ASPECTS OF FUNCTIONAL CONSTIPATION - A TRIAL OF AN OPIOID ANTAGONIST AS A TREATMENT AND AN INVESTIGATION OF THE COLONIC MICROBIOTA ASSOCIATED WITH THE ILLNESS.

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AUTHOR'S DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of the University of East Anglia. The work is original except where indicated by special reference in the text and no part of the thesis has been submitted for any other academic award. Any views expressed in this thesis are those of the author and in no way represent the University of East Anglia

Dr E. Katherine Kemsley and Dr Jack Dainty at the Institute of Food Research (IFR) completed all multivariate analysis and Dr Adrain Tett, also at the IFR, performed downstream analysis of the pyrosequencing data.

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ABSTRACT

INTRODUCTION

Functional constipation (FC) is a common condition which affects patients' quality of life (QoL), is of uncertain pathophysiology, and is poorly treated. The aims of this research were to conduct a randomised, placebo-controlled trial investigating an opiate antagonist (Naloxone - Nalcol™) on symptom relief in FC and secondly to describe the colonic microbiota in FC and determine the effect of Nalcol™ on it.

MATERIAL AND METHODS

The trial consisted of; a 2 week screening period (Period I), a 4 week trial period of Nalcol™ vs. Placebo (Period II), and a 4 week open label period with all patients taking Nalcol™ (Period III). The primary outcome was patients' assessment of '*satisfactory improvement in the preceding 2 weeks' after* each period. The secondary outcomes were: changes in QoL, stool type and frequency, and transit time. A subset of patients donated stool samples at the end of each period for microbiological analysis which were compared with controls without FC. Faecal samples were analysed using denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridisation (FISH), and pyrosequencing.

RESULTS

41 females were recruited (median age 45, range 23-76 years) .There were no significant differences in '*satisfactory improvement in the preceding 2 weeks'*, (Nalcol™ vs. Placebo, 20% vs. 24%, p=1.00, n=41) or any secondary outcomes. Nalcol™ use was associated with more bloating (40% vs. 5%, p=0.009). Patients with FC had significant reductions in the *Firmicutes* phylum (25% vs. 45%, p=0.004) and *Bifidiobacteria* spp. (0.67 vs. 0.88 log10 cell/g, p=0.03), with increases in the *Bacteroidetes* phylum (66% vs. 41%, p=0.002) compared to controls.

CONCLUSION

Nalcol™ had no clinical benefits for managing FC in this trial and cannot be recommended in this group of patients. Differences in the colonic microbiota in FC warrant further investigation to see how it is implicated in the pathophysiology of FC.

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CHAPTER 1

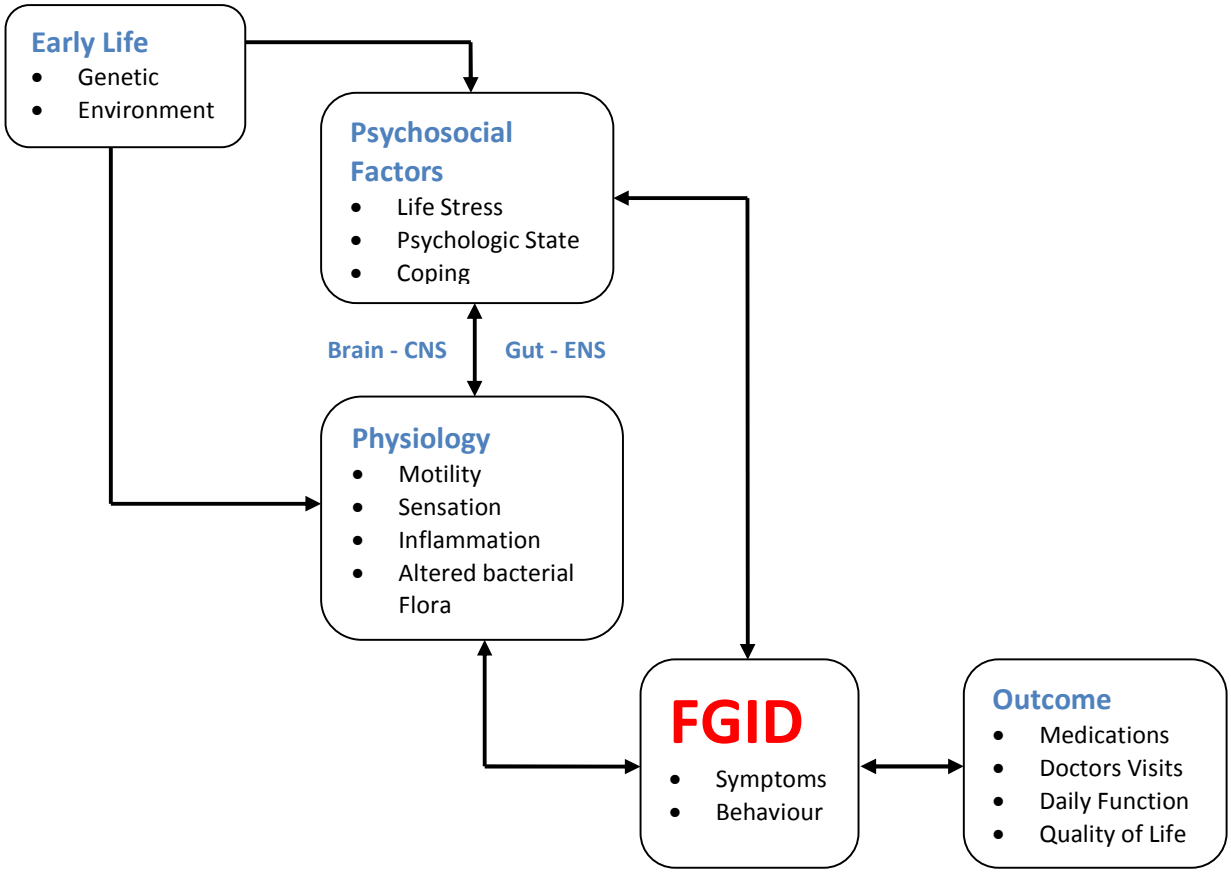
PATHOPHYSIOLOGY AND MANAGEMENT OF FUNCTIONAL CONSTIPATION

1.1 DEFINITION OF CONSTIPATION

Constipation is defined as defecation that is unsatisfactory because of infrequent stools, difficult stool passage, or seemingly incomplete defecation. Stools are often hard and dry, and may be abnormally large or small. Constipation is a subjective symptom and therefore attempts have been made to define constipation using objective criteria, most notably the Rome III criteria[1] (see below). Constipation is a common condition with an estimated prevalence of 12-19% in the US[2] and 29-35%[3,4] in Europe and the prevalence increases with age. The exact prevalence is difficult to determine due to significant underreporting by the population who often manage the condition with over the counter remedies and the differing criteria used to define constipation. The condition is commoner in women and the elderly and there is some correlation with low socioeconomic class. Constipation has a significant economic burden and in the US in 2004 was responsible for 6.3 million healthcare visits with a cost of $1.7 billion[5]. In England, in 2009 almost 14 million prescriptions were filled for laxatives, at a cost of almost £60 million[6].

1.2 FUNCTIONAL GASTROINTESTINAL DISORDERS (FGID) AND FUNCTIONAL CONSTIPATION

Constipation can be divided into two subtypes; functional or primary constipation and secondary constipation. Functional constipation is one of a collection of disorders known as functional gastrointestinal disorders (FGID) where there is no structural explanation to explain the patient's symptoms. There are significant psychosocial and physiological factors which interact to manifest clinically in FGIDs which are summarised in the figure 1.1. Gastrointestinal transit time is often delayed in healthy women around the time of the menses[7-9] and this has often been used to explain why FGID such as irritable bowel syndrome (IBS) and functional constipation are more prevalent in women. However, not all women are affected by FGID and therefore the sex hormones and their variation over the menstrual cycle cannot be the sole cause for the symptoms of FGID and are more likely a component of a multifactorial problem.



Constipation probably involves a combination of genetic, physiological, environmental, and psychosocial factors.

Figure 1.1 - The relationships between psychosocial and physiological factors, functional GI symptoms, and clinical outcome for FGID[14].

ROME III CRITERIA FOR FUNCTIONAL CONSTIPATION

1. Must include two or more of the following:
    a. Straining (during at least 25% of defecations).
    b. Lumpy or hard stools (on at least 25% of defecations).
    c. Sensation of incomplete defecation (on at least 25% of defecations).
    d. Sensation of anorectal obstruction/blockage (on at least 25% of defecations).
    e. Manual manoeuvres to facilitate defecation (on at least 25% of defecations).
    f. Fewer than three defecations a week.
2. Loose stools rarely present unless induced by laxatives.
3. Would not normally include patients who satisfy criteria for IBS-C

Genetic factors may predispose some patients to FGID by altering levels of neurotransmitters such as serotonin (5-HT) or noradrenaline with subsequent effects on gut motility. Functionally distinct alpha adrenoceptor and serotonin transporter polymorphisms are associated with constipation and high somatic symptoms in patients with lower FGID[10]. The finding that FGID cluster in some families and that 1st degree relatives often exist with similar symptoms to the patient[11-13] support the implication of genetic factors in the aetiology of FGID.

Whilst not specific to the FGIDs, psychological and social influences can affect the patient's perception of their symptoms and their subsequent outcome and health care use. Children learn behaviours from parents who have FGIDs which puts them at a higher risk of developing FGIDs than from genetics alone[15]. Environmental stresses, emotional and sexual abuse in childhood and adulthood also predispose to FGIDs, especially in women, with associated poor health-related quality of life and an increase in health care use[16]. There is also an increased prevalence of FGIDs in those patients who suffer with depression and/or anxiety compared to healthy controls[17].

1.2.1 FUNCTIONAL CONSTIPATION

In the absence of structural or metabolic causes to explain constipation, functional constipation is divided into three broad categories: (i) normal transit, (ii) slow transit, and (iii) evacuation disorders. There is however significant overlap. In a study of 1009 patients, 59% had normal colonic transit and normal pelvic floor function, 25% had pelvic floor dysfunction, 13% had slow transit constipation and 3% had both slow transit constipation and pelvic floor dysfunction[18].

1.3 INVESTIGATION OF FUNCTIONAL CONSTIPATION

In current clinical practice, investigation is limited to the assessment of intra-luminal transit and the assessment of pelvic floor function. The use of colonic manometry to assess contractile activity is slowly gaining clinical acceptance.

1.3.1 TRANSIT STUDIES.

Transit studies address the question: 'Does the patient have normal colonic transit?' Two techniques are widely used for the assessment of colonic transit time, namely: radio-opaque markers and radionuclide scintigraphy. The former involves the ingestion of radio-opaque markers and then assessing their progress through the GI tract by plain X-rays, and were first described by Hinton et al in 1969[19]. There are two widely accepted techniques. The first of which is the 'simple' radio-opaque marker test. A single gelatine capsule containing 20-50 markers is swallowed on day 0 and a single X-ray is taken on day 5. The 'multiple markers' study involves ingestion of a capsule on 3 consecutive days, each containing a different set of shapes, with a plain X-ray taken on day 5[9]. The simple method allows the distinction between normal transit and slow transit whereas the multiple marker study allows assessment of the residence times of the markers in defined colonic regions. This is of potential significance if segmental resection is to be contemplated (see 'surgical management of constipation', section 1.7.5, page 40).

The second method, radionucleide scintigraphy, assesses transit time by monitoring the progress of a radioisotopic chemical through the GI tract using a gamma camera and is based on a technique described by Krevsky et al[20]. Intra-luminal movement is expressed by calculating the geometric centre of the isotope mass with a low centre implying most of the marker is in the caecum, with a high value indicating that the marker has been expelled. The progression of the geometric centre over time can be calculated as the time the marker resides in specific areas of the colon. Radio-opaque markers are cheap, simple to perform and are widely available. Radionucleide scintigraphy allows for more precise quantification and is physiologically more accurate, but analysis is more complex and time consuming than radio-opaque markers, with the test often only available in specialist centres.

More recently techniques that use MRI have been reported which compare well with the radio-opaque marker studies but have the advantage of not using ionising radiation in a population of which a substantial proportion are women of child bearing age[21]. However it is not yet widely available and requires greater exertise that the traditional radio-opaque marker studies

1.3.2 ANORECTAL PHYSIOLOGY.

There are a number of methods for assessing anorectal structure and function, however in clinical practice this routinely consists of anorectal manometry (Figure 1.2) and defecating proctography.

1.3.2.1 Anorectal Manometry

Anorectal manometry is a widely available tool and encompasses a series of measurements designed to test for;

I. Deficits in anal sphincter function.

II. Presence or absence of rectoanal reflexes.

III. Rectal sensory function and compliance.

Testing allows evaluation of both incontinence and constipation and may be useful as an indicator for biofeedback, assessment prior to surgery, and as an objective measurement of treatment efficacy.

The equipment consists of four major components: a thin intraluminal pressure catheter, pressure transducers, a balloon for inflation in the rectum and an amplification-recording-display system[22]. Equipment must be accurately calibrated to yield both reproducible and accurate results. Unfortunately, the lack of consistency in equipment used and the technique performed makes comparison of results from different laboratories difficult and this is compounded by the lack of normative data stratified for age and gender[23,24]. A detailed protocol for the conduction of anorectal manometry is outlined below[24]:

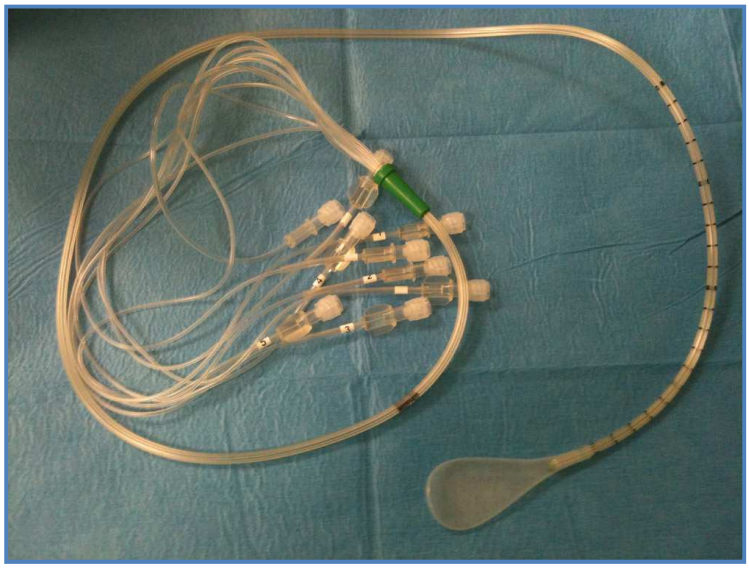
I.	**Patient preparation**	Bowel preparation and consent is optional. Patients should be placed in the left lateral position.
II.	**Probe placement**	Once the probe is inserted a rest period of 5 minutes should be allowed for the patient to relax and sphincter tone to return to basal levels.
III.	**Squeeze (Anal)**	For a maximum of 30 seconds, rest, then repeat.
IV.	**Cough reflex test**	Tests the reflex increase in anal sphincter pressure during abrupt changes in intrabdominal pressure.
V.	**Attempted defecation**	Assesses the rectal and anal sphincter response**.**
VI.	**Rectoanal inhibitory reflex**	The rectoanal reflex is the transient relaxation of the internal anal sphincter and contraction of the external anal sphincter on distension of the rectum. Functionally it allows the anorectum complex to assess the contents of the rectum. Rectal balloon is rapidly distended with 50 ml of air. The reflex is typically absent in Hirschsprung's disease.

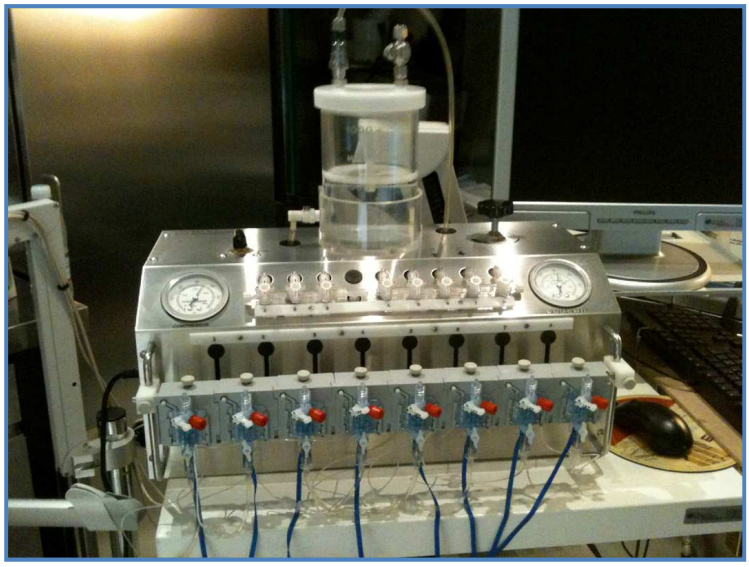
VII.	**Rectal sensation**	Intermittent balloon distension of the rectum to provide an assessment of rectal sensation, rectoanal inhibitory reflex and rectal compliance.
VIII.	**Balloon expulsion test**	Balloon is filled with 50 mls of water and expulsion is attempted (within 3 minutes).
IX.	**Simulated defecation**	Performed if the balloon expulsion test is abnormal and is an assessment in suspected pelvic floor dyssynergia.

Rao et al recommended that sufferers of constipation undergo resting pressure measurement, attempted defecation, assessment of the rectoanal inhibitory reflex and rectal sensation, and balloon expulsion[24].

1.3.2.2 Defecating Proctography

This involves the use of video fluoroscopy whilst the patient evacuates barium paste of stool consistency. Barium-soaked guaze may be inserted into the vagina and paste also added to the perineum to aid in assessing perineal descent and the anorectal angle. Furthermore the small bowel may be opacified with oral contrast to assess for enteroceles. Defecating proctography assesses anorectal structure but also gives real-time assessment of function. The most clinically important feature of the assessment is the duration and extent of evacuation rather than the presence of anatomical changes such as rectoceles. Normal evacuation should be 90% complete. If this is less then the presence of structural abnormalities are clinically significant[25].
Some centres use dynamic MRI rather than video fluoroscopy. Using modified sequences pelvic floor motion can be imaged by comparison at rest and on straining and has less interobserver error than barium studies whilst obviating the use of radiation. More recently the role of dynamic three-dimensional ultrasonography compares well with the other techniques described but is cheaper and better tolerated.





The catheter (diameter 0.5 cm) with a balloon at its tip is inserted into the rectum. The catheter has lateral openings arranged radially at the tip that detect the pressure exerted by the rectal wall. The principle on which this system is based is that the pressure exerted by the rectum on the catheter has an impact on the water column present along the capillaries up to the transducers, which detect the pressure and transmit it to the computer that determines the pressure profile.

Figure1.2 – Anorectal manometry catheter and transducer

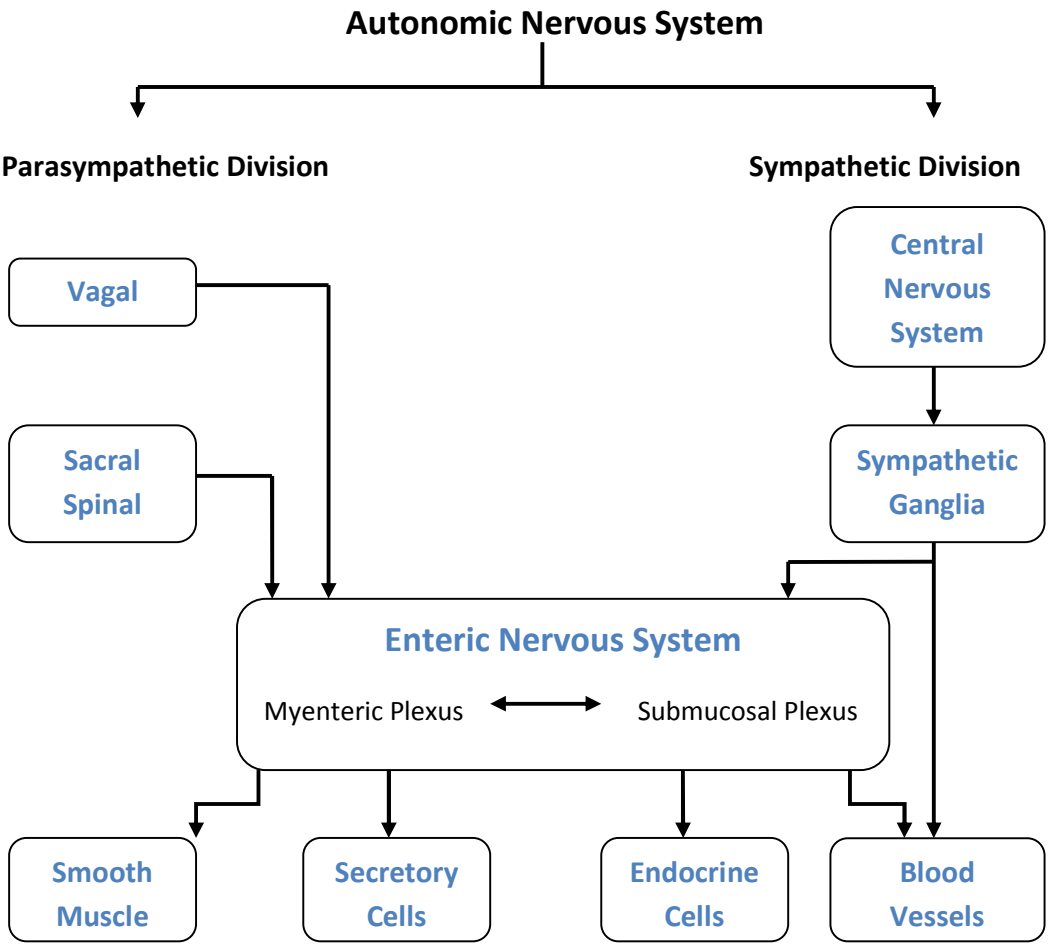
1.4 PHYSIOLOGY OF COLONIC MOTILITY

To understand the pathophysiology of functional constipation it is first necessary to understand the normal physiology of the colon. The principal function of the colon is to absorb water and electrolytes from small bowel contents to form solid faeces and to store and expel faecal matter. Effective function of the colon depends upon a functioning muscular system under enteric neuronal control with both sympathetic and parasympathetic modulation.

1.4.1 INNERVATION OF THE COLON

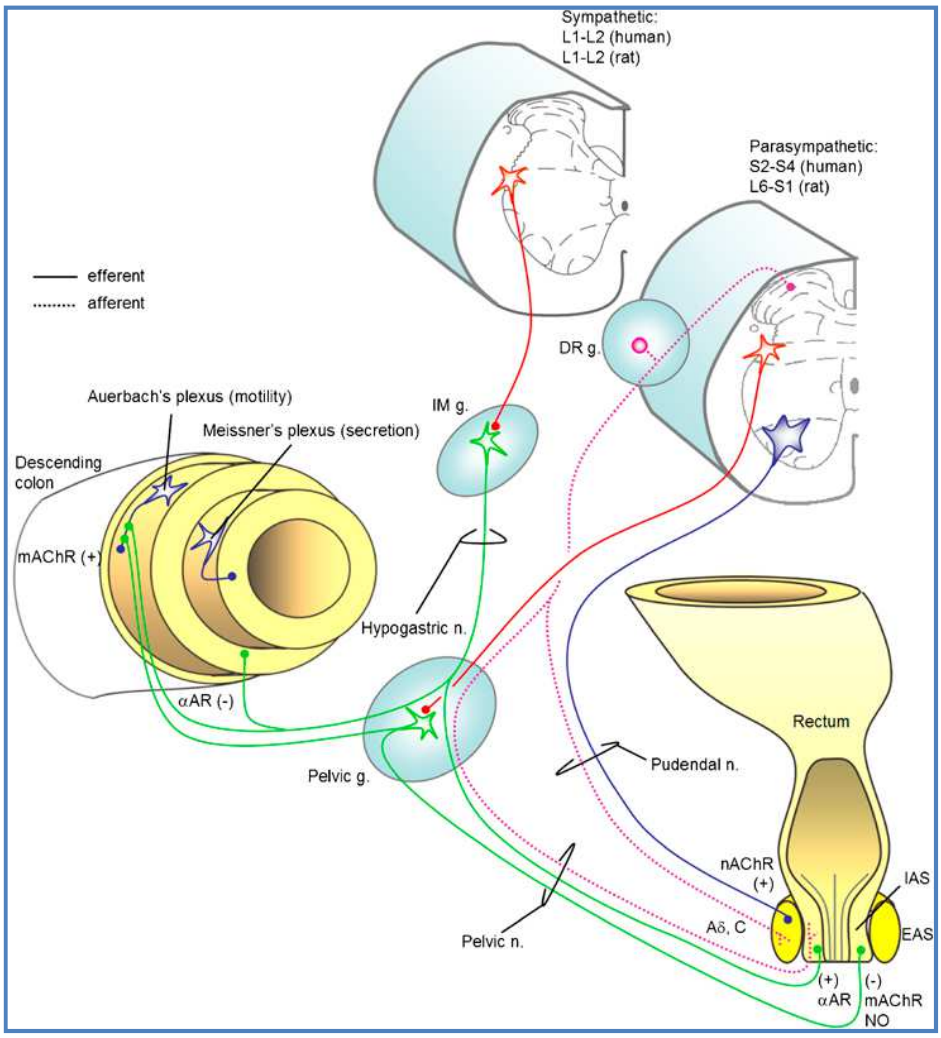
The colon is innervated by the sympathetic and parasympathetic nervous systems (extrinsic innervation) and the enteric system (intrinsic innervations) (Figures 1.3 and 1.4). The sympathetic fibres, via the splanchnic nerves (T5-L2), do not directly innervate structures in the GI tract but instead terminate on neurones in the intramural plexuses where they release noradrenaline. Noradrenaline inhibits colonic motility through the inhibition of smooth muscle contraction. Sympathetic fibres also cause vasoconstriction of mesenteric and submucosal blood vessels and modulate secretory activity. Parasympathetic neurones consist of both the vagus nerve and the pelvic nerves. The vagus nerve innervates the upper GI tract to the proximal half of the colon with the pelvic nerves (hypogastric plexus) supplying the distal colon and rectum. The parasympathetic system, in general, is responsible for an enhancement in colonic motility through an increase in excitability of enteric neurones via nicotinic cholinergic synapses.

However, the great majority of neural control of gut function is through the enteric nervous system. This lies entirely in the wall of the gut and is organised into the myenteric and submucosal plexuses. The myenteric plexus lies between the circular and longitudinal muscle layers, extending the length of the intestine. Stimulation increases muscular tone of the wall, peristaltic wave frequency and the intensity and rhythm of contractions. The submucosal plexus controls local secretory and absorptive activity. These plexuses consist of afferent and efferent fibres and ganglion cell bodies which are connected via interneurons. This allows



Dysfunction in these neurological pathways are likely to be involved in the aetiology of constipation through, as yet, undetermined mechanisms.

Figure 1.3 – The major features of the autonomic innervations of the GI tract. (Modified from Berne and Levy, 2005[26])



Abbreviations: Aδ, C, mechanosensitive primary afferents; αAR, α-adrenergic receptors; DR g, dorsal root ganglion; EAS, external anal sphincter; g, ganglion; IAS, internal anal sphincter; IM g, inferior mesenteric ganglion; mAChR, muscarinic cholinergic receptors; n, nerve; nAChR, nicotinic cholinergic receptors; NO, nitric oxide; (+) denotes excitatory synapses; (-) denotes inhibitory synapses. (Taken from Inskip et al[27])

Figure 1.4 -Innervation of the distal gastrointestinal (GI) tract.

coordinated activity in the absence of extrinsic innervations through the monitoring of wall tension and changes in luminal content by releasing numerous neurotransmitters from the enteric nerve endings. The principal excitatory agent is acetylcholine which acts through muscarinic receptors on smooth muscle cells whilst noradrenaline produces gut relaxation. Other neurotransmitters include dopamine, serotonin, vasoactive intestinal peptide, substance P, endorphins, Nitric oxide, carbon monoxide and amines. Gastrointestinal hormones also affect motor activity. The role of serotonin and endorphins will be discussed in more detail (see 'Management of Functional Constipation', section 1.7, page 28).

Extrinsic sensory innervation is split between the vagus nerve (proximal colon) and spinal afferent neurones (distal colon and rectum). These afferents may give rise to the pain felt from distension of the colon and are responsible for the conscious awareness of rectal distension, playing an important role in preserving continence.

1.4.2 COLONIC MOVEMENTS

Colonic movements are divided into mixing (segmental) and propulsive (peristaltic). Mixing movements occur mainly in the caecum and ascending colon. Approximately 2.5 cm of circular muscle contracts almost to the point of occluding the lumen and this is followed by contraction on the longitudinal muscle. These contractions occur for 30 seconds then disappear after 60 seconds and then reoccur nearby. The repeated action causes the faecal matter to be 'dug into and rolled over' exposing it to the mucosal surface. The second movement is propulsive and known as a 'mass movement'. These occur from the mid transverse colon to the rectum and consist of a wave of contraction. A constrictive ring appears in response to a distended or irritated point in the colon. Twenty or more cm distal to this there is contraction as a unit forcing the faecal matter in this segment en masse distally. The contraction lasts for 30 seconds and is then followed by 2-3 minutes of relaxation. There are then further mass contractions distally and this is continued for 10-30 minutes. This occurs every half to one day and when the rectum is full defecation occurs (see 'Normal Defecation', section 1.4.4, page 22).

1.4.3 COLONIC CONTRACTILE PATTERNS

To achieve colonic movements three complex motility patterns exist; segmental (non-propagating), propagated and rectal motor complexes.

1.4.3.1 Segmental activity

This is also known as low amplitude propagating contractions (LAPCs). This constitutes most of the overall colonic motility producing frequent, low pressure waves resulting in slow propulsion of faecal matter distally allowing optimal time for absorption of water, electrolytes, short chain fatty acids and bacterial metabolites[28]. The electrical activity promoting contraction of the colon is generated by the interstitial cells of Cajal (ICCs), an internal pacemaker within the GI musculature. These cells act as an electrical syncytium to allow action potentials and pacemaker potentials to spread via gap junctions between muscle fibres and accounts for the self-excitable characteristics of the muscle[29]. The action of the ICCs is blocked by inhibitory motor neurones to prevent disordered contraction of muscle, with the circular muscle only responding to the pacemaker when the inhibitory motor neurones are inactivated. In this way the slow peristaltic wave of contraction is regulated and ordered, forming the basis of colonic motility.

1.4.3.2 Propagated Activity

This is also known as high amplitude propagated contractions (HAPCs)[28]. HAPCs occur infrequently but are of a much higher pressure and correspond to the concept of 'mass movements'. These powerful contractions are strongly associated with defecation, preceding the expulsion of stools, and are considered one of the driving events of defecation[28,30].

1.4.3.3 Rectal motor complexes

Within the sigmoid and rectum periodic contractile activity predominates, although this activity can be recorded throughout the colon. These are known as the 'rectal motor complex'. They

consist of strong sustained contractions with frequencies of three to six a minute. They last for 3-30 minutes, occurring every 80-90 minutes in the day, and more frequently at night when there is less central nervous system influence. Their significance is unknown, but they are triggered by pressure waves from the proximal colon and by the arrival of stool or gas into the rectum, suggesting a role in defecation. Their number and frequency is reduced in women with slow transit constipation (see 'Pathophysiology of Functional Constipation', section 1.5, page 23).

1.4.4 NORMAL DEFECATION

The process of normal defection is initiated by rectal distension and occurs involuntarily in response to faeces filling the rectum. Passive distension occurs until the threshold is reached whereby conscious awareness is triggered. As the rectum continues to fill the urge to defecate increases. Distension of the rectum causes it to contract, the internal anal sphincter (IAS) to relax, and the external anal sphincter (EAS) to contract. This allows passage of rectal contents into the upper anal canal where their nature can be determined.

For defecation to occur the intrarectal pressure must exceed the anal canal pressure and this is achieved by relaxation of puborectalis resulting in straightening of the anal canal and descent of the pelvic floor, combined with relaxation of the EAS. The valsalva manoeuvre assists with defecation to a variable extent by increasing intrarectal pressure. Contraction of the colonic and rectal smooth muscle is necessary to expel the stool. This 'mass movement' correlates with an increase in HAPCs which have been clearly associated with defecation and faecal expulsion[30,31]. There is also an increase in the frequency and amplitude of LAPCs prior to defecation[30].

Once the faeces have been expelled the puborectalis contracts restoring the anorectal angle and the EAS temporarily contracts. This is joined by contraction of the IAS and passive distension of the anal cushions to close the anal canal and maintain continence.

Disturbances of these physiological processes may result in the symptoms of constipation and are discussed next.

1.5 PATHOPHYSIOLOGY OF FUNCTIONAL CONSTIPATION

The pathophysiology of functional constipation is poorly understood. However, there are demonstrable differences in both histology and physiology between normal volunteers and sufferers of chronic constipation which allows a possible mechanism be formulated to explain the pathophysiology of functional constipation.

1.5.1 ABNORMALITIES IN HISTOLOGY

Changes in histology in patients with functional constipation allow an understanding of the possible mechanisms involved.

1.5.1.1 Conventional histology

Routine light microscopy of the ENS using Hematoxylin and Eosin staining (H&E) has not revealed any marked differences between the normal colon and that of sufferers of STC except for the presence of melanosis coli, a black/brown discolouration of the colonic mucosa. This condition is related to the use of anthraquinone laxatives rather than abnormalities of the ENS[32].

1.5.1.2 Silver Staining techniques

The addition of silver as a stain to highlight proteins has revealed that colons from STC sufferers have neuronal and axonal abnormailites, with less argyrophilic neurones in the myenteric plexus. Preston et al[33] reported that this occurred in 90% of colons from patients with STC, findings which were confirmed by a semi-blind, controlled trial of 12 patients[34].

1.5.1.3 Immunohistochemistry (IHC)

The use of IHC combined with neuronal markers has given conflicting results with some reports noting no change in neuronal numbers, whilst others demonstrating a reduction. These differences probably represent a lack of homogeneity in sample selection[35].

1.5.1.4 Glial Cells

Enteric glial cells (EGC) make up 80% of the ENS with the remainder comprising the enteric neurones. Whilst first thought of as supportive tissue for the neuronal elements it has since been discovered that they have specific functions in maintaining ion and neurotransmitter concentrations and thereby promote the health of neurones. This is supported by the finding that if the glia is disrupted there is neuronal degeneration[36].

The role of EGC in the aetiology of STC was demonstrated by Bassotti et al[37] who, in a controlled study of 26 patients who underwent surgery for STC, showed a significant reduction in EGC cell number in the myenteric and submucosal plexuses. There was also a decrease in enteric neurones and ICCs. Further work by Bassotti et al[36] reported a similar loss of EGC, but not of ICCs, in patients with obstructed defecation. There is no clear hypothesis to explain the reduction in EGC number and hence it is uncertain whether the decrease in cell number is the cause of the functional constipation or the result of it. A reduction in gilal cells is seen in other gastrointestinal conditions associated with constipation that have different pathophysiologies to FC suggesting that the loss of EGC is secondary to a reduction in transit time[38-40]. This reduction in EGC number could be explained by the change in luminal content associated with prolonged transit which has been demonstrated to modulate the EGC population[41]. Therefore whilst it is clear that a reduction in EGC number is associated with FC, further work is needed to fully explain their role, if any, in the pathophysiology of the disease.

1.5.1.5 Interstitial Cells of Cajal

ICCs are important in controlling gastrointestinal motility and thus fewer numbers could be an aetiological factor in reduced colonic motility and functional constipation. The first study to demonstrate such a reduction in patients with functional constipation revealed that whilst the

ICCs were present in the same layers of the sigmoid colon, the number was significantly reduced[42] (see table 1.1). This was associated with a blunting of ICC processes and a reduction in neuronal structures compared to healthy controls. Results have been confirmed by subsequent studies[43,44]. One study[45] was unable to detect any difference between controls and sufferers of constipation, but this may be explained by the methodology used. Here the samples were formalin fixed and paraffin embedded and this leads to an underestimate of ICC numbers compared to fixation in paraformaldehyde solution[46].

Despite good evidence to support the hypothesis of reduced numbers of ICCs in functional constipation it is unclear what causes the decrease and the associated loss of enteric nerves. Whether a common factor is responsible or whether one cell type is dependent on the other is unknown.

1.5.2 PHYSIOLOGICAL DIFFERENCES IN FUNCTIONAL CONSTIPATION

Physiological abnormalities in functional constipation have been demonstrated by colonic manometry. Developments in fibre-optic sensing technology have seen a large increase in the number of sensors from 6-10 @ 7-15 cm intervals in solid-state colonic catheters to up to 120 sensors @ 1cm intervals. This has allowed more detailed recording and analysis to be undertaken.

1.5.2.1 Non-propagating motor activity (Segmental activity) (LAPCs)

These make up the bulk of colonic activity and are thought to be associated with mixing and propulsion of colonic contents. In constipated subjects these are increased in the rectum and sigmoid[47,48], and decreased more proximally[49], and this uncoordinated activity distally may impede propulsion.

Layer of sigmoid colon	Control		Functional Constipation	
	He et al[42]	Lyford et al[43]	He et al[42]	Lyford et al[43]
Longitudinal Muscle	5.5% +/- 0.7	4.7% +/- 0.4	1.5% +/- 0.2	0.8% +/- 0.3
Myenteric Plexus	21.3% +/- 1.9	20.3% +/- 1.8	7.9% +/- 1	8.0% +/- 1.4
Circular Muscle	7% +/- 1.3	4.4% +/- 0.4	2.5% +/- 0.3	2.6% +/- 0.4
Submucosa	10% +/- 1.4%	6.3% +/- 1.0	2.8% +/- 0.7	2.0% +/- 0.7

ICCs are important in controlling gastrointestinal motility and thus fewer numbers could be an aetiological factor in reduced colonic motility and functional constipation. ICC are decreased in all layers of the colonic wall in sufferers of functional constipation compared to healthy controls but it is unclear what causes this decrease in cell numbers.

Table 1.1 – c-Kit positive immunoreactive structures in the sigmoid colon in functional constipation and healthy controls as a percentage of total volume.

1.5.2.2 Propagating motor activity (HAPCs)

HPACs are associated with powerful contractions stimulating luminal transit in the colon, which are decreased or absent in functional constipation. Bassotti et al[49] demonstrated that in slow transit constipation the average number per subject per day was significantly decreased compared to controls (0.62 +/- 0.2 vs. 5-5 +/- 0.78, $p<0.001$), but there were no differences in HPAC amplitude. A similar finding demonstrated a reduction from 6 a day in healthy volunteers to 1.9 in sufferers of functional constipation[50]. Along with a decrease in frequency and the amplitude of HPACs, Dinning et al[51] have also demonstrated a reduction in the linkage of HPACs in an organised spatiotemporal pattern throughout the colon. This linkage of HPACs along the bowel ensures that they span the entire colon and are important for the transport of contents over longer distances.

1.5.2.3 Meal Response and Diurnal Variation

In healthy individuals there is an increase in the frequency of HPACs in response to a meal[52] and these are reduced or absent in functional constipation[53]. Colonic motor activity at night is suppressed[54] and early morning waking results in an increase in colonic activity in the healthy. This circadian pattern is altered in patients with functional constipation, with a general decrease in contractile activity throughout the day but normal activity at the night. There is an absent response to early morning waking[54]. This lack in diurnal variation, probably mediated via the CNS in the healthy, may support a neuropathic cause for the symptoms of sufferers of FC.

1.6 HYPOTHESIS TO EXPLAIN FUNCTIONAL CONSTIPATION

From the evidence above, it can be proposed that some sufferers of functional constipation have histological abnormalities in the enteric neuronal networks leading to a decrease in propagating colonic motor activity promoting the symptoms of constipation. However, this hypothesis does not explain whether the histological abnormalities are inherited or acquired or whether they are the cause of FC or the consequence of it. Further work is needed to fully understand the pathophysiology of this complex and probably multi-factorial disease.

1.7 MANAGEMENT OF FUNCTIONAL CONSTIPATION

The management of functional constipation can be divided into six categories;

I. lifestyle modifications.
II. biofeedback .
III. conventional pharmacological interventions.
IV. sacral nerve stimulation.
V. surgery.
VI. novel pharmacological interventions.

1.7.1 LIFESTYLE MODIFICATION

There is a commonly held belief that increases in dietary fibre, water, and exercise improves the symptoms of constipation. These are often indicated as first line treatments but there is little clinical evidence to justify their use. Stool from sufferers of constipation has less water compared to healthy volunteers[55], thereby changing its consistency by increasing the water content may alleviate the symptoms of constipation. A clinical study by Anti et al[56] reported an improvement in those patients who increased their fluid intake to more than 2 litres a day compared to controls. However, the water group drank mineral water containing magnesium and this may have actually been responsible for the symptomatic improvement. Lindeman et al[57] and Chung et al[58] did not demonstrate an increase in stool output with fluid consumption of greater than 2 litres supporting the earlier results of Klauser et al[59]. They demonstrated that

a fluid restriction of less than 500 mls was associated with a decrease in stool volume and therefore recommended that low fluid intakes should be corrected. Therefore an increase in fluid intake over 2 litres is unlikely to improve symptoms of constipation and this is supported by two recent reviews[60,61].

Fibre supplementation decreases colonic transit time, holds water within the stool, and increases stool weight, resulting in reduced intracolic pressures and softer stools[62-64]. This is felt to benefit constipation sufferers even though there is no difference in the amounts of dietary fibre in their diets compared to controls[65]. Evidence for the use of bran comes from studies in healthy volunteers and although a meta-analysis by Muller-Lissner[66] confirmed the above effects of fibre in healthy volunteers, it did not show that bran was an effective treatment for constipation. Badiali et al[67] failed to show an improvement with bran compared to placebo and two other randomised studies reported minimal[68] or absent[69] effect on stool frequency and moisture. These findings may be explained by the fact that increasing dietary fibre is associated with bloating and flatulence and therefore some constipated patients may not ingest enough to alleviate their symptoms[70]. Probably it is more likely that in but all of the most mild cases, constipation has a more complex aetiology than simply a lack of fibre. Therefore it would be appropriate to recommend fibre to patients with a diet in which it is lacking but should not be recommended to patients with functional constipation with normal fibre consumption as it is unlikely to benefit and may indeed exacerbate symptoms.

The effect of exercise on colonic transit times is unclear. Studies in healthy volunteers have shown that exercise both increases and decreases colonic motility, but the discrepancies may be related to the existing fitness of subjects and the intensity of exercise prescribed[71-73]. In one study regular exercise did not improve the constipation indices in sufferers of constipation leading the authors to conclude that 'regular exercise does not play a role in the management of chronic constipation'[74]. Despite the lack of evidence to confirm exercise as an effective treatment it should still be recommended because it improves general quality of life and has other beneficial health effects including improved cardiovascular health and prevention of obesity and osteoporosis.

1.7.2 BIOFEEDBACK

The aim of biofeedback is for the patient to learn to relax the pelvic floor muscle and respond appropriately to physiological stimuli. The steps involved in biofeedback vary depending on the clinical laboratory but there is a growing consensus that six sessions of 45 minutes each are required, led by well trained and experienced therapists to produce clinical benefits. Patients must be motivated to increase their chances of a response.

Whitehead et al[75] recommended in a review article that biofeedback should consist of the following steps:

I. Patient education – explanation of normal defecatory physiology and what the patient may be doing incorrectly.

II. Straining Training – Patient is taught to increase intra-abdominal pressure when attempting to defecate. Feedback on rectal balloon pressures or abdominal wall EMG during straining.

III. Pelvic Floor Relaxation training – through the use of electronic feedback on anal canal pressures or pelvic floor EMG, the patient learns to relax the pelvic floor muscles whilst straining abdominal muscles.

IV. Stimulated Defecation – patient practice to evacuate a water-filled rectal balloon whilst the therapist slowly withdraws it from the rectum. The amount of assistance provided is decreased as the patient relearns the sensations associated with defecation.

V. Sensory Retraining – by distending a rectal balloon above and below the pressures needed to elicit the sensation of defecation the patient can be taught to identify weaker sensations for defecation.

The use of visual stimuli during biofeedback has been debated. Koutsomanis et al[76] studied 60 adult patients referred for biofeedback. Subjects were randomised to 'muscular coordination

training' (MCT) or to biofeedback with the use of EMG tracing. Both groups had significant improvement after the treatment periods in physiological parameters and subjective measurements and these responses were similar. The MCT group needed slightly fewer sessions to achieve adequate training. This treatment does not require a physiological laboratory, is less expensive than biofeedback treatment and can be done by paramedical personnel.

Biofeedback is effective in the management of constipation secondary to pelvic floor dyssynergia but debate exists over its role in the management of slow transit constipation. Heymen et al[77] demonstrated that patients with pelvic floor dyssynergia who underwent biofeedback had significantly more unassisted bowel movements at follow-up compared to patients in the placebo group and those treated with diazepam. Rao et al[78] undertook a randomised controlled trial comparing biofeedback against both sham feedback (placebo) and standard constipation treatments. This demonstrated that biofeedback was more likely to correct dyssynergia and produced more complete spontaneous bowel movements compared to other groups. The colonic transit time was also improved in those patients who underwent biofeedback and this finding was supported by the work of Wang et al[79] and Chiotakakou-Faliakou et al[80]. Generally biofeedback is of benefit in all patients who suffer with functional constipation. However, the improvement of transit times in these studies may be due to the treatment of the pelvic floor dyssynergia (PFD) rather than direct effects on colonic motility, i.e. these patients may not suffer with true slow transit constipation, but it may be secondary to pelvic floor dyssynergia. This view is supported by results of a randomised controlled trial of 52 patients with slow transit constipation on transit studies[81]. These were divided into three groups based on their response to anal manometry and balloon defecation testing. The three groups consisted of patients with STC only (n=12), STC and PFD (n=36), and a group with STC and an element of PFD who did not meet the criteria for a diagnosis of PFD (n=6). After a course of biofeedback there was significant improvement in those patients with PFD compared to their baseline measurements and when compared to the STC only group. Satisfaction was reported by 71% of the PFD group and 76% had greater than 3 bowel movements per week compared to 8% and 8% in the STC group ($P < 0.001$). Whilst there was no improvement noted in the STC arm in any of the outcomes measured, the improvements in the PFD group were maintained after 24 months of follow-up. This led the authors to conclude that biofeedback is only successful in PFD and that transit studies are unreliable in patients with PFD, as the delay

is most likely secondary to PFD. Further work is needed to better understand the pathophysiology of functional constipation and a recent study by Singh et al[82] attempted this. Using 24 h ambulatory colonic manometry, sufferers with slow transit constipation were classified as having normal manometry, a colonic myopathy or a colonic neuropathy. Clinical response to pharmacological, biofeedback and surgical management at 1 year was correlated with manometric findings. This reported that 65% of patients with colonic myopathy or normal manometry, versus 15% with colonic neuropathy, improved with medical/biofeedback therapy ($p<0.01$). Almost three-quarters of the study population had either normal manometry or a colonic myopathy. Given the cost, time, and facilities needed for anorectal manometry and the potential embarrassment to the patient it seems reasonable to perform biofeedback therapy on all patients referred to secondary care who have failed simple medical management.

1.7.3 CONVENTIONAL PHARMACOLOGICAL INTERVENTION

Pharmacological intervention can be divided into the traditional laxatives and the newer pharmacological agents whose mode of action is through influencing colonic receptors. Currently these are principally 5-HT_4 agonists, Chloride (Cl^-) channel blockers, opiate receptor antagonists and more recently Guanylate Cyclase C (GC-C) activators.

There are a number of traditional laxatives (listed below) that are available over the counter. The evidence to support their use is often limited and rarely derived from randomised, double-blind, placebo-controlled trials.

Bulk agents	e.g. Bran, Ispaghula Husk, Psyllium Husk (Fybogel).
Emollient Stool Softeners	e.g. Docusate Sodium, Arachis Oil.
Stimulant Laxatives	e.g. Senna, Sodium Picosulphate (SPS) and Bisocodyl (Dulcolax).
Osmotic Laxatives	e.g. Lactulose, Polyethylene Glycol (Movicol).

1.7.3.1 Bulk Agents

Their mechanism of action is through increasing stool weight and water-absorbent properties of the stool with a resultant improvement in stool consistency. Of three placebo-controlled trials of bulk laxatives[83-85] two reported an improvement in stool frequency, consistency and ease of evacuation[83,84], whilst one failed to show this, but noted a trend towards an increase in stool frequency[85]. The study by Ashraf et al[84] also measured transit time with colonic manometry and found that psyllium husk did not alter the colonic transit time and proposed that its' primary action was through facilitating the defecatory process.

Psyllium husk has been compared to other traditional treatments with a mixed response. A study by Attaluri et al[86] compared psyllium with dried prunes in an 8 week single-blind, randomised cross-over study with the subjects using each treatment for 3 weeks with a 1 week washout period. The authors demonstrated that prunes resulted in a significant improvement in complete spontaneous bowel movements (CSBM)/week (3.5 +/- 0.2 vs. 2.8 +/- 0.2, p= 0.006) and stool consistency scores compared to psyllium (3.2 vs. 2.8, p=0.02). A CSBM is defined as a spontaneous bowel movement (SBM) with a complete sense of evacuation. However, this cross-over study was flawed by its short duration of 3 weeks in each arm as it has been proposed that psyllium takes at least 14 days to have any beneficial effect. When compared to lactulose[87] and sodium docusate[88] psyllium improved the symptoms of constipation, which was enhanced with the addition of senna compared to psyllium alone[89]. These studies are all limited by their short trial periods of a maximum of 4 weeks and as such there is little evidence on the long-term efficacy of bulking agents. Common side effects demonstrated in all studies were flatulence, bloating and abdominal distension, and very rarely colonic obstruction.

The role of bran has been described earlier but there is little evidence to support its current usage in anything but the mildest forms of constipation.

1.7.3.2 Stool Softeners

Stool softeners are anionic surfactants of which sodium docusate is the most commonly used. They allow water to interact more effectively with solid stool thereby softening it. Their efficacy relates to their ability to bind to stool and they often only have a modest effect. There

is limited data to support their use. Whilst one placebo controlled trial showed a benefit from docusate in improving stool frequency[90] a second study did not support these findings[91]. A further single blind study[92] compared Docusate at two strengths and was only able to show a non-significant slight increase in stool frequency compared to placebo. However stool softeners have minimal adverse events and are therefore often combined with other laxatives.

1.7.3.3 Stimulant Laxatives

These agents stimulate the colonic myenteric plexus to increase peristalsis and subsequent colonic motility[93], but also activate sensory nerve endings with the unwanted effect of colic. They may also act by inhibiting water absorption by the colon. In practice they consist of senna, bisocodyl and sodium picosulphate (SPS). Bisocodyl and SPS are prodrugs which are converted into the same active metabolite and will be discussed together.

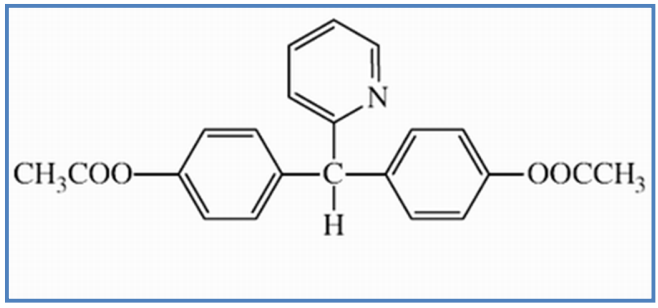
1.7.3.3.1 Senna

Senna and its main active metabolites, the sennosides, are effective in the treatment of functional constipation, but there are no double-blind, placebo-controlled trials to support its use. Senna has been shown in elderly residents, in long-term hospital or nursing home care, to be an effective treatment[94,95] with an improvement in stool consistency and ease of evacuation, whilst being well tolerated. However, in both of these studies senna was combined with fibre and it therefore cannot be determined what effect the senna contributed to. The beneficial effect of combining senna with psyllium was investigated earlier when Martlett et al[89] compared psyllium and senna with psyllium alone. Whilst both groups demonstrated a subjective relief from the treatments there was a significant improvement in objective measures (attributed to an increase in water content of the stool) from the combined preparation compared to psyllium alone. When senna was compared directly to lactulose in a multicentre trial[96] lactulose resulted in 58% of patients passing a normal stool by day 7 compared to 42% in those taking senna ($p=0.04$), with a prolonged carry-over effect in the lactulose group.

Senna, although well tolerated, may cause melanosis coli[91] and concern has been raised about the incidence of carcinogenicity and cathartic colon following prolonged administration[98,99]. However, Morales et al[100] were unable to establish the following from a review of the current literature:

I. Any convincing evidence to suggest that chronic use of senna resulted in structural +/- functional alteration of enteric nerves of the smooth intestinal muscle.
II. A link between senna extract and GI tumours in rats, even after a 2 year daily dose.

1.7.3.3.2 Bisacodyl/Sodium Picosulphate (SPS)



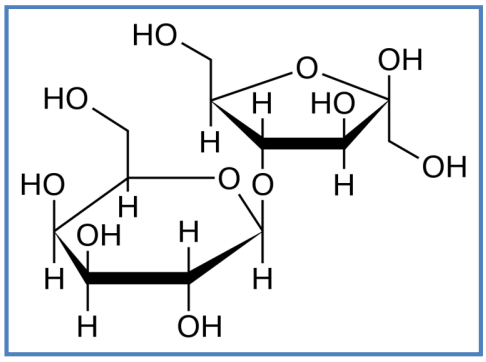
The evidence for the use of bisacodyl and SPS is better than that for senna. A double-blind, randomised controlled trial[101] demonstrated that bisacodyl significantly improved the mean number of stools per day (1.8 vs 0.95, p=0.006) and also stool consistency whilst being well tolerated. The study was limited by its short duration of only 3 days and therefore information on long-term efficacy and safety were not assessed. Importantly, the participants were recruited from primary care and while efficacy is confirmed in this group it is uncertain whether the results are applicable to patients treated in either secondary or tertiary care who have more severe symptoms. This study does support the use of bisacodyl as a bowel preparation in procedures such as colonoscopy and as a rescue treatment in other constipation treatement trials[102,103]. A further randomised, double-blind, placebo-controlled study recruiting 368 patients in 2011[104] confirmed the short-term effects seen by Kienzle-Horn et al[101] and proved that they continued over a 4 week period, with a significant improvement in overall

PAC-QOL score, whilst remaining well tolerated. Two randomised, double-blind, placebo-controlled trials for SPS[105,106] have also confirmed an improvement in stool frequency and consistency over placebo. The results of Wulkow et al[105] are again limited by the short trial period of 3 days, but the study by Mueller-Lissner[106] involved 367 patients and ran for 4 weeks. This confirmed that SPS treatment was also associated with a significant increase in quality of life and was well tolerated. There was no clinical difference between bisacodyl and SPS with both treatments being well tolerated and effective[107] with abdominal pain and diarrhoea reported as common side-effects.

