Slow transit constipation: clinical and aetiological studies.

Knowles, Charles H

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SLOW TRANSIT CONSTIPATION: CLINICAL AND AETIOLOGICAL STUDIES

by

Charles H Knowles BChir., FRCS.

St Bartholomew's and the Royal London
School of Medicine and Dentistry

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ABSTRACT

Constipation is the second most commonly self-reported gastrointestinal symptom. On the basis of anorectal physiological investigations and colonic transit studies, a sub-group of patients with severe intractable symptoms, but without organic disease, will be found to have slow transit constipation (STC). STC is a condition of gut dysmotility which predominantly affects young women, and may result in surgical intervention with variable, often unsatisfactory results. The aetiology remains elusive.

New aetiological hypotheses for STC were examined following full clinical and pathophysiological characterisation of a large cohort of 130 patients referred to our institution over the last 10 years. Aspects of nerve and muscle dysfunction were studied.

A new scoring system demonstrated some ability of multiple symptoms to discriminate STC from other forms of constipation. Detailed clinical and gastrointestinal physiological studies confirmed the heterogeneity of STC patients. Some significant physiological differences were detectable between clinically defined sub-groups of patients and refuted previous assumptions based on smaller numbers. Detailed neurophysiological studies, including quantitative peripheral sensory and autonomic testing, provided evidence of a small fibre neuropathy in a proportion of patients with STC. Mutational screening of some early-onset cases for a possible congenital pathogenetic mechanism, based on the observation that some STC patients had relatives with Hirschsprung's disease demonstrated that mutation of 2 important genes now implicated in this disorder were not a frequent cause of STC. Serum immunoprecipitation assays showed that anti-neuronal ion channel autoantibodies may have an as yet unrecognised role in the development of STC in a small proportion of acquired cases. An inclusion body myopathy was identifiable in colonic tissue of patients with STC, and this appeared to arise secondary to denervation. Further knowledge of the single or multiple pathogenetic mechanisms leading to this clinical condition may allow more rational or directed therapies aimed at the correction of the disease process or processes themselves.

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To my parents

STATEMENT OF ORIGINALITY

The author wishes to certify that all the work presented in this thesis is original, and that all the experiments, the acquisition and analysis of resulting data and the subsequent production of this manuscript were performed by himself unless clearly stated otherwise.

In particular, the following specialised techniques were observed, but not performed by the author:

Chapter 2 & 3	Anorectal physiological tests
	Prolonged ambulatory small bowel manometry
Chapter 5	Nerve conduction studies
Chapter 8	Routine and some histochemical stains

PUBLICATIONS

Some of the results presented in this thesis have already been published, in part, in the following journals:

PAPERS

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6. **Knowles CH,** Eccersley AJP, Scott SM, Walker SM, Reeves B, Lunniss PJ. Linear discriminant analysis of symptoms in patients with chronic constipation: validation of a new scoring system. *In press: Dis Colon Rectum 2000.*

7. **Knowles CH**, Scott SM, Williams NS, Lunniss PJ. Clinical and physiological

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1. **Knowles CH**, Wellmer AC, Scott SM, Misra VP, Rogers J, Pilot M-A, Williams NS, Anand P. Slow transit constipation: a sporadic or hereditary neuropathy? (abstr.). *Br J Surg* 1997; **84**: 1601.

2. Wellmer A, Sharief MK, **Knowles CH**, Misra VP, Kopelman P, Ralph D, Anand P. Quantitative sensory and autonomic testing in impotent diabetic patients (abstr.). *J Neurol Neurosurg Psychiatry*. Proceedings 1997; 699.

3. **Knowles CH**, Scott SM, Wellmer AC, Pilot M-A, Williams NS, Anand P. Slow transit constipation: a sporadic or hereditary neuropathy? (abstr.). *J Neurol Neurosurg Psychiatry*. Proceedings 1997; 699-700.

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6. **Knowles CH**, Misra VP, Wellmer AC, Monson JP, Anand P. Peripheral sensory dysfunction in a kindred of Multiple Endocrine Neoplasia Type 2A with a codon 634 mutation of the *RET* proto-oncogene (abstr.) *J Neurol Neurosurg Psychiatry* Proceedings 1998; 425.

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muscle inclusion bodies in gastrointestinal motility disorders (abstr.). *Neuropathol Appl Neurobiol* 1999; **25**: 148.

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9. **Knowles CH,** Gayther SA, Scott SM, Ramus S, Anand P, Williams NS, Ponder BAJ. Idiopathic slow transit constipation is not associated with mutations of the RET proto-oncogene or GDNF (abstr.). *Br J Surg* 1999; **86** (Suppl. 1): 17.

10. Scott SM, **Knowles CH**, Lunniss PJ, Newell M, Garvie N, Williams NS. Disordered rectal evacuation or rectal sensory dysfunction does not influence severity of colonic transit disturbance in patients with chronic idiopathic slow transit constipation (STC) (abstr.). *Gastroenterology* 1999; **116**: A-1080.

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PRESENTATIONS TO LEARNED SOCIETIES

1. **Knowles CH**, Wellmer AC, Scott SM, Misra VP, Rogers J, Pilot M-A, Williams NS, Anand P. Slow transit constipation: a sporadic or hereditary neuropathy? *SRS (Patey prize plenary session), Nottingham, May 1997.*

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3. **Knowles CH**, Scott SM, Wellmer AC, Pilot M-A, Williams NS, Anand P. Sensory and autonomic neuropathy in patients with idiopathic slow transit constipation. *Gastroenterology (1998) AGA, New Orleons, June 1998.*

4. **Knowles CH**, Misra VP , Wellmer AC, Monson JP , Anand P. Peripheral sensory dysfunction in a kindred of Multiple Endocrine Neoplasia Type 2A with a codon 634 mutation of the RET proto-oncogene. *ABN, Leeds, September 1998.*

5. Eccersley AJP, **Knowles CH**, Scott SM, Metcalf KB, Walker SM, Lunniss PJ. A new discriminatory scoring system for constipation (KESS). *Association of Coloproctology of Great Britain and Ireland, Jersey, June 1998.*

6. **Knowles CH,** Scott SM, Newell M, Garvie , Lunniss PJ. Colonic scintigraphy highlights differences in regional colonic transit in sub-groups of patients with slow-transit constipation. *AGA, New Orleons, June 1998.*

7. **Knowles CH,** Gayther SA, Scott SM, Ramus S, Anand P, Williams NS, Ponder BAJ. Idiopathic slow transit constipation is not associated with mutations of the *RET* proto-oncogene or GDNF. *Joint SRS / ASGBI, Brighton, May 1999.*

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1 INTRODUCTION, LITERATURE REVIEW AND AIMS

1.1 CONSTIPATION

1.1.1 DEFINITION

Con.sti.pat.ion [Latin. *constipatio* a crowding together]. Confinement of the bowels: a state of the bowels in which the evacuations are obstructed or stopped (Burchfield & Robert, 1987).

A symptom is a term that people use to describe a sensation or bodily function that they perceive as abnormal. Constipation is a symptom reported by patients who believe that there is a disturbance of the events that they perceive to comprise normal defaecation. As such, patients may associate a wide variety of symptoms with the term constipation, including those apparently directly related to defaecation e.g. infrequency of bowel action, loss of urge to defaecate, straining, incomplete, painful or unsuccessful evacuation, or more diverse symptoms such as abdominal pain, bloating or nausea. Without agreement over what constitutes normality or abnormality in terms of the type or frequency of symptoms reported by patients, clearly the definition of constipation is highly subjective. In practice, patients present when their personal situation is unsatisfactory.

Attempts have been made to define constipation by objective criteria (Drossman *et al.*, 1982; Whitehead *et al.*, 1991; Thompson *et al.*, 1992). An international workshop (Whitehead *et al.*, 1991) defined constipation as the presence of fewer than 2 bowel movements per week on average for at least 12 months, or the presence of 2 or more of the following complaints for at least 12 months:

- Straining on at least 25% of bowel movements when not taking laxatives.
- Feeling of incomplete evacuation after at least 25% of bowel movements when not taking laxatives.
- Stools less frequent than 3 per week without laxatives.

A simpler, but also acceptable definition may be that proposed by Drossman *et al.* (Drossman *et al.*, 1982): "two or fewer bowel movements per week and / or straining at stool more than 25% of the time".

1.1.2 EPIDEMIOLOGY OF CONSTIPATION AND COST

Constipation is the second most commonly self-reported gastrointestinal symptom, affecting between 2-34% of populations studied (Drossman *et al.*, 1982; Sonnenberg & Koch, 1989; Whitehead *et al.*, 1991; Camilleri *et al.*, 1994). The lack of consistency in the description of constipation may be responsible for such vast discrepancies in estimation of prevalence and epidemiological factors associated with constipation (Sonnenberg *et al.*, 1994a). When definitions such as those outlined above are employed, the prevalence is probably about 2% (Sonnenberg & Koch, 1989). At all ages, there appears to be an increased rate of physician visits by women, while, for both sexes, visits increase with age (Sonnenberg & Koch, 1989).

Most of the economic costs incurred for constipation relate to this high frequency of occurrence rather than its medical severity. Annually, constipation results in at least 2.5 million visits to healthcare professionals in the United States, the largest proportion of which are physicians (Sonnenberg & Koch, 1989). Eighty-five percent of patients receive at least one prescription for a medication, most commonly laxatives. Based on United States' data, the average direct cost ($73) and indirect cost ($6) of constipation per patient per year led to a total cost in 1985 of over $350 million (Sonnenberg *et al.*, 1994a). If the costs of self-prescribed medication and evacuation aids (estimated to be used by 12% of the population) had been added to this figure, the total would have exceeded $650 million per year. This figure is increasing: in 1994, over $725 million

were spent in the United States on laxatives alone (Wald, 1999).

1.1.3 CAUSES OF CONSTIPATION

There are a wide range of causes of constipation (Table 1.01a), and numerous methods of their classification. In general, patients can be divided into those whom routine clinical practice (clinical / radiological / endoscopic examination with routine biochemical tests) defines a cause, leaving a sub-group of patients with intractable symptoms but no definable organic pathology. This second group constitutes what is sometimes referred to as "idiopathic constipation" in recognition of the absence of a defined cause, and represents a major part of a group of gastrointestinal conditions loosely termed "functional bowel disease". This group itself may also be subdivided on the basis of specialist investigations (Table 1.01b).

1.2 SLOW TRANSIT CONSTIPATION (STC)

1.2.1 DEFINITION

Slow transit constipation is a physiological description based on the observation in a proportion of patients with severe constipation that there is a reduction in the rate of progress of colonic intraluminal contents as demonstrated by transit studies (below). The disorder encompasses patients with idiopathic constipation (the majority), as well as some with a defined systemic cause. The condition is probably synonomous with "chronic intestinal stasis" first described by Arbuthnot Lane at the turn of the century (Lane, 1908 & 1909), and possibly with some subsequent descriptions of atonic bowel (Trumble, 1934; Trumble, 1935; White *et al.*, 1940).

Whilst disturbances of transit do occur with megacolon and megarectum, the term slow transit constipation is used for patients with a normal calibre colon (Preston *et al.*, 1985b; MacDonald, 1993).

Table 1.01

a. Classification of defined structural or systemic causes of constipation

GASTROINTESTINAL CAUSES		
Colorectal	Mechanical obstruction	Benign and malignant neoplasms, other strictures: e.g. inflammatory
	Megacolon or megarectum	Hirschsprung's disease
		Idiopathic
		Neurological or other
Anorectal	Anal atresia or malformation	
	Hereditary internal anal sphincter hypertrophy	
	Anal stenosis	
	Rectal prolapse	
	Large rectocele	
	Solitary rectal ulcer syndrome	
EXTRAGASTROINTESTINAL CAUSES		
Endocrine /	Hypothyroidism	
metabolic	Hypercalcaemia	
	Porphyria	
Neurological	Degenerative CNS diseases	
	Spinal lesions	
	Damage to sacral parasympathetic nerves	
	Autonomic neuropathy	
Psychological	Severe endogenous depression	
	Eating disorders	
Drugs	Opiates	Anticholinergics
	Antidepressants	Anticonvulsants

b. Classification of functional disorders causing constipation

Simple constipation ("reversible")	*Lifestyle*	exercise
		mobility
	Diet	fibre
		hydration
Outlet obstruction	*Dynamic structural abnormalities*	rectocele
		intussusception
	Idiopathic outlet obstruction	anismus
Ineffective straining	*Failure of coordinated abdominal / diaphramatic contraction to raise intrapelvic pressure*	
Normal transit constipation	*"constipation predominant irritable bowel syndrome"*	
Idiopathic slow transit constipation	*"colonic inertia"*	

The fact that a diagnosis of STC is entirely dependent on the results of a single physiological observation cannot be over-stressed. Such a definition might in reality be guilty of pigeonholing patients with multiple pathologies into a single group, either for descriptive convenience or to aid clinical decision making. Of particular concern are observations made in man and experimental animals that painless rectal distension can inhibit proximal intestinal motility and transit (Pearcy & Van Liere, 1926; Youmans & Meek, 1937; Kreulen & Szurszewski, 1979; Youle & Read, 1984; Kellow *et al.*, 1987; Klauser et al, 1989; Brugere *et al.*, 1991; Bojö & Cassuto, 1992; Wingate, 1993; Gué *et al.*, 1995). Conversely, the cut-offs used to denote abnormality on transit estimation

might simply select for the severe end of a continuous spectrum of biologically similar patients, placing these into one group, whilst excluding others. Nevertheless, the assumption that STC represents a separate clinical entity is now so well accepted in the literature and clinical practice, that for the purposes of current studies, including those within this thesis, it has been regarded as a *fait accompli*.

1.2.1.1 Slow transit constipation: a group disorder

It is well recognised that heterogeneity of clinical presentation exists amongst patients with STC (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988; MacDonald, 1993). In the majority of patients, symptoms arise de-novo in childhood, and these have been labelled chronic and idiopathic (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988). A proportion of patients with intractable slow transit constipation present in later life. Some of these patients will have no obvious trigger for their complaint (Waldron *et al.*, 1988), whereas others follow events such as hysterectomy (Roe *et al.*, 1988; Vierhout *et al.*, 1993) or childbirth (MacDonald *et al.*, 1997). STC has similarly been demonstrated in patients with endocrine (Iber *et al.*, 1993; Maleki *et al.*, 1998), CNS (Devroede *et al.*, 1979; Weber *et al.*, 1987; Beuret-Blanquart *et al.*, 1990; Keshavarsian *et al.*, 1995; Leduc *et al.*, 1997; DeLooze *et al.*, 1998) and connective tissue diseases (Basilisco *et al.*, 1993). The term STC therefore probably encompasses a range of disorders, grouped for convenience by the finding, in a normal calibre large bowel, of a definable, measurable abnormality i.e. prolonged transit time. Whilst the clinical end-point is almost indistinguishable between patients with STC (MacDonald, 1993), heterogeneity, including that of aetiology, is likely to exist, and STC should be probably be considered as a "group disorder".

1.2.1.2 Transit studies

Methods used to measure gut transit may be classified as radiological, calorimetric, particulate, chemical, and isotopic (Hinton *et al.*, 1969). Historically, bismuth subnitrate and barium sulphate have been used as radiological markers (Hurst, 1919,

Barclay, 1936), insoluble coloured powders and coloured glass beads (Alvarez & Freelander, 1924) as particulate markers, barium, chromium and copper compounds as chemical markers (Alvarez & Freelander, 1924), and ^{51}Cr-labelled sodium chromate as an isotope marker (Hansky & Connell, 1962; Hinton *et al.*, 1969).

Radioopaque marker studies

The first description of methodology, employing radioopaque polythene cylindrical pellets, was that of Hinton *et al.* (Hinton *et al.*, 1969) who measured the disappearance of 20 such markers from the gut and their appearance in the stool by serial radiographs. They were able to establish that this marker was not absorbed, and was completely recoverable in the stool. Transit rate of the markers was thought comparable to that of food residue because of similar density of the markers to stool, and there was no evidence to suggest that the markers affected gut activity (a criticism made of previous methodologies). In addition, they determined a normal range for males, who passed the first marker by 3 days, and 80% of the markers by 5 days. Normal ranges for both men and women have subsequently been determined, with abnormal transit defined as retention of more than 20% of markers on a single plain radiograph at 5 days (Bassotti *et al.*, 1988; Evans *et al.*, 1992; Roberts *et al.*, 1993) (Figure 1.01a).

The study of marker retention with a single abdominal radiograph allows the distinction between those patients in whom the transit is normal and those in whom it is prolonged (Hinton *et al.*, 1969; Evans *et al.*, 1992; Roberts *et al.*, 1993). Attempts have been made to extend the technique to assess segmental colonic transit times using a series of abdominal radiographs following a single ingestion of markers (Arhan *et al.*, 1981), as first described by Martelli *et al.* (Martelli *et al.*, 1978). The method has been simplified and the radiation exposure of daily X-ray films reduced by giving either different shaped (Metcalf *et al.*, 1987), or even simpler, identical markers (Chaussade *et al.*, 1989) on three successive days, and taking an X-ray on the 4th day after ingestion. The utility of such studies in the determination of the pattern of colonic transit has been shown in healthy volunteers (Metcalf *et al.*, 1987), but

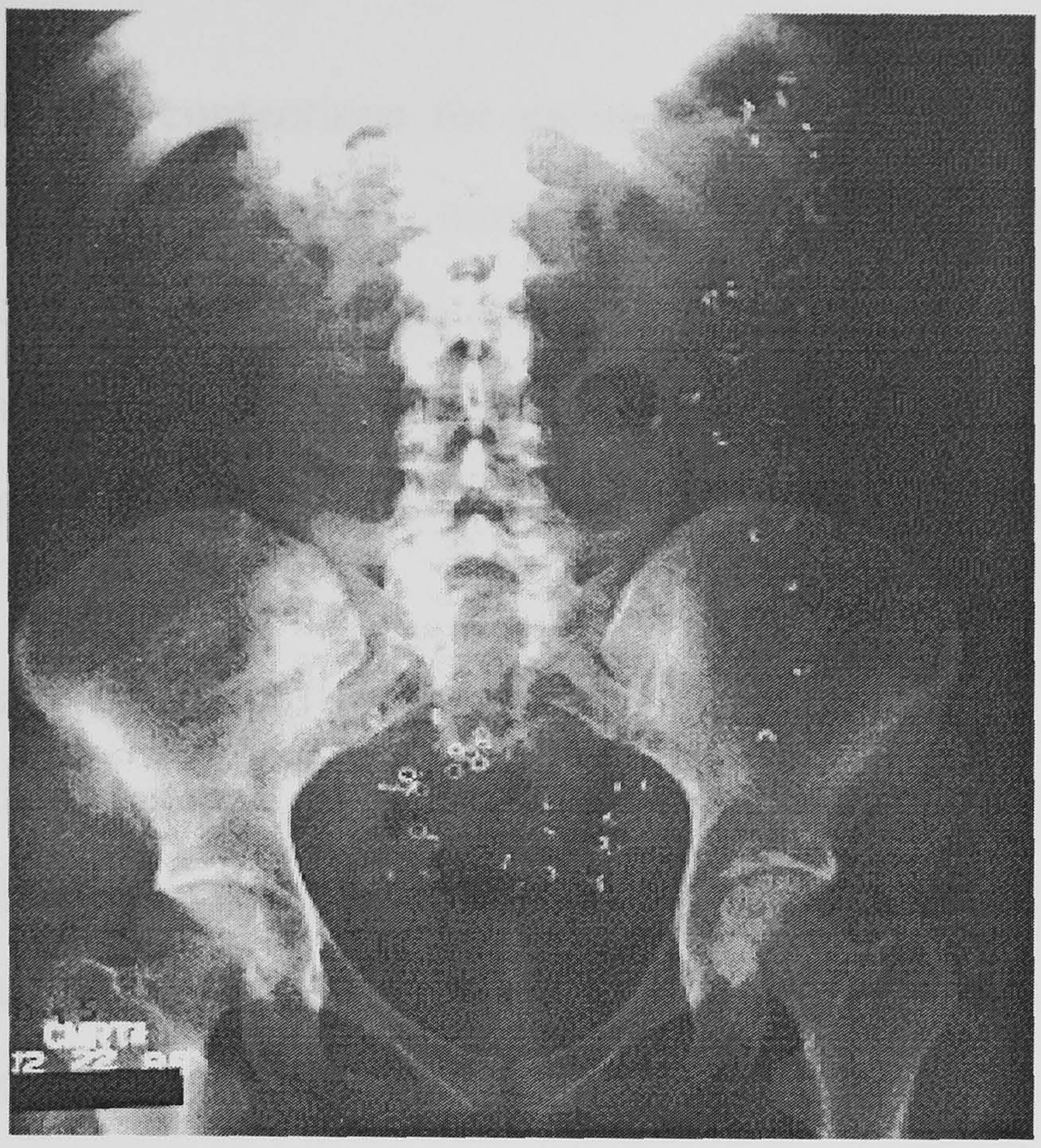


Figure 1.01 (a) Radio-opaque marker study of a patient with STC. All 50 markers (shapes) are retained at 96 hours.

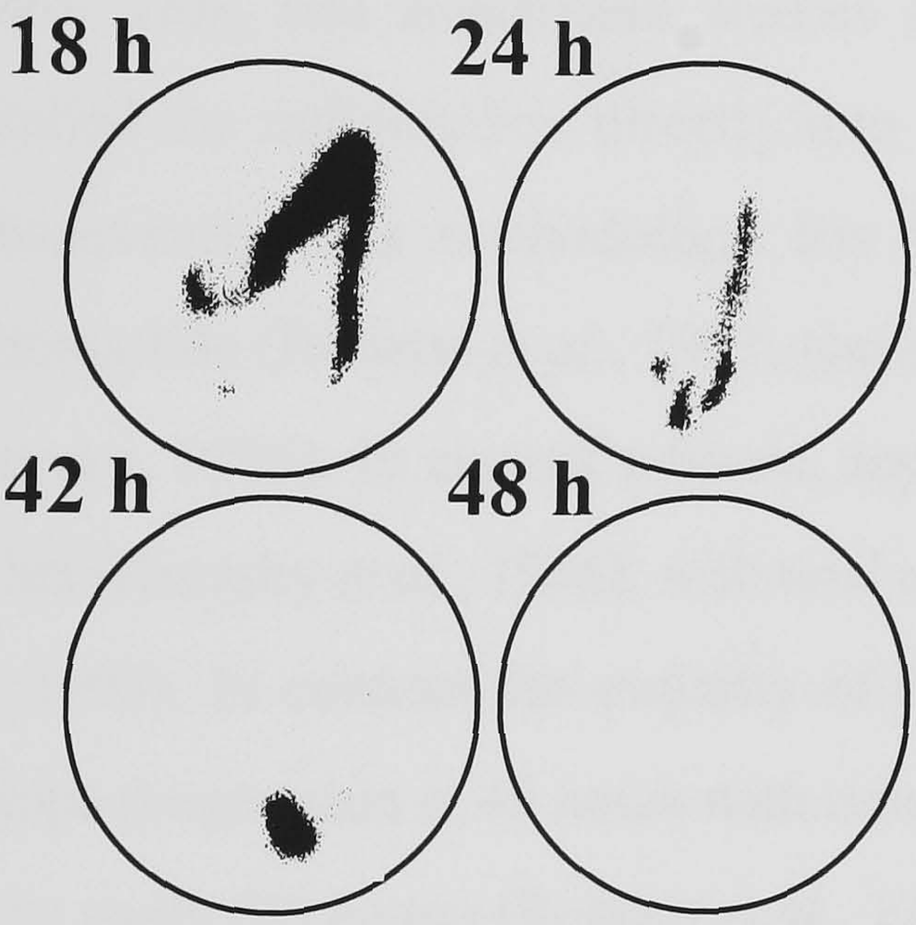
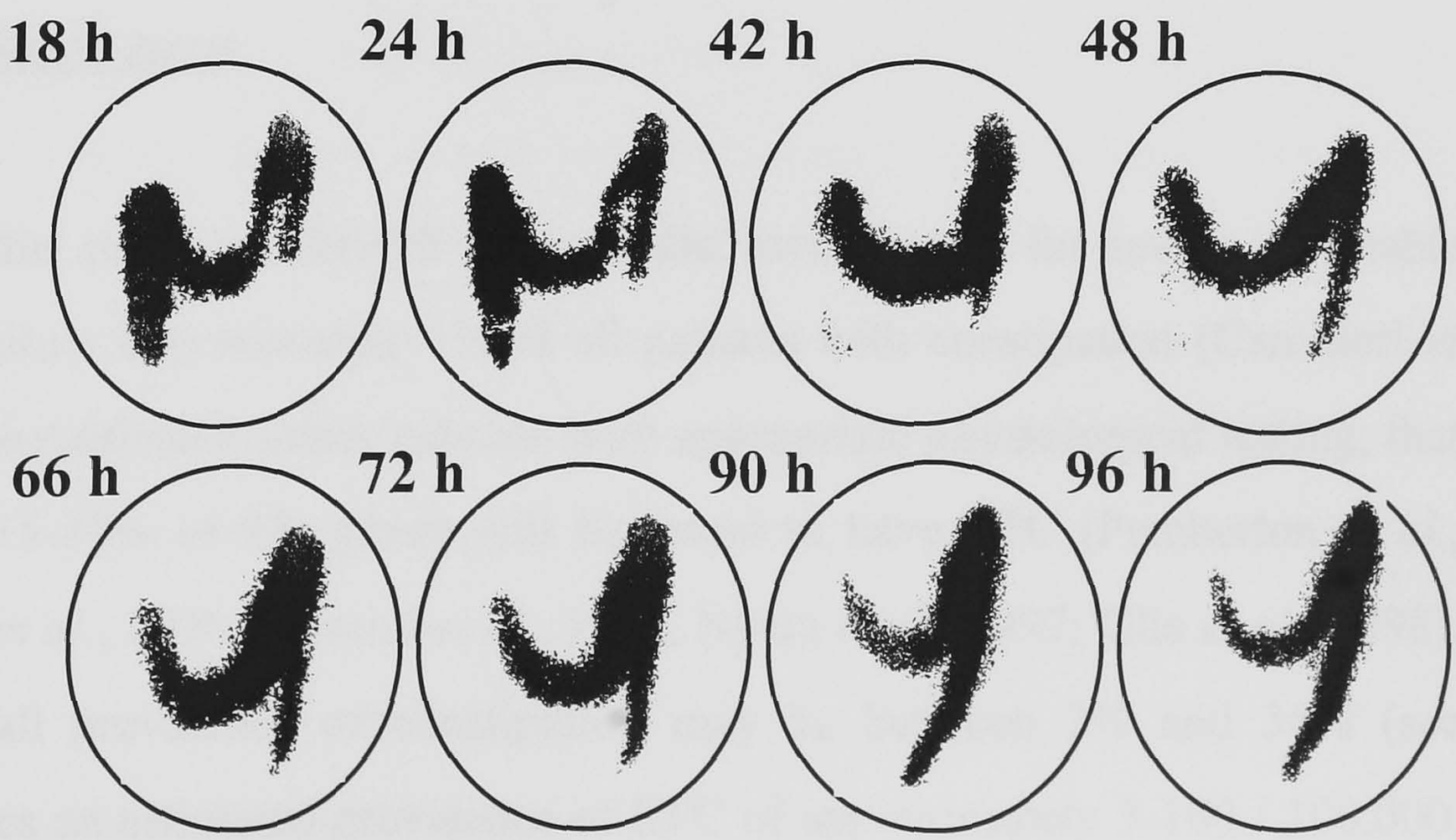


Figure 1.01 (b) ^{111}In-DTPA radioisotpe scintigraphy. Normal healthy control with complete isotope excretion by 48 hours, and a patient with STC, in which isotope progresses slowly around the whole colon with no focal areas of hold-up.



remains contentious for patients with constipation (van der Sijp *et al.*, 1993). In particular, because mass movements are infrequent events in patients with constipation (Bassotti *et al.*, 1988; see below), a large number of frequent serial radiographs would be required to adequately monitor segmental transit, and would be unacceptable with respect to radiation exposure (van der Sijp *et al.*, 1993).

