qualitative exploration into experiences of living with Crohn's anal fistula. *J. Crohn's Colitis*. 2018;12:S568–S569.
520. Ritchie J, Lewis J. QUALITATIVE RESEARCH PRACTICE A Guide for Social Science Students and Researchers.
521. Creswell J, Clark V, Plano Clark V. *Designing and Conducting Mixed Methods Research*.

3rd ed. SAGE; 2007.
522. Rothrock NE, Kaiser KA, Cella D. Developing a Valid Patient-Reported Outcome Measure. *Clin. Pharmacol. Ther.* 2011;90:737–742.
523. Cheung WY, Garratt AM, Russell IT, et al. The UK IBDQ-a British version of the inflammatory bowel disease questionnaire. development and validation. *J. Clin. Epidemiol.* 2000;53:297–306.
524. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet (London, England)*. 1980;1:514.
525. Bennebroek Evertsz' F, Hoeks CCMQ, Nieuwkerk PT, et al. Development of the patient Harvey Bradshaw index and a comparison with a clinician-based Harvey Bradshaw index assessment of Crohn's disease activity. *J. Clin. Gastroenterol.* 2013;47:850–6.
526. Marx RG, Bombardier C, Hogg-Johnson S, et al. Clinimetric and psychometric strategies for development of a health measurement scale. *J. Clin. Epidemiol.* 1999;52:105–11.
527. Alrubaiy L, Cheung W-Y, Dodds P, et al. Development of a Short Questionnaire to Assess the Quality of Life in Crohn's Disease and Ulcerative Colitis. *Orig. Artic.* 2015;4:66–76.
528. Deyo RA, Inui TS, Wood R, et al. *Articles Toward Clinical Applications of Health Status Measures: Sensitivity of Scales to Clinically Important Changes*.
529. Bindman AB, Keane D, Lurie N. Measuring health changes among severely ill patients. The floor phenomenon. *Med. Care*. 1990;28:1142–52.
530. Dibley L, Norton C, Cotterill N, et al. Development and initial validation of a disease-specific bowel continence questionnaire for inflammatory bowel disease patients: The ICIQ-IBD. *Eur. J. Gastroenterol. Hepatol.* 2016;28:233–239.
531. Marx RG, Bombardier C, Hogg-Johnson S, et al. Clinimetric and psychometric strategies for development of a health measurement scale. *J. Clin. Epidemiol.* 1999.
532. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet (London, England)*. 1986;1:307–10.
533. Giavarina D. Understanding Bland Altman analysis Lessons in biostatistics. *Biochem. Medica*. 2015;25:141–51.
534. Kwong E, Neuburger J, Black · Nick. Agreement between retrospectively and contemporaneously collected patient-reported outcome measures (PROMs) in hip and knee replacement patients. *Qual. Life Res.* 2018;27:1845–1854.
535. Sailer M, Bussen D, Debus ES, et al. Quality of life in patients with benign anorectal disorders. *Br. J. Surg.* 1998;85:1716–1719.
536. García-Aguilar J, Davey CS, Le CT, et al. Patient satisfaction after surgical treatment for fistula-in-ano. *Dis. Colon Rectum*. 2000;43:1206–12.
537. Wong S, Solomon M, Crowe P, et al. Cure, continence and quality of life after treatment for fistula-in-ano. *ANZ J. Surg.* 2008;78:675–682.
538. Ferrer-Márquez M, Espínola-Cortés N, Reina-Duarte A, et al. Design and psychometric evaluation of the quality of life in patients with anal fistula questionnaire. *Dis. Colon Rectum*. 2017;60:1083–1091.
539. Williams HRT, Cox IJ, Walker DG, et al. Characterization of Inflammatory Bowel Disease With Urinary Metabolic Profiling. *Am. J. Gastroenterol.* 2009;104:1435–1444.
540. Nicholson JK, Holmes E, Kinross JM, et al. Metabolic phenotyping in clinical and surgical environments. *Nature*. 2012;491:384–392.
541. Garcia-Olmo D, Phd M, Phd G-A, et al. Expert Opinion on Biological Therapy Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease (2008) Expanded adipose-derived stem cells for the treatment of

complex perianal fistula including Crohn's dis. *Expert Opin. Biol. Ther.* 2017;8:1417–1423.
542. García-Olmo D, García-Arranz M, Herreros D, et al. A phase I clinical trial of the treatment of crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis. Colon Rectum*. 2005;48:1416–1423.
543. Dietz AB, Dozois EJ, Fletcher JG, et al. Autologous Mesenchymal Stem Cells, Applied in a Bioabsorbable Matrix, for Treatment of Perianal Fistulas in Patients With Crohn's Disease. *Gastroenterology*. 2017;153:59-62.e2.
544. Hagen SJ Van Der, Baeten CG, Soeters PB, et al. Autologous platelet-derived growth factors (platelet-rich plasma) as an adjunct to mucosal advancement flap in high cryptoglandular perianal fistulae: A pilot study. *Color. Dis.* 2011;13:215–218.
545. Ciccocioppo R, Bernardo ME, Sgarella A, et al. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut*. 2011;60:788–798.
546. Halfvarson J, Cummings F, Grip O, et al. Inflammatory bowel disease registries for collection of patient iron parameters in Europe.
547. Shawihdi M, Razanskaite V, Cummings F, et al. OTU-008 GROWTH IN PARTICIPATION, REGISTRATIONS AND DATA MATURITY IN THE UK IBD REGISTRY: FOCUS ON BIOLOGICS 1.
548. Dobson L, Bloom S, Cummings F. IBD Registry News – August 2018. 2018. Available at: https://www.bsg.org.uk/resource/ibd-registry-news-august-2018.html [Accessed August 21, 2019].
549. Gika HG, Theodoridis GA, Wingate JE, et al. Within-day reproducibility of an HPLC-MS-based method for metabonomic analysis: application to human urine. *J. Proteome Res.* 2007;6:3291–303.
550. Sarafian MH, Gaudin M, Lewis MR, et al. Objective set of criteria for optimization of sample preparation procedures for ultra-high throughput untargeted blood plasma lipid profiling by ultra performance liquid chromatography-mass spectrometry. *Anal. Chem.* 2014;86:5766–74.
551. Veselkov KA, Vingara LK, Masson P, et al. Optimized Preprocessing of Ultra-Performance Liquid Chromatography/Mass Spectrometry Urinary Metabolic Profiles for Improved Information Recovery. *Anal. Chem.* 2011;83:5864–5872.
552. FDA. *Bioanalytical Method Validation Guidance for Industry Biopharmaceutics - Nonbinding Recommendations*. 2018.
553. Trygg J, Holmes E, Lundstedt T. Chemometrics in Metabonomics. *J. Proteome Res.* 2007;6:469–479.