1.7.3.4 Osmotic Laxatives

Osmotic laxatives retain water in the lumen since the laxative contains poorly absorbed ions or molecules which create an osmotic gradient within the lumen of the colon promoting water retention. This leads to softer stools and improved propulsion. Both lactulose and polyethylene glycol (PEG) have been shown to accelerate colonic transit but this effect is greater following administration of PEG[108] (see section 1.7.3.4.3 for further discussion).

1.7.3.4.1 Lactulose



Lactulose has long been used to treat the symptoms of constipation and its therapeutic effect is dose dependent[109,110]. Despite lactulose having a proven benefit over fibre[111] it performs less well compared to bulk laxative combined with senna[94,95,112] with no difference compared to sorbitol[113].

1.7.3.4.2 Polyethylene Glycol

Of all the conventional laxatives PEG has been the most extensively studied in five randomised, double-blind, placebo-controlled studies[114-118]. In each study stool frequency and consistency was significantly improved. PEG was more effective than either tegresod ($5HT_4$ agonist – see later) with a better side effect profile[119] and isphaghula husk[120]. In an open label study conducted over 12 months, PEG was shown to be safe with continued efficacy[119]. Similar to other laxatives, PEG is associated with diarrhoea and loose stools, abdominal distension, flatulence, and nausea. The incidence of GI symptoms in the trials ranged from 12-40% and although they were usually rated from mild to moderate, the withdrawal rate varied between 0-7%.

1.7.3.4.3 PEG vs Lactulose

When compared to lactulose, a Cochrane review[121] presented data from all ten RCT and concluded that PEG had better outcomes of stool frequency/week, form of stool, relief of abdominal pain and need for additional products compared to lactulose. This was seen in both adults and children and is not associated with the osmotic load of lactulose and PEG[108]. It has been proposed that both the metabolism of lactulose by the colonic microbiota resulting in a lower osmotic effect and the production of short chain fatty acids after lactulose metabolism, which inhibits colonic transit, are the reasons for the reduced clinical response of lactulose compared to PEG[108]. PEG has also been shown to be more cost-effective than lactulose with a greater likelihood of both treatment success and quality-adjusted life years[122,123].

1.7.4 SACRAL NERVE STIMULATION (SNS)

SNS has long been used as a treatment for neurogenic bladder dysfunction and faecal incontinence and it was through its use in the management of neurogenic bladder dysfunction that a role in functional constipation was noted. SNS is still in its infancy and there is a paucity of robust clinical trials with the evidence based on prospective studies with small numbers and a few randomised controlled trials. An example is shown in figure 1.5.

The initial small prospective studies reported mixed success with rates ranging from 25%[124] to 75%[125]. Both studies had small numbers (8 and 12 respectively) and therefore any benefits

could be due a placebo effect. There has been only one, small, double-blind placebo-controlled crossover study published by Kenefick et al[126]. Two women with severe resistant functional constipation were managed successfully with SNS. During this time, the stimulators were switched 'on' and 'off' for two two-week intervals to which the patient and investigator were both blinded. When the stimulator was switched off, bowel frequency, abdominal pain and bloating all reverted to baseline levels before the stimulators were inserted, suggesting that benefit patients derived was from sacral nerve stimulation alone.

Further evidence has supported the use of SNS. Kamm et al[127] undertook a large multi-centre prospective study which recruited patients with functional constipation of various aetiologies. Here 45 of the 62 (73%) patients recruited gained benefit from temporary SNS and therefore underwent permanent sacral nerve stimulator implantation. Treatment success was achieved in 39 patients (89%) after a median follow up of 28 months (1-55 months). This study demonstrated SNS to be effective and that the effects were long-lasting. Govaert et al[128] reported on 117 patients who were eligible for trial of a SNS. This retrospective review of prospectively collected data demonstrated a 58% (68 patients) improvement in symptoms after the insertion of the temporary SNS. This benefit continued after a permanent SNS was inserted in 61 of these 68 patients (90%) with a median follow-up of 37 months (range 4-96 months). However, not all studies report a benefit. One by Holzer et al[129] reported that temporary SNS was successful in 42% of patients (n=8/19) and that the benefit continued after permanent SNS in the first month after implantation. However after 12 months only 5 patients derived benefit and on an ITT basis this equated to 25% compared to 63% (39/62)[127] and 52% (61/117)[122] in other trials. This lower ITT figure was recently shown by Ortiz et al[130] in their retrospective review where 29% of patients (n=14) still had a successful outcome a median of 2 years after insertion (range 6-96 months) with 6 of the 14 patients (43%) still requiring the use of laxatives. This discrepancy in the results reporting the success of SNS is unsurprising given the multifactorial nature of functional constipation and the heterogeneity between subjects recruited into the various studies and the outcome measures used.

SNS is not without adverse events. Maeda et al[131] reported that 22 adverse events in 38 patients whilst Kamm et al[127] reported 101 adverse events in 48 patients. Furthermore Maeda et al[132] reported adverse events in 150 of 176 patients (85%) who had a SNS for faecal

SNS has long been used as a treatment for neurogenic bladder dysfunction and faecal incontinence and it was through its use in the management of neurogenic bladder dysfunction that a role in functional constipation was noted. A sacral nerve stimulator is inserted under general anaesthetic and activates the S3 nerve root to exert its effect.

Figure 1.5 - A sacral nerve stimulator (Image used with permission from Medtronic Inc, USA)

incontinence. The majority of adverse events in these 3 trials were related to a loss or lack of efficacy (up to 66%) with the next most frequently reported complication being pain or discomfort which may have required explantation of the stimulator.

There is also limited data on the precise mechanism of SNS. A study by Dinning et al[133] utilised pancolonic manometry to demonstrate that the clinical improvement that patients noticed correlated with an increase in antegrade and retrograde PS frequency and HAPCs frequency and amplitude in an antegrade direction. Further work by the same author[134] confirmed the ability of SNS to stimulate HAPCs but only at a suprasensory level (i.e. a voltage above that perceived by the patient) and not at a subsensory level. This important point needs to been taken into account in study design to avoid inappropriately misinterpreting SNS as ineffective.

SNS appears to be a promising treatment modality in patients who have failed conventional treatment. However, further RCTs are needed to confirm this and also to identify which patients SNS will be useful for. Further research needs to be done to understand the mechanisms by which SNS brings about symptomatic improvement. SNS, however, has the advantage over colonic resection since it can be reversed and although the adverse events are common they are not as significant has those associated with surgery (see next section). Although it is not always locally available, it should therefore be considered prior to colectomy.

1.7.5 SURGERY

Surgery is often the last clinical option in the management of functional constipation. The two commonest procedures are either subtotal colectomy with ileorectal anastomosis (STC + IRA) or segmental colectomy. Although data are available for both techniques from a number of studies, comparison between these is difficult due to different selection criteria and outcome measures used. This has led to wildly different success reported for colectomy in review articles ranging from 40% to 100%[135,136]. Irrespective of the surgical procedure, patient selection is critical to good success rates.

1.7.5.1 STC + IRA

STC +IRA increases bowel frequency but there is debate as to whether this actually translates into an increase in quality of life. Studies are often of a small size and are of retrospective or prospective design with no randomised controlled trials published to date. Following surgery the incidence of diarrhoea (>3 bowel movements/day) ranges from 46% -100%[137-140] with incontinence being a significant problem in up to 50% of patients who report diarrhoea[137]. Despite this, Quality of life (QoL) assessments in patients post-operatively report an improvement comparable to healthy subjects[139,140]. STC+IRA, however, does not always improve abdominal pain and bloating with some studies reporting post-operative incidences up to 40%[137,138]. Furthermore, open resection is associated with a mortality rate of 1% and morbidity secondary to; anastomotic leak (6%), revision of the anastomosis or further resectional surgery (7%) and adhesional small bowel obstruction (20%)[137,138]. One study, with data different from others, demonstrated a mortality rate of 15% and 32 surgical interventions performed in the follow-up period on the 20 original patients. The majority of these were for adhesional small bowel obstruction[141]. The authors acknowledged that the inclusion of patients who had had previous abdominal surgery may bias the results but state that the high morbidity and mortality coupled with the poor outcomes in their 20 patients did not support the use of STC+IRA for the management of constipation. Laparoscopic surgery has not reduced early post-operative complications and does not appear to lower re-operation rates for adhesional small bowel obstruction, but the follow up time and patient numbers are small[142,143].

1.7.5.2 Segmental Resection.

Segmental resection, tailored to segmental transit time measurements, aim to reduce the unwanted side effects of faecal incontinence and watery diarrhoea. Kamm et al[144], with just 2 patients, demonstrated good functional outcomes after 3 years following segmental resection. There have been subsequent larger studies since with encouraging results. De Graaf et al[145] compared tailored left hemicolectomy with STC and IRA with mixed results. Whilst the segmental group had a lower incidence of constipation and abdominal discomfort, the incidence of diarrhoea and faecal incontinence was equal in both groups. You et al[146] reported

in a cohort of 40 patients the efficacy of segmental resection. All patients had left or right side delay with normal transit at the sigmoid and rectum. At 3 months all had improvements in the symptoms of constipation with 93% (37/40) remaining well at 2 years. Of the 3 patients in whom the segmental resection was unsuccessful, STC and IRA was performed with good outcomes reported at 3 months. Lundin et al[147] undertook segmental resection in 28 patients based upon the results of 111-Indium – DTPA Scintigraphy. At 50 months 82% (23/28) of patients were pleased with the outcome with an increase in stool frequency and a reduction in the number of hard stool and straining and concluded that segmental resection was comparable to STC and IRA for symptom relief with less severe side effects.

Segmental resection with good patient selection appears to offer comparable symptomatic relief with fewer side effects compared to STC and IRA. At surgery there is the advantage of only needing to mobilise one flexure which is of benefit when performed laparoscopically. Robust randomised controlled trials with strict patient selection and measurable outcomes between studies are needed now to demonstrate if a clear advantage between the two procedures exists.

1.7.6 NOVEL PHARMACOLOGICAL INTERVENTIONS

Although there are many treatment options for constipation, their lack of benefit in many patients means new interventions are required. The use of novel pharmaceutical agents which target colonic receptors has been developed as the next generation of treatments for functional constipation.

1.7.6.1 The Role of Serotonin and Serotonin Agonists

Serotonin (5-hydroxytryptamine [5-HT]), a signalling molecule that exerts its actions via seven main receptor subtypes (5-HT_{1-7}), is involved in increasing GI motility, secretion and sensation. Approximately 95% of the body's 5-HT is found in the GI tract, 90% of which is synthesised by the enterochromaffin (EC) cells and the remaining 10% by nerves in the myenteric plexus[148]. 5-HT released from EC cells in response to luminal factors stimulates extrinsic (vagal or spinal) or intrinsic primary afferent neurons (IPANs) by binding to 5-HT receptors, principally 5-$HT_{3,4\text{ and }7}$. Extrinsic afferents transmit signals to the brain and are thought to convey feelings of satiety, nausea, pain and discomfort. IPANs, through the release of Ach and calcitonin gene related peptide (excitatory) and nitric oxide (inhibitory), act locally and depending on the site are responsible for nausea and vomiting, increased luminal secretions and peristalsis[149,150]. After release extra 5-HT is principally inactivated by the serotonin reuptake transporter (SERT) located on some enterocytes with the remainder, which enters the bloodstream, by circulating platelets.

The role of 5-HT in the control of large bowel function is poorly understood due to the large numbers of receptors present in the GI tract. However, evidence has been gained from the use of selective agonists and antagonists on the 5-HT_3 and 5-HT_4 receptors. The 5-HT_3 receptor is a ligand gated ion channel that causes a rapid and transient excitatory response when activated by 5-HT. The receptor is located on the intrinsic and extrinsic sensory neurones and the use of 5-HT_3 antagonists, such as ondansetron and granisetron, have been demonstrated to decrease nausea and vomiting in clinical practice including in highly emetogenic chemotherapy[151,152]. Ondansetron reduces the colonic transit time in healthy volunteers[153] and alosetron, which is 10 times more potent than ondansetron, has been developed for the treatment of IBS-D. In a clinical study by Delvaux et al[154] alosetron increased the compliance of the colon to distension, without an associated increase in nociception. Subsequent randomised controlled trials reported a significant improvement in stool frequency and abdominal discomfort in suffers of IBS-D[155,156]. Thus activation of the 5-HT_3 receptor will increase GI motility, whilst antagonism results in a decrease in nausea and vomiting, nociception and motility.

The 5-HT_4 receptor is a G protein-coupled receptor whose activation leads to a prolonged excitatory response, but whose distribution is not yet clearly defined. Activation of presynaptic 5-HT_4 receptors appears not to elicit a motor response but instead to augment motor function

within the intestine[157,158]. Thus luminal stimulation releases 5-HT which activates the 5-HT_4 receptor to increase colonic motility. This physiological effect has been utilised in the treatment of slow transit constipation.

Cisapride was among the first 5-HT_4 receptor agonists to be developed for gastro-oesophageal reflux disease and gastroparesis. However, drug-drug interactions and more importantly QT interval prolongation and cardiac arrhythmias led to its withdrawal in 2000[159]. Subsequently Tegaserod was approved by the FDA in 2002. Tegaserod is a selective 5-HT_4 agonist which accelerates gastrointestinal transit[160,161], stimulates intestinal secretion[162], and improves visceral hypersensitivity[163]. This has been translated into clinical benefits in patients with functional constipation. Two, phase III RCTs comparing tegaserod (2 mg and 6 mg doses) and placebo demonstrated a significant increase in complete spontaneous bowel movements (CBSMs) in patients taking tegaserod compared to the placebo group (37-41% vs. 25-27%, 2 mg dose vs. Placebo, $p<0.0001$; 40-43% vs 25-27%, 6 mg dose vs placebo, $p<0.0001$). This benefit was maintained over the 12 week study period, but was lost in the 4 week withdrawal period[164,165]. The benefit was confirmed in a trial using male patients only[166] and in studies the use of Tegaserod was associated with an improvement in abdominal pain and bloating. A 13 month safety, tolerability, and efficacy study[167] using patients from the work by Kamm et al[165] reported that tegaserod was safe and well-tolerated, whilst patient satisfaction with treatment was maintained. However, a cross-study analysis found that 13 of 11,600 patients treated with tegaserod had ischaemic cardiovascular events compared to one event in the placebo group[168]. The mechanism for this is not fully understood, but it is most likely related to poor selectivity of tegaserod for 5-HT receptors and perhaps from its affinity for 5-HT_{1B} receptors[169].

Therefore, a more highly selective 5-HT_4 receptor agonist, prucaloride, has been developed. Prucalopride is a highly-selective, high-affinity 5-HT_4 receptor agonist which has been demonstrated in animal models to stimulate GI motility and transit throughout the length of the GI tract[170,171]. Prucalopride has been extensively evaluated in the management of functional constipation. Three large, randomised, double-blind, placebo-controlled, 12 week, phase III trials have assessed the efficacy of prucalopride[172-174] involving almost 2000 patients, all of whom had severe constipation resistant to conventional treatments. All three studies compared placebo, prucalopride 2 mg and 4 mg doses. Each trial showed a significant improvement in patients who had ≥3 CSBM a week and also in those patients who had an

increase of 1 or more CSBM a week compared to their pre-trial baseline (Table 1.2). There were also significant improvement in symptom severity scores and quality of life scores as measured by PAC-SYM and PAC-QOL health surveys.

Each of the trials reported no significant adverse or cardiac events with the main complaints being headache, abdominal pain and diarrhoea. There were no differences in the rate of these adverse events between the placebo group and the treatment groups. A subsequent study in the elderly assessed prucalopride in over 300 patients aged over 65 years of age with fewer than 3 SCBM / week. Again the drug was significantly more effective than placebo and more importantly there were no changes in laboratory, cardiovascular, or ECG variables over the 4 week study period[175]. These findings were supported by a smaller study by Camilleri et al[176], but both studies were limited by their short follow up of only 4 weeks. These results have been replicated in an Asian-Pacific population over a 12-week period with similar results[177] and this beneficial effect with minimal adverse events has been shown to continue in an open follow up study of the participants from the three randomised controlled trials listed above[178]. Improvement in PAC-QOL scores was observed up to 18 months with 40-50% of patients not requiring additional laxatives during this period. The most frequent adverse events resulting in discontinuation were gastrointestinal (3.3%) and headache (1.0%). Assessment of cardiac safety was more extensively evaluated in 120 healthy volunteers in a double-blind, double-dummy, placebo- and active-controlled trial. There was no significant change in the QT interval between the placebo, 2mg, and 10mg prucalopride groups, but the use of prucalopride was associated with a mean increase of 2-5 beats per minute. No ventricular arrhythmias were noted during the study period[179]. There has been one randomised controlled trial comparing prucalopride with PEG 3350+electrolytes[180]. Both treatments demonstrated an improvement in CSBMs, SBMs, stool weight, and colonic transit time compared to the run-in period, although these improvements were greater in the PEG group. PEG was more commonly associated with a loose and watery stool (Type of 5-6 on the Bristol stool chart) whilst prucalopride was associated with a normal stool constistency (Type 3-4) but this did not translate into lower patient satisfaction with a lower withdrawal rate (0 vs. 3 pts) and greater patient satisfaction in the PEG group.

The success of prucalopride has led to the development of further 5-HT_4 agonists which are currently at varying stages of development, the most promising of which is called velustrag.

		2mg Prucalopride	4mg Prucalopride	Placebo	p-Value
Camilleri [172]	≥ 3 CSBM / Wk	30.9%	28.4%	12%	Both <0.001
	Increase of 1 or more CSBM / Wk	47.3%	46.6%	25.8%	Both <0.001
Quigley [173]	≥ 3 CSBM / Wk	24%	24%	12%	Both <0.01
	Increase of 1 or more CSBM / Wk	43%	47%	28%	Both <0.001
Tack [174]	≥ 3 CSBM / Wk	19.5%	23.6%	9.6%	2mg - <0.01 4mg - <0.001
	Increase of 1 or more CSBM / Wk	38%	44%	20.9%	Both <0.01

Prucalopride is a highly selective, high affinity 5-HT_4 receptor agonist which stimulates GI motility and transit throughout the length of the GI tract. In each of the three large randomised controlled trials prucalopride significantly improved the outcomes measured compared to the placebo demonstrating that prucalopride was an effective treatment in functional constipation.

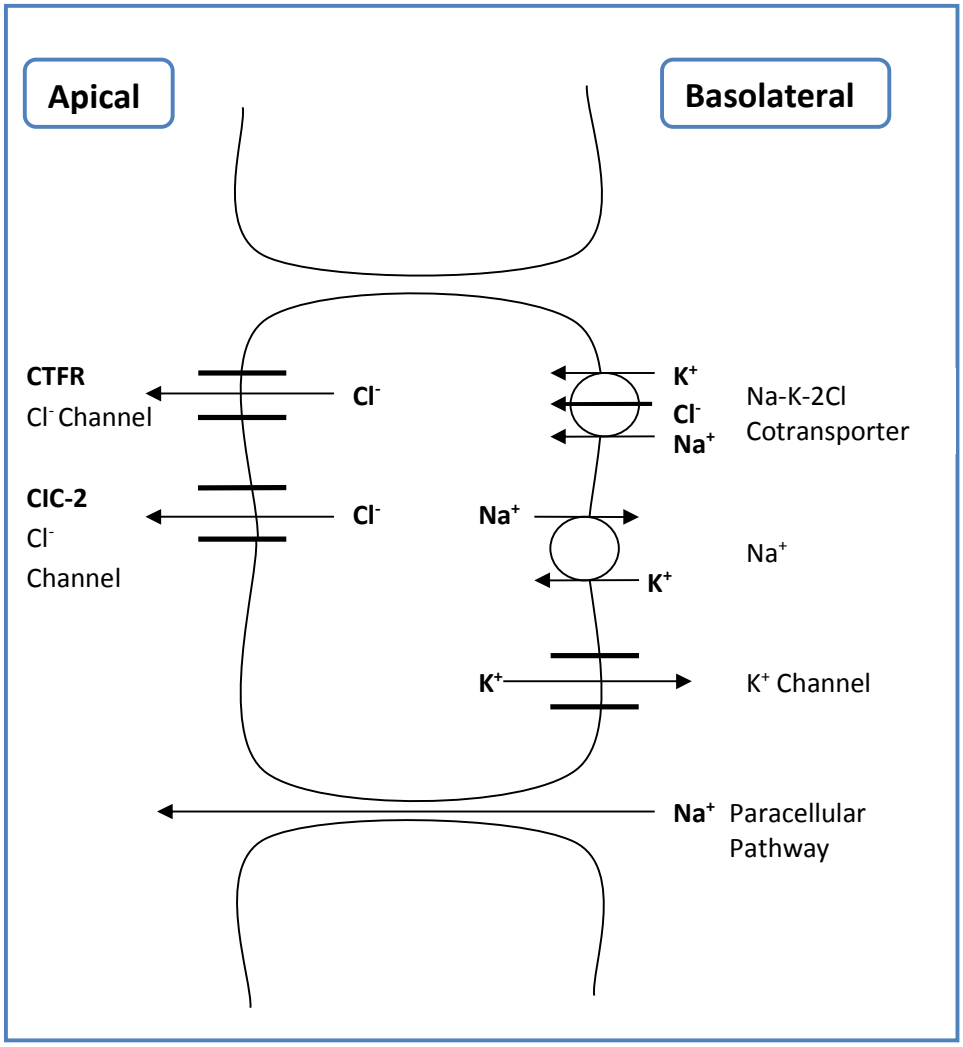
Table 1.2 - The percentage of participants in each of the three trials of prucalopride who had ≥3 CSBM/wk and an increase of 1 or more CSBM/wk.

This drug is a potent agonist of 5-HT_4 receptors which has efficacy *in vitro* and *in vivo* and is highly selective for the 5-HT_4 receptor over other 5-HT receptors which may help in limiting its side effects[181]. The drug increases the transit time in healthy volunteers in a dose-dependent manner[182] and in a 4 week clinical trial, patients receiving 15, 30 and 50 mg doses achieved statistical significance in CSBM/week compared to the placebo group ($P<0.0001$)[183].

1.7.6.2 Chloride Channels and Lubiprostone

A second novel pharmacological agent has been developed which acts through chloride channels. Chloride channels (CIC) are found throughout the body and are responsible for the transport of chloride ions across membranes[184]. In the gastrointestinal tract they play a critical role in regulating fluid transport and maintenance of both cell volume and intracellular pH since chloride ions are followed by the passage of sodium ions and water (Figure 1.6). Nine separate CIC have been identified however only chloride channel 2 (CIC-2) is sensitive to lubiprostone[185]. CIC-2 is distributed throughout the GI tract on the apical cell membrane and is a transmembrane protein that is highly selective for chloride ions. Lubiprostone is a CIC activator which is derived from prostaglandin E_1 which increases intestinal fluid secretion and chloride ions[185]. It is through this mechanism that lubiprostone, in healthy volunteers, decreases small bowel and colonic transit times[186]. Subsequent work has shown that the decrease in colonic transit time is not as a result of an increase in HAPCs and as such lubiprostone does not directly affect motility[187].

Despite the uncertainty about the precise mechanisms by which lubiprostone produces laxation it has shown to be efficacious in two randomised controlled trials[188,189]. In total, these two trials enrolled 479 patients (242 and 237 respectively) who were studied for 4 weeks and received either lubiprostone 24 mcg b.d. or placebo. Both trials reported a significantly greater number of SBMs/wk in the lubiprostone group and more of these patients also had a SBM within 24 h compared to the placebo group. These benefits were sustained over the 4 week period with the commonest adverse events being nausea and headache. A third, similar trial of 170 Japanese patients, suffering with both functional constipation and IBS-C, confirmed these findings[190]. The benefits of lubiprostone were shown in both patients with functional



Cl^- enters from the basolateral membrane across the Na-K-2Cl co-transporter. The cystic fibrosis transmembrane regulator (CTFR) and the CIC-2 chloride channel are situated on the apical membrane and allow Cl^- to leave the cell. Lubiprostone activates the CIC-2 channel whilst the CTFR channel is activated by a rise in cGMP and is the basis of action of Linaclotide.

Figure 1.6 - Model of Cl^- transport in intestinal epithelial cells.
Taken from Cuppoletti et al[185].

constipation[191] and IBS-C[192] for up to 48 weeks. Both trials showed continued improvement in symptom score and that the drugs were safe. The commonest adverse events were diarrhoea (10%) and nausea (10% - 20%). Despite this the number of withdrawals due to AEs was low (21/520 in the IBS-C group and 33/248). There were no reported SAEs in the IBS-C group; there were 16 in the constipation group and no mortalities in either trial.

1.7.6.3 Guanylate Cyclase C (GC-C) Activators (Linaclotide)

The GC-C receptor is found on the apical surface of the intestinal epithelium and when it is activated by circulating endogenous peptides there is a resultant anion efflux into the intestinal lumen with associated fluid secretion[193]. Linaclotide is a 14 amino acid peptide which activates the GC-C receptor and results in an increase in intra- and extracellular cyclic guanosine monophosphate (cGMP). The clinical benefits in 42 patients with functional constipation were an improvement in CSBMs/wk, stool consistency, straining, abdominal pain and bloating with a concomitant improvement in quality of life[194]. The results of two randomised controlled phase III trials were presented by Lembo et al[195]. A total of 1276 patients were recruited, with approximately 20% of subjects who received linaclotide having three CSBM/week compared to 3.3-6% in the placebo group. The secondary endpoints of stool consistency, straining, abdominal discomfort and bloating also improved compared to the placebo group. Furthermore, significant improvements in the constipation-related quality of life were also seen.

Additionally the rise in extracellular cGMP has been demonstrated to ameliorate visceral hypersensitivity in animal models by a direct action on afferent nerve endings in the gut[196]. This may have an important clinical role in the amelioration of pain associated with IBS[197].

1.7.6.4 The Opioid Receptor and Opioid Antagonists

There are 3 opioid receptors, μ-, κ-, δ-, which are located throughout the body including in the gastrointestinal tract[198]. These belong to a family of membrane bound receptors that are linked to G-proteins which act as second messengers to activate potassium channels, inhibit calcium channels, produce membrane hyperpolarisation and reduce production of cyclic adenosine monophosphate[199]. Opioids are synthesied by the enteric neurones to act as transmitters

notably from myenteric neurons projecting on to the circular muscle and in neurons of descending enteric pathways[198,200]. Activation of these receptors reduces neuronal excitability with an overall inhibitory effect on peristaltic activity and can occur as a result of endogenous or exogenous opioids[201]. This has been demonstrated in colon models after the administration of both opiate agonists and antagonists which have inhibited or augmented peristaltic activity respectively[202-204]. The inhibitory mechanism on peristalsis arises from interruption of transmission within enteric nerve pathways where the presynaptic site of action attenuates the release of acetylcholine and other excitatory transmitters[205]. This was demonstrated in animal models that activation of opioid receptors also resulted in attenuation in the secretion of electrolytes and water[206]. This effect has been disputed in human colons and that the reduced water content of the stool is secondary to prolonged contact with the colonic mucosa.

The overall effect of endogenous colonic opioids is to slow colonic transit and, through prolonged contact of the stool with mucosa and possibly the interruption of prosecretory enteric reflexes, facilitate the net absorption of water (Figure 1.7). The overall effect is constipation as seen by the exogenous administration of opiates.

1.7.6.4.1 Opioid Induced Constipation (OIC) and the role of Naloxone

OIC is a major debilitating side effect of opiate use and patients may discontinue their pain treatment due to constipation. In a survey it was reported that only 46% of opioid-treated patients achieved desired relief of OIC greater than 50% of the time[207]. The effects of opiates on gastrointestinal motility have been discussed and that their use leads to OIC. The colonic opiate receptors are thus a potential target for treatment in OIC. OIC can be treated in a similar way to functional constipation and these agents, including the more novel agents, have varying success. However, local antagonism of the opiate receptor in the gastrointestinal tract offers a more attractive option and this has been achieved through the use of opioid antagonists naloxone and methylnaltrexone (a quaternary ammonium derivative of naltrexone, an opioid antagonist similar to naloxone).

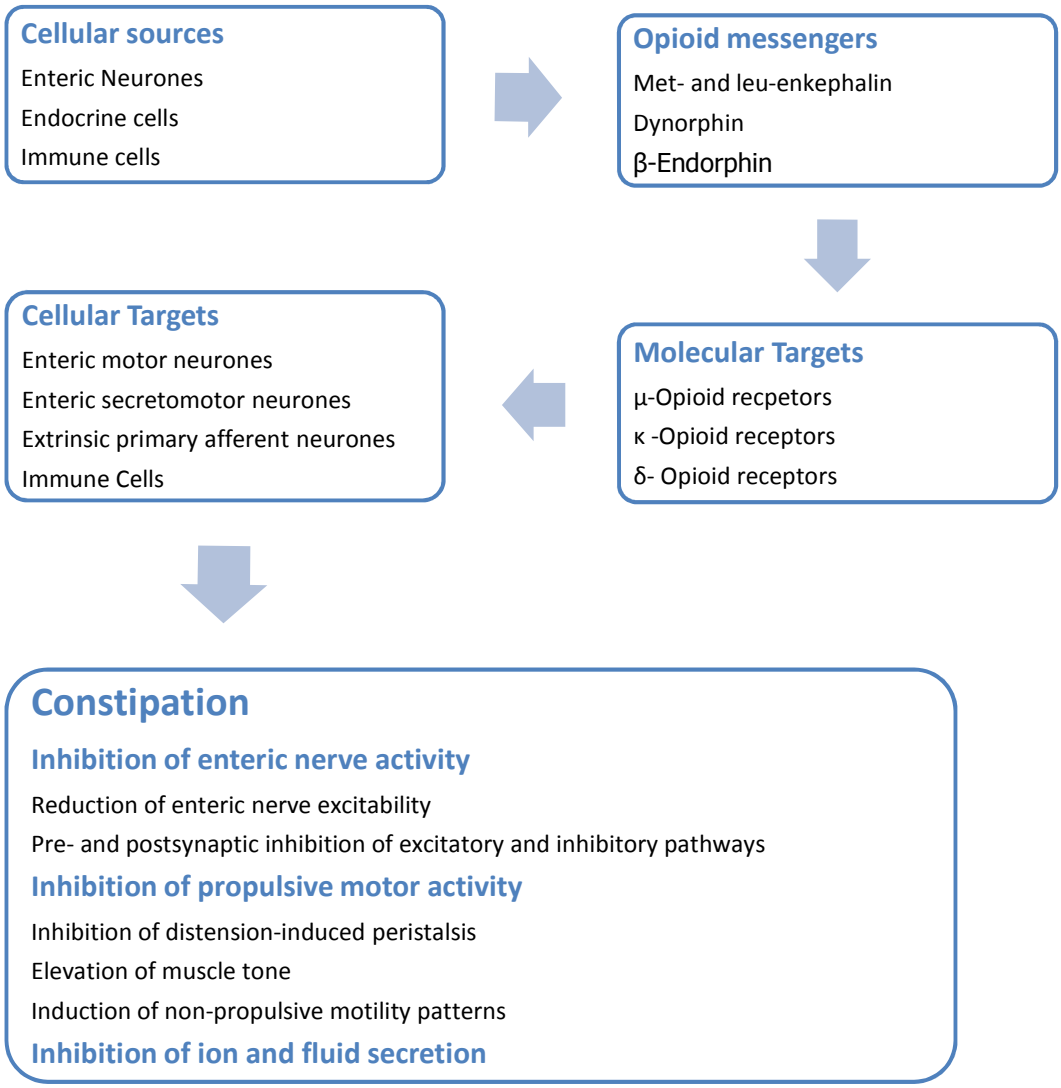
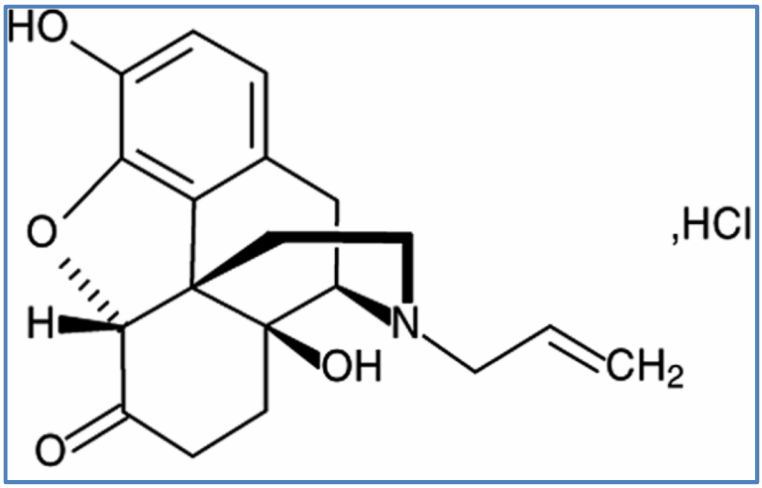


Figure 1.7 – Overview of the gastrointestinal opioid system. (From Holzer[208])

Opioid receptors, activated by circulating opoids, produced by the enteric neurones, inhibit peristalsis to prolong colonic transit and facilitate the net absorption of water.

1.7.6.4.2 Naloxone and its' effects on colonic motility



Empirical Formula of Naloxone: C19 H21 NO4 HCL, 2H20

Naloxone hydrochloride is an established active substance whose chemical structure is shown below. The pharmacological activity of naloxone on the gut has been evaluated in both in *vitro* and in *vivo* studies. Liu et al[204] demonstrated that naloxone induced a concentration-dependent elevation of electricity-stimulated contraction amplitude of cathartic colon strips. The cathartic colon rat model attempts to replicate the cathartic colon seen after excessive use of stimulant laxatives and in the rat model in this study this was induced by the feeding the rats rhubarb or phenolphthalein for 3 months. Each concentration of naloxone (0.05 mmol/L, 0.10 mmol/L, 1.00 mmol/L) induced a significant elevation of the contractile response, which showed the contraction amplitude was significantly elevated ($P<0.01$) in its presence (Table 1.3). Krevsky et al[209] had performed an in *vivo* study in felines and measured colonic transit using scintigraphy. An intramuscular dose of 0.3 mg/kg of naloxone accelerated emptying of the caecum and ascending colon with an increased filling of the transverse colon.

Naloxone in healthy volunteers reverses the delays in colonic transit seen after the administration of opiates[210,211]. Importantly Netzer et al[211] demonstrated that the administration of naloxone did not reverse the analgesic effects of morphine, whilst Hawkes et al[210] demonstrated that naloxone alone in healthy volunteers reduced overall transit time and proposed it may be of benefit in the treatment of functional constipation.

Concentration of naloxone(mmoL)	Basic contraction amplitude without naloxone (mm)	Contraction amplitude with naloxone (mm)
0.05	11.40±0.21	13.18±0.93 [b]
0.10	11.40±0.21	15.87±0.98 [ab]
1.00	11.40±0.21	19.46±1.79 [bd]

a - P<0.05 vs 0.05 mmol/L,
b - P<0.01 vs without Naloxone,
d - P<0.01 vs 0.05 mmol/L

The addition of naloxone in isolated colon strips results in a significant dose-dependent increase in contraction amplitude compared to controls in the presence of electrical stimulation (4 ms duration, 10Hz and 70V). This explains the reduction in transit time seen with the administration of naloxone in healthy volunteers and in sufferers of opioid induced constipation

Table 1.3 - Effect of naloxone on electricity-stimulated contractile response of cathartic colon strips (mean±SD)[204].

Naloxone has a low systemic bioavailability (2%) due to extensive 1st pass metabolism in the liver. Despite this it is widely distributed in the central nervous system reversing analgesic effects or resulting in opioid withdrawal[212]. The development of a prolonged-release (PR) preparation has overcome these issues. PR naloxone has been combined with PR oxycodone (a strong, semi-synthetic opioid) with excellent results in the management of OIC. Nadstawek et al[213] compared oxycodone PR + Placebo with Oxycodone PR + Naloxone PR (10 mg, 20 mg, and 40 mg/day). Successful management of chronic pain was not reduced with the administration of naloxone PR, and this was independent of the naloxone dose. Effective analgesia was confirmed in a pooled prospective analysis of two randomised, double-blind, parallel-group, multicentre studies involving 587 patients[214]. The analysis also assessed bowel function through the Bowel Function Index (BFI) (a mean scored based on scores (0-100) for defecation, feeling of incomplete evacuation, and judgement of constipation with a low score indicating better bowel function) and laxative use. BFI scores were similar at baseline but there was a significant improvement in score over the 12 week period in the oxycodone/naloxone group and this was supported by a lower laxative intake ($p<0.0001$). Open-label extension studies demonstrated its' long-term efficacy and tolerability over a 52 week period with a continued reduction in the BFI scores[215]. Similar effects were seen with methylnaltrexone[216,217].

The use of naloxone in the management of constipation was reported in a small case study of two patients by Kreek et al[218]. Both patients responded with an increased frequency of passage of faeces and also an increase in the wet and dry weight of the faeces. To date, there are no clinical trials designed to study the use of naloxone in functional constipation, but it has been used in IBS-C sufferers and although not significant, the results tended towards an improvement in pain, bloating, straining, and urgency to defecate[219].

1.7.7 PROPOSED MANAGEMENT PATHWAY FOR PATIENTS WITH INTRACTABLE CONSTIPATION

A proposed diagnostic and treatment pathway is shown in figure 1.8. Patients are investigated to exclude organic causes and evacuatory disorders and these should be managed appropriately. Once a diagnosis of functional constipation is made then the patients should be stared on one sachet of Movicol (polyetheleneglycol) twice a day and this titrated to achieve a satisfactory response. Failure of movicol should alert the clinician to consider the use of a novel

agent such as prucalopride. Failure at this level will require further to a tertiary centre for consideration of a trial of a SNS followed by surgery if unsuccessful. All treatments are guided by the patient's co-morbidites and wishes.

1.8 COLONIC MICROBIOTA AND THEIR ROLE IN FUNCTIONAL CONSTIPATION

The term colonic microbiota describes the microorganisms that populate both the lumen and the mucosa of the colon. There are approximately 3000 identified species, but this estimate could possibly be as high as 36000[220,221], with only 11 of the known 55 bacterial divisions represented (see later). Two types of organism are present; autochthonous which are resident and allochthonous that are transient. The gastrointestinal microbiota performs a number of key biological functions which are beneficial for the host, some examples of which are listed here:

- Biosynthesis of essential nutrients e.g. Vitamin K and B12.
- Biotransformation of conjugated bile acids.
- Degradation of dietary oxalates.
- Extraction of useable calories from indigestible polysaccharides.
- Production of butyrate (formed as an end product of fermentation and has a role in the metabolic welfare of colonocytes).
- Colonisation Resistance against pathogens

1.8.1 COMPOSITION OF THE COLONIC MICROBIOTA

The GI tract is an 'open environment' with different populations of microbiota existing along its length due to rapid turnover of intestinal epithelium and mucus, different exposure to peristaltic activity, different environmental conditions and exposure to bacteria from the oral cavity. Attempts have been made to identify the composition of the microbiota and the

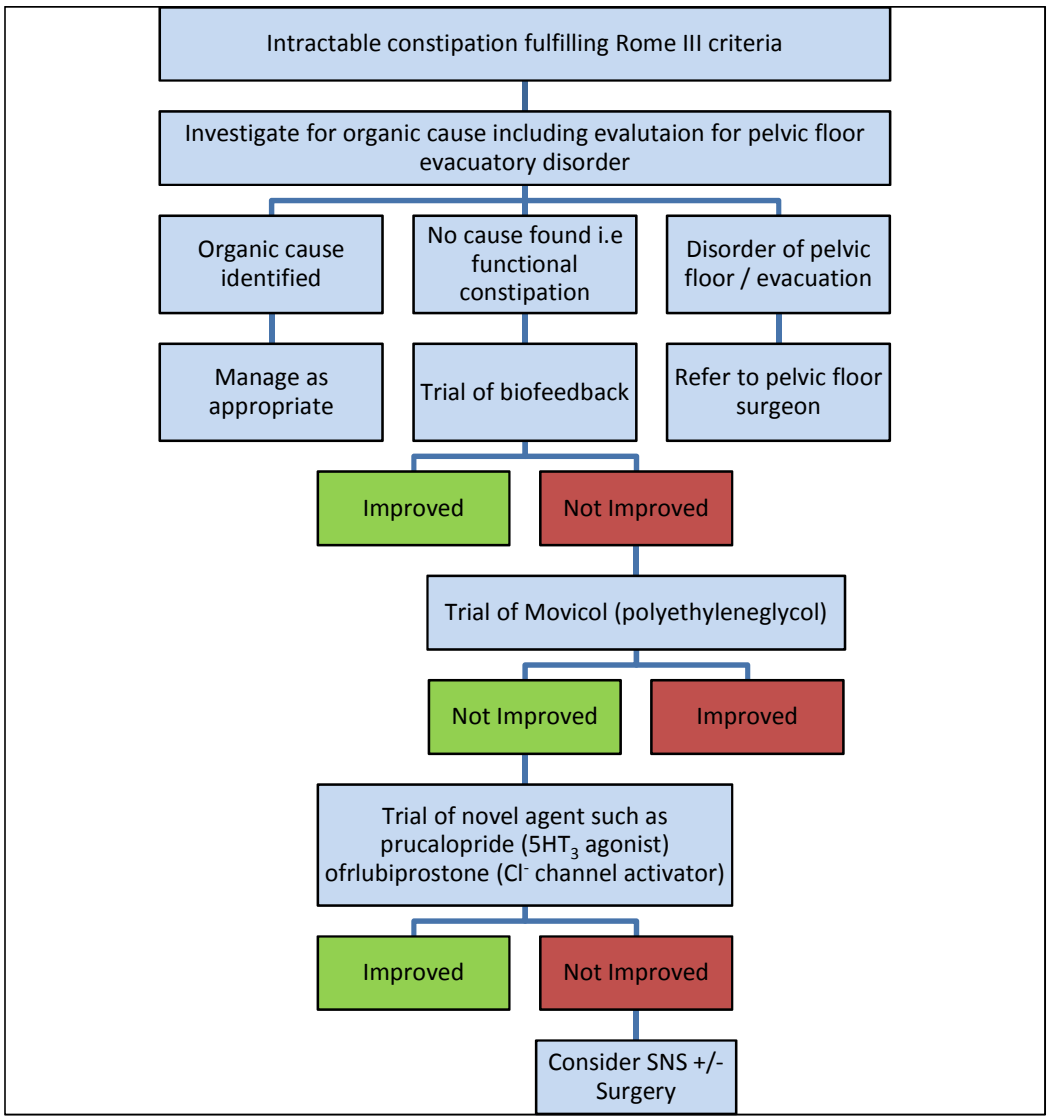


Figure 1.8 – Proposed management pathway for patients with intractable constipation

reliability of such results in part depends of the methods used in detection. Early attempts used 'culture-dependent' techniques which rely on the ability for bacteria to be cultured so that they can be counted and recorded. Subsequently it has been estimated that these techniques may only detect 20-40 species (60-80% of species) when compared to the use of a DNA stain to detect the total number of bacteria present[222]. Reasons for the inability to culture all the bacteria are:

- Obligate syntrophs (Cannot be grown in isolation).
- Need for strict anaerobiosis (Lost during sampling and plating).
- Lack of specific culture media.
- Intracellular adherence may decrease the number of organism within faecal samples.

Therefore, culture-independent methods have been developed which are based on analysis of the 16S rRNA within the ribosome of the bacterium. The 16S rRNA gene has conserved regions which enable the development of universal primers and variable regions which allow differentiation and identification of species. Techniques involved included real-time PCR, Fluorescent in-situ Hybridisation (FISH), and Denaturing Gradient Gel Electrophoresis (DGGE), and 454 sequencing. FISH, DGGE, and 454 sequencing are fully discussed later in the methods section along with more detail on the 16S ribosome.

Numerous major studies have used gene sequencing techniques to further the knowledge on the normal colonic microbiota. Of the 11 bacterial divisions represented in the colonic microbiota, two make up 99% of the bacterial population; *Cytophaga-Flavobacterium-Bacteroides* (CFB) and *Firmicutes*[223-225]. The *firmicutes* comprise 50 – 65% of the detected phylotypes whilst the CFB division comprises a further 23-48% of the remaining detected[223,225]. *Clostridia* comprise the bulk of the *firmicutes* (95%), most of which are divided between sub cluster XIVa (*Clostridium-Coccoides* gp) and sub cluster IV (*Clostridium-Leptum* gp). The next two most populous groups are the *Mollicutes* (4.5%) and the *Bacilli* (0.2%). Of the CFB division, *Bacteroidetes* make up 48% of the total with B.*vulgatus (31%)* and B.*thetaiotaomicron* (13%) appearing most dominant. From these culture-independent techniques it has been discovered that the abundance of *Bifidobacterium* and Lactic Acid bacteria, reported on earlier studies based on culture techniques, was wildly inaccurate. Langendijk et al[226] compared *Bifidobacterium sp* counts from culture and from FISH from the same samples and found that whilst *Bifidiobacterium*

sp may account for up to 10% of the total culturable population, this estimate was incorrect by a factor of 10 with *Bifidiobacterium sp* only representing 0.8% +/- 0.4% of the total population. A similar finding was presented by Harmsen et al[227] who using a *Lactobacillus/Enterococcus* probe estimated that only 0.01% of the total microbiota was comprised of *Lactobacillus and Enterococcus sp* compared to the 2% estimation from culture-dependent techniques.

1.8.2 FACTORS AFFECTING THE COMPOSITON OF COLONIC MICROBIOTA IN STUDIES

1.8.2.1 Site of Sampling

Not only is the method used for sampling important but also the type of sample used when determining the makeup of the colonic microbiota. Most work has been done using faecal samples as these are easiest to collect and it is assumed that they are representative of the microbiota within the colon. Eckberg et al[223] took mucosal biopsies from 6 regions from the colon at colonoscopy, and a further stool sample 1 month later from 3 individuals. Zoetendal et al[228] took 3 biopsies and a stool sample from 10 individuals. Although differences in the microbiota existed between individuals in both studies, in the same individual the microbiota from the mucosal biopsies were similar, regardless of site, and differed from the microbiota of the stool sample suggesting:

1. The faecal microbiota represents a combination of shed mucosal bacteria and a separate non-adherent luminal population and;
2. A homogenous bacterial population within the mucosa along the length of the colon.

The second possibility has been disputed in other studies which showed a variation between the microbiota identified in the caecum and the proximal sigmoid, in samples taken from the lumen[229] and mucosa[230]. However, the first point is likely to be more significant as those organisms related to the mucosa may have a more significant impact on the host and an imbalance being related to GI pathology.

1.8.2.2 Age of subjects

There are obvious striking changes to the microbiota of the whole of the GI tract following birth and also into childhood as a result of breast or formula feeding and the introduction of solid foods when weening. However, there are further alterations in adulthood and this may be due to continued changes in diet, changes in the function of the immune system, and increased exposure to GI tract infections. The commonest changes seen are increases in Enterobacteria, with decreases in Bacteroidetes and *Bifidiobacteria*[231,232].

1.8.2.3 Diet

Although the exact impact of diet on colonic microbiota is largely unknown it is well established that diet plays a significant role in its composition. Subjects who are vegan and vegetarian have been shown to have lower counts of *Bifidobacterium* spp., *Escherichia coli* and *Enterobacteriaceae* spp. compared to omnivore controls and this difference is more marked in a vegan diet[233]. In a separate analysis *Clostridium* cluster XIVa was more prevalent in omnivores[234]. Neither of these studies was able to identify the actual food groups responsible for the changes or the impact this may have on the volunteers clinically.

1.8.2.4 Role of Antibiotics

Although antibiotics are used to treat pathogenic bacteria they will also affect the normal microbiota of the patient and the colonic microbiota is no exception. A five day course of oral amoxicillin with clavulanic acid (Augmentin) in healthy volunteers reduced both total bacterial (10.7 +/- 0.1 log 10 vs. 11.1 +/- 0.1 log 10, p=0.003) and *bifidobacteria* concentrations (8.1 +/- 0.5 log10 vs. 9.4 +/- 0.3 log 10, p=0.003) in stool samples compared to their baseline readings and that these effects persisted for over 2 months following completion of the course[235]. In a study using mice three different antibiotics were given for a total of 21 days. This resulted in a reduction in *Clostridium*-like and *Bacteroides* species which increased but did not return to baseline values after 14 days. Despite the effects on bacterial communities, bacterial numbers were not affected indicating that the changes in a relatively small number of taxa were disproportionately favoured by antibiotics[236]. These changes in colonic microbiota with the favouring of some taxa over others may result in increased susceptibility to pathogenic bacteria and explain the long known clinical finding

that the use of antibiotics, especially cephalosporins, is associated with *Clostridium difficile* diarrhoea[237,238].

1.8.3 THE COLONIC MICROBIOTA IN DISEASE

1.8.3.1 Obesity

Striking changes in the microbiota are associated with obesity and these alterations revert to a more normal composition with weight loss. Ley et al[224] studied obese individuals and normal controls and found that in the former there were increased numbers of *firmicutes* and decreased *bacteroidetes* compared to controls and that this reverted with sustained weight loss. This was confirmed in mice given a high fat diet (HFD) for 8 weeks[240]. The *Firmicutes* to *Bacteriodetes* ratio increased with an increase in the growth of *Enterobacteriaceae*. Furthermore a HFD induced colonic inflammation and accelerated obesity and this was confirmed by Lam et al[241] who demonstrated an increase in macrophage infiltration, TNF-α, and IL-6. It has been shown that germ-free mice are protected from obesity that develops from a Western diet compared to mice with gut microbiota[242]. This Western-diet causes a restructuring of the distal gut microbial community with increases in the numbers of *mollicutes* at the expense of *bacteroidetes*[243]. It has been proposed that an individual's colonic microbiota can increase the capacity for harvesting sugars whilst modulating the host ability to process and store fats.

1.8.3.2 Inflammatory Bowel Disease (IBD)

IBD is characterised by recurrent intestinal inflammation of unknown aetiology which can be managed medically or surgically which involves an inappropriate and relapsing and remitting activation of the mucosal immune system. There are two major subtypes; ulcerative colitis (UC) and Crohn's disease (CD). UC is distributed within the mucosa of the colon and rectum whilst CD can occur at any site within the GI tract and involves the whole thickness of the bowel wall. IBD is multifactorial with the concordance rates for IBD between monozygotic twins being less than 50% and this is lower in CD[244]. Therefore a complex interplay between genetic environmental factors exists with one factor, the gut

microbiota, attracting increasing interest with the advent of next generation sequencing techniques.

The evidence to suggest that the gut microbiota may play a role in IBD comes from the observation that faecal diversion may induce remission in CD and that restoration of flow has the opposite effect of increasing inflammation[245,246]. The administration of antibiotics in animal models of colitis have completely prevented disease[247-249] and whilst the aerobic colony counts are unchanged, there is a dramatic decrease in the anaerobic colony counts[247,249], which suggests a colonic dysbiosis in IBD. Furthermore, the use of antibiotics in clinical trials has been shown to be comparable to mesalazine and superior to placebo in treating the symptoms of CD[250,251].

Analysis of mucosal-associated and faecal bacteria have demonstrated a dysbiosis in patients with IBD. As with all microbial analyses certain factors need to be taken into account such as the age and diet of subjects (as discussed earlier) and the secondly the site of the bacterial sample. There is an important point to consider with respect to the age of the study population. There is an age-related variation in the distribution of IBD with a peak at 15-30 years with smaller peaks under the age of 10 years and around 60 years of age. Therefore the age-related changes seen with increasing age may compound any changes in the microbiota seen in the different age groups with IBD compared with each other and with healthy controls. The site of biopsy has been shown to affect the microbial composition. Whilst there are differences between lumen and mucosal composition there does not appear to be any regional differences between mucosal sites in the ileum and colon[252-255].

Taking these factors into account there are disturbances in the composition of the microbiota compared to healthy subjects. There is a reduced biodiversity with, at a phyla level, decreased levels of *Firmicutes* and *Bacteroidetes* and increased numbers of *Gamma Proteobacteria* and *Enterobacteriaceae* [225,252,255-260]. Differences are also noted at higher taxomic levels and offer some insight into the pathophysiology of IBD. Levels of *E.coli/Shigella* are seen to increase in IBD, especially ileal CD and are more pronounced in mucosal samples compared to faecal samples[257,258,261]. The invasive nature of *E.coli*

pathovar is more abundant in ileal CD and has been shown to induce granuloma formation, a key characteristic of CD[262]. *Enterobacteriaceae* induces pro-inflammatory cytokine pathways[257] and a rise in number, particularly *Escherichia coli*, is associated with a younger age to surgery and, a higher incidence of abscess formation in those subjects with CD. Higher levels of serum reactivity toward microbial antigens such as *Escherichia coli* outer membrane porin C and Pseudomonas fluorescens I2 sequence result in a greater frequency of strictures, internal perforations, and small bowel surgery[263,264].