Isotope transit studies

Radioisotopes are well suited to the study of regional transit in constipated patients because they allow unlimited numbers of observations at a low radiation dose. Whilst the use of isotopes (radionuclides) to study intestinal transit were first described using ^{51}Cr-labelled sodium chromate in 1962 (Hansky & Connell, 1962), the first description using ^{111}In DTPA was made in 1986 by Kreksky *et al.* (Krevsky *et al.*, 1986). This, and subsequent studies (Kamm *et al.*, 1988; Krevsky *et al.*, 1989), installed the radionuclide directly into the large bowel by intubation of the caecum. Subsequently, this methodology has been replaced by oral administration of the radionuclide (Roberts *et al.*, 1993; van der Sijp, 1993; McLean *et al.*, 1995; Maurer & Krevsky, 1995). In normal subjects, approximately 70% of radioisotope is excreted by 48 hrs (Krevsky *et al.*, 1986), with total excretion by approximately 70 hours (Roberts *et al.*, 1993). In contrast the majority of patients with STC show significant slowing of isotope progression at 48 hours with minimal faecal elimination occuring in the duration of the study (96 hours) (Roberts *et al.*, 1993) (Figure 1.01b).

1.2.2 EPIDEMIOLOGY

It is estimated that patients refererred for specialist investigation for severe intractable symptoms constitute approximately 1% of all patients with constipation (Camilleri *et al.*, 1994). Large published series indicate with appropriate physiological testing, that approximately 15-30% of this group will be found to have STC (Pemberton *et al.*, 1991; de Graaf *et al.*, 1996; Surrenti *et al.*, 1995; Nyam *et al.*, 1997; Glia *et al.*, 1998). Since the overall prevalence of constipation may be between 2% and 34% (see above), this gives an estimated prevalence of STC of approximately 3-100 / 100,000.

STC predominantly affects women, comprising 70-100% of published series (Read *et al.*, 1986; Preston *et al.*, 1986; Chaussade *et al.*, 1989).

1.2.3 CLINICAL FEATURES AND ROUTINE INVESTIGATIONS

The clinical history elicits any triggering events (e.g. pelvic surgery). The long duration of symptoms usually noted at presentation reflects the nature of onset, which is in the majority of patients in early childhood or teenage years. The main symptoms usually include difficulties with defaecation itself, and abdominal pain and bloating. Whilst generally there has been poor correlation between specific symptoms and the presence of slowed colonic transit as compared with other causes of constipation (Grotz *et al.*, 1994), patients with STC often report a loss of defaecatory urge with a marked infrequency of bowel opening, with many days, or even weeks between successive bowel actions. In addition, a high proportion complain of general malaise and lassitude, and some have upper gastrointestinal symptoms such as nausea. These symptoms have commonly come to dominate the patient's life and greatly impair its quality (Preston and Lennard-Jones, 1986). Patients with STC often give a past history of previous pelvic and abdominal surgery, and a family history of constipation is present in about half of cases (Chaussade *et al.*, 1989). Most patients with STC will have tried several laxatives with little or short-lived benefit, and several will have tried high doses of powerful stimulant laxatives.

Abdominal examination may reveal faecal loading, or gaseous distension, but this and examination of the pelvis by digital rectal examination and sigmoidoscopy are usually unremarkable. Abdominal plain X-ray and barium studies may show a long redundant colon (Brummer *et al.*, 1962) and the latter will exclude megabowel (Preston *et al.*, 1985b). It is customary to perform routine blood tests to exclude an endocrine or metabolic cause of constipation, although these are rarely present.

1.2.4 MANAGEMENT

1.2.4.1 Current therapies for STC

Non-surgical treatment

In the first instance, patients should be given reassurance and advice that dangerous disease has been excluded, and attention should be made to lifestyle, fluid intake, and dietary alterations as well to psychological or behavioural aspects.

Laxatives are effective for some patients especially in the short-term, but long-duration high dose stimulant laxatives should be used with caution because of the unproven, but potentially deleterious effect on the colon (see section 1.6.6). Bulking agents (bran, ispaghula, sterculia) are usually not effective in severe idiopathic constipation. Stool softeners (arachis oil, docusate sodium, liquid paraffin, given as an enema) may be of value in assisting evacuation of stool. Stimulant laxatives induce secretion and propulsive movements in severely constipated patients (Kamm *et al.*, 1992), and fall into 3 groups: anthranoids e.g. senna, aloe and danthron; polyphenol derivatives e.g. phenolphthalein, bisacodyl and sodium picosulphate, and miscellaneous compounds e.g. docusate sodium (also a stool softener). Another group of compounds are the osmotic laxatives (mannitol, lactulose, magnesium salts etc.), which increase the fluid content of the stool and facilitate expulsion of the bolus by lubication and softening.

Biofeedback, a form of behavioural therapy, has been used for a long time to strengthen the pelvic floor in patients with faecal incontinence (Engel *et al.*, 1974). More recently, it has been utilised in patients with obstructed defaecation with variable success rates from 8 – 100% (Rieger *et al.*, 1997; Gilliland *et al.*, 1997). Inappropriate pelvic floor contraction in many of these patients during attempted defaecation formed the original focus for behavioural therapies, and most groups have restricted the use of biofeedback to patients with normal transit and paradoxical puborectalis contraction during straining (Wexner *et al.*, 1992; Karlbom *et al.*, 1997).

Such therapy may however be equally effective in symptom reduction for patients with STC (Chiotakakou-Faliakou *et al.*, 1998), and may even normalise measured transit time (Koutsomanis *et al.*, 1994). Indeed, the St Mark's group (Chiotakakou-Faliakou *et al.*, 1998) use biofeedback as a first-line treatment for all patients presenting with intractable constipation (*Michael Kamm; personal communication*).

Psychotherapy has been shown to have a role in the treatment of normal-transit constipation (IBS) (Camilleri, 1999), and may have been a contributory factor in the success of some trials of behavioural therapies such as biofeedback, especially for pelvic floor disorders. In STC, the role of psychogenic factors is small in comparison with these pathophysiological groups (Wald *et al.*, 1989; Grotz *et al.*, 1994; see 1.6.7 for detail). No specific data exist to confirm or refute the effect of psychotherapy for STC patients and treatment is generally directed at possible modification of the disordered transit itself (Wald *et al.*, 1989).

Surgery for slow colonic transit

Because most medical lines of management are relatively ineffectual, surgery may be contemplated. Since the beginning of the century, surgery for STC has been loosely based on a concept of the pathology (colonic stasis) (Albuthnot Lane, 1908; Albuthnot Lane, 1909). Colectomy and ileorectal anastomosis has remained the treatment of choice for STC in preference to other surgical options, such as limited resection (Preston *et al.*, 1984a). The outcome from such surgery is highly variable, with widespread variability in satisfaction rates, incidences of post operative complications and functional outcome. In a review performed by the author (Knowles *et al.*, 1999), 32 studies (1981-1998) which had fulfilled the entry criteria of (1) publication in the English language, (2) description of outcome of $\geq$ 10 patients with STC, (3) excision of all or part of the colon, with restoration of bowel continuity by primary anastomosis, i.e. subtotal or segmental colectomy, were analysed. Overall documented patient success / satisfaction rates varied greatly from 39% (Hawagewa *et al.*, 1999) to 100% (Pemberton *et al.*, 1991). Post-operative morbidity rates from small bowel obstruction (with or without re-operation) varied from 2% to 71% (median 18%), and resulted in re-operation in 0% to

50% of patients (median 14%). Functional outcome measures were often poorly documented, and were also highly variable. Poor functional outcome related principally to the incidence of diarrhoea: range 0 - 46% of patients (median 14%), incontinence: range 0 - 52% (median 12%), recurrent constipation: range 0 - 33% (median 7%), and abdominal pain: range 0 - 90% (median 39%). As a result of poor functional outcome, permanent ileostomy was formed in up to 28% of patients (Hasegawa *et al.*, 1999), (median 5%, range 0 - 28%). It is now generally agreed that surgery for STC should probably only be considered in a highly selected group of patients who fulfil certain clinical and physiological criteria (Pfeiffer *et al.*, 1996; Knowles *et al.*, 1999)

1.2.4.2 Novel therapies for STC

The search continues for pharmacological agents that might be able to regulate colonic motility. Initial enthusiasm for the known enterokinetic erythromycin (Minocha *et al.*, 1995), has been tempered by its failure to have a prokinetic effect on distal colonic motility (Bassotti *et al.*, 1998). The prostaglandin analogue, misoprostol has been shown to be of some value (Soffer *et al.*, 1994), and continues to be used, especially in the U.S.A (*Arnold Wald; personal communication*), although side-effects are a problem (Roarty *et al.*, 1997). Other recently considered agents have included cholecystokinin-like peptides, and opioid antagonists (see section 1.6.5), neither of which have shown great future promise (Briejer *et al.*, 1999). 5-hydroxytryptamine (5-HT) agonists continue to receive much attention. Cisapride, a gastrointestinal motility stimulant and 5-HT_4 receptor agonist, accelerates colonic transit and is beneficial in a subset of constipated patients (Longo *et al.*, 1995), but its effects are only moderate and therefore it is not considered a standard treatment (Muller-Lissner, 1995). Novel, more selective 5-HT_4 receptor agonists, such as prucalopride are currently undergoing evaluation. Prucalopride has been shown to induce propulsive contractions in a dose-dependent manner in dogs (Briejer *et al.*, 1997), accelerate transit and increase stool frequency in healthy subjects (Emmanuel *et al.*, 1998), and reduce symptoms in patients with chronic constipation including STC (Felt-Bersma, 1999).

Resarch into the pharmacology of cardiac arrhythmias has focussed on drugs that affect voltage-gated channels involved in generating diastolic depolarisation. Ion channels specifically involved in the regulation of intestinal motility, especially slow-wave activity, have not as yet been identified, but it seems likely that such pharmacological modification of intestinal pacemaker activity may be possible in the future (Huizinga *et al.*, 1997). The importance of interstitial cell function is discussed below, and that of ion channels in chapter 7.

1.3 NORMAL COLONIC CONTRACTILE ACTIVITY

Gastrointestinal (GI) motility is a term used to describe the mixing and propulsive movements of the alimentary canal, responsible for the transit, digestion and absorption of intraluminal food contents. In patients with STC, normal defaecation is compromised by an abnormal slowing of colonic contents (transit). Since transit is dependent upon the normal contractile functions of the colon, STC should be thought of as a motility disorder.

In man, the contractile activity of a viscus can be measured by recording changes in intraluminal pressure i.e. manometry, and information regarding the ability of the intestine to propel its contents, inferred from this data. Whilst a variety of techniques are available, the most commonly employed are perfused tubes or catheter mounted strain gauges. Advances in the placement and positioning of new devices by colonoscopy, and in the use of solid state transducers linked to portable recording systems with enhanced memory capacities, now enable 24 hour ambulatory studies of colonic motility to be performed. However, the majority of studies to date have been carried out in the left side of the colon only, under circumstances where basal physiological conditions were not usually observed (non-ambulant subjects, prepared bowel) (e.g. Richie *et al.*, 1962; Frexinos *et al.*, 1985). Monitoring of proximal colonic motility has rarely been achieved (Soffer *et al.*, 1989; Lémann *et al.*, 1995), and prolonged recordings of colonic motility are scant (Narducci *et al.*, 1987; Soffer *et al.*, 1989; Crowell *et al.*, 1991; Lémann *et al.*,

1995). The reproducibility and methods used for interpretation of data produced by such studies still require validation.

The term motility encompasses various patterns of motor activity in the digestive tract. In general, the colon can be considered to have 3 broad categories of motor activity, which are considered to be the motor correlates of the movements required to subserve the functions demanded by this organ.

1.	Individual phasic contractions (short and long duration)	Mixing (pendicular or segmental) movements
2.	Organised groups of contractions e.g. migrating & non-migrating motor complexes	Propulsion over short distances or further mixing
3.	Specialised high amplitude propagated contractions	Mass movements / defaecation

Normal colonic motility is characterised by a relative background quiescence with a general infrequency of motor events, providing an optimal environment for water and electrolyte absorption. Phasic contractions are the basic unit of contractile activity throughout the GIT. In the colon, the individual phasic contractions are of 2 types: short-duration (<15 secs), and long-duration (40-60 secs) (Sarna, 1991). Such contractions are primarily non-propagating or propagate only short distances, and these appear to facilitate mixing and mucosal absorption. The role of organised (periodic) groups of contractions in the colon (Narducci *et al.*, 1987) is less clear than in the small intestine, but these probably spread faeces over a short distance, predominantly in the caudad direction (Sarna 1991). The colon also has specialised high amplitude propagated contractions (HAPCs), which are the motor correlates of mass movement waves (Holzknect, 1909; Richie *et al.*, 1962; Hardcastle & Mann, 1968; Torsoli, 1971; Narducci *et al.*, 1987), and which propel intraluminal contents rapidly over large

distances. These occur spontaneously, approximately 3 – 6 times per day (Narducci *et al.*, 1987; Soffer *et al.*, 1989; Crowell *et al.*, 1991; Lémann *et al.*, 1995), originate mainly in the proximal colon, and also accompany most episodes of spontaneous defaecation (Karaus & Sarna, 1987; Scott *et al.*, 1995). In addition, the colon displays tonic contractile activity (Steadman *et al.*, 1991). In health, phasic activity, tonic activity, and HAPCs increase in frequency after awakening from sleep, or eating (Narducci *et al.*, 1987; Soffer *et al.*, 1989; Crowell *et al.*, 1991).

1.4 THE CONTROL OF COLONIC MOTILITY

The mechanisms that control gastrointestinal motility have been studied more extensively in the foregut than hindgut, and more completely in experimental animals than in man. Nevertheless, the following general points can be made about the control of human colonic motility.

Movements of the colon have underlying patterns of contractile activity which are the "motor correlates" of each movement type. These motor correlates in turn have underlying electric events which are termed the "myoelectrical correlates" of motor activity. Such electrical events arise on a background of spontaneous, intrinsic "pace making" electric activity which is omnipresent (Figure 1.02).

Three main control mechanisms co-operate in the regulation of the normal transit of contents through the gastrointestinal tract. These are

Myogenic	Control systems lying within the musculature itself
Neural	Enteric, autonomic, and central nervous system influences
Chemical	Neurotransmitters, hormones and other peptides

Although they are often considered individually for convenience (including in the description below), these control systems do not act independently, but are closely functionally intertwined.

Whilst some motor events are spontaneous, many are activated by intraluminal distension or chemical stimuli. Such reflex events are of primary importance to the transit of colonic contents, and have been studied in health and disease.

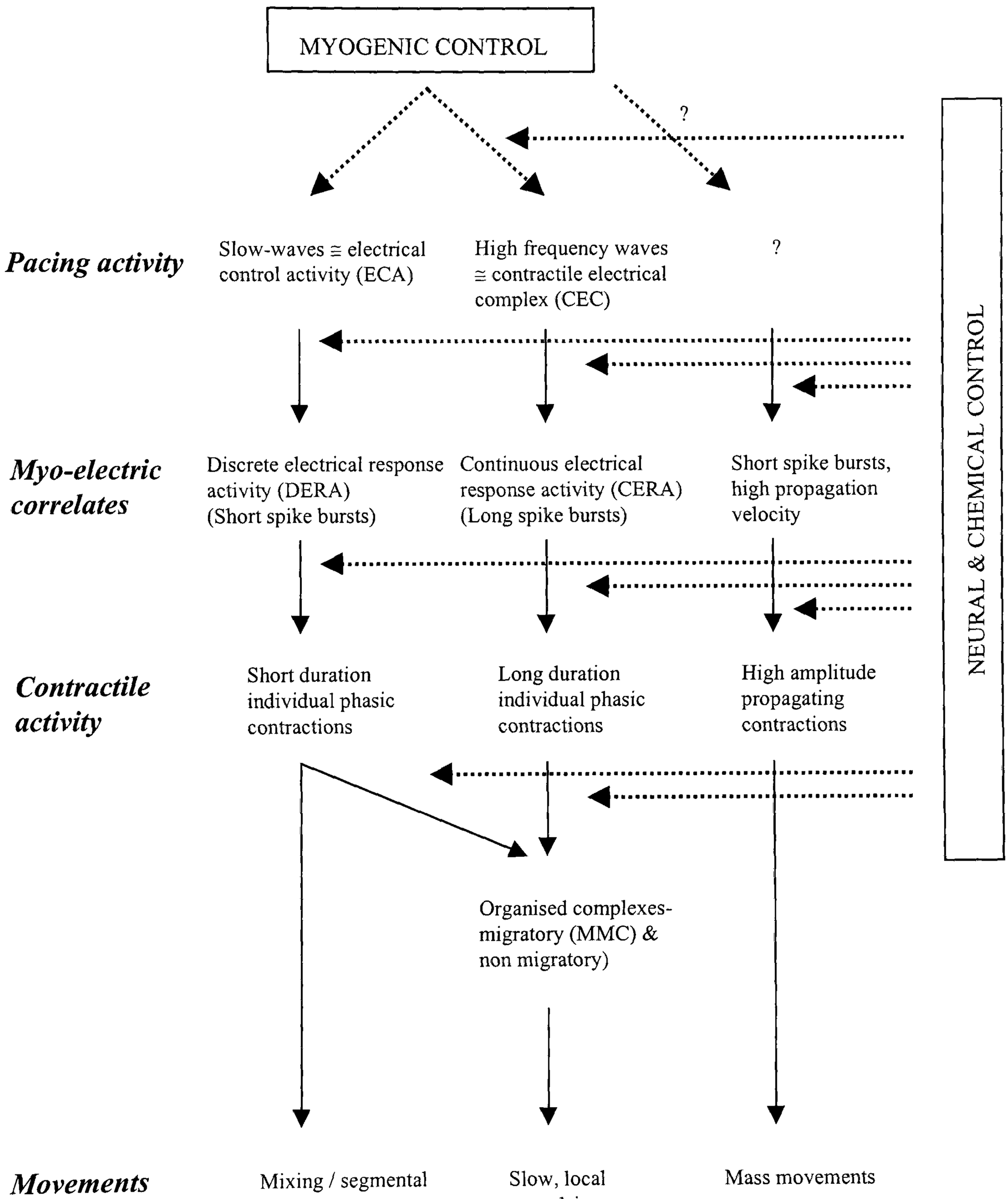


Figure 1.02: Schematic overview of the control of colonic motility:

KEY: ? = unknown. *See text for description.*

1.4.1 MYOGENIC CONTROL MECHANISMS

The muscle coat of the gut is a continuous structure made of smooth muscle. Intestinal muscle cells are small uninucleate, spindle shaped cells with a high surface area to volume ratio, and which contain the necessary organelles and filaments (actin and myosin) for the conversion of chemical energy into shortening. Muscle cells lie approximately parallel to each other forming sheet-like layers. In the colon, two layers are superimposed, with their cells orientated at right angles to each other to form the circular (inner), and longitudinal (outer) musculature. The latter is very attenuated except in 3 regions around the circumference (taeniae), and the circular muscle forms the bulk of the muscularis propria. Whilst the longitudinal muscle and circular muscle layers have been shown *in vitro* to have different myoelectric and motor activities (El-Sharkawy 1983; Huizinga *et al.*, 1983), it is likely that they function as a syncytium *in vivo* (Huizinga *et al.*, 1983), and are considered as such in this description.

The gastrointestinal tract, when activated by intestinal contents or neural activity, produces local contractions lasting several seconds, each of which is followed by relaxation in a regular periodic manner. These contractions cause a local, partial luminal occlusion, which propagates in an arboral direction and moves gut contents anally. The frequency and propagation characteristics of the contractions are characteristic for each organ, and are governed by intrinsic omnipresent, electrical membrane-potential oscillations, the so-called "slow-waves" or electric control activity (ECA) (frequency = 2-13 / min in human colon) (Sarna, 1991). The slow-waves represent the pacemaker activity activity of the gastrointestinal contractile activity, and translate an excitatory, usually neural, stimulus into orderly peristaltic movements.

Slow-waves are generated by specialist cells, the interstitial cells of Cajal (ICCs) (Thuneberg, 1982; Sanders *et al.*, 1989; Huizinga *et al.*, 1995; Sanders, 1996). These cells were first described more than 100 years ago (Cajal, 1893) as small fusiform or stellate cells that formed networks in gastrointestinal tissues. Subsequent work (Christensen *et al.*, 1987) has established these to be unique specialised fibroblast-like

cells which lie between the axonal plexuses of the gut and the smooth muscle itself. In the large intestine they are found particularly in relation to a dense axonal plexus which lies in the submucosa, immediately adjacent to the muscularis propria (Stach's plexus externus extremus) (Stach, 1972), but are also found in association with the myenteric plexus. [An additional plexus is found within the circular muscle layer in the small intestine].

The slow-wave activity generated by ICCs controls short-duration contractions. Such temporally and spatially discoordinated activity, which predominates in the colon, is perfectly suited to fulfil the colonic motor function of mixing of intraluminal contents (Sarna, 1991). The significance of slow-wave activity to transit has been shown in murine models in which slow wave-driven peristalsis is absent (Ward *et al.*, 1994; Huizinga *et al.*, 1998). In addition to slow waves, high frequency waves are also generated, the so called contractile electrical complex (CEC) (frequency = 25-40 / min) (Sarna, 1991). These are intermittent rather than omnipresent, and probably require neural or chemical stimulation to be present (Huizinga and Daniel, 1986; Sarna, 1991). Unlike the ECA, the CEC controls long duration contractions.

1.4.2. NEUROGENIC CONTROL MECHANISMS

Neural control mechanisms are integral to the generation of propulsive activity in the gastrointestinal tract. Afferent nerves generate and convey, via specialised endings, visceral sensory information. Efferents control motility but also have modulatory roles in secretory and absorptive functions.

The enteric nervous system is a collection of neurones that control the motility, exocrine and endocrine secretions and microcirculation of the gastrointestinal tract (Furness & Costa, 1987, Wingate, 1993). It can further be considered to have 2 components: intrinsic neurones whose components are intramural, and lie in plexuses which consist of ganglia (clusters of tightly packed nerve cell bodies and glial cells), connected by intraganglionic fascicles of nerve fibres arising principally from the

nerve cells, and extrinsic neurones whose cell bodies lie extra-intestinally, though they may subsequently follow an intramural course. Extrinsic neurones are generally considered to be part of the autonomic nervous system (ANS), which is in turn influenced by the central nervous system (CNS). The hierarchy of neural control in the colon, like the rest of the gastrointestinal tract, may be the ENS > ANS > CNS (Wingate, 1993) (Figure 1.03).

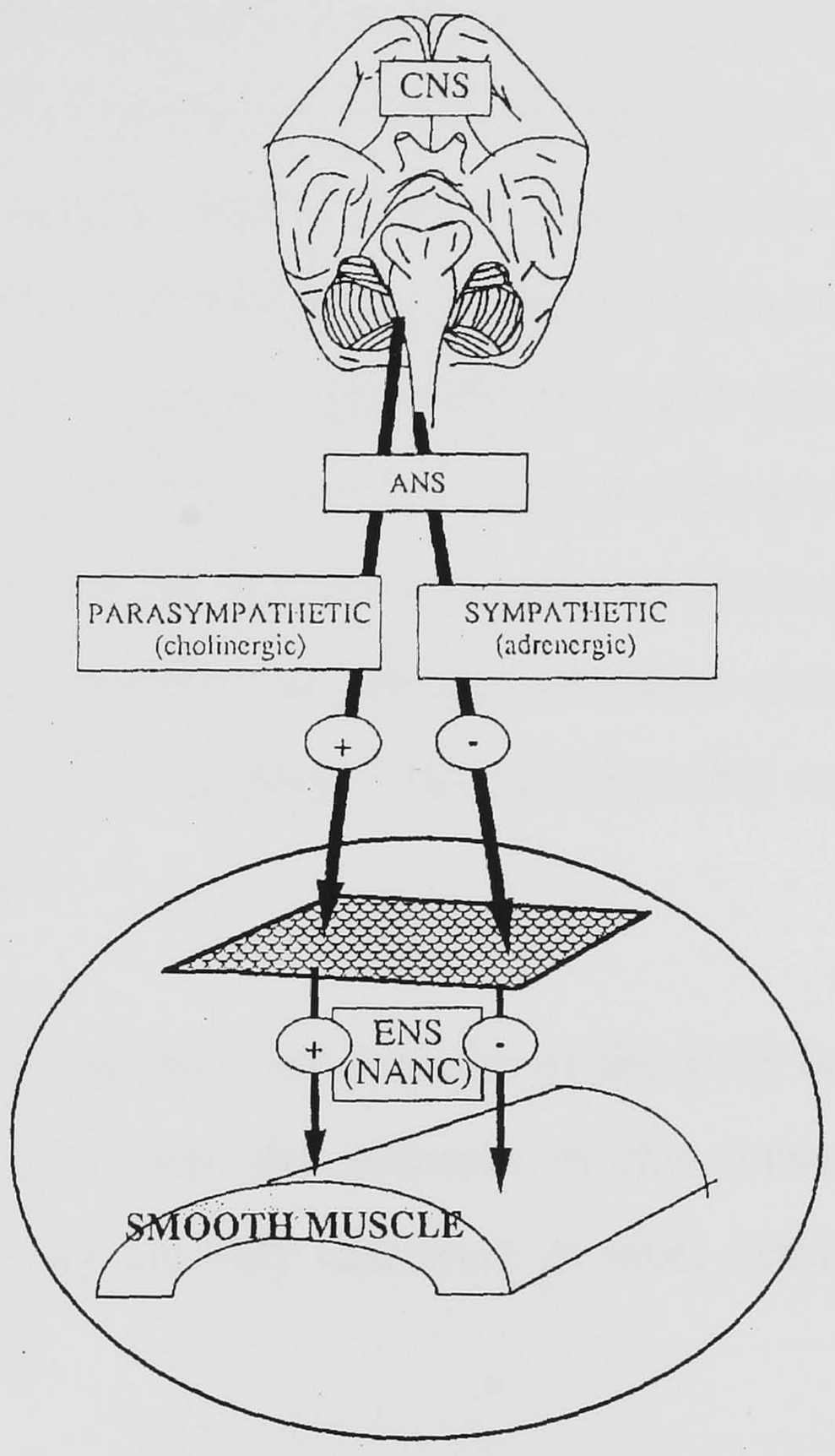


Fig 1.03: Autonomy of the ENS in the control of motility. Motor programmes are generated within the ENS and the contribution of extrinsic innervation is limited to modulation. The hatched plane represents the ganglionic layer of the myenteric plexus. The majority of intrinsic, post-ganglionic fibres are now known to be non-adrenergic, non-cholinergic (NANC) as shown. *With kind permission from Wingate, 1993.*

The ENS exerts almost continuous spatial and temporal control of colonic contractions, whereas the ANS and CNS chiefly modulate ENS activity, apart from during defaecation, where the CNS input is transmitted directly through autonomic nerves (Sarna, 1991, Wingate, 1993). The ENS participates in the reflex activity which is of particular importance to the generation of propulsive contractile activity.