A second group, the genus *Fusobacterium*, may be involved in the pathogenesis of UC. *F.varium* numbers are increased in the colonic mucosa in patients with UC (61%) compared with CD (15%) and healthy controls (29%, $p<0.001$) where it increases the products of pro-inflammatory pathways[265,266]. The instillation of butyric acid, a product of *F.varium*, by enema in mice has induced mucosal erosions similar to those seen in UC[267.] *F.nucleatum* has been demonstrated to stimulate rowth of colorectal cancer by activating inflammatory and oncogenic responses[268] and this may represent a link between UC and the increased incidence of colon cancer seen associated with UC.

Decreased levels of *Faecalibacterium prausnitzii* are reduced in patients with UC but also in unaffected relative compared with healthy controls[269]. In patients with ileal CD who have undergone resection those patients with reduced numbers of *Firmicutes* (*Faecalibacterium prausnitzii and C.coccoides*) have a higher incidence of endoscopic recurrence compared with patients who were disease free at 6 months[270]. A proposed mechanism to account for this is a decrease in butyrate production. Butyrate, a short chain fatty acid (SCFA), is the primary energy source for the intestinal epithelial cells and is involved in the synthesis of tight junction proteins which act to reinforce the colonic barrier. Also butyrate inhibits cytokine pathways resulting in an anti-inflammatory effect. Other SCFA-producing bacteria including *odoribacter* are reduced in UC whilst *Roseburia* are reduced in CD.

It therefore seems logical that alterations of the microbiome may be of therapeutic benefit. As mentioned earlier the use of antibiotics has been shown to improve clinical symptoms of IBD. Antibiotics have an essential role in treating the septic complications of IBD e.g. intra-abdominal and perianal abscesses, inflammatory masses, fistulae, toxic megacolon and post-operative infections. The role of antibiotics in the management of

primary is more controversial. The use of ATM (amoxicillin, tetracycline, metronidazole), an antibiotic combination therapy, produces an improvement in endoscopic, histological, and clinical scores with prolonged remission and steroid withdrawal in active UC compared to placebo for up to 12 months[271,272] and that this likely resulted from an alteration in the intestinal microbiota[273]. Rifaximin and ciprofloxacin induce remission in the management of luminal and perineal CD[274,275] however not all studies are promising and it appears antibiotic therapy is better used to maintain remission alongside conventional agents[276].

The use of probiotics has also been widely investigated with a mixed response in CD. Steed at al[277] demonstrated an improvement in clinical symptoms and reduced TNF-α expression at 3 months but not at 6 months with a symbiotic comprising B.longum. Malchow et al[278] found that *E.coli* Nissle was superior to placebo in prevention of relapse after induction of remission with standard medical therapy. However *Lactobacillus* GG[279] or *Saccharomyces boulardii*[280] were unable to prevent relapse of disease compared to placebo. Again mixed results have been seen with UC. *Lactobacillus* combined with sulphasalazine resulted in a reduction of inflammation both histologically and immunologically (decrease in calprotectin and TNF-α) compared to sulphasalazine alone in both children and adults[281,282]. However Groeger et al were unable to demonstrate a reduction in inflammatory biomarkers after administration of *B.infantis* 35624 despite it having effects in other non-gastrointestinal inflammatory conditions[283] confirming the results of Wildt et al who could not demonstrate a significant remission rate compared to placebo[284.] It therefore appears likely that the species of probiotic used plays a significant part in the therapeutic benefit seen.

The dysbiosis seen in IBD offers an exciting insight into disease progression but also into the control of the disease. Further work is needed to fully understand the role the microbiome plays, whether the changes seen are the causative agent or merely reflect the luminal mileau created by the mucosal inflammation. Once this is understood restoring the dysbiosis may lead to a new therapeutic armamentarium to control IBD.

1.8.3.3 Irritable Bowel Syndrome (IBS)

IBS is the commonest of the FGIDs, characterised by abdominal pain in association with an improvement in pain on defectation or a change in stool frequency or form. Diagnosis is based on the Rome III criteria for IBS[1] (see below) and is divided into 3 categories; Diarrhoea predominant (IBS-D), Constipation predominant (IBS-C), and an alternating or mixed pattern (IBS-M). Reflecting the heterogenous nature of this condition there are a number of possible pathophysiological mechanisms of which a disturbance in the GI microbiota is one.

Rome III Criteria for IBS

Recurrent abdominal pain or discomfort** at least days/month in the last months associated with two or more of the following:

- Improvement with defecation
- Onset associated with a change in frequency of stool
- Onset associated with a change in form (appearance) of stool

Criterion fulfilled for the last months with symptom onset at least months prior to diagnosis

** "Discomfort" means an uncomfortable sensation not described as pain.

In pathophysiology research and clinical trials, a pain/discomfort frequency of at least 2 days a week during screening evaluation is recommended for subject eligibility

Would not normally include patients who satisfy criteria for Functional Constipation

The change in the microbiota in IBS has been widely demonstrated (Table 1.4) although no consistent change has been identified. This inconsistency between studies may reflect different analytical techniques used or whether analysis is done for the IBS group as a whole, or divided into different subsets. There is also no assessment of the diet taken by the subjects in the studies. There are likely to be dietary differences between the two groups as IBS patients are known to modify their diets in an attempt to control their symptoms. These inconsistencies, however, may reflect the heterogenous nature of the condition and that IBS can alternate from one subset to another and as such have a greater

variability of their microbiota over time. Therefore, better designed, longitudinal trials are needed to accurately quantify the changes in the microbiota so that targeted treatments can be implemented.

1.8.3.4 Functional Constipation

Despite the incidence and associated reduction in quality of life of patients with functional constipation, very little is known about the colonic microbiota compared with that of IBS. The existing information is from culture-dependent studies and therefore must be interpreted with caution for the reasons discussed earlier. Celik et al[291] implied that colonic microbiota had an important role in functional constipation after they demonstrated that the administration of the antibiotic, vancomycin, to sufferers of FC improved stool frequency, consistency, ease of defecation, and the amount of stool patients produced. However, they were unable to show an improvement in objective measures and since there was no control arm a profound placebo effect could not be excluded. Zoppi et al[292] compared 42 children, 28 patients with FC and 14 healthy controls, as part of a trial assessing the efficacy of calcium polycarbophil as a treatment for FC. A secondary outcome of the trial assessed the colonic microbiota of the participants measured in faecal samples. Compared to the healthy participants, sufferers of FC demonstrated significantly elevated levels of *clostridia* ($p<0.001$) and *bifidiobacterium* ($p<0.002$), but following treatment with calcium polycarbophil showed no change in the colonic microbiota towards that of the normal subjects. Khalif et al[293] compared the faecal microbiota in patients with FC before and after treatement with bisacodyl. Levels of *bifidiobacteria* and *lactobacillus* were significantly lower in constipated subjects with bisacodyl treatment resulting in the normalisation of the faecal flora. This led the authors to suggest that constipation caused these changes in the microbiota as opposed to a derangement in microbiota as a cause for FC.

Further changes in the microbiota have been proposed by the increased levels of methane production that occurs in FC. Attaluri et al[294], using methane breath testing, reported that sufferers of FC who had slow transit (STC) had greater numbers of methanogenic flora (defined as ≥ 3ppm) compared to either constipated patients with normal transit and normal controls. STC patients had a significantly higher methane response compared to

patients with normal transit and normal controls after administration of a carbohydrate substrate. These changes were supported in a wider population which also showed that subjects with diarrhoea had a significantly lower methane production compared to controls[295]. Changes in methane production may have an impact on colonic transit with higher levels causing an increase in transit time and hence constipation and vice versa. Based on these studies it is unclear, however, whether the clinical disorder is secondary to these changes or as a result of the disorder itself. However animal studies have shown that methane attenuates the contractile amplitude of colonic longitudinal muscle strips[296] and slow transit time in small bowel[297] supporting the hypothesis that an increase in methane producing colonic microbiota results in constipation but the explanation for the initial disturbance in these microbiota remains unclear.

1.9 Hypothesis and Aims of this Thesis

Functional constipation is a challenging condition with limited treatment options. As our understanding of gut physiology increases it is possible to develop new pharmacological treatments tailored to act on the colonic receptors and improve the symptoms of constipation. I propose to use naloxone hydrochloride (opiate antagonist), as a slow release preparation (Nalcol™) to treat functional constipation. The hypothesis is that Nalcol™ will antagonise the opiate receptors and decrease colonic transit by the mechanisms discussed above. This will result in an increase in weekly stool frequency and an amelioration of associated symptoms. From a subset of patients stool samples will be collected for microbial analysis to determine the differences in colonic microbiota between sufferers of functional constipation and healthy subjects using culture-independent analytical methods. I will investigate if these changes are corrected after successful treatment with Nalcol™. It is hypothesised that there will be quantifiable differences in the colonic microbiota and that successful treatment with Nalcol™ will normalise this.

	Subjects	Mode of Analysis	Observation	Notes
Malinen [285]	IBS – 27 (IBS-D -12, IBS-C -9, IBS-M – 6) Controls – 22	qPCR	IBS –D ↓*Bifidobacterium* ↓*Lactobacillus* IBS-C ↑*Veillonella* ↑*Lactobacillus* IBS ↓C.*coccoides* ↓B.*catenulatum*	10/27 took regular IBS medication
Kassinen [286]	IBS – 24 (IBS-D -10, IBS-C -8, IBS-M – 6) Controls – 23	16S ribosomal sequencing with confirmation with qPCR on a subset	IBS-D ↓Bifidobacterium ↓*Collinsella* IBS-C ↓*Collinsella* IBS-M ↑Bacteroidetes All Groups ↓lactobacillus	
Kerckhoffs [287]	41 IBS (IBS-D -14, IBS-C -11, IBS-M – 16) Controls – 26	FISH qPCR	↓Bifidobacterium (greater ↓ in B.*catenulatum* in IBS-C and IBS-M)	Controls were a younger population
Noor[260]	11 IBS 22 Control	DGGE	↓Bacteroidetes	
Tana [288]	26 IBS (IBS-D -8, IBS-C -11, IBS-M – 7)	Culture qPCR	↑*Veillonella* (qPCR) ↑*lactobacillus* (Culture)	No yoghurt 2 weeks prior to sampling. Also investigated faecal organic acids and found link to symptom severity
Rajilić-Stojanović [289]	62 IBS (IBS-D -25, IBS-C -18, IBS-M – 19)	qPCR Phylogenetic microarray	↓*Bifidobacterium* ↑Firmicutes ↓Bacteroides	
Chassard [290]	IBS-C – 14 Controls – 12	FISH Culture	FISH ↓*Roseburia* (Firmicute) ↓*Bifidobacterium* Culture ↑Enterobacteriaceae ↓*lactobacillus* ↓*Bifidobacterium*	

All samples were faecal in origin. Kerckhoffs et al[287] also took duodenal brushes. All subjects fulfilled the Rome criteria for IBS except for 3 subjects in the study by Kassinen et al[286].

Table 1.4 – Summary of the relative changes seen to the colonic microbiota in IBS compared to healthy controls.

CHAPTER 2

A RANDOMISED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL OF NALOXONE PROLONGED RELEASE (NALCOL™) AS A TREATMENT FOR FUNCTIONAL CONSTIPATION

2.1 TRIAL METHODOLOGY

2.1.1 OVERVIEW OF THE CLINICAL TRIAL

A single-centre double-blind, randomised, placebo controlled study investigating the efficacy and safety of Nalcol™ given to patients with refractory constipation (Table 2.1.1 and Figure 2.1.1). The trial consisted of three periods.

- Period I was a two week period. Patient suitability and health were assessed to ensure that the inclusion criteria were met, to confirm short-term symptom stability, and to confirm patient compliance with diary completion. Patient demographics, clinical details, and pre-treatment data were collected. Consent was taken at the start of Period I.
- Period II was the key treatment period and lasted 4 weeks. Nalcol™ was given to relieve symptoms which may vary from day-to-day and patients were randomised to Nalcol™ or a placebo. Clinical data over the four weeks of treatment was collected.
- A final period of four weeks (Period III) allowed all patients in the trial, regardless of initial randomization, to evaluate Nalcol™ and provided further clinical observation over a longer time.
- All patients were seen by Mr Mark Bignell (MB), the principal investigator, in a clinic room at the NNUH with a dedicated clinic nurse. The appointments were further standardisation as they all followed the protocol set out in the clinical record file (CRF).

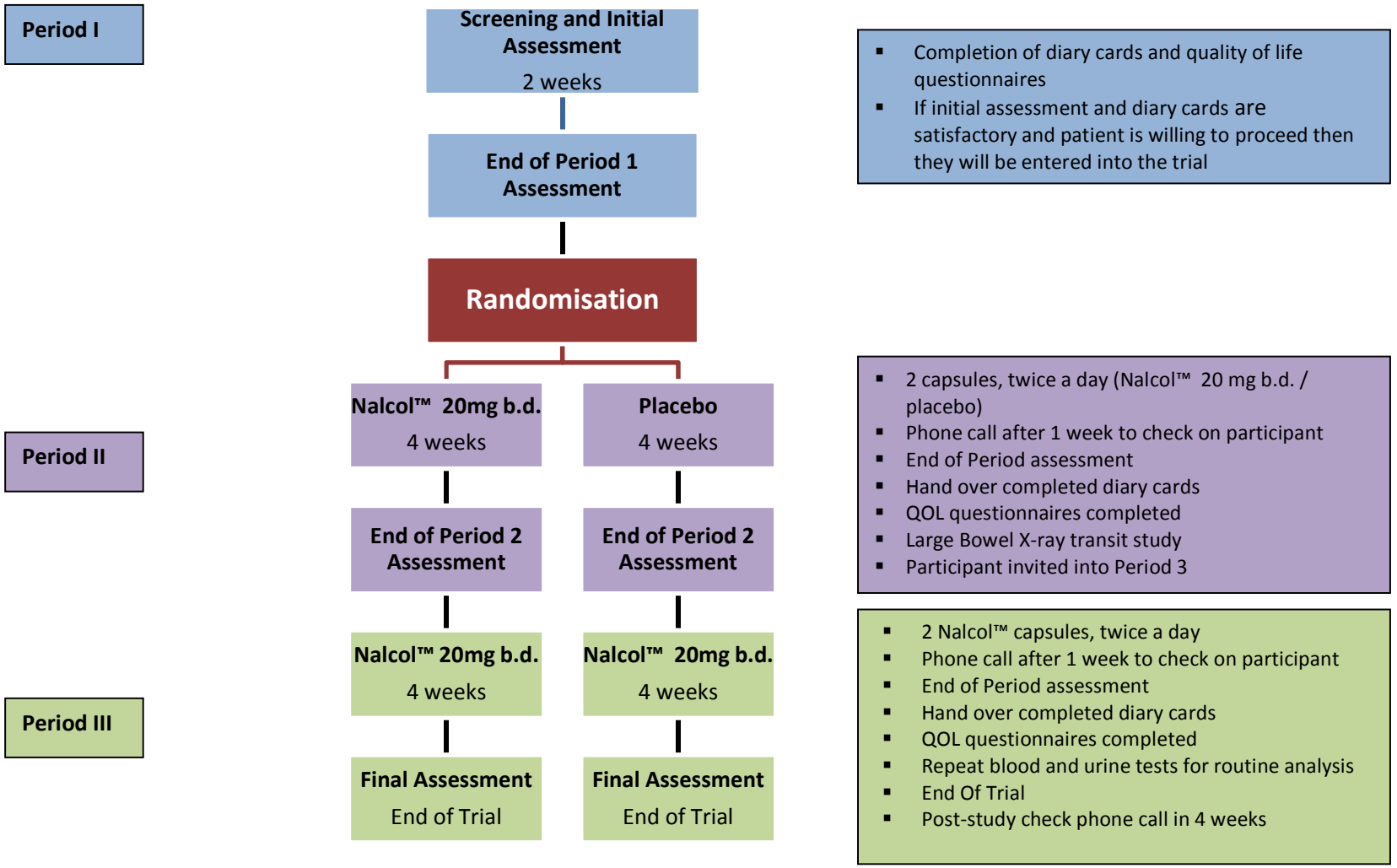
All patients fulfilled the Rome III criteria for functional constipation (page 11) and were managed at a specialist gastroenterology or colorectal clinic at the Norfolk and Norwich University Hospital NHS Trust (NNUH). They may have undergone radiological or endoscopic investigation for their constipation and their basic management, including dietary and lifestyle advice and modification of laxatives, had been instigated.

Period	Week	Visits	Treatments
I Screening	1-2	2 visits at the end of week 1 and week 2	None (Screening and assessment period)
II RCT	3-6	Phone call at end of week 3 to check progress Visit at the end of week 6	Randomised to Nalcol™ or placebo
III Open-label	7-10	Phone call at the end of week 7 to check progress Visit at the end of week 10	All patients received Nalcol™
Follow up phone call at 4 weeks after exiting the trial to look for delayed serious adverse events and adverse events.			

Over the ten week period there were four visits and three telephone calls and all were undertaken by Mr M Bignell. Period I consisted of 2 visits and allowed for screening and consent of patients and lasted 2 weeks. Period II and III lasted 4 weeks each. In period II patients were randomised to either Nalcol™ or placebo whilst in period III all patients received Nalcol™.

Table 2.1.1 – Outline of the three trial periods and the contact made with each trial participitant.

Figure 2.1.1 - The 3 Periods within the Nalcol Study



The primary trial endpoint was a patient-derived assessment of *'satisfactory improvement of symptoms'* and was assessed at the end of each period. Secondary endpoints were change in stool type and frequency recorded on diary cards, disease specific questionnaires, and objective improvements in transit times on repeat transit studies. Patients were assessed at the end of each period except for the transit study which was repeated once, at the end of period II. Only patients who had been initially investigated by means of a transit study would have these repeated at the end of period II. The full clinical trial protocol is located in appendix 1 (page 184).

2.1.2 PATIENT RECRUITMENT

2.1.2.1 Study Population

Adults referred to the gastroenterology or colorectal clinics specialising in chronic constipation at the NNUH and who had persistent symptoms despite initial treatment with diet and laxatives were approached. The definition of constipation was according to the Rome III criteria for functional constipation (see below). Attempts to exclude IBS-constipation (IBS-C) were made on clinical grounds based on the clinical assessment of the principal investigator. IBS-C was diagnosed if either abdominal pain was the most prevalent feature or if there was a history of alternating diarrhoea and constipation.

2.1.2.2 Inclusion Criteria (all must be met)

- Age > 18 years.
- Male or Female.
- Satisfy Rome III criteria for functional (slow transit) constipation.
- No medical or surgical cause for constipation after investigation.
- Symptoms for greater than 6 months.
- Symptoms not relieved by diet and laxatives after 6 months.

Rome III Criteria for Functional Constipation

1. Must include two or more of the following:

    a. Straining (during at least 25% of defecations)
    b. Lumpy or hard stools (on at least 25% of defecations)
    c. Sensation of incomplete defecation (on at least 25% of defecations)
    d. Sensation of anorectal obstruction/blockage (on at least 25% of defecations)
    e. Manual manoeuvres to facilitate defecation (on at least 25% of defecations)
    f. Fewer than three defecations a week

2. Loose stools rarely present unless induced by laxatives

3. Would not normally include patients who satisfy criteria for IBS-C

2.1.2.3 Exclusion Criteria

- Severe cardiac, renal or hepatic impairment*
- Severe psychiatric disturbance*
- Mental disorder preventing adequate informed consent*
- Dilatation of the bowel (megarectum or pseudo-obstruction)
- Concomitant medication with drugs known to cause constipation
- Known pregnancy, suspected pregnancy, or trying to conceive*
- Currently Breast Feeding*
- Currently participating (or within 1 month) in any other study*

- * These are standard exclusion criteria for drug trials studying novel, receptor-based laxatives and not specifically related to Nalcol™.

2.1.2.4 Recruitment

Patients with functional constipation were recruited from three possible sources.

I. Direct referral from consultants in gastroenterology and colorectal surgery who managed these patients. Most participants would be recruited from clinics which specialise in functional gastrointestinal disorders.
II. Recruitment from the department of physiology at the NNUH. They have a database of patients who had undergone biofeedback training for constipation.
III. Recruitment from the department of radiology at the NNUH. A search dating back ten years (2001 to 2011), on the electronic radiology reporting system, identified patients who had undergone transit studies for investigation of functional constipation. Since we were recruiting patients who had failed conventional treatments for a chronic condition, a retrospective approach was justified.

There would be some overlap of suitable cases between the three groups. The potential participants were sent a letter detailing the study, inviting them to take part with a follow-up phone call, one week after the letter, to answer any questions. A follow up phone call aimed to increase recruitment. Arrangements were then made to screen those interested in participating with a view to trial entry. The recruitment process was all conducted by MB.

2.1.3 SCREENING (PERIOD I)

The first phase of the study was a decision about whether patients were suitable for the study. The period lasted 2 weeks and this was an adequate time to assess disease stability. There were two visits during this period:

I. At the initial interview eligibility was assessed against the inclusion/exclusion criteria, with medical and social background considered. At this point informed consent was sought.

II. At the end of Period 1 after review of the initial blood and urine tests and on successful completion of the diary card and questionnaires over the two week period.

If for any reason the patient was considered unsuitable at the end of Period I then they were not randomised to Period II of the trial and were not included in the analysis. This is discussed fully in the results section (2.3.1.1).

Screening procedures involved during visit I:

- Explanation of all the procedures involved.
- Recording of all current medical therapy including those used to manage constipation.
- Current and past medical history – if necessary, referring to the patient's medical notes.
- Blood sample for routine clinical biochemistry and heamatology tests.
- Urine for routine urinanalysis.
- General, abdominal and digital rectal examination.
- Blood Pressure measurement and ECG.
- Sigmoidoscopy and stool sample if consent was obtained (to be performed ONLY if accepted into the trial). The stool samples were stored on ice and at the end of the screening clinic transferred by MB to the Institue of Food Research (IFR), Norwich for preparation prior to freezing. All samples were identified by the participant's identification number (see methodology section for faecal analysis).

2.1.3.1 Consent and Randomisation (Visit II)

Patients seen at the start of Period I had the trial discussed and, if willing, were consented for the trial. At the end of the first visit they underwent routine blood tests and were instructed to complete the first section of the diary card. At the second screening visit two weeks later, the diary card was reviewed to ensure completion and assuming normal routine blood tests the participant was entered into the trial. The participant was entered into period II and randomised (1:1) to receive one of the two treatment regimes, using a computer –generated

randomization sequence. St Mary's Pharmacy Unit in Cardiff was responsible for the packaging and randomization of the trial capsules, both Nalcol™ and Placebo. The capsules were stored and dispensed by the pharmacy at the NNUH, which also ensured that both the participant and the researchers remained blinded to the treatment.

2.1.4 INTERVENTIONS

Trial interventions were undertaken in Periods II and III. In period II patients were randomised to either Nalcol™ 20mg b.d. or a placebo (2 capsules b.d.). In period III all subjects were given Nalcol™20mg b.d. on a open label basis.

2.1.5 TRIAL ENDPOINTS

2.1.5.1 Primary Endpoint

The primary endpoint to assess the effectiveness of Nalcol™ was a patient derived global assessment of satisfactory improvement of symptoms derived from Dunger-Baldauf et al[298] who used it in patients with IBS. This global assessment asked the participant the statement *'During the last 2 weeks I have had satisfactory improvement in my symptoms of constipation'* An affirmative response indicated subjective satisfactory improvement. This was assessed at the end of each period.

2.1.5.2 Secondary Endpoints

- Change in stool frequency and stool type assessed from daily diaries.
- Change in symptom scores from a patient-completed questionnaire (PAC-SYM).
- Change in disease specific quality of life from a patient-completed questionnaire (PAC-QOL).
- Objective improvements in transit time on repeat X-ray transit studies.

2.1.6 TRIAL ASSESSMENTS

Throughout the study patients were asked to complete a diary, recording details related to their bowel frequency, laxative use and any adverse events. These were reviewed at the end of each trial period to confirm adequate completion. The timing of assessments is shown in table 2.1.2 (Page 80) and a copy of the diary card is documented in the appendices 2 (page 248).

2.1.6.1 Period I

At the end of Period I patients completed the two validated questionnaires[299,300];

- Patient assessment of their symptoms of constipation (PAC-SYM).
- Patient assessment of their quality of life secondary to their constipation (PAC-QOL).

The returned diary card was reviewed to check satisfactory completion and if acceptable the patient was randomised into the trial i.e. to start Period II. The global assessment question was included on the diary card to assess the primary endpoint. At this stage it was expected that this would be a negative response. Patients were also asked to undergo rigid sigmoidoscopy for stool sampling at the end of visit I, but consent to this was not necessary for entry into the clinical trial.

2.1.6.2 Period II

At the end of Period II these assessments were completed:

- The diary cards were checked for satisfactory completion over the last 4 weeks of Period II including answering the global assessment question. Adverse events were noted.
- PAC-SYM and PAC-QOL Questionnaires.
- Compliance in taking medication was assessed against capsules returned.
- Repeat stool sample in subset of patients.
- Large bowel X-ray transit study in patients in those patients who had documented slow transit as part of their initial work up and these were used as a comparison.

Patients were phoned after the first week of period II to ensure they understood the requirement of participation in the trial and to answer any questions.

2.1.6.3 Period III

At the end of the study the following procedures were completed:

- The diary cards were checked for satisfactory completion over the last 4 weeks of Period III including answering the global assessment question. Adverse events were also noted.
- PAC-SYM and PAC-QOL Questionnaires.
- Compliance in taking medication was assessed against capsules returned.
- Repeat stool sample in subset of patients.
- Repeat blood pressure recordings.
- Repeat blood and urine samples for follow-up haematology and biochemistry testing.

Patients were telephoned at the end of the first week of period III, as per Period II. The timing of assessments are summarised in table 2.1.2.

2.1.7 ANALYSIS OF TRIAL DATA

The primary analysis was a comparison of the response to treatment during Period II of the study (weeks 3-6) in the treatment group and those in the placebo group. Response was defined as those participants who give an assessment of *'satisfactory improvement'* over the last two weeks of the Period II to the 'global question' on the diary card.

A number of secondary analyses aimed to further assess the response to Nalcol™ treatment:

- Comparison of stool frequency and type and of laxative use between treatment and placebo groups during Period II of the study.
- Comparison of PAC-SYM and PAC-QoL scores at the end of period II. PAC-SYM will be used to provide an overall symptom score; but each component will also be analysed separately to identify changes to individual symptoms.
- Comparison between the pre and post trial transit study as an objective marker of transit times.
- Graphical representation (line or bar charts) of the temporal changes in stool frequency and type and analysis to assess any drop-off of effect after eight weeks of use of Nalcol™. i.e comparison of period II and Period III in the Nalcol™ arm.
- A comparison of pre-treatment versus treatment symptom levels in the whole study population (n=120) i.e. Period 1 vs. Period 3.

Note: Usual laxatives and other lifestyle measures were taken throughout with no new laxatives started during the study period. Patients who developed diarrhoea were instructed to call MB who advised about any changes needed. In this scenario, it would be advised that participants reduce their 'usual' laxative dosage and rely on the trial capsule if they appeared to be working. This would be taken into account when analysing the diary cards.

PHASE / ASSESSMENT	Pre-Treatment (Period I)		Treatment Period (Period II)				Post Treatment Period (Period III)			
	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10
Global assessment		X				X				X
Diary Card	X	X	X	X	X	X	X	X	X	X
PAC-SYM		X				X				X
PAC-QOL		X				X				X

The main outcome measure, the global assessment question, was recorded at the end of weeks 2, 6, and 10. The diary card was completed everyday and recorded stool type and frequency and also laxative usage. PAC –SYM and PAC-Qol are validated questionnaires used to asses constipation focusing on symptoms and quality of life respectively.

Table 2.1.2 – The timing of assessments over the ten week trial period.

2.1.7.1 Statistical Analysis

The primary outcome was analysed as a 2x2 contingency table using Fisher's exact test. This was also used for the data generated from the transit studies as appropriate. Continuous variables from diary card data were analysed using the mean or median according to the distribution of the data and the appropriate statistical test (Student t-test or a Mann-Whitney U test), with comparisons made between baseline and treatment periods. Pre-treatment symptom levels and quality of life at the end of period I were compared to symptom and quality of life levels at the end of the study (end of period III) using the above tests, again according to the distribution of the data.

2.1.7.2 Patient Numbers and Power of the study

The primary endpoint was to identify responders and non-responders according to the global question. Recent clinical trials in this condition have shown placebo response rates ranging from 15-35%[172,216,301,302] and as such we chose a placebo response rate of 25%. A worthwhile treatment response would be 32% greater than the placebo which was considered to be clinically significant given the resistant nature of constipation to medical management. For a study with a power of 95%, this increase in response could be detected at a significance level of 0.05 if the total number included was 120 (60 in each group). This estimate was based on using a Binomial Test and was confirmed using Fishers Exact Test.

2.1.8 ANALYSIS OF FAECAL SAMPLES

We aimed to obtain faecal samples for analysis from a minimum of 30 participants at the end of each trial period. At least 200mg of fresh stool was needed but it was anticipated that a greater quantity would be collected. The stool was initially stored in faecal sample pots in polythene bags placed over ice and then transferred by MB to the IFR, Norwich (i.e. within 4 hours of collection). Here they were divided into 5 batches of approximately 200mg and frozen. The methodology used to analyse the stool samples is explained later in the 'Analysis of colonic microbiota methodology' section.

Provision of a stool sample was **NOT** an absolute requirement for entry into the trial, although all participants were approached until there were sufficient numbers for analysis.

2.1.9 PATIENT WITHDRAWAL, MONITORING, AND SAFETY

2.1.9.1 Withdrawal from the trial

Withdrawal from the trial was defined as any patient who failed to complete all three periods. Patients were withdrawn from the trial if they failed to adequately complete the diary cards or if they did not attend for the appointments once period II had commenced. Patients could voluntarily withdraw for any reason without this affecting their subsequent clinical management.

On withdrawal from the study patients were asked to complete:

- PAC-SYM and PAC-QOL questionnaires.
- Diary cards for review to check compliance and adverse effects.
- Routine blood and urine for analysis.

They were also followed-up by phone four weeks after withdrawal to ensure no delayed adverse events had occurred.

2.1.9.2 Monitoring

The study was monitored by the trial investigators and the Norfolk and Norwich Hospital (sponsor) in accordance with the guidance in section 5.18 of the ICH Harmonised Guidance for Good Clinical Practice (www.ichgcp.net). During the trial the Medicines and Healthcare Products Regulatory Agency (MHRA) conducted an inspection with the only major concern related to timing of the consent process, which was moved to the 1st visit of Period I from the 2nd visit. The inspection was otherwise favourable for the running of the clinical trial (see 'Bringing a Clinical Trial to Fruition', section 2.2, page 85).

2.1.9.3 Safety Evaluation

Potential adverse events were reported to the investigators at trial visits by the participant. Certain symptoms were specifically enquired about, namely; abdominal pain, diarrhoea, bloating, and dizziness. These were then evaluated by the investigators and the sponsor informed of any serious adverse events. All serious adverse events and suspected unexpected serious adverse events were reported in accordance with the ICH Harmonised Guidance for Good Clinical Practice (section 4.11, www.ichgcp.net). Full details of the safety evaluation of the trial are outlined in section 7 of the protocol which is located in appendix 1 (page 215). Both the Chief and Principal investigators had received full training in GCP.

2.1.10 ETHICAL CONSIDERATIONS

Written informed consent was taken by the principal investigator with a third party witness present. Patients were informed that they could withdraw at anytime. The use of a patient information sheet facilitated the process of informed consent. After receiving the information sheet the patients did not have a time limit to decide on whether to participate in the trial. All trial data was confidential and identification within the study was by a coded number to ensure anonymity. The subject's involvement in the clinical trial was made clear in the notes through the use of a sticky label. The trial was approved by the MHRA, Cambridgeshire Research and Ethics Committee 4, and the Research and Development Department at the NNUH, the latter also acted as sponsors. Full details of ethical considerations are listed in section 8 of the protocol which is located in appendix 1 (page 219).

2.1.11 FUNDING

The trial was funded by a research fund held by Mr Michael Rhodes at the NNUH. This paid for labarotory consumables, the work carried out at St Mary's Pharmacy Unit and any costs incurred from the NNUH for blood sampling etc. The Nalcol™and placebo were supplied by SLA Pharma (Watford, UK) at no cost. Mark Bignell was funded by working at the Spire Hospital, Norwich, and the NNUH.

2.2 BRINGING A CLINICAL TRIAL TO FRUITION IN THE NHS – A PERSONAL PERSPECTIVE

This was the first time I had undertaken research within the NHS. There are significant processes involved in setting up and running a clinical trial in this setting. This is increased where the trial involves an investigational medicinal product (IMP). A timeline for the Nalcol™ trial is shown below.

2.2.1 GAINING APPROVAL

As with all research, approval must be sought from the local research and ethics committee (REC) and the hospital research and development department (R&D). In the case of a trial involving an IMP approval must also be sought from the medicines and healthcare products regulatory agency (MHRA). Clinical trials in the UK are regulated by The Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 1031) as amended. These regulations implement Directive 2001/20/EC ('The Clinical Trials Directive'). According to the Clinical Trials Directive, clinical trials of medicinal products in human subjects require authorisation by the competent authority (MHRA in the UK) and a favourable opinion by an ethics committee. This authorisation is granted in the form of a clinical trial authorisation (CTA). The regulations only apply to trials of medicinal products. All trials involving human subjects must abide by the principles of good clinical practice (GCP). GCP is an international quality standard that is provided by International Conference on Harmonisation (ICH), an international body that defines standards, which governments can transpose into regulations for clinical trials involving human subjects. GCP guidelines are designed to protect subjects in a clinical trial and also provide assurance of the safety and efficacy of the research undertaken. GCP guidelines include standards on how clinical trials should be conducted; define the roles and responsibilities of clinical trial sponsors, clinical research investigators, and monitors, and how to act upon adverse events.

Applications must be made to all these bodies and in January 2009 a new web based application system, IRAS (Integrated research application system), was introduced. As its' name suggest, IRAS allows for one application form to be filled and once completed the programme produces the required fields to be amalgamated for each regulatory body to avoid duplication. Prior to completing the application form it is important to take the time to fully develop the protocol and anticipate any possible amendments that maybe needed once the trial is running before the application is approved. The benefit of this is that any amendment made after approval often need to be submitted to all three regulatory agencies before it can be implemented. The turnaround time for this is up to 35 days and can add significant delays to the progress of the trial. To aid in this it is important to have liaised with all parties involved in the running of the trial at the outset. Aspects of this advice were not heeded at the start of this trial. The research team felt whilst designing the protocol that the Norfolk and Norwich University hospital (NNUH) should act as sponsor since it would recruit NHS patients at a NHS institution. The R&D department were approached before ethics and MHRA approval was sought but, due to a lack of experience in their department with acting as a sponsor, they felt that the trial should be sponsored by the UEA and Mr Michael Rhodes. The sponsor is the individual or institution that takes responsibility for the initiation, management and financing (or arranging the financing) of the study. The sponsor must satisfy itself that the study meets the relevant GCP standards and ensure that arrangements are put in place for management, monitoring and reporting. The sponsor is often involved in the indemnification of the trial. This trial recruited NHS patients and the NHS indemnity scheme therefore applied. Furthermore SLA pharma provided further indemnity. It was only after submitting to R&D, having gained approval from the REC was it felt that this trial should be sponsored by the NNUH requiring a substantial amendment to REC before the trial had even started. This highlights the importance of good communication from the outset and is something that is gained from running IMP trials which neither I nor the NNUH R&D department had.

2.2.2 RUNNING A CLINICAL TRIAL

Running a clinical trial is not simply recruiting patients and recording the data generated. Any clinical trial requires a trial master file (TMF) to be created and maintained, the contents of which are listed in the appendices. Any documentation pertaining to the trial is filed in the TMF. Should any amendment to the trial need to be made then an application to both REC and the MHRA needs to be submitted and this needs to be recorded in the amendment log, as well as the documentation being filed in the TMF. Maintaining the TMF is an important part of running a trial. This acts as an audit trail to ensure that the trial is run in accordance with the principles of GCP and forms the basis of monitoring visits. Monitoring visits are usually conducted monthly by the sponsor and the TMF forms the basis of the visit. Any action that needs to be undertaken after a monitoring visit is recorded in a report which must be completed by the time of the next monitoring visit The work generated from these visits depends on the complexity of the trial but also on the experience of the research team. Those who are less experienced are more likely to be unaware of all the documentation that needs to be archived. The TMF for this trial consisted of five A4 ring binders. Alongside the monitoring visits it is important to have regular meetings with the sponsor (+/- R&D), chief investigator and other closely involved departments, in this case pharmacy. The minutes of these meetings are recorded.

2.2.2.1 Inspection by MHRA

Any clinical trial where approval by the MHRA was sought is liable to inspection by the MHRA as is any institution involved in the running or sponsoring of the clinical trials. In January 2011 the NNUH was inspected by the MHRA who randomly selected three trials to be examined in detail, with this trial being selected. The preparation required for the visit by the investigators and the R&D department was extensive since a poor review could result in the trial being suspended or at worst the NNUH having to suspend all clinical trials if a serious breach was identified. To prepare for the inspection the TMF was fully reviewed and a number of issues were found and dealt with. Attendance at a 'GCP and how to survive a MHRA inspection visit' was compulsory to help in the preparations. The inspection was conducted over three days with an interview with the inspectors for the principal investigators of the three trials selected

on the final day. The interview dealt with issues that had arisen over the course of the inspection and in my opinion was a stressful experience. Fortunately the report from the MHRA was positive and the inspection report was favourable.

2.2.3 CLOSURE OF A CLINICAL TRIAL

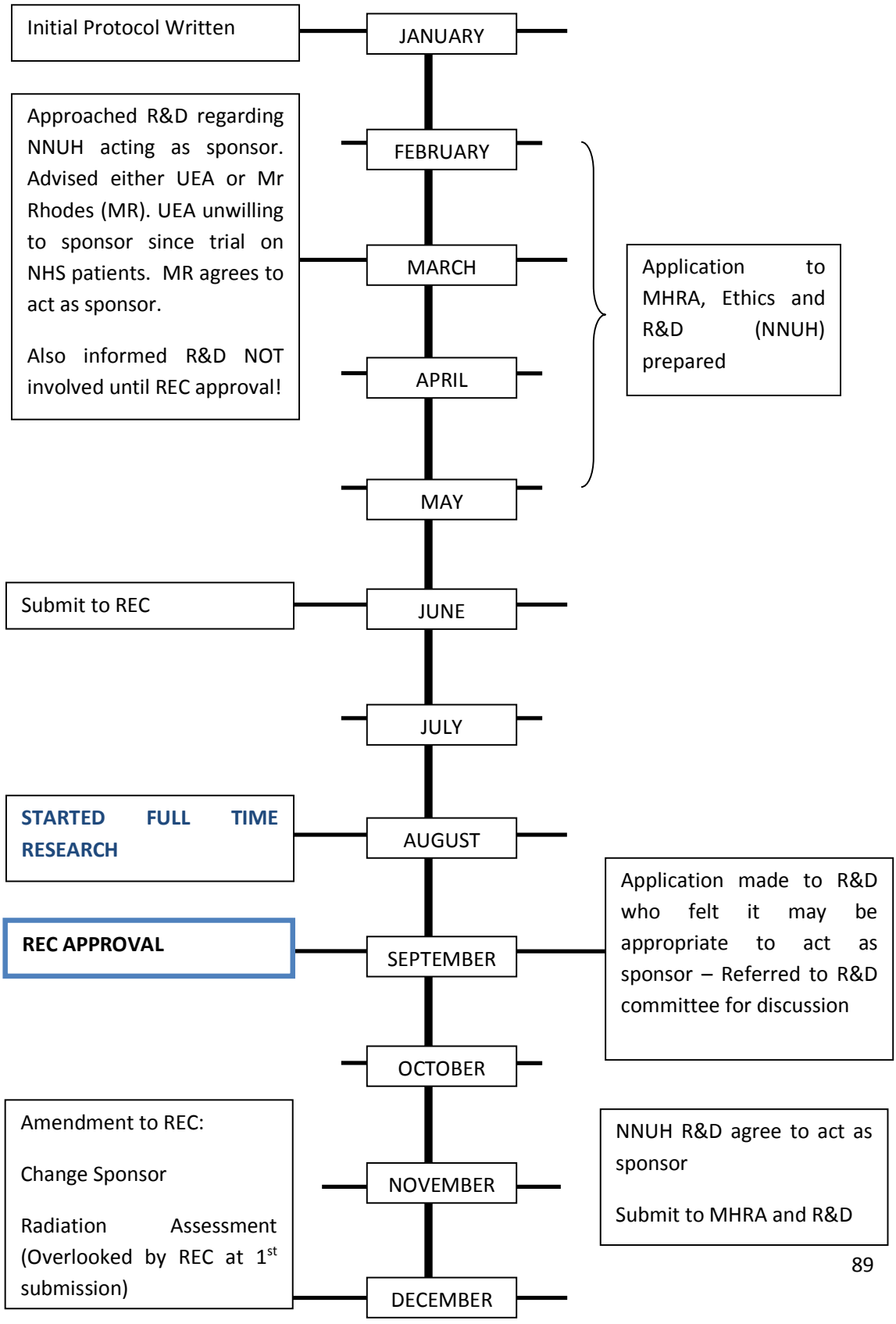
Closure of a clinical trial is relatively straight forward. All the documentation including the clinical record files and database need to be archived and reports sent to each of the regulatory bodies. It is also courteous to inform the family doctor of the participants that the trial had ceased and any further follow-up or procedures which need to be undertaken.

2.2.4 LESSONS LEARNT

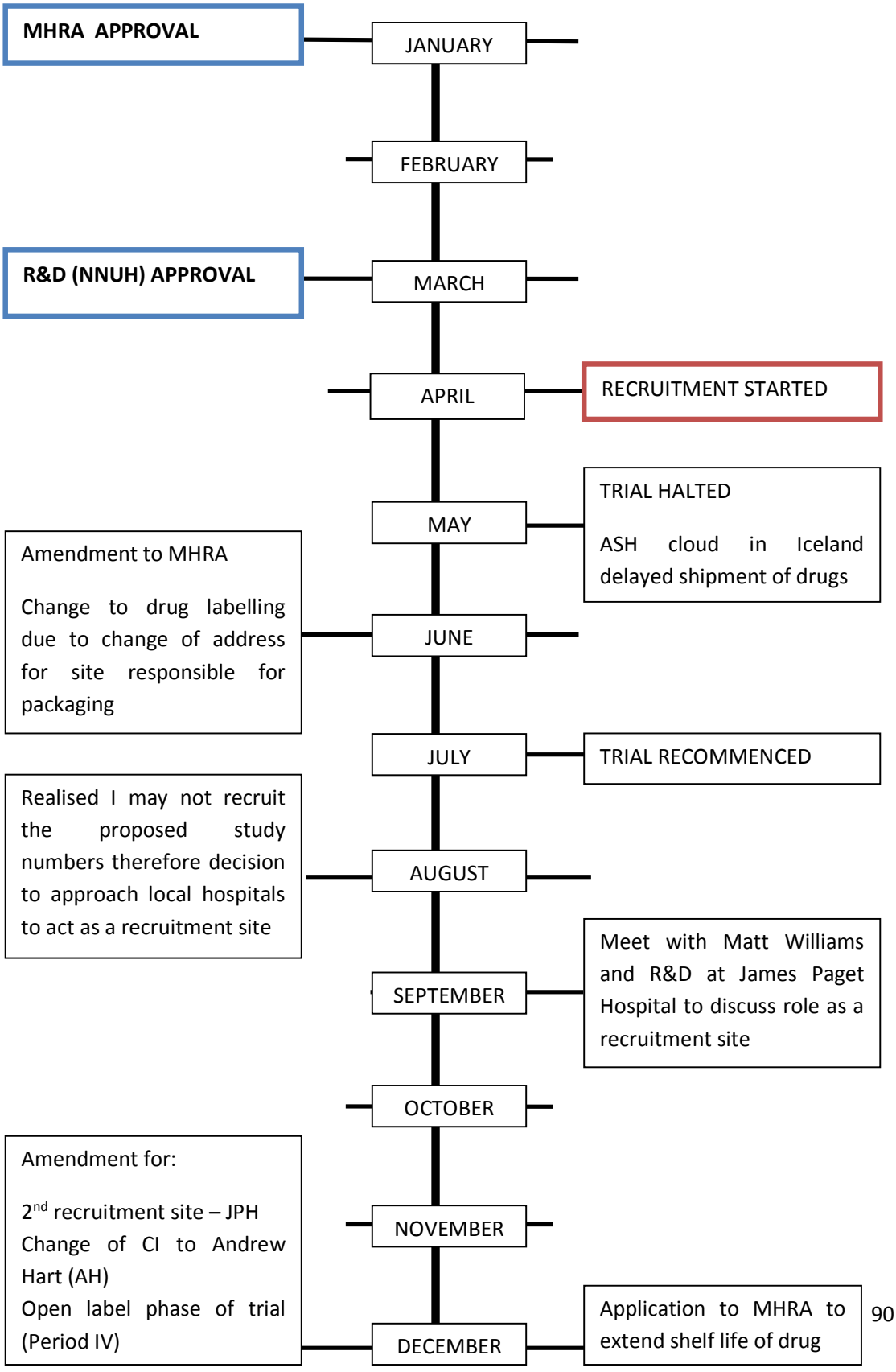
At the start of the trial I had very limited experience of clinical research and looking back over the last two years there are a two areas that I would do differently. Firstly the importance of good protocol design and the involvement of the sponsor and other parties involved in running the trial is key. This allows the trial to run smoothly without the delays encountered that accompany any amendment made. Secondly it is apparent that it is unrealistic for a clinical trial to be run without the involvement of the clinical research and trials unit based at the hospital. At a minimum, advice can be sought but I think the use of a dedicated research nurse is essential to aid not only in participant interventions but to also ensure that the administration is also kept up-to-date.

Running a clinical trial has been a worthwhile experience and given me skills that I will continue to use on my return to clinical medicine. I will continue to be involved in clinical research and the experience gained in this trial will stand me in good stead to ensure that further trials are run efficiently and in accordance with the standards of GCP.

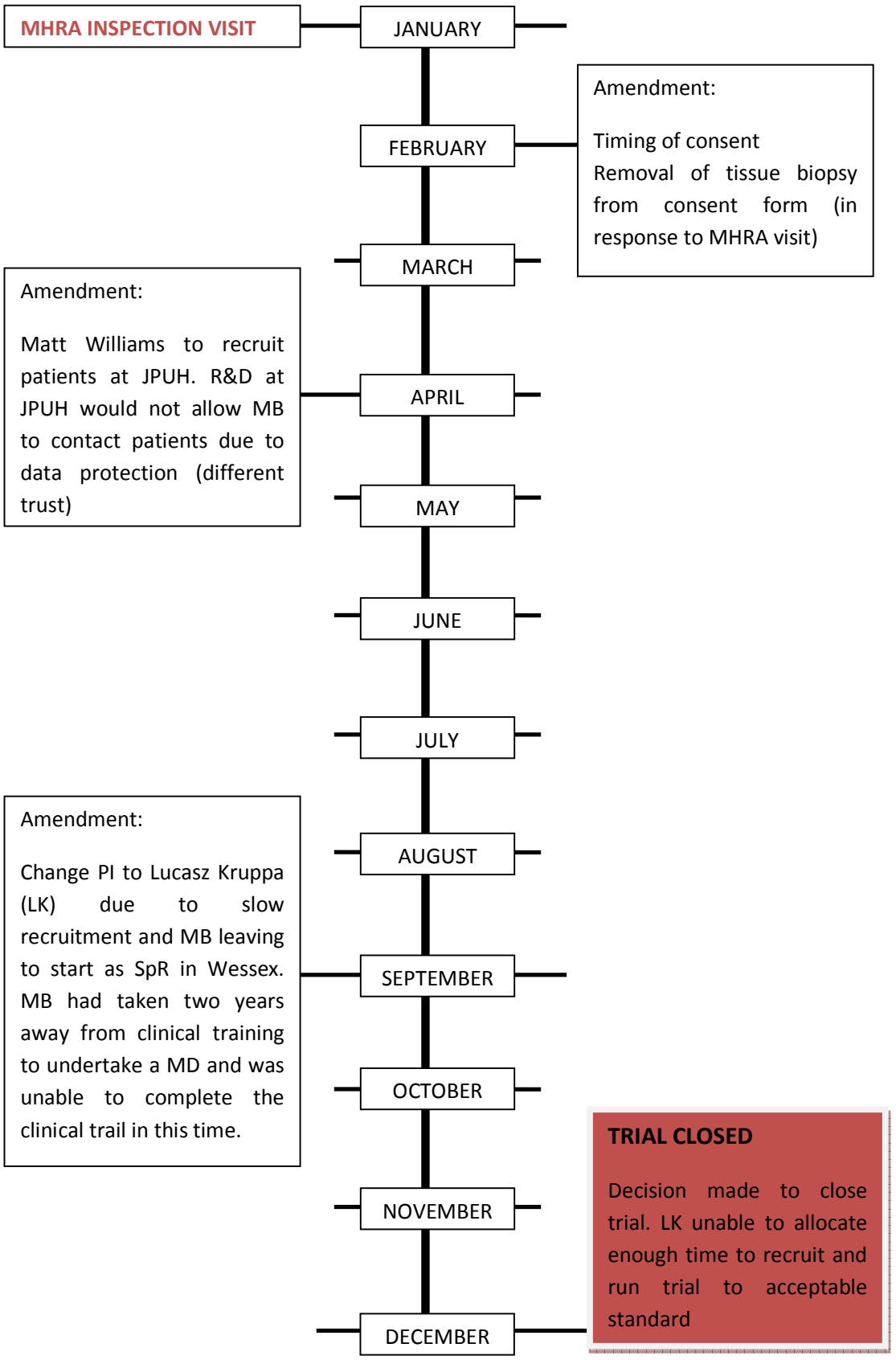
2.2.5 TIMELINE OF CLINICAL TRIAL – Year 1 - 2009



TIMELINE OF CLINICAL TRIAL – Year 2 - 2010



TIMELINE OF CLINICAL TRIAL – Year 3 - 2011



2.3 RESULTS OF THE NALCOL™ CLINICAL STUDY

The results are presented according to the three trial periods; Period I (Screening), Period II (Nalcol™ vs. Placebo), and Period III (Open Label).

2.3.1 PERIOD I (SCREENING FOR ELIGIBILITY PERIOD)

This was an initial two week period designed to assess, demographics, baseline symptoms and clinical characteristics, and eligibility for the clinical study.

2.3.1.1 Patient Recruitment

During the study 42 females and no males attended for screening. One patient completed the initial interview but was ineligible for entry as symptom control on her current treatment had improved over the preceding few weeks. She was therefore excluded from subsequent analysis leaving 41 participants who were enrolled and randomised. The 41 patients had a median age of 45 yrs (range 23-76 years) with symptoms for a median of 20 years (Range, 3-55; IQR, 12-30). The demographic and clinical investigations and treatments are described in Table 2.3.1. Twenty were randomised to the active treatment phase and twenty-one to the placebo phase (Figure 2.3.1). It was the aim of the study to recruit 120 subjects (60 in each group) and this was not achieved.

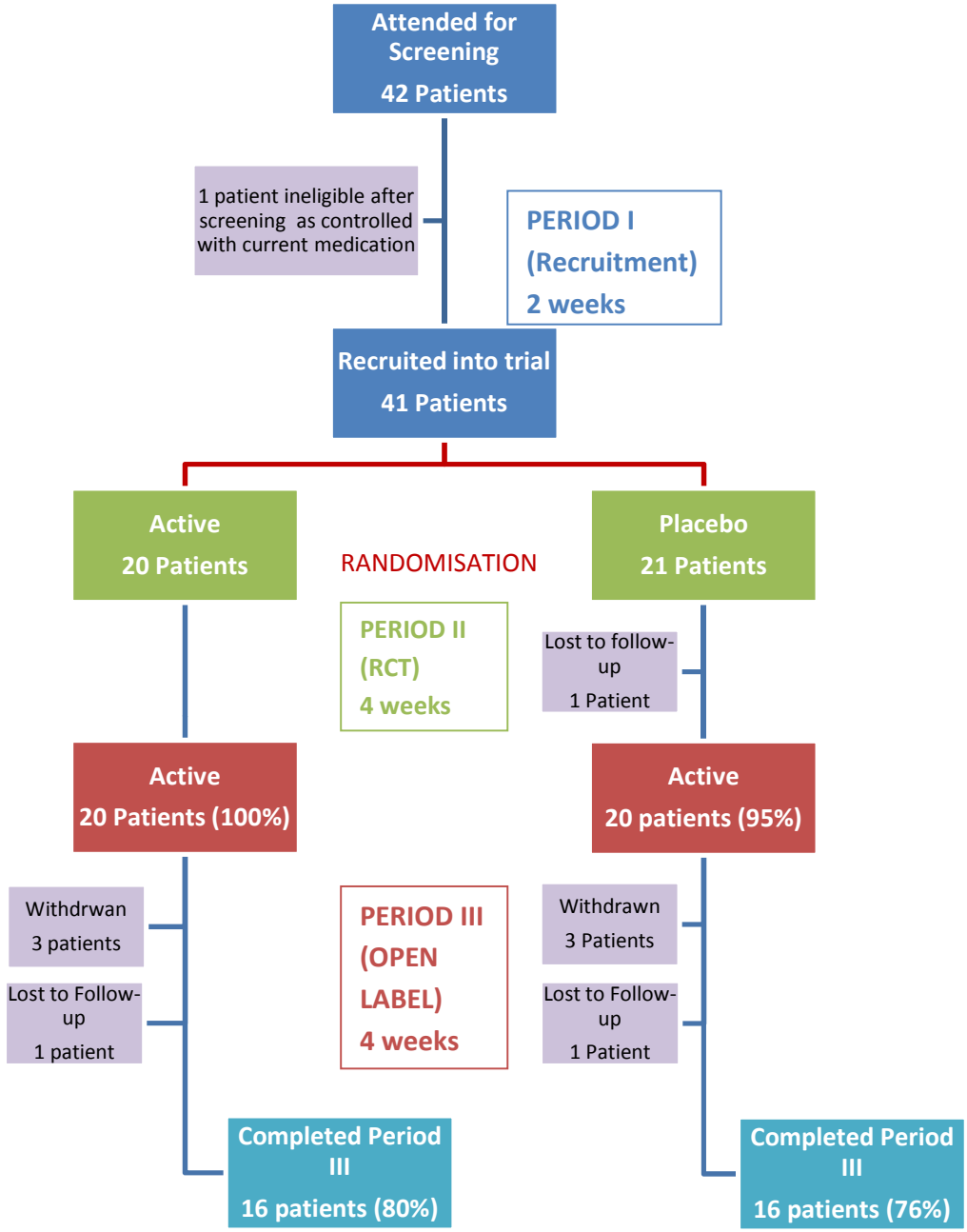
Transit studies were part of the initial workup in 34 patients (83%) of which 27 (66%) had documented slow transit. There were 18 patients (44%) who had undergone biofeedback with either no initial response or no continued response. Thirty-two patients (78%) were taking laxatives without satisfactory symptom resolution.

		Active (n=20)	Placebo (n=21)	P value
Median Age (Yrs) (range, IQR)		47 (25 – 76, 39 – 53)	42 (23 – 67, 38 – 55)	0.66
Sex (F)		20	21	n/a
Median Duration of symptoms (yrs) (range, IQR)		20 (3 – 45, 9 – 27)	23 (3 – 55, 13 – 30)	0.28
Previous Investigations	Transit Study	15 (75%)	19 (90%)	0.24
	Barium Enema	12 (60%)	8 (38%)	0.22
	Colonoscopy	10 (50%)	8 (38%)	0.54
	Biofeedback	9 (45%)	9 (43%)	0.76
Current treatment	None	3 (15%)	5 (24%)	0.70
	Laxatives	17 (85%)	15 (71%)	0.45
	Rectal Irrigation	0	1 (5%)	1.00
Concomitant opiate Use		4 (20%)	2 (10%)	0.41

The 41 patients had a median age of 45 yrs (range 23-76 years) with symptoms for a median of 20 years (Range, 3-55; IQR, 12-30). Transit studies were part of the initial workup in 34 patients (83%) of which 27 (66%) had documented slow transit. There were 18 patients (44%) who had undergone biofeedback with either no initial response or no continued response. Thirty-two patients (78%) were taking laxatives without satisfactory symptom resolution. There were no significant differences between either treatment arm

Table 2.3.1 – Characteristics of patients screened into clinical trial (Period I)

FIGURE 2.3.1 – PROGRESSION OF PARTICIPANTS THROUGH THE STUDY



42 patients attended for screening and these were all female. One patient was ineligible therefore 41 patients were entered into the trial. One patient was lost to follow up in period II and two were lost in period III. There were six withdrawals in period III. In Period II the number of patients completing each arm was similar (Nalcol – 20/20, Placebo 20/21).

2.3.2 PERIOD II – NALCOL™ VS. PLACEBO

During period II patients were randomised and received either oral Nalcol™ (20mg, b.d.) or Placebo for 4 weeks. There were 20 participants randomised to the active medication in Period II whilst the remaining 21 took the placebo with one patient lost-to-follow up in the placebo arm. The patient who was lost to follow up was a 23yr old woman who had had symptoms for 7 years. She had documented slow transit and had no response from biofeedback

2.3.2.1 Adverse Events and Reasons For Withdrawal

The reported adverse events throughout period II are listed in Table 2.3.2. There were 32 adverse events in 23 patients (56%). In the placebo group, 11 adverse events occurred in 9 patients (43%) compared to 21 events in 14 patients (70%) in the active group (p=0.21). The commonest adverse events were abdominal pain/cramps and bloating. There was no difference in the number of patients with abdominal pain (8/20 (40%) Vs 7/21 (33%), p=0.75) between treatment arms, however there were significantly more patients in the Nalcol™ group who complained of bloating than in the placebo group (8/20 (40%) Vs 1/21 (5%), p=0.009). There were no withdrawals, in either arm, in Period II.

2.3.2.2 Response to the Global Improvement Question (Table 2.3.3)

This was the primary outcome of the trial. In both groups all responses were 'No' to the global question - 'Did you have a satisfactory improvement in your symptoms over the last 2 weeks?' at the end of the screening period (Period I). There was no difference between the two groups at the end of period II in response to the global question (Nalcol™ vs. Placebo; 20% vs. 24%, p=1.00). Further sub analysis of those patients who had had a transit study as part of their initial work-up revealed no statistical difference in the number of affirmative responses between those who had documented slow transit and those with normal transit (1/12 (8%) vs. 3/8 (38%), p=0.26).

	Period II				Period III			
	Abdominal Pain	Dizziness	Bloating	Other	Abdominal Pain	Dizziness	Bloating	Other
Active (n=20)	8 (40%)	0	8 (40%)	Anxiety Flatus x2 Diarrhoea x2	9 (45%)	1 (5%)	9 (45%)	Flatus x2 Migraine Anxiety
Placebo (n=21)	7 (33%)	1 (5%)	1 (5%)	Rectal Bleed Migraine	4 (19%)	0	5 (24%)	Headache Palpitations

In period II there were 32 adverse events in 23 patients. In the placebo group 11 adverse events occurred in 9 patients compared to 21 events in 14 patients in the active group. In period III (open label) there were 34 adverse events in 20 patients.

Table 2.3.2 – Adverse events reported throughout the study period for all patients

2.3.2.3 Interpretation Of The Symptom Diary Cards (Tables 2.3.4 and 2.3.5 and Figure 2.3.2)

Completed diary cards were available for 37 of the 41 (90%) participants with two participants in each group failing to return diary cards at the end of the 10 week trial (end of period III). At completion of Period II there were no differences in the median stool type (Nalcol™ vs. Placebo; 3 vs. 3, p=0.91) or the median stool frequency (Nalcol™ vs. Placebo; 1 vs. 1, p=0.69) between the two groups. There was also no difference in the scores for each group compared to the baseline scores at the end of period I. When the patients who had stool types 5-7 were excluded (diarrhoea secondary to laxative use or rectal irrigation, n=7; placebo=5) there was a non-significant improvement in the median stool type from 2.5 to 2 at the end of period II (p=0.60) in the active group and from 2.5 to 2 in the placebo group (p=0.60) however there was no significant difference between the two treatment arms at the end of period II (P=0.92). There was no difference in the median stool frequency between periods I and II in either the active or placebo group (p=0.96 and 0.75 respectively). Of the 41 patients enrolled eight patients (20%) were not using laxatives to manage their constipation (four in each group). There was no difference at the end of period II between the two groups in the amount of laxative used compared to the end of Period I (Figure 2.3.2).

2.3.2.4 Results Of The Repeat Transit Studies (End Of Period II)

Repeat transit studies were requested on all patients who had previously had documented slow transit (n=27, 66%). This was performed at the end of period II, with any change to normal transit providing an objective marker of improvement. Only 33% (4/12) of patients randomised to Nalcol™ attended for these and 47% (7/15) in the placebo group (p=0.70) (Table 2.3.6). One patient in the placebo group forgot to attend for her transit study at the end of period II and instead attended at the end of period III (patient is shown in italics). No patients had a reduction in their transit time at the end of period II, irrespective of treatment arm, and in spite of 2 patients having a clinical response in the placebo group.

	Period II	Period III
Active (n=20)	4 (20%)	7 (35%)
Placebo (n=21)	5 (24%)	9 (43%)

Table 2.3.3 – Number of affirmative responses to the global question at the end of Period II and III (Primary Outcome)

Stool frequency / day	Week 2 Period I	Week 6 Period II	Week 10 Period III
Active	1 (0 – 4)	1 (0 – 2)	1 (0 – 2)
Placebo	1 (0 – 3)	1 (0 – 2)	1 (0 – 2)

Table 2.3.4 - Median stool frequency /day (range) at the end of each trial period for both groups

Stool type	Week 2 Period I	Week 6 Period II	Week 10 Period III
Active	2 (1 – 5)	2.5 (1 – 6)	3 (1 – 7)
Placebo	3 (1 – 7)	2.5 (1 – 5)	3 (1 – 5)

Table 2.3.5 - Median stool type at the end of each trial period for both groups as determined by the Bristol Stool Chart (range)

Completed diary cards were available for 37 of the 41 participants. None of the parameters listed in the tables above were significant

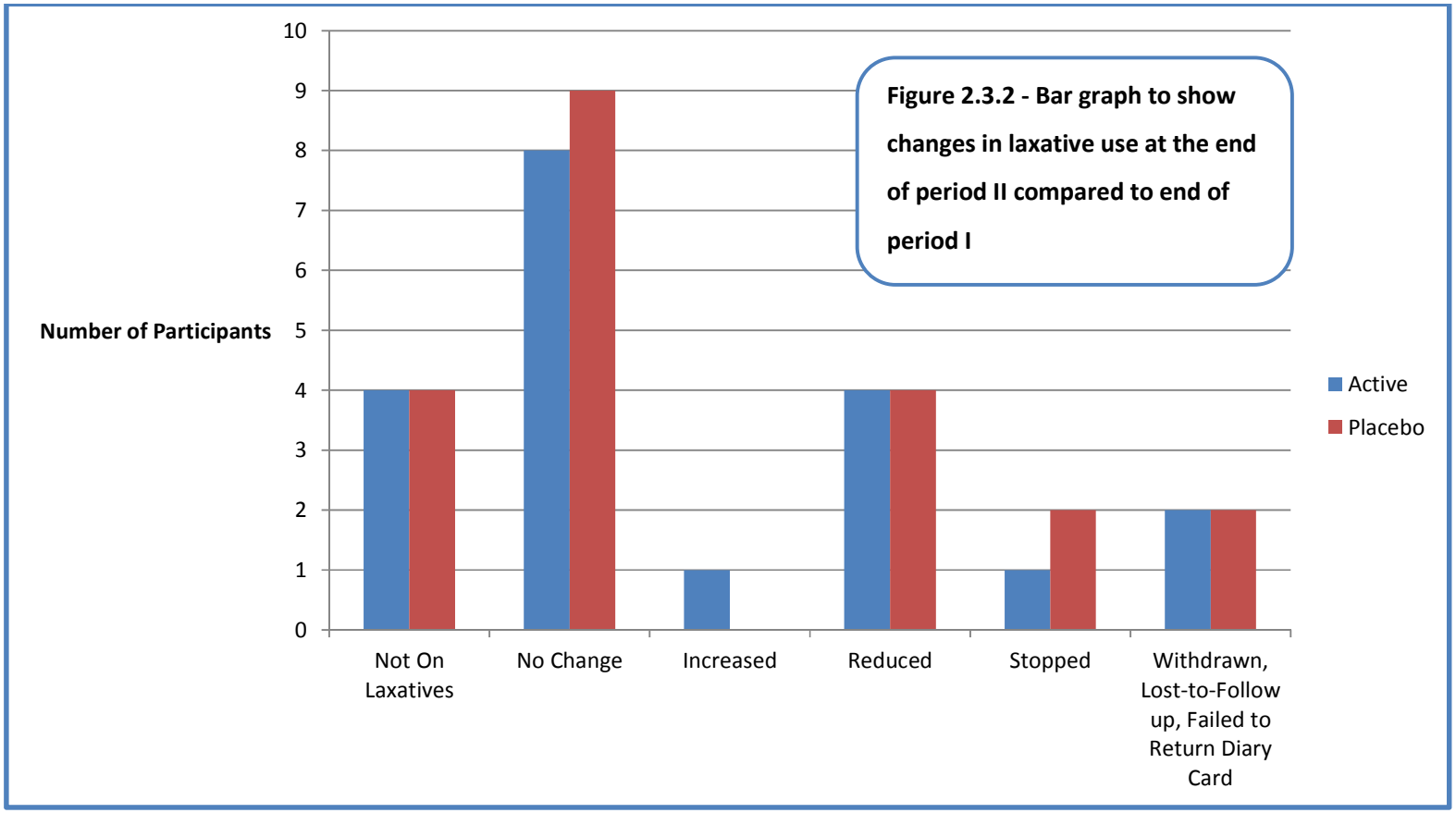


Figure 2.3.2 - Bar graph to show changes in laxative use at the end of period II compared to end of period I

Of the 41 patients enrolled eight patients (20%) were not using laxatives to manage their constipation (four in each group). There was no difference at the end of period II between the two groups in the amount of laxative used compared to the end of Period I

	Active	Placebo
Patients who had transit study as part of initial work-up	15 (75%) (n=20)	19 (90%) (n=21)
Number of patients with documented slow transit	12 (80%) (n=15)	15 (79%) (n=19)
Number of patients who had a repeat transit study	4 (20%) (n=15)	7 (37%) (n=19)
Number of patients with slow transit at end of period II	4	6
Number of patients with an affirmative response to the global question (1° outcome) at the end of period II	0	3 *1 patient had developed normal transit with a clinical response in both periods II and III*

Repeat transit studies were requested on all patients who had previously had documented slow transit (n=27, 66%). This was performed at the end of period II, with any change to normal transit providing an objective marker of improvement. Only 33% (4/12) of patients randomised to Nalcol™ attended for these and 47% (7/15) in the placebo group (p=0.70) (Table 2.3.6). One patient in the placebo group forgot to attend for her transit study at the end of period II and instead attended at the end of period III (patient is shown in italics). No patients had a reduction in their transit time at the end of period II, irrespective of treatment arm, and in spite of 2 patients having a clinical response in the placebo group.

Table 2.3.6 – Patients who had a repeat transit study at the end of period II

2.3.2.5 Analysis Of The Disease-Specific Questionnaires (PAC-SYM And PAC-QOL)

In total 34 of the 41 participants (83%) completed questionnaires for each of the study periods, 16 (80%) in the active group and 18 (86%) in the placebo group (p=0.70).

2.3.2.5.1 Patient Assessment of Constipation – Symptoms (PAC-SYM)

The PAC-SYM consists of 12 questions that are assigned to 3 subsets:

- Abdominal Symptoms (4 questions)
- Rectal Symptoms (3 questions)
- Stool Symptoms (5 questions)

The responses were recorded on a 5-point likert scale ranging from 0 'absent' to 4 'very severe' and recalled symptoms over the preceding two weeks. A low score indicates a low symptom severity and vice versa. The median symptom scores for period II are show in table 2.3.7 and figure 2.3.3. There was no significant difference between the scores for the groups at the end of periods I and II, except for the rectal symptoms in Period II. Here the score was lower in the placebo group; 3 Vs 0.5 (p=0.01). However, this value was of borderline significance at the end of Period I; Placebo vs. Nalcol™, 3 Vs 1 (p=0.06)

Comparing the two groups longitudinally over the study period the symptom scores were not significantly different at the end of period II compared to period I in either group.

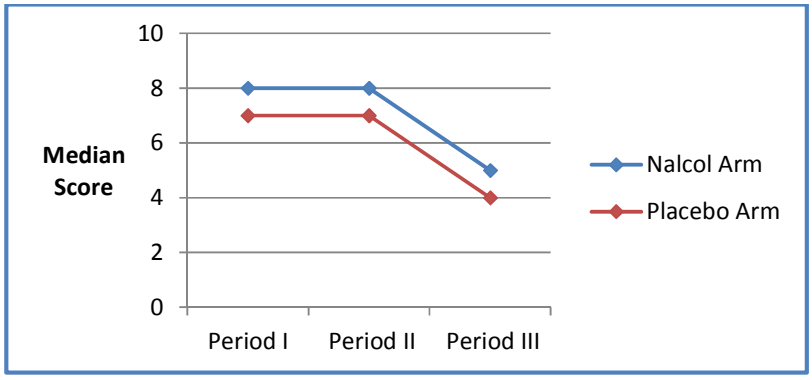
The mean difference in the scores for each component of the PAC-SYM (Abdominal, Rectal, Stool) was -0.06, 0, and 0.13 respectively in the active group compared to -0.22, -0.89, and -0.56 in the placebo group (figure 2.3.4). There were no significant differences between the scores in the active and placebo groups (p=0.89, 0.69, and 0.58 respectively). At the end of period II there had been a slight improvement in the abdominal symptoms of the active group all scores improved in the placebo group, however, the difference in scores between the active and placebo group were not significant.

	Period I	Period II	Period III
Abdominal Symptom Scores			
Active	8 (0-15)	8 (0-16)	5 (0-14)
Placebo	7 (0-16)	7 (0-14)	4 (0-15)
Rectal Symptom Scores			
Active	3 (0-9)	3 (0-10) *	2 (0-10)
Placebo	1 (0-11)	0.5 (0-7)	0 (0-7)
Stool Symptoms Scores			
Active	8 (2-16)	9 (0-17)	7 (1-19)
Placebo	7.5 (0-19)	6.5 (1-18)	4.5 (0-18)

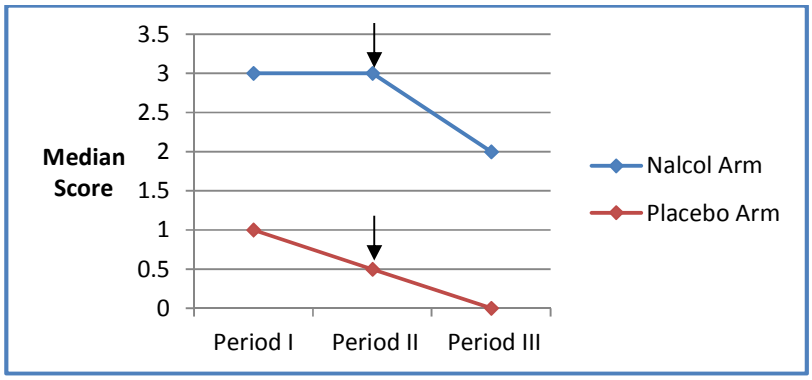
The PAC-SYM consists of 12 questions that are assigned to 3 subsets. The responses were recorded on a 5-point likert scale ranging from 0 'absent' to 4 'very severe' and recalled symptoms over the preceding two weeks.

A higher numerical score represents a high symptom severity (subjectively).The only significance difference (*) was between the rectal symptom scores for the end of period II (3 vs 0.5, p=0.01).

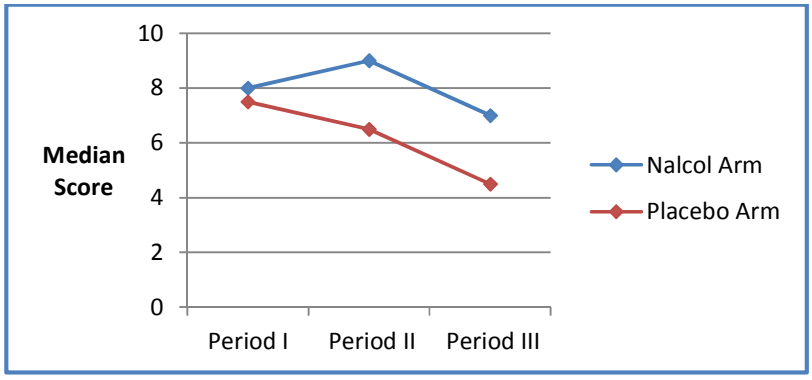
Table 2.3.7 – Table to show the median scores (range) for each component of the PAC-SYM for both active and placebo groups at the end of each study period.



Abdominal Symptoms



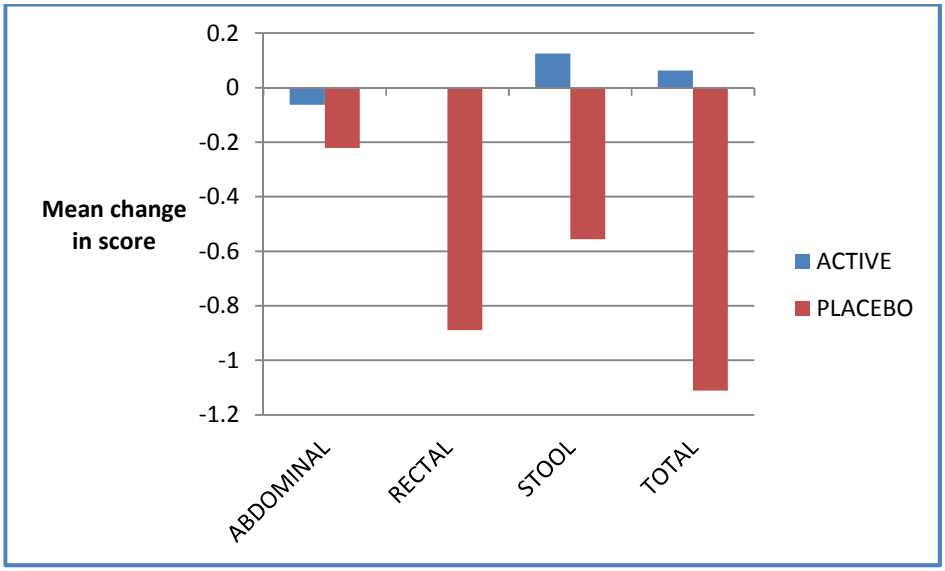
Rectal Symptoms



Stool Symptoms

The only significance difference was between the rectal symptom scores for the end of period II, indicated by the arrow (3 vs 0.5, p=0.01). The cause for this is unclear and unexpected but suggests that the placebo group may have had patients whose FC consisted of an element of pelvic floor dysfunction.

Figure 2.3.3 - Line plots to show the median score for each component of the PAC-SYM for each treatment arm over the study periods



The PAC-SYM consists of 12 questions that are assigned to 3 subsets. The responses were recorded on a 5-point likert scale ranging from 0 'absent' to 4 'very severe' and recalled symptoms over the preceding two weeks. There was no significant difference between the scores in the active and placebo groups

Figure 2.3.4 - Bar Graph to show the mean differences in scores for each component of the PAC-SYM for active and placebo groups at the end of Periods I and II.

2.3.5.5.2 Patient Assessment of Constipation – Quality of Life (PAC-QOL)

The PAC-QOL consists of 28 questions that are assigned to 4 areas:

- Physical Discomfort (4 questions)
- Psychosocial Discomfort (8 questions)
- Worries and Concerns (11 questions)
- Satisfaction with QoL (5 questions)

The responses were recorded on a 5-point likert scale ranging from 0 'none of the time / not at all' to 4 'all of the time / extremely' and recalled symptoms over the preceding two weeks. A low score indicates a low symptom severity and vice versa, except for satisfaction where a low score reflects poor satisfaction. The median symptom scores for each three periods are show in the table 2.3.8 and figure 2.3.5.

At the end of period II the QoL was not-significantly better in the placebo group compared to the Nalcol™ group when assessed for 'physical discomfort' and 'worries and concerns' with no difference in the 'psychosocial' component and a worse score for 'patient satisfaction with symptoms'.

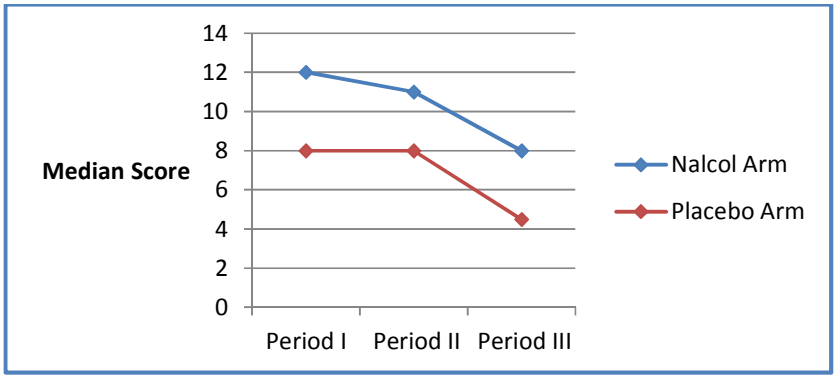
At the end of period II, in those participants who took Nalcol™, all scores except for 'satisfaction' had improved, but not significantly. In the placebo group there was no change in the 'physical' component with a worsening in the 'psychosocial' component and an improvement in 'worries and concerns'. Again 'satisfaction' scores had worsened, but these were not significant.

The mean difference in the scores for each component of the PAC-SYM (Physical Discomfort, Psychosocial Discomfort, Worries and Concerns, Satisfaction with QoL) was -1, -2.56, -2.56, and 0.13 respectively for the active group compared to -0.72, -1.28, -3, 0.94 respectively in the placebo group (figure 2.3.6). At the end of period II there had been improvements in all categories except for satisfaction for both the placebo and active groups. However this difference in scores between the active and placebo group were not significant (p=0.84, 0.51, 0.89, and 0.54 respectively).

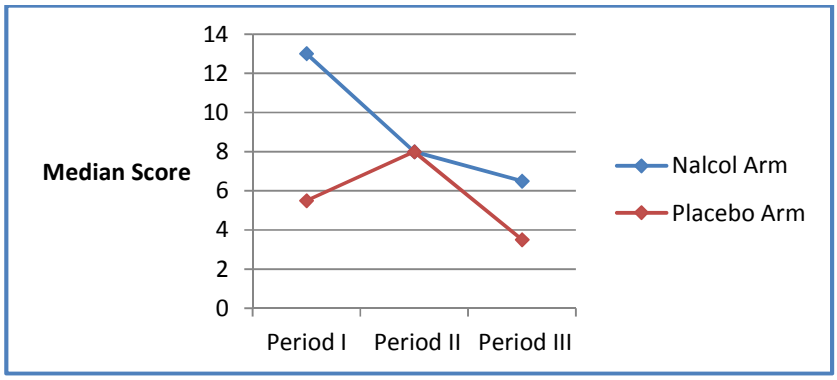
	Period I	Period II	Period III
Physical			
Active	12 (0-15)	11 (1-17)	8 (0-15)
Placebo	8 (0-15)	8 (0-13)	4.5 (1-14)
Psychosocial			
Active	13 (0-27)	8 (0-28)	6.5 (0-22)
Placebo	5.5 (0-24)	8 (0-20)	3.5 (0-20)
Worries and Concerns			
Active	23.5 (1-37)	20.5 (3-41)	15.5 (0-41)
Placebo	19.5 (6-44)	14.5 (3-27)	13 (4-29)
Satisfaction			
Active	4.5 (2-14)	5 (3-9)	6.5 (3-16)
Placebo	4* (1-13)	6 (2-13)	7* (3-16)

The PAC-QOL consists of 28 questions that are assigned to 4 areas: The responses were recorded on a 5-point likert scale ranging from 0 'none of the time / not at all' to 4 'all of the time / extremely' and recalled symptoms over the preceding two weeks. A low score indicates a low symptom severity and vice versa, except for satisfaction where a low score reflects poor satisfaction. The only significance difference was for 'satisfaction with quality of life' in those who were randomised to the placebo arm at the end of period III (4 weeks of Nalcol™, open label) compared to period I (7 vs. 4, p=0.01).

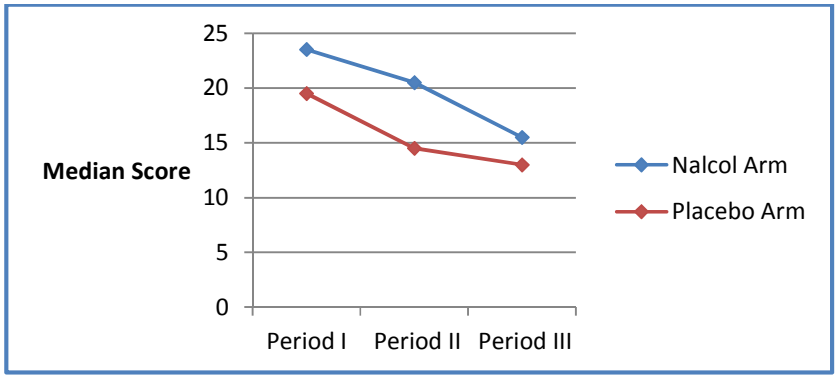
Table 2.3.8 – The median scores (range) for each component of the PAC-QOL for both active and placebo groups at the end of each study period.



Physical

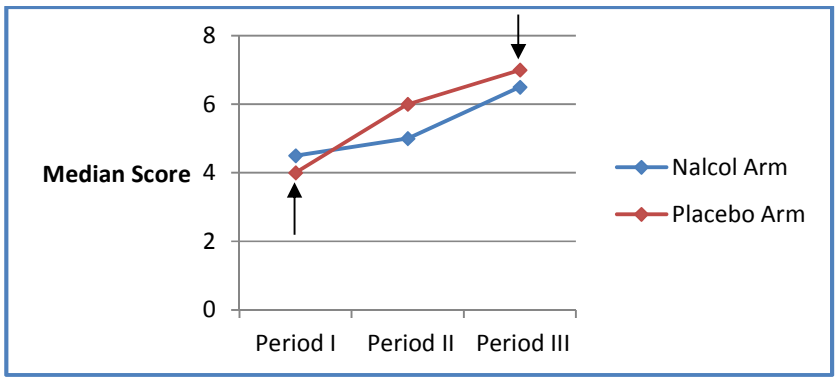


Psychosocial



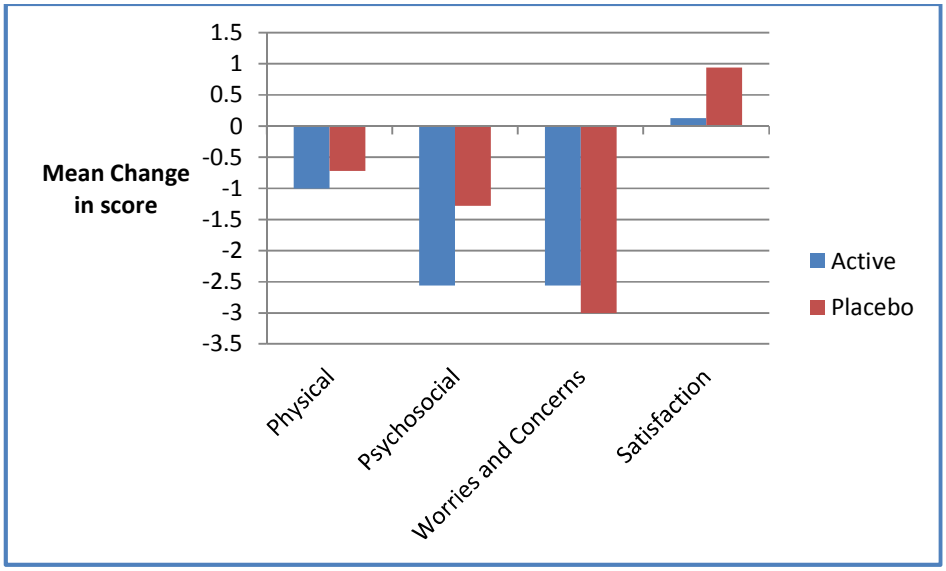
Worries and Concerns

Figure 2.3.5 - Line charts to show the changes in the median score for each component of the PAC-QOL for each treatment arm during the study period



The PAC-QOL consists of 28 questions that are assigned to 4 areas: The responses were recorded on a 5-point likert scale ranging from 0 'none of the time / not at all' to 4 'all of the time / extremely' and recalled symptoms over the preceding two weeks. A low score indicates a low symptom severity and vice versa, except for satisfaction where a low score reflects poor satisfaction. The only significant difference was for satisfaction with quality of life in those who were randomised to the placebo arm at the end of period III (4 weeks of Nalcol™, Open label) compared to Period I as indicated by the arrows (7 Vs 4, p=0.01).

Figure 2.3.5 (Cont'd) - Line charts to show the changes in the median score for each component of the PAC-QOL for each treatment arm during the study period



The PAC-QOL consists of 28 questions that are assigned to 4 areas: The responses were recorded on a 5-point likert scale ranging from 0 'none of the time / not at all' to 4 'all of the time / extremely' and recalled symptoms over the preceding two weeks. A low score indicates a low symptom severity and vice versa, except for satisfaction where a low score reflects poor satisfaction. There was no significant difference in the scores between the active and placebo groups.

Figure 2.3.6 - Bar Graph to show the mean difference in scores for each component of the PAC-QOL for active and placebo groups at the end of Periods I and II

2.3.3 Period III – Open label Period

This was a 4 week open label period where all participants were prescribed Nalcol™ i.e. patients who took Nalcol™ in period II continued Nalcol™ (8 weeks in total) and those who took the placebo in period III started Nalcol™. The purpose of this phase was to monitor for adverse events over an eight period and also allow the opportunity for all participants to take the trial medication. There were 20 patients in each arm at the start of period III. In each arm there was 1 patient lost-to-follow up and three patients who withdrew. Therefore 32 patients (78%) finished the trial in total (16 in each arm) (Figure 2.3.1, page 94).

2.3.3.1 Adverse Events And Reasons For Withdrawal

The reported clinical adverse events during period III are listed in Table 2.3.2 (page 96). In period III (open label) there were 34 adverse events in 20 patients. The commonest events were again abdominal pain/cramps and bloating (79% n=27). There was no significant difference at the end of period III in the abdominal pain (9/20 vs. 4/21; p=0.10) or bloating (9/20 vs. 5/21; p=0.18) in those who had taken Nalcol™ for eight weeks compared to four weeks. This was despite a reduction in abdominal pain and an increase in bloating in those who started Nalcol™ (previously placebo). There was no difference in abdominal pain, dizziness, or bloating between period II and period III. Six patients withdrew from the study and these all occurred during period III. The reasons for which were:

Patient 1	Anxiety and Bloating
Patient 2	Pain and Bloating
Patient 3	Headaches
Patient 4	Headaches
Patient 5	Palpitations
Patient 6	Abdominal Pain

None of the patients who withdrew had any improvement in their symptoms at the time of withdrawal. All three patients who had been on the placebo in period II withdrew in week 9, i.e. after 2 full weeks of Nalcol™ treatment. Of the patients who had been prescribed Nalcol™ in period II, one withdrew in week 8 (after taking Nalcol for a further week), one in week 9 and the final patient in week 10.

2.3.3.2 Response To The Global Question (Table 2.3.3, page 98)

Both groups reported an increase in the number of affirmative responses at the end of period III (35% vs. 43%, p=0.75) to the global question - 'Did you have a satisfactory improvement in your symptoms over the last 2 weeks?', but this difference was not significant. Between periods II and III there was an increase in affirmative response in the Nalcol™ arm of 15% (20% to 35%, p=0.48) compared to an increase of 19% (24% to 43%, p=0.33) in the placebo arm.

2.3.3.3 Interpretation Of The Diary Cards (Tables 2.3.4 And 2.3.5, page 98)

There was no improvement in the stool frequency or stool type at the end of period III compared to either the end of Period I or Period II.

2.3.3.4 Analysis Of The Disease-Specific Questionnaires

In total 34 of the 41 participants (83%) completed questionnaires for each of the study periods, 16 (80%) in the active group and 18 (86%) in the placebo group (p=0.70).

2.3.3.4.1 Patient Assessment of Constipation – Symptoms (PAC-SYM)

All three subsets which assessed the symptoms of constipation had improved by the end of period III compared to their initial scores at the end of period I and this almost reached borderline significance for abdominal symptoms (Nalcol™ arm, p=0.11; Placebo arm, p=0.10).

There was no difference in the scores at the end of period III between the active and placebo group (Table 2.3.7, page 102 and figure 2.3.3, page 103).

2.3.3.4.2 Patient Assessment of Constipation – Quality of Life (PAC-QOL)

At the end of period III, the QoL was better in the placebo group compared to the active group when assessed except for patient satisfaction with symptoms although these differences were not significant. The QoL scores at the end of period III for each component were not significantly better than the scores at the end of period I and period II except for 'satisfaction'. This component worsened throughout the whole trial in both arms and this was significant in the placebo group at the end of period III (p=0.01) (Table 2.3.8, page 106 and figure 2.3.5, page 107-108).

2.4 DISCUSSION OF CLINICAL TRIAL

2.4.1 SUMMMARY OF FINDINGS

The hypothesis of this clinical trial was that Nalcol™ would improve the symptoms of functional constipation compared to the placebo over a four week period. The results do not support the hypothesis since there was no improvement as judged by the primary and secondary outcome measures. In period II, in response to the primary outcome (the global patient satisfaction question), 20% of participants who took Nalcol™ felt that they had satisfactory improvement in their symptoms over the preceding two weeks compared to 24% in the placebo group (p=1.00). Furthermore, there was no difference in the stool type or frequency at the end of period II between the two groups or longitudinally when period I was compared with period II. This lack of difference was supported by the PAC-SYM and the PAC-QOL scores at the end of period II. The only significant difference in period II was for 'rectal symptoms' which were significantly better in the placebo arm compared to the Nalcol™ arm (3 vs. 0.5,p=0.01). There was no significant difference in the other component scores of the PAC-SYM or PAC-QOL between the two treatment arms at the end of period II and whilst the scores for both questionnaires improved at the end of period III compared to period I this was not significant. This suggests that any benefits perceived by the individuals are likely to be the result of a placebo effect. This finding is supported by the repeat transit studies which showed no improvement in transit time at the end of period II, regardless of treatment response, except for 1 patient who was in the placebo group. However, this data is limited by the small number of participants who completed a repeat transit study. Therefore in summary no clinical benefits of Nalcol™ could be demonstrated in functional constipation.

Despite Nalcol™ appearing to have no therapeutic benefit it was associated with an increased frequency of bloating in period II compared to the placebo arm (8/20 vs. 1/21, p=0.01). In the placebo arm the frequency of bloating increased in period III when Nalcol™ was started suggesting it was related to the trial medication. It is however, difficult to determine if abdominal pain and bloating are related to the trial medication or just reflect symptoms associated with functional constipation. Irrespective of the aetiology, two patients withdrew as a result of abdominal pain and bloating and a third from abdominal pain alone and this would certainly be a limitation of a medication which in this trial had little or no proven benefit. Except

for dizziness, the other adverse events were not predictable from the two previous studies that have used Nalcol™[210,219] but have been seen in a report combining the data from two RCTs using 20-80mg / day of naloxone PR in combination with oxycodone in the treatment of chronic, non-malignant pain[214]. They reported a frequency of diarrhoea of 5% and headaches of 4% compared to 4% and 4% respectively in this study. They did not report any incidence of cardiac adverse events but these are a well recognised, rare complication of the use of prolonged release naloxone[303].

There were no withdrawals during period II with one patient lost-to-follow-up and only during the open-label period, period III, did any withdrawals happen. It is interesting that patients who had been taking Nalcol™ in period II should develop symptoms after taking the drug for 4 weeks without incident. Possibly four weeks is not long enough to evaluate the full effects of Nalcol™ and has implications for further trial design. In healthy volunteers effects from Nalcol™ were noted over a 9 day period[202] and in the report by Löwenstein et al[214] benefits from naloxone were noted within 4 weeks even though the trial ran for 12 weeks. However Hawkes et al[219] used Nalcol™ for 8 weeks in patients with IBS and did not achieve a significant improvement in symptoms but a 'satisfactory improvement in symptoms' was noted at 4 weeks in 4/14 increasing to 6/14 in the Nalcol™ group compared to 3/12 and 3/11 in the placebo group (p=1 and p=0.68). However, this study was open to all patients with IBS-C and IBS-M and not specifically those with IBS-C and this may have impacted on the response rates.

2.4.2 STRENGTHS AND LIMITATIONS OF THE STUDY

The biggest strength of the trial was that it was randomised, double-blinded, and placebo-controlled. Treatment trials for any FGID have been shown to be associated with a placebo response rates up to 85% depending on the FGID investigated[304,305] and this highlights the importance of blinding both the participants and investigators. The trial was well designed in that a two week screening period allowed disease stability to be assessed and the 4 week trial period (Period II) is of an accepted duration[306] to assess short-term efficacy in FGID. This trial also included a further 4 week open label period meaning that some patients will have taken Nalcol™for 8 weeks. The evidence supporting the use of a 4 week trial period has been discussed in the preceding paragraph in more detail. The use of a global symptom question as a primary

outcome measure of satisfactory symptom relief has been used in IBS[307,308]. This outcome measure was used in this trial for its ease, simplicity, and lack of specificity. Constipation consists of a heterogenous collection of symptoms where patients place differing importance on each symptom. Therefore using a global question relating to symptomatic improvement is appropriate for the group as a whole. The secondary outcomes were able to target specific symptoms such as frequency of defecation or abdominal pain and the use of validated, disease-specific questionnaires further enhanced the strength of the trial design with analysis of both primary and secondary outcomes done on an intention-to-treat basis.

There are however several potential limitations to the study. The first and foremost was the lower than expected recruitment into the trial. Over 200 patients were identified as potentially suitable for the trial but approximated 50% of these were symptom free or unable to be contacted, a limitation of using retrospective databases. Of those contacted only 40% were interested in taking part in the study. The participants were not reimbursed for their time and enrolment in the study required 4 visitors to the NNUH which serves a wide geographic area and this may have been a reason for the low uptake. Only a few patients were recruited prospectively from specialist clinics. To improve recruitment in further work it would be appropriate to pay for travel expenses but more importantly to involve other centres in patient recruitment, either as patient recruitment centres or as a multi-centred trial. A multi-centre trial would reduce the distance patients need to travel but at the expense of increasing the administrative side of the clinical trial. It was attempted in this trial to use the James Paget University Hospital (JPUH) as a recruitment site in August 2010 when it was realised that recruitment would fall short in a single-centre trial. Approval from local ethics and the MHRA was obtained but the R&D department of the JPUH could not allow the principal investigator access to clinical notes without patient approval first. Therefore, a gastroenterologist at the JPUH was happy to contact potential participants but was unsuccessful due to a lack of administration time. The power calculation to detect a 35% response required the enrolment of 120 patients, but only a third of this target was achieved. Therefore any possible benefit from Nalcol™ may not have been seen. However the statistical analysis showed no difference between the Nalcol™ and placebo arms with a p-value that was not close to significance ($p=1.00$). So whilst it cannot be proved that Nalcol™ is ineffective in the treatment of functional constipation it is felt that if a benefit exists it is probably very small with the number of patients in our work only able to detect a very large benefit. Two pilot studies using linaclotide

(guanylate cyclase-C receptor agonist) as a treatment for functional constipation and IBS-C recruited 42 patients[194] and 36 patients[309] respectively with significant results in favour of the trial drug. This has also been seen for the pilot studies of prucalopride ($5HT_4$ agonist)[310] and lubiprostone (Chloride channel 2 agonist)[311] which recruited 53 and 129 patients respectively. The lubiprostone trial recruited 33 patients into the placebo arm and 30, 32, and 34 patients into 3 different lubiprostone doses (24mcg, 48mcg, and 72mcg). This built upon work from healthy volunteers recruiting 30 and 26 volunteers which demonstrated that 24mcg was an effective dose to increase bowel movements in healthy volunteers[186,312]. This lends weight to our suggestion that if a real difference did exist between the Nalcol™ and the placebo arms, then this should be noted in a trial such as ours recruiting 42 subjects.

Heterogenous patient selection may further limit this work. All patients fulfilled the Rome III criteria, but there was great variability in the pre-trial investigations. Imaging of the colon by barium enema or colonoscopy was achieved in 68% (n=28) of patients thus excluding mechanical causes for obstruction. Of the remaining 13 patients, the mean age was 39 Yrs (+/- 9.6 Yrs), only 3 had no investigation at all with the remaining 10 patients having either a transit study or biofeedback. In total thirty-four patients (83%) underwent transit studies but only 66% of patients (27/41) had documented slow transit and further to this no patient had undergone formal investigation for pelvic evacuatory disorder (PED) which is a cause of slow transit demonstrated on shape studies. This lack of heterogeneity combined withthe poor recruitment is likely to further underpower the study supporting the hypothesis that only a large benefit would be detected in this work. Even then it would be difficult to draw valid conclusions. Ideally if the trial was repeated all patients, as part of screening, would undergo a transit study and a defecating proctogram to characterise the nature of the constipation and exclude patients with PED. Those patients with slow transit could then undergo repeat transit study as in this study to act as an objective secondary outcome but since normal transit demonstrated on the initial transit study would not exclude patients from the trial it would need careful consideration whether the increase in radiation exposure is justified. The use of a defecating proctogram as part of the initial screening investigations however, would need careful consideration. The proctogram would be a point of embarrassment for most patients and would most likely reduce patient recruitment further in what has already been shown in our work to be difficult. The enrolment of patients with IBS-C is another potential limitation. IBS is defined by the presence of abdominal pain with or without bowel dysfunction. Any patient who suffered predominately

with abdominal pain, irrespective of the presence of constipation, or any patient who had an alternating bowel habit prior to laxative use was excluded from the trial and therefore the number of IBS-C patients enrolled was likely to be low. The enrolment of IBS-C subjects cannot be totally avoided due to the overlap in symptoms with functional constipation, however the use of lubiprostone in IBS-C has been shown to improve the symptoms of constipation similar to that in functional constipation[188,313], suggesting that recruitment of these patients may not affect the end result.

The use of a binary response (Yes/No) to the primary outcome is also a limitation. Given that 'satisfaction in symptom relief' is highly subjective and dependant on what the subject feels is satisfactory it is likely that only large benefits will be noted and subtle improvements will be underreported. A continuous assessment scale, such as the visual analogue score, would have been better as this will still assess satisfaction with bowel function but detect a response that would not produce a 'yes' in a binary outcome assessment.

The final limitation of the study was the decision to keep the participants on their usual laxative regimen. This decision was based on the limited clinical information available and to aid in recruitment. As detailed in the introduction (section 1.7.6.4.1, page 50) the data available for the use of naloxone in opiate induced constipation is well documented but there is limited data on its use in functional constipation. The use of naloxone in healthy volunteers reduced gut transit time from 53.1 to 42.1h ($p=0.005$)[210], whilst its use in IBS-C showed an improvement in symptomatic relief, pain, bloating, straining, and urgency to defecate but this was not significant[219]. Since the population recruited for this trial had used laxatives extensively without full benefit it was deemed appropriate to see if Nalcol™ could augment their usual laxative regimen and if possible replace it. It was also thought that this would be more appealing to the participants and increase enrolment.

However, the concomitant use of laxatives has two flaws. First it is impossible to confirm that the subjects did not alter their usual laxative regieme to gain symptom relief and failed to record this accurately on the diary card or that lifestyle changes were made that could have impacted on the results. Secondly interpretation of the patient data more difficult, specifically the stool frequency and stool type over the study period and also the laxative use which was obtained from data from the diary cards. Patient use of laxatives could make it harder to detect any significant benefit of Nalcol™. In this study 20% (8/41) of the participants had stool type 5 or

above (diarrhoea) as a result of their laxative use. Involvement of these patients in stool analysis would skew stool type towards the normal range of 3-4. The median stool frequency/day is compounded by laxative use but also by the nature of constipation itself. A subset of patients with constipation has stool types I and II, but have to defecate multiple times a day to achieve a satisfactory response. This would skew the frequency towards '1' (i.e. normal) but belie the extent of the symptoms. Therefore these factors may hide any response to Nalcol™ when using the outcome measures of stool type and frequency. Finally stool type is a highly subjective outcome with high intra- and inter-observer variance.

The use of CSBM as in indicator of response is therefore a better outcome measure than stool frequency and type. A CSBM is defined as a spontaneous bowel movement (SBM) with a complete sense of evacuation and the use of this nullifies the increased frequency associated with type I and II stool and the impact that type 5-7 stool has. ≥3 CSBM/wk or an increase in 1 CSBM/wk have been used as outcome measures in the large, multi-centred trials already quoted[172-174] and is therefore an accepted outcome measure. Its use here would have allowed for easier analysis of treatment effect whilst allowing for comparison between trials. The use of the primary outcome measure used in this trial has been justified here and if the trial was repeated it would remain as the primary outcome measure with the CSBM replacing stool frequency and type as a secondary outcome. They could be used in conjunction as the global question is simple and assesses the patient as a whole whilst the CSBM assesses just one facet of the symptomatology of constipation.

2.4.3 EXPLANATIONS FOR LACK OF EFFICACY

Why did Nalcol™ not have any therapeutic benefit over a placebo in this particular patient group? There are four possible explanations:

I. The length of time Nalcol™ was prescribed for was insufficient
II. Nalcol™ had a small effect and was not seen in the small sample size of this trial
III. Incorrect dosage used
IV. Nalcol™ does not affect colonic motility in vivo as expected in functional constipation

Explanations I and II have been discussed earlier, however points III and IV warrant further discussion.

2.4.3.1 Incorrect Dosage

The dosage chosen was greater than the dosage used in the previous trials of Nalcol™, 20mg b.d compared with 10 mg b.d[210,219]. This increase was chosen due to the severity of the constipation and the possibility existing that the dose may be too great, with the resultant diarrhoea an indication of this. Using a dose that may be potentially too great allows the trial dose to be reduced to an effective and tolerable dose whereas too low a dose would result in the scenario of 'too low a dose' vs. 'Nalcol™ not having a physiological effect in vitro in functional constipation' should no response be seen. The use of naloxone prolonged release (naloxone PR) has been extensively studied in combination with oxycodone in the management of OIC. Meissner et al[217] reported the bowel function index (BFI) score decreased as the naloxone PR dose increased and that 40mg/day of naloxone PR brought about a significant result compared to placebo (27.9 vs. 43.3, p=0.0004) earlier than 20mg/day, which brought about significant improvements by the end of the trial (27.9 vs. 34.2 vs. 43.3, 40mg vs. 20mg vs. Placebo). 10mg/day of naloxone PR however did not bring about any significant reduction in the BFI scores compared to placebo. This effect of naloxone PR was also reported by Nadstawek et al[213] who again found that treatment efficacy improved with increasing dose of naloxone PR. 43.5 % of patients in the placebo group described efficacy as 'good' or 'very good' compared to 50%, 67.4%, and 72.5% in the 10mg, 20mg, and 40mg/day naloxone PR dose groups respectively. It

therefore seems likely that a dose of 40mg/day, as chosen for this study, should be adequate to elicit a response and would be used again if the trial was to be repeated.

2.4.3.2 Nalcol™ does not affect colonic motility in vivo as expected in functional constipation

If the dose of Nalcol™ was adequate then why did the patients not respond in a positive manner? The answer may lay in the role that endogenous opioids play in controlling colonic motility. The effects of opiates on colonic motility have been studied *in vivo* and *in vitro* and have often used opiate anatogonists to confirm responses. Van Neuten[314] in 1977 demonstrated that the administration of met-enkephalin produced a dose-dependent inhibition of rhythmic peristaltic activity induced by increasing the distending pressure. This inhibitory effect of peristaltic activity by opiates is on opioid receptors. When morphine is administered to μ-receptor knock-out mice there is no effect on transit time compared to heterozygous or wild-type mice where the expected increase in transit time is seen[315]. This has been confirmed by the use of selective μ, κ, and δ receptor agonists. Shahbazian et al[316] demonstrated that μ- and κ-opioid receptor agonists dampened peristaltic performance whilst Liu et al[317] confirmed that μ and κ agonists decreased contractile amplitude. The ability of naloxone to antagonise μ and κ agonists and to promote peristaltic activity when used alone supports the role of opiates and opioid receptors in the role of decreasing peristaltic activity. Whilst this explains why opioid analgesic such as morphine induce constipation and why naloxone can act as a therapeutic agent in OIC it does not explain the role of endogenous opiates in normal gut function.

Endogenous opiates suppress both excitatory and inhibitory neuronal pathways to GI muscle producing either muscle relaxation or spasm respectively. Suppression of excitatory pathways reduces neuronal excitability and neurotransmitter release at both the pre and post-synaptic sites with a resultant decrease in the levels of acetylcholine (ACh) and substance-P producing a blockade of distension-induced peristalsis. Ach and naloxone were able to reinitiate peristaltic activity in isolated guinea pig ileum but that this effect was inhibited by pre-treatment with normorphine[201]. It was subsequently shown that normorphine caused depression of the excitatory post synaptic potential (EPSP) by increasing potassium conductance following activation of μ receptors and that this effect was bocked by administration of naloxone[318]. Suppression of inhibitory pathways results in decreased levels of nitric oxide (NO), vasoactive intestinal peptide (VIP), and adenosine 5'-triphosphate (ATP) with an elevation of muscle tone and non-propulsive motility patterns as a consequence. This overall suppressive function of

opiates was demonstrated by Kadlec and Horacek[319] who stressed guinea pig ileum by the use of longitudinal stretch and luminal distension for 2 minutes. This resulted in an inhibition of peristalsis that outlasted the length of the stimulus and which was abolished or shortened when the experiment was repeated in the presence of naloxone. The role of endogenous opiates in suppressing peristalsis was demonstrated using guinea pig ileum and measuring met-enkephalin levels at rest and during peristaltic activity[320]. When guinea pig ileum was maintained with an intraluminal pressure of 0cm of H_2O met-enkephalin was released into the bathing fluid. As the luminal pressure was increased, peristalsis was induced, and met-enkephalin levels reduced. However, following prolonged distension, the peristaltic activity became intermittent and the levels of met-enkephalin rose compared to the ileum that continued to peristalse. It had been shown several years earlier that the addition of naloxone to intermittently peristalsing ileum as a result of prolonged distension resulted in an increase in peristaltic activity leading the authors to conclude that naloxone reversed fatigued ileum[321]. What is more likely is that endogenous opioids are protective in periods of prolonged stress by abolishing peristaltic activity and increasing muscle tone and non-propulsive muscle activity.