Neural control provides the necessary stimulation, by the release of neurotransmitters, for the depolarisation of the muscle membrane to yield electrical complexes, which control propulsive contractions (Figure 1.02). Postganglionic highly branched axons reach the effector smooth muscle where they become beaded or varicose. Neurotransmitters are released from these varicosities during impulse conduction. Co-transmission i.e. the release of more than one transmitter from neurones (Burnstock, 1976), is very common in the ENS, and it is likely that many of these substances have neuromodulatory effects on the release or actions of primary transmitters, and / or have trophic roles. Whilst nerves can directly stimulate smooth muscle cells, ICCs also function in conveying (Thuneberg, 1982), and possibly amplifying (Publicover *et al.*, 1993) neural stimuli.

The morphology, neurochemistry and function of the ENS is a complex and rapidly expanding subject in itself. For the purposes of this thesis, neural influences on colonic contractile activity are only discussed in more detail with relevance to the pathophysiology of STC.

1.4.3 CHEMICAL CONTROL MECHANISMS

Chemical control of intestinal motility refers to the modulation of contractile activity via the release of chemical substances from nerve varicosities or endocrine-paracrine cells. An ever expanding number of such chemicals have been isolated, which may act directly on smooth muscle, pre-synaptic or post-synaptic enteric neurones, paravertebral or prevertebral ganglia, the spinal cord, or the CNS, to stimulate or inhibit contractions of the gut. They may work as endocrine, neurocrine, or paracrine

substances to modify contractile activity. The chief substances include established neurotransmitters, amines, peptides and fatty acid derivatives, such as prostaglandins and sex steroids. Such chemical modulators of motility are discussed with respect to postulated alterations in their function that might underly the aetiology of STC.

1.5 PATHOPHYSIOLOGY OF SLOW TRANSIT CONSTIPATION

1.5.1 PHYSIOLOGICAL ABNORMALITIES IN STC

The finding of an overall reduction in colonic transit, which defines STC, has been discussed. Some further information may also be attained from transit studies. It is possible to distinguish between a generalised colonic or distal colonic delay (Krevsky *et al.*, 1989; Roberts *et al.*, 1993), and in patients with the same pattern of transit disturbance, the progression of the radionuclide marker is slower in some than others (Kamm *et al.*, 1988; Krevsky *et al.*, 1989; Roberts *et al.*, 1993; van der Sijp *et al.*, 1993). The significance of these observations with respect to either the pathophysiology of STC, or its treatment, especially in terms of choice of surgical resection remains unclear.

1.5.1.1 Colonic contractile activity

Whilst transit studies are a relatively safe and simple method of estimating colonic propulsive contractile function, they must be regarded as an indirect measure of such activity. The use of manometric techniques (1.3) has allowed the direct study of colonic contractile activity in patients with STC. The paucity of data in man with respect to the normal pancolonic measurement of contractile activity, especially in the ambulant state has been noted and care must be taken therefore in reaching conclusions about studies in patient groups.

Pancolonic studies

Few publications have specifically selected constipated patients for study of pan-colonic motility on the basis of delayed transit (Bassotti *et al.*, 1993; Bassotti *et al.*, 1994; Bassotti *et al.*, 1998; Bassotti *et al.*, 1999a; Bassotti *et al.*, 1999b). These studies have shown a reduction in the number, amplitude and duration of propulsive high amplitude waves (HAPCs) in patients with STC, although these findings had also been observed in constipated patients with severe idiopathic constipation in which the transit was not specified (Kamm *et al.*, 1988), and also in constipated patients with normal transit (Bassotti *et al.*, 1994).

The gastrocolonic response has been shown to be impaired in patients with severe idiopathic constipation (Bassotti *et al.*, 1988; Bazzocchi *et al.*, 1990). In one study, the early post-prandial motor response was absent in patients with slow transit (Kamm *et al.*, 1988). In contrast, no differences have been observed in the motor response to sudden awakening (Bassotti *et al.*, 1999a). A reduced colonic motor response to intraluminal bisacodyl has been demonstrated in some patients with STC (Kamm *et al.*, 1988). This observation, has however been refuted by more recent studies of patients with severe idiopathic constipation (Kamm *et al.*, 1992) and with proven STC, in which, the majority (88%) had an intact bisacodyl response (Bassotti *et al.*, 1999b). Motor responses following intravenous administation of the anticholinesterase, edrophonium, were reduced in patients with STC (Bassotti *et al.*, 1993).

Rectosigmoid studies

There have been a number of studies of rectosigmoid motility in intractable constipation, although few papers specifically selected only patients with STC. These and other studies had paradoxical findings: in some, overall motility (Waldron *et al.*, 1990; Ferrara *et al.*, 1994), post prandial response (Reynolds *et al.*, 1987; Grotz *et al.*, 1993; Ferrara *et al.*, 1994; responses to awakening (Ferrara *et al.*, 1994) and responses to cholinergic stimulation (Grotz *et al.*, 1993) and intrarectal bisacodyl (Preston & Lennard-Jones, 1985; Shouler & Keighley, 1986) were all reduced. However, other studies have shown normal basal levels of rectosigmoid motility (Shouler & Keighley,

1986; Reynolds *et al.*, 1987; Waldron *et al.*, 1988), and a normal response to cholinergic stimulation (Reynolds *et al.*, 1987) or bisacodyl (Kamm *et al.*, 1992). A reduced tone in the left colon has been suggested by one study (O'Brien *et al.*, 1996).

1.5.1.2 *In-vitro* physiological studies of colonic tissue

The results of organ bath studies of colonic muscle from patients with constipation have been conflicting. Hoyle *et al.* (Hoyle *et al.*, 1992) found little difference overall between the electrical properties of the circular smooth muscle of the sigmoid colon from patients with idiopathic STC and those of controls. Slater *et al.* (Slater *et al.*, 1997) suggested that denervation hypersensitivity and an intrinsic myopathy were present, on the basis of altered carbachol responses with or without the addition of cisapride. In addition to the inclusion of only 5 patients, the interpretations reached by this study are not clear. Further *in vitro* studies have found alterations in the release of neurotransmitters to field stimulation with a reduced release of acetylcholine (ACh) (Burleigh *et al.*, 1988), and increased release of inhibitory neurotransmitters (NO, ATP) (Mitolo-Chieppa *et al.*, 1998). Alterations in the contractile responses to neurotransmitter application have similarly been demonstrated with a reduced contraction in response to ACh, and increased relaxation after inhibitory neurotransmitter application (Mitolo-Chieppa *et al.*, 1998).

1.5.1.3 Anorectal and pelvic floor physiological abnormalities

Abnormalities of rectal emptying with anatomical abnormalities (Turnbull *et al.*, 1988; Wald *et al.*, 1990; Karlbom *et al.*, 1995; MacDonald *et al.*, 1997), rectal hyposensation (Read *et al.*, 1986; Akervall *et al.*, 1988; Waldron *et al.*, 1988; Kamm & Lennard-Jones, 1990), or paradoxical puborectalis contraction (Turnbull *et al.*, 1986, Shouler & Keighley; 1986; Wald *et al.*, 1990; Miller *et al.*, 1991) have been well documented in patients with STC. Similarly, abnormalities of sphincteric function have been reported using anorectal manometry (Read *et al.*, 1986; Wald *et al.*, 1990). These abnormalities may equally be found in patients with constipation without STC (Turnbull *et al.*, 1986;

Wald *et al.*, 1990; Miller *et al.*, 1991; Wald *et al.*, 1993; Karlbom *et al.*, 1995), and indeed, also in subjects with no evacuatory symptoms (Shorvon *et al.*, 1989; Voderholzer *et al.*, 1997).

1.5.1.4 Upper gastrointestinal motility

The presence in some patients of upper gastrointestinal symptoms prompted the investigation of patients with STC for upper gastrointestinal motor abnormalities. Dysmotility is now well documented in idiopathic STC, with motor abnormalities (usually reduced overall motility) demonstrated in the oesophagus (Watier *et al.*, 1983; Reynolds *et al.*, 1987; Redmond *et al.*, 1995; Ghosh *et al.*, 1996) stomach (Reynolds *et al.*, 1987; Van der Sijp *et al.*, 1993; Ghosh *et al.*, 1996; MacDonald *et al.*, 1997), duodenum (Bassotti *et al.*, 1996; Glia & Lindberg, 1998; Mollen *et al.*, 1999), ileum (Panagamuwa *et al.*, 1994) and gallbladder (Hemingway *et al.*, 1997). The term "generalised intestinal disorder" (GID) has been used to describe patients with STC who have extracolonic gastrointestinal dysmotility (Redmond *et al.*, 1996)

1.5.1.5 Extragastrointestinal physiological abnormalities

Studies of patients with idiopathic STC have demonstrated sensory or motor abnormalities of the urinary tract (Abdel-Rahman *et al.*, 1981; Watier *et al.*, 1983; Bannister *et al.*, 1988; Kerrigan *et al.*, 1989). Evidence of autonomic dysfunction has been found by peripheral sweat-testing in patients with STC (Altomare *et al.*, 1992), with similar abnormalities also reported in other functional gastrointestinal disorders (Camilleri & Fealey, 1990; Bharucha *et al.* 1993; Camilleri *et al.*, 1993).

1.5.1.6 Discussion

The results of physiological studies in STC pose some problems in interpretation. In the colon, studies demonstrating a minimal colonic motor response to intraluminal bisacodyl (a surface laxative) (Preston & Lennard-Jones, 1985; Shouler & Keighley,

1986; Kamm *et al.*, 1988) might be indicative of an intrinsic neuronal problem, since this effect can be blocked by lignocaine (Hardcastle & Mann, 1968). However, the precise receptor, site and mode of action of this laxative remain unclear, and further reports have refuted this observation (Bassotti *et al.*, 1999b). In general, both *in vivo*, and *in vitro* studies point towards an altered cholinergic innervation. e.g. *in vivo*, the reduced reponses to intravenous edrophonium (Bassotti *et al.*, 1993; Grotz *et al.*, 1993), and increased responses to surface application of choline esters such as bethanecol (Waldron *et al.*, 1988; Slater *et al.*, 1997). However, such responses to edrophonium have not been observed by all studies (Reynolds *et al.*, 1987), and the increased response to choline esters, which are said to be indicative of cholinergic denervation supersensitivity (Cannon, 1934; Lapides *et al.*, 1962) are at variance with the *in vitro* reduced response to surface application of ACh itself (Mitolo-Chieppa *et al.*, 1998). Whether the demonstration of an altered release of neurotransmitters in response to field stimulation (Burleigh *et al.*, 1988; Mitolo-Chieppa *et al.*, 1998) supports an intrinsic neuronal problem is arguable (especially for ACh, which is both an intrinsic and extrinsic excitatory neurotransmitter). Similarly a disturbed gastrocolonic or awakening response could theoretically be indicative of extrinsic or intrinsic denervation, or a myopathy.

1.5.2 ABNORMALITIES OF SMOOTH MUSCLE

The important role of smooth muscle in the control of motility, as well as being the final effector of gastrointestinal motor activity has been described (1.4.1). In terms of human disease, recent advances have identified some (albeit rare) underlying smooth muscle structural and morphological abnormalities, which may be primary or secondary (see reviews: Smith & Milla 1997; Martin *et al.*, 1999).

Most reported myopathies are characterised by intestinal dilatation e.g. intestinal pseudoobstruction, although some patients may present with constipation. Morphological changes seen include varying degrees of fibrosis, hypertrophy or atrophy of muscle fibres, vacuolation of myocytes (Smith & Milla, 1997; Schuffler *et*

al., 1977; Martin *et al.*, 1990), alteration in the immunohistochemical staining pattern of myocyte contractile proteins (Smith *et al.*, 1992), abnormal layering of the muscle (Smith & Milla, 1997), and the presence of intracellular inclusion bodies (Martin *et al.*, 1990).

In contrast, consistent myopathic findings have never been demonstrated by light microscopy in patients with idiopathic constipation (Dyer *et al.*, 1969; Smith *et al.*, 1977; Hughes *et al.*, 1981; Krishnamurthy *et al.*, 1985; Kamm *et al.*, 1988; Ghosh *et al.*, 1996), or STC (Pemberton *et al.*, 1991; Benson *et al.*, 1992; Redmond *et al.*, 1995; Pluta *et al.*, 1996). One study (Park *et al.*, 1995) demonstrated an increased ratio of the circular layer to longitudinal layer of the muscularis propria in the descending and sigmoid colon. Similarly, *in-vitro* physiological studies have had conflicting results (Hoyle *et al.*, 1992; Slater *et al.*, 1997; Mitolo-Chieppa, 1998). Whilst a single ultrastructural study has observed some changes in patients with STC i.e. muscle cell degeneration in the muscularis propria and increased collagen in the longitudinal muscle layer (Benson *et al.*, 1992), detailed ultrastructural studies of smooth muscle have generally not been performed (unlike, for instance, cases of childhood pseudoobstruction). Inclusion bodies have recently been reported in small numbers of patients with STC (Martin *et al.*, 1999).

1.5.3 ENTERIC NEURONAL ABNORMALITIES

A multitude of studies have provided evidence for morphological abnormalities of colonic enteric neurones in patients with constipation. The number of studies in which there is documentation of slow transit for all patients is more limited, but their inclusion may be inferred in others. The results of such studies have been tabulated for completion, but are excluded from prolonged discussion in the text.

1.5.3.1 Routine and silver staining (Table 1.02)

In colonic tissue from patients with STC, routine light microscopy has failed to identify

a persistent abnormality other than melanosis coli (Redmond *et al.*, 1996; Preston *et al.*, 1984b; Kamm *et al.*, 1988), although 2 studies found variable nerve abnormalities by H&E staining in 3 / 9 (Watier *et al.*, 1983) and 5 / 24 (Pluta *et al.*, 1996) STC colons respectively. Described changes include axonal vacuolation, loss of myenteric neurones, and non specific plexus degeneration.

Studies have reported morphological abnormalities of the colonic innervation using the silver staining method first described by Smith (Smith *et al.*, 1967). A number of studies of patients with severe idiopathic constipation without proven STC had previously reported argyrophilic degeneration of the myenteric plexus, with or without associated Schwann cell hyperplasia (Dyer *et al.*, 1969; Smith *et al.*, 1977; Sninsky *et al.*, 1984; Yoshioka and Keighley, 1989; Krishnamurthy *et al.*, 1985). Notably, Krishnamurthy *et al.* (1985) in their widely-cited, semi-blinded and controlled study of 12 patients noted a reduction in the total number of argyrophilic neurones coupled with morphological abnormalities. Preston *et al.* (Preston *et al.*, 1984b) suggested that there was a loss of the argyrophil plexus in 9 / 10 patients with STC, with associated Schwann cell hyperplasia. Two subsequent studies have confirmed Krishnamurthy's findings using silver staining in un-blinded studies of 12 and 25 colons respectively (Zenilman *et al.*, 1989; Pemberton *et al.*, 1991).

1.5.3.2 Immunohistochemistry and assay studies

Neuronal associated antigens

Pan-neuronal markers used in studies of the colon in patients with STC include neuron-specific enolase (NSE), protein gene product 9.5 (PGP 9.5), and the satellite cell marker S100 protein. Neurofilaments, which provide the basis for silver staining (Gambetti *et al.*, 1981), are found exclusively in neurones as part of the cytoskeleton and therefore antibodies raised against them predominantly stain the myenteric plexus. Neuronal markers have been used to demonstrate a variety of conflicting findings in STC (Table 1.03).

Table 1.02: Morphological findings by routine and silver staining in patients with idiopathic constipation

Author	Year	Number	STC only	Mega-colon	Melanosis coli	Abnormalities Neuronal	Abnormalities Axonal	SCH
H & E staining only								
Hughes *et al.*	1981	10	n.s	0	0	N	N	N
Walsh *et al.*	1987	19	some	2	11	N	N	N
Kamm *et al.*	1988	44	some	0	16	N	N	N
Ghosh *et al.*	1996	21	n.s	4	6	variable in 4	n.s	n.s
Watier *et al.*	1983	9	Y	0	n.s	variable in 3	n.s	1
Preston *et al.*	1984b	21	Y	0	5	n.s	n.s	n.s
Redmond *et al.*	1995	34	Y	0	7	N	N	N
Park *et al.*	1995	14	Y	0	4	N	N	N
Pluta *et al.*	1996	24	Y	n.s	n.s	variable in 4	2	n.s
Porter *et al.*	1998	15	Y	0	1	N	n.s	n.s
Silver staining								
Dyer *et al.*	1969	1	N	0	n.s	*	*	Y
Smith *et al.*	1977	4	N	2	1	*	n.s	N
Sninsky *et al.*	1984	5	n.s	n.s	n.s	degeneration	n.s	n.s
Krishmanurthy	1985	12	N	3	4	* in 10	* in 1	N
Yoshioka	1989	13	some	8	5	* in all	n.s	n.s
Preston *et al.*	1984b	10	Y	0	some	variable loss MP in 9		Y
Zenilman *et al.*	1989	12	Y	0	4	* number n.s	dec no	N
Pemberton *et al.*	1991	25	Y	n.s	1	* in 15	n.s	n.s

Key: SCH = Schwann cell hyperplasia, N = none, n.s = not stated, dec = decreased, inc = increased, * = decreased number and morphological changes, MP = myenteric plexus.

Table 1.03: Morphological findings by immunostaining for neuronal associated antigens in patients with STC

Author	Year	N	Immunostain used	Main results
Park *et al.*	1995	14	PGP 9.5 / S100	Normal myenteric plexus ↑ neural supporting tissue ↑ small fibres muscularis propria
Porter *et al.*	1998	15	NSE	Normal
Schouten *et al.*	1993	39	NF_2F_{11}	Patchy axonal loss 29/39
Benson *et al.*	1992	12	S100 / NSE / NF_2F_{11}	↑ small fibres muscularis propria
Romanska *et al.*	1996	6	NCAM	Normal

Neurotransmitters

In relation to the putative effects of an imbalance of neurotransmitters, a number of studies have used immunostaining, immunoassay, or both to demonstrate an alteration in the expression of one or more neurotransmitters (the majority of which are neuropeptides) or their enzyme markers in the large intestine of patients with constipation. Studies have been performed on neurotransmitters thought to be predominantly inhibitory: VIP (Dolk *et al.*, 1990; Milner *et al.*, 1990; Cortesini *et al.*, 1995; Romanska *et al.*, 1996; Tzavella *et al.*, 1996; Sjolund *et al.*, 1997); NO (Cortesini *et al.*, 1995; Porter *et al.*, 1998), NPY (Dolk *et al.*, 1990; Milner *et al.*, 1990; Tzavella *et al.*, 1996; Porter *et al.*, 1998); or excitatory: SP (Goldin *et al.*, 1989; Dolk *et al.*, 1990; Milner *et al.*, 1990; Romanska *et al.*, 1996; Tzavella *et al.*, 1996; Sjolund *et al.*, 1997; Porter *et al.*, 1998), ACh (Porter *et al.*, 1998), opioid peptides (Dolk *et al.*, 1990; Romanska *et al.*, 1996; Sjolund *et al.*, 1997; Porter *et al.*, 1998).

Table 1.04 demonstrates the wide inconsistency of results with the most frequently studied neuropeptides (SP, VIP, NPY and 5-HT). For example, considering only studies with physiological selection for STC, decreased (Romanska *et al.*, 1996),

increased (Sjolund *et al.*, 1997), and unchanged (Tzavella *et al.*, 1996) levels of VIP have been demonstrated by immunoassay, and similarly, decreased (Romanska *et al.*, 1996), increased (Cortesini *et al.*, 1995) or unchanged (Dolk *et al.*, 1990; Porter *et al.*, 1998) VIP immunoreactivity has been shown by immunostaining.

Table 1.04: Immunohistochemical (IHC) and immunoassay studies of neuropeptides in colon tissue from patients with either severe idiopathic constipation or proven STC.

Author	*Year*	*Blind*	*Number*	*STC only*	*Specimen site(s)*	*Immuno-histochemistry*	*Immunoassay*
Substance P							
Goldin *et al.*	1989	n.s	24	n.s	rectal biopsy	NP	↓
Milner *et al.*	1990	n.s	8	n.s	sigmoid colon	→	→
Dolk *et al.*	1990	n.s	7	Y	colon levels	→	NP
Tzavella *et al.*	1996	Y	22	Y	rectal biopsies	NP	↓
Sjolund *et al.*	1997	Y	18	Y	colon levels	↑ *	↑ (AC only)
Porter *et al.*	1998	n.s	15	Y	colon levels & ileum	↓	NP
VIP							
Koch *et al.*	1988	n.s	4	Y	descending colon	↓ †	↓ §
Milner *et al.*	1990	n.s	8	n.s	sigmoid colon	→	↓ §
Dolk *et al.*	1990	n.s	7	Y	colon levels	→	NP
Cortisini *et al.*	1995	n.s	5	Y	colon levels	↑ ↓ +	NP
Tzavella *et al.*	1996	Y	22	Y	rectal biopsies	NP	→
Sjolund *et al.*	1997	Y	18	Y	colon levels	↑ (TC only)	↑ (AC only)
Porter *et al.*	1998	n.s	15	Y	colon levels & ileum	→	NP
NPY							
Milner *et al.*	1990	n.s	8	n.s	sigmoid colon	→	→
Dolk *et al.*	1990	n.s	7	Y	colon levels	→	NP
Sjolund *et al.*	1997	Y	18	Y	colon levels	↑*	NP

Porter *et al.*	1998	n.s	15	Y	colon levels & ileum	→	NP
5HT							
Dolk *et al.*	1990	n.s	7	Y	colon levels	→	NP
Lincoln *et al.*	1990	n.s	9	Y	sigmoid colon	→	↑‡
Sjolund *et al.*	1997	Y	18	Y	colon levels	↑$	NP

Key: n.s: not stated, NP: not performed, AC: ascending colon, TC: transverse colon, * : descending colonic myenteric plexus only, †:circular muscle only, +: decreased in myenteric plexus, increased in circular muscle, $: endothelial cells of mucosa of descending colon only, §: muscularis propria only, ‡: increase in circular muscle, no change in myenteric plexus.

Changes in assayed indole levels merit comment because of increasing interest in their role in colonic motor activity, especially with reference to new directed enterokinetic therapies (see discussion below). Lincoln *et al.* (Lincoln *et al.*, 1990) demonstrated a significant increase in the total level of indoles (sum of 5-HT and its breakdown product 5-HIAA) in the mucosa and circular muscle (but not in the combined muscle and plexuses sections), of sigmoid colonic tissue from patients with STC. Differences were most marked for mucosal indole levels (a finding also made by Sjolund *et al.* (Sjolund *et al.*, 1997), and may have reflected differences in enterochromaffin cell content which demonstrated strong immunostaining. No consistent differences were demonstrated in the pattern of immunostaining for 5-HT in this study, and in one other (Dolk *et al.*, 1990).

1.5.3.3 Variability of pathological findings

The results described demonstrate the variability of pathological findings between studies of STC. This may have resulted from the heterogeneous mixture of patients in many studies e.g. chronic idiopathic *vs.* other causes of STC, or may have arisen as a result of differences in tissues studied both with respect to the level in the colon, or areas

of the colonic wall studied. Some variability is implicit with routine staining or immunohistochemical methodology when attempts are made to quantify changes in density of staining. Such error may also be compounded by observer bias, and it should be noted that few studies were documented as blind in design (Krishnamurthy *et al.*, 1985; Tzavella *et al.*, 1996; Sjolund *et al.*, 1997) (see table 1.04).

Argument still exists (see below) as to the contribution of laxatives on changes seen in argyrophil staining (Smith, 1968; Smith, 1973; Preston *et al.*, 1984b), ultrastructural analysis (Reimann *et al.*, 1980), and neuropeptide levels (Milner *et al.*, 1992; Tzavella *et al.*; 1995). In addition, ageing may have had an effect on density of subsets of certain neurochemically coded neurones. Neuronal loss has been demonstrated with age in experimental animals (Gabella *et al.*, 1989), and in the myenteric plexus of the small (de Souza *et al.*, 1993), and large (Gomes *et al.* 1997) intestine of man. Indeed, the only study utilising age matched controls showed an isolated decrease in tachykinin and leu-enkephalin-coded neurones with an otherwise normal density of neural elements (Porter *et al.*, 1998). Sensory neuropeptides have been shown to be lost in the obstructed human bladder (Chapple *et al.*, 1992), but the question of the effect of prolonged colonic stasis on neuropathological findings has not been answered because no human study has controlled for these variables, and no animal model currently exists for STC. Study design in STC continues to improve, and it is anticipated that future blinded studies with better defined clinical groups, which are adequately controlled for age, will yield better quality data.