Therefore, it would seem logical that the addition of local acting naloxone would block the endogenous opiates and allow colonic peristalsis to continue unabated and thus be beneficial in functional constipation. However, naloxone failed to initiate contractions of guinea pig ileum in the absence of a distension stimulus[201] and had no effect on EPSP amplitude when used alone but prevented depression of the EPSP as a result of treatment with opioid agonists[318]. In the same year Schang et al[322] demonstrated that in healthy volunteers who had been given only naloxone, non significant changes in colonic myoelectric activity were recorded compared to the controls. In those volunteers given morphine there was an increase in rhythmic stationary bursts and a decrease in propagating bursts which was reversed when naloxone was given. It has subsequently been shown that oral naloxone (40-80mg daily in two or three divided doses) in an open study failed to reduce the whole gut transit time in 4 patients with chronic constipation[323]. The endogenous opioid system, therefore, may act as a 'brake', protecting the GI tract from inappropriate or adverse conditions. Although the pathophysiology of functional constipation is poorly understood (section 1.5, page 23) it is accepted that there is a decrease in peristaltic activity resulting in an increased transit time. Blockade of the opioid system, as in this study, is ineffective as the GI tract is not under inappropriate or adverse conditions and hence the opioid system is not active. If it is imagined that a car represents the GI tract and the engine represents

colonic activity, removal of the handbrake (the opioid system) will allow the car to pull forward. In the case of constipation, however, it is possible that the engine does not work and therefore removal of the handbrake is insufficient to allow the car to go forward. From this we can hypothesise that the opioid system is not involved in the pathophysiology of functional constipation. That said it is still possible that naloxone may play a role in the management of functional constipation if combined with an agent, such as prucalopride, which increases colonic motility, i.e. *gets the engine running*.

2.4.4 SUMMARY

The trial found no effect of Nalcol™ in clinical practice. The reasons for this may be due to underpowering of the study, incorrect dosage and short duration, or a lack of appreciation of the physiological mechanisms of colonic motor control. This work suggests that future studies should be conducted with the aim of determining the effective dose and duration, if any, of Nalcol™ in a larger sample group and to investigate the role of endogenous opiates in chronic constipation.

CHAPTER 3

COMPARISON OF COLONIC MICROBIOTA IN SUFFERERS OF FUNCTIONAL CONSTIPATION AND HEALTHY CONTROLS

3.1 SUMMARY OF THE LABAROTORY METHODOLOGY USED

3.1.1 CULTURE INDEPENDENT ANALYSIS AND THE 16S RIBOSOME

The classical method for determining the quantitative composition in a faecal sample is microbial culture. This is a labour intensive process which involves dilution of the faecal sample and then plating on a specific medium. Taking into account the dilution bacterial populations can be determined if the number of colonies is calculated. This method has two major flaws. Firstly the reliability of the bacterial count relies on the ability of the culture medium to allow the growth of the bacterial species. If the species is poorly cultured then an underestimation of the proportion that the particular species makes up in the total microbiota will occur. Secondly it well known that not all 'specific' media are totally specific with the result that some bacterial species may be counted more than once. Therefore, DNA based culture independent analysis has been developed and these methods have been applied to the faecal samples from participants in the Nalcol trial with the primary aim of determining if any difference exists between healthy subjects and those suffering with constipation. A secondary objective was to determine if any changes in the microbiota were noted should the trial medication prove to be effective.

3.1.1 16S Ribosomal RNA

Ribosomes play a key role in the synthesis of protein in any cell. Bacterial ribosomes consist of a 50S and a 30S subunit (Figure 3.1.1) and these subunits consist of RNA and proteins. The 30S subunit contains the 16S rRNA subunit which, in bacteria, is a molecule containing 1500 nucleotides. The significance of the 16S rRNA subunit is that it has different degrees of variability between species and this variability can be harnessed to identify specific bacterial groups and species.

A number of such 16S based molecular profiling methods have been developed in the last ten years. The selection of the methods will depend on the specificity and the depth of phylogenic

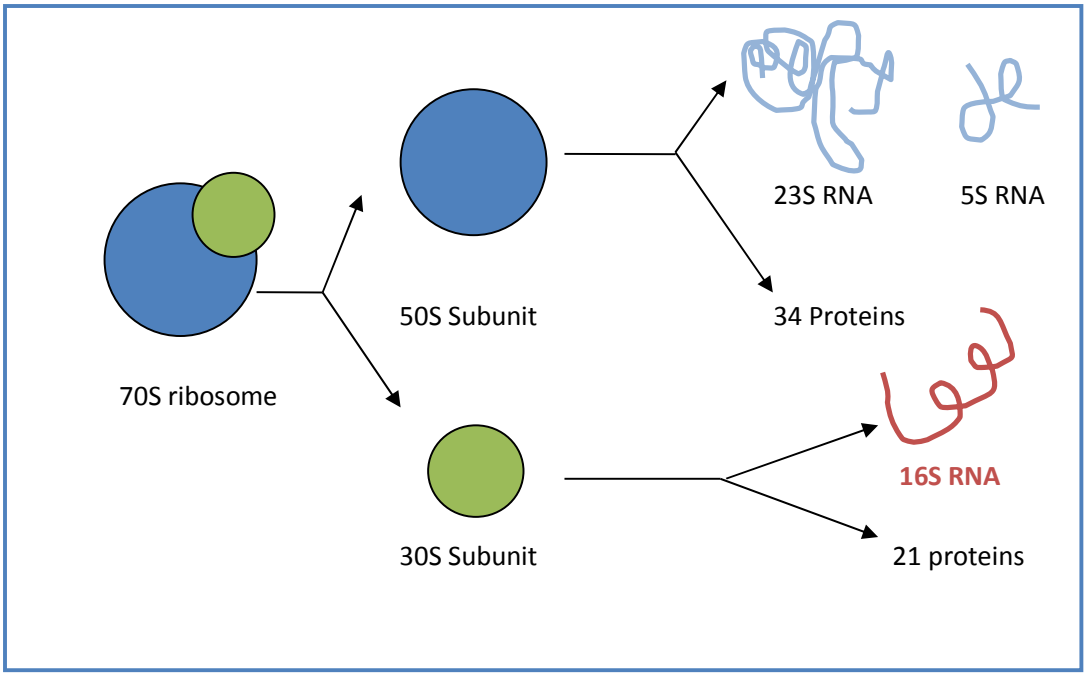


Figure 3.1.1 - Components of a bacterial ribosome.

Ribosomes play a key role in the synthesis of protein in any cell. Bacterial ribosomes consist of a 50S and a 30S subunit (Figure 3.1.1) and these subunits consist of RNA and proteins. The 30S subunit contains the 16S rRNA subunit which, in bacteria, is a molecule containing 1500 nucleotides. The significance of the 16S rRNA subunit is that it has different degrees of variability between species and this variability can be harnessed to identify specific bacterial groups and species.

information that is required. In this thesis I have employed the following three forms of analysis which will be described later in the section:

1. Polymerase Chain Reaction – Denaturating Gel Gradient Electrophoresis (PCR-DGGE)
2. Fluorescence in Situ Hybridisation (FISH)
3. 454 pyrosequencing

3.1.2 POLYMERASE CHAIN REACTION – DENATURING GRADIENT GEL ELECTROPHORESIS (PCR – DGGE)

PCR was developed by Kary Mulis et al[324] and allows sequences of DNA or even whole DNA strands to be replicated and billions of copies to be made *in vitro* and this is the first step in PCR-DGGE. DGGE is an electrophoresis gel based methods originally developed by Muyzer[325] and the principle behind DGGE is that single stranded or partially denatured DNA migrates more slowly than double-stranded DNA during electrophoresis. The application of a constant heat and an increasing gradient in the denaturing agents in the polyacrylamide gel (Formamide (0-40%) and Urea 0-7mM) results in the DNA molecules unwinding. The DNA molecules move through the pores of the polyacrylamide gel to the positive electrode and each separate DNA molecule, due to differing DNA sequences, unwind at a specific point along the gel gradient. This is the point at which the DNA strand is said to have 'melted', it stops from further migration and appears as a band on the gel after staining. The differences in the different melting points of the DNA strands depend on the base sequences which unwind at different concentrations and this slows the migration over time. G-C pairs which have 3 hydrogen bonds are more stable than A-T pairings which have 2 hydrogen bonds. This means that PCR amplicons which have different sequences will migrate different distances along the gel and this can allow different bacterial species to be separated and detected. An important component of the PCR step is the GC Clamp. One of the PCR primers contains an extended sequence of multiple G bases. The GC clamp formed during PCR has a high melting domain and prevents the DNA strand from completely dissociating during the electrophoresis.

3.1.3 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

DNA probes have been developed which consist of 18-22 nucleotides that hybridise to specific regions of the 16S rRNA molecule. These probes are fluorescently labelled and are directed at different phylogenetic levels and allow identification of bacterial species within the gut microbiota. These can then be viewed under a microscope using green light and from this an estimate of the total number in the faecal microbiota for that subject can be calculated.

3.1.4 PYROSEQUENCING

Pyrosequencing is a technique whereby DNA sequences are determined by the generation of pyrophosphate which produces detectable light following an enzymatic cascade using luciferase (Figure 3.1.2)[326]. The process in its current form was developed by Margulies et al[327] and follows four main steps which are illustrated in Figures 3.1.2 and 3.1.3:

- Generation of a single stranded template DNA Library
- Emulsion-Based PCR clonal amplification of the library
- Data generation via pyrosequencing
- Data analysis

3.1.4.1 Generation of a single stranded template DNA Library

The whole genome or target DNA is first prepared. The double helix is fragmented into 400-600 base pair fragments (not necessary for small non-coding RNA or PCR amplicons) which are then ligated to adapters and then separated into single strands. The adaptors are used for purification, amplification, and sequencing steps and the single-stranded fragments with attached adaptors comprise the library used in the downstream processes.

3.1.4.2 Emulsion-Based PCR clonal amplification of the library

Each single-stranded DNA fragment from the library is then bound to an individual capture bead which, under certain condition, allows only one DNA fragment to bind to it. The bead-DNA complexes are then emulsified and the water-in-oil emulsion is subjected to emulsion PCR to produced approximately 10 million identical copies that are immobilised onto each bead.

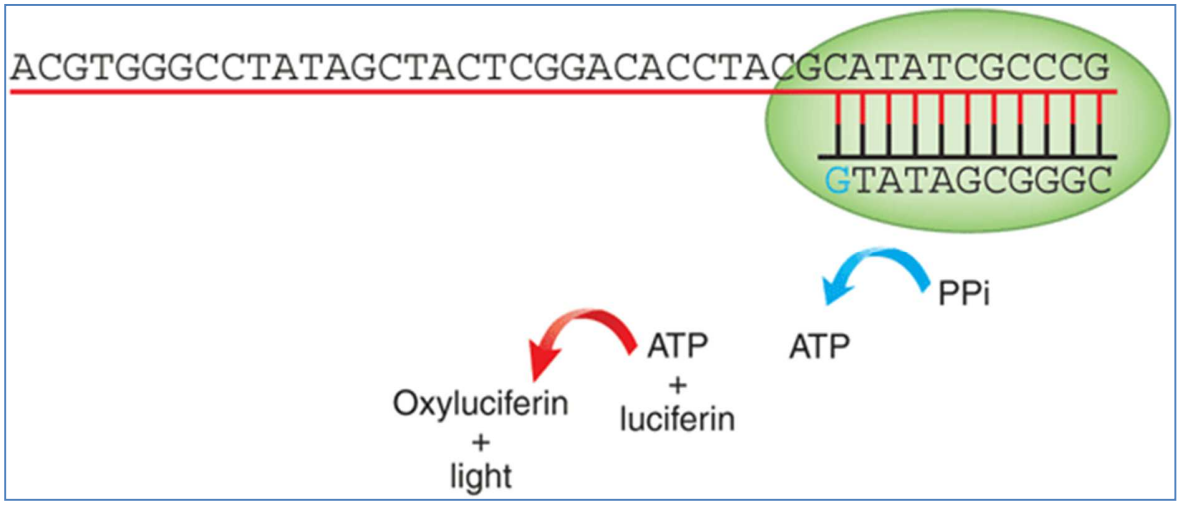
3.1.4.3 Data Generation via pyrosequencing

The bead-DNA complexes are placed onto the wells of a fibre-optic slide. The diameter of the wells is designed so that only one bead-DNA complex will fit into each well. Beads containing the enzymes required for the pyrosequencing are also added to each of the well. Once loaded into the sequencer the slide is washed sequentially with the four nucleotide bases which are incorporated onto the DNA strands and in doing so convert the pyrophosphate generated into light which is detected. The bases are passed in the same order a number of times to allow construction of the complementary DNA strand.

The light signal is detected by a charge-coupled device (CCD) camera with the intensity of the light generated varying with the number of complimentary nucleotide bases added i.e. three consecutive A's (A-A-A) has three times the light intensity as a single A nucleotide. At the start of sequenceing the machine is calibrated with a TCAG sequence.

3.1.4.4 Data analysis

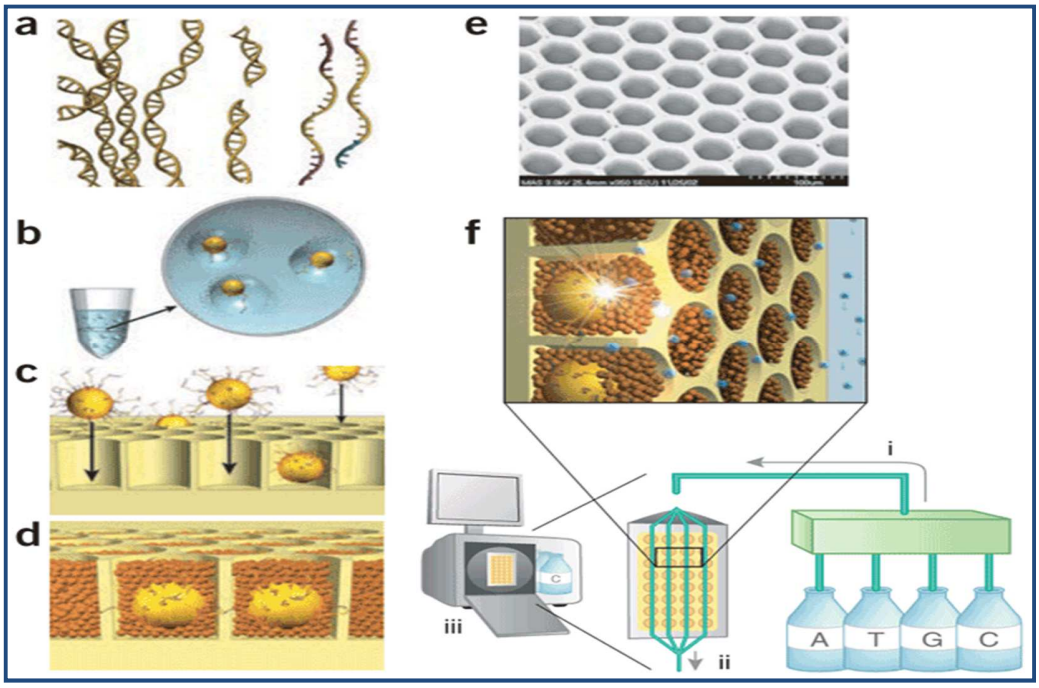
The combination of signal intensities allows the DNA sequences to be determined in each well. Bioinformatic software can be used to reconstruct the DNA templates which are then mapped against reference sequences.



Pyrosequencing is a technique whereby DNA sequences are determined by the generation of pyrophosphate which produces detectable light following an enzymatic cascade using luciferase. The template strand is represented in red, the annealed primer is shown in black and the DNA polymerase is shown as the green oval. Incorporation of the complementary base (the blue "G") generates inorganic pyrophosphate (PPi), which is converted to ATP by the sulfurylase (blue arrow). Luciferase (red arrow) uses the ATP to convert luciferin to oxyluciferin, producing light.

Taken from Rothberg and Leamon[328].

Figure 3.1.2 – Diagram of the pyrosequencing process



(**a**) Genomic DNA is isolated, fragmented, ligated to adapters and separated into single strands.

(**b**) Fragments are bound to beads under conditions that favour one fragment per bead, the beads are isolated and compartmentalized in the droplets of a PCR-reaction-mixture-in-oil emulsion and PCR amplification occurs within each droplet, resulting in beads each carrying ten million copies of a unique DNA template.

(**c**) The emulsion is broken, the DNA strands are denatured, and beads carrying single-stranded DNA templates are enriched (not shown) and deposited into wells of a fibre-optic slide.

(**d**) Smaller beads carrying immobilized enzymes required for a solid phase pyrophosphate sequencing reaction are deposited into each well.

(**e**) Scanning electron micrograph of a portion of a fibre-optic slide, showing fibre-optic cladding and wells before bead deposition.

(**f**) The 454 sequencing instrument consists of the following major subsystems: a fluidic assembly (object i), a flow cell that includes the well-containing fibre-optic slide (object ii), a CCD camera-based imaging assembly with its own fibre-optic bundle used to image the fibre-optic slide (part of object iii), and a computer that provides the necessary user interface and instrument control (part of object iii).

Figure 3.1.3 – Overview of the 454 Pyrosequencing Technology
(Taken from Rothberg and Leamon[328]).

3.2 LABORATORY METHODOLOGY

The Nalcol™ clinical study was given ethical approval by the Cambridgeshire REC 4, the MHRA, the R&D department at the NNUH and the IFR. Full details are found in the Methodology section of the clinical trial (Chapter 2, Section 1, page 69). The trial protocol is also fully detailed in appendix 1 (page 189) but is summarised here. Patients enrolled into the microbiota analysis were taking part in a single-centre double-blind, randomised, placebo controlled study investigating the efficacy and safety of Nalcol™ given to patients with refractory constipation. The trial consisted of three periods (Figure 3.2.1 and Table 3.2.1);

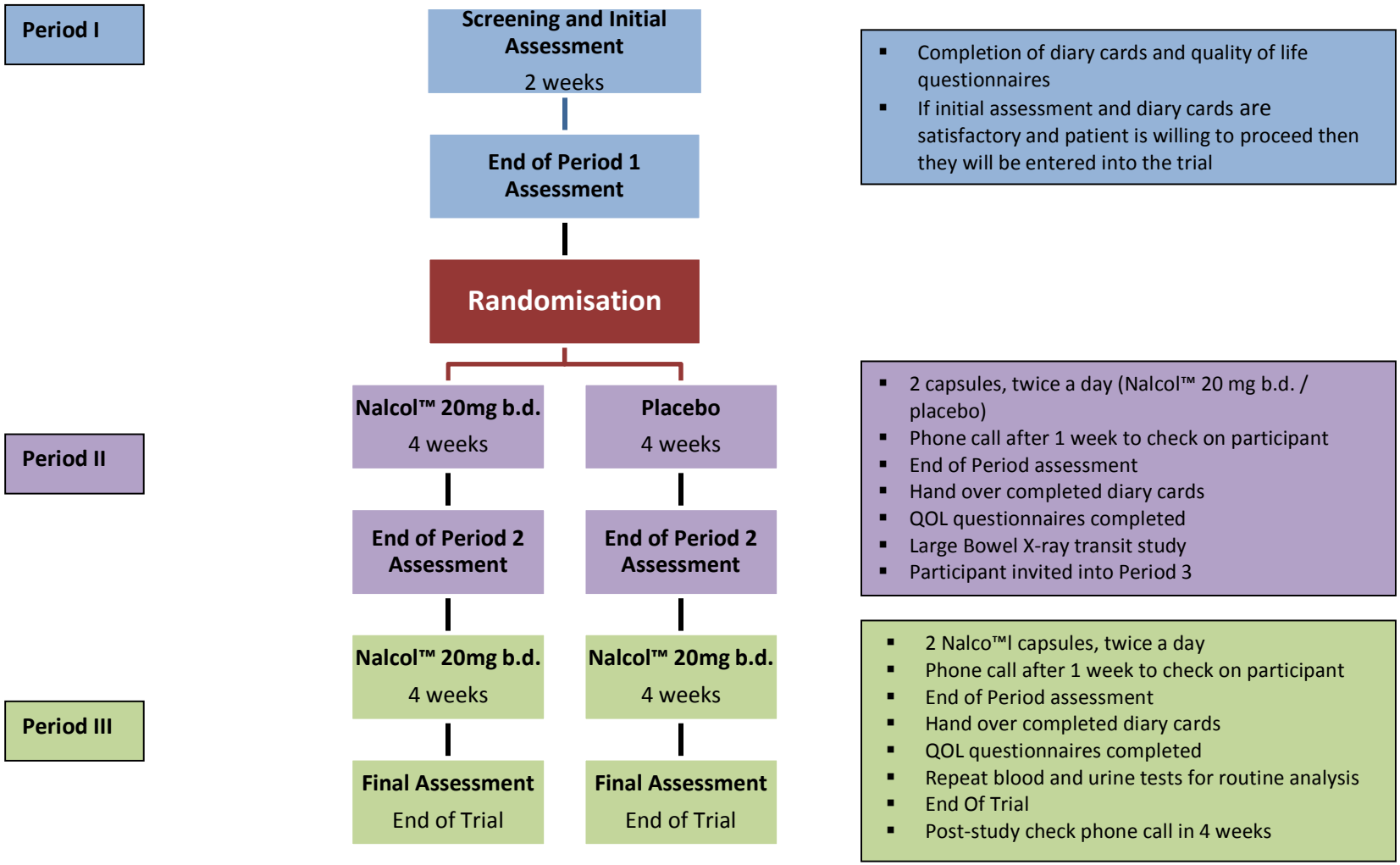
- Period I was a two week period. Patient suitability and health were assessed to ensure that the inclusion criteria were met, to confirm short-term symptom stability, and to confirm patient compliance with diary completion. Patient demographics, clinical details, and pre-treatment data were collected. Consent was taken at the start of Period I.

- Period II was the key treatment period and lasted 4 weeks. Nalcol™ was given to relieve symptoms which may vary from day-to-day and patients were randomised to Nalcol™ or a placebo. Clinical data over the four weeks of treatment was collected.

- A final period of four weeks (Period III) allowed all patients in the trial, regardless of initial randomization, to evaluate Nalcol™ and provided further clinical observation over a longer time.

All patients fulfilled the Rome III criteria for functional constipation (page 11) and were managed at a specialist gastroenterology or colorectal clinic at the Norfolk and Norwich University Hospital NHS Trust (NNUH). They may have undergone radiological or endoscopic investigation for their constipation and their basic management, including dietary and lifestyle advice and modification of laxatives, had been instigated.

Figure 3.2.1 - The 3 Periods within the Nalcol™ Study



PHASE / ASSESSMENT	Pre-Treatment (Period I)		Treatment Period (Period II)				Post Treatment Period (Period III)			
	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10
Global assessment		X				X				X
Diary Card	X	X	X	X	X	X	X	X	X	X
PAC-SYM		X				X				X
PAC-QOL		X				X				X

The main outcome measure, the global assessment question, was recorded at the end of weeks 2, 6, and 10. The diary card was completed everyday and recorded stool type and frequency and also laxative usage. PAC –SYM and PAC-Qol are validated questionnaires used to asses constipation focusing on symptoms and quality of life respectively.

Table 3.2.1 – The timing of assessments over the ten week trial period.

3.2.1 COLLECTION AND STORAGE OF FAECAL SAMPLES

Samples were taken at the end of each trial period. The samples from period I would be compared against healthy controls to determine if a difference existed between the colonic microbiota of healthy and constipated individuals. The samples at the end of period I also acted as a baseline for which changes in period II and III could be compared against. Consent was obtained from participants of the Nalcol™ trial for faecal sampling. In the left lateral position faecal material was taken from the patient through the use of a rigid sigmoidoscope. Samples were placed into a faecal sample specimen container and immediately labelled and placed onto ice. At the end of the clinic, the samples were transferred to the IFR from the NNUH. The maximum time a sample was kept on ice before freezing was 180 minutes. At the IFR the faecal samples were aliquoted into 200 mg samples, placed into 2 ml eppendorf tubes and stored in a -80^{o}C freezer until further processing.

3.2.2 EXTRACTION OF DNA FROM FAECAL SAMPLES

DNA was extracted from one 200 mg aliquot using the QIAamp DNA Stool Mini Kit (Qiagen®, UK.). The full protocol is listed in appendix 3 section but the methodology is summarised here with the adjustments that were made to the protocol.

The frozen sample was taken and 10x vol/wt of Buffer ASL was added (200µl). The sample was then homogenised using a hand-held homogeniser for approximately one minute. The sample was then placed in a water bath at 90^{o}C and then vortexed for a further 30 seconds. After centrifugation for 1 minute at 14000 x g 1.2 ml of supernatant was transferred to a new 2 ml snap-lock tube and 1 tablet of InhibitEX was added. This was centrifuged for 6 minutes, the supernatant then removed and centrifuged for a further 3 minutes at 14000 x g. 15 µl of proteinase K was added to 200 µl of supernatant and then 200 µl of Buffer AL was added before incubating at 70^{o}C for 10 minutes. Following this 200µl of 96-100% ethanol was added before placing in a QIAamp spin column and centrifuged at 14000 x g for 1 minute. The spin column was then washed with 500 µl of washing buffer AW 1 and then by 500 µl of washing buffer AW2. 100 µl of elution buffer AE was then used to elute the DNA which was then stored at -80^{o}C or in the fridge at 4^{o}C depending on the time interval for downstream processing.

3.2.3 PCR AMPLIFICATION

PCR amplification for DGGE analysis was based on the method described by Tourlomousis et al (2010)[329]. PCR amplification was undertaken using as the template the DNA extracted from the faecal samples as previously described. The variable V4 region of the 16S rRNA gene was amplified by using the primers U968-GC-f (5'-CGC-CCG-GGG-CGC-GCC-CCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAA-CGC-GAA-GAA-CCT-TAC-3') and U1401-r (5'-CGG-TGT-GTA-CAA-GAC-CC-3')(primers were supplied from Eurofins MWG Operon, Ebersberg, Germany).

PCR amplification was performed with the following mixture:

HotMaster Taq DNA Polymerase (5-Prime, Nottingham, UK), (5U/µl)	0.2µl
dNTP Mix 100mM, Concentration 25mM (Bioline, London, UK)	0.4µl
Hotmaster Taq Buffer with magnesium, 10X (5-Prime, Nottingham, UK)	5µl
Forward Primer (20 mM)	1µl
Reverse Primer (20 mM)	1µl
BSA (Sigma, UK) (1% w/v)	1µl
Ultrapure water	40.4µl
Extracted DNA (50-100ng)	1µl
TOTAL Volume	**50µl**

The use of BSA as a PCR enhancer had already been described in this laboratory[329] and was added since PCR amplification was suboptimal without its addition.

The mixture was amplified using a Tprofessional standard gradient thermocycler (Biometra®) with the following program:

94°C	5 minutes	
94°C	30 seconds	35 cycles
50°C	20 seconds	
72°C	40 seconds	
72°C	7 minutes	

The presence of PCR products was confirmed through electrophoresis using a standard 0.7% (wt/vol) agarose gel and visualised using a dark reader after staining with Ethidium Bromide. The products were then cleaned using the E.Z.N.A.® Cycle-Pure Spin kit (Omega Bio-tek, USA) and their concentration was measured via spectophotometry (ND1000, Nanodrop Technologies, Wilmington, USA).

3.2.4 DGGE METHODOLOGY

DGGE was carried out using the Bio-Rad D-Code system (Bio-Rad, Hemel Hempsted, UK) and the full protocol is described in appendix 4. The acrylamide gels were created using appropriate volumes of 0%, 40%, and 60% acrylamide solution (Severn Biotech ltd., UK). 200 ng of each PCR product along with 5 µl of loading buffer were loaded into the wells of the gel and DGGE was then undertaken using 1xTAE and run at 50V for 16 hours. Gels were stained in SYBR Green for 45 minutes then washed in 300 ml of dH_2O for 15 minutes. Images were obtained after scanning via Pharos FX molecular imager (Bio-Rad, Hemel Hempsted, UK)

Each gel was loaded with a marker at the beginning and end and with one to two markers in between depending on the number of samples loaded. The marker was created from the PCR product of a healthy sample was felt to have a wide spread of bands when a pilot DGGE was run. The use of this marker was critical for final analysis of the DGGE gels.

3.2.4.1 Analysis of DGGE Gel images

DGGE Lanes were analysed using Total Lab 120 V2006 (Phoretix 1D Advanced Software, NonLinear Dynamics, Newcastle, UK) and subjected to a number of steps which were applied to all gels. Firstly lanes were automatically detected and then manually corrected. Background was then subtracted and bands automatically detected. Three parameters were adjustable for band detection:

Minimum slope	how pronounced the band should be from its surrounding area
Noise Reduction	degree to which small peaks are ignored
Percentage maximum peak	parameter which discards peaks of under a certain size in relation to the highest peak on the gel

In all analyses the parameters above were set at '100', '5' and '5' respectively. After automatic detection the lanes were checked to confirm correct detection of bands with any artefacts detected by the software deleted. The next step was Retardation Factor (Rf) calibration. This is a measurement of the position of a band along the length of the lane. Horizontal lines were added and 'locked' across bands from each of the marker lanes and given arbitrary values ranging from 0 to 1. Once this was completed bands in the non-marker lanes were matched to the marker lanes.

Output from TL120 comprised of Rf values of all detected bands, their intensities, and their corresponding positions (measured in pixels) in the original profiles. The data along with the original profiles was then transferred to Matlab R2008a (The Mathworks, Inc., Cambridge, MA) for further analysis. The use of Matlab was developed by this laboratory in conjunction with the Bioinformatics and Statistics Partnership at the IFR[329]. In brief the data was first aligned and then normalised. Alignment was carried out to a set of 10 reference bands and common to all of the reference lanes of the 3 gels. The alignment method used was the same as in earlier DGGE work carried out at the IFR[329]. After this the data were normalised by setting the minimum of each lane to 0; and for subsequent statistical analyses, by setting the integrated area under the curve (lane) to unity. Supervised modelling and univariate analyses were then undertaken to look for differences between the samples.

3.2.5 FISH ANALYSIS

Faecal samples were subjected to FISH using the protocol detailed in appendix 5. The samples were prepared and then stored at -18^{o}C in a PBS/ethanol mixture until needed. When needed samples were defrosted and hybridised with the appropriate volume of hybridisation buffer and probe (see table in FISH protocol). Samples were then washed by adding the hybridised sample to washing buffer and DAPI. These were then filtered on 0.2µm paper and fixed with slow fade before a glass slide was applied. Slides were stored at 4^{o}C in the dark to minimise fading. Using a Nikon electron microscope with a Fluor 100 lens the bacteria were located with green light (UV light for DAPI) and the cells counted manually. 15 random fields from each slide were counted and each sample was prepared and counted twice.

3.2.6 454 PYROSEQUENCING

DNA was extracted from the faecal samples using QIAamp DNA extraction protocol and then cleaned up using an ENZAcycle pure spin protocol as outlined below. The extracted DNA was quantified and approximately 100ug of the DNA was sent to Veterinary Laboratiory agency for 454 pyrosequencing. The method for the pyrosequencing is fully explained in the paper by Ellis et al[330]. The DNA was amplified with universal primers for the V4 and V5 regions of the 16S rRNA gene. The primers used permitted amplification of both bacterial and archaeal ribosomal gene regions, whilst providing the best possible taxonomic resolution based on published information. Amplification was performed with FastStart HiFi Polymerase (Roche Diagnostics Ltd, UK) and the amplicons were purified using Ampure XP magnetic beads (Beckman Coulter). The concentration of each sample was measured using the fluorescence-based Picogreen assay (Invitrogen). Concentrations were normalized before pooling samples in batches of up to 16, each of which would be subsequently identified by its unique MID. Pooled samples were then subjected to unidirectional sequencing from the forward primer on the 454 GS FLX Titanium platform according to the manufacturer's instructions (Roche Diagnostics Ltd, UK). The sequencing data received from VLA were analysed at IFR using Qiime software[331] with help of Dr Adrian Tett.

3.3 COMPARATIVE ANALYSIS OF THE COMPOSITON OF THE FAECAL MICROBIOTA IN PATIENTS WITH CHRONIC CONSTIPATION AND IN HEALTHY SUBJECTS

3.3.1 PATIENTS

There were 42 patients enrolled into the clinical trial comparing the effect of Nalcol™ to placebo in the management of functional constipation and 20 of these patients gave at least one stool sample via rigid sigmoidoscopy as described in the trial methodology (Section 2.1.3, page 74). The patient characteristics are summarised in table 3.3.1. The number of samples donated and the patient outcomes in the clinical trial at the end of period II (Nalcol™ vs. Placebo) are also reported. All the subjects were female with a median age of 46.5 years (Range; 23-67 years, IQR; 36.5-55.5 years). At the time of sampling, in period I, 25% of subjects (5/20) were not taking laxatives with 1 patient (5%) using rectal irrigation. All other patients were taking laxatives as listed in table 3.3.1. The subject who used rectal irrigation did so after faecal sampling as required. At the end of period II only 15 patients (75%) were able to provide a further stool sample; of the eight patients randomised to take Nalcol™ during period II only one of these failed to provide a stool sample at the end of the period compared to four patients in the placebo group (p=0.60). This number reduced further at the end of period III with only eight of the original 20 patients providing a sample, three patients who took Nalcol™ in period II and five patients who took placebo during period III. There were three responses to the Nalcol™ and no responses in the placebo group (Nalcol - 3/7, Placebo – 0/8, p=0.077) in those who provided a sample at the end of period II.

The healthy controls were all female, were from the local region, and had no history of gastrointestinal disease or laxative use.

Subject number	Age	Laxative Use	Active/ Placebo	Outcome at end of Period II	Stool sample at the end of ..... (5 x 200 mg aliquots unless stated)		
					Period I	Period II	Period III
NAL001	55	Picolax	Placebo	Response	X (4)	-	-
NAL002	61	Lactulose and Senna	Active	No Response	X	X	X
NAL003	40	Senna	Placebo	No Response	X (2)	X	X (3)
NAL004	48	Picolax	Active	No Response	X	X	-
NAL005	62	Movicol and Magnesium Hydroxide	Placebo	No Response	X (2)	X (4)	X
NAL008	39	Diary card not returned	Active	No Response	X	X	-
NAL010	52	Nil	Placebo	No Response	X	X	X
NAL013	50	Nil	Active	No Response	X (4)	X	-
NAL015	57	Bisacodyl	Placebo	No Response	X	-	X
NAL016	25	Laxido	Active	Response	X	X	X
NAL017	23	Laxido and Dulcolax	Placebo	No Response	X	X (4)	X
NAL019	39	Movicol	Placebo	No Response	X	X	-
NAL021	67	Rectal Irrigation	Placebo	No Response	X	X (1)	-
NAL023	45	Movicol	Active	Response	X (3)	X	-
NAL026	31	Lactulose	Active	Response	X	X	X
NAL028	32	Movicol	Placebo	No Response	X (2)	X	-
NAL029	38	Nil	Placebo	No Response	X	X	-
NAL031	49	Nil	Active	No Response	X	-	-
NAL032	58	Lactulose	Placebo	No Response	X	-	-
NAL033	32	Nil	Placebo	Response	X (4)	-	-

Table 3.3.1 - Patient characteristics of those participants who underwent stool sampling

3.3.2 ANALYSIS OF MICROBIOTA COMPOSITION USING DGGE

DGGE analysis was undertaken using 20 healthy controls and samples from all 20 constipated subjects (taken in period I). The gels can be seen in figure 3.3.1. No profile was produced for one of the healthy controls despite all other samples on that gel running without incident. After lane alignment was performed, supervised modelling was used to look for evidence of difference between the groups. A cross-validated, discriminate, Partial-Least-Squares method was used (Figure 3.3.2). This is a multivariate pattern recognition method that uses the whole profile of the sample and the relative band intensity information is retained (as opposed to converting to presence-absence data). Using this method it was shown that a difference existed between the two groups with a cross-validated success rate of 77% ($p<0.01$, y-scrambling permutation test, 10 000 permutations, a test used to determine if variables in two groups are from the same distribution). Looking specifically at the bands (Figure 3.3.3) it was shown that one band was most associated with the 'patient group (constipated)' as indicated by the red arrow. This band was weak in most of the healthy controls and was the only band to show a significant difference between groups in a univariate test (Kruskal-Wallis, figure 3.3.4). Analysis of the bands using presence/absence plots (not shown) showed no significant difference, even at different intensity thresholds. This was the same for similarity methods using presence/absence data and band position. Therefore statistical difference was only seen between the two groups when band intensity information was preserved and pattern recognition modelling was used.

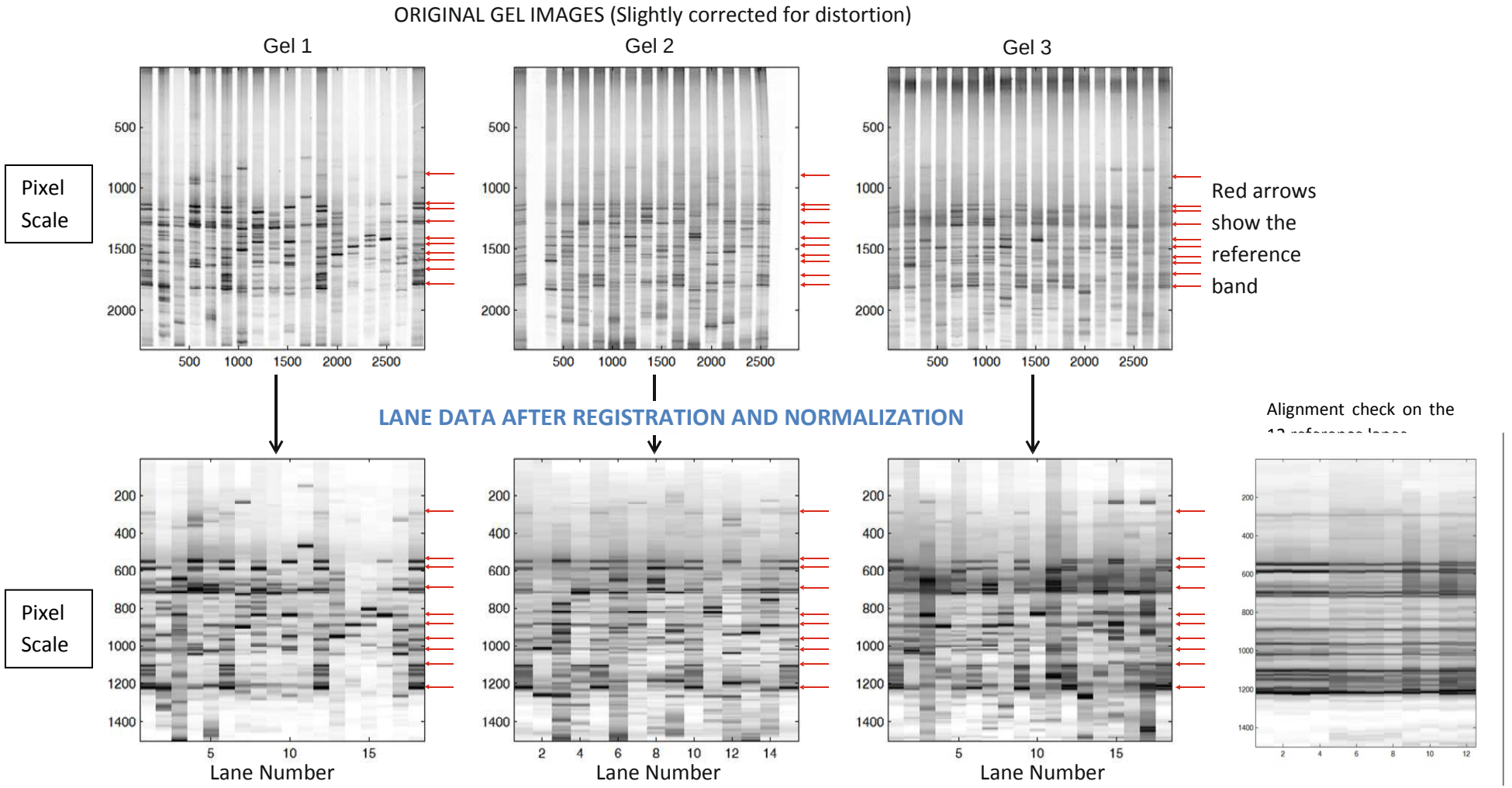
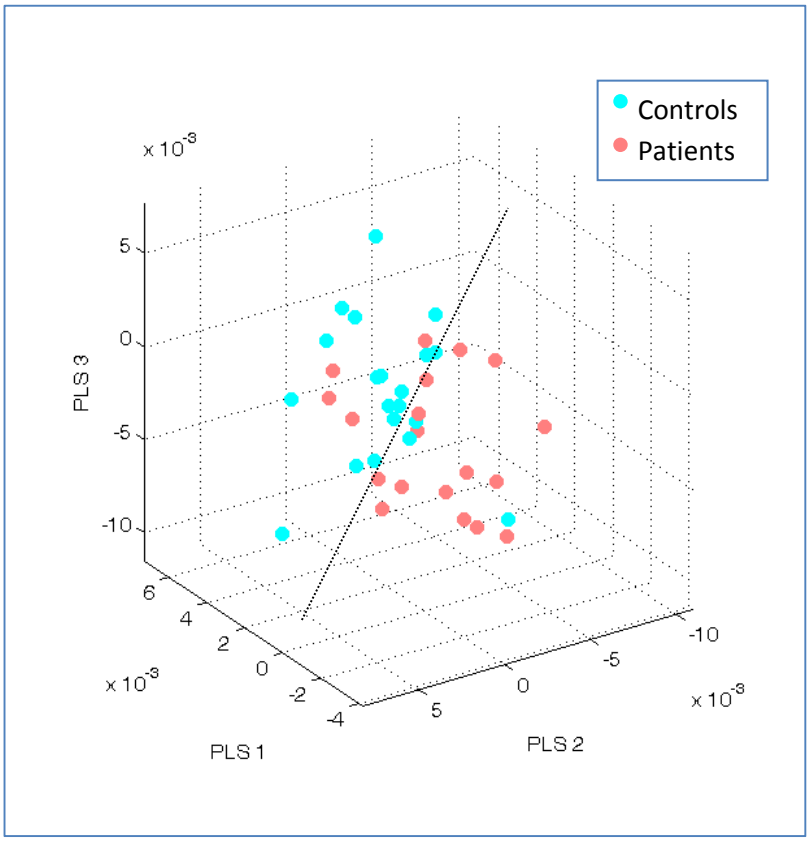


Figure 3.3.1 - Alignment and normalization of data generated from PCR-DGGE of healthy and constipated faecal samples

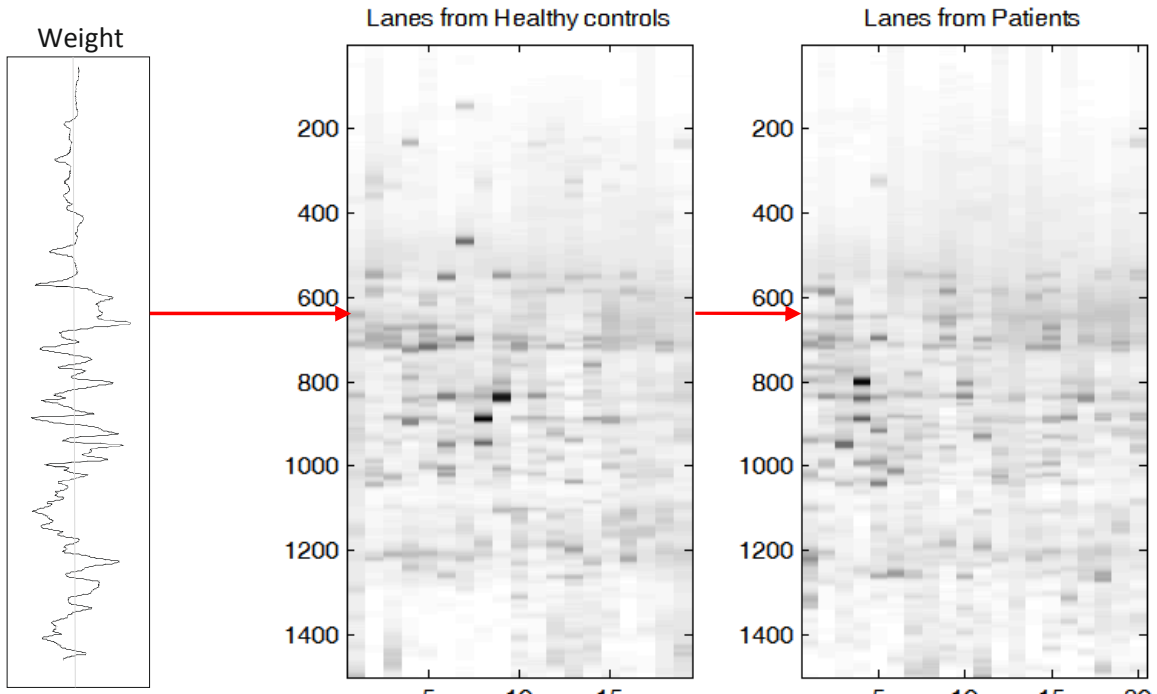


The cross validated success rate in classification = 77%

Figure 3.3.2 – Cross-validated PLS scores (normalised dataset) of DGGE-PCR Data.

The control group consisted of 19 healthy female subjects vs. 20 subjects with constipation. One lane in the healthy group failed to run correctly and was excluded

The band most associated with the 'constipated' group is indicated with the red arrows (at index ~650). This band is very weak in most of the healthy subjects. It is also the only band to show a significant ($p<0.01$) difference between groups in a univariate test

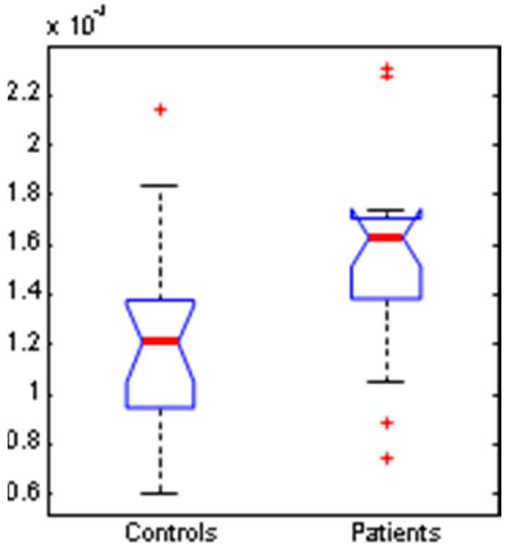


-ve 0 +ve

Indicates bands associated more with the 'healthy' group

Indicates bands associated more with the 'constipated' group

Figure 3.3.3 – Comparison of band intensity between the two groups following PCR-DGGE



A Kruskal-Wallis test looking for differences between groups was applied separately to each row in the profile matrices. The only band to show even an uncorrected p-value less than 0.01 was that at index 644

Figure 3.3.4 – Box-Plot for data at index 644 as described in Figure 3.3.3

3.3.3 ANALYSIS OF MICROBIOTA COMPOSITION USING FISH

Samples from eight healthy volunteers were compared with samples from eight subjects with constipation who had given samples for each of the trial periods. Three of the eight trial participants had taken Nalcol™ and two of these had a positive response in both periods. Six patients took the placebo with no response at the end of period II with only one participant having a response after four weeks of Nalcol™ at the end of period III.

The mean count for each of the bacterial probes was standardised since different volumes of probes were used and then converted to a logarithmic scale with the results presented as 'log10 cell/g faeces'. The value for each probe was then converted to a ratio:

$$\frac{\log_{10} \text{ cells/g of faeces for the target probe}}{\log_{10} \text{ cells/g of faeces of DAPI}}$$

This allowed comparison between subjects and time periods and the values are shown in table 3.3.2.

3.3.3.1 Healthy Controls vs. Constipated subjects (Period I – Screening)

Constipated subjects had a lower ratio of *bifidobacteria* compared to healthy subjects (0.67 vs. 0.88, p=0.03) with five of the constipated subjects having bacterial counts below the threshold for detection. These were given a mean count of 0.07 per sample (one bacteria detected over 15 counts) to allow for analysis. If these patients are excluded the ratio changes to 0.93 compared to 0.88 in the healthy group (p=0.26). There was no statistical difference in the counts for the other probes used between the two groups (Bac, p=0.29; Lab, p=0.21; Clos p=0.30). This is summarised in table 3.3.2 and figure 3.3.5.

		BIF 164	BAC 303	LAB 158	EREC 482
Healthy Controls (n=8)		0.88 (+/- 0.04)	0.92 (+/- 0.02)	0.82 (+/- 0.02)	0.86 (+/- 0.11)
Constipated Subjects (n=8)	**Period I**	0.67 (+/- 0.23)	0.93 (+/- 0.02)	0.81 (+/- 0.02)	0.9 (+/- 0.03)
	Period II	0.70 (+/- 0.15)	0.93 (+/- 0.01)	0.81 (+/- 0.09)	0.91 (+/- 0.04)
	Period III	0.75 (+/- 0.16)	0.94 (+/- 0.01)	0.81 (+/- 0.04)	0.91 (+/- 0.01)

DAPI - Total DNA, BIF 164 - *Bifidobacterium* spp., BAC 303 - *Bacteroides* spp.,

LAB 158 - *Lactobacillus / enterococcus*, Erec 482 - *C.coccoides-Eubacterium* rectale gp.

Table 3.3.2 – ratio of probe/DAPI (+/-s.d.) for each period following FISH analysis comparing healthy samples (n=8) against sufferers of functional constipation enrolled in the Nalcol™ drug trial (n=8).

Constipated subjects had a lower ratio of *bifidobacteria* compared to healthy subjects (0.67 vs. 0.88, p=0.03) with five of the constipated subjects having bacterial counts below the threshold for detection. There were no significant changes to any of the counts over the three study periods.

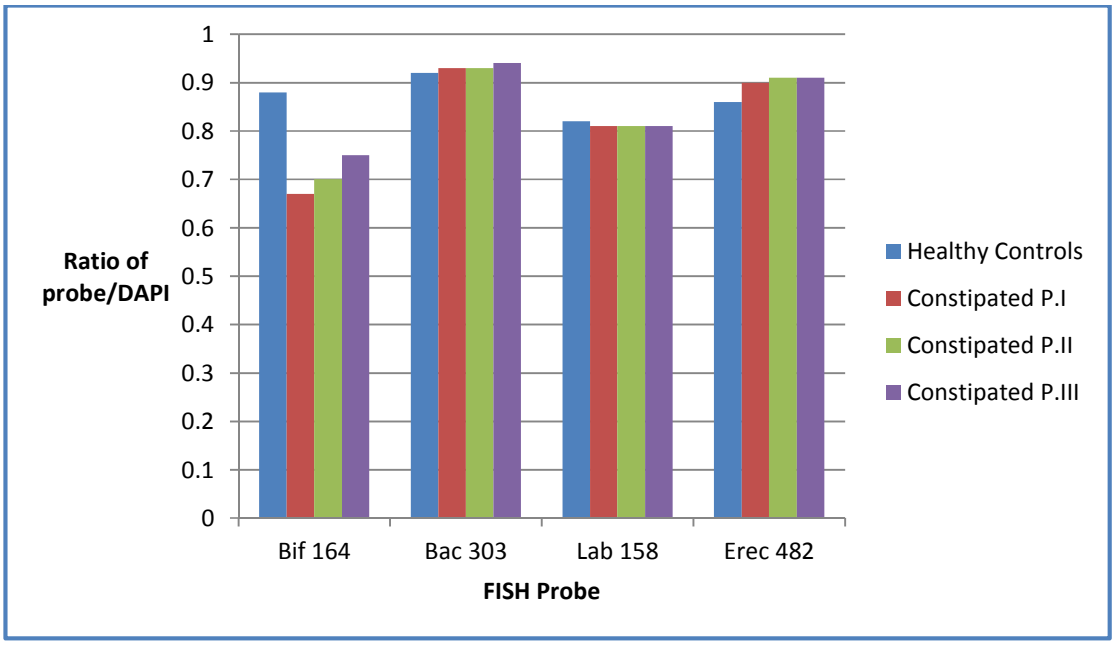


Figure 3.3.5 - Ratio of probe/DAPI for each period following FISH analysis comparing healthy samples against sufferers of functional constipation enrolled in the Nalcol™ drug trial.

3.3.3.2 Controls vs. Nalcol vs. Placebo (Period II)

This was a four week period where patients were randomised to either Nalcol™ or Placebo in a double blind fashion. To assess if the trial drug had any impact on the microbiota of the subjects, a comparison was made between healthy controls, subjects who took Nalcol™ for four weeks, and those who were randomised to take the placebo (Figure 3.3.6 and Table 3.3.3). The three patients who had taken Nalcol, two of whom had a response, had a *bifidobacteria* ratio closer to that of the healthy subjects (0.82 vs. 0.88, p=0.62) compared to those on the placebo (0.61 vs. 0.88, p>0.0001). However these *bifidobacteria* ratios were not significantly different to the baseline ratios taken from the samples given at the end of Period I (Nalcol, 0.79 vs. 0.81, p=0.88, Placebo, 0.57 vs. 0.61, p=0.65). There was no significant difference in the ratios for the other probes at the end of period II.

3.3.3.3 Controls vs. Nalcol™ (Period III)

This was an open label period where all subjects had taken Nalcol for at least 4 weeks at the end of period III. There were no significant changes in the *bifidobacteria* / DAPI ratios at baseline compared to the end of period III. There was no significant change in the other probes analysed (Figure 3.3.7)

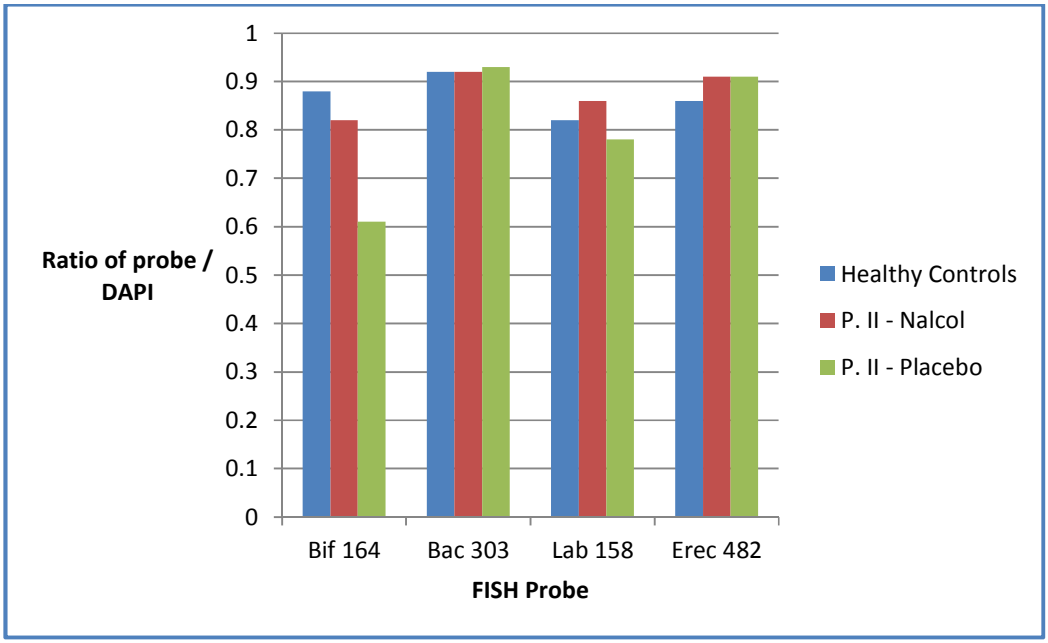


Figure 3.3.6 - ratio of probe/DAPI following FISH analysis comparing healthy samples against subjects who had taken Nalcol™ for 4 weeks and those who took placebo (Period II)

The three patients who had taken Nalcol, two of whom had a response, had a *bifidobacteria* ratio closer to that of the healthy subjects (0.82 vs. 0.88, p=0.62) compared to those on the placebo (0.61 vs. 0.88, p>0.0001). However these *bifidobacteria* ratios were not significantly different to the baseline ratios taken from the samples given at the end of Period I (Nalcol, 0.79 vs. 0.81, p=0.88, Placebo, 0.57 vs. 0.61, p=0.65). There was no significant difference in the ratios for the other probes at the end of period II.

	BIF 164	BAC 303	LAB 158	EREC 482
Healthy Controls	0.88 (+/- 0.04)	0.92 (+/- 0.02)	0.82 (+/- 0.02)	0.86 (+/- 0.11)
Baseline count for patients who took Nalcol in period II	0.79 (+/- 0.27)	0.92 (+/- 0.01)	0.80 (+/- 0.02)	0.88 (+/- 0.02)
Baseline count for patients who took Placebo in period II	0.57 (+/- 0.19)	0.93 (+/- 0.02)	0.82 (+/- 0.02)	0.91 (+/- 0.03)
Randomised to Nalcol in Period II	0.81 (+/- 0.18)	0.92 (+/- 0.01)	0.86 (+/- 0.01)	0.91 (+/- 0.05)
Randomised to Placebo in Period II	0.61 (+/- 0.01)	0.93 (+/- 0.02)	0.79 (+/- 0.12)	0.92 (+/- 0.03)

Table 3.3.3 - ratio of probe/DAPI (+/- s.d.) following FISH analysis comparing healthy samples against subjects who had taken Nalcol for 4 weeks and those who took placebo during Period II

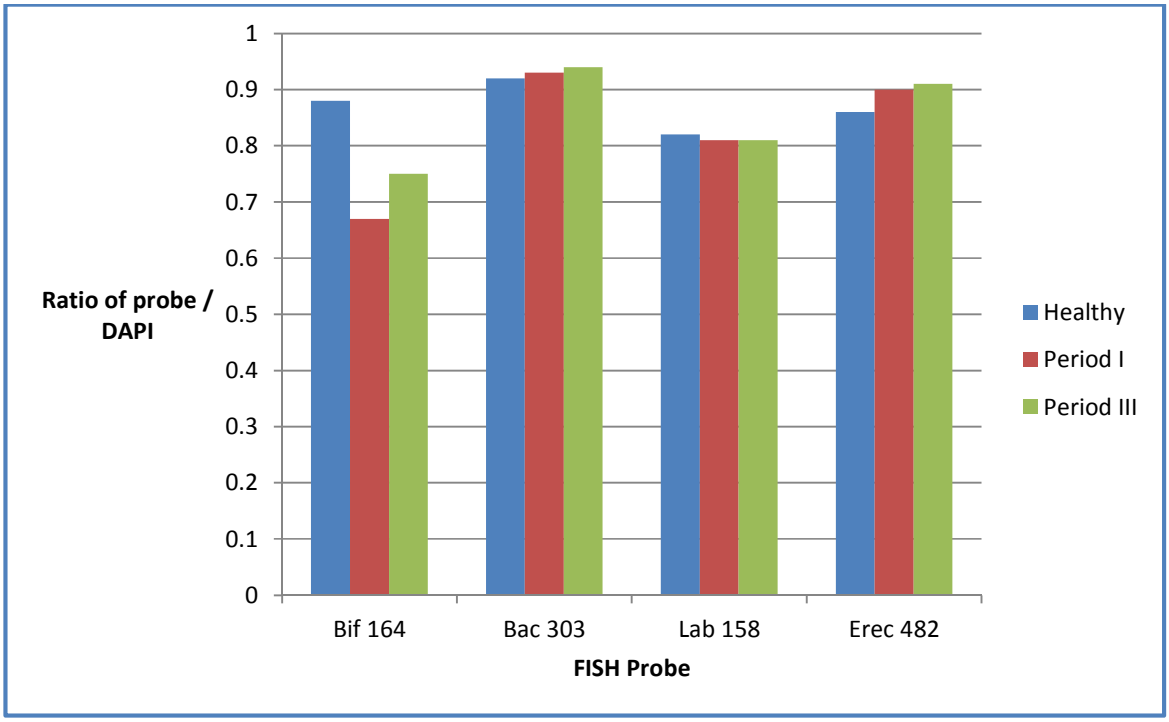


Figure 3.3.7 – ratio of probe/DAPI following FISH analysis comparing healthy samples against periods I and III of the Nalcol™ drug trial

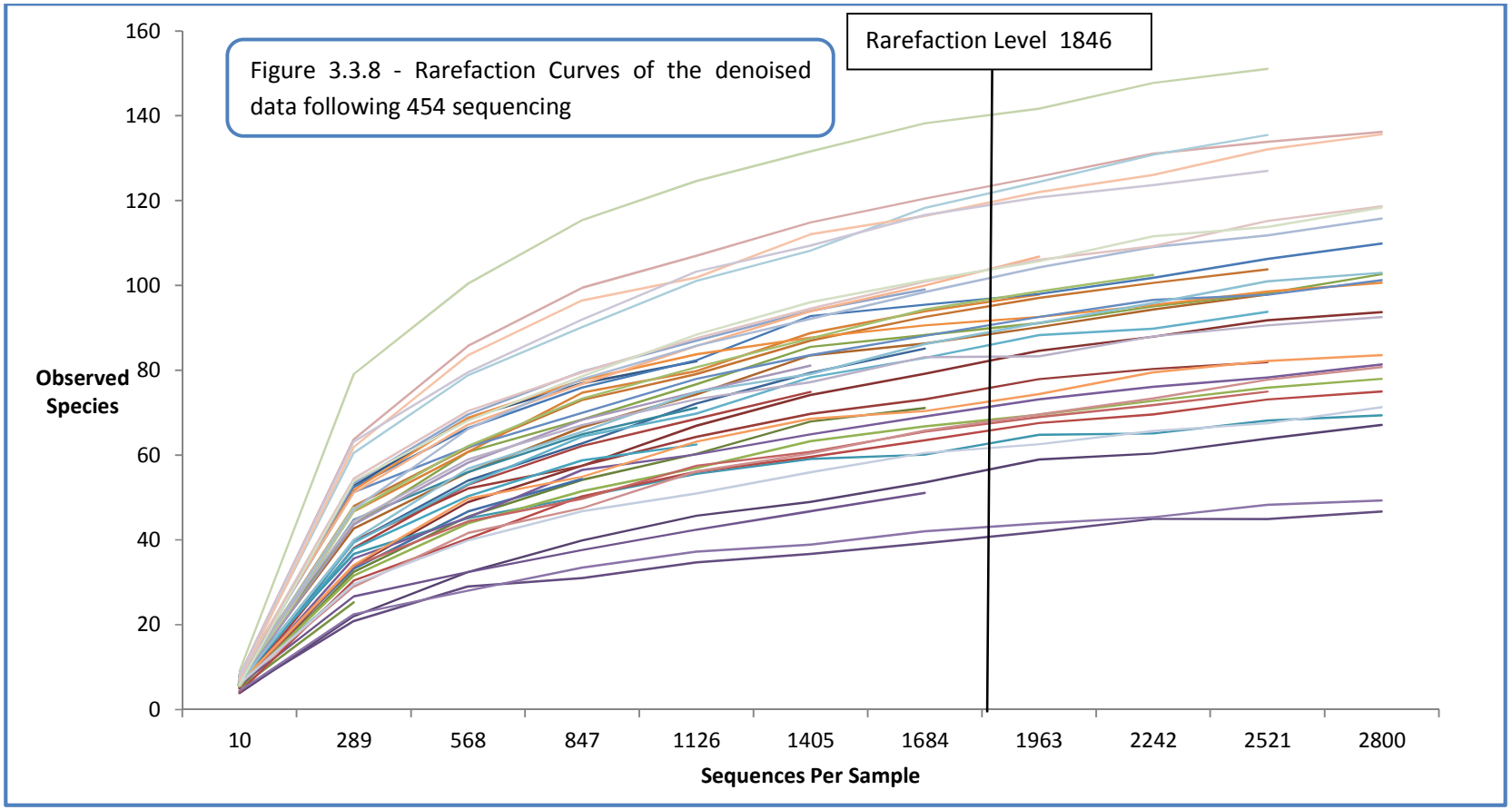
This was an open label period where all subjects had taken Nalcol for at least 4 weeks at the end of period III. There were no significant changes in the *bifidobacteria* / DAPI ratios at baseline compared to the end of period III. There was no significant change in the other probes analysed.

3.3.4 ANALYSIS OF MICROBIOTA COMPOSITION USING 454 PYROSEQUENCING

In total 48 samples were sent for 16S rDNA sequencing which included 16 healthy samples, 16 constipated subjects taken at the end of Period I, eight samples taken at the end of period II and eight at the end period III. Rarefaction curves were plotted to determine sample richness and adequacy of sampling in the population (figure 3.3.8) and the data was denoised in an attempt to reduce sequencing errors. After analysis of the curves it was decided to use a rarefaction level of 1846. This excluded five samples from the constipated group sampled at the end of period I and two samples from the healthy group.

At the phylum level there was a significant decrease in the proportion of *Firmicutes* (25% vs. 45%, p=0.004) and a significant increase in the proportion of *Bacteroidetes* (66% vs. 41%, p=0.002) in the constipated group compared to healthy individuals (Table 3.3.4, Figure 3.3.9). There were no other differences seen between the two groups at the phylum level. The *Bacteroidetes* and *Firmicutes* made up 91% of the phyla detected in the constipated subjects and 86% of those in the healthy population (p=0.38). The difference in the constipated subjects in the *Firmicutes* phylum was secondary to a significant reduction in the *clostridia* class (25% vs. 45%, p=0.004) whilst the difference seen in the *bacteroidetes* phylum was due to a significant increase in the *bacteroidia* class compared to healthy volunteers (66% vs. 41%, p=0.002). Other differences were noted between the *ML615J-28*, *mollicutes*, and *Erysipelotrichi* classes of the *tenericutes* phylum in the two groups but these were either not significant or were of a low abundance (Table 3.3.5).

At the order level the *bacteroidales* (phylum – *bacteroidetes*) is significantly greater in the constipated subjects compared to the healthy individuals (66% vs. 41%, p=0.002) with the decreases seen in the *firmicute* phylum in constipated subjects are due to a reduction in the *clostridiales* order (24% vs 45%, p=0.003). Again other differences between the two groups were noted but were either not significant or of low abundance (Table 3.3.6). Analysis at the family level demonstrated that both *porphyromonadaceae* (3.5% vs. 1.6%, p =0.007) and *rikenellaceae* (22% vs. 9%, p=0.010) were responsible for the increase in the *bacteroidetes* phylum (Table 3.3.7, Figure 3.3.10). The decrease in the *clostridia* (continued on page 163.....)



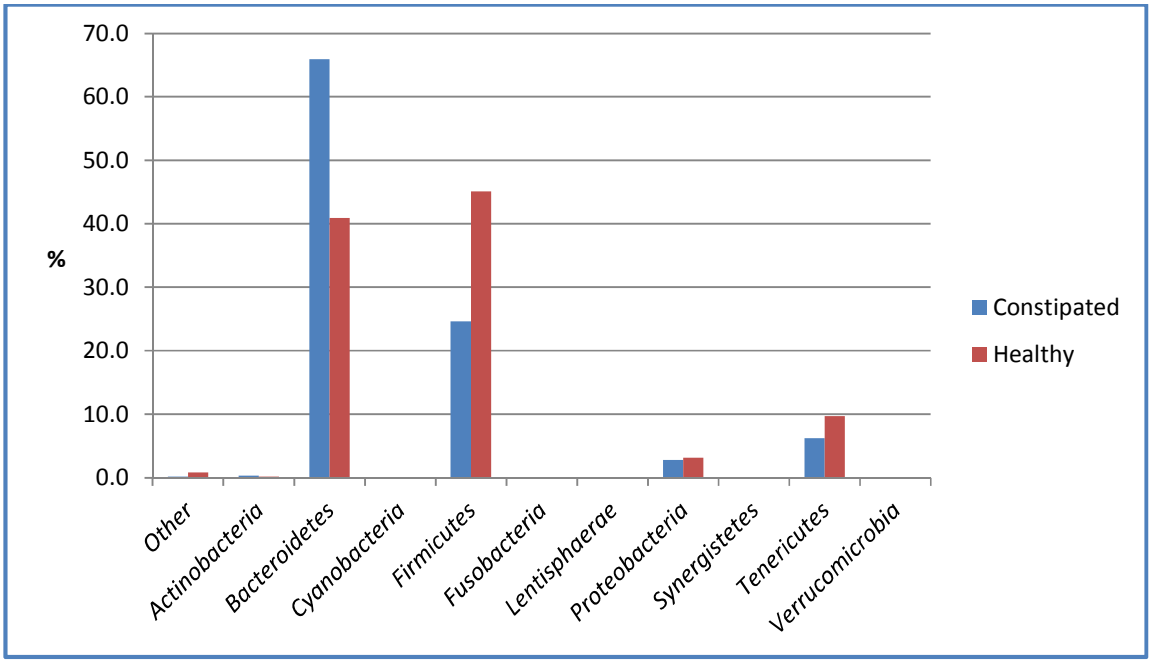
Rarefaction curves were plotted to determine sample richness and adequacy of sampling in the population. After analysis of the curves it was decided to use a rarefaction level of 1846. This excluded five samples from the constipated group sampled at the end of period I and two samples from the healthy group

Phyla	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P value
Other	0.2 (+/- 0.3)	0.8 (+/- 1.5)	0.18
Actinobacteria	0.3 (+/- 0.8)	0.2 (+/- 0.2)	0.56
*Bacteroidetes**	65.9 (+/-17.5)	40.9 (+/- 18.0)	**0.002**
Cyanobacteria	0.1 (+/- 0.2)	0.1 (+/- 0.2)	0.71
*Firmicutes**	24.6 (+/- 12.9)	45.1 (+/- 18.6)	**0.004**
Fusobacteria	0.0 (+/- 0.00)	0.0 (+/- 0.0)	0.34
Lentisphaerae	0.0 (+/- 0.00)	0.1 (+/- 0.0)	0.11
Proteobacteria	2.8 (+/- 3.6)	3.1 (+/- 4.6)	0.83
Synergistetes	0.0(+/- 0.00)	0.0 (+/- 0.0)	0.34
Tenericutes	6.2 (+/- 7.7)	9.7 (+/- 7.3)	0.25
Verrucomicrobia	0.0 (+/- 0.00)	0.0 (+/- 0.0)	0.87

*p<0.005. The increase in the *Bacteroidetes* phylum and the decrease in Firmicutes phylum in constipated subjects were significant (p=0.002 and 0.004 respectively).

Table 3.3.4 – Comparison of the percentage of bacteria (%) at the phylum level in healthy and constipated subjects who underwent pyrosequencing.

Samples from 16 healthy individuals and 16 constipated individuals were sent for 16S rDNA sequencing taken at the end of Period I but after exclusion based on the rarefaction curve five samples were excluded from the constipated group



The increase in the Bacteroidetes phylum and the decrease in Firmicutes phylum in constipated subjects were significant (p=0.002 and 0.004 respectively).

Figure 3.3.9 - Comparison of the percentage of bacteria (%) at the phylum level in healthy and constipated subjects who underwent pyrosequencing.

Samples from 16 healthy individuals and 16 constipated individuals were sent for 16S rDNA sequencing taken at the end of Period I but after exclusion based on the rarefaction curve five samples were excluded from the constipated group (n=11) and two from the healthy population (n=14)

	Phyla	Class	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	p-value
1	Other	Other	0.2 (+/- 0.3)	0.8 (+/- 0.015)	0.18
2	*Actinobacteria*	*Actinobacteria*	0.3 (+/- 0.8)	0.2 (+/- 0.002)	0.56
3	*Bacteroidetes**	*Bacteroidia*	65.9 (+/- 17.5)	40.9 (+/- 0.180)	**0.002**
4	*Cyanobacteria*	*4C0d-2*	0.1 (+/- 0.2)	0.1 (+/- 0.002)	0.62
5	*Cyanobacteria*	*Chloroplast*	0.0 (+/- 0.0)	0.0 (+/- 0.000)	0.34
6	*Firmicutes*	*Bacilli*	0.1 (+/- 0.1)	0.1 (+/- 0.001)	0.65
7	*Firmicutes**	*Clostridia*	24.5 (+/- 12.9)	45.0 (+/- 0.185)	**0.004**
8	*Fusobacteria*	*Fusobacteria*	0.0 (+/- 0.0)	0.0 (+/- 0.000)	0.34
9	*Lentisphaerae*	*Lentisphaerae*	0.0 (+/- 0.0)	0.1 (+/- 0.002)	0.11
10	*Proteobacteria*	*Alphaproteobacteria*	1.5 (+/- 3.1)	1.3 (+/- 0.029)	0.90
11	*Proteobacteria*	*Betaproteobacteria*	1.0 (+/- 0.9)	1.5 (+/- 0.019)	0.38
12	*Proteobacteria*	*Gammaproteobacteria*	0.2 (+/- 0.5)	0.2 (+/- 0.005)	0.99
13	*Synergistetes*	*Synergistia*	0.0 (+/- 0.0)	0.0 (+/- 0.000)	0.34
14	*Tenericutes*	*Other*	0.0 (+/- 0.0)	0.0 (+/- 0.000)	0.34
15	*Tenericutes*	*Erysipelotrichi*	2.7 (+/- 4.6)	1.0 (+/- 0.010)	0.25
16	*Tenericutes**	*ML615J-28*	0.0 (+/- 0.1)	0.4 (+/- 0.007)	**0.037**
17	*Tenericutes*	*Mollicutes*	3.5 (+/- 6.3)	8.3 (+/- 0.065)	0.07
18	*Verrucomicrobia*	*Verrucomicrobiae*	0.0 (+/- 0.0)	0.0 (+/- 0.000)	0.87

* p< 0.05. The difference in the constipated subjects in the *firmicute* phylum was secondary to a significant reduction in the *clostridia* class (25% vs. 45%, p=0.004) whilst the difference seen in the *bacteroides* phylum was due to a significant increase in the *bacteroidetes* class compared to healthy volunteers (66% vs. 41%, p=0.002).

Table 3.3.5 - Comparison of the percentage of bacteria (%) at the class level in healthy and constipated subjects who underwent pyrosequencing

	Phyla	Class	Order	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P value
1	Other	Other	Other	0.2 (+/- 0.3)	0.8 (+/- 1.5)	0.18
2	Actinobacteria	Actinobacteria	Actinomycetales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.49
3	Actinobacteria	Actinobacteria	Coriobacteriales	0.3 (+/- 0.8)	0.2 (+/- 0.2)	0.59
4	Bacteroidetes*	Bacteroidia	Bacteroidales	65.9 (+/- 1.75)	40.9 (+/- 18)	**0.002**
5	Cyanobacteria	4C0d-2	YS2	0.1 (+/- 0.2)	0.1 (+/- 0.2)	0.62
6	Cyanobacteria	Chloroplast	Streptophyta	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
7	Firmicutes	Bacilli	Bacillales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.06
8	Firmicutes	Bacilli	Lactobacillales	0.1 (+/- 0.0)	0.1 (+/- 0.1)	0.77
9	Firmicutes	Bacilli	Turicibacterales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
10	Firmicutes	Clostridia	Other	0.0 (+/- 0.1)	0.0 (+/- 0.1)	0.56
11	Firmicutes*	Clostridia	Clostridiales	24.5 (+/- 12.9)	45.0 (+/- 18.5)	**0.003**
12	Fusobacteria	Fusobacteria	Fusobacteriales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
13	Lentisphaerae	Lentisphaerae	Victivallales	0.0 (+/- 0.0)	0.1 (+/- 0.2)	0.11
14	Proteobacteria	Alphaproteobacteria	-----	1.5 (+/- 3.1)	1.3 (+/- 2.9)	0.90
15	Proteobacteria	Betaproteobacteria	Burkholderiales	1.0 (+/- 0.9)	1.5 (+/- 1.9)	0.38
16	Proteobacteria	Gammaproteobacteria	Enterobacteriales	0.2 (+/- 0.5)	0.0 (+/- 0.0)	0.17
17	Proteobacteria	Gammaproteobacteria	Pasteurellales	0.0 (+/- 0.0)	0.2 (+/- 0.5)	0.12
18	Proteobacteria	Gammaproteobacteria	Pseudomonadales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
19	Synergistetes	Synergistia	Synergistales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
20	Tenericutes	Other	Other	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
21	Tenericutes	Erysipelotrichi	Erysipelotrichales	2.7 (+/- 4.6)	1.0 (+/- 1.0)	0.25
22	Tenericutes*	ML615J-28	-----	0.0 (+/- 0.1)	0.4 (+/- 0.7)	**0.037**
23	Tenericutes	Mollicutes	Anaeroplasmatales	0.0 (+/- 0.0)	0.0 (+/- 0.1)	0.54
24	Tenericutes	Mollicutes RF39	-----	3.5 (+/- 6.3)	8.3 (+/- 6.5)	0.08
25	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.87

Table 3.3.6 Comparison of the percentage of bacteria at the order level in healthy and constipated subjects who underwent pyrosequencing. * p>0.05. Significant differences were seen in the bacteroidales order (66% vs. 41%, p=0.002) and the Clostridales order (25% vs. 45%, p=0.003).

Table 3.3.7 – Comparison of the percentage of bacteria at the family level in healthy and constipated subjects (Divided into three parts on pages 159 – 161).

	Phyla	Class	Order	Family	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P value
1	Other	Other	Other	Other	0.2 (+/- 0.3)	0.8 (+/- 1.5)	0.18
2	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.76
3	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
4	Actinobacteria	Actinobacteria	Coriobacteriales	----	0.3 (+/- 0.8)	0.1 (+/- 0.2)	0.47
5	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	0.0 (+/- 0.0)	0.1 (+/- 0.1)	0.09
6	Bacteroidetes	Bacteroidia	Bacteroidales	Other	0.7 (+/- 1.0)	0.5 (+/- 0.6)	0.53
7	Bacteroidetes*	Bacteroidia	Bacteroidales	-----	3.3 (+/- 3.3)	6.6 (+/- 5.3)	0.07
8	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	36.3 (+/- 22.3)	20.2 (+/- 13.7)	**0.05**
9	Bacteroidetes*	Bacteroidia	Bacteroidales	Porphyromonadaceae	3.5 (+/- 1.8)	1.6 (+/- 1.3)	**0.007**
10	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.1 (+/- 0.1)	2.9 (+/- 6.4)	0.13
11	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	21.9 (+/- 12.7)	9.2 (+/- 7.3)	**0.010**
12	Cyanobacteria	4C0d-2	YS2	-----	0.1 (+/- 0.2)	0.1 (+/- 0.2)	0.62
13	Cyanobacteria	Chloroplast	Streptophyta	-----	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
14	Firmicutes	Bacilli	Bacillales	Other	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.06
15	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	0.0 (+/- 0.0)	0.0 (+/- 0.1)	0.79
16	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
17	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	0.0 (+/- 0.0)	0.0 (+/- 0.1)	0.74
18	Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
19	Firmicutes	Clostridia	Other	Other	0.0 (+/- 0.1)	0.0 (+/- 0.1)	0.56
20	Firmicutes	Clostridia	Clostridiales	Other	0.5 (+/-0.8)	0.7 (+/- 1.1)	0.60
21	Firmicutes	Clostridia	Clostridiales	-----	3.3 (+/- 3.8)	5.0 (+/- 6.0)	0.40

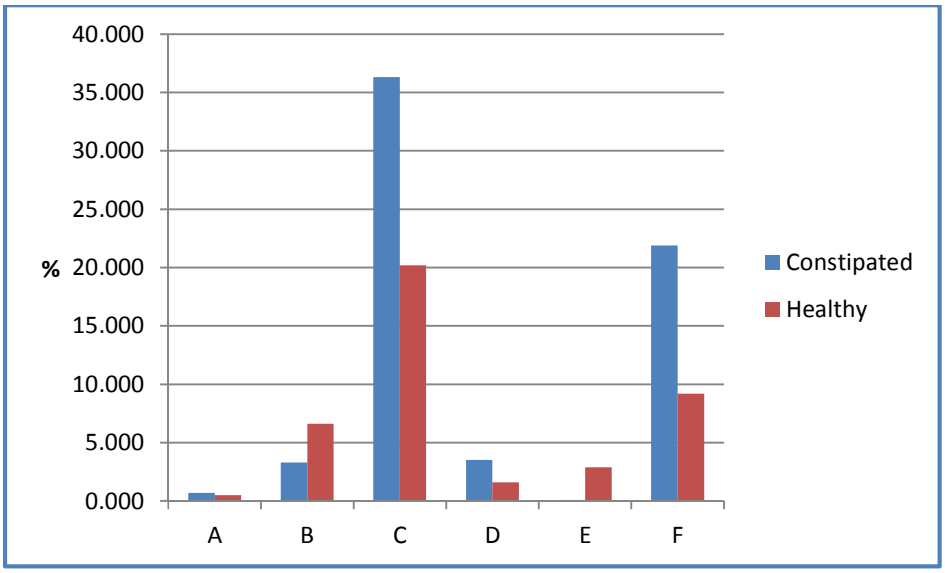
Continuation of table 3.3.7

	Phyla	Class	Order	Family	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	p value
22	Firmicutes	Clostridia	Clostridiales	Catabacteriaceae	0.4 (+/- 0.5)	1.1 (+/- 1.5)	0.11
23	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	0.1 (+/- 0.4)	0.2 (+/- 0.3)	0.50
24	Firmicutes	Clostridia	Clostridiales	Clostridiales Family XI. Incertae Sedis	0.0 (+/- 0.1)	0.0 (+/- 0.0)	0.47
25	Firmicutes	Clostridia	Clostridiales	Clostridiales Family XIII. Incertae Sedis	0.0 (+/- 0.1)	0.1 (+/- 0.2)	0.06
26	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae	0.0 (+/- 0.0)	0.0 (+/- 0.1)	0.09
27	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.43
28	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	8.5 (+/- 6.7)	12.0 (+/- 6.9)	0.23
29	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	0.0 (+/- 0.1)	0.0 (+/- 0.0)	0.90
30	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.22
31	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	11.0 (+/- 5.1)	14.4 (+/- 5.5)	0.12
32	Firmicutes*	Clostridia	Clostridiales	Veillonellaceae	0.6 (+/- 0.9)	11.4 (+/- 14.7)	**0.016**
33	Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
34	Lentisphaerae	Lentisphaerae	Victivallales	Other	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
35	Lentisphaerae	Lentisphaerae	Victivallales	Victivallaceae	0.0 (+/- 0.0)	0.1 (+/- 0.2)	0.10
36	Proteobacteria	Alphaproteobacteria	-----	-----	1.5 (+/- 3.1)	1.3 (+/- 2.9)	0.90
37	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	1.0 (+/- 0.9)	1.5 (+/- 1.9)	0.40
38	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	0.0 (+/- 0.0)	0.1 (+/- 0.1)	0.15
39	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	0.2 (+/- 0.5)	0.0 (+/- 0.0)	0.17
40	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	0.0 (+/- 0.0)	0.2 (+/- 0.5)	0.12
41	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
42	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.34
43	Tenericutes	Other	Other	Other	0.0 (+/- 0.0)	0.0 +/- (0.0)	0.34

Continuation of table 3.3.7

	Phyla	Class	Order	Family	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	p value
44	Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	2.7 (+/- 4.6)	1.0 (+/- 1.0)	0.25
45	Tenericutes	Erysipelotrichi	Erysipelotrichales	vadinHA31	0.0 (+/- 0.0)	0.0 (+/- 0.1)	0.24
46	Tenericutes*	ML615J-28	-----	-----	0.0 (+/- 0.1)	0.4 (+/- 0.7)	**0.037**
47	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	0.0 (+/- 0.0)	0.0 (+/- 0.1)	0.54
48	Tenericutes	Mollicutes RF39	-----	-----	3.5 (+/- 6.3)	8.3 (+/- 6.5)	0.08
49	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	0.0 (+/- 0.0)	0.0 (+/- 0.0)	0.87

* - $p < 0.05$. In the bacteroidetes phylum, *Porphyromonadaceae* and *Rikenellaceae* were significantly increased in constipated subjects. In the clostridia phylum, *Veillonellaceae* was significantly reduced in constipated subjects compared to healthy subjects.



A	*k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Other*
B	*k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__*
C	*k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae*
D	*k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae*
E	*k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae*
F	*k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae*

k – Kingdom, p – Phyla, c – Class, o – Order, f - Family

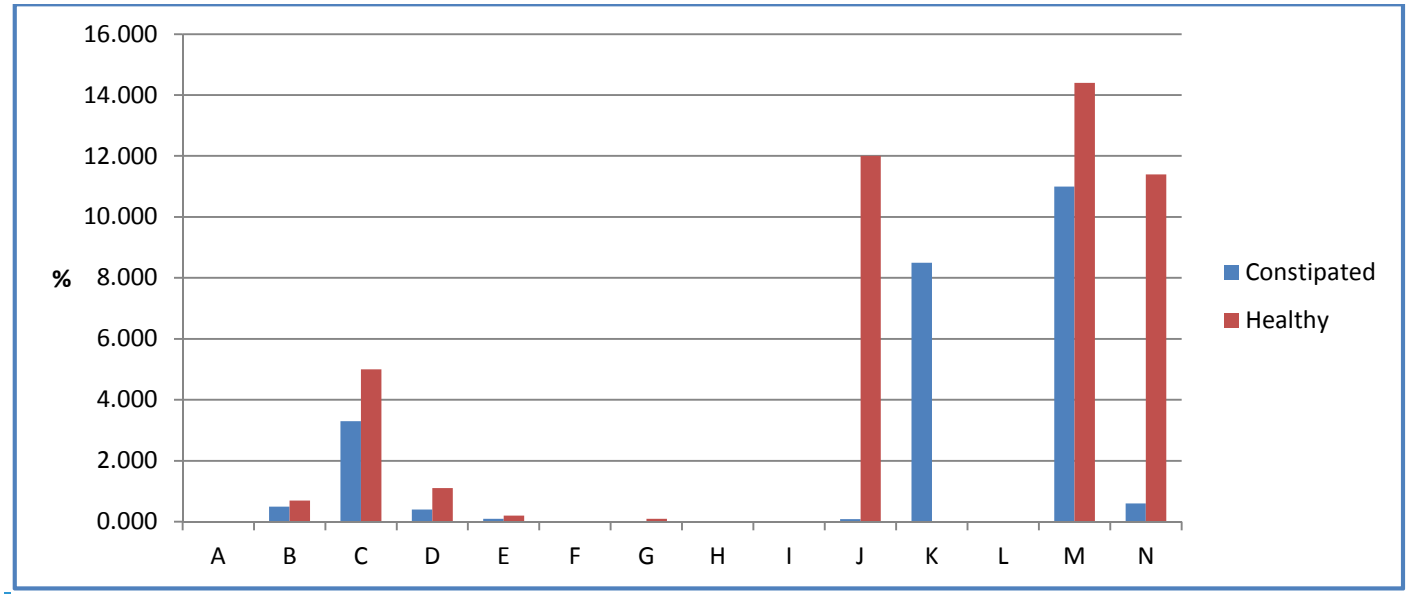
Figure 3.3.10 – Comparison of the mean percentage of the *bacteroidetes* phylum in constipated and healthy subjects when viewed at the family level who underwent pyrosequencing.

In the bacteroidetes phylum, *Porphyromonadaceae (3.5% vs. 1.6%, p=0.007)* and *Rikenellaceae (2.2% vs. 9.2%, p=0.01)* were significantly increased in constipated subjects.

class in constipated subjects was as a results of an almost 20x decrease in *veillonellaceae* (0.6% vs. 11%, p=0.016), however all family members of the *clostridia* class are either lower or equal in constipated subjects compared to the healthy population (Table 3.3.7, Figure 3.3.11). Again there were no significant differences except in the *tenericutes* phyla but these were of low abundance.

There were several genera that were significantly reduced or increased in the constipated group compared to the healthy group. In the *bacteroidetes* phylum of constipated subjects there were significant increases in the *Odoribacter* (% vs. 0.55%, p=0.02), *Parabacteroides* (2.21% vs. 1.10%, p=0.04), and *Alistipes* (20.05% vs. 8.16%, p=0.01) genera. In the *Firmicutes* phylum there were significant decreases in the *Oscillospira* (0.52% vs. 1.33%, p=0.007) and *Dialister* (0.44% vs. 11.14%, p=0.02) genera. Other changes in the microbiotra at a genus level are shown in table 3.3.8.

Analysis was undertaken on the samples provided from the constipated subjects who gave a sample at the end of each period (n=8). The baseline samples did not differ in these 8 subjects from the constipated group as a whole (n=11) and as such there was no difference other than that already described above when compared to healthy subjects. There was also no change in the composition of the microbiota of the subjects over the 10 week trial period, despite the use of Nalcol for in all subjects for four weeks during period III. The data for these analyses is not shown.



A	*k__Bacteria;p__Firmicutes;c__Clostridia;Other;Other*
B	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other*
C	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__*
D	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Catabacteriaceae*
E	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae*
F	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XI. Incertae Sedis*
G	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis*
H	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae*
I	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae*
J	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae*
K	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae*
L	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae*
M	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae*
N	*k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae*

k – Kingdom, p – Phyla, c – Class, o – Order, f - Family

Figure 3.3.11 - Comparison of the mean percentage of the *clostridia* class in constipated and healthy subjects after pyrosequencing when viewed at the family level

In the clostridia phylum, *Veillonellaceae (0.6% vs. 11.4%, p= 0.016)* was significantly reduced in constipated subjects compared to healthy subjects.

Table 3.3.8 – Comparison of the percentage of bacteria at the genus level in healthy and constipated subjects (Divided into five parts on pages 165 – 169).

Phyla	Class	Order	Family	Genus	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P Value
Other	Other	Other	Other	Other	0.16 (+/- 0.32)	0.73 (+/- 1.41)	0.16
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Actinomyces*	0.00 (+/- 0.02)	0.01 (+/- 0.02)	0.70
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*	0.01 (+/- 0.05)	0.00 (+/- 0.00)	0.34
Actinobacteria	*Actinobacteria*	*Coriobacteriales*	-----	-----	0.32 (+/- 0.81)	0.13 (+/- 0.17)	0.47
Actinobacteria	*Actinobacteria*	*Coriobacteriales*	*Coriobacteriaceae*	*Adlercreutzia*	0.02 (+/- 0.04)	0.06 (+/- 0.13)	0.30
Actinobacteria	*Actinobacteria*	*Coriobacteriales*	*Coriobacteriaceae*	*Collinsella*	0.00 (+/- 0.00)	0.00 (+/- 0.01)	0.34
Actinobacteria	*Actinobacteria*	*Coriobacteriales*	*Coriobacteriaceae*	*Eggerthella*	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Other*	*Other*	0.02 (+/- 0.06)	0.05 (+/- 0.10)	0.32
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	-----	-----	4.06 (+/- 4.09)	7.00 (+/- 5.54)	0.14
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	36.35 (+/- 22.75)	20.08 (+/- 13.56)	0.05
*Bacteroidetes**	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Odoribacter*	1.38 (+/- 0.93)	0.55 (+/- 0.49)	**0.02**
*Bacteroidetes**	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Parabacteroides*	2.21 (+/- 1.44)	1.10 (+/- 0.96)	**0.04**
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Porphyromonas*	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	-----	0.00 (+/- 0.00)	0.41 (+/- 1.55)	0.34
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	0.07 (+/- 0.07)	2.62 (+/- 6.76)	0.18
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	-----	1.53 (+/- 1.51)	1.10 (+/- 1.33)	0.46
*Bacteroidetes**	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*	20.05 (+/- 12.47)	8.16 (+/- 6.76)	**0.01**

Continuation of Table 3.3.8

Phyla	Class	Order	Family	Genus	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P Value
Cyanobacteria	*4C0d-2*	*YS2*	------	-----	0.04 (+/- 0.13)	0.10 (+/- 0.21)	0.38
Cyanobacteria	*Chloroplast*	*Streptophyta*	------	-----	0.01 (+/- 0.03)	0.01 (+/- 0.02)	0.85
Euryarchaeota	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*	0.05 (+/- 0.02)	0.05 (+/- 0.01)	0.87
*Firmicutes**	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	0.00 (+/- 0.00)	0.04 (+/- 0.06)	**0.04**
Firmicutes	*Bacilli*	*Turicibacterales*	*Turicibacteraceae*	*Other*	0.01 (+/- 0.03)	0.00 (+/- 0.00)	0.19
Firmicutes	*Clostridia*	*Other*	*Other*	*Other*	0.03 (+/- 0.07)	0.01 (+/- 0.04)	0.45
Firmicutes	*Clostridia*	*Clostridiales*	*Other*	*Other*	0.49 (+/- 0.76)	0.49 (+/- 1.11)	1.00
Firmicutes	*Clostridia*	*Clostridiales*	------	-----	3.21 (+/- 3.79)	4.84 (+/- 5.86)	0.41
Firmicutes	*Clostridia*	*Clostridiales*	*Catabacteriaceae*	-----	0.40 (+/- 0.53)	1.10 (+/- 1.43)	0.11
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	0.13 (+/- 0.30)	0.28 (+/- 0.50)	0.36
Firmicutes	*Clostridia*	*Clostridiales*	*ClostridialesFamilyXI.Incertae Sedis*	-----	0.03 (+/- 0.07)	0.02 (+/- 0.05)	0.66
Firmicutes	*Clostridia*	*Clostridiales*	*ClostridialesFamilyXI.Incertae Sedis*	*Peptoniphilus*	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34
Firmicutes	*Clostridia*	*Clostridiales*	*ClostridialesFamilyXIII.Incertae Sedis*	*Other*	0.00 (+/- 0.00)	0.09 (+/- 0.20)	0.10
Firmicutes	*Clostridia*	*Clostridiales*	*ClostridialesFamilyXIII.Incertae Sedis*	-----	0.02 (+/- 0.04)	0.06 (+/- 0.08)	0.09
Firmicutes	*Clostridia*	*Clostridiales*	*ClostridialesFamilyXIII.Incertae Sedis*	*Eubacterium*	0.04 (+/- 0.06)	0.03 (+/- 0.05)	0.70
Firmicutes	*Clostridia*	*Clostridiales*	*Dehalobacteriaceae*	*Dehalobacterium*	0.00 (+/- 0.02)	0.02 (+/- 0.05)	0.19
Firmicutes	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Anaerofustis*	0.00 (+/- 0.00)	0.01 (+/- 0.03)	0.19
Firmicutes	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Eubacterium*	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34

Continuation of Table 3.3.8

Phyla	Class	Order	Family	Genus	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P Value
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Other*	0.59 (+/- 0.67)	0.82 (+/- 1.24)	0.56
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	-----	0.64 (+/- 0.91)	1.87 (+/- 2.27)	0.08
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Blautia*	0.75 (+/- 0.99)	0.26 (+/- 0.31)	0.14
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Clostridium*	1.99 (+/- 1.3)	2.57 (+/- 01.62)	0.33
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*	0.43 (+/- 0.53)	0.46 (+/- 0.35)	0.91
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Eubacterium*	0.37 (+/- 0.52)	0.29 (+/- 0.27)	0.66
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnobacterium*	0.03 (+/- 0.11)	0.23 (+/- 0.5)	0.19
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospira*	1.39 (+/- 2.05)	2.61 (+/- 3.02)	0.24
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*	1.91 (+/- 1.83)	2.43 (+/- 2.54)	0.56
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Ruminococcus*	0.48 (+/- 0.84)	0.16 (+/- 0.18)	0.24
Firmicutes	*Clostridia*	*Clostridiales*	*Peptococcaceae*	-----	0.04 (+/- 0.07)	0.04 (+/- 0.06)	0.91
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Other*	0.95 (+/- 0.50)	1.41 (+/- 1.26)	0.23
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	-----	2.50 (+/- 1.37)	4.24 (+/- 3.44)	0.10
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Acetivibrio*	0.00 (+/- 0.00)	0.06 (+/- 0.17)	0.23
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Anaerotruncus*	0.06 (+/- 0.08)	0.04 (+/- 0.04)	0.48
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Clostridium*	0.50 (+/- 0.60)	0.25 (+/- 0.27)	0.22
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Eubacterium*	0.29 (+/- 0.63)	0.06 (+/- 0.10)	0.27
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Faecalibacterium*	3.72 (+/- 2.81)	3.36 (+/- 3.37)	0.78
Firmicutes[*]	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillospira*	0.52 (+/- 0.62)	1.33 (+/- 0.76)	**0.007**
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	1.02 (+/- 1.83)	1.20 (+/- 1.78)	0.80
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Subdoligranulum*	1.54 (+/- 2.06)	2.64 (+/- 3.91)	0.37

Continuation of Table 3.3.8

Phyla	Class	Order	Family	Genus	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P Value
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	------	0.09 (+/- 0.29)	0.15 (+/- 0.46)	0.70
*Firmicutes**	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Dialister*	0.44 (+/- 0.85)	11.14 (+/- 14.69)	**0.02**
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Veillonella*	0.02 (+/- 0.04)	0.09 (+/- 0.14)	0.10
Fusobacteria	*Fusobacteria*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*	0.01 (+/- 0.02)	0.00 (+/- 0.00)	0.17
Lentisphaerae	*Lentisphaerae*	*Victivallales*	*Victivallaceae*	-----	0.00 (+/- 0.020	0.07 (+/- 0.14)	0.12
Proteobacteria	*Alphaproteobacteria*	-----	-----	-----	1.48 (+/- 2.97)	1.36 (+/- 2.88)	0.92
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	-----	0.49 (+/- 0.79)	0.36 (+/- 0.41)	0.62
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Sutterella*	0.55 (+/- 0.86)	1.11 (+/- 1.72)	0.30
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Oxalobacter*	0.03 (+/- 0.10)	0.06 (+/- 0.12)	0.54
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Bilophila*	0.00 (+/- 0.02)	0.00 (+/- 0.01)	0.87
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Other*	0.17 (+/- 0.55)	0.00 (+/- 0.00)	0.34
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia*	0.07 (+/- 0.10)	0.02 (+/- 0.03)	0.14
Proteobacteria	*Gammaproteobacteria*	*Pasteurellales*	*Pasteurellaceae*	*Haemophilus*	0.01 (+/- 0.03)	0.23 (+/- 0.49)	0.13
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Xanthomonas*	0.00 (+/- 0.00)	0.00 (+/- 0.01)	0.34
Synergistetes	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*Pyramidobacter*	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34
*Tenericutes**	*Other*	*Other*	*Other*	*Other*	0.00 (+/- 0.00)	0.02 (+/- 0.03)	**0.04**
Tenericutes	*Erysipelotrich*	*Erysipelotrichales*	*Erysipelotrichaceae*	-----	1.65 (+/- 4.11)	0.52 (+/- 1.01)	0.40
Tenericutes	*Erysipelotrich*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Clostridium*	1.00 (+/- 0.68)	0.46 (+/- 0.65)	0.06

Continuation of Table 3.3.8

Phyla	Class	Order	Family	Genus	Mean percentage of bacteria in faecal samples of constipated subjects (+/- s.d) N=11	Mean percentage of bacteria in faecal samples of healthy subjects (+/- s.d) N=14	P Value
Tenericutes	Erysipelotrich	Erysipelotrichales	Erysipelotrichaceae	Coprobacillus	0.01 (+/- 0.02)	0.00 (+/- 0.00)	0.17
Tenericutes	Erysipelotrich	Erysipelotrichales	Erysipelotrichaceae	Holdemania	0.06 (+/- 0.06)	0.02 (+/- 0.03)	0.06
Tenericutes	Erysipelotrich	Erysipelotrichales	vadinHA31	RFN20	0.00 (+/- 0.00)	0.02 (+/- 0.06)	0.19
Tenericutes*	ML615J-28	-----	-----	-----	0.03 (+/- 0.11)	0.50 (+/- 0.79)	**0.05**
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	-----	0.00 (+/- 0.00)	0.04 (+/- 0.16)	0.34
Tenericutes	Mollicutes	RF39	-----	-----	3.43 (+/- 6.22)	8.30 (+/- 6.49)	0.07
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	0.00 (+/- 0.02)	0.00 (+/- 0.00)	0.34

* p>0.05. In the *bacteroidetes* phylum there were significant differences in the *Odoribacter* (1.38% vs. 0.55%, p=0.02), *Parabacteroides* (2.21% vs. 1.10%, p=0.04), and *Alistipes* (20.05% vs. 8.16%, p=0.01) genera. In the *Firmicutes* phyla there were significant differences in the *Oscillospira* (0.52% vs. 1.33%, p=0.007) and *Dialister* (0.44% vs. 11.14%, p=0.02) genera.

3.4 DISCUSSION OF THE COMPARATIVE ANALYSIS OF THE COMPOSITION OF THE FAECAL MICROBIOTA IN PATIENTS WITH CHRONIC CONSTIPATION AND IN HEALTHY SUBJECTS

3.4.1 SUMMARY OF RESULTS

The analysis of the samples provided has shown that differences exist between the microbiota of healthy and constipated subjects. DGGE has shown that this difference exists in the abundance of different organisms but not in a complete absence of one particular organism. This was confirmed by supervised modelling carried out on the 454 sequencing data. FISH and 454 Sequencing has highlighted the differences that exist at various taxonomic levels. FISH revealed that constipated subjects have a lower number of *bifidobacterium* spp. compared to healthy subjects with no significant difference in C.*coccoides-Eubacterium rectale* sp., *Lactobacillus* sp., *Enterococcus* sp. or B*acteroides spp.* 454 sequencing revealed that constipated subjects had a significantly lower proportion of the *Firmicutes* phylum, specifically the *veillonellaceae* family (*Clostridia* order), and a significant increase in the *bacteroidetes* phylum, specifically the *porphyromonadaceae* and *rikenellaceae* families. There were no significant changes in the microbiota of the constipated subjects over the ten week trial period on 454 sequencing or FISH analysis. This demonstrates that the microbiota was stable over the trial period and implies that Nalcol™ had no impact on this balance. It may be hypothesised that Nalcol™ was not used selectively by some organisms as a substrate or that Nalcol™ had no effect on colonic motility and thus further supports the results of the clinical trial.

3.4.2 STRENGTHS AND LIMITATIONS OF THE METHODOLOGY

The strengths and limitations of methodology are divided into those relating to study design and those related to the specific techniques.

3.4.2.1 Study Design

The patients with functional constipation all fulfilled the Rome III criteria (page 11) and had undergone investigation to confirm the diagnosis. They represented a closely homogenous disease group. Whilst there may be some features consistent with IBS-C in the group this has been minimised through the strict screening criteria set out within the clinical trial. The healthy controls had all donated samples previously at the IFR, were female, and over the age of 18 years. All healthy subjects were free of GI disease. The median age of the healthy control group is not known and if there was a significant age difference between the two groups this may confound the microbiota analysis. Although age-related changes occur in the microbiota these are more striking with advanced age (age > 60 years) and therefore the impact may be less significant if the median ages are less than 60 years[332,333]. The use of antibiotics in the healthy group and the individual diet of both groups have also not been controlled for. No patient with functional constipation who donated a sample took antibiotics either 4 weeks before the trial commenced or during the trial period but this is not certain in the healthy volunteers. With respect to the individual diets, all subjects were from the Norfolk area and likely consumed produce from the local area or from similar food outlets and whilst there may be some individual differences it is unlikely that significant differences exist between the groups which have an impact on the microbial analysis.

Recruitment of case-matched controls would remove the confounding factor of age and control for antibiotic use. Control of diet would be more difficult. As discussed in the introduction (section 1.8.2.3, page 59), diet modification is a simple measure to improve the symptoms of functional constipation which all patients are aware of. Stipulating a change in their diets as a prerequisite for stool sampling would be met with resistance and result in a reduction of volunteers and therefore not be feasible. Furthermore, controlling for these factors is only likely to have an impact on individuals and at the lower orders of the taxonomic classification (genus and species). Comparison of the groups and interpretation of the results at the phylum and class level are therefore valid.

The use of laxatives may also impact on the results although to what extent is unclear. Increases in *bifidobacteria* and *Eubacterium* / Clostridium – coccoides groups have been seen following the administration of lactulose in rats and healthy human subjects[334,335] whilst administration of PEG to rats is associated with a significant decrease in the contribution of

Firmicutes (p=0.01) and an increased contribution of *Verrucomicrobia* (p=0.01) to the faecal flora following analysis with pyrosequencing techniques[336]. The results from this work however showed a reduction in the contribution of *bifidobacterium* to the colonic microbiota and only 3 subjects were taking lactulose and therefore its impact is unlikely to be significant. The use of PEG may be of more importance. PEG was taken by a 1/3 of subjects (n=6) and this may have contributed to the reduction in the proportion of *Firmicutes* seen in the constipated subjects compared to the healthy subjects. When the patients who took PEG were removed from the phyla analysis there was still a significant reduction in the number of Firmicutes (24% vs. 45%, p=0.004) demonstrating that the reduction in numbers cannot be explained by the use of PEG alone.

3.4.2.2 Techniques used to analyse the colonic microbiota

There are inherent limitations to the techniques used and each has their advantages over the next.

3.4.2.2.1 Extraction of DNA from Stool Samples

All the techniques are limited by the ability to isolate the DNA from the stool sample. The extraction of DNA for downstream PCR was carried out with the QIAamp® DNA Stool Mini Kit (Qiagen™). This kit has been shown to as effective as the bead-beating method described by Stahl et al[337] when the product was used for PCR-DGGE analysis[338] and in a comparison of five methods for extracting bacterial DNA (including 4 commercial DNA extraction kits) it was the most effective extraction method[339]. It is less effective than commercial kits that incorporated a bead-containing lysis matrix[340] and therefore use of a hand-held homogeniser aimed to compensate for this. In preparation for FISH analysis the use of the hand-held homogeniser was also used to further enhance the breakup of the stool samples and dissolution into the PBS solution

3.4.2.2.2 Inherent Bias in PCR Amplification

PCR amplification can be a major source of bias. The quality and the amount of the DNA template used, whether is high[341] or low[342] can adversely affect the PCR product. The number of cycles involved in the PCR reaction is also important with a high number of cycles responsible for a loss in the original ratios of the initial concentrations of the template DNA[343]. Low cycle numbers with the lowest annealing temperature possible to allow a reaction that is specific and where unspecified products are not observed should also be used[344]. Optimisation of the primer concentrations, annealing temperature, number of amplification cycles and the quality of the DNA template will result in a reduction in the number of side products, chimeras, heteroduplexes, and single-stranded DNA molecules which will bias further downstream analysis[341,345,346]. Despite optimisation of the above factors there may still be selective amplification of DNA with a low-G+C content. High-G+C genes dissociate into single-stranded molecules with lower efficacy than low-G+C templates therefore low-G+C templates may be over-represented in the population[343]. This may be remedied by the addition of acetamide to the PCR mix to reduce the melting point of the template hybrids and therefore allow high-G+C genes to compete more effectively[341,347]. The methodology for the PCR reaction had been used previously in similar work carried out at the laboratory in the IFR with good outcomes. Whilst the factors could have been optimised it was not done so for two reasons. Firstly there were time constraints that limited the development of a new PCR method and secondly the results of the DGGE analysis were qualitative and not quantitative. Any error or bias introduced by the PCR reaction would either dilute or exaggerate the differences between the two groups and this would be done in an homogenous way such that the overall difference would still be apparent. This is also applicable for the downstream analysis performed with pyrosequencing such that whilst the differences will be preserved the actual components of each microbiota may be influenced.

3.4.2.2.3 Strengths and Limitations of DGGE

DGGE allows rapid analysis of multiple samples and estimations of diversity. It allows the monitoring of shifts in communities over time and the fragments excised can be cloned and sequence for identification[325,348,349]. It is able to detect a constituent that represents only 1% of the total population[325]. Using the intensity of the bands it is also able to detect the relative abundance of the constituents of the population. For these reasons it was suitable to look for a difference in the diversity between the two groups and to allow for analysis of the microbiota over the trial period.

DGGE is not without its limitations however. The technique is difficult to learn and it can be difficult to replicate results between gels, furthermore the band position does not provide reproducible taxonomic identification. DGGE is also susceptible to the biases inherent in PCR as described earlier. These may result in an over estimation of bacterial diversity. Bacterial species may display microheterogeneity, i.e. have multiple copies of the 16S rRNA gene. This will be represented on DGGE by multiple bands and biasing the true diversity of the sample[350,351]. Also it has been demonstrated that heterologous sequences may migrate the same distance i.e. bands at the same position are not necessarily phylogenetically related[352,353]. With these limitations in mind interpretation of the DGGE analysis needs to be taken with some care. Whilst the gels have shown a small but statistical difference it is possible that this is due to the biases mentioned above or due to the wide variability seen in the colonic microbiota of humans. However the samples were randomly distributed amongst the 3 gels and it would be expected that any biases would apply to all sequences evenly. In addition the results of the pyrosequencing have also shown a clear difference and it can therefore be assumed with a degree of confidence that a difference does exist in the composition of the microbiota between healthy and constipated subjects based on DGGE analysis.