1.5.3.4 Discussion

A substantial proportion of studies of STC (Preston *et al.*, 1984b; Zenilman *et al.*, 1989; Pemberton *et al.*, 1991; Schouten *et al.*, 1993) and severe idiopathic constipation (Yoshioka & Keighley 1989; Krishmanurthy *et al.*, 1985) have found degeneration of neurones in the myenteric plexus. Krishnamurthy *et al.* (Krishnamurthy, 1985) suggested that this pathology was definitive of a single disorder, probably congenital, and distinct from the pathology of chronic idiopathic

intestinal pseudoobstruction (CIIP) (Schuffler & Jonak, 1982). Likewise, a number of studies highlighting variation in immunoreactivity or assay levels of a number of intrinsic neurotransmitters have concluded that the results are representative of primary intrinsic neuronal disease (Goldin *et al.*, 1989; Dolk *et al.*, 1990; Lincoln *et al.*, 1990; Milner *et al.*, 1990; Tzavella *et al.*, 1996; Sjolund *et al.*, 1997; Porter *et al.*, 1998). The premise of such studies is that a generalised degeneration of intrinsic neurones, or a disturbance in the balance of intrinsic neurones with different functional and neurochemical characteristics could lead to an alteration in colonic motility manifest as STC. Accepting the enormous variability of the findings from these studies, it is still difficult to equate specific changes in neurotransmitter immunoreactivities with the physiological findings in STC. Whilst recent attempts have been made to link the function of subsets of ENS neurones with their chemical coding, projections and morphology in normal colon (Wattchow *et al.*, 1997; Porter *et al.*, 1997), further knowledge of the role of individual subsets of neurones in local enteric reflex activity is probably required to understand how degenerative changes may contribute to the observed patterns of motility in the human colon in disease. For instance, the finding of increased assay levels of 5-HT in patients with STC (Lincoln *et al.*, 1990), does not equate with the observations that 5-HT, acting at 5-$HT_{3/4}$ receptors initiates peristaltic reflexes in the intestine (Grider *et al.*, 1998), including the colon (Jin *et al.*, 1999), and that 5-HT_4 receptor agonists increase transit in healthy volunteers (Emmnauel *et al.*, 1998) (see section 1.2.5: novel therapies).

1.5.4 EXTRINSIC (AUTONOMIC) NEURONAL INFLUENCES

There are 3 principal extrinsic nerve supplies to the colon derived from the autonomic nervous system (ANS).

1.	Pelvic nerves	parasympathetic
2.	Vagus nerves	parasympathetic
3.	Mesenteric nerves	sympathetic

Their role in the control of normal colonic motility, and potential role in the aetiology of STC is considered here.

1.5.4.1 Parasympathetic dysfunction

1.5.4.1.1 Pelvic nerve dysfunction

Functional importance of the pelvic parasympathetic innervation of the colon

Morphological studies of various species including man, indicate that the pelvic nerves innervate most or all of the colon. (Schmidt, 1933; Fukai & Fukuda, 1984; Christensen & Rick, 1987; Christensen, 1991), and there is no doubt as to the importance of the pelvic nerves in colonic motor function. In experimental animals, bilateral pelvic nerve section effects a fall in colonic tone and a decrease in spontaneous motor activity (Garry, 1933; Scott & Cantrell, 1949; De Groat & Krier, 1976), with loss of mass defaecation and passage of pellet-like stools. Matsushima showed that high-amplitude propagated contractions (HAPCs), and therefore colonic "mass movements" were abolished following bilateral pelvic nerve section (Matsushima, 1989). Similar decreases in motility, which did not recover during the 6 month duration of the study, have been shown in the proximal colon in dogs (Ishikawa *et al.*, 1997). Conversely, stimulation of the pelvic nerves causes contractions of the colon powerful enough to expel the entire luminal contents (Langley & Anderson, 1895; Bayliss & Starling, 1900; Elliott & Barclay-Smith, 1904; Gray *et al.*, 1955; Garry & Gillespie, 1955; Hultén *et al.*, 1969; De Groat & Krier, 1976).

Pelvic nerves also provide the pathways for extracolonic and intramural reflexes (Wingate, 1993). Inhibition of proximal colonic motility has been shown to be invoked by distending the distal colon or rectum: the colo- or recto-colonic inhibitory reflex (Brugere *et al.*, 1991; Pearcy & Van Liere, 1926; Kreulen & Szurszewski, 1979; Gué *et al.*, 1995). Reflex inhibition of motility is not limited to the proximal colon, however, as painless rectal distension also inhibits contractile activity and delays intraluminal transit

in various organs of the upper gastrointestinal tract (Pearcy & Van Liere, 1926; Youmans & Meek, 1937; Wingate, 1993).

Observations on pelvic nerve injuries in humans

Small numbers of patients with constipation have been described following established neural injuries to the pelvic parasympathetic outflow. Observations were similar to those observed in animals with loss of mass defaecation and passage of pellet-like stools (Trumble, 1935), and atonic bowel with loss of peristaltic rush waves (fluorometric equivalent of mass movement waves) (White *et al.*, 1940). Two subsequent studies of infra-conal, i.e. *cauda equina* injury (Devroede *et al.*, 1979; Beuret-Blanquart *et al.*, 1990) have demonstrated delayed transit. Devroede and Lamarche (1974) described one patient who underwent resection of the *nervi erigentes* for multiple sclerosis related urinary symptoms, resulting in the immediate loss of rectal sensation and ability to defaecate. Subsequent physiological study demonstrated retardation of transit throughout the colon (Devroede and Lamarche, 1974). Similar findings have been demonstrated by 2 further studies (Gunterberg *et al.*, 1976; Sun *et al.*, 1995).

Sacral nerve stimulation can facilitate defaecation in humans (Binnie *et al.*, 1991; MacDonagh *et al.*, 1990; Varma, 1992), concomitant with a decrease in oro-anal transit time (Binnie *et al.*, 1991). An increase in colonic motility indices, especially with S3 stimulation, has been demonstrated in the left and transverse colon (Varma, 1992).

Relationship of STC to pelvic surgery and childbirth

Many studies have demonstrated changes in bowel function after hysterectomy (Taylor *et al.*, 1989; Smith *et al.*, 1990; Heaton *et al.*, 1993; van Dam *et al.*, 1997) and a significant group of patients with proven STC have symptoms that started at or after pelvic surgery, particularly hysterectomy (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988; Roe *et al.*, 1988; Vierhout *et al.*, 1993), but also after other operations such as tubovarian surgery (Waldron *et al.*, 1988) and even appendicectomy (Preston & Lennard-Jones, 1986). Similarly, STC can follow childbirth (Waldron *et al.*, 1988),

especially in patients with a prolonged second stage of labour and instrument assisted delivery (MacDonald *et al.*, 1997).

The assumption that nerve injury might follow pelvic surgery or childbirth, whilst gaining general acceptance (Christensen and Schulze-Delrieu, 1985; Smith *et al.*, 1990; MacDonald *et al.*, 1993), has never been anatomically proven. The possible sites of nerve injury during pelvic surgery have been discussed with particular reference to sexual (Long & Bernstein, 1959) and urinary dysfunction (Smith & Ballantyne, 1968). The preganglionic parasympathetic pelvic splanchnic nerves (*nervi erigentes*) leave the spinal cord in the ventral roots at levels S_2-S_4 in humans (Gonella *et al.*, 1987; Williams *et al.*, 1989; Christensen, 1991), and pass forward as visible condensations, expanding into the inferior hypogastric plexuses (left and right: also known collectively as the pelvic plexus). In females, each plexus is lateral to the rectum, uterine cervix, vaginal fornix and the posterior part of the bladder, extending into the uterine broad ligament (Williams *et al.*, 1989), and is presumably susceptible during hysterectomy, especially any nerves lying in the broad ligament which may be ligated with the uterine arteries. Whether a similar nerve injury can occur, possibly by mechanical stretching of nerves during prolonged, or even 'normal' childbirth is less clear. Certainly permanent changes in the pelvic floor anatomy and physiology have been observed following childbirth (Snooks *et al.*, 1990; Karasick & Spettell, 1997), and similarly the effect of stretching of the pudendal nerves with subsequent sphincteric dysfunction is well recognised (Snooks *et al.*, 1990; Sultan *et al.*, 1993; Jorge & Wexner, 1993). No study in human or higher mammals has been performed to morphologically demonstrate that nerve injury occurs following either pelvic surgery or childbirth, but degeneration of pelvic ganglia posterolateral to the vagina has now been demonstrated in a simulated birth injury model in the rat (Lin *et al.*, 1998).

It should also be noted that symptoms attributed to pelvic surgery might have been present before surgery, but given heightened significance afterwards, as has been shown with urological symptoms and hysterectomy (Parys *et al.*, 1989). Prospective study of IBS symptomatology (Prior *et al.*, 1992) suggests that symptoms arise at the time of

hysterectomy in only 10% of patients, other patients having pre-existing symptoms, which were either made worse or better by surgery. Indeed, it is possible that a number of patients undergoing hysterectomy do so for problems such as pelvic visceral pain that may have been primarily related to bowel dysfunction.

Evidence for pelvic denervation in patients with chronic idiopathic STC

Since patients with STC following pelvic surgery and childbirth are clinically indistinguishable from the chronic idiopathic group in whom symptoms arose *de-novo* (MacDonald *et al.*, 1993), pelvic nerve damage might be a plausible hypothesis for the aetiology of chronic idiopathic STC, in which similar pelvic parasympathetic dysfunction might occur by way of a non-traumatic, possibly degenerative process.

In support of this hypothesis, physiological studies demonstrate many similarities between patients with STC following pelvic surgery or childbirth and those with chronic idiopathic symptoms (Abdel-Rahman *et al.*, 1981; Baldi *et al.*, 1982; Watier *et al.*, 1983; Bannister *et al.*, 1986; Turnbull *et al.*, 1988; Roe *et al.*; 1988; de Medici, 1989; Kerrigan *et al.*, 1989; Kamm & Lennard-Jones, 1990; Wald *et al.*, 1990; Smith *et al.*, 1990; Varma, 1992; Bassotti *et al.*, 1994). The co-existence of bladder and rectal dysfunction in patients with chronic idiopathic STC (Abdel-Rahman *et al.*, 1981; Watier *et al.*, 1983; Bannister *et al.*, 1988; Kerrigan *et al.*, 1989), and increased pelvic visceral responses to the parasympathomimetic choline esters (supersensitivity) (Watier *et al.*, 1983; Slater *et al.*, 1997), as seen in patients with pelvic nerve injury (Smith *et al.*, 1990), is consistent with a denervation hypothesis (Cannon, 1934; Lapides *et al.*, 1962).

Direct study of colonic motility in patients with chronic idiopathic STC demonstrates a reduction in the frequency, amplitude and duration of high-amplitude propagated contractions (HAPCs) (Bassotti *et al.*, 1988; Bassotti *et al.*, 1994), and reduced tone in the left colon (O'Brien *et al.*, 1996). These findings are similar to those made in animals following experimental pelvic denervation (above) (Garry, 1933; Scott & Cantrell, 1949; De Groat & Krier, 1976; Matsushima, 1989).

Morphological findings reported in the distal colon, i.e. neuronal degeneration with

morphological changes and Schwann cell hyperplasia, in patients with chronic idiopathic STC (Krishnamurthy *et al.*, 1985; Preston *et al.*, 1984b; Zenilman *et al.*, 1989; Pemberton *et al.*, 1991) are identical to those of patients with surgical section of the pelvic nerves (Devroede & Lemarche, 1974), and *cauda equina* injury (Devroede *et al.*, 1979).

Pelvic nerve injury and STC: pathogenetic mechanisms

Several mechanisms may co-operate in the pathogenesis of observed retardation of colonic transit.

i) Severance of the extramural pelvic nerves has been shown experimentally to result in degeneration of the ascending colonic nerves in dogs and cats (Fukai & Fukuda, 1984; Christensen & Rick, 1987), and may involve the entire colon (Schmidt, 1933). In the human, at least 80% of the colon might be affected by such degeneration (Christensen, 1991) with resultant hypomotility coincident apon a direct loss of parasympathetic excitatory influence.

ii) Following degeneration of ascending colonic nerves, further changes in motility might be explained by reorganisation of efferent pathways. Experimental work has shown that, following injury to the innervation of other pelvic organs such as the bladder, subsequent re-innervation of denervated parasympathetic ganglion cells by sympathetic nerves may contribute to the dysfunction (de Groat & Kawatani, 1989). It has long been recognised that the sympathetic nerves of the autonomic nervous system exert a tonic inhibitory influence on the colon (Lister, 1858).

iii) Alternatively, left sided stasis may contribute to proximal slowing of colonic transit by reflex inhibition occurring secondary to distal distension. The recto-colonic inhibitory reflex has been well defined in experimental animals (Pearcy & Van Liere, 1926; Kreulen & Szurszewski, 1979; Brugere *et al.*, 1991; Gué *et al.*, 1995), but has rarely been reproduced in man due to technical limitations (Klauser *et al.*, 1990; Warren *et al.*, 1994). Reflex inhibition of transit or motility in more proximal regions of the

gastrointestinal tract is well documented during rectal distension, however (Youle & Read, 1984; Kellow *et al.*, 1987; Bojö & Cassuto, 1992; Tjeerdsma *et al.*, 1993), and may explain the upper gastrointestinal findings in some patients with STC (Watier *et al.*, 1983; Bassotti *et al.*, 1996; MacDonald *et al.*, 1997; Mollen *et al.*, 1999).

1.5.4.1.2 Vagal dysfunction

The vagal nerves innervate at least the proximal, and possibly all of the colon, although the density of innervation decreases aborad (Fukai & Fukuda, 1984). Experimental evidence has demonstrated that interruption of the vagi causes a significant decrease in contractions of proximal, middle and distal colon segments in monkeys in both fasted and fed states (Esser *et al.*, 1989; Dapoigney *et al.*, 1992), and that a colonic excitatory vago-vagal reflex pathway exists which is mediated by both cholinergic and non-cholinergic mechanisms (Dapoigney *et al.*, 1992; Collman *et al.* , 1984).

Truncal vagotomy performed with gastric drainage procedures is well known to cause diarrhoeal syndromes in a significant proportion of patients by a number of mechanisms, and can cause constipation (Hines *et al.*, 1975). The effect of vagotomy on colonic motility alone in humans is not known, and no studies have been directed to examining a possible vagal role in the causation of idiopathic constipation.

1.5.4.2 Sympathetic dysfunction

Sympathetic nerve section causes an increase in colonic motility (Lister, 1858; Learmonth & Markowitz, 1930; Garry, 1933; De Groat & Krier, 1979). Conversely, stimulation of pre- and post-ganglionic extrinsic sympathetic nerve fibres (lumbar splanchnics and lumbar colonics) inhibits spontaneous contractions (Langley & Anderson, 1895; Bayliss & Starling, 1900; Garry & Gillespie, 1955; Hultén *et al.*, 1969), and reduces or blocks the stimulatory response from pelvic nerve stimulation (below) (Hultén *et al.*, 1969; Gillespie & Khoyi, 1977; Hedlund *et al.*, 1985). These

observations suggests that sympathetic nerves have a predominantly inhibitory influence on the gut, exerted by suppressing the parasympathetic nerve-derived excitatory drive.

Since immunoassay levels or immunostaining for the sympathetic neurotransmitters NA, NPY, and dopamine β-hydroxylase appear to be normal (Lincoln *et al.*, 1990; Milner *et al.*, 1990; Dolk *et al.*, 1990; Porter *et al.*, 1998), there is little morphological evidence to suggest that extrinsic sympathetic denervation occurs in STC. This is in accord with the predominantly inhibitory role of the sympathetic innervation to the intestine. The role of sympathetic reorganisation, which might occur following parasympathetic denervation has been discussed.

1.5.5 CENTRAL NEURONAL INFLUENCES

It is probable that the CNS modulates normal colonic function only under particular circumstances e.g. defaecation, and that the moment-to-moment control is provided by the ENS (Sarna, 1991; Wingate, 1993). Whether there is any continuous central input to the colon is still questionable. Indirect evidence that cerebral influences modify colonic motility may be implied from the behavioural response of mammals to stressful situations, such as anxiety or fear (Truelove, 1966), and the changes in motility observed with sleep and awakening (decreased and increased contractile acitvity respectively) (Narducci *et al.*, 1987; Soffer *et al.*, 1989). Colonic motor activity is enhanced after exposure to short-term mental or physical stress (Narducci *et al.*, 1985; Stam *et al.*, 1995). More direct evidence that the CNS modulates colonic motor activity is derived from observations that intracerebroventricular administration of various chemicals can influence change in colonic contractility (e. g. Bueno *et al.*, 1985).

There is no conclusive physiological or morphological evidence for CNS dysfunction in patients with STC, but, indirect clinical evidence does exist. Epidemiologically, there is concordance of occurrence of constipation and central nervous system disorders

(Johanson *et al.*, 1992): STC occurs in some patients with diseases of the brain and spinal cord e.g. spinal cord transection (Keshavarsian *et al.*, 1995; Glickman & Kamm, 1996); Parkinson's disease (Edwards *et al.*, 1994), and multiple sclerosis (Weber *et al.*, 1987).

In particular, it is well established that disordered defaecation can follow supraconal spinal cord injury (Glickman & Kamm 1995). Clearly other factors have a role in the association of constipation with such injuries e.g. recumbency and opiate use, but colonic transit is invariably delayed in patients with spinal cord transection (Devroede *et al.*, 1979; Nino-Murcia *et al.*, 1990; Beuret-Blanquart *et al.*, 1990; Keshavarsian *et al.*, 1995; Leduc *et al.* 1997; De Looze *et al.*, 1998). A possible role for psychogenic influences is discussed below (1.6.7).

1.6 PROPOSED AETIOLOGIES

1.6.1 PRIMARY NEURONAL OR SMOOTH MUSCLE DISEASE

The onset of STC in infancy or early childhood in some patients, which may be familial, suggests the possibility of a primary, possibly congenital aetiology for STC. At present, evidence is lacking, but the hypothesis that STC might have a genetic basis has never been formally tested.

1.6.1.1 Primary neuronal dysfunction

The neural-crest-derived precursors of the ENS migrate along defined pathways to colonise the bowel. Recent studies of the sequential actions of essential growth and transcription factors have revealed that enteric neuronal development involves a complex interaction of lineage-determined and microenviromental elements. The genetic signals that are critical for normal crest migration and differentiation have been identified by studying natural and targeted mutations in mice, and include the normal transcription of genes such as the *RET* proto-oncogene (Schuchardt *et al.*,

1994), as well as genes encoding other neurotrophins, their receptors, and associated neuropoietic cytokines (Gershon, 1997; Chalazonitis *et al.*, 1998). Hirschsprung's disease is an example of a human disorder caused by failure of these complex processes, and mutations of genes involved in neural crest development have now been described in this condition (Lyonnet *et al.*, 1993; Puffenberger *et al.*, 1994; Salamon *et al.*, 1996; Hofstra *et al.*, 1996). Candidate genes are also being characterised for other gastrointestinal pseudo-obstructive phenotypes (Auricchio *et al.*, 1996; Verma *et al.*, 1997). The finding of intrinsic neuronal abnormalities, coupled with the onset in infancy or early childhood of symptoms in many patients (chronic idiopathic STC) might suggest that STC is a primary ENS disorder arising during development.

1.6.1.2 Primary smooth muscle dysfunction

It is conceivable that some developmental morphogenic abnormalities could result from defective genetic control of the complex sequential events in gut development (Pitera *et al.*, 1999), although other mechanisms such as congenital viral infection or intrauterine vascular defects / accidents may also operate. Abnormality in any of the up-stream or down-stream genes involved in smooth muscle migration, maturation and programmed cell death could result in impairment of these functions and thereby lead to the muscle defects seen in some enteric myopathies. There has been much recent interest in key regulatory proteins, termed transcription factors that are involved in determining the eventual phenotype of mesenchymal cells early in development. One important class are the homeodomain proteins, that when mutated cause alterations in the body plan of the fruit fly called homeotic changes. Expression patterns of homeodomain proteins are critical for defining the structural blueprint of the body plan and the identity of cells in many organisms, from flies to humans. A number of homeobox genes are expressed in complex patterns in mesoderm-derived cells in the hindgut tube (Pitera *et al.*, 1999), and experimental abnormalities have been shown to affect its structure (Wolgemuth *et al.*, 1989; Roberts *et al.*, 1995). Whilst such gross abnormalities have been demonstrated in mice, a genetic component to certain human visceral myopathies is also known to exist

in some families with autosomal dominant and recessive (Mitros *et al.*, 1982), and X-linked (Smith & Milla, 1997) modes of inheritance. A greater understanding of the pathogenesis of the skeletal muscular dystrophies is being rapidly achieved, particularly in relation to membrane associated or linked cytoskeletal proteins, and similar mechanisms are likely to be discovered in gut leiomyodystrophies, and possibly STC.

1.6.2 INTERSTITIAL CELL DYSFUNCTION

Because of their potential importance throughout the gastrointestinal tract in motility (section 1.4.1), abnormalities in the density and distribution of ICCs have been sought and described in human aganglionosis (Vandervinden *et al.*, 1996), some cases of hypoganglionosis, idiopathic neonatal (Kenny *et al.*, 1998a) and adult pseudoobstruction, and in high anorectal malformations with refractory constipation (Kenny *et al.*, 1998b). Although germline mutations of the *C-KIT* proto-oncogene were absent in patients tested with STC (Malik *et al.*, 1998), and evidence for a decreased density or distribution of ICC in the colon of patients with STC is contradictory (Hagger *et al.*, 1997; He *et al.*, 1998), it remains feasible that a disruption in motility in humans could result from ICC dysfunction, as has been shown in murine models (Ward *et al.*, 1994; Huizinga *et al.*, 1995; Huizinga *et al.*, 1998).

1.6.3 AUTOIMMUNITY

1.6.3.1 Autoantibody mediated neuronal damage

Antibodies against antigenic determinants on enteric nerve cells have been found in pseudoobstruction associated with small-cell lung carcinoma (Lennon *et al.*, 1991). High levels of circulating IgG enteric neuronal antibodies were reported in small numbers of patients who presented with abdominal pain and constipation in late childhood with acquired intestinal aganglionosis, but without other neurological problems or neoplasia (Smith *et al.*, 1997). Similarly, anti-myenteric autoantibodies have been demonstrated in progressive systemic sclerosis (scleroderma) (Howe *et al.*,

1994; Eaker *et al.*, 1999), a disorder which can cause STC (Basilisco *et al.*, 1993). Autoantibodies may also mediate the gastrointestinal manifestations of certain infections, for instance in patients with Chagas' disease, in which antibodies have been detected to myenteric neurones (Wood *et al.*, 1982), and more recently to specific neuronal muscarinic receptors (Goin *et al.*, 1999). Antibodies to enteric neuronal epitopes have not been investigated in idiopathic STC, and therefore remain an aetiological possibility. This hypothesis is supported by Lindberg *et al.* (Lindberg *et al.*, 1999), who have recently described a lymphocytic epithelioganglionitis, i.e. ganglionitis accompanied by intraepithelial lymphocytosis and mild chronic mucosal inflammation in intestinal diseases characterised by severe motor dysfunction, including a group of patients with STC.

1.6.3.2 Autoantibody mediated smooth muscle damage

Cases of intestinal pseudoobstruction caused by an autoimmune myopathy characterised by a dense lymphocytic myositis, and circulating IgG-class autoantibodies against smooth muscle have been described in children (Smith & Milla, 1997). Intestinal pseudoobstruction may similarly be a feature of other recognised secondary myopathic disorders such as the collagen vascular diseases e.g. systemic lupus erythematosus (Cacoub *et al.*, 1993).

1.6.4 INFECTIVE AGENTS

The aetiology of STC might be explained by a systemic infection acting permanently to alter normal peristaltic activity via effects on the extrinsic enteric innervation, myenteric plexus, as is seen in Chagas' disease (see above), or directly on enteric smooth muscle. Sporadic cases of chronic idiopathic intestinal pseudoobstruction (CIIP) may follow a poorly defined illness, and recent evidence has linked a viral aetiology (herpetoviridae) to this condition (Debinski *et al.*, 1997). In addition, previous clinical case reports of CIIP had reported tissue localisation of viral inclusions in the myenteric plexus for EBV (Vassallo *et al.*, 1991), Varicella (Chang *et al.*, 1978) and CMV (Sonsino *et al.*, 1984).

Varicella zoster virus has also been identified in patients with oesophageal motility disorders (Robertson *et al.*, 1993). A clear mechanism of causation of disease has not been shown for these agents, and may relate to a direct cytopathic effect or damage resulting from the host immune response, as is seen in Chagas' disease (Wood *et al.*, 1982). Of interest with respect to the latter is the observation that in both CIIP (Camilleri & Fealey, 1990; Bharucha *et al.* 1993; Camilleri *et al.*, 1993) and STC (Altomare *et al.*, 1992) there may be associated abnormalities of the autonomic nervous system in approximately one third of cases. Acquired selective cholinergic dysautonomia leading to CIIP has been shown to occur following acute infectious mononucleosis (Vassallo *et al.*, 1991). A role for infective agents in STC has not been tested.

1.6.5 ROLE OF ENDOGENOUS OPIOIDS

Opioid peptide immunoreactivity, principally for enkephalins, but also for endorphins and dynorphins is present in the normal human gut, principally in elements of the myenteric plexus (Polak *et al.*, 1977), and opiate receptors are present in the intestinal wall both in the submucosa and in the myenteric plexus (Ahmad *et al.*, 1990). The effect of opiates on motility is mediated both centrally and peripherally (Bueno *et al.*, 1985) by 3 subtypes of opiate receptor, μ, δ and κ, with the relative inhibitory or excitatory effects of stimulation of each receptor subtype being species, site and opioid dependent (Porreca *et al.*, 1984; Hoyle *et al.*, 1990). In man, the combined effects of opiate receptor activation produce a stimulation of colonic contractions with reinforcement of tonic activity and a decrease in propulsive activity leading to an overall decrease in transit (Bueno & Fiormonti, 1988).

Constipation is a regular and established sequel to treatment with opiates. Experimentally, morphine slows colonic transit (Kaufman *et al.*, 1988), by increasing phasic contractile, but decreasing propagating activity (Frantzides *et al.*, 1992), and these motility changes may be reversed by opiate antagonists (Sykes, 1991). Since naloxone also speeds colonic transit in healthy volunteers (Kaufman *et al.*, 1988), it is

possible that endogenous opioids exert an effect on the colon of patients with STC.

Studies of colonic opioid immunoreactivity in STC have shown no abnormalities of met-EnK (Dolk *et al.*, 1990; Sjolund *et al.*, 1997) and dynorphin (Dolk *et al.*, 1990), but have demonstrated a reduced density of leu-EnK containing neurones (Porter *et al.*, 1998). A reduced activity of enkephalins on inhibitory neurones in patients with constipation has also been suggested (Hoyle *et al.*, 1989). The potential influence of such alterations remains unclear, and indeed a controlled study of patients with STC showed that neither naloxone nor a more potent opioid antagonist, nalmefene, were able to correct the transit abnormality (Fotherby *et al.*, 1987).