3.4.2.2.4 Strengths and Limitations of FISH

FISH is a simple technique that can detect single species in an ecosystem and quantify their number within it. It can also be used to follow changes in the target organism over time. Its reliance on probes however limits its use. The human gut has a complex microbiota which is difficult to study and its diversity limits analysis at a species level using FISH due to a lack of probes. Diversity can therefore only be reliably measured at a higher taxonomic levels, and even then the diversity of the probes is still limited[354,355]. Furthermore probes are not always specific. To detect the bacteria of the CFB phylum, Manz et al[356] designed four probes which could only discriminate at a group or genus level and with one probe binding to both the *Bacteroides* and *Preveotella* genera within the *bacteroides* group. This is also seen with the *Eubacterium* and *Clostridium* genera which are intertwined and thus genus-specific probes cannot be designed. Bacteria need to be present at levels of greater than 10^6 / ml[357] and this, along with the lack of available probes, means that only 2/3rd of normal flora can be detected using FISH[358].

The methodology of FISH can also limit the reliability of the results for a given probe and these are the hybridisation of the probe and the subsequent detection of the hybridised bacteria. For the bacteria to be detected the probe needs to hybridise successfully to the 16S rRNA and this can be a problem in the cell wall of gram-positive bacteria. The concentration of formamide used in the hybridisation step and the concentration of NaCl in the wash solution in the post hybridisation step can significantly influence performance[359]. This, the authors felt, partly explained the difference in detection of bacteria in aquatic ecosystems with the EUB338 oligonucleotide probe from 1% - 100% in the 105 studies they reviewed. Whilst it has been demonstrated that variation exists between sample-to-sample measurements due to uneven fluoresence and that variation even exists between cell measurements on different fields on the same slide[360] this was not a factor in the differences described by Bouvier et al[359]. The differences in FISH protocols amongst the studies in the literature make comparison between studies difficult and the results from this work are no different. However the results of this study do allow comparison between healthy and constipated subjects and comparison of the samples from the constipated subjects over the three periods of the clinical trial. Since the same methodology was used for all the samples any bias inherent in the methodology would apply equally to all the groups and therefore any difference seen is true.

3.4.2.2.5 Technical considerations of pyrosequencing

Pyrosequencing is a high throughput analysis that allows multiple samples to be sequenced simultaneously, generating hundreds of thousands of sequence reads of up to 500 bases which can describe the microbiota of the sample down to species level in a short time frame. This allows in depth analysis of the sample that the previous methods are unable to perform. Read lengths are limited by the number of flow cycles. The 454/Roche GS FLX Titanium platform used here is limited to 200 flow cycles which produces average read lengths of 400 bases. The limitation in read lengths is due to the limitations imposed by the efficiency of the polymerases and luciferases. The simultaneous generation of large numbers of sequence reads from multiple samples can be obtained for a relatively low cost compared to other sequencing techniques and this cost continues to fall. The advantage of this high number of sequence reads is that it allows greater investigation into the sample diversity. This investigation of diversity is dependent on the depth of sequencing (number of sequences per sample) and the breadth of samples (number of samples sequenced). A greater depth of sampling will permit better coverage of the sample and allow identification of rare species whilst a greater breadth of sampling allows more samples to be examined and gives greater statistical confidence on the results obtained. However, it is unclear to what depth samples need to be sequenced to accurately determine microbial diversity. The abundance of the microbial population is comprised of a few species with the majority of the species present in low numbers[361] and as a result deep sequencing with up to 400 000 sequences is needed to determine diversity or differences in an individual sample or closely related community[362-364]. However if gross differences are required or analysis at the higher taxonomic levels then sequencing to a depth of 100 – 1000 reads will be sufficient[216,217,364,365] but this will negate the effect of the rarer species in what are collectively known as the 'rare biosphere'[366].

The concept of the 'rare biosphere' is one that warrants further discussion and the ability to sequence at depths is why pyrosequencing is the best tool for the investigation of population diversity. Sorgin et al[366] demonstrated that the microbial diversity in sea water (through pyrosequencing of the V6 hypervariable region) was dominated by a small number of different populations and that thousands of low abundance populations accounted for the majority of the diversity seen. This rare biosphere, they argued, was largely unexplored and needed

further investigation to determine their relationship with the more dominant flora. However it is unclear to what extent this 'rare biosphere' represents a previously unknown population or rare phylotypes as opposed to sequencing errors inherent in the sequencing process. Sequencing errors lead to an over estimation of the actual diversity and this was demonstrated by Kunin et al[367] who used *Escherichia coli* MG1655 alone as a reference template thereby ensuring that all sequence reads from the pyrosequencing should be allocated to the *Escherichia coli* MG1655 phylotype. The downstream analysis correctly assigned 99.97% of the sequence data but 3 operational taxonomic units (OTUs) were incorrectly assigned to members of the *Bacteroidetes, Proteobacteria,* and *Firmicutes* phyla.

Pyrosequencing is therefore potentially limited by inherent errors in the methodology which are broadly categorised into errors introduced by the PCR step and errors introduced by the pyrosequencing itself. Factors in the PCR step involve PCR polymerases, chimera formation, experimental contamination, and 'mixed beads'. The PCR polymerases typically have error rates of one substitution per $10^5 – 10^6$ bases[368] and the rate of chimera formation, where incomplete PCR products serve as primers to amplify related fragments, is thought to range from 5 – 45%[369]. However there are a number of chimera-checking software programs that form part of the downstream processing that are aimed to remove these before final analysis[369-371]. Experimental contamination of the PCR mixture was thought to be responsible for the errors introduced in the study by Kunin et al[367] and supports the work of Tanner et al[372] which demonstrated that PCR undertaken with no DNA templates still produced rDNA which was thought to be secondary to contamination of the reagents used. Following PCR, single templates are combined with the emulsion mixture before being deposited in separate wells on the picotiter plate. A fraction of these beads will, however, contain multiple copies of the PCR templates and the result will be a sequence that does not reflect a true molecule. It is again possible to filter these reads out in downstream processing.

The majority of errors, approximately 40%, introduced during pyrosequencing are due to inaccurate reads of homopolymers[373-375]. Homopolymers are lengths of sequence comprised of only one base and there can be ambiguity of the length due to difficulty in resolving the intensity of the luminescence produced. This is more likely for large polymers (7 or more bases)[327]. Errors also occur if there are excess or insufficient nucleotides for each flow and are collectively known as CAFIE (CArry Forward Incomplete Extension). Carry forward errors occur

when flushing between two flows is not sufficient and leftover nucleotides remain within the well. These can then bind to the template with luminescence, but for a different nucleotide. Incomplete extension occurs when there are insufficient nucleotides within a flow to complete the sequence causing a misread. It is estimated that the error rate following pyrosequencing approximates at 0.5% comprising insertions (0.18% – 0.27%), deletions (0.13% – 0.23%), mismatches (0.02% - 0.09%) and ambiguous base (0.01% - 0.09%)[363,373]. It is proposed that the majority of errors seen are introduced before pyrosequencing and that the basic methodology of pyrosequencing is sound[373,376]. The error rate for test fragments (have not undergone PCR) is lower than experimental reads, despite test fragments having extensive homopolymers and being difficult to read. Furthermore, the errors in experimental reads are not randomly distributed amongst the reads. 86% of reads contained no errors whilst nearly 50% of the errors detected were in sequences that differed by more than 4% from the reference sequence[373]. Huse et al[373] also showed that the test fragments had a more random distribution of errors and demonstrated the difference between experimental and test templates was due to 'mixed beads' and that these were responsible for the majority of the sequencing errors.

The detection and removal of the sequencing errors, whatever the cause, are therefore important to attain a true picture of the diversity of the sample and the development of bioinformatics tools attempts to remove these low-quality reads or 'noise'. There are three general approaches to reduce noise and its' subsequent effects.

1. Remove sequences reads that had features suggestive of sequencing errors such as ambiguous base calls or shorter or longer reads than expected. Huse et al[373] adopted this approach and were able to reduce the observed error rate to 0.0016 whilst Schloss et al[377] reduced the rate to 0.0056 which resulted in the removal of 16% of the sequences
2. Trim the regions with low quality scores. Kunin et al[367], who sequenced the 16S RNA gene of E.coli only, removed regions with an average quality score of less than 27 reducing the number of OTUs from 16 to the expected 1
3. Denoising algorithms. A number of algorithms have been introduced which aim to reduce errors associated with both PCR amplification and pyrosequencing. These include pyronoise[378], Amplicon Noise[370], QIIME[331,] and mothur[379]. These have all been compared extensively by Gaspar and Thomas[380] who concluded that whilst the effects

of noise are removed it is at the potential expense of changing some of the OTUs from 1 species to another.

Using a combination of the above methods and management of errors secondary to PCR amplification it is possible to reduce the sequencing error rate by 30-fold (0.006 to 0.0002) and the number of chimeric sequences by 10-fold (8% to 1%)[379]. Despite this however, chimeras that could not be detected were largely responsible for the identification of spurious OTUs and that the number of spurious OTUs increased with sequencing effort.

What remains unclear is how the metagenomic study is altered by attempts to remove noise. It is likely that denoising produces a substantial decrease in the diversity at both the OTU level and in terms of phylogenetic diversity[381] but prevents inflated estimates of OTU number and a bias towards rare taxa[370]. It is most likely that the role of denoising needs to be conducted on an individual level. It is unlikely to impact on the higher taxonomic levels and may not be needed but for a more detailed analysis on diversity denoising needs to be combined with more reads on a greater number of samples.

There are two factors that may have influenced the results of the pyrosequencing undertaken in this thesis. Firstly was the hypervariable used and secondly the decision to 'denoise' the sequence reads. The 16S rRNA gene consists of conserved and hypervariable (V1 – V9) regions with the length of the hypervariable regions varying between 50 and 100 bases. The human microbiome project[382] used the V3-V5 region whilst Schmalenberger[383] determined that the V4 – V5 region was the best for detecting members of the CFB group, thought to predominate in the colonic microbiota. This is contrary to the findings of Chakravorty et al[384] who demonstrated that V2, V3, and V6 were the best regions for detecting the 110 bacterial species they aimed to identify. Further work has shown that the V3 and V6 regions in combination are comparable to full sequence reads[385] despite other evidence suggesting that the V6 – V9 regions yield the lowest proportion of calls at genus level, albeit these regions were similar at higher taxonomic levels and the authors admited potentially poor coverage of the V6 region[385] . Whilst it is clear that no region has received universal acceptance a two-region approach is most effective for identification[377,386] and the hypervariable region is unlikely to be significant when analysing the results at the higher taxonomic levels but may be of importance when comparisons are made at lower levels with other studies.

The data in this thesis was denoised using QIIME and this may be a source of error. QIIME results in 3.5x as many changes as Amplicon Noise and Mothur, which altered the number of reads the least[380]. This may impact on the analysis of the reads at the lower taxonomic levels however this is felt to be unlikely. When the data was compared to data that had not been denoised there were no statistical differences at the taxonomic levels and this is consistent with the results of Reeder and Knight[381].

3.4.3 COMPOSITION OF THE COLONIC MICROBIOTA IN HEALTHY SUBJECTS

Analysis of the pyrosequencing data in this study at a phylum level has demonstrated that 85% of colonic microbiota in healthy subjects is comprised of *Bacteroidetes* (40%) and *Firmicutes* (45%). This is consistent with other studies[223,225,387-390]. However in all of these studies the proportion of *Bacteroidetes* and *Firmicutes* differs with the proportion of *Bacteroidetes* ranging from 10%[388] to 80%[389] and the converse is true for the *Firmicutes*. In this study similar ratios of *Bacteroidetes:Firmicutes* is seen ranging from 67%:28% to 8%:74% respectively. This wide variation has been demonstrated in the Eldermet project[391] where the ratio of B*acteroidetes:Firmicutes* ranged from 90% *Bacteroidetes* to 85% *Firmicutes*, highlighting the importance of a large cohort size. As with other studies proteobacteria was the other predominant phyla but actinobacteria were low in number. The Tenericutes comprised 10% of the total microbiota.

Interpretation of the data at a genus level must be done with reservation for the reasons outlined in the section above (Technical considerations of pyrosequencing, page 170). The predominant genus determined in this study was *bacteroides* (phylum *Bacteroidetes*) and this is consistent with that found in other studies[388-391]. Although the other genera detected in this study were also detected in the above studies the relative proportions are different. This difference reflects the difficulty in comparing results between studies with different populations, different ages, small numbers, and sequencing of different hypervariable regions. The clinical significance of these results is unknown.

3.4.4 CHANGES IN THE GUT MICROBIOTA IN CONSTIPATION

The PCR-DGGE analysis demonstrated that a difference exists between the microbiota of the two groups but was unable to show specifically which bacteria were responsible for this difference. The results of FISH studies demonstrated that the numbers of *bifidobacteria* were significantly reduced in constipated subjects but this could not be confirmed by the results of pyrosequencing which did not detect the *bifidobacterium* genus and noted no significant difference in the proportion of *actinobacteria* (Constipated vs. Healthy; 3% vs. 2%). This reduction in *bifidobacterium* was due to an inability to count any hybridised bacteria in five of the eight samples suggesting that the number of bacteria was less than 10^6 / ml. These results cannot be blamed on the methodology as it did not occur in all the constipated subjects and in any of the healthy controls. All were subjected to the sample methodology and reagents. Therefore it can be reported that constipated subjects do suffer with reduced level of *bifidobacteria*, consistent with other published work. It is a widely held belief that *bifidobacterium* spp. are associated with gut health[392] and have been shown to be reduced in number in constipated subjects compared to healthy controls[293], despite contrary evidence showing increased numbers in constipation in children[292]. The supplementation of *bifidobacteria* in both healthy and constipated subjects has been shown to decrease transit time and improve the symptoms of constipation[392-396]. These effects on transit were not always associated with a change in faecal mass or bile acid content suggesting a direct effect on colonic motility. The precise mechanism however is not clear. *Lactobacillus* spp. have also been shown, when used as a probiotic, to improve transit time or have a positive effect on the symptoms of constipation[397], however this study was unable to demonstrate any difference in *lactobacillus* counts following FISH or pyrosequencing and confirmed the findings of Banaszkiewicz and Szajewska [398].

Contrary to the FISH data which was unable to show any further differences in the probes selected for analysis the pyrosequencing analysis was able to quantify the changes in the microbiota in more detail. This lack of difference following FISH may be due to the low numbers of subjects (n=8) compared to pyrosequencing (n=16) and also to the limitations of FISH as discussed above (Strengths and Limitations of FISH, page 169). Compared to healthy controls there was a significant reduction in the proportion of *Firmicutes* (24% vs. 45%) with an increase in *Bacteroidetes* (66% vs. 40%) at a phylum level. The two phyla still made up 90% of

the total bacteria sequenced with no other significant differences seen. At a genus level there were significant increases in *bacteroides* and *alistipes* (Phylum *Bacteroidetes*) with reductions in *Dialister* (Phylum *Firmicutes*).

Unfortunately there are no previous studies using pyrosequencing which compare constipated subjects with healthy controls and therefore direct comparisons cannot be made. However investigation of other conditions, in particular IBS, has been carried out and comparison with these is useful. Multiple studies have been unable to demonstrate uniform changes between IBS subjects and healthy controls. Reductions in the numbers of *bifidobacteria*[287,289,290], *Bacteroidetes*[260,289] whilst increases in *Firmicutes*, specifically *veillonella* spp.[285,289] have been noted. The decrease in *bifidobacteria* confirms the changes seen in the FISH analysis however the results of the pyrosequencing do not support the changes in numbers seen in the above studies. It is likely that the patients used in the IBS studies are too heterogenous, having included patients with all forms of IBS, including IBS-D. The subjects in whom diarrhoea is induced by osmotic laxatives have a reduction in the richness of the colonic microbiota with decreases in *Firmicutes* and *Bacteriodetes* with an increase in *Proteobacteria*[399] and is not unreasonable therefore to assume that the microbiota of patients with IBS-C and IBS-D will also be different. This will limit comparison with the subjects involved in this work.

There are two areas of interest surrounding the microbiota in constipation and these are the roles of hydrogen sulphide (H_2S) and methane (CH_4). Endogenous synthesis of H_2S in the colon follows the metabolism of the amino acid L-Cysteine by the enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS)[400, 401]. Exogenous production is secondary to sulphate reducing bacteria (SRB) which have been identified in human faeces[402] and which metabolise hydrogen and short chain fatty acids (SCFA) into H_2S. The role of H_2S is unclear but it has been implicated in increasing colonic secretions and intestinal motility whilst having involvement in nociception[403-405]. It therefore seems logical that an absence or reduction of SRB may have a role to play in chronic constipation. SRB however were not detected by pyrosequencing in either the constipated or healthy groups and therefore this study cannot support this hypothesis. CH_4 is produced by colonic anaerobic microflora which ferments unabsorbed carbohydrates to produce hydrogen, carbon dioxide, CH_4, SCFA, and sulphites. CH_4 was thought to be physiologically inert but methane production in healthy subjects has been shown to inversely correlate with stool frequency and GI transit[297, 406] whilst in IBS patients methane

production during a lactulose breath test was reported to correlate with the degree of constipation[407]. In patients with slow transit compared to normal transit constipation, CH_4 production was higher following a glucose breath test and this was used a surrogate marker for methanogenic flora[294]. This was confirmed in a meta-analysis of nine studies (including Attaluri et al[294]) which concluded that the presence of CH_4 on breath testing was significantly associated with IBS-C and functional constipation[408]. What could not be demonstrated was causality but only an association between CH_4 and constipation. In this study only methanobrevibacter (Phylum Euryarchaeota) could be detected and the proportion was both small and detected in one subject in each group (0.053% each) suggesting that the result could have been artefactual. There was no significant difference between the two groups but it must be noted that the constipated subjects contained patients with both slow and normal transit constipation.

3.4.5 SUMMARY

The analysis of faecal samples in this work has shown clear differences in the microbiota of constipated subjects compared to healthy subjects and these changes differ on the method of analysis employed. FISH demonstrated that constipated subjects had a decrease in *Bifidobacterium spp* whilst pyrosequencing demonstrated that constipated subjects had a reduced abundance of *Firmicutes* and an increase in *Bacteroidetes* at a phyla level. These changes were seen down to a genus level. Due to the lack of efficacy of Nalcol™ in clinical practice it was not possible to determine if the changes seen in constipation were reversed if constipation was improved clinically.

CHAPTER 4

SUMMARY OF FINDINGS AND FURTHER WORK

4.1 SUMMARY OF CLINICAL TRIAL

The trial found no effect of Nalcol™ in clinical practice. The reasons for this may be due to underpowering of the study, incorrect dosage and short duration, or a lack of appreciation of the physiological mechanisms of colonic motor control. Nalcol™ was associated with adverse effects of abdominal pain and bloating. This work suggests that future studies should be conducted with the aim of determining the effective dose and duration, if any, of Nalcol™ in a larger sample group and to investigate the role of endogenous opiates in chronic constipation.

4.2 FURTHER WORK WITH NALCOL™

To confirm, with certainty, that the dose of Nalcol™ used was sufficient would be to undertake a dose-ranging study. There is no reason why the original patients could not be recruited. The trial would still be double-blinded and randomised to address the likely high placebo effect. The trial would be conducted without the concomitant use of laxatives and the primary outcome measure of the 'number of patients with ≥3 CSBM/wk' would be used as this would also allow comparison with other trials. All patients would undergo a transit study at the start of the screening period and also at the end of the trial which would last for 10 weeks (2 week screening period and 8 week trial period). It is suggested that doses of 10mg, 20mg, and 40mg b.d would be used along with a placebo (i.e. 4 treatment arms). As this would be a pilot study it would be reasonable to enrol 20 patients into each group to determine if a difference existed as these numbers have been sufficient in other pilot studies as detailed above. It would also be interesting to study these patients using colonic manometry and determine if naloxone had any effect on the contractile patterns over a 24h period and this would support the outcome of the dose-ranging study. Should a test dose be shown to be effective then this would then be used for further work, but to recruit the 120 patients that this trial required would need a multi-centred approach.

A second line of study would be to combine Nalcol™ with a second proven motility agent such as prucalopride or lubiprostone. Releasing the endogenous braking system in the presence of these motility agents may improve treatment rates in constipated subjects above those seen with the individual agents alone. Any increase in response rate with the combined approach

would also support the hypothesis that the endogenous opiate system is not responsible for the pathophysiolgy of functional constipation.

4.3 SUMMARY OF THE ANALYSIS OF THE COLONIC MICROBIOTA IN CONSTIPATION

The analysis of faecal samples in this work has shown clear differences in the microbiota of constipated subjects compared to healthy subjects and these changes differ on the method of analysis employed. FISH demonstrated that constipated subjects had a decrease in *bifidobacterium spp* whilst pyrosequencing demonstrated that constipated subjects had a reduced number of Firmicutes and an increase in Bacteroidetes at a phyla level. These changes were seen down to a genus level. Due to the lack of efficacy of Nalcol™ in clinical practice it was not possible to determine if the changes seen in constipation were reversed when constipation was improved clinically.

4.4 FURTHER WORK TO INVESTIGATE THE CHANGES SEEN IN THE MICROBIOTA OF CONSTIPATED SUBJECTS

The results of this study demonstrated a clear difference between healthy and subjects with constipation but was undertaken using different techniques which could not be cross validated or were not in complete agreement with the current literature. Due to limitations in the methodology of FISH it would be worthwhile to repeat the analysis of the both healthy and constipated subjects but with a larger cohort at a greater depth using pyrosequencing to fully elucidate the changes in constipation. The changes seen could be further investigated to assess the role they may have on colonic and systemic physiology. If these changes could be confirmed at a genus or species level then this could lead to further targeted trials in the treatment of constipation using pre- and probiotics.

The rationale of surveying the colonic microbiota over the course of a clinical trial in tandem with clinical improvement could have delivered interesting results and it is unfortunate that

this could not be done here. It remains unclear what effect the changes in colonic microbiota seen in constipation have both locally and systemically and what changes in the microbiota occur if symptomatic relief is achieved. The advantage of Nalcol™ was that it is receptor-based and was therefore thought not to have any impact on the colonic microbiota and although the colonic microbiota remained stable over the three study periods it cannot be said that Nalcol has no effect on the colonic microbiota and further work would be needed. There is no reason to assume that prucalopride would have any effect on the colonic microbiota as it is also receptor-based and therefore the study could be repeated with prucalopride and the colonic microbiota monitored using the same protocol used in this study. The only potential issue would be the recruitment of adequate numbers of subjects both to power the clinical trial or the microbiota analysis. This could be achieved with either the enrolment of subjects with IBS-C or to conduct a multicentred trial. Both have potential limitations however. As discussed earlier the microbiota of patients with IBS-C and that of constipation are different but note must be made of the heterogenous patient selection in the IBS trials which may have biased the results. A multicentred trial will pose numerous difficulties including stool collection and quality control of the sample until it is received at the labarotory. Monitoring the changes in the microbiota over time and comparing with clinical outcome will further identify potential microbiota involved in the pathophysiology and aetiological of clinical symptoms of constipation.

CHAPTER 5

APPENDICES

APPENDIX 1 CLINICAL TRIAL PROTOCOL

CLINICAL STUDY PROTOCOL

NALOXONE HYDROCHLORIDE SR GASTRO-RESISTANT SUSTAINED RELEASE CAPSULES AS A TREATMENT FOR FUNCTIONAL CONSTIPATION: A RANDOMISED, DOUBLE BLIND CONTROLLED TRIAL IN SECONDARY CARE

Version 7

October 2011

PROTOCOL APPROVAL PAGE

Study Drug:	Nalcol
Protocol Title:	Nalcol treatment for functional constipation: a randomised, double blind controlled trial in secondary care.
Protocol Number:	NAL COL 01
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I, the undersigned, have reviewed this protocol, including Appendices, and will conduct the clinical study as described and will adhere to the ethical and Regulatory Considerations stated. I have read and understood the contents of the Study Drug Investigator Brochure.

Investigator: _____

Investigator signature: _____

_____

Date: _____

CONTENTS

PROTOCOL

Outline Protocol

Title: Naloxone Hydrochloride SR Gastro-Resistant Sustained Release Capsules as a treatment for functional constipation: a randomised, double blind controlled trial in secondary care.

Medicinal Product: Naloxone Hydrochloride SR Gastro-Resistant Sustained Release (Nalcol) in a delayed release oral preparation at a dose of 20mg bd.

Study Centre: Norfolk and Norwich University Hospital

Clinical Phase: Phase II

Objective: The primary objective of this study is to assess the efficacy of Nalcol when used as an adjunct to usual laxatives in the treatment of patients with refractory chronic constipation.

Study design: This is a single-centre, parallel group study involving 120 patients. They will be patients who have been referred to secondary care for management of functional constipation as defined by Rome III criteria. The Rome Criteria has been developed to diagnose and classify functional gastrointestinal disorders. The criteria for functional constipation are listed in the introduction and 2 or more of the criteria must be met for a diagnosis of functional constipation to be made. The study group will comprise three consecutive periods. **Period 1** will be a two week observation period where subjects will undergo screening to assess suitability for the trial. **Period 2** will last for four weeks. A phone call will be made at 1 week and an assessment at the end of the period with participants randomised to receive either Nalcol or identical placebo capsules. **Period 3** is a post trail period of observation, lasting four weeks, in which all the patients will be invited to take part and take active Nalcol. Again there will be an assessment by phone at 1 week with a face-to-face assessment at the end of the period. **Period 4** will be open to those participants in whom the Nalcol has had an obvious benefit. It will be a prospective, open follow-up period consisting of questionnaires sent by post.

Sample Size: 120 patients (n=60 per group), which will include both male and female, but we would anticipate most will be female.

Patient Recruitment: Consenting patients, aged 18 years and over, suffering from functional constipation, refractory to standard therapy (diet and laxatives) and satisfying inclusion/exclusion criteria. These patients will have been referred by their GPs to specialist clinics and will have been thoroughly investigated with either a barium enema, transit study or perhaps both. Some patients may have undergone biofeedback training.

Treatment Schedule: Patients will be advised to continue with the measures they normally use for constipation (diet and medication) throughout the study. If patients completed their diary cards satisfactorily during Period 1 and are willing to enter the trial then they will be admitted to the trial proper. This takes places during Period 2 and the subject will be randomised to receive either active drug or placebo. All patients will be required to take two capsules (Nalcol - 10mg / Placebo) twice a day. A note will be made of the total number of capsules used during each of the weeks of period 2 and any medication taken for constipation will be recorded on the diary.

Patients will be invited to take part in Period 3 when they will all be given the active trial capsules i.e. two 10mg capsules twice a day. The format for this period is as for Period 2.

Assessments and Analysis: Patients will be recruited from Gastroenterology and surgical clinics specialising in chronic constipation and standard assessments done as part of clinic investigations will be recorded. They will be counselled and entered into the study by MB, who will see them before entry into the trial, at the start of Periods 2 and 3, and at the end of the trial. The primary outcome measures will be the patient's assessment of 'satisfactory improvement' and a responder to the treatment will be defined by the experience of satisfactory improvement on at least 50% of occasions. Diary cards will be used each day and details recorded of the patient's bowel frequency and medication. Disease specific symptom severity and quality of life will be assessed at the end of each trail Period using PAC-SYM and PAC-QOL questionnaires. Analysis of this data will comprise the secondary outcomes. Faecal bacterial analysis will be carried out on a subset of patients who volunteer to have faecal sampling via rigid sigmoidoscopy after each of the trial periods i.e. Periods 1, 2 and 3. Those patients, who had transit studies as part of their initial work-up, will have these repeated at the end of Period 2.

1. Introduction and Rationale

Constipation: Incidence and Prevalence

Constipation is present in between 10 and 15 percent of the UK population, and as many as 1% of women have chronic functional constipation, which often develops when they are young, and is unresponsive to dietary manipulation or laxatives.

Thirteen million general practitioner prescriptions were written for laxatives in England in 2004[1]. Prevalence data are limited by small samples, problems with definition and the under reporting of constipation to health professionals.

Functional Constipation

First characterised by Preston et al[2], patients are usually female with symptoms since childhood or early adulthood. Symptoms are often severe and intractable, failing to respond to a high fibre diet, high fluid intake, or laxatives. The quality of life is often severely affected by symptoms, which together with feelings of anxiety and embarrassment cause many to be incapacitated to the extent that they are unable to work or carry out social and domestic activities. Whilst much is written about the cause, it is poorly understood and no clearly defined pathogenic mechanism has been identified.

Though there is no universal definition of constipation, there are widely accepted criteria defined by international consensus (Rome III)[3] as shown below.

Rome III Criteria for Functional Constipation

1. Must include two or more of the following:
    a. Straining (during at least 25% of defecations)
    b. Lumpy or hard stools (on at least 25% of defecations)
    c. Sensation of incomplete defecation (on at least 25% of defecations)
    d. Sensation of anorectal obstruction/blockage (on at least 25% of defecations)
    e. Manual manoeuvres to facilitate defecation (on at least 25% of defecations)
    f. Fewer than three defecations a week
2. Loose stools rarely present unless induced by laxatives
3. Would not normally include patients who satisfy criteria for IBS-C

Therapeutic concepts based on 5-HT and opioids in the gut

The enteric nervous system, through several mediators, plays a major role in the regulation of normal gut motility. One of these – serotonin (5-hydroxytryptamine, 5-HT) has receptor sites throughout the gut. Recent developments in the pharmaceutical industry have explored the role of agonists of 5-HT in patients with constipation and 5-HT_4 receptor partial agonists have been shown to be of some value in patients with constipation and IBS.

Opioids are known to have a marked effect on both gut motility and secretion – producing a delay in intestinal transit and constipation. The receptor sites for opioids in the enteric nervous system are closely interrelated with those for 5-HT, and the functional consequences of this relationship determine that similar changes in bowel motility can be achieved by opioid antagonists and 5-HT agonists. This role of opioid receptors in the enteric nervous system underpins the basis for use of naloxone, an opioid antagonist, in patients with constipation.

NALOXONE: Pharmacological effects

Naloxone is a specific opioid antagonist that is used intravenously to reverse the side effect of respiratory depression following treatment with opioids. In the gut, opioids reduce intestinal secretion and motility – both of these effects are reversed by the antagonist naloxone[4,5]. Even in large doses, the naloxone does not produce troublesome adverse effects and can therefore be given safely. In a pilot study of normal volunteers, 40mg daily was occasionally associated with abdominal discomfort and urgency of defecation[5].

NALOXONE: Adverse effects and safety issues

Naloxone has been used as an intra-venous preparation to treat opioid overdose for many decades, with an excellent safety record. Cases of anaphylaxis are rare, severe irreversible side effects have not been reported, and there are no reports of permanent disability as a result of the drug. It is thought to be safe to the foetus. Furthermore, the formulation of Nalcol used in this study produces colonic release of the drug with greatly reduced absorption and a high first pass effect such that only approximately 2% of the ingested drug is systemically available.

Possible adverse effects are allergic type reactions, nausea, vomiting and colicky abdominal pain. In the two previous studies of Nalcol there have been no serious adverse events, and no effect on pulse or blood pressure recordings[5,6].

Rationale for topical (colonic) delivery of naloxone

The role of opioids and opioid antagonists in the gut is thought to be a consequence of their local effect – mediated by systemic levels of the drug. It is thought that this local effect can also be produced topically by release of the drug in that part of the gut where the effect is desired. Release of naloxone in the terminal ileum and first part of the colon, slowly over 6 hours, will influence colonic function. The first pass metabolism of naloxone in the liver removes 97% of the drug and as a result only very low levels reach the systemic circulation. The slow release of naloxone in the colon, in contrast with a bolus release, will limit rises in the systemic level of the drug.

Oral formulation of naloxone – Nalcol

Naloxone has been formulated in a gelucire, which is not unlike a wax matrix, and produces a linear release of the drug over 6 hours. The gelucire is encapsulated and the capsule itself also coated with a polyacrylic resin (Eudragit 4/S) to delay release of its contents until the terminal ileum. Each capsule contains 10mg of naloxone hydrochloride sustained release and two capsules are given twice daily (total of 4 capsules daily).

Rationale for stool sampling

There is extensive investigation into the cause of constipation. It has been shown that as humans age the bacterial content of the colon decreases, especially in the number of bifidobacteria and this predisposes the elderly to constipation and other gastrointestinal disease[7]. Normalisation of these bacterial counts can restore bowel habit to normality in elderly populations[8,9] and some laxatives e.g. lactulose can be thought of as prebiotic[10]. This does not explain whether the decrease in the bacterial counts is the primary event for the

development of increased transit time and subsequent constipation. It could be hypothesised that the development of constipation and slowing of transit time for other reasons promotes an increase in toxic metabolites which are not removed satisfactorily. These toxic metabolites may then alter the environment locally such that the bacterial counts drop. Although it has been shown that the use of laxatives can alter bacterial flora[10] these laxatives act within the bowel lumen and it is not possible to say whether their beneficial effects in constipation are due to their effects on the bowel wall and increase in transit time or on the bacterial content of the lumen by restoring normal colonic flora.

However Nalcol should have no direct effect on bacterial flora as its' proposed mechanism of action is on the opioid receptor. Therefore any change in the bacterial population would be as a direct effect of a decrease in transit time. If there is no change in the bacterial population despite a decrease in transit time it may be reasonable to assume that an alteration away from the normal bacterial population is contributory to the aetiology of constipation.

We would aim to take faecal samples from a subset of patients at the start of Period 1 and at the ends of Periods 2 and 3 performing bacterial analysis on these samples. This will determine if there is any difference in the use of Nalcol over 8 weeks compared with 4 weeks as some of these patients will be randomised to the placebo group initially. The faecal samples would be obtained via rigid sigmoidoscopy, a procedure with little in the way of complications[11]. The benefit of sampling the faeces in this way allows us to guarantee the conditions in which the faecal sample will be stored after collection. This is important as incorrect storage may result in changes to the bacterial content and thus invalidate the data collected. The sampling would be voluntary and is not required from entry into the trial proper.

2. Overview of Study Design

This is a single-centre double-blind placebo controlled study of the efficacy and safety of Nalcol given to patients with refractory constipation attending a specialist clinic. The study is principally a phase II trial of short-term (4 weeks) effect. It is suspected that Nalcol will have a moderate effect, and is used here as an adjuvant to regular laxatives

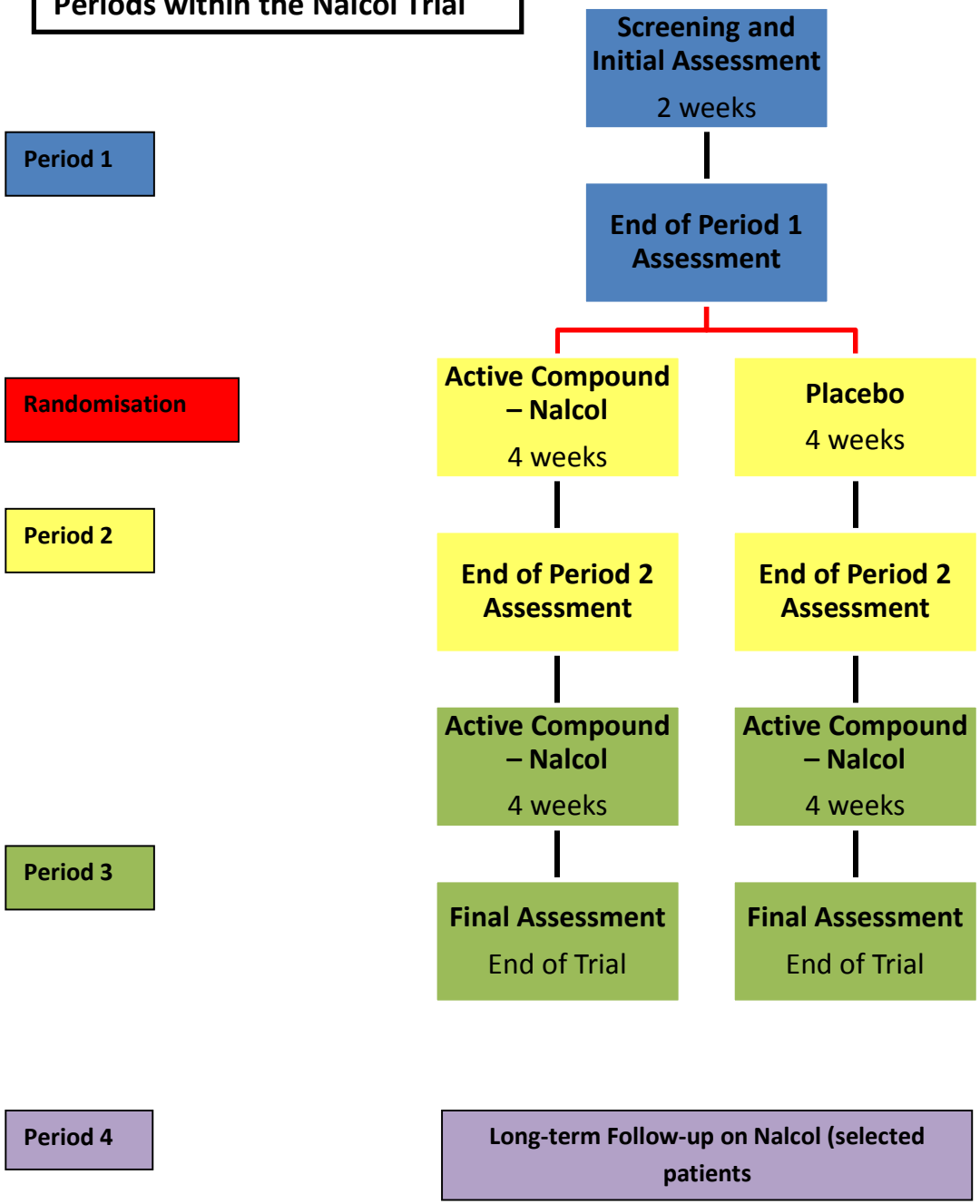
The patients will all fulfill criteria for functional constipation (as above) and will have attended a specialist gastroenterology or colorectal clinic at the Norfolk and Norwich University Hospital. They will have undergone investigation for their constipation with barium enemas and also transit studies and their basic management, including dietary and lifestyle advice and modification of laxatives, will have been instigated.

The key treatment phase (Period 2) will last 4 weeks and data over four weeks of treatment will be available. Two weeks of pre-treatment data will be collected during the screening period before randomisation (Period 1). This will allow an assessment of patient suitability and health to ensure that the inclusion criteria are met and permit examination with a rigid sigmoidoscope to obtain a stool sample. This initial period will ensure short term symptom stability and confirm patient compliance with diary completion. It will allow secondary analyses comparing pre-treatment and treated symptom scores and QoL data. A final phase of four weeks (Period 3) will allow all patients in the trial to evaluate the treatment and provide further observation over a more prolonged duration. Period 4 will be open to those patients in whom the Nalcol has had an obvious benefit. Nalcol will be continued and the patients asked to complete two questionnaires every month so that long-term efficacy can be assessed. This period will continue until the trial has been completed. The length of time that each participant spends in this period will therefore vary. Patients will need to attend the hospital to collect their Nalcol each month when the completed questionnaire can be returned. Patients will be contacted on a monthly basis to check on their progress and monitor for adverse events and will be asked to attend the hospital for an outpatient appointment every three months to undergo three monthly BP measurement, blood testing and urinanalysis.

Nalcol is given to relieve symptoms, which may be variable from day-to-day. The tolerability of Nalcol tablets is expected to be comparable to that of commonly dispensed stimulant laxatives;

with no significant systemic side effects. From previous studies[5,6] it is known that 10mg, twice a day is effective in increasing gut motility in normal subjects and a group of patients have taken 40mg daily and occasionally up to 60mg daily for severe symptoms. 40mg daily has therefore been chosen to be given to patients in this trial because of the very troublesome symptoms of constipation from which they suffer.

Diagram to Summarise the 3 Periods within the Nalcol Trial



- Inclusion and exclusion criteria assessed
- Medical history assessed
- Examination including rectal examination and rigid sigmoidoscopy
- **Consent into trial**
- Blood tests for routine analysis

- Completion of diary cards and quality of life questionnaires
- If initial assessment and diary cards are satisfactory and patient is willing to proceed then they will be entered into the trial

- 2 capsules, twice a day
- Phone call after 1 week to check on participant
- End of Period assessment
  - Hand over completed diary cards
  - QOL questionnaires completed
  - Large Bowel X-ray transit study
- Participant invited into Period 3

- 2 Nalcol capsules, twice a day
- Phone call after 1 week to check on participant
- End of Period assessment
  - Hand over completed diary cards
  - QOL questionnaires completed
- Repeat blood and urine tests for routine analysis
- End Of Trial
- Post study check phone call in 4 weeks

- PAC-SYM and PAC-QOL monthly by post
- Blood test, BP and urinanalysis 3 monthly at hospital
- Monthly phone call to check on patients progress and monitor for adverse events

3. Patient Recruitment

Study Population

Adults who have been referred to the gastroenterology or colorectal clinics specialising in chronic constipation at Norfolk and Norwich University Hospital and remain unsatisfied with their symptoms despite initial treatment with diet and laxatives will be considered for inclusion into the study. The definition of constipation will be based on the criteria for functional constipation (Rome III) as stated in section 1.2 above.

Inclusion Criteria (all must be met)

- Age > 18 years
- Male or Female
- Satisfy Rome III criteria for functional (slow transit) constipation
- Symptoms not relieved by diet and laxatives

Exclusion Criteria

- Severe cardiac, renal or hepatic impairment
- Severe psychiatric disturbance
- Mental disorder preventing adequate informed consent
- Dilatation of the bowel (megarectum or pseudo-obstruction)
- Concomitant medication with drugs known to cause constipation
- Known pregnancy, suspected pregnancy, or trying to conceive
- Currently Breast Feeding
- Currently participating (or within 1 month) in any other study

Patient Numbers

The primary endpoint will identify responders and non-responders. Recent clinical trials in this condition have shown placebo response rates ranging from 15-35%[12-15] and as such we have chosen a placebo response rate of 25%. A worthwhile treatment response would be 32% greater than the placebo given the resistant nature of constipation to medical management. A study with a power of 95% that will detect this difference would require 120 patients randomized to two equal groups (see Section 7 for further details).

4. Endpoints

Endpoints to assess the value of Nalcol as additional medication will be:

Primary Endpoint

Patient derived assessment of satisfactory improvement of symptoms measured at weekly intervals. A responder will have 'satisfactory' improvement on at least two of the four weeks in the treatment phase[16]

Secondary Endpoints

- Stool Frequency and type from daily diaries
- Laxative use from daily diaries
- Symptom scores from a weekly patient-completed questionnaire (PAC-SYM)
- Disease specific quality of life measured weekly (PAC-QOL)
- Objective improvements in transit time on repeat X-ray transit studies
- Normalisation in faecal bacterial counts after treatment with Nalcol (in subset of patients)

5. Study Procedures

Recruitment

Patients with functional constipation will be recruited from three possible sources. Firstly there will be referral from consultants within gastroenterology and colorectal surgery who see these patients as part of their normal working practice. It is envisaged that the majority will be referred from clinics which specialise in functional gastrointestinal disorders. Secondly we will recruit patients from the department of physiology at the Norfolk and Norwich Hospital. They have a record of the patients who have undergone biofeedback training for constipation. Thirdly patients will be recruited from the department of radiology at the Norfolk and Norwich Hospital. A search dating back five years will identify patients who have undergone transit studies for functional constipation. It is expected that there will be some overlap between the three groups.

The recruited patients will then be sent a letter detailing the study and inviting them to take part with a follow-up phone call one week after the letter is sent to answer any questions. It is hoped that a follow up phone call will increase recruitment. Arrangements will then be made to screen those who are keen to participate with a view to entering into the trial.

Screening

Screening and a decision about whether patients are suitable for the study will be undertaken

- At the initial interview on the inclusion/exclusion criteria, with medical and social background considerations. At this point consent will be taken.
- At the end of Period 1 after reviewing initial blood and urine tests and on successful completion of the diary card and questionnaires

If for any reason the patient is considered unsuitable at the end of Period 1 then they will not be randomised to Period 2 of the trial; they will not be included in the analysis.

Screening procedures will include:

- Explanation of all the procedures involved in the study
- Details of all current medical therapy including those used to manage constipation
- Current and past details of medical history – if necessary, referring to the patient's medical notes
- Provision of a blood sample for routine clinical biochemistry and heamatology tests
- Urine for routine tests
- General, abdominal and digital rectal examination
- Blood Pressure measurement and ECG.

- Sigmoidoscopy and stool sample if consent is obtained (to be performed ONLY if accepted into the trial). The stool samples will be stored on ice and at the end of the screening clinic transferred by MB to the IFR for preparation prior to freezing. All samples will be identified by the participant's identification number.

Consent and Randomisation

Patients seen at the start of Period 1 will have the trail discussed and if willing consented into the trial. They will undergo routine blood tests and complete a diary card.

On satisfactory completion of the diary cards and normal routine blood tests the participant will be entered into the trial and randomized (1:1) to receive one of the two treatment regimes, using a computer –generated randomization sequence. St Mary's Pharmacy Unit in Cardiff will be responsible for the packaging and randomization of the trial capsules. The capsules will be stored and dispensed by the pharmacy at the NNUH and this will ensure the capsules are appropriately stored and prepared prior to dispensing and ensure both the participant and the researchers remain blind to the treatment.

Treatment Schedule

Period 1:	Week 1-2:	No treatment – Screening and assessment period
Period 2:	Weeks 3-6:	Randomised to active treatment or placebo
		Phone call at end of week 3 to check progress
Period 3:	Weeks 7-10:	All patients to receive active drug
		Phone call at end of week 7 to check progress
Period 4:	Week 11 onwards:	Ongoing Nalcol treatment
		Questionnaires x2 each month
		Blood tests. BP and urinanalysis every 3 months.

Usual laxatives and other lifestyle measures are taken throughout. No new laxatives to be started during the study period. Should patients develop diarrhoea then they will be instructed to call MB who will advise about any changes needed. It is thought best to reduce their 'usual' laxative dosage and rely more on the trial capsule if they appear to be working

Schedule of Assessments

Phases	**Pre-Treatment**		**Treatment Period**				**Post-Treatment (Nalcol)**			
Assessments	**Wk 1**	**Wk 2**	**Wk 3**	**Wk 4**	**Wk 5**	**Wk 6**	**Wk 7**	**Wk 8**	**Wk 9**	**Wk 10**
Global Assessment										
Diary										
PAC-SYM										
PAC-QOL										

Assessments, Patient Diary Cards and Questionnaires

Throughout the study patients will be asked to complete a diary, recording details related to their bowel frequency, laxative use and any adverse events.

Period 1

At the end of Period 1 patients will be asked to complete two standard questionnaires –

- One documenting symptoms related to constipation (PAC-SYM)
- And one quality of life questionnaire (PAC-QOL)

A global assessment question will be included on the diary card. Their completed diary card for the Period 1 will be checked prior to randomisation into the trial proper at the end of Period 1

They will also be asked to undergo rigid sigmoidoscopy for stool

Period 2

At the end of Period 2 the following will be completed:

- Their completed weekly diary cards for the Period 2 will be checked including the global assessment question and adverse events noted
- Complete Questionnaires PAC-SYM and PAC-QOL
- Check compliance against capsule returned
- Repeat blood pressure recordings
- Repeat stool sample in subset of patients
- Large Bowel X-ray transit study in subset of patients

Patients will be phoned after the first week. This will ensure that they have understood what is expected and answer any queries that they may have.

Period 3

At the end of the study the following procedures will be completed:

- Review of final diary cards, global assessment question and check for adverse events
- Complete Questionnaires PAC-SYM and PAC-QOL
- Check compliance against capsule returned
- Repeat stool sample in subset of patients
- Repeat blood pressure recordings
- Repeat blood and urine samples for follow-up heaematology and biochemistry testing

Patients will be called at the end of the first week as per Period 2.

Those patients completing the study who would like to continue taking the Nalcol in the longer term will be given further supplies and arrangements made through the family practitioner.

Participants will be followed-up by phone four weeks after finishing the trial.

Period 4

Patients will be asked to complete a PAC-SYM and PAC-QOL questionnaire every month and undergo blood tests, BP testing and urinanalysis at hospital every 3 months. The patients will be contacted on a monthly basis to ensure that they are tolerating the Nalcol and for any adverse events to be reported and will be reviewed in clinic every third month. The participants will have the contact details of the MB should they need advice or wish to be seen at the hospital sooner.

Premature Withdrawal

On premature withdrawal from the study patients will be asked to complete:

- PAC-SYM and PAC-QOL questionnaires
- Diary cards for review to check compliance and adverse effects
- Routine blood and urine for analysis

They will be followed-up by phone four weeks after withdrawal.

Analysis of Faecal Samples

We aim to study faecal samples from a minimum of 30 participants. A minimum of 200mg of fresh stool will be needed but it is anticipated that a greater quantity can be easily collected. The stool will initially be stored in polythene bags placed over ice until the end of the research clinic and then transferred by M.B. to the IFR (i.e. within 4 hours of collection). Here they will be divided into 4 batches and frozen. The first sample will be used to determine fresh and dry weights, the second to run denaturing gradient gel electrophoresis (DGGE) which will allow the bacterial population i.e. the bacterial geni to be determined. The third sample will be prepared prior to freezing to allow Fluorescent in situ hybridization (FISH) to take place. This allows the bacteria to be counted. The metabolic activity of the bacterial population will also be assessed through the analysis of short-chain fatty acids on the fourth sample. These analyses will be performed on each of the stool sample taken at the differing stages of the trial and the result compared and correlated with the clinical findings.

Provision of a stool sample is **NOT** an absolute requirement for entry into the trial.

6. Analysis

The primary analysis will be a comparison of the responders during Period 2 of the study (weeks 3-6) in the treatment group and those in the placebo group. Response is defined as those participants who give an assessment of 'satisfactory improvement' on at least 50% of occasions over the 4 week trial period (Period 2) to the global question on the diary card.

A number of secondary analyses will be performed to further assess the response to the Nalcol treatment:

- Comparison of stool frequency and type, laxative use, PAC-SYM, and PAC-QOL between treatment and placebo groups during Period 2 of the study. PAC-SYM will be used to provide an overall symptom score; but each component will also be analysed separately to identify changes to individual symptoms. Comparison will be made between the pre and post trial transit study as an objective marker of a reduction in transit time.
- Graphical representation of the temporal changes in stool frequency and type and analysis to assess any drop-off of effect after eight weeks of use in those receiving Nalcol for 8 weeks or in the whole group.
- A comparison of pre-treatment versus treatment symptom levels in the whole (n=120) i.e. Period 1 vs. Period 3
- Comparison of stool bacterial counts between the treatment and placebo groups in the selected subset after each Period.

Statistical Analysis and Power of the Study

The trial is powered in relation to the primary outcome measure of "satisfactory improvement". Previous papers[12-15] indicate that the proportion of participants who can be expected to show a positive response after placebo is of the order of 25%. An increase in response of 32%, to 57% of the participants, is considered to be clinically significant for the treatment group. For a power of 95%, this increase in response could be detected at a significance level of 0.05 if the total number in the study was 120 (60 in each group). This estimate was based on using a Binomial Test and was confirmed using Fishers Exact Test.

With regard to symptom outcomes, ANOVA models will be constructed to observe any changes in symptoms and quality of life at the end of study periods 1, 2, and 3, with the treatment group entered into the model as a factor. Where appropriate, data from variables collected prior to Period 2 will be included as covariate values where ignoring these may be to the detriment of the model.

Diary data will be initially presented via descriptive statistics, with any subsequent statistical analysis being in accordance with the nature of the data.

Finally, pre-treatment symptom levels will be compared to symptoms levels at the end of the study using appropriate statistical tests, i.e. either ANOVA based (t-test) or non-parametric according to the nature of the data. Prior to the analysis of the continuous data, distributions (of differences) will be examined within groups to assess normality. Where data fails to meet the assumptions of normality, non-parametric equivalents will be applied. However the ANOVA model described above is known to be fairly robust to non-extreme departures from normality.

7. Patient Withdrawal, Monitoring, and Safety.

Participant withdrawal criteria

No specific withdrawal criteria have been defined for this study. If a participant discontinues from the study prematurely (i.e. prior to completion of the protocol), the primary reason will be determined and recorded. In all cases the investigator will ensure that the participant receives medical follow-up as necessary. Withdrawn participants will not be replaced.

If a participant discontinues from the study prematurely, every effort will be made to perform an early termination visit. This will include retention of any diary information, completion of PAC-SYM and PAC-QOL questionnaires and repeat haematology and biochemical tests.

Monitoring

The study will be monitored by Messrs Mark Bignell and Michael Rhodes in accordance with the guidance in section 5.18 of the ICH Harmonised Guidance for Good Clinical Practice.

Safety evaluation

The safety of Nalcol in the treatment of patients with constipation will be evaluated by examining the occurrence of all adverse events, abnormal laboratory findings, the use of concomitant medications and physical examination findings. Follow-up of each adverse event should continue until the event or its sequelae resolve or stabilize at a level that is acceptable to the investigator.

An ‘adverse event’ (AE) is any untoward medical occurrence in a trial participant, which does not necessarily have to have a causal relationship with the treatment. In order to elicit details of any AEs, at each visit the participant will be asked a non-leading question: ‘Do you feel different in any way since the last visit?’

An 'adverse reaction' (AR) is an untoward or unintended response to an investigational medicinal product related to any dose administered.