1.6.6 EXOGENOUS NEURONAL TOXINS: LAXATIVE MEDIATED MYENTERIC DAMAGE

The idea that laxatives might play a role in the pathogenesis of severe constipation is not new, the "cathartic colon" being first described more than 50 years ago (Heilbrun, 1943). However, no case of this radiologically characterised distinct entity has been published of a patient whose laxative intake started after 1960. An outdated laxative, such as podophyllin, a known systemic neurotoxin, was therefore the most likely candidate (Muller-Lissner, 1996). Similarly, melanosis coli, which is not an infrequent finding in patients with or without chronic constipation, and which is caused by accumulation of pigment laden macrophages following colonic epithelial cell apoptosis, appears to be a harmless condition which is indicative of, but not specific to (Byers *et al.*, 1997), chronic laxative, especially anthraquinone ingestion (Mengs & Rudolph, 1993).

Current laxatives

Whether anthraquinone laxatives given in the long-term to patients with constipation can cause adverse functional or structural changes in the intestine is debatable. Smith (Smith, 1968) demonstrated abnormalities in colonic innervation, both in a patient, and in mice given senna experimentally. Neuronal abnormalities were confirmed on light

microscopy in humans (Smith, 1973), and also by a controlled blinded ultrastructural study (Reimann *et al.*, 1980). However, subsequent experimental evidence in rodents given sennosides for up to one year did not demonstrate neural changes by light microscopy (Dufour & Gendre, 1988), and it is possible that Smith's original findings were caused by free anthroquinones rather than sennosides (Dufour & Gendre, 1988). Free anthroquinones have been shown to cause ultrastructural changes in the mouse (Dufour & Gendre, 1984), but not light microscopical changes in the dog (Case *et al.*, 1977). A case controlled study of the human submucous plexus following one year of anthroquinone ingestion showed no difference on electron microscopy (Riecken *et al.*, 1987). In addition, it should be noted that the neuronal morphological changes seen in some of these studies may be as indicative of a primary pathology leading to constipation as nerve damage from laxatives. Anthraquinones may, however, be responsible for some of the variations seen in neuropeptide immunoreactivity in studies of idiopathic constipation (Milner *et al.*, 1992; Tzavella *et al.*, 1995). Like the anthroquinones, polyphenolic laxatives lead to apoptosis of colonic epithelial cells with accumulation of macrophages containing colonic cell remnants, but these are not pigmented (Mengs *et al.*, 1993). There is no current evidence to suggest that polyphenolic compounds cause adverse effects on long-term use (Gebboes *et al.*, 1993).

At least with respect to one year of laxative use, scientific evidence does not support the commonly held belief amongst the public and the medical profession alike, that long-term laxative use results in a "lazier" bowel. Whether patients taking unprescribed high doses of laxatives for a much longer duration do damage to their bowel is not known.

1.6.7 PSYCHOGENIC FACTORS

The role of psychological factors in the pathophysiology of severe chronic constipation is unclear. Personality may influence stool weight and frequency (Tucker *et al.*, 1981), and a coexisting psychiatric disorder is common in patients with functional bowel disorders (Young *et al.*, 1976; Johansen *et al.*, 1992). Although early studies demonstrated that psychometric scores may be abnormal in patients with STC

(Devroede *et al.*, 1989), the association between abnormal psychological symptom scores and STC is much less clear than in patients with normal transit constipation (Wald *et al.*, 1989; Wald *et al.*, 1992; Heyman *et al.*, 1993; Grotz *et al.*, 1994).

There are 3 possible explanations for the commonly perceived association between psychological abnormalities and STC.

Psychological disorders cause STC

Youle and Read (Youle & Read, 1984) demonstrated in healthy volunteers that painless rectal distension could slow gastric emptying and small bowel transit. Klauser *et al.* (Klauser *et al.*, 1989) likewise demonstrated that healthy volunteers could substantially delay defecation when paid to do so, and that such delays were associated with some slowing of transit in the rectosigmoid and right colon. It is therefore possible that chronic rectal distension by faeces, as a result of chronic inhibition of defaecation as a learned behaviour, perhaps in childhood (Whitehead *et al.*, 1992; Leroi *et al.*, 1995) could lead to STC. However, in chronically constipated patients, there is little relation between symptoms (Wald *et al.*, 1989), or physiological evidence of obstructed defaecation (Wald *et al.*, 1990; Wald *et al.*, 1993; Pezim *et al.*, 1993; Grotz *et al.*, 1994; Karlbom *et al.*, 1995), and transit findings. Likewise, psychological profiles do not correlate with anorectal sensory or motor function (Wald *et al.*, 1989). In addition, the premise that anxiety-induced paradoxical puborectalis contraction might cause constipation by outlet obstruction is largely discredited by the finding that such sphincteric contraction is common in healthy controls, and may therefore have been a testing artefact (Voderholzer *et al.*, 1997).

Despite an excess incidence of constipation symptoms in patients with psychotic depression (Sonnenberg *et al.*, 1994b; Parker *et al.*, 1997), no study has proven a clear association between depression and slow colonic transit (Gorard *et al.*, 1996). Likewise, a true association between STC and anorexia and bulimia nervosa is also questionable (Kamal *et al.*, 1991; Chun *et al.*, 1997); the possible role of laxative abuse-related myenteric damage, that can theoretically occur in such patients, has been discussed.

Constipation may lead to psychological disorders

Whilst no objective evidence exists to prove such a causal link, it is possible that patients suffering from a chronic disorder characterised by severe symptoms including pain, for whom no adequate explanation or treatment exists, might develop secondary psychological problems (Wright *et al.*, 1995). This may be especially true for those patients who acquire symptoms in adulthood, having previously been accustomed to a "normal" bowel habit.

Coexistence of psychological disorders leads to self-selection for treatment.

Self-selection for treatment has been shown to contribute to the association between psychological distress and irritable bowel syndrome (Whitehead *et al.*, 1988; Drossman *et al.*, 1988). However, anxiety and depression are found equally in constipated patients who have not consulted physicians as well as in those who have consulted (Whitehead, 1994), implying that the association of constipation with psychological disorders is not explained by a greater tendency of psychologically distressed individuals to present to doctors.

1.6.8 INTESTINAL ABSORPTION AND STC

Patients with STC invariably complain of hard stools, and hard stools contain less water than normal stools (Read & Timms, 1986). In addition, certain laxatives (e.g. magnesium salts) work at least in part by exerting an osmotic effect that causes water to be retained in the intestinal lumen (Izzo *et al.*, 1996).

1.6.8.1 Colonic absorption

Two studies of large intestinal absorption in constipated subjects contained small numbers of widely heterogeneous patients without prior physiological selection (Nava *et al.*, 1973; Devroede & Soffie, 1973). Using a colonic perfusion technique, they concluded that water, sodium & chloride absorption were increased in the symptomatic

group, but that such excessive absorption was secondary to stagnation as a result of slowed colonic transit and / or variation in large bowel volumes rather than abnormal absorptive capacity of the large bowel. No defect in colonic mucosal absorption has been demonstrated to date in STC.

1.6.8.2 Small intestinal absorption

Low faecal bile acid losses have been reported in a few patients with severe constipation (Iser *et al.*, 1977). Bile acids shorten intestinal transit experimentally (Kruis *et al.*, 1986), and have been used to treat constipation (Hepner *et al.*, 1973). Furthermore, anion exchange resins that bind bile acids may be used to treat some patients with diarrhoea. Van Tilburg *et al.* (Van Tilburg *et al.*, 1990) demonstrated, using an *in vitro* preparation of brush border membrane vesicles, that this low faecal bile acid loss was associated with a low sodium dependent ileal bile acid transport. Hosie *et al.* (Hosie *et al.*, 1992), having validated a slightly different *in vitro* methodology utilising an intact ileal mucosa preparation, found no significant differences in bile acid absorption between patients with proven STC, cancer and ulcerative colitis.

1.6.9 HORMONAL ABNORMALITIES AND STC

There has been a special interest in the role of hormones in the aetiology of idiopathic constipation because of the well known constipating effects of some common endocrinopathies e.g. hypothyroidism, as well as the female predominance of the condition.

1.6.9.1 Non-colonic gut hormones

A number of substances are released by cells in the mucosa (and in some cases nerve endings) of the gastrointestinal tract, where they have a role in postprandial colonic motor activity by their participation in gut reflexes. These include the peptides: neurotensin, somatostatin, gastrin and cholecystokinin, and also motilin.

The first studies to measure such hormones in constipated patients showed an impaired release of circulating motilin, pancreatic polypeptide and gastrin in response to the standard stimulus of drinking water (Preston *et al.*, 1985a), or demonstrated reduced basal and stimulated levels of motilin (Sjolund *et al.*, 1986). Recent studies have had similarly conflicting results. Van der Sjip (van der Sjip *et al.*, 1998) demonstrated no difference in circulating levels of a panel of upper gastrointestinal hormones including neurotensin, motilin, gastrin and pancreatic polypeptide, showing alterations only in somatostatin (basal increased but stimulated incremental level decreased) and glucagon (decreased). A subsequent study of 8 STC patients showed normal basal, but decreased postprandial patterns of motilin (absent), cholecystokinin, neurotensin and somatostatin (Peracchi *et al.*, 1999). These discrepancies may result from the small numbers of patients studied, and further studies are required.

1.6.9.2 Sex hormones

Because severe idiopathic constipation predominantly effects females, and a high proportion of patients have co-existent disorders of menstruation and previous gynaecological surgery (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988), it is possible that a gynaecological or hormonal abnormality is associated with STC. Additionally, during pregnancy (Winship, 1975, Baron *et al.*, 1993), and the menses (Heitkemper & Jarrett, 1992) there is an increased incidence of intestinal symptoms including constipation.

Sex hormones are known to have a role in the control of colonic contractility, although their significance remains unclear. *In vitro* studies of canine and human colonic tissue have demonstrated a relaxant effect of progesterone on smooth muscle (Kumar, 1962, Gill *et al.*, 1985). Similarly, the *in-vitro* responsiveness of the colon to ACh is reduced in the presence of progesterone (Scott & DeFlora, 1989). A similar effect is seen for progesterone in the upper gastrointestinal tract (Bruce & Behsudi, 1979). Both oestrodiol and progesterone have been shown to influence gastrointestinal transit in the

rodent (Ryan & Bhojwani 85).

However, whilst steroid hormone abnormalities have been detected in severely constipated women (Kamm *et al.*, 1991), it remains unclear as to whether sex hormones have an effect on intestinal transit *in vivo* in the human female either in normals or in constipated patients (Rees & Rhodes, 1976, Wald *et al.*, 1981, Hinds *et al.*, 1989, Turnbull *et al.*, 1989), and whether such alterations in circulating hormone concentrations may also reflect an alteration in their enterohepatic metabolism caused by stasis or surgery (Kamm *et al.*, 1991).

1.6.10 TRAUMATIC NEURONAL INJURY (IATROGENIC)

The role that pelvic trauma or surgery and childbirth might have in some patients with STC has been discussed, and is widely acknowledged.

1.7 SUMMARY

Definition

Slow transit constipation is defined by transit studies, and is a group disorder in which there is considerable patient heterogeneity. Currently used definitions may artificially select patients with multiple distinct biological entities, whilst excluding others.

Pathophysiology

Studies of colonic contractile activity in STC are extremely limited, and have shown some variability in findings. Whilst the failure of these studies to observe basal physiological conditions should be noted, patients with STC appear to have a reduction in the number, amplitude and duration of colonic HAPCs. Other findings include variable abnormalities of rectosigmoid motility, and evidence of a generalised intestinal disorder in a proportion of cases. Few consistent observations have been made of colonic tissue from patients with STC, and this variability has been discussed. In general, the majority of studies point to a degenerative neuronal process, although

myopathic changes may co-exist.

Proposed aetiologies

Previously tested hypotheses (laxatives, absorption, hormones, psychological abnormalities, endogenous opioids etc.) have not provided conclusive results. Accepting variability between studies, pathological and physiological evidence exists for a neuronal (particularly intrinsic neuronal or extrinsic pelvic parasympathetic neuronal) and / or smooth muscle disease process in the pathogenesis of STC. Primary and secondary aetiologies leading to such abnormalities may be responsible for the similar clinical end-point of STC.

1.8 AIMS

The primary aim of this study was to examine new aetiological hypotheses for STC following full clinical and pathophysiological characterisation of all patients with proven STC referred to our institution over the last 10 years.

1.8.1 SPECIFIC AIMS

1. to clinically and physiologically classify a group of patients with STC for the purposes of further aetiological studies. Areas of interest include:
   a. findings from clinical history taking in relation to symptoms and their onset.
   b. findings on gastrointestinal physiological testing.
   c. the subclassification of patients on the basis of clinical and physiological findings.

2. to investigate a potential role of neuronal dysfunction in STC. Specifically:
   a. to look for **peripheral sensory or motor abnormalities** using routine neurophysiology, and quantitative peripheral sensory and autonomic tests.

b. to examine colonic tissue for **morphological abnormalities** of neurones or neural supporting elements suggestive of intrinsic or extrinsic neuronal dysfunction.

c. to examine the possible **pathogenesis of neuronal dysfunction** including:

i. congenital mechanisms: germline mutations

ii. acquired mechanisms: autoantibodies to neuronal ion channels

3. to investigate a **potential role of smooth muscle dysfunction** in STC. Specifically:

a. to examine colonic and small intestinal smooth muscle for the presence of inclusion bodies.

b. to test whether such inclusions are a specific and unique feature of STC, or reflect a secondary cellular response to denervation or other tissue injuries.

c. to establish the composition of such inclusions.

2 MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter covers the recruitment and selection of patients for participation in clinical and tissue based studies of STC. The methodology of gastrointestinal physiological investigations required for selection of patients for further studies, i.e. the physiological entry criterion defining STC, is discussed in detail. Likewise general methodology relating to the preparation of the thesis is included. As a result of the diversity of the studies included in the thesis, specific methodology pertaining to each study and the background of such methods are discussed separately within each appropriate chapter. Control and comparison subjects are described in the chapters to which they refer, and listed in appendix 2.01.

The latter part of the chapter presents a summary of all the clinical data collected for STC patients, which is tabulated.

2.2 ETHICS APPROVAL

The use of patient and control human subjects, for clinical studies (Chapter 5), and tissue studies (Chapters 6-8) was approved by the East London and the City Health Authority (ELCHA) Research Ethics Committee. The following ethics committee references cover the body of work contained within this thesis.

ELCHA no: P97062

ELCHA no: P97338

ELCHA no: P98258

2.3 STC PATIENTS

2.3.1 RECRUITMENT

Patients for study were recruited by written invitation from the author, or directly approached in person at times of clinical presentation to the Royal London Hospital. Initial selection of patients for recruitment was based on information provided from surgical colleagues, the Gastrointestinal Physiology Unit (GIPU) (Dr Mark Scott), and from lists of patients used in previous research studies of this condition (Dr Marie-Anne Pilot; Dr Mark Scott). In all cases, where participation was invited for further research investigations, appropriate consent was obtained. The GIPU investigates an almost exclusively adult population.

2.3.2 SELECTION

2.3.2.1 Inclusion criteria

Patients with STC were identified from those referred consecutively to the Royal London Hospital for lower gastrointestinal physiological investigation of constipation between 1988 and 1999. Patients were included on the basis of:

1. Fulfilment of clinical criteria for idiopathic constipation. Constipation was defined according to agreed criteria (see chapter 1) (Whitehead *et al.*, 1991), as the presence of fewer than 2 bowel movements per week for at least 12 months, or the presence of 2 or more of the following complaints for at least 12 months:

- Straining on at least 25% of bowel movements when not taking laxatives.
- Feeling of incomplete evacuation after at least 25% of bowel movements when not taking laxatives.
- Stools less frequent than 3 per week without laxatives.

2. Demonstration of a reduction in the rate of progress of colonic intraluminal contents by radio-opaque marker studies, ^{111}In DTPA isotope scintigraphy, or both (see CHAPTER 1 and below).

3. Proof of the above, regardless of the time or mode of onset of symptoms. Patients were included if they had idiopathic STC, or symptoms were coincident temporally with any defined event i.e. pelvic surgery, neurological disease. Such divisions did however effect subsequent study inclusion and analysis of data (see chapter 3).

2.3.2.2 Exclusion criteria

Patients were specifically excluded for the following.

1. Failure of proof of STC. i.e. severe intractable constipation without transit study, or with a negative study. Some such patients were however included in chapter 4, and were used as positive controls in chapter 8. This group predominantly included patients with isolated rectal evacuation disorders (RED).
2. Inadequate data available. These included patients who were known historically to have had a positive transit study, but in whom the patient was lost to follow-up, and the notes unavailable for review.
3. Isolated functional rectosigmoid hold-up, as demonstrated by isotope scintigraphy, with proctographic evidence of significant mechanical pelvic outlet obstruction. These patients were excluded on the basis that they do not fulfil the criteria for STC (Krevsky *et al.*, 1989; McLean *et al.*, 1995).
4. Evidence of megabowel (megacolon or megarectum). This is defined by increased bowel calibre on contrast studies (Preston *et al.*, 1985b), and is a separate clinical (Gattuso & Kamm, 1997), and probably pathological (Gattuso *et al.*, 1994) condition from STC. These patients were however included as disease controls in chapter 7.
5. Histological evidence of Hirschsprung's disease (HSCR). Patients with equivocal rectoanal inhibitory reflexes (see below) underwent rectal biopsy to exclude HSCR.

[No such patients were referred].

6. Evidence of an obstructive organic cause for constipation i.e. neoplasia / stricture.

[No such patients were referred].

2.3.3 DATA COLLECTION

2.3.3.1 Methods of collection

Clinical information was obtained by interview and review of records (patient hospital notes and computerised records (patient administration system, PAS). Data were collected:

1. Prospectively	Patients presenting during the course of the thesis study period i.e. October 1996 to October 1999.
2. Retrospectively	Patients seen before October 1996 a. Patients recalled for interview or participation in research protocols. b. Patients unavailable for recall; notes review only.

2.3.3.2 Data storage

All data, regardless of method of acquisition, were stored on a dedicated, password protected database, which was established at the start of the study, and modified subsequently as required. Data were stored on Microsoft ® Access 97 for Windows 95 (Microsoft Corporation, Santa Rosa, CA, USA). Subsequent data manipulation was performed by executing 'queries'. Hard copies of data were produced using 'report' functions.

2.3.3.3 Clinical data

A full clinical history, including the nature of the patients presenting symptoms, mode of onset, personal past medical / obstetric / psychiatric / treatment history, family history, and systematic enquiry was obtained, and included the variables listed in table 2.01

Table 2.01: Clinical data collected for STC patients

Basic details	***Name / Age / D.O.B / Address / Occupation***	
Duration	***Age of onset***	
	Age at presentation	
	Duration (years)	
Mode of onset / precipitating events	***Obstetric episode / pelvic surgery***	
	Neurological disease / injury	
	Physical / sexual abuse	
	Other	
	None	
Presenting symptoms	***Defaecatory symptoms***	Frequency of bowel action
		Straining
		Time spent per attempt
		Incomplete evacuation
		Unsuccessful evacuation
		Painful evacuation
		Stool consistency
		Incontinence
		Sensation of rectal prolapse
		Digitation: rectal / vaginal
		Rectal bleeding
	Other gastrointestinal symptoms	Abdominal pain
		Abdominal bloating

		Dyspepsia
		Nausea / vomiting
Management	***Drugs***	Laxatives
		Suppositories / enemata
		Prokinetics
	Behavioural therapy	Biofeedback
	Surgery	Anorectal or abdominal
Personal History	***Surgical history***	
	Obstetric & gynaecological history	
	Past medical history	Metabolic / endocrine
		Neurological / spinal disease
		Rheumatological
		Autoimmune
		Other
	Pyschiatric history	
Drug history		
Family history	***Constipation / other***	
Systems enquiry	***Urological symptoms***	
	Neurological symptoms	Central nervous system
		Peripheral nervous system
		Autonomic nervous system
	Rheumatological symptoms	

Patients had previously undergone routine clinical examination performed in an outpatient setting by a surgeon or physician of consultant grade. This included general and abdominal examination, and pelvic examination by digital rectal examination, proctoscopy and sigmoidoscopy. Such examination is directed primarily at excluding organic disease, and these data were not specifically collected for the purposes of this study.

2.3.3.4 Routine investigations

Patients had previously undergone blood tests to exclude metabolic or endocrine disease (thyroid function tests, serum calcium, and random blood glucose). An anatomical study of the large intestine was performed using either double contrast barium enema and / or colonoscopy.

2.4 GASTROINTESTINAL PHYSIOLOGICAL TESTS

A number of tests have been developed, principally in the last 30 years, to examine the physiological function of the colon and anorectum. An introduction to transit studies has been made in chapter 1, and the contractile activity of the colon and rectosigmoid in health and disease, as tested by manometric studies has also been discussed. Such manometric tests predominantly remain a research tool. This section specifically describes the tests used routinely to assess patients presenting with constipation to the GIPU. Whilst some other tests may have been performed for some patients, including anorectal electromucosal sensitivity testing, pudendal nerve motor latencies and anal ultrasound, these had not been uniformly performed, and are not presented in this thesis. The data from the following tests are included in this thesis:

1. Transit studies	Radio-opaque marker and / or radioisotope
2. Anal manometry	Anal sphincter resting and squeeze pressures
	Rectoanal inhibitory reflex testing
3. Rectal sensory testing	First constant sensation
	Defaecatory desire volume
	Maximum tolerated volume
4. Evacuation proctography	
5. Upper gastrointestinal studies	Prolonged ambulatory small bowel manometry

2.4.1 TRANSIT STUDIES

2.4.1.1 Radio-opaque markers

Laxative medication and opiate analgesics were stopped 24 h prior to the start of the study and avoided until its completion. Patients remained on their normal diet during the study period. Studies were carried out as previously described (Roberts *et al.*, 1993), with a single plain abdominal radiograph performed at 100 h after administration of a gelatin capsule (broken down rapidly in the stomach) containing 50 radio-opaque markers, cut from a length of 2.5 mm (external diameter) diameter radio-opaque vinyl tubing (SIMS Portex Ltd., Hythe, UK). A positive study was defined as >20% of 50 administered markers remaining at 100h (Hinton *et al.*, 1969; Evans *et al.*, 1992; Roberts *et al.*, 1993).

2.4.1.2 ^{111}In-[DTPA] isotope colonic scintigraphy

Laxative medication and opiate analgesics were stopped 24 h prior to the start of the study and avoided until its completion. Patients remained on their normal diet during the study period. Colonic scintigraphy was carried out as previously described (Roberts *et al.*, 1993). 3.7 MBq of [^{111}In]DTPA (Amersham International Plc, Amersham, UK), equivalent to an effective whole body dose of 1.1 mSv in normal individuals, was ingested orally in a small quantity of water on the afternoon of day 1 ($Time_{0\ h}$). Anterior (supine) and posterior (prone) abdominal scans were taken with a gamma camera (Scintiview; Siemens Plc., Bracknell, UK) twice a day for the following four days at $T_{18,\ 24,\ 42,\ 48,\ 66,\ 72,\ 90\ \&\ 96\ h}$. Any bowel movement that occurred following ingestion of the radioisotope was collected and the activity counted.

Data Processing and Analysis

Seven regions of interest (ROIs) were drawn around computer generated images of the anterior and posterior abdominal scans for each patient, namely: (1) caecum and ascending colon, (2) hepatic flexure, (3) transverse colon, (4) splenic flexure, (5)

descending colon, (6) sigmoid colon and rectum, and (7) excreted faeces (Roberts *et al.*, 1993). The percentage activity (of total ingested activity) in each ROI was calculated at each time point by customised computer software (Micas V Nucmed software; Park Medical Systems (UK) Ltd., Farnborough, UK) which corrected scans for decay, and averaged corresponding anterior and posterior scans to correct for tissue attenuation (geometric mean). Time-activity curves for each ROI were then generated, and the geometric centre of isotope mass (GCI) at each time point plotted (Figure 2.01) (Krevsky *et al.*, 1986; Roberts *et al.*, 1993). GCI is synonymous with MAP (mean activity position: (McLean *et al.*, 1995), COM (centre of mass: van der Sijp *et al.*, 1993), or geometric centre (Krevsky *et al.*, 1986; Stivland *et al.*, 1991).

STC was defined by scintigraphy as a failure of total excretion of isotope by approximately 70 hours (based on control data from the Radioisotope Department, Royal London Hospital) (Roberts *et al.*, 1993). In practice, patients showed significant slowing of isotope progression at 48 hours with minimal faecal elimination occurring in the duration of the study (96 hours) (Roberts *et al.*, 1993). Delineation of differential patterns of colonic transit was based on previous published guidelines: a delay throughout the entire colon (generalised delay), based on a GCI of < 3.6 at 48 h (Roberts *et al.*, 1993; Maurer & Krevsky; 1995) or a distal colonic delay only, based on a GCI of ≥ 3.6 at 48 h (Roberts *et al.*, 1993).