These may be:

Intensity classification:

- Mild: Symptoms do not alter participant's normal functioning
- Moderate: Symptoms produce some degree of impairment to function, but are not hazardous, uncomfortable of embarrassing to the participant.
- Severe: Symptoms definitely hazardous to the well being, significant impairment of function or incapacitation

Causality classification

- Probable: Reports including good reasons and sufficient information to assume a causal relationship in the sense that it is plausible, conceivable, or likely.

- Possible: Reports containing sufficient information to indicate the possibility of a causal relationship in the sense of it not being Impossible and not unlikely, although the connection may be uncertain or doubtful (e.g. due to missing data, insufficient evidence, etc)

- Unlikely: Reports of a clinical event, including laboratory test abnormality, with a temporal relationship to drug administration which make a causal relationship improbable, and in which other drugs, chemicals or underlying disease provide plausible explanations

- Not related: Reports excluding the possibility of a relationship between the event and the drug treatment, i.e. no reasonable suspected causal relationship to study drug administration

- Unclassified: Reports of a clinical event, including laboratory abnormality, reported as an AE, about which more data are essential for a proper assessment

Serious adverse events (SAE)

All SAEs regardless of treatment group or suspected relationship to the study drug will be reported immediately (within 24h) by telephone to the research coordinator Mr. Mark Bignell. SAEs will also be reported immediately to SLA Pharma.

A SAE is any adverse drug experience occurring at any dose that:

- Results in death
- Is life-threatening
- Results in inpatient hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability or incapacity
- Results in congenital abnormality or birth defect

Important medical events may not result in one of the above may still be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include pregnancy, allergic bronchospasm requiring intensive treatment in the Emergency Department or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalisation, or the development of drug dependency or abuse.

Regardless of the above (serious) criteria, any additional adverse experiences that an investigator considers serious will be immediately (within 24h of the investigator becoming aware) reported. SAEs will be included in the SLA Pharma SAEs database system.

The minimum information required from the investigator when reporting a SAE is as follows:

- Protocol identification number
- Investigator's identification (name and centre number)
- Subject identification number
- SAE description including criteria for seriousness and the immediate outcome

The chief investigator will also report all SAEs to the MHRA, REC, Eudravigilance component of the EudraCT database within the required reporting timelines. A written acknowledgment is required from the ethics committee to confirm that they have received this notification.

SUSAR

In accordance with the EU directive the Mr. Mark Bignell will report SUSARs (Suspected Unexpected Serious Adverse Reactions) to SLA Pharma, Mr. Michael Rhodes, his/her local Trust R&D department and the sponsor within 24 hours of becoming aware of the event. The chief investigator and sponsor will report SUSARs to the MHRA, REC, Eudravigilance component of the EudraCT database within 7 days if the event was fatal of life threatening or 15 days if the event was not fatal of life threatening.

8. Ethical Considerations

Informed consent

Subjects will be required to give written informed consent witnessed by a third party. The investigator will explain verbally and in writing, the exact nature of the study and the known side effects which they might expect. Information regarding the research study will be provided to potential participants during discussion in clinic and by a patient information leaflet. They will not be expected to make decisions about involvement at this stage, but will be encouraged to take the information home and discuss it with their family. Patients will be allowed to deliberate for at least 24 hours after the initial discussions before the consent process is completed. Patients will be given as much time as they need to decide whether they wish to participate in the research study or not. Agreement to participate will be documented using a consent form (copies will be available for the investigators, the participant and the medical records).

Patients will be advised that they are free to withdraw from the study at their own request. It will be explained that the study has been designed following the edicts of the International Conference of Harmonisation – Good Clinical Practice (ICH-GCP) and that they are protected by the 2000 Declaration of Helsinki to ensure their rights, safety and well being. Arrangements will be made to ensure adequate consent for participants who may have difficulty on understanding English or who have impairments (e.g. visual or hearing) that could influence the consent process. Independent witnesses will be available to confirm consent in those unable to do so in writing.

Confidentiality

Identification within the study will be by a pseudonymous coded number effectively ensuring anonymity. However, using this number the principle investigators will be able to identify subjects rapidly to react to research related information that may influence a patient's management or involvement in the study. A subject's inclusion in the study will be made clear in their medical notes. Other medical professionals involved in non-research related care of the

subjects will be able to use the information recorded in the notes about study participation and contact the principle investigators if needed.

The main objective of the blood tests is to help identify any adverse effects on the bone marrow, liver function or kidney function, so it is vital they are linked to the donor. The DNA samples will be anonymous, but the code will be available to the principle researcher so that future analysis can be linked to trial outcomes. Once the study is completed, it would be acceptable to break the link between data and subject identity so that future genetic analysis could not be linked to the individuals concerned. This would be subject of a further LREC application and at this stage approval only is sought for storage of samples.

Ethics committee approval

This protocol will be submitted to the Local Research Ethics Committee in Norwich. The study will not begin until ethics approval has been obtained. Any changes or revisions to the study protocol will be submitted to the ethical committee as appropriate.

Information to the patient's general practitioner

General practitioners will be informed of their patient's decision to participate in the study. This letter will provide information on the test drug, the nature of the study and possible side effects. The GP will be invited to contact the investigator at the hospital if they have any enquiries or objections to the subject taking part.

Indemnity

All patients will be recruited from NHS sites and the NHS indemnity scheme or professional indemnity will apply. The drug naloxone is already very extensively used but this trial constitutes a new application with a new formulation of the drug. Once the project Review Board approves the study, indemnity will be provided by the trust as per the Trust's Clinical Negligence Scheme for non-negligent harm. This ensures that indemnity issues are adequately

covered. Participants will be made aware of the arrangements for potential compensation before agreeing to take part. This information is included in the consent form.

APPENDICES TO TRIAL PROTOCOL

Appendix 1 – Summary of events in the trial/study

Recruitment

1. Patient referred to Constipation Clinic at Norfolk and Norwich University Hospital: history, examination and full investigation including barium enema and transit study
2. Standard treatments (laxatives and life style modification, biofeedback)
3. Patients not responding to standard treatment offered entry into Nalcol trial
4. Patients given information sheet
5. Discussion with investigators and entry into trial at patient request

Pre-treatment Phase – Period 1 (weeks 1-2)

1. Inclusion and exclusion criteria confirmed and medical history assessed.
2. Patient consented into the trial
3. Digital rectal examination, rigid sigmoidoscopy with rectal biopsy and stool sample
4. ECG and blood pressure recordings
5. Blood and urine samples taken for routine analysis
6. Blood stored for DNA sampling
7. Patient completes diary cards and questionnaire at end of second week

Treatment/Placebo Phase – Period 2 (weeks 3-6)

1. If above tests and diary card are acceptable on review then proceed with entry into study.
2. Patient randomised to treatment or placebo group
3. Patients given capsules – 2 capsules, twice a day (total of 40mg).
4. Patient phoned at end of week 3
5. Patients complete diary cards for each week
6. At end of Period 2 (week 6) patients complete 2 questionnaires
7. End of Period 2
    - Repeat Stool Sample as applicable
    - Repeat blood pressure recordings
    - Diary cards reviewed and AEs noted
    - Compliance checked against number of capsule returned and total number of capsules used
    - Repeat Transit study as applicable
    - Patient invited into Period 3

Post-Treatment Phase – Period 3 (weeks 7-10)

1. All patients are now invited to Receive Nalcol treatment
2. Patients given 20mg nalcol (2 capsules) twice a day.
3. Patients proceed as for Period 2 completing their diary cards weekly and questionnaires at the end of the Period (week 10)
4. End of Study
    - Repeat Stool Sample
    - Repeat blood pressure recordings
    - Diary cards reviewed and compliance checked
    - Repeat Haematological and biochemical testing
    - Patients are invited to continue taking Nalcol
    - Post study check by phone in 4 weeks time

Nalcol Continuation - Follow-up Period – Period 4

1. Patients in whom Nalcol is obviously beneficial are invited to continue taking Nalcol whilst followed up for long term efficacy and adverse events
2. PAC-SYM and PAC-QOL carried out monthly
3. Monthly telephone call to monitor progress
4. 3 monthly blood tests, BP and urinanalysis at hospital

Appendix 2 – Initial screening of patients for the study

Inclusion/Exclusion Criteria **Subject Number ……..**

Date _ _ / _ _ / _ _ _ _ **Initials……**

This document is to be used on the first contact with patients to decide whether or not to proceed. If patients fulfill the criteria for inclusion then they can be given the appropriate information literature.

This document is to be filed in the patient's Clinical Record Form.

Inclusion Criteria

Subject must fulfill the 'Rome III' criteria which define constipation.

When you are not taking laxatives, at least two of the following six criteria must be fulfilled (tick Box)

1) Passage of less than 3 bowel movements per week ☐

2) Passage of small hard stools on more than 25% of occasions ☐

3) Straining at stool on more than 25% of occasions ☐

4) Feeling of incomplete rectal evacuation for more than 25% of the time ☐

5) Sensation of anorectal blockage/obstruction on at least 25% of defecations ☐

6) Manual manouvres on more than 25% of occasions to help defecation (digital evacuation, support of pelvic floor) ☐

If at least 2 of the above are fulfilled the following must all be answered in the affirmative to proceed

1) Age 18yrs or more ☐

2) Troubled with constipation for at least 6 months ☐

3) No medical reason to explain constipation ☐

4) Failure of standard treatments (laxatives, lifestyle alteration, biofeedback) ☐

5) Normal barium enema and abnormal transit studies ☐

6) Ability and willingness to co-operate with those conducting the study, including satisfactory completion of diary cards and availability for interviews. ☐

Exclusion Criteria

1) Subjects with sensory impairment or any other reason which may lead to poor compliance in the study (poor vision for example may make it difficult for them to recognize the appearance of their motions)

2) Unable to complete the diary satisfactorily

3) Severe psychiatric disease

4) Very poor general health caused or complicated by cardiac, respiratory, hepatic or renal failure

5) Where applicable, women pregnant or breast feeding, and fertile, sexual active women who are not practicing effective contraception

Appendix 3 Patient Information Sheet

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Department of General Surgery
Colney Lane, Norwich NR4 7UY

Patient Information Sheet

Why have I been chosen?

You have been invited to take part because you have severe constipation that has not responded well to standard treatments.

What is the purpose of the study?

The main purpose of the study is to look at the effectiveness of Nalcol capsules in the treatment of constipation which does not respond to standard treatments. Nalcol will be used in addition to your normal laxatives.

What is Nalcol and how does it work?

The active drug in Nalcol is called naloxone. Naloxone is not a new drug. It has been used routinely across the world for decades as it is the antidote to morphine overdose. Morphine and morphine-like drugs such as codeine and Tramadol are painkillers known as opioids. Opioids act by binding to opioid receptors found throughout the body, including the gut. The body produces its' own natural opioids, called endorphins. Endorphins also act on opioid receptors. When endorphins and other opioids bind to gut opioid receptors they increase the transit time of the gut. The transit time of the gut is the time taken for stool to reach the end of the colon (the rectum) from the start (the caecum). An increase in this time leads to constipation. Naloxone prevents this action. This will reduce the transit time and improve the symptoms of constipation.

What about safety and possible side effects?

Unlike many drug trials, this study is not assessing a new drug, but a new way of using a drug that has been widely used for many years. The safety record of naloxone is especially good. Nalcol is designed so that the drug is released in the large bowel with very little being absorbed into the main blood circulation. In the two studies where Nalcol was used no serious side effects were noted. We therefore feel that the risk of serious harm is very low. Minor temporary side effects which may occur include skin rashes (hypersensitivity), nausea (feeling sick), vomiting, and abdominal cramps. These would settle on reducing the dose or on complete cessation of the drug.

Recent data has shown that another opioid anatagonist, Olvimopan (Entereg) has been associated with cardiovascular side effects, particularly low blood pressure. The systemic absorption of naloxone is expected to be very low (2-3%). The possibility of cardiovascular adverse events with naloxone needs to be evaluated further in clinical trials but has been reported as common in trials involving Targinact, a fixed combination tablet containing naloxone. As such blood pressure monitoring will be carried out each time the participant attends for review.

What will happen to me if I take part?

The study will last 10 weeks for each person taking part in the trial. There will be three periods of assessment during the study with a fourth period if the Nalcol is effective.

Period 1 – this will be a two week period of observation of your usual symptoms with your usual treatment.

Period 2 - The second period will consist of four weeks. During this period you will take the 'trial' capsules. The trial capsule is either the active drug (Nalcol) or a placebo (dummy capsule). You will not be told which capsule you are taking and will be randomised to either Nalcol or the placebo.

Period 3 - this will also consist of four weeks where every patient in the study will receive Nalcol capsules.

Period 4 - This is a continuation period for those patients in whom the Nalcol is effective.

Throughout the study you will be required to keep a simple diary. This will take 2-3 minutes a day to complete. At the end of each period you will also be asked to complete questionnaires about your symptoms and quality of life. These will take approximately 10 minutes to complete. You will need to make a minimum of four visits to the hospital to see the research doctor during the study.

You will have blood taken at the beginning and the end of the study for routine tests. This allows us to assess the function of the bone marrow, liver and kidneys. The volume of blood taken will be no more than 45mls (the equivalent of three desert spoons). A rectal examination will be performed at the start of the study. This is to exclude any obstruction to your rectum and anal canal that would exclude you from the study.

We would like to repeat the transit studies on those patients who have had them done as part of their initial assessment by their consultant. This would take place at the end of the Period 2.

The Large Bowel Transit Study

This test gives a measure of whether or not the passage of food through the gut (colon) is slow or normal. The test itself is simple – you will be asked to swallow some capsules (containing tiny “markers” that show up on x-ray) and then have an x-ray of your abdomen a few days later. The distribution of the markers in your colon shows whether your bowel transit is normal or slow. Normal transit of contents from the mouth to the anus is less than 72 hours for the majority of patients. You will have had a transit study which had been organised by your consultant when you were initially assessed and this has shown that you have slow transit constipation. The trail capsule aims to reduce your transit time and this will be confirmed by repeating the transit study. The results of this investigation will be compared with your diary card to look at the effects of the trial capsule.

The transit study has very little in the way of risks. The 'markers' used are completely safe and pass through the body unchanged. The abdominal X-ray which is taken a few days later is equivalent to 2 months of 'background radiation'. 'Background radiation' is normally occurring radiation which we are all exposed to. Radiation is all around us. It comes from radioactive substances including the ground, the air, building materials and food. For example a 50 years old woman will have been exposed to background radiation for 50 years or 600 months (50 years x 12 months). That is to say that the abdominal X-ray is a small amount of radiation compared to what we are exposed to in our daily life and therefore a single X-ray is unlikely to be harmful.

What will happen to the results of my tests?

The results of your investigations will be kept confidentially in a special folder. The results will be available to your GP and specialist. We will analyse the results of the tests and questionnaires and discuss the results with you if you wish. We may write a report about our findings and attempt to publish this in a medical journal. In this report the results will be anonymous with no personal details such as name, date of birth or addresses. If you decide to withdraw from the study we will keep the results of your investigations. Any results from the study will be kept securely and confidentially in the hospital for a maximum of five years. After this time they will be destroyed.

Will my details be kept confidential?

The results and information collected in the study will be discussed outside the hospital at scientific meetings and will be published in scientific journals. However any information about you will have the name and address removed so that you cannot be recognised by it.

What are the benefits of taking part in the study?

The experience in other hospitals around the world suggests that investigations such as these give useful information about the management of severe constipation. It is hoped that such information will help patients now and in the future who have similar problems. If the drug is found to be effective, the results of this study may lead to it becoming licensed for prescription. This means many other patients can be helped. If the drug personally helps you then you will be entered into follow-up period where you will be able to continue taking the Nalcol which you will need to collect from the hospital pharmacy at the Norfolk and Norwich Hospital. During this time you will be required to complete two questionnaires each month and undergo 3 monthly blood test, urine tests, and blood pressure monitoring at the hospital when you will also need to attend for a clinic appointment. The lead researcher, Lukasz Kruppa, will also contact you monthly by phone to monitor your progress. When the trial has finished this follow-up period will also finish. The drug company who make the drug will continue to supply it for you. This will continue until a license for the drug is obtained (unless the drug is withdrawn for reasons of safety or lack of effect).

What are the disadvantages of taking part in the study?

We do not anticipate any serious side effects or complications. There is always a small degree of risk when starting any new drug and the long-term effects of this drug are uncertain. The study will involve a small amount of inconvenience with diaries to fill in and visits to the hospital. It is also important that you appreciate that you may take a placebo (dummy tablet) for four weeks with no benefit.

Will I be paid to take part in the study?

Participants will not be paid to enter the study and unfortunately we are unable to reimburse participants for travel to and from the hospital. However, as mentioned earlier, you will be able to continue taking the Nalcol if it proves effective in improving your symptoms.

Do I have to take part in the study and can I withdraw?

It is up to you to decide whether to take part. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Routine blood and urine analysis will need to be performed when you withdraw and a phone call will be made 1 month after withdrawal as routine follow-up. Withdrawal from the trial will not affect the standard of care you receive now or in the future. Being involved in the study is completely voluntary.

What if I am upset or inconvenienced by taking part in the study?

If you feel upset at anytime during your involvement or you wish to complain about any aspect of the way you have been approached or treated during the study, the normal National Health Services complaints processes will be available to you. You will also be able to discuss any concerns you have at any time with Mr. M Bignell or Mr. M Rhodes.

Who is coordinating the study?

Mr. M Bignell, Dr L Kruppa and Dr A. Hart are coordinating the study. Mr. M Bignell will perform the assessments during the study and this work will go towards Mr. Bignell earning a Doctorate of Medicine (MD).

Has the design of this study been checked by other doctors or scientists?

Before the study could take place it was checked by pharmaceutical advisors of the Medicines and Healthcare products Regulatory Agency (MHRA), who have principally assessed the safety of the product. It has been approved by the Project Review Committee of the hospital trust and the Local Ethics Committee. They have checked the aims and design of the study. The Committee is made up of nurses, doctors, lay people, and possibly scientists. Their job is to

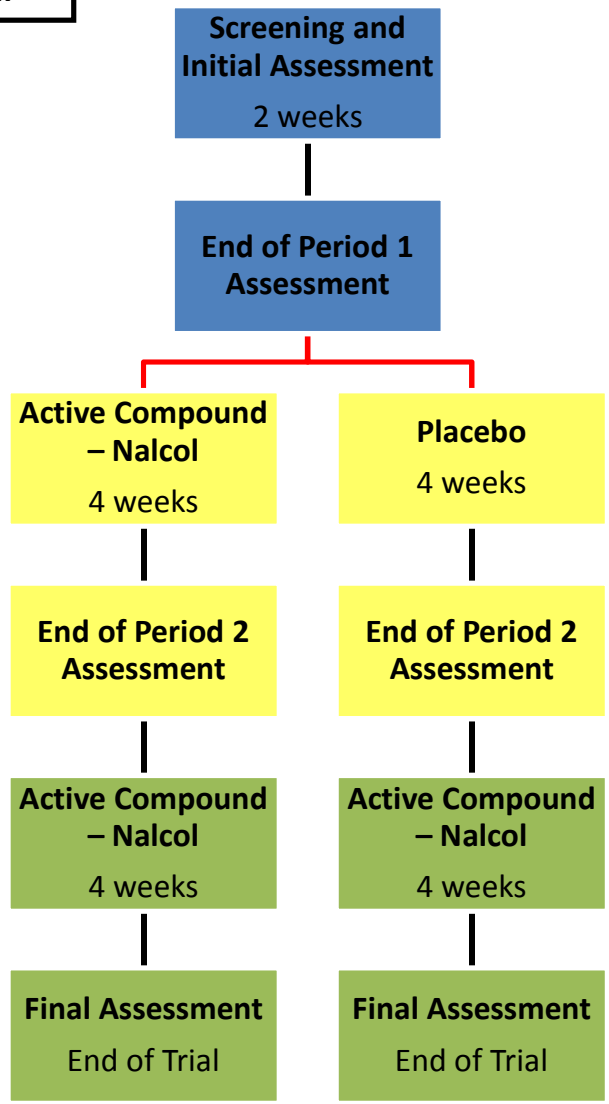
make certain that any studies involving patients in the UK are carried out safely and sensibly and in a way that will be beneficial to patients now and in the future.

What do I do now?

If you would like to help with the study please contact your consultant who will organise for you to meet Mr. M Bignell or Dr L Kruppa to discuss the trial further or contact Mr. Bignell directly on 01603 286 418 or 07921 004 585.

Thank you for your cooperation.

Diagram to Summarise the 3 Periods within the Nalcol Trial



Period 1

- Inclusion and exclusion criteria assessed
- Medical history assessed
- Examination including rectal examination and rigid sigmoidoscopy
- Consent into trial
- Blood tests for routine analysis

Randomisation

- Completion of diary cards and quality of life questionnaires
- If initial assessment and diary cards are satisfactory and patient is willing to proceed then they will be entered into the trial

Period 2

- 2 capsules, twice a day
- Phone call after 1 week to check on participant
- End of Period assessment
  - Hand over completed diary cards
  - QOL questionnaires completed
  - Large Bowel X-ray transit study
- Participant invited into Period 3

Period 3

- 2 Nalcol capsules, twice a day
- Phone call after 1 week to check on participant
- End of Period assessment
  - Hand over completed diary cards
  - QOL questionnaires completed
- Repeat blood and urine tests for routine analysis
- End Of Trial
- Post study check phone call in 4 weeks

Period 4

- PAC-SYM and PAC-QOL monthly by post
- Blood test, BP and urinanalysis 3 monthly at hospital
- Monthly phone call to check on patients progress and monitor for adverse events

Appendix 4 – GP Letter

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Department of General Surgery
Colney Lane Norwich NR4 7UY

Date _ _ / _ _ /_ _ _ _

Dear Dr ……,

Your patient ……of …….

Has kindly agreed to participate in a ten week placebo controlled trial investigating the potential role of oral naloxone (Nalcol) for constipation, where current measures used by the patient are not entirely satisfactory.

Naloxone is an opioid antagonist that has a beneficial effect in patients with constipation. The drug affects bowel motility and intestinal secretion with a shortening of the whole gut transit time. A small group of patients with constipation have had improvement in their symptoms whilst taking the formulation of naloxone. The only side effects encountered have been some urgency with defecation and slight abdominal discomfort.

The ten week study will comprise of three periods. In Period 1 (2 weeks) patients will continue with the measures they usually use to manage constipation. In Period 2 (4 weeks) they will also be given the trial capsule (randomised to either the active drug or the placebo) and continue with other measures. In Period 3 (4 weeks) they will all be treated with the active drug.

Period 4 will run after completion of the trial for those patients in whom Nalcol has been effective. They will be given the opportunity to continue taking the Nalcol under

supervision of the hospital. They will be asked to complete a monthly questionnaire and will be telephoned each month to monitor their progress. Every three months they will attend an outpatient clinic at the hospital where they will undergo routine blood testing, blood pressure measurement, and urinanalysis. Once both trials have concluded we will then make arrangement for the patients to continue the Nalcol on a named patient basis in agreement with the pharmaceutical company.

The dose of naloxone in each capsule is 10mg, and the total number of capsules used in Periods 2 and 3 will be two capsules, twice a day. Throughout the ten weeks, patients will keep a diary card of their symptoms and complete questionnaires at the end of each period.

We do not anticipate any serious adverse effects from this treatment, but have warned about the possibility of hypersensitivity, nausea, vomiting and abdominal cramps.

We have provided the patient with information sheets and a contact phone number, but if you require any further information, please do not hesitate to contact me.

Patients who wish to continue taking Nalcol after the study will be given the opportunity to do so via SLA Pharma, the pharmaceutical company supplying the drug.

Yours sincerely

Mark Bignell

Research Coordinator

Appendix 5 – Consent Form

CONSENT FORM

Patient Identification Addressograph

Title of project

NALOXONE HYDROCHLORIDE SR GASTRO-RESISTANT SUSTAINED RELEASE CAPSULES AS A TREATMENT FOR FUNCTIONAL CONSTIPATION: A RANDOMISED, DOUBLE BLIND CONTROLLED TRIAL IN SECONDARY CARE

Name of Researchers: Mr. Mark Bignell, Dr. L Krupa and Dr. A Hart

Please initial box

- I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

- I understand that sections of any medical notes may be looked at by responsible individuals from Norfolk and Norwich University NHS Foundation Trust or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

- I understand that the investigators would like to inform my GP of my participation in the study. I give permission for my GP to be informed. ☐

- I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Name of Researcher	Date	Signature

1 copy for patient; 1 for researcher; 1 to be kept with the hospital notes

Appendix 6 PAC-SYM – Patient Assessment of Constipation

PAC-SYM © PATIENT ASSESSMENT OF CONSTIPATION

This questionnaire asks you about your constipation symptoms in the **past week.** Answer each question according to your symptoms, as accurately as possible. There are no right or wrong answers.

For each symptom below, please indicate **how severe** your symptoms have been during the **past week**. If you have not had the symptoms during the past week, tick 0. if the symptom seemed mild, tick 1. If the symptom seemed moderate, tick 2. If the symptom seemed severe, tick 3. If the symptom seemed very severe, tick 4. Please be sure to answer every question.

How severe have each of these symptoms been in the past week?	**Absent 0**	**Mild 1**	**Moderate 2**	**Severe 3**	**Very Severe 4**
Discomfort in your stomach					
Pain in your stomach					
Bloating in your stomach					
Stomach cramps					
Painful bowel movements					
Rectal burning during or after a bowel movement					
Rectal bleeding or tearing during or after a bowel movement					
Incomplete bowel movement, as though you didn't 'finish'					
Stools that were too hard					
Stools that were too small					
Straining or squeezing to try to pass stools					
Feeling like you had to pass a stool but you couldn't (false alarm)					

Appendix 7 PAC-QOL – Patient Assessment of Constipation

PAC-QOL © PATIENT ASSESSMENT OF CONSTIPATION

The following questions are designed to measure the impact constipation has had on your daily life **during the past week.** For each question, please tick one box.

The following questions ask you about the intensity of your symptoms. To what extent, during the past week….	**Not at all 0**	**A little bit 1**	**Moderately 2**	**Quite a bit 3**	**Extremely 4**
Have you felt bloated to the point of bursting?					
Have you felt heavy because of your constipation?					

The next few questions ask you about the effects of constipation on your daily life. How much time, during the past week….	**None of the time 0**	**A little of the time 1**	**Some of the time 2**	**Most of the time 3**	**All of the time 4**
Have you felt any physical discomfort?					
Have you felt the need to open your bowel but not been able to?					
Have you felt embarrassed to be with other people?					
Have you been eating less and less because of not being able to have bowel movements?					

The next few questions ask you about the effects of constipation on your daily life. To what extent, during the past week….	**Not at all 0**	**A little bit 1**	**Moderately 2**	**Quite a bit 3**	**Extremely 4**
Have you had to be careful about what you eat?					
Have you had a decreased appetite?					
Have you been worried about not being able to choose what you eat (for example, at a friend's)?					
Have you been embarrassed about staying in the toilet for so long when you were away from home?					
Have you been embarrasses about having to go to the toilet so often when you were away from home?					
Have you been worried about having to change your daily routine (for example, traveling, being away from home)?					

The next few questions ask you about your <u>feelings</u>. How much of the time, during the past week….	**None of the time 0**	**A little of the time 1**	**Some of the time 2**	**Most of the time 3**	**All of the time 4**
Have you felt irritable because of your condition?					
Have you been upset by your condition?					
Have you felt obsessed by your condition?					
Have you felt stressed by your condition?					
Have you been less self-confident because of your condition?					
Have you felt in your control of your situation?					
Have you been worried about not knowing when you are going to open your bowels?					
Have you been worried about not being able to open your bowels when you needed to?					
Have you been more and more bothered by not being able to open your bowels?					

The next questions ask about your <u>life with constipation</u>. How much of the time, during the past week….	**None of the time 0**	**A little of the time 1**	**Some of the time 2**	**Most of the time 3**	**All of the time 4**
Have you been afraid your condition will get worse?					
Have you felt that your body was not working properly?					
Have you had fewer bowel movements than you would like?					

The next questions ask you about <u>how satisfied</u> you are. To what extent, during the past week….	**Not at all 0**	**A little bit 1**	**Moderately 2**	**Quite a bit 3**	**Extremely 4**
Have you been satisfied with how often you open your bowels?					
Have you been satisfied with the regularity with which you open your bowels?					
Have you been satisfied with your bowel function?					
Have you been satisfied with your treatment?					

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APPENDIX 2 DIARY CARD

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Study of Nalcol Therapy in Functional Constipation

DIARY CARD

Should you wish to have advice on issues at any time during the study or wish to withdraw you may contact the trial co-ordinator, **Mr Bignell**, on **07928 941828** between the hours of 08.30hrs and 17.00hrs, Monday to Friday. In case of emergency please contact your GP or local A&E with a copy of the patient information leaflet and this diary card.

Explanation of Terms used in the Diary Card

1. Frequency - Number of stools each day

2. Type of stool – As per the stool chart (next page)

Type of Stool Chart

	**Type 1:** Stools appear in separate, hard lumps, similar to nuts.
	**Type 2:** Stools are sausage-like in appearance but lumpy with deep cracks separating the hard lumps.
	**Type 3 (Normal):** Stools come out similar to a sausage but with cracks in the surface.
	**Type 4 (Normal):** Stools are smooth and soft in the form of a sausage or snake.
	**Type 5:** Stools form soft blobs with clear-cut edges, and easily pass through the digestive system. Soft diarrhoea.
	**Type 6:** Stools have fluffy pieces with ragged edges. Considered mushy stools, they indicate diarrhoea;
	**Type 7:** Stool is mostly liquid with no solid pieces. Passed quickly through the colon; is indicative of severe diarrhoea.

Look at the stool in the toilet before you use toilet paper, and note the type of stool you have passed. Types 1,2, and 3 are the most common in people with constipation

Explanation of Diary Card for Weeks 1 and 2

- The purpose of this week is to identify your usual bowel habit and weekly requirement of laxative usage.

- Please complete the diary at the end of each day as accurately as possible recording any laxatives used during that day.

Week 1 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 1 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Explanation of Diary Card for Weeks 3-6

- For the next four weeks you should take the trial capsule twice a day.
- Continue to take your regular laxative medication as needed.
- Please complete the diary at the end of each day as accurately as possible recording any laxatives used during that day.

Week 3 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 4 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 5 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 6 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Explanation of Diary Card for Week 7-10

- In weeks 3 to 6 you have either been taking the active drug, Nalcol, or a placebo. You are now invited to take the active medication, Nalcol, for four weeks

- Continue to take your regular laxative medication as needed.

- Please complete the diary at the end of each day as accurately as possible recording any laxatives used during that day.

Week 7 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 8 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 9 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

Week 10 Pre-Trial

Date _ _ / _ _ / _ _ _ _

Patient Number _____

Patient Initials _____

Sex - MALE / FEMALE

DOB _ _ / _ _ / _ _ _ _

Day	1	2	3	4	5	6	7
Frequency							
Type of Stool							

Day	Name of medication taken on any day for constipation with any comments you may wish to make
1	
2	
3	
4	
5	
6	
7	

APPENDIX 3 PROTOCOLS FOR DNA EXTRACTION AND PURIFACATION FOR DOWN STREAM ANALYSIS

QIAamp® DNA EXTRACTION PROTOCOL

1. Weigh 180-220mg stool in a 2ml microcentrifuge tube (not provided) and place tube on ice.

2. Add 1.4ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenised.
   It is important to vortex the samples thoroughly. This helps ensure maximum DNA concentration in the final eluate.

3. Heat the suspension for 5 min at 70^{o}C.
   This heating step increases total DNA yield 3- to 5-fold and helps to lyse bacteria and other parasites. The lysis temperature can be increased to 95^{o}C for cells that are difficult to lyse (such as Gram-positive bacteria)

4. Vortex for 15 seconds and centrifuge sample at full speed for 1 minute to pellet stool particles.

5. Pipet 1.2ml of the supernatant into a new 2ml microcentrifuge tube and discard the pellet.

6. Add 1 InhibitEX tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

7. Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX matrix.

8. Pipet all the supernatant into a new 1.5ml centrifuge tube and discard the pellet. Centrifuge the sample at full speed for 3 min.

9. Pipet 15 µl proteinase K into a new 1.5ml microcentrifuge tube.

10. Pipet 200 µl supernatant from step 8 into the 1.5 ml microcentrifuge tube containing proteinase K.

11. Add 200 µl Buffer AL and vortex for 15 s.
    Note: Do not add proteinase K directly to Buffer AL
    It is essential that the sample and Buffer AL are thoroughly mixed to form a homogenous solution.

12. Incubate at 70^{o}C for 10 min.

13. Add 200 µl of ethanol (96-100%) to the lysate, and mix by vortexing.

14. Label the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully apply the complete lysate from step 13 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2ml collection tube, and discard the tube containing the filtrate.
    Close each spin column in order to avoid aerosol formation during centrifugation
    If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.

15. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2ml collection tube, and discard the tube containing the filtrate.

16. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.

Note: Residual buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

17. Recommended: Place the QIAamp spin column in a new 2 ml collection tube and centrifuge at full speed for 1 min.
    This step helps to eliminate the chance of possible Buffer AW2 carryover.

18. Transfer the QIAamp spin column into a new, labelled 1.5 ml microcentrifuge tube. Carefully open the QIAamp spin column and pipet 200 µl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate at room temperature, then centrifuge at full speed for 1 min to elute DNA.

E.Z.N.A.® CYCLE-PURE SPIN PROTOCOL

1. Perform agarose gel/ethidium bromide electrophoresis to analyse PCR Product.

2. Determine the volume of the PCR reaction. Transfer the sample into a clean 1.5 ml microcentrifuge tube and add 4-5 volumes of CP buffer. For PCR products smaller than 200 bp, add 6 volumes of CP buffer.

3. Vortex thoroughly to mix. Briefly spin the tube to collect any drops from inside of the lid.

4. Place a HiBind DNA Mini Column into a provided 2ml collection tube.

5. Add the mixed sample from step 3 to the HiBind DNA Mini Column and centrifuge at 13 000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the HiBind Mini Column back into the same collection tube.

6. Add 700 µl of DNA Wash Buffer and centrifuge at 13 000 x g for 1 minute. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.
   DNA Wash Buffer must be diluted with absolute ethanol before use. If refrigerated, DNA Wash Buffer must be brought back to room temperature before use.

7. Add 500 µl of DNA Wash Buffer and centrifuge at 13 000 x g for 1 minute. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

8. Centrifuge the empty HiBind DNA Mini Column for 2 min at maximal speed (≥13 000 x g) to dry the column matrix.
   Do Not skip this step. It is critical for the removal of ethanol from the HiBind DNA Column
9. Place the HiBind DNA Mini Column into a new, clean 1.5 ml microcentrifuge tube. Depending on the desired concentration of the final product, add 30-50 µl of Elution Buffer (10mM Tris, pH 8.5) or water directly onto the centyre of the column matrix. Let it sit at room temperature for 2 minutes. Centrifuge for 1 minute at 13 000 x g to elute the DNA.
   This represents approximately 80-90% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

ELECTROPHORESIS WITH AGAROSE GEL

1. 0.7g agarose powder mixed with 100mls of 0.5x TBE buffer and weigh.

2. Heat to ensure agarose dissolves and then add TBE buffer if necessary.

3. Pour into electrophoresis box to form gel plate.
   - Place combs into box.
   - Pour slowly to avoid bubbles.
   - Use small pipette tip to remove bubbles.

4. Once gel has set:
    - Cover gel with TBE buffer.
    - Carefully remove comb.
    - Add DNA to wells (4µl DNA, 2µl Loading Dye).
    - Use 4µl of ladder in first well.

5. Connect to power.
    - Band runs black to red (-ve to +ve).
    - 80-90V, 20mA.

6. Once gel has run transfer to Ethidium Bromide and leave for 30 minutes.

7. Remove and Wash.

8. Place on camera, filter II, UV transillumination.

9. Take photo, print and save.

APPENDIX 4 DGGE PROTOCOL BIORAD SYSTEM

Add dH_2O to the cylinders of the gradient maker and switch on the stir and mixing systems and the pump at the maximum speed (9.9mL/min). Refill the cylinders with water one more time to rinse them. Make sure there is a beaker for the waste.

Clean the glass plates (2 large and 2 small), the combs and the spacers with detergent. Dry and clean them with 70% ethanol.

Switch off the stir and mixing system and the pump and dry the cylinders.

Prepare the platform for the gels

Place grey strips on the white platform

Prepare the large plate with the spacers, and place the small plate on top of it

Place the clamps both sides of the glass plates as the arrows indicate

Make sure the spacers are completely aligned on the bench

Place the cardboard between the 2 plates and make sure it can be moved

Adjust the screws until they cannot be moved (don't tighten the screws too much since the glass plates and clamps might break)

Check again that the spacers are completely aligned with the plates

Place the gel sandwich on the white platform

Prepare the Ammonium persulfate (APS) 10%

This is the catalyst that can be kept frozen. Weigh 200mg and dissolve in 2ml dH_2O

Preparation of the Gradient gel

Collect TEMED and the 60%, 40%, and 0% acrylamide solutions from the fridge

Put a new needle on the tube

Switch on the stirrer and make sure the connection is closed. Set the pump speed to 4.0mL / min

Add 11ml of 60% acrylamide to the cylinder in the left (closed to pump), and 11ml of 40% acrylamide to the cylinder in the right

Add 15µl of TEMED (take care, toxic) and 45µL of 10% APS to each cylinder. When TEMED and APS get added the acrylamide starts polymerising therefore do not wait too long before continuing.

Place the needle in the middle between the glass plates of the gel sandwich, start the pump and immediately after you see the acrylamide in the white tube open the connection.

Once the cylinders are empty remove the needle from the tube and put it in the beaker. Add H_2O to the cylinders to wash them and increase the pump speed to 9.9mL/min. When the cylinders are empty close the connection and stop the pump. Dry the cylinders with a tissue.

Preparation of the Stacking Gel

Start the stirrer and make sure the connection between both cylinders is closed

Fill the left hand cylinder with 7ml of 0% acrylamide

Add 11µL of TEMED and 30µl of 10% APS to the cylinder

Start the pump and once all the water has been removed from the tube select a pump speed of 1.0mL/min and place the needle between the glass plates to pour the stacking gel

Remove the needle, switch of the pump, and quickly place the comb

Rinse the tube with dH_2O

Repeat the same process for the other gel

Leave the gels at least 2-3 hours to polymerise

Wash the bench with ethanol

Preparing the buffer and loading the gels

Fill the gel tank with 1x TAE until the 'fill' line (approx 7 litres)

Top up with 20ml of 50X TAE to 1L of dH_2O

Pre-heat the buffer to 60^{o}C at least 1 hour before starting to load the gel

Mix the samples in a microtitre plate and then mix with 5µl of loading buffer

Remove the gels from the white platform and put them in a yellow one

Place the gel in the yellow platform into the bath making sure the red button is on the right hand side

Remove the comb in the back gel and load it with a syringe. Then do the same with the front gel

Switch on the pump and let the buffer reach the black part in the top of the bath

Run the electrophoresis at 50V for 16hours

Staining the gels

Prepare 2 trays with 300ml of 1x TAE

Switch off the DGGE and take out the gels

Remove the spacers carefully and also the small glass plate (upper plate)

Put the transparency on the gel, turn it and remove the large glass plate

Put the gels in the tray with the TAE

Add 5µL of SYBr Green to each tray (1 spots in each corner) and leave them approximately 45 minutes on the shaker at the minimum speed in the dark

Wash the gels for 15 min in 300ml of dH_2O in the dark

Scan the gels using 'PHAROS' scanner (Bio-Rad) and using the manufacturer's instructions

APPENDIX 5 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH) PROTOCOL

Faecal sample preparation (not *lactobacillus/enterococcus* probes)

Weigh the sample and add PBS to make a 1 / 10 (w/v) solution

Homogenise the sample in an Eppendorf with 0.5mls of PBS

Transfer to a 7ml plastic tube and washout Eppendorf with the remaining PBS for the sample

Repeat homogenisation until complete then split sample equally between two 2ml Eppendorf tubes

Centrifuge for 2 min at 1500 rpm to remove particulate matter

Remove 375µl of supernatant and add 1125µl of filtered 4% paraformaldehyde solution in a 1.5ml microcentrifuge tube.

Always add paraformaldehyde then supernatant and paraformaldehyde needs to be cold (4^0C prior to use)

Mix and store overnight at 4^0C

Take the 1.5ml of fixed sample and centrifuge at 13000 rpm for 5 min

Remove the supernatant and resuspend the pellet in 1ml of filtered PBS

Repellet by centrifuging at 13000 rpm for 5 min

Wash the pellet a second time and repeat the above step

Remove as much supernatant as possible and resuspend the pellet in 150µl of filtered PBS

Add 150µl of 96% ethanol, mix well and store at -20^0C for at least 1 hour

THE ETHANOL/PBS SAMPLES COULD BE STORED FOR UPTO 3 MONTHS

Faecal sample preparation (*lactobacillus/enterococcus* probes)

Weigh the sample. A volume of PBS will be required to make a 1 / 10 (w/v) solution

Homogenise the sample in an Eppendorf with 0.5mls of PBS

Transfer to a 7ml plastic tube and washout Eppendorf with the remaining PBS for the sample

Repeat homogenisation until complete then split sample equally between two 2ml Eppendorf tubes

Centrifuge for 2 min at 1500 rpm to remove particulate matter

Remove 375 µl of supernatant and add 1125µl of filtered 4% paraformaldehyde solution in a 1.5 ml microcentrifuge tube.

Always add paraformaldehyde then supernatant and paraformaldehyde needs to be cold (4^0C prior to use)

Mix and store overnight at 4^0C

Take the 1.5ml of fixed sample and centrifuge at 13000 rpm for 5 min

Remove the supernatant and resuspend the pellet in 1ml of filtered PBS

Repellet by centrifuging at 13000 rpm for 5 min

Wash the pellet a second time and repeat the above step

Remove as much supernatant as possible and resuspend in 145µl of *Lactobacillus* enzyme buffer

Incubate at 37^0C for 2 hours

Add 5µl of 4% paraformaldehyde solution and leave at 4^0C for 10 minutes

Repellet by centrifuging at 13000 rpm for 5 min

Remove the supernatant and resuspend the pellet in 1ml of filtered PBS

Repellet by centrifuging at 13000 rpm for 5 min

Wash the pellet a second time and repeat the above step

Remove as much supernatant as possible and resuspend the pellet in 150µl of filtered PBS

Add 150µl of 96% ethanol, mix well and store at -20^{0}C for at least 1 hour

Hybridisation (all probes)

In a 0.5ml eppendorf tube:

Add 16µl of the fixed cells (PBS/EtOH @-20) to 264µl of filtered hybridisation buffer (prewarmed in the oven)

It is important that the above mixture is close to the hybridisation temperature before it is added to the probe. If it has cooled it should be rewarmed in the oven to the appropriate temperature (see later table)

Take a 2nd 0.5ml microcentrifuge tube and add the appropriate volume of the probe (50ng/µl, 20^{0}C). Then as quick as possible pipette 90µl of the above mixture into the probe tube, vortex and return to the hybridisation oven. Ensure that the lids are firmly closed to prevent evaporation.

The volume of hybridisation mix may not be the same for all probes (see table 1)

Leave overnight to hybridise

Washing (all probes)

Take a 7ml Sterilin tube and add 5ml of filtered prewarmed washing buffer and 20µL of DAPI (4,6-diamidino-2-phenylindole, 500ng/µl)

Add hybridised sample and leave Sterilin tubes in the oven for 30 min. Any remaining hybridised sample should be returned to the oven if the dilution is not known (max. 24h).

Set up the apparatus using a wet filter (0.2µm), matt side up

Pour the sample mixture onto the filter and switch on the vacuum pump

Rinse the Steriliin tube and glass tube of the filtering apparatus with prewarmed washing buffer using a syringe

Remove the filter and place it onto a glass slide

Put a drop of Slow Fade on top of the filter and place a glass cover slip onto this

Store the slide in the dark at 4^0C to minimise fading

Counting (all probes)

Place the slide onto a microscope, add a drop of immersion oil and use the Flour 100 lens

Start with UV light to find DAPI stained bacteria then switch to the green light to count the organisms hybridised with the probe

Count the number of organisms inside the target box, focusing up and down to see all of the bacteria. On average there should be 20-30 cells. If more or less then dilutions will need to be adjusted.

Count 15 random fields then dispose of the slide

Uncounted slides should be stored in the dark at 4^0C

Quantities of hybridisation mixture

The volume of hybridisation mixture prepared depends on the volume planned to be used the next day during the washing stage. (e.g if 100µl is required then make sure that at least 120µl are prepared to allow for some evaporation)

The volume of hybridisation mixture used during the washing stage depends on how many bacteria you expect to find in the sample. In general higher bacterial numbers correspond to lower volumes (dilutions and vice versa).

The volume of the hybridisation mix has an upper limit that depends on the total number of bacteria count (DAPI count)

The ratio of hybridisation mixture (fixed cells and hybridisation buffer) and probes is ALWAYS 1:10

Avoid using small volumes (5:45) because of overnight evaporation

Probe	Target Genus	Temp (^{0}C)	Probe Volume (µl)	Sample Volume (µl)	Hybridised Mixture Volume (µl)	Optimum Hybridisation Time (h)
Bac 303	*Bacteroides* spp.	45	10	90	20	
Bif 164	*Bifidobacterium* spp.	50	10	90	20	15-17
Erec 482	*C.coccoides* *Eubacterium* rectale gp.	52	10	90	20	**Max time - 24**
Chis 150	C.histolyticum gp.	50	15	135	100	
Ec 1531	E.*coli*	37	15	135	100	
Lab 158	*Lactobacillus* spp. *Enterococcus* spp.	45	15	135	100	
Srb 687	*Desulfovibrio* spp.	48	15	135	100	3
Eub 338	Total Bacteria	48	10	90	10	**Max time – 4**
DAPI	Total DNA	Any	10	90	5	Nil

Table 2 – Probes, Temperatures, Hybridisation Mixture Volumes and Times.

In the case of DAPI HPLC (filtered) water is used instead of a probe. These volumes are for faecal samples. They are not absolute values but are starting points that may have to be adjusted according to samples and test substrates.

Solutions and buffers

All solutions and buffers were made up as described below;

PBS**:** 1 PBS tablet was added into 100ml of distilled water and then autoclaved. The pH was adjusted to 7.4 and filtered through 0.2µm filter paper and then stored at 4^0C

4% PARAFORMALDEHYDE: A 16% paraformaldehyde vial (10ml) was dilute to 4% with filtered PBS solution (above)

LACTOBACILLUS ENZYME BUFFER: 23mM Tris-HCl, 585mM Sucrose, and 5mM $CaCl_2$ were combined and then stirred until dissolved. 10mM EDTA and 30mg Taurocholic Acid were then added and dissolved. The pH was adjusted to 7.6 and then the solution was filtered through 0.2µm filter paper and stored at room temperature. Prior to use 2mg/ml lysosyme (50 000U) and 1mg/ml lipase (100-400U, Porcine Pancreas Type II) were added.

HYBRIDISATION BUFFER (NOT EUB 338, SRB 687, OR EC 1531 PROBES): 30mM Tris-Hcl, 1.36 M NaCl, and 1.5ml of 10% SDS solution were combined. The pH was adjusted to 7.2 and the solution filtered through 0.2µm filter paper and store at room temperature. Prior to use it was stored in hybridisation oven

HYBRIDISATION BUFFER (EC 1531 PROBE): 40mM Tris-HCl, 1.8M NaCl, and 2ml of 10% SDS solution were combined and the pH adjusted to 7.2. The solution was filtered through 0.2µm filter paper and stored at room temperature. Prior to use 35% formamide was added and the solution filtered through 0.2µm filter paper and store in hybridisation oven.

HYBRIDISATION BUFFER (*DESULFOVIBRIO* spp. AND EUB 338 PROBES): 20mM Tris-HCl, 0.9 M NaCl, and 100µl of 10% SDS solution were combined and the pH adjusted to 7.2. The solution was then filter through 0.2µm filter paper and stored at room temperature. Prior to use 10% formamide was added for *Desulfovibrio* spp., filtered through 0.2µm filter paper and store in the hybridisation oven whilst 20% formamide was added for total bacteria, filtered through 0.2µm filter paper and stored in the hybridisation oven.

WASHING BUFFER (NOT EUB 338 OR SRB 687 PROBES): 20mM Tris-HCl and 0.9 M NaCl were combined, the pH adjusted to 7.2, and the solution filtered through 0.2µm filter paper and stored at room temperature. Prior to use the solution was stored in the hybridisation oven.

WASHIING BUFFER FOR SRB 687 PROBE: 20mM Tris-HCl, 0.386 M NaCl, and 1ml of 10% SDS were combined and the pH adjusted to a pH of 7.2. The solution was then filtered through 0.2µm filter paper and stored at room temperature. Prior to use it was stored in the hybridisation oven.

WASHING BUFFER FOR EUB 338 PROBE: 20 mM Tris-HCl, 0.166 M NaCl, and 1ml of 10% SDS were combined and the pH adjusted to a pH of 7.2. The solution was then filtered through 0.2µm filter paper and stored at room temperature. Prior to use it was stored in the hybridisation oven.

6. ABBREVIATIONS

5-HT	5-HYDROXYTRYPTAMINE (SEROTONIN)
ACH	ACETYLCHOLINE
ATP	ADENOSINE 5'-TRIPHOSPHATE
BAC 303	*Bacteroides* spp
Bif 164	*Bifidobacterium* spp.
BFI	BOWEL FUNCTION INDEX
CCD	CHARGED COUPLE DEVICE
CFB	CYTOPHAGA-FLAVOBACTERIUM-BACTEROIDES
cGMP	CYCLIC GUANOSINE MONOPHOSPHATE
CIC	CHLORIDE CHANNELS
CNS	CENTRAL NERVOUS SYSTEM
CSBM	COMPLETE SPONTANEOUS BOWEL MOVEMENT
CTA	CLINICAL TRIAL AUTHORISATION
CTFR	CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR
DGGE	DENATURING GRADIENT GEL ELECTROPHORESIS
DNA	DEOXYRIBONUCLEIC ACID
EAS	EXTERNAL ANAL SPHINCTER
EC	ENTEROCHROMAFFIN
ECG	ELECTROCARDIOGRAM
EGC	ENTERIC GLIAL CELLS
EMG	ELECTROMYOGRAM
ENS	ENTERIC NERVOUS SYSTEM
EPSP	EXCITATORY POST SYNAPTIC POTENTIAL
Erec 482	*C.coccoides-Eubacterium* rectale gp
FGID	FUNCTIONAL GASTROINTESTIINAL DISORDERS

FC	FUNCTIONAL CONSTIPATION
FISH	FLUORSCENT IN-SITU HYDRIDISATION
GC-C	GUANYLATE CYCLASE C
GCP	GOOD CLINICAL PRACTICE
GI	GASTROINTESTINAL
H&E	HEMATOXYLIN AND EOSIN
HAPCs	HIGH AMPLITUDE PROPAGATING CONTRACTIONS
HFD	HIGH FAT DIET
IAS	INTERNAL ANAL SPHINCTER
IBD	INFLAMMATORY BOWEL DISEASE
IBS	IRRITABLE BOWEL SYNDROME
IBS-C	IRRITABLE BOWEL SYNDROME – CONSTIPATION
IBS-D	IRRITABLE BOWEL SYNDROME – DIARRHOEA
IBS-M	IRRITABLE BOWEL SYNDROME – MIXED
ICC	INTERSTITIAL CELLS OF CAJAL
ICH	INTERNATIONAL CONFERENCE ON HARMONISATION
IFR	INSTITUE OF FOOD RESEARCH
IHC	IMMUNOHISTOCHEMISTRY
IL	INTERLEUKIN
IMP	INVESTIGATIONAL MEDICINAL PRODUCT
IPANs	INTRINSIC PRIMARY AFFERENT NEURONES
IQR	INTERQUARTILE RANGE
IRAS	INTEGRATED RESEARCH APPLICATION SYSTEM
ITT	INTENTION TO TREAT
JPUH	JAMES PAGET UNIVERSITY HOSPITAL
LAB 158	*Lactobacillus / enterococcus*
LAPCs	LOW AMPLITUDE PROPAGATING CONTRACTIONS

MCT	MUSCULAR COORDINATION TRAINING
MHRA	MEDICINES AND HEALTHCARE PRODUCTS REGULATORY AGENCY
NNUH	NORFOLK AND NORWICH UNIVERSITY HOSPITAL
NO	NITRIC OXIDE
OIC	OPIOID INDUCED CONSTIPATION
PAC-QOL	PATIENT ASSESSMENT OF CONSTIPATION – QUALITY OF LIFE
PAC-SYM	PATIENT ASSESSMENT OF CONSTIPATION – SYMPTOMS
PCR	POLYMERASE CHAIN REACTION
PED	PELVIC EVACUATORY DISORDER
PEG	POLYETHYLENE GLYCOL
PFD	PELVIC FLOOR DYSSYNERGIA
PR	PROLONGED RELEASE
PS	PROPAGATING SEQUENCES
qPCR	QUANTITATIVE POLYMERASE CHAIN REACTION
QOL	QUALITY OF LIFE
R&D	RESEARCH AND DEVELOPMENT
RCT	RANDOMISED CONTROLLED TRIAL
REC	RESEARCH AND ETHICS COMMITTEE
rRNA	RIBOSOMAL RIBONUCLEIC ACID
Rf	RETARDATION FACTOR
SBM	SPONTANEOUS BOWEL MOVEMENT
s.d.	STANDARD DEVIATION
SERT	SEROTONIN REUPTAKE TRANSPORTER
SNS	SACRAL NERVE STIMULATOR
SPS	SODIUM PICO SULPHATE
STC	SLOW TRANSIT CONTIPATION
STC + IRA	SUBTOTAL COLECTOMY AND ILEORECTAL ANASTOMOSIS

TMF	TRIAL MASTER FILE
TNF-α	TUMOUR NECROSIS FACTOR ALPHA
UEA	UNIVERSITY OF EAST ANGLIA
VIP	VASOACTIVE INTESTINAL PEPTIDE

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