2.4.2 OTHER PHYSIOLOGICAL TESTS

2.4.2.1 Anal manometry

Satisfactory measurements of anal canal pressures and anal sphincter responses can be obtained with open-tipped or side-opening water-perfused catheters, direct online solid-state transducers, or air- or water-filled balloons of various sizes and configurations. Normal anal canal pressures vary according to sex, age and technique used (see AGA Technical review, Diament *et al.*, 1999). In general, pressures are higher in men and younger people (Jameson *et al.*, 1994), and are reduced by

Region of interest (ROI)

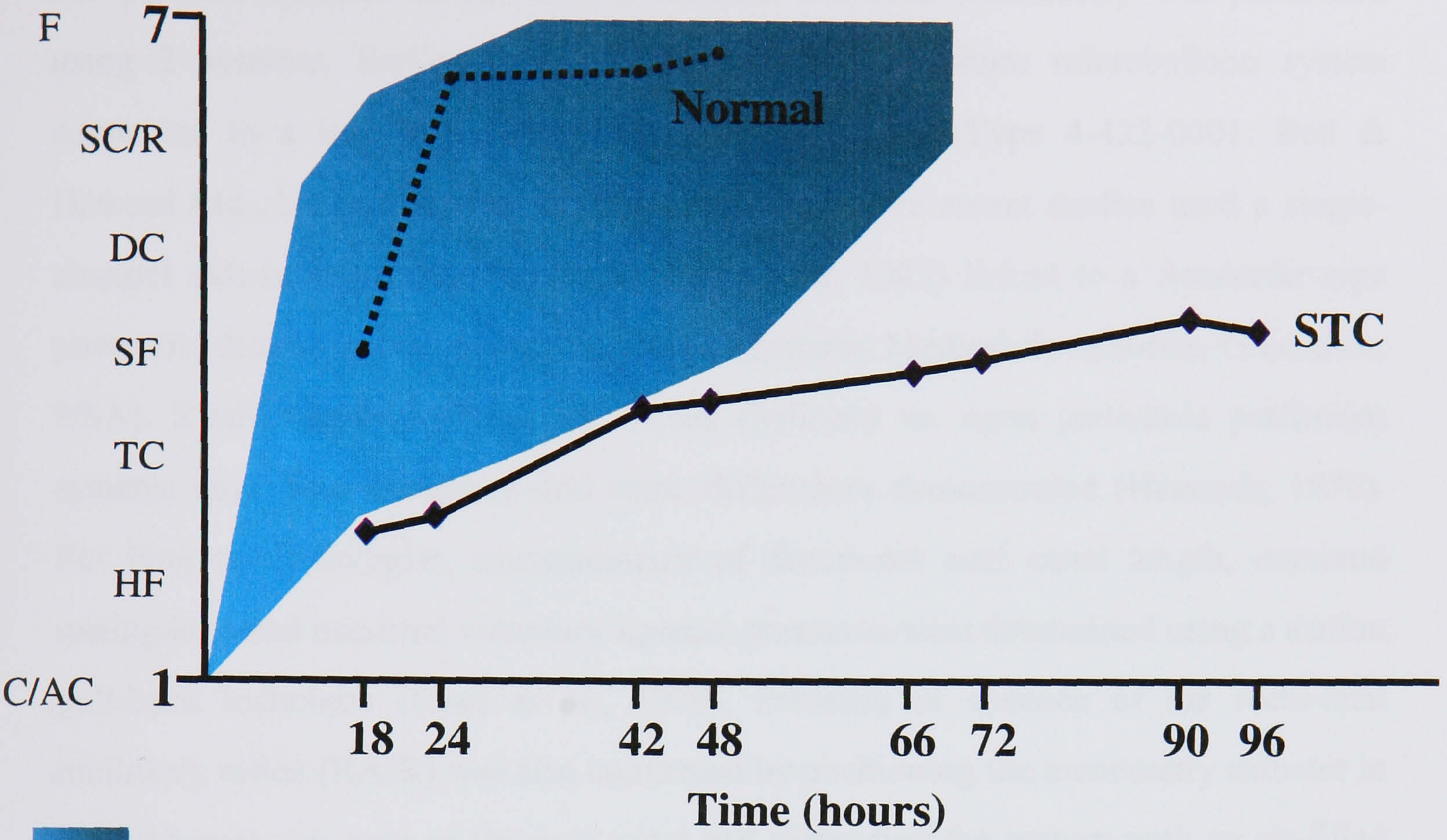


Figure 2.01: Isotope scintigraphic transit study. Progress of the geometric centre of isotope mass (GCI) through the colon with time. Seven regions of interest (ROIs) are defined:

C/AC= caecum / ascending colon;

HF = hepatic flexure;

TC = transverse colon;

SF = splenic flexure;

DC = descending colon;

SC/R = sigmoid colon / rectum;

F = faeces.

The graph shows a normal control, control range, and a typical trace of a patient with generalised STC.

childbirth (Sultan *et al.*, 1993).

For patients included in this thesis, standard anorectal manometry was performed using 2 systems. Earlier studies used a water-filled 4mm microballoon system connected to a Bell and Howard pressure transducer (Type 4-422-0001, Bell & Howard Ltd., UK) (Hallan *et al.*, 1989), whereas more recent studies used a single-channel side-hole catheter (McHugh & Diament, 1987) linked to a Arndorfer-type pneumohydraulic water perfusion system (Arndorfer Medical Specialities, Greendale, USA). Similar methodologies i.e. closed (balloon) vs. open (side-hole perfusion) systems have been compared and some differences demonstrated (Hancock, 1976). For both methodologies, measurements of functional anal canal length, maximal resting tone and maximal voluntary squeeze pressures were determined using a station pull-back technique (Read *et al.*, 1979). Presence or absence of the recto-anal inhibitory reflex (RAIR) was also confirmed by positioning the manometry catheter in the high-pressure zone of the anal canal and distending the rectum with an air-filled balloon tied to a Foley-catheter (Farthing & Lennard-Jones, 1978). For the closed system departmental control values for women < 50 years of age were used to define abnormal results (Hallan *et al.*, 1989): resting pressure (40 – 110 cm H_2O), squeeze increment (40 – 160 cm H_2O). For the perfusion system, no departmental control values were available, and control values were taken according to data using the same methodology from St Mark's Hospital: resting pressure (50 – 110 cm H_2O), squeeze increment (50 – 160 cm H_2O). Such values are comparable to those obtained from the literature for this technique (Read *et al.*, 1979; Read *et al.*, 1986). Reference values, stratified for age and parity were not available for this study, and any conclusions derived from these findings have been accordingly treated with caution (Chapter 3).

2.4.2.2 Rectal sensory testing

Rectal sensation was assessed by inflating the same rubber party balloon with air at 1 ml/sec and determining the threshold volumes for first constant sensation, defaecatory desire and maximum toleration (Farthing & Lennard-Jones, 1978). Values obtained

for each sensory threshold were compared with normal ranges matched for age and sex (Jameson *et al.*, 1994).

2.4.2.3 Evacuation proctography

Evacuation proctography was performed by instilling barium paste (artificial stool) into the rectum to the previously determined maximum tolerable volume and allowing the patient to evacuate under fluoroscopy (Mahieu *et al.*, 1984; Womack *et al.*, 1985). The semisolid contrast medium was prepared by mixing barium sulphate and scotch porridge oats at body temperature to make a thick paste. The contrast was injected into the rectum using a wide tipped syringe via a proctoscope. With the patient seated on a radiolucent commode, lateral fluoroscopy was performed using an image intensifier (Siemens Plc., Bracknell, UK). The amount of contrast voided, and the time taken to void were recorded. Any radiological abnormalities in the rectum were identified.

The definition of what constitutes normality or a rectal evacuation disorder (RED) with respect to evacuation proctography is difficult because (a) control ranges for time and volume do not exist; (b) the amount voided is estimated by eye only; (c) proctographic "abnormalities", especially small rectoceles occur in subjects without defaecatory symptoms (Shorvon *et al.*, 1989); (d) The traditional barium paste used for performing defaecography may not accurately represent normal rectal evacuatory function, when in the case of patients with STC the stool often has a consistency of hard pellets (89% of patients in this series: see appendix 2.04), and (e) embarrassment may inhibit defaecatory effort. Attempts have been made to use more "realistic" artificial stool types, but this has usually been at the expense of visualising proctographic filling abnormalities (Pelsang *et al.*, 1999). For the purposes of this thesis, the presence of a functional rectal evacuation disorder was made by a consultant coloproctologist with experience in gastrointestinal physiological investigation, based loosely on the inability of the patient to evacuate the "majority" (approx. 80%) of the contrast in a reasonable time period (approx. less than 2 min),

and the absence of an anatomical proctographic obstruction which retained contrast. Patients with everting prolapse were defined as having an RED regardless of their ability to evacuate.

2.4.2.4 Small bowel manometry

Prolonged proximal small bowel manometry was used to establish the presence or absence of a generalised intestinal disorder (Redmond *et al.*, 1995). Twenty-four hour ambulatory studies were performed in the Gastrointestinal Science Research Unit of the Royal London Hospital (GISRU) according to protocol (Castillo PhD Thesis, 1994; von Schonfeld *et al.*, 1997). Studies used a thin, flexible manometric catheter incorporating 3-6 strain-gauge micro-transducers [type: CTG/L3] (Gaeltec Ltd., Dunvegan, Isle of Skye), and a central air channel and 10ml balloon. Catheters were pre-cleaned and calibrated. Patients were intubated in the sitting position with the use of a water-soluble lubricant, and xylocaine spray to anaesthetise the nasal and oral mucosa. Advancement of the tip of the catheter was facilitated by asking the patient to swallow water. Catheter position was confirmed using fluoroscopy, with measures taken to limit radiation exposure. Once the middle catheter had reached the ligament of Trietz the balloon was deflated, the catheter securely taped to the side of the face and ear, and the connector plugged into a Flex 3000 multichannel intestinal data logger (IDL) (Oakfield Instruments, Oxford, UK). At the end of 24 hour recording, the catheter was removed, and data transferred to an IBM PC.

Analysis was performed by Dr Mark Scott and Dr Etsuro Yasaki using specialised software developed in the GISRU (Benson *et al.*, 1993). The computer algorithm used to analyse the large numbers of pressure events over the recording period has been shown to be superior in reliability and reproducibility, as well as considerably faster, than manual visual analysis (Benson *et al.*, 1993). In particular, the algorithm overcomes the problems of variable baseline and sudden changes in pressure due to body movements that are unavoidable in prolonged recording from the small bowel of ambulant subjects.

Interpretation of data analysed by the computer algorithm was performed by Professor David L Wingate (GISRU). Interpretation reviewed 22 main parameters of phasic activity of the intestinal interdigestive migrating motor complex (IMMC) for daytime and nocturnal recording periods, comparing numeric activity values with departmental controls (Castillo PhD Thesis, 1994). Patterns of abnormality, said to be indicative of neuropathic or myopathic disease have been previously described (Soffer & Thongsawat, 1996), and continue to be further validated (Wingate, 1999; *manuscript in preparation*)

2.5 DATA ANALYSIS

2.5.1 SOFTWARE

Following database entry, data were generally exported following appropriate execution of 'queries' into a spreadsheet application (Microsoft ® Excel 97, SR-1, Microsoft Corporation, Santa Rosa, CA, USA). After manipulation within this format, further statistical analysis and graphic presentation of data were performed using a computerised statistical package (GraphPad Prism v. 2.0, GraphPad Software, Inc., San Diego, CA, USA), unless otherwise stated (see chapter 4: Systat v. 8.0, SPSS, Inc. Chicago, USA).

2.5.2 STATISTICAL ANALYSIS

Specific statistical analyses are described within the chapters to which they refer. Deviations from a Gaussian distribution were tested using the Kolmogorov-Smirnov test with a p value calculated from Dallal and Wilkinson's approximation to Lilliefor's method (Dallal & Wilkinson, 1986). In general, most data comparison was performed with non-parametric analyses based on the distribution of data points.

Comparing numerical data between groups

The Mann-Whitney U-test was used for comparison of 2 independent populations of

non-parametric numeric data, and the Wilcoxon rank-sum test for paired sets of data. For studies comparing 3 or more groups, an analysis of variance (ANOVA) was performed using the Kruskal-Wallis test +/- a post test for multiple comparison of individual groups (Dunn's multiple comparison post test).

Contingency analyses

Contingency analyses were performed using Fisher's exact (2 rows and columns in contingency table) or chi-square tests (more than 2 rows or 2 columns in contingency table). A two-tail *p* value was calculated in all cases.

Correlation

Linear correlation or regression was used to compare the covariation of 2 numeric variables. In simple terms, regression was chosen when X values were controlled e.g. age. When linear regression analysis was used, 95% confidence intervals (CI), goodness of fit (r^2), and residuals were calculated. A *p* value testing whether the slope is significantly different from zero was calculated from an F test. These parameters are shown on the graphs, as is now the recommended convention (Porter AM, 1999). When correlation was applied, parametric (Pearson correlation) or non-parametric (Spearman correlation) methods were used as appropriate.

The measure of inter-rater or inter-method agreement for categorical data was presented as the kappa (κ) statistic to correct proportional agreement for chance (Altmann, 1996). The interpretation of the κ value was based on published guidelines (Landis & Koch, 1977).

For all tests, $p < 0.05$ was considered to show a significant difference.

2.6 LITERATURE REVIEW AND REFERENCING

References within this thesis were obtained by literature review, including the use of Medline searches from: Ovid for Windows v. 3.0, release 7.05 (Ovid technologies

Inc, New York, USA), and Pubmed, National Center for Biotechnology Information [www.ncbi.nlm.nih.gov].

All references have been prepared in accordance with the "Uniform requirements for manuscripts submitted to medical journals" developed by the international Committee of Medical Journal Editors (*N Engl J Med* 1991; **324**: 424-428), based on formats for bibliographical references first developed by the Vancouver Group.

2.7 STC PATIENT DATA SUMMARY

2.7.1 SELECTION OF THE STUDY POPULATION

Inclusion (see 2.3.2.1)

Of 1365 patients referred for lower gastrointestinal investigation during the 10 year period, 1989 to 1999, 485 presented with symptoms of severe intractable constipation. Of these, 146 patients were found to have slow colonic transit.

Exclusion (see 2.3.2.2)

Ten patients were excluded for lack of information. Four patients were found to have megarectum and are excluded from this data. Two further patients were excluded on the basis of the demonstration by scintigraphy of isolated functional rectosigmoid hold-up, coincident with a significant mechanical RED, leaving a total of 130 patients. No patient was found to have HSCR or an obstructive organic cause for constipation.

The final figure of 130 was equal to 9.6% of the total, and 26.8% of constipated patients referred to the GI physiology Unit for investigation. Patients with normal colonic transit (73.2%) had constipation attributable to other causes (e.g. isolated rectal evacuation disorders, constipation-predominant irritable bowel syndrome). The proportion of patients with STC as a function of the total presenting with intractable constipation is higher than that reported from the Mayo Clinic in an equally large series (Nyam *et al.*, 1997), but comparable with European series (de Graaf *et al.*, 1996). This difference

from series in the USA may reflect an increased presentation or prevalence of IBS in this population, or may simply reflect referral pattern to our institution which historically has a surgical bias. Indeed, some patients were referred for further specialist investigation on the basis that STC had already been proven at the referring centre.

2.7.2 RECRUITMENT DATA (see 2.3.3.1)

Fifty patients were recruited prospectively. A further 35 patients were recalled for interview, leaving 45 whose data were collected by notes review only.

2.7.3 DEMOGRAPHIC DATA (APPENDIX 2.02)

The wide age range reflects the heterogeneity of the population (Chapter 3), and is comparable with previous series (Kamm *et al.*, 1988; Yoshioka & Keighley, 1989; Piccirillo *et al.*, 1995; Lubowski *et al.*, 1996; Platell *et al.*, 1996; Nyam *et al.*, 1997). The strong female predominance (32: 1) is also recognised (Preston & Lennard-Jones, 1986; Kamm et al, 1988; Chaussade et al, 1989). The interpretation of the strong caucasian predominance is difficult. The majority of patients from Greater London were residents of the London Borough of Tower Hamlets which has a large ethnic proportion of the population. The relatively low incidence of ethnic minorities may reflect cultural differences with respect to the decision to report constipation, or may be a true reflection of a lower incidence in these populations. Large demographic studies in the USA have not shown differences in self-reporting of symptoms of constipation between such populations (Johanson & Sonnenberg, 1990). The geographical make-up of patients reflects the referral practice of consultants within and without the Royal Hospital's catchment area.

2.7.4 AGE AND MODE OF ONSET (APPENDIX 2.03)

Data with respect to the age and mode of onset are discussed in detail in chapter 3. Commonly, the patient was unable to ascribe an exact age to the start of their

symptoms. In such cases, a notional age was given dependent on the patient's response to this question. Where the patient described the onset as "a baby" or "in infancy", the age of onset was taken to be 1 year; onset "in early childhood", 5 years; "in childhood" (unspecified), 8 years; and in teenage, 15 years. Duration of symptoms was then calculated by subtracting the age of onset from the age at presentation. Patients in whom symptoms were present from childhood, but who presented only after a significant worsening of their symptoms in adulthood (n = 11) usually as a result of pelvic surgery, are discussed in chapter 3. These patients are not shown in the appendix. Median age of initial onset in these patients was 8 years (range 4 – 8), and median age of symptomatic worsening was 40 years (range 22 – 50). The wide rage of ages of onset overall reflects the heterogeneity in the STC population, and has been shown in previous series (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988). Symptom duration was similarly highly variable: median 20 years, range (1 – 71).

The mode of onset i.e. idiopathic vs. following a recognised precipitating event e.g. pelvic surgery is discussed in detail in chapter 3. The main precipitating factors are listed in appendix 2.04.

2.7.5 PRESENTING SYMPTOMS (APPENDIX 2.05)

The frequency of bowel action was taken as the time numerically in days between successive bowel actions (with laxatives if these were being taken) at the time of presentation. The value was based on the mean of the extremes when ranges were given. In some patients a numeric value could not be obtained on questioning. The median bowel frequency was 8 days (range 1 – 49 days) in the 115 patients where a value was recorded, and is comparable with previous series (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988). Abdominal bloating was the commonest symptom (after constipation), occurring in 97% of patients. Incomplete evacuation and abdominal pain were similarly very common (95%). Less common symptoms included incontinence of faeces (self-defined by the patient: 20%), a feeling of rectal prolapse (14.5%), and the need for digitation (either vaginal or rectal: 24%).

Interestingly, upper gastrointestinal symptoms, particularly nausea or vomiting were reported by over half the patients. The frequencies of these symptoms were again comparable to previous studies (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988).

2.7.6 MANAGEMENT (APPENDIX 2.06)

Ninety percent of patients were taking, or had taken laxatives for the treatment of constipation. Multiple laxative preparations had commonly been tried, and included bulk forming laxatives (commonly ispaghula husk), stimulant laxatives (commonly bisacodyl, danthron, senna and sodium picosulphate), osmotic laxatives (commonly lactulose). A smaller proportion used suppositories (usually glycerine) (39%) or enemata (usually phosphates) (28%). Only 7% had been prescribed a prokinetic (cisapride in all cases).

A wide variety of surgical interventions had been used. These included anorectal procedures for the treatment of mechanical obstructions to defaecation such as rectopexy for prolapse, and rectocele repair. A proportion of patients had undergone colonic resections, most commonly subtotal colectomy and ileorectal anastomosis (Preston *et al.*, 1984) (n = 15, 12%). Colonic conduit formation is a relatively new operation (Williams *et al.*, 1994) to allow anterograde colonic irrigation through a continent stoma, and was performed in 6 patients. In general, the results of surgery were variable: of the total of 8 ileostomies formed, 4 were performed electively as a first procedure, but 4 were performed for poor outcome from colonic surgery.

Two patients had been included in a trial of botulinum injections for a diagnosis of "anismus" (see Hallan *et al.*, 1988). Interestingly, despite the demonstration of the efficacy of behavioural therapy in some centres (Chiotakakou-Faliakou *et al.*, 1998), only 2 recently investigated patients had been further referred for biofeedback therapy. This small number probably reflects the lack of availability of this service in the East London Region.

2.7.7 PERSONAL HISTORY (APPENDIX 2.07)

2.7.7.1 Surgical history

Common general surgical operations included appendicectomy (11%) and cholecystectomy (4%). In general, a history of abdominal surgical procedures was much less frequent than gynaecological procedures with only 31% of patients having undergone one or more procedure.

2.7.7.2 Obstetric history

Over 2 / 3rd of patients were parous. The distribution of parity is shown with the rates of instrumentation (venteuse or forceps) and caesarean section. Rates of episiotomy and tears (of any degree), and of prolonged second stage of labour were recorded for some patients, but not specifically questioned in the majority. These data have not therefore been tabulated. Overall, approximately 10% of patients had one or more caesarean section, and 7% had one or more instrument assisted deliveries.

2.7.7.3 Gynaecological history

Commonly reported gynaecological conditions and interventions are shown in the appendix. Approximately one third of patients had a diagnosed coexistent gynaecological disease. Over half (64 / 113) of patients had undergone some gynaecological surgical intervention, with some having multiple procedures. This figure is comparable with other STC series in which the coincidence of procedures was similarly high (Preston *et al.*, 1984; Kamm *et al.*, 1988), and may reflect a role either of such surgery in the causation of STC (20 patients attributed origin or worsening of symptoms to gynaecological surgery, especially hysterectomy) (see chapter 1.4.1.1), or that gynaecological surgery was performed for symptoms (especially pelvic and abdominal pain) that were actually related to STC. Hysterectomy was the most commonly performed procedure (38 patients, 34% total).

The low incidence of recorded gynaecological disorders (especially in comparison with gynaecological surgery) reflects the fact that the majority of surgery was performed on the basis of symptoms rather than an underlying diagnosis i.e. menorrhagia, dysfunctional uterine bleeding and dysmenorrhea. Although such symptoms were commonly reported, data were not specifically collected, and have therefore not been included.

2.7.7.4 Past medical history

The numbers of patients with metabolic / endocrine, cardiorespiratory, neoplastic, neurological and spinal conditions are shown in the appendix. No patient had diabetes mellitus, and although 5 patients had thyroid disease, all these had been successfully treated. Both patients with malignant disease (malignant ocular melanoma, pulmonary carcinoid) had been successfully treated, and at the time of review were in remission from disease. Reported neurological conditions were uncommon, but spinal disease (13%) or spinal surgery (8%) were relatively common. This reflects in part, the 7 patients whose bowel symptoms arose as a result of CNS injury.

2.7.7.5 Psychiatric history

A history of psychiatric illness was present in 23% of patients. It should be noted that this figure included patients with a disorder at any time in the past regardless of severity, or need for specific therapy. For instance, whilst depressive illness was relatively common (13%), only 7% of patients (see below) were currently taking anti-depressant medication. Other disorders e.g. anxiety states, behavioural disorders were less common.

2.7.8 DRUG HISTORY (APPENDIX 2.08)

Current (at the time of study), physician or self-prescribed medications have been tabulated. Analgesics were most commonly used (22%). Eleven patients were taking

opiate-containing medications, and this group included several post-colectomy patients using codeine for its anti-diarrhoeal properties as well as those taking opiates for analgesia. Antidepressants or antianxiolytics were only used by 9 patients. With the exception of 2 patients (see above: drug abuse) the use of opiates post-dated diagnosis of STC. Antidepressants may have influenced constipation, but no patient was taking any other drugs that are known to be neurotoxic, or induce constipation e.g. verapramil, anticholinergics.

2.7.9 FAMILY HISTORY (APPENDIX 2.09)

Of the total of 91 patients in which data were recorded (37 data not available, 2 adopted), 39 patients (43%) gave a history of constipation in one or more family relative. Seventeen patients (19%) had relatives who had consulted medical practitioners for their problem, and included patients who had undergone investigation and surgery for constipation (n = 3). One patient had an identical (monzygotic) twin with severe symptoms comparable with her own. Four index cases had one or more relative with HSCR who are further discussed in chapter 6. A family history of autoimmune and rheumatological conditions was elicited in those patients tested for ion channel autoantibodies (see chapter 7).

2.7.10 SYSTEMATIC ENQUIRY (APPENDIX 2.10)

A high proportion (39%) of patients had coexistent urological symptoms. Commonly reported symptoms included urinary frequency (24%) and incontinence (stress and urge) (19%). Such urinary problems are known to commonly occur in patients with STC (Abdel-Rahman *et al.*, 1981; Watier *et al.*, 1983; Bannister *et al.*, 1988; Kerrigan *et al.*, 1989). Enquiry for neurological symptoms, including those suggestive of peripheral and autonomic dysfunction was undertaken for patients having neurophysiological studies (Chapter 5). Similarly, symptoms suggestive of autoimmune or rheumatological disease are included in chapter 7.

3 SLOW TRANSIT CONSTIPATION: RESULTS OF PHYSIOLOGICAL STUDIES; CLINICAL AND PHYSIOLOGICAL HETEROGENEITY

3.1 INTRODUCTION

Patients with STC may be considered for surgical intervention, including subtotal colectomy and ileo-rectal anastomosis or segmental resection (Preston *et al.*, 1984). The highly variable outcomes of such surgical procedures in this group of patients (Preston *et al.*, 1984; Kamm *et al.*, 1988; Piccirillo *et al.*, 1995; Nyam *et al.*, 1997; Bernini *et al.*, 1998; Hasegawa *et al.*, 1999) have been described. These may be due to the lack of effectiveness of such interventions as a whole, or may simply reflect heterogeneity within this group of patients. However, difficulty exists in comparing outcomes after surgery because of lack of detail regarding patient selection (Knowles *et al.*, 1999). In particular, most publications do not detail clinical history beyond duration of symptoms, and uniform or complete pre-operative physiological work-up is not always documented.

It is well recognised that heterogeneity of clinical presentation exists amongst patients with STC. In the majority of patients, symptoms arise *de-novo* in childhood, and these have been labelled chronic and idiopathic (Preston & Lennard-Jones, 1986). The aetiology of such idiopathic cases remains unclear, and is probably itself heterogeneous, with attribution to extrinsic autonomic, intrinsic neuropathic or myopathic dysfunction, and even psychogenic causes.

A proportion of patients with intractable slow transit constipation present in later life. Some of these patients will have no obvious trigger for their complaint (Waldron *et al.*, 1988), whereas others follow events such as hysterectomy (Roe *et al.*, 1988, Vierhout *et al.*, 1993) or childbirth (MacDonald *et al.*, 1993; MacDonald *et al.*, 1997). An additional

sub-group includes those patients whose constipation appears to follow a recognised acute or chronic neural injury. This sub-group includes presumed myenteric damage e.g. diabetes (Iber *et al.*, 1993; Maleki *et al.*, 1998), progressive systemic sclerosis (Howe *et al.*, 1993; Eaker *et al.*, 1999), Chagas' disease (Wood *et al.*, 1982; de Oliveira *et al.*, 1998; Goin *et al.*, 1999), spinal injury (trauma, tumour) (Devroede *et al.*, 1979; Beuret-Blanquart *et al.*, 1990; Keshavarsian *et al.*, 1995; Leduc *et al.*, 1997; DeLooze *et al.*, 1998), and CNS disease (Parkinson's, CVA, demyelination e.g. MS) (Weber *et al.*, 1987).

It is similarly recognised that, in addition to the finding of a colonic transit disturbance, other gastrointestinal and extra-gastrointestinal physiological abnormalities may co-exist, including: (a) disorders of rectal evacuation, either anatomical (Waldron *et al.*, 1990; Wald *et al.*, 1990; Nyam *et al.*, 1997; Bernini *et al.*, 1998; Hasegawa *et al.*, 1999), sensory (Akervall *et al.*, 1988; Waldron *et al.*, 1990; Kamm & Lennard-Jones, 1990), or functional (Read *et al.*, 1986; Wald *et al.*, 1990); (b) upper gastrointestinal dysmotility (Watier *et al.*, 1986; Panagamuwa *et al.*, 1994; Bassotti *et al.*, 1996; Ghosh *et al.*, 1996; Glia & Lindberg, 1998); and (c), bladder dysfunction (Abdel-Rahman *et al.*, 1981; Watier *et al.*, 1986; Kerrigan *et al.*, 1989; Ghosh *et al.*, 1996).

In this chapter:

1. The physiological data collected from all STC patients are presented.

2. Patients have been classified into sub-groups based on clinical findings, especially with respect to those features outlined above i.e. clinical presentation. The results of gastrointestinal physiological investigations have then been analysed to see if differences exist between these sub-groups. The chapter focuses principally on the 2 main sub-groups (patients with chronic idiopathic STC and those whose symptoms arose following pelvic surgery or childbirth).

3.2 STC: PHYSIOLOGICAL RESULTS

3.2.1 TRANSIT STUDIES

3.2.1.1 Radio-opaque marker studies (Table 3.01)

A positive marker study was defined as ≥ 20% of 50 markers retained at 96 hours (Hinton *et al.*, 1969; Evans *et al.*, 1992; Roberts *et al.*, 1993). The majority (93%) of patients had documentation of a positive study.

Table 3.01: Results of radio-opaque marker studies

	Number
Not known (but positive scintigraphy)	3
Not performed (but positive scintigraphy)	3
Negative c/o diarrhoea at the time of study (but positive scintigraphy)	3
Positive	121 (93%)
Distribution known	108 / 121
Median marker retention [range]	43 / 50 (86%) [20 - 100%]
Retention of all markers	45 / 108 (42%)

The distribution of markers was right-sided in 4 patients, generalised in 51, left-sided in 29, left-sided and rectum in 24, and not-known (film not available for review of distribution, but study reported positive study) in 13.

3.2.2.1 ^{111}In-DTPA isotope scintigraphy

A total of 80 patients underwent radioisotope scintigraphy. The remaining patients did not undergo scintigraphy because some had attended before the introduction of this investigation, and others were unable to attend because of time constraints or

geographical reasons. A delay throughout the entire colon was diagnosed in 53 patients (66%), and a left-sided (i.e. distal colonic delay) in 27 (34%). For the 71 patients undergoing scintigraphy with a prior positive radio-opaque marker study, 61 x-ray films were available for quantitation (see 2.4.1) of the centre of markers at 96 hours. Table 3.02 demonstrates that in the prediction of a generalised or left-sided delay, the radio-opaque marker studies were poorly predictive of the final pattern as evidenced by scintigraphy (inter-method agreement: $\kappa = 0.31$, CI 0.28 – 0.34, 31% misclassified). Assuming scintigraphy to be the gold standard, this is in keeping with previous reports that have suggested that a single radio-opaque marker study is only adequate in screening for STC, not for determining pattern of transit, especially if surgery is considered (van der Sijp *et al.*, 1993; Lennard-Jones, 1994).

Table 3.02: Cross tabulation of pattern of transit by radio-opaque markers and radioisotope scintigraphy

Prediction by radio-	***Pattern as evidenced by radioisotope study***		
opaque marker study	Generalised	Left-sided	Total
Generalised*	31	7	38
Left-sided*	12	11	23
Total	43	18	61

* defined by C.O.Markers (see text)

3.2.2 ANAL MANOMETRY

The results of anal manometry are shown in table 3.03. Test results were available in 124 patients, and compared with control values for women, or men ≤ or > 50 years of age, obtained from the literature (see chapter 2). Reduced resting anal canal pressures were uncommon, occurring in only 7% of patients. That three patients had demonstrable anal hypertonia is consistent with a previous report in a cohort of patients with chronic constipation (Meunier, 1986).

Table 3.03: Results of anal manometry

	Normal	Reduced	Increased	Not known
Resting pressure (cmH_2O)*	112	9 (7%)	3	6
Squeeze pressure(cmH_2O)†	81	41 (33%)	-	6

* based on a lower limit of normal of 40 or 50 cm H_2O dependent on method used, and upper limit of normal of 110 cm H_2O.

† based on a lower limit of normal of 40 or 50 cm H_2O dependent on method used, and an upper limit of normal of 160 cm H_2O (see CHAPTER 2.4.2.1: Modified from Hallan *et al.*, 1989; Read *et al.*, 1979; Read *et al.*, 1986)

A total of 41 (33%) of patients had reduced anal squeeze pressures. The proportion of patients with reduced squeeze pressures increased with parity (Table 3.04). Further comment on the pathophysiological significance, (for instance in keeping with a generalised neuropathological or myogenic disorder), of these findings in respect of STC can not be made because:

1. There is no uniform agreement of what pressure values constitute normality. In female subjects, parity must be considered as a further independent variable since this is known to affect both squeeze and resting pressures (Snooks *et al.*, 1990; Sultan *et al.*, 1993; Jameson *et al.*, 1994) regardless of any documentation of instrumentation or other complication during delivery (Snooks *et al.*, 1990), or proven obstetric-related sphincter defects which further reduce pressures (Sultan *et al.*, 1993). The control values used (Read *et al.*, 1979; Read *et al.*, 1986; Hallan *et al.*, 1989) do not distinguish values by parity, and those studies which do (Sultan *et al.*, 1993; Jameson *et al.*, 1994), do not use identical methodology, nor give sufficient data to be used as control ranges. There was a decrease in squeeze pressures with parity in the female STC patients (Table 3.04) consistent with these observations.

2. The absence of data from testing of pudendal nerve motor latencies and endosonographic visualisation of the sphincter complex in the majority of patients (techniques only introduced recently) prevented identification of the cause of sphincteric dysfunction. It is likely, based on the number of patients with episiotomies, tears and instrumented deliveries (Appendix 2.06) that a proportion will have obstetric-related mechanical disruption of the sphincter complex.

Table 3.04: Anal canal maximum squeeze pressures *vs.* parity

	Anal canal squeeze pressures	
Parity	Normal	Reduced
0	27	7 (26%)
1	10	5 (33%)
2	23	13 (36%)
3	6	7 (54%)
≥4	5	7 (58%)

3.2.3 RECTAL SENSORY TESTING

The results for rectal sensory thresholds to balloon distension were available in 121 patients, and are shown in table 3.05. Overall, 62 patients (51%) had a raised threshold to one or more of the 3 thresholds tested compared with control figures (Jameson *et al.*, 1994). Two or more thresholds were raised in 41 patients (34%), and all three in 16 patients (13%). A raised threshold for defaecatory desire was the most common finding. This has been previously reported (Read *et al.*, 1986; Shouler & Keighley, 1986), and may underlie the common complaint of loss of urge in STC patients. There was no correlation between the presence of rectal hyposensation, and the number of radio-opaque markers retained on transit studies.

Table 3.05: Results of rectal sensory testing

Threshold	*Median (range)*	*Normal Range**	*Number normal*	*Number abnormal*	*Not known*
Minimum perceived Volume (MPV)	40 (10 - > 400)	0 – 75	87	30 (26%)	13
Defaecatory desire Volume (DDV)	135 (20 - > 400)	21-141	60	48 (44%)	22
Maximum tolerated Volume (MTV)	200 (50 - > 400)	61-265†	86	36 (30%)	8

KEY: * based on values for women ≤ 50 years of age (from Jameson *et al.*, 1994). † 304 ml if > 50 years of age (from Jameson *et al.*, 1994). All values in ml.

3.2.4 EVACUATION PROCTOGRAPHY

Evacuation proctography was performed in a total of 120 patients. Common proctographic abnormalities occurred in all but 29 patients tested (76%), and are listed in table 3.06. These figures are comparable with previous studies of STC, and are unlikely to be of overall significance for reasons discussed in chapter 2 (Shorvon *et al.*, 1989).

A rectal evacuation disorder (RED) by any cause was present in 55 (46%) of patients undergoing proctography (n = 120). Median contrast evacuated was 90% (0 – 100%). The "main" causes of failure to evacuate are listed in table 3.07 (see notes, Chapter 2). Patients labelled as having mechanical obstruction included a proportion without proctographic abnormality, but who failed to relax and open the anal canal on straining with or without, in addition, failure to open the anorectal angle. Such patients have been variably referred to as having "idiopathic or functional" outlet obstruction (Duthie &

Bartolo, 1992). When puborectalis muscle fibre recruitment has been demonstrated by EMG, this condition is often described as "anismus" (Preston & Lennard-Jones, 1985b). It now seems likely, on the basis of subsequent studies, that this latter physiological entity has been overdiagnosed (Miller *et al.*, 1991; Voderholzer *et al.*, 1997).

Table 3.06: Proctographic abnormalities

Proctographic abnormality	***type***	***Number***
Intussusception	Anterior	18
	Posterior	7
	Large circumferential	4
	Unspecified	8
	Sub-total	***37 (31%)***
Rectal prolapse (any)		2
Rectocele	Small	35 (29%)
	Moderate	21
	Large	16
	Unspecified	4
	Sub-total	***76 (63%)***
Any abnormality	***Total***	***91 (76%)***
Nil		29
Not known		10

A prolonged discussion of this topic is not relevant to this thesis, but based on our own observations and those of others *(personal communication: Professor DZ Lubowski)*, some of these patients may be unable to evacuate actually as a result of loss or rapid adaptation of urge i.e. rectal hyposensation. When further contrast was installed in the rectum of 6 such patients, evacuation was rapid and complete. This physiological situation is in accord with the description of "pseudoanismus", which may be secondary

to disordered reflex colorectal motility (Bampton *et al.*, 1998; *DZ Lubowski, personal communication*).

Table 3.07: Causes of failure of rectal evacuation

Mechanical obstruction	Functioning rectocele / intussusception / prolapse	33
	Failure anal canal opening	20*
	Sub-total	***46***
Failure to strain	Embarrassment	2
	Pain	2
	Unable	5
	Sub-total	***9***
Total with RED		**55 (46%)**
No RED		65 (54%)
Not known		10

KEY: RED = rectal evacuation disorder, * includes 9 patients labelled as having "anismus" i.e. paradoxical puborectalis contraction, in isolation, or in combination with other causes of mechanical obstruction, and 11 patients with gross rectal hyposensation and verbally-reported loss / rapid adaptation of urge to evacuate. Some were able to subsequently evacuate after installation of further contrast (see text). 7 / 20 patients also had proctographic evidence of other abnormalities.

3.2.5 SMALL BOWEL MANOMETRY

A total of 29 patients underwent investigation; intubation failed in two subjects. Patients were initially selected for study because of upper gastrointestinal symptoms (6 / 27), although subsequently, this investigation had been performed prospectively on all patients newly diagnosed with STC (21 / 27). Overall, small bowel manometry

revealed the following main abnormalities: increased overall contraction frequency, altered overall contraction frequency (increased or decreased), increased nocturnal phase II contraction frequency, and perturbances of nocturnal phase III activity (duration, cycle length and velocity). Based on expert interpretation (see chapter 2), 8 (28%) patients were defined as having definitely abnormal small bowel motility (3 or more abnormalities of measured parameters): these included 6 of the 21 (29%) patients investigated prospectively and 2 of the 6 (33%) patients referred for study because of upper GI symptoms. A further 6 (22%) patients were described as having minor abnormalities or borderline normal motility (usually based on the presence of 2 abnormalities). Of the patients with clear abnormalities, phase III abnormalities e.g. diminished velocity, prolongation and abnormal diurnal pattern, and nocturnal phase II abnormalities were strongly suggestive of a neuropathological process similar to that seen in Chagas' disease and neuropathic intestinal pseudoobstruction (Soffer & Thongsawat, 1996). No patient had a recording suggestive of myopathic abnormalities. The total figure (definite + minor abnormalities; 28 + 22%) of 50% STC patients with manometric abnormalities is in keeping with data from five hour (Mollen *et al.*, 1999), and prolonged antroduodenal studies (Glia & Lindberg, 1998), and shorter studies of more distal small bowel motility (Bassotti *et al.*, 1996).

3.3 CLINICAL AND PHYSIOLOGICAL HETEROGENEITY OF PATIENTS WITH STC

3.3.1 PATIENTS AND METHODS

One hundred and twenty patients were placed into sub-groups based on clinical history. [10 patients were excluded because they had not undergone complete physiological assessment, defined for the purposes of this study as a minimum of anorectal manometry, rectal sensory thresholds to balloon distension, and evacuation proctography]. Patients were classified with particular reference to the age and mode of onset of constipative symptoms. The sub-groups included those previously described (see introduction to this chapter).

The results of physiological investigations (scintigraphic pattern of colonic transit, co-existence of extracolonic disturbances, including sphincteric dysfunction, rectal hyposensation, rectal evacuatory disorder (RED), or abnormal small intestinal motility) were then re-analysed by sub-group.

3.3.2 RESULTS

3.3.2.1 Sub-groups of patients based on clinical history

Patients were placed into one of 6 sub-groups on the basis of clinical presentation, and included those previously described (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988; Roe *et al.*, 1988; Keshavarzian *et al.*, 1995; MacDonald *et al.*, 1997). The sub-classification of patients is shown in figure 3.01, and the main demographic data for individual sub-groups, in table 3.08. Abbreviations have been assigned to each sub-group for brevity of further use.

The largest and best recognised sub-groups were (1) patients whose symptoms arose *de-novo* in childhood or teenage years, classified as chronic (early-onset) idiopathic STC (**CIST**: n = 64, 53%) (ages of onset: < 5 years, n = 9; 5–10 years, n = 37; 11-15 years, n = 11; 16-20 years, n = 7); and (2), patients who presented following events including hysterectomy (n = 5), childbirth (n = 12, complicated in 7 cases) or other pelvic procedures (2 tubo-ovarian, 1 appendicectomy, 1 uterine myomectomy, 1 laser treatment of endometriosis), which were classified as post pelvic intervention STC (**PIST**: n = 22, 18%).

Four other sub-groups were apparent. These included: patients whose symptoms arose *de-novo* in adulthood (after the age of 20 years) i.e. adult (late-onset) idiopathic STC (**AOIST**: n = 13, 11%) (Waldron *et al.*, 1988); patients whose symptoms arose in childhood but were substantially worsened by pelvic surgery or childbirth (obstetric in 2, hysterectomy in 8, urological surgery in 1) (**C/PIST**: n = 10, 8%); patients with

central neural (especially spinal) injuries (spina bifida (n = 1) or trauma (n = 1), discogenic disease (n = 2), infection (n = 1) or tumour (n = 1)) (**NIST**: n = 6, 5%) (Kesharvarsian *et al.*, 1995; Weber *et al.*, 1987); and patients with other miscellaneous attributable factors e.g. drug abuse (Kaufmann *et al.*, 1988) or anorexia nervosa (Chun *et al.*, 1997) (**MIST**: n = 5, 4%)

Table 3.08: Main demographic data of STC sub-groups

Sub-group	*Number (%)*	*age / years med. (range)*	*sex F: M*	*duration med. (range)*	*Parity med. (range)*
CIST	64 (53.3%)	39 (13-79)	32:1	29 (2-71)*	1 (0-5)
AOIST	13 (10.8%)	39 (24-60)	13:0	6 (1-10)	2 (0-6)
PIST	22 (18.3%)	35 (20-69)	22:0	6 (1-51)	2 (0-5)
C/PIST	10 (8.3%)	46 (29-66)	9:0	7 (3-23)	3 (2-5) †
NIST	6 (5.0%)	59 (22-64)	5:1	6 (1-20)	1 (0-4)
MIST	5 (4.2%)	39 (27-69)	4:1	6 (1-51)	0 (0-2)
Total	120 (100%)	39 (13-79)	122:3	1-71 (19)	2 (0-6)

Key: * Kruskal Wallis ANOVA: $p < 0.0001$

† Kruskal-Wallis ANOVA: $p = 0.001$

There was no significant difference in age distribution between the 2 main sub-groups, but the NIST, and C/PIST sub-groups tended to have a slightly higher age range. The duration of symptoms was longer in the CIST sub-group, reflecting the childhood onset in the majority. The 3 male patients in the study were distributed 1 in the CIST, and one each in the MIST, and NIST sub-groups.

3.3.2.2 Physiological assessment

Physiological data were collected and analysed for all sub-groups, but the emphasis of presentation and discussion of results has been focussed on the 2 largest clinical sub-groups: chronic idiopathic STC (CIST) and post pelvic intervention STC (PIST).

Pattern of colonic transit

The pattern of transit abnormality was characterised in all 76 patients who had an isotope study. The results of scintigraphy for each main clinical sub-group are shown in table 3.09. Overall, a generalised delay was much more common than a left sided segmental delay (50 vs. 26 patients) (66% generalised), and this held true for the 2 main clinical sub-groups, including PIST (77% generalised). Although there was some variation in the smaller sub-groups, there was no difference in contingencies (p = 0.18, chi-square test).

Table 3.09: Results of transit studies by sub-group

	Number	**Isotope scintigraphy**		
		number	***generalised***	***left-sided***
CIST	64	41	26 (63%)	15 (27%)
AOIST	13	12	5 (42%)	7 (58%)
PIST	22	13	10 (77%)	3 (23%)
C/PIST	10	4	4 (100%)	0 (0%)
NIST	6	3	2 (67%)	1 (33%)
MIST	5	3	3 (100%)	0 (0%)
Total	**120**	**76**	**50 (66%)**	**26 (33%)**

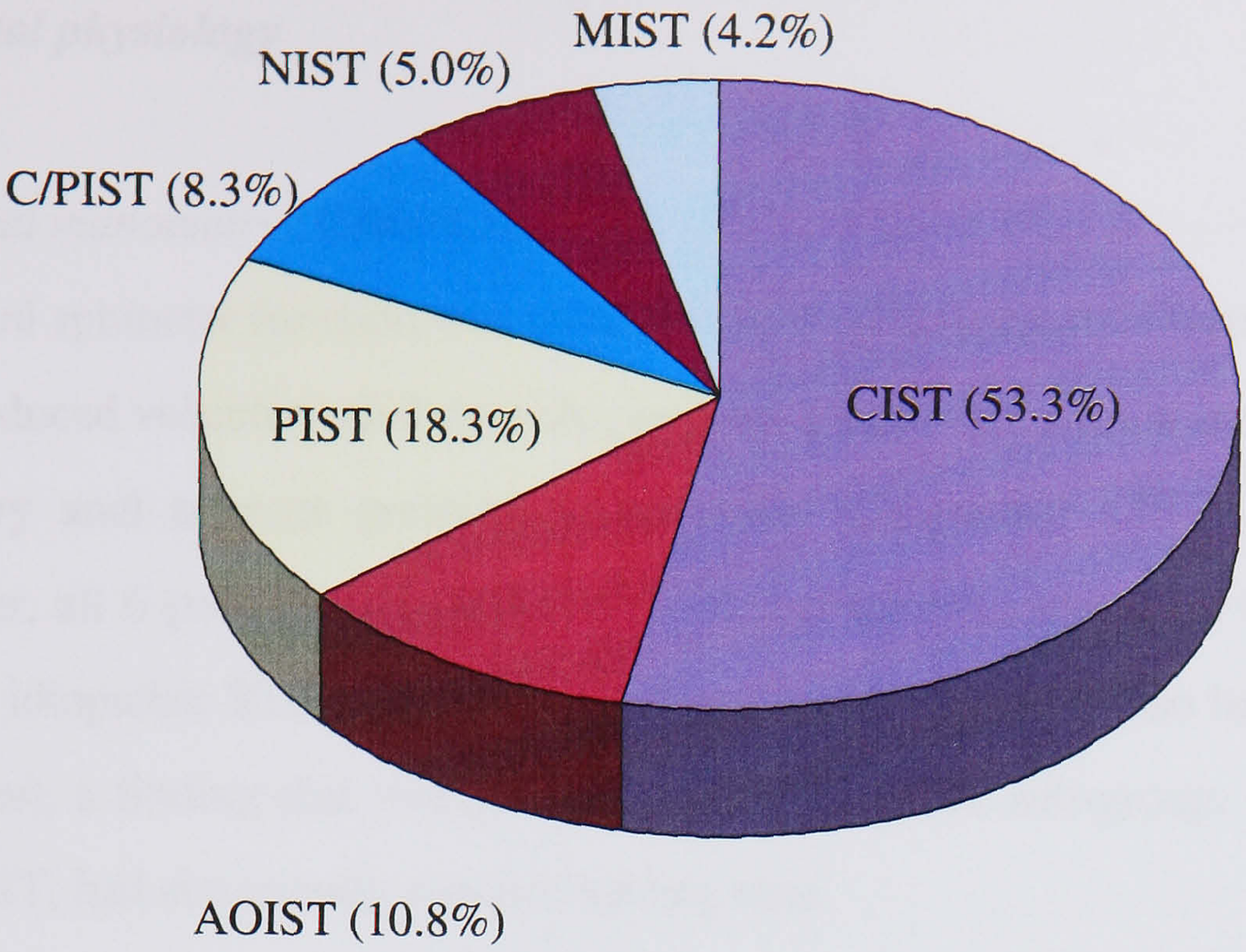


Fig 3.01. Sub-classification of 120 patients with slow transit constipation by clinical history. The pie chart demonstrates the percentage of patients falling into each clinical sub-group. **Key**: CIST: chronic idiopathic slow transit. PIST: Post pelvic intervention slow transit, AOIST: adult onset idiopathic slow transit, C/PIST: constipation from early childhood (chronic), becoming significantly worse following pelvic intervention, NIST: neural injury slow transit, MIST: miscellaneous slow transit.

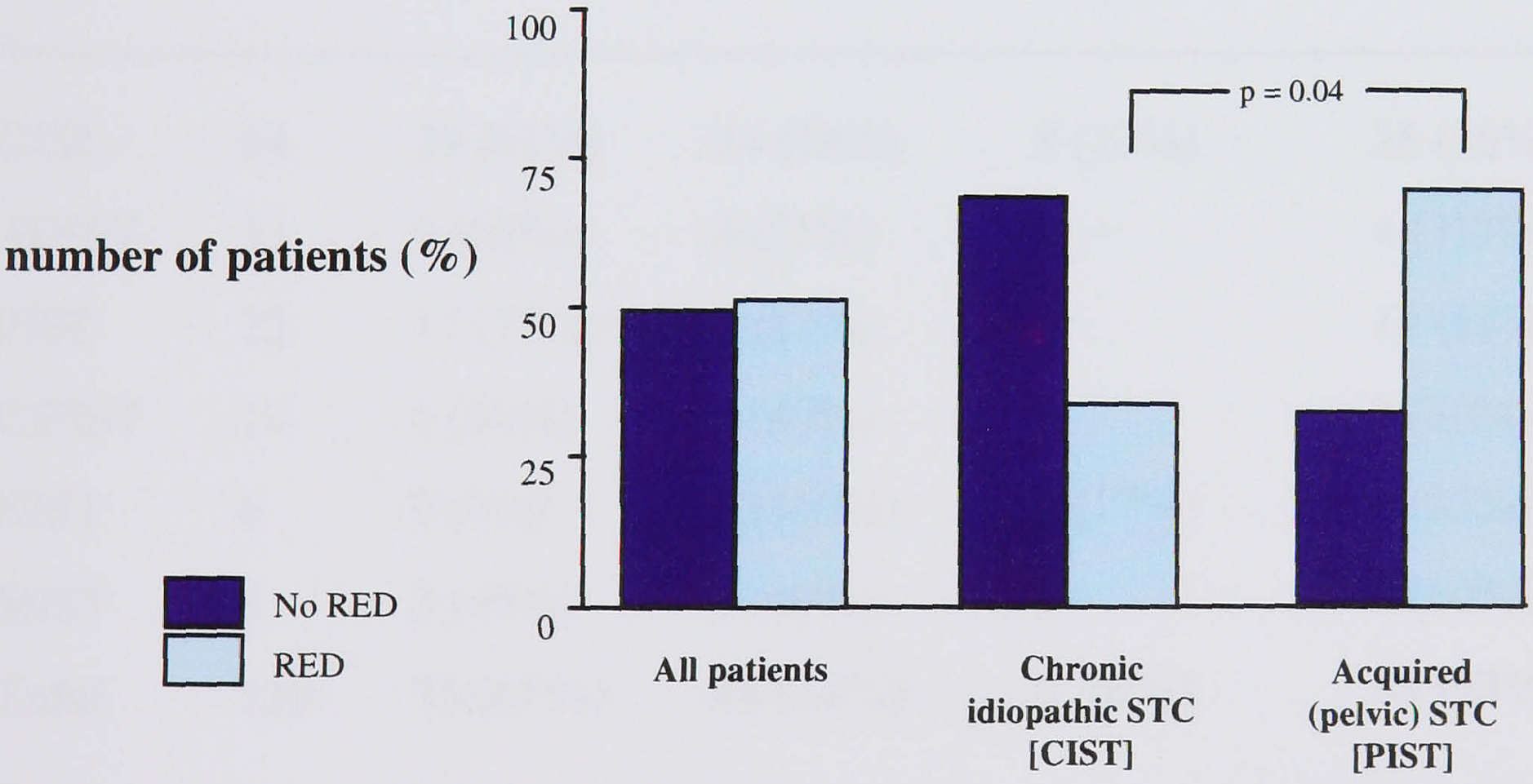


Fig 3.02. Bar chart showing the percentage co-existence of a rectal evacuation disorder (RED) with slow transit constipation. Overall approximately 50% of patients had evidence of RED, but the incidence was significantly higher in the PIST than in the CIST sub-group (Fisher's exact test: $p = 0.04$).

Anorectal physiology

Anorectal manometry (Table 3.10).

Anorectal sphincter function was normal in 61% of all patients. Overall, 38% of patients had a reduced voluntary anal squeeze pressure. There were similar incidences of reduced voluntary anal squeeze pressure in the main sub-groups: CIST: 38%, PIST: 23%. However, all 6 patients with NIST had reduced squeeze pressures. Eight patients with chronic idiopathic STC had reduced resting tone (7 of which also had reduced squeeze pressures), a finding that was not observed in the PIST sub-group. Two patients, both with PIST, had abnormally elevated resting tone.

Table 3.10: Results of anal manometry and rectal sensory testing by sub-group

a.	**N**	**Anal manometry**			**rectal hyposensation**
		normal pressure	***red squeeze***	***red resting tone***	
CIST	64	39 (61%)	24 (38%)	8 (13%)	36 (56%)
AOIST	13	9 (69%)	4 (31%)	0	4 (31%)
PIST	22	17 (77%)*	5 (23%)	0	12 (55%)
C/PIST	10	6 (60%)	4 (40%)	0	3 (30%)
NIST	6	0 (0%)	6 (100%)	1 (17%)	5 (83%)
MIST	5	2 (40%)	3 (60%)	0	3 (60%)
Total	**120**	**73 (61%)**	**46 (38%)**	**9 (8%)**	**63 (53%)**

Key: red squeeze = reduced maximum squeeze pressure

red resting = reduced maximum resting pressure

* = 2 had increased resting pressures with hypersensitivity

Rectal sensory testing (Table 3.10).

Overall, 63 patients (53%), had raised thresholds to rectal balloon distension (hyposensation), and the incidence did not vary significantly between the 2 main sub-groups. However, hyposensation was present in 5 / 6 NIST patients (83%).

Evacuation proctography (Table 3.11).

On evacuation proctography, a rectal evacuatory disorder (RED) was found in 53 / 120 (44%) patients (Figure 3.02). Overall, mechanical obstruction (e.g. rectocele, intrarectal intussusception, non-relaxing striated pelvic floor muscles) was the most common cause of RED, occurring in 37 / 53 (70%) patients. Eighteen of these 37 patients with a mechanical evacuatory obstruction also had rectal hyposensation. A further 11 patients (21%) were unable to evacuate, apparently as a result of gross rectal hyposensation alone (verbally reported loss of urge in addition to abnormal sensory thresholds). The remaining 5 patients had no demonstrable reason for evacuatory difficulty, presumably due to lack of evacuatory effort, caused in most cases by embarrassment or pain.

Table 3.11: Results of evacuation proctography by sub-group

Group	*N*	*abnormal result*	*mechanical only*	*hypo-sensation*	*both*	*no cause found*
CIST	64	22 (34%)	7 (32%)	8 (36%)	4 (18%)	3 (14%)
AOIST	13	5 (38%)	3 (60%)	1 (20%)	1 (20%)	0
PIST	22	15 (68%)*	4 (27%)	1 (7%)	9 (60%)	1 (7%)
C/PIST	10	6 (60%)	5 (83%)	1 (17%)	0	0
NIST	6	2 (33%)	0	0	2 (100%)	0
MIST	5	3 (60%)	0	0	2 (67%)	1 (33%)
Total	**120**	**53 (44%)**	**19 (36%)**	**11 (21%)**	**18 (34%)**	**5 (9%)**

(* p = 0.04, Fisher's exact test).

Notably, there were marked differences in the incidence of RED between certain sub-groups. Only 34% (22 / 64) of patients in the CIST sub-group had problematic evacuation, compared to a significantly greater proportion of patients in the PIST sub-group (15 / 22 = 68%; p = 0.04, Fisher's exact test).

Upper gastrointestinal physiology

Of the 25 patients investigated, 12 (48%) had abnormalities: these included 10 of the 19 (53%) patients investigated prospectively and 2 of the 6 (33%) patients referred for study because of upper gastrointestinal symptoms (Table 3.12). Clear abnormalities were demonstrated in 7 (28%), and minor abnormalities in a further 5 (20%). Of 9 patients tested with CIST, 5 were reported as normal studies, 2 had abnormalities which were characteristic of neuropathic disorder, and 2 had borderline abnormalities. Of 7 patients tested with PIST, 3 had neuropathic-type abnormalities, 2 had borderline abnormalities, and only 2 were normal. Nine further patients included 6 with AOIST, of which 2 (33%) had abnormal studies. The other 3 patients fell one each into the NIST, MIST and C/PIST sub-groups, of which only the C/PIST patient had a borderline study.

Table 3.12: Results of small bowel manometry by sub-group

	Number	**Small bowel manometry**			
		number	***abnormal***	***borderline***	***normal***
CIST	64	9	2	2	5
AOIST	13	6	2	0	4
PIST	22	7	3	2	2
C/PIST	10	1	0	0	1
NIST	6	1	0	0	1
MIST	5	1	0	1	0
Total	**120**	**25**	**7 (28%)**	**5 (20%)**	**13 (52%)**

Table 3.13 summarises all the physiological results between the 2 main sub-groups (CIST and PIST).

Table 3.13: Summary of physiological results between the 2 main sub-groups

Investigation		***Chronic Idiopathic STC***	***Acquired STC (Pelvic)***
^{111}In DTPA Colonic Scintigraphy	Generalised pattern	27 (68%)	10 (77%)
	Left-sided delay	13 (32%)	3 (27%)
Anal Manometry	Normal	39 (61%)	17 (77%)†
	Reduced Resting	8 (13%)*	0 (0%)
	Reduced Squeeze	24 (38%)	5 (23%)
Rectal Hyposensation	Normal	28 (44%)	10 (45%)
	Abnormal	36 (56%)	12 (55%)
Evacuation Proctography	Normal	42 (66%)	7 (32%)
	Abnormal	22 (34%)	15 (68%)‡
Ambulatory Small Bowel Manometry	Normal	6 (67%)	2 (29%)
	Abnormal	3 (33%)	5 (71%)

* 7 patients had combined reduced anal resting and squeeze pressures

† 2 patients had elevated anal resting tone

‡ p = 0.04 (Fisher's exact test)

3.3.3 DISCUSSION

3.3.3.1 Summary of results

Heterogeneity within patients with documented STC is well recognised (Preston *et al.*, 1984; Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988; Roe *et al.*, 1988; MacDonald *et al.*, 1997; Keshavarzian *et al.*, 1995). In addition to the 2 largest sub-groups, chronic idiopathic (CIST) (Preston & Lennard-Jones, 1986; Waldron *et al.*, 1988), and post pelvic intervention (PIST) (Roe *et al.*, 1988; MacDonald *et al.*, 1997), we have, on a similar basis of clinical history, incorporated 4 other sub-groups. We have compared the results of physiological tests between these sub-groups, in a large cohort of 120 patients with proven STC.

We have provided evidence that the pattern of colonic transit abnormality is similar between STC sub-groups, with the majority (approximately 70%) of patients suffering from a generalised delay throughout the large bowel (Krevsky *et al.*, 1989; Roberts *et al.*, 1993), regardless of history, and a smaller proportion with delay localised to the left colon (Krevsky *et al.*, 1989; Stivland *et al.*, 1991; Roberts *et al.*, 1993; McLean *et al.*, 1995). It has generally been considered that in patients with STC following pelvic surgery or childbirth (PIST), colonic motor disturbance is limited to the distal / left-side, perhaps secondary to rectal-pelvic floor dysynergia (MacDonald *et al.*, 1993; MacDonald *et al.*, 1997), whereas patients with chronic idiopathic symptoms are thought to suffer primarily from generalised colonic inertia (MacDonald *et al.*, 1993). However, previous studies, having clearly demonstrated differential colonic transit patterns in patients with chronic constipation, have not equated findings with mode of onset (Kamm *et al.*, 1988; Krevsky *et al.*, 1989; Roberts *et al.*, 1993; van der Sijp *et al.*, 1993). Likewise, reports of physiological differences between patient groups with varying clinical histories e.g. with regard to upper gastrointestinal and rectal function, have not considered the pattern of colonic transit itself (Roe *et al.*, 1988; Smith *et al.*, 1990; MacDonald *et al.*, 1997).

Anorectal manometric findings were similar between the 2 main sub-groups. A proportion of patients had either reduced resting tone, reduced squeeze pressure, or both, in keeping with changes expected within a female population of variable age and parity (Sultan *et al.*, 1993; Jameson *et al.*, 1994). The abnormalities of resting tone which were almost exclusive to a small group of patients in the CIST sub-group, might be said to be indicative of a primary myopathic process (Vaizey *et al.*, 1997), but further comment can not be made regarding this observation because of lack of endosonographic data.

Approximately 50% of all patients had rectal hyposensation, consistent with previous reports (Akervall *et al.*, 1988; Waldron *et al.*, 1988; Kamm & Lennard-Jones 1990). Although this incidence did not vary between the main sub-groups, nearly all of the small number of patients with NIST had hyposensation and this has been previously documented in patients with spinal cord injuries (MacDonagh *et al.*, 1992; Sun *et al.*, 1995; De Looze *et al.*, 1998). There were differences between the sub-groups with respect to the results of evacuation proctography. Although half of the patients with STC overall had a co-existent RED, a significantly higher proportion of patients with acquired (pelvic) STC had an RED compared to those with chronic idiopathic STC.

The rectal sensory findings in this study concur with those of Smith *et al.* (Smith *et al.*, 1990) who studied patients with constipation occurring after hysterectomy (transit unspecified), but are in contrast to those of Roe *et al.* (Roe *et al.*, 1988) who described rectal hyposensation only in chronic idiopathic patients. The latter study reported that rectal evacuatory problems, as assessed by ability to expel a balloon, were seen at a higher frequency, although not significantly so, in chronic idiopathic patients than those with post-hysterectomy STC.

The finding that 3 / 9 of CIST patients had abnormal small bowel motility equates reasonably well with previous larger studies of chronic idiopathic cases, in which the presence of upper gastrointestinal dysmotility in approximately half of patients is well

accepted (Bassotti *et al.*, 1996; Glia & Lindberg, 1998; Mollen *et al.*, 1999). No study has previously examined whether such abnormalities can follow pelvic surgery, and only one has examined patients whose symptoms followed childbirth (MacDonald *et al.*, 1997). This latter study measured gastric emptying, and suggested that abnormalities only occurred in the chronic idiopathic group. In fact, although it is not discussed in the text, the figure for solid-phase gastric emptying clearly demonstrates that at least one patient with STC following childbirth had an emptying time outside the normal range presented.

This is the first study to use current sensitive methodology i.e. prolonged ambulatory small bowel manometry in patients clearly defined to each of these groups. Our results might be treated with a degree of caution because of the initial selection bias, but it is important to note that only 6 of the 25 patients were initially investigated purely on the basis of having upper gastrointestinal symptoms. Indeed, a greater proportion of those patients studied prospectively, regardless of upper GI symptomatology, had evidence of a upper gastrointestinal dysmotility, including 5 / 7 patients with STC acquired following pelvic intervention. These results demonstrate, therefore, that in some cases at least, a generalised gastrointestinal disturbance can follow pelvic surgery or childbirth. This confirms the view that whilst some patients clearly have an isolated disorder of the colon, others have a more diffuse disorder of the gastrointestinal tract.

In general, the results of physiological testing were similar between patients with adult onset idiopathic slow transit (AOIST) and those with CIST. In contrast, the C/PIST sub-group, albeit small, had similar findings on anorectal physiology to the PIST sub-group. Naturally these observations must be interpreted with caution in view of the lack of physiological data pre-dating the pelvic event in the C/PIST sub-group, and the relatively small number of patients. The findings in patients with central (spinal) neural injuries differed from other sub-groups i.e. almost uniformly affected sphincteric and rectal sensory function.

Regardless of the implications derived from the smaller sub-groups in this study, this

study has demonstrated the following important findings with respect to the comparison of patients with chronic idiopathic STC (CIST) and those with acquired (pelvic) STC (PIST):

1. The pattern of colonic transit is similar in both groups i.e. predominantly generalised.
2. The incidence of rectal evacuatory disorder is significantly higher in patients acquiring STC after pelvic surgery or childbirth
3. Small bowel motility disturbances occur in both groups, and are not limited to patients with chronic idiopathic STC.

These 3 observations are in contrast to previously published reports which were all based on smaller numbers of patients studied.

3.3.3.2 Pathogenetic considerations

Although a plethora of aetiological theories have been proposed for chronic idiopathic slow transit constipation, including hormonal, laxative abuse, endogenous opioids, absorption, psychogenic etc., the cause or causes remain elusive. The role of muscle and nerves in control of gut motility is well established, and it is likely that a disorder of nerve, muscle or both is implicated. A substantial proportion of studies of chronic idiopathic STC have found degeneration of neurons in the myenteric plexus, said to be suggestive of a distinct, possibly congenital, intrinsic neuropathy (1.5.3.4). In contrast, STC acquired following pelvic intervention (PIST) may provide a model of acquired extramural pelvic parasympathetic damage (1.5.4.1.1). The high incidence of rectal evacuatory problems in patients with post pelvic intervention STC in this study may complement the pelvic nerve dysfunction hypothesis. The pathogenesis in this sub-group of a generalised disturbance of colonic transit, and in some patients of small intestinal dysmotility as well, might be explained either by degeneration of ascending colonic nerves, with or without sympathetic re-innervation, as occurs in the bladder, or by reflex inhibition of proximal motility by distal stasis and distension. These theories have been

addressed in detail in the introduction (1.5.4.1.1). Whilst the pathogenesis might be different, the final physiological and clinical consequences could thus become indistinguishable. The finding of patients with pre-existent symptoms, which were worsened by a pelvic event (CPIST) supports the hypothesis that multiple independent or additive events might play a part in the aetiopathogenesis of STC. An underlying abnormality made clinically apparent by the effects of pelvic surgery or childbirth can similarly not be excluded in the PIST sub-group.

To our knowledge, no morphological study has presented findings by such clinical sub-groups, and the lack of uniform histopathological findings in STC, methodological differences apart, might therefore also reflect biological heterogeneity.

3.3.3.3 Management considerations

Reported patient satisfaction rates after colectomy for STC range between 39% and 100% in recent studies (Knowles *et al.*, 1999). The co-existence of certain physiological abnormalities is known to influence surgical outcome. Some studies have demonstrated a deleterious effect of untreated disorders of rectal evacuation (Bernini *et al.*, 1998; Hasegawa *et al.*, 1999) or rectal hyposensation (Akervall *et al.*, 1988; Pluta *et al.*, 1996). Others have treated co-existent abnormalities of the pelvic floor by pre-operative retraining (Nyam *et al.*, 1997), or rectopexy at the time of colectomy (Piccirillo *et al.*, 1995) with apparently excellent results. It is generally accepted that patients with a generalised gastrointestinal disorder rather than an isolated disorder of colorectal dysmotility have poor outcomes in comparison with other cases (Redmond *et al.*, 1995). The influence of pattern of colonic motility disturbance itself (i.e. generalised *vs.* left segmental) on outcome after surgical intervention remains unclear (de Graaf *et al.*, 1996).

Regardless of the importance of physiological differences, it is also unclear what independent effect a variation in pathogenesis of STC might have on outcome, i.e. the response to surgery might be expected to be different for diseases with diverse

pathogenesis. The influence that these differences might have on outcome from surgery for STC is not known because studies have not stratified results by such sub-groupings (Knowles *et al.*, 1999). The results of this chapter continue to emphasise the need for thorough investigation of patients with STC before specific management, including surgery, is advised.

3.3.3.4 Sub-classification

The exercise of clinical sub-classification of patients with STC is implicitly dependent on factors such as the patients' recall of events, and the necessity to define a cut off between sub-groups which may be arbitrary (e.g. the age of 20 for AOIST, and the severity of symptoms accepted as indicating constipation preceding the event in patients with C/PIST), but we have demonstrated that all patients included in this study could be placed without difficulty into a single sub-group by this system.

Whether such a classification system has a clinical role cannot be addressed by this study. However, this classification system is used for subsequent chapters of this thesis in which aetiological hypotheses are addressed: 5,6,7 and 8.

4 LINEAR DISCRIMINANT ANALYSIS OF SYMPTOMS IN PATIENTS WITH CHRONIC CONSTIPATION: VALIDATION OF A NEW SCORING SYSTEM (KESS)

4.1 INTRODUCTION

Investigation of patients presenting with severe chronic constipation reveals a sub-group who have underlying physiological abnormalities. On the basis of these investigations, patients may be further subdivided into those whose underlying physiological abnormality is either a rectal evacuatory disorder (RED), slow transit constipation (STC), or a mixture of the two (Keighley & Schouler, 1984; Nyam *et al.*, 1997; Koch *et al.*, 1997; Halverson & Orkin, 1998; Bernini *et al.*, 1998; Hasegawa *et al.*, 1999). Distinguishing these sub-groups may be important in planning treatment and predicting prognosis. For example, subtotal colectomy and ileorectal anastomosis can be successful in the treatment of STC (Pemberton *et al.*, 1991; Wexner *et al.*, 1991; Piccirillo *et al.*, 1995; Lubowski *et al.* 1996; Nyam *et al.*, 1997), but is less satisfactory if the underlying cause of constipation is related to a disturbance in rectal evacuation (Kamm *et al.*, 1984; Yoshioka & Keighley, 1989; Pfeifer *et al.*, 1996). Conversely, patients with outlet obstruction but normal colonic transit may respond favorably to pelvic floor retraining, biofeedback (Wexner *et al.*, 1992; Karlbom *et al.*, 1997) or surgery to restore normal anatomy (Jorge *et al.*, 1994; Pfeifer *et al.*, 1996).

Previous studies have investigated the symptoms associated with constipation, and scoring systems have been used in its diagnosis (Agachan *et al.*, 1996). Some groups have attempted to identify patho-physiological sub-groups by analysis of symptoms prior to investigation, with application of various statistical methods, with variable results (Grotz *et al.*, 1994; Karlbom *et al.*, 1995; Agachan *et al.*, 1996; Koch *et al.*, 1997; Mertz *et al.*, 1999). We aimed to devise and validate a new scoring system, utilizing appropriate statistical analyses, which might be able both to diagnose cases

of constipation and to discriminate between the important patho-physiological sub-groups.

4.2 METHODS

Questionnaire Design

A structured interviewer-led questionnaire consisting of eleven questions was devised by incorporating internationally agreed criteria (Whitehead *et al.*, 1991) for constipation and previous reported relevant symptoms of constipation (Agachan *et al.*, 1996). This questionnaire (Figure 4.01) was designed to be simple enough to be completed in under five minutes. Each question had four or five possible answers which were scored on an unweighted linear integer scale to produce a range of between 0 and 3 or 0 and 4 points. Lower scores represented symptom-free states and higher scores, increased symptom severity. The total KESS (**K**nowles-**E**ccersley-**S**cott-**S**ymptom) score was the sum of all scores gained on individual questions with a maximum possible of 39 points. The answers to each question were worded such that any patient who fitted agreed criteria (Agachan *et al.*, 1996) for constipation would be likely to score at least 1 point per question.

Patient recruitment and investigation

Seventy-one patients (66 women) with intractable constipation who had been referred from specialist colorectal surgical clinics, and who underwent full investigation of their constipation during the period 1997-1999, were recruited. In addition, 20 normal controls (18 women) of similar age distribution, with no history of constipation, were recruited from staff and visitors to the unit. Prior to referral, all patients had been fully assessed in the outpatients clinic, endocrine or metabolic abnormalities excluded, and colonic imaging by either barium enema or colonoscopy performed to exclude structural abnormalities. Patients with radiological evidence of megabowel were excluded from the study.

Figure 4.01: The KESS questionnaire

1. Duration of constipation

0-18 months	0
18 months to 5 years	1
5-10 years	2
10-20 years	3
>20 years (or all life)	4

2. Laxative use

None	0
Laxatives prn or for short duration	1
Laxatives regular, long duration	2
Laxatives long duration, ineffective	3

3. Frequency of bowel movement
(using current therapy)

1-2 times / 1-2 days	0
2 or less times / week	1
Less than once per week	2
Less than once per 2 weeks	3

4. Unsuccessful evacuatory attempts

Never / rarely	0
Occasionally	1
Usually	2
Always = manual evacuation	3

5. Feeling incomplete evacuation

Never	0
Rarely	1
Occasionally	2
Usually	3
Always	4

6. Abdominal pain

Never	0
Rarely	1
Occasionally	2
Usually	3
Always	4

7. Bloating

Never	0
Perceived by patient only	1
Visible to others	2
Severe causing satiety or nausea	3
Severe with vomiting	4

8. Enemas / Digitation

None	0
Enemata / suppositories occasionally	1
Enemata / suppositories regular	2
Manual evacuation occasionally	3
Manual evacuation always	4

9. Time taken
(minutes in lavatory / attempt)

< 5 minutes	0
5-10 minutes	1
10-30 minutes	2
> 30 minutes	3

10. Difficulty evacuating
(causing a painful evacuation effort)

Never	0
Rarely	1
Occasionally	2
Usually	3
Always	4

11. Stool consistency
(without laxatives)

Soft / loose/ normal	0
Occasionally hard	1
Always hard	2
Always hard, usually pellet-like	3

KEY

Rarely	**= < 25% of time**
Occasionally	**= 25-50% of the time**
Usually	**= > 50% of the time**

The questionnaire was completed prospectively (before any investigations) in 51 consecutive patients. In 20 further patients, some investigations had already been performed prior to the completion of the KESS questionnaire. A researcher who was unaware of the patients' referral source, previous history or investigation results conducted a fully structured interview to assess each persons' current bowel symptoms. The interviewer was instructed to avoid discussion with the interviewee, and to ask which single answer to each question was most appropriate at the time of the interview.

At the same time that the KESS questionnaire was completed, the constipation scoring system questionnaire (Agachan *et al.*, 1996), previously used at the Cleveland Clinic was also completed (Cleveland Clinic score: CCS).

All 71 patients were fully investigated according to our standard protocol which included anorectal manometry, anorectal sensory function testing, evacuation proctography, and assessment of colonic transit by radio-opaque marker studies (see chapter 2). The results of these investigations were reviewed by a surgeon of consultant status with a special interest in lower gastrointestinal dysfunction who, unaware of the questionnaire results or of the patients' identities, assigned each patient to one of the three patho-physiological sub-groups: STC, RED and mixed (STC & RED).

Delayed colonic transit was diagnosed if more than 20% of 50 radio-opaque markers were still present on a single abdominal radiograph taken 100 hours after ingestion (Hinton *et al.*, 1969; Roberts *et al.*, 1993). A rectal evacuatory disorder (mechanical or functional) was diagnosed by evacuation proctography, performed after filling the rectum with semi-solid barium paste to the previously measured maximum tolerated volume (Chapter 2).

Data Analysis

In order to measure the agreement between KESS and CCS scores for each patient,

the scores were expressed as percentages of the totals for each scoring system (total KESS = 39, total CCS = 30). Agreement between the two scoring systems was described by subtracting CCS percentage scores from KESS percentage scores and calculating the 95% limits of agreement as 1.96x the standard deviation of the differences (Bland & Altman, 1986). Any tendency for patients' scores to be higher using one or other system was tested using a paired t-test.

As the scores for each individual question were ordinal, non-parametric methods of analysis were used. Hence median KESS scores were compared between controls and constipated patients, and between patho-physiological groups, using Mann Whitney and Kruskal Wallis tests respectively (GraphPad Prism v. 2.0, GraphPad Software Inc. San Diego, USA). Linear discriminant analysis (Systat v. 8.0, SPSS Inc. Chicago, USA) was used to investigate the extent to which symptoms could be used to classify patients into the three sub-groups. Discriminant analysis using maximum likelihood methods (the bases of most implementations of discriminant analysis in statistical software) assumes that variables used to predict group membership are continuous and normally distributed. The robustness of the method using other kinds of variables is uncertain (Moore, 1973; Krzanowski, 1975; Titterington *et al.*, 1981; Krzanowski, 1995; Reeves *et al.*, 1997) and the definitive test of any classification algorithm is its performance on a new dataset. In the present study, the sample size was not sufficient to validate classification performance by dividing the data into 'train' and 'test' samples and cross-validation was used instead to correct the performance estimates based directly on the data (Reeves *et al.*, 1997).

4.3 RESULTS

Correlation of KESS with CCS

Scores were distributed normally on both scoring systems and were highly correlated (Pearson $r = 0.90$). The 95% limits of agreement were $\pm$14%. Scores for some patients on one CCS question were not available (unsuccessful evacuation attempts per 24 hours, see discussion). However, the limits of agreement were unaffected whether the

CCS percentage for these patients was calculated out of a total of 26, (i.e. excluding the question for which a response was not available), or out of 30. The extent of agreement appeared to be independent of the mean of the KESS and CCS scores (Figure 4.02). Patients scored marginally (mean difference 3%) higher on KESS score than CCS (95% confidence interval = 1.0% to 5.0%, p = 0.003).

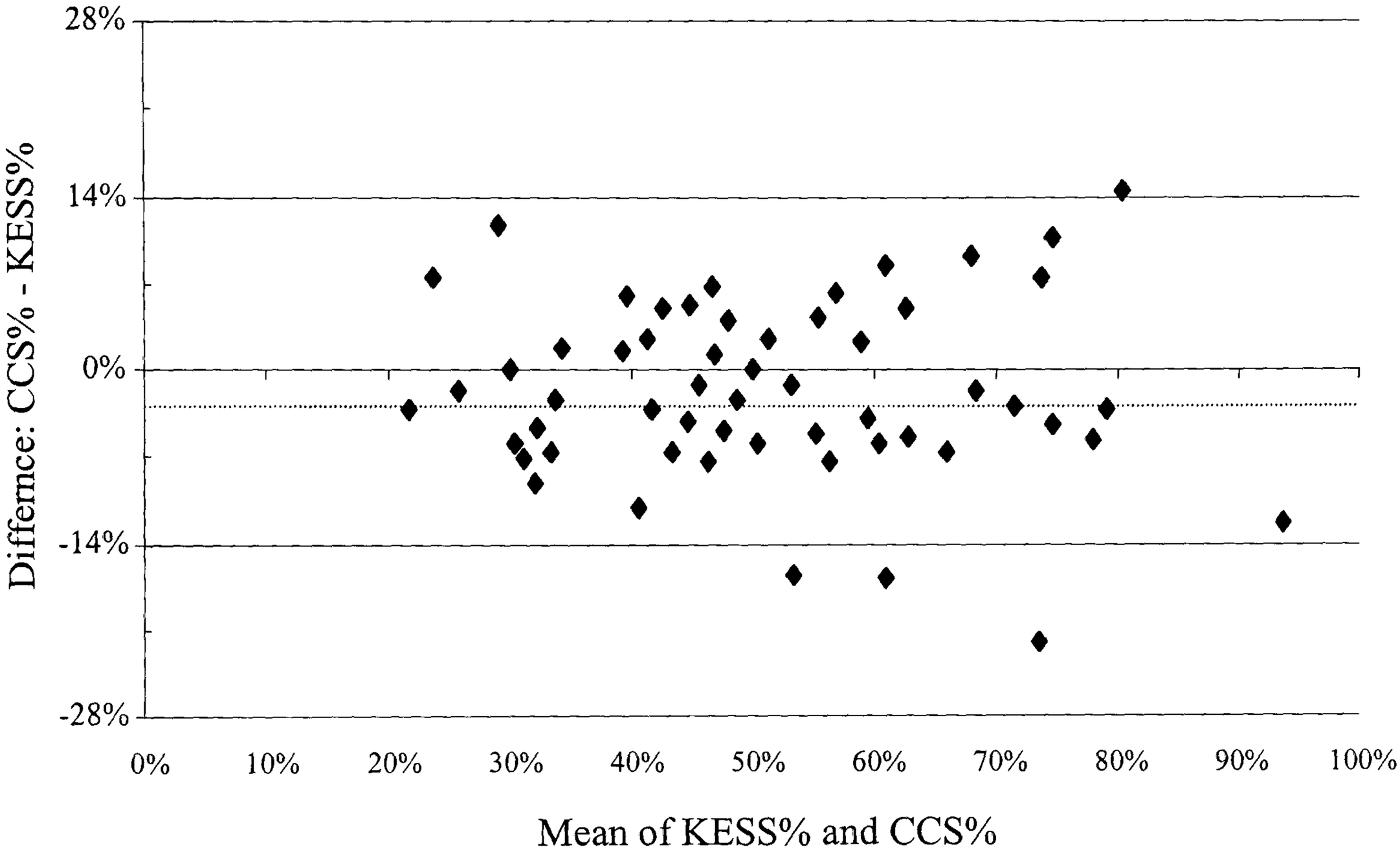


Figure 4.02: Bland-Altman plot showing the range of agreement between KESS and CCS scores as a function of increasing symptoms of constipation.

KESS score delineates constipated patients from normal controls

The median total KESS scores were significantly different between patients and normal controls: median 20 (range 11-35) *vs.* median 2 (0-6), $p < 0.0001$. Moreover, as demonstrated by figure 4.03, there was no overlap at all between the scores of constipated and control patients. Using a cut-off criterion of ≥ 10, the total KESS score had a sensitivity of 100% (95% CI=95-100%) and a specificity of 100% (95% CI=63-100%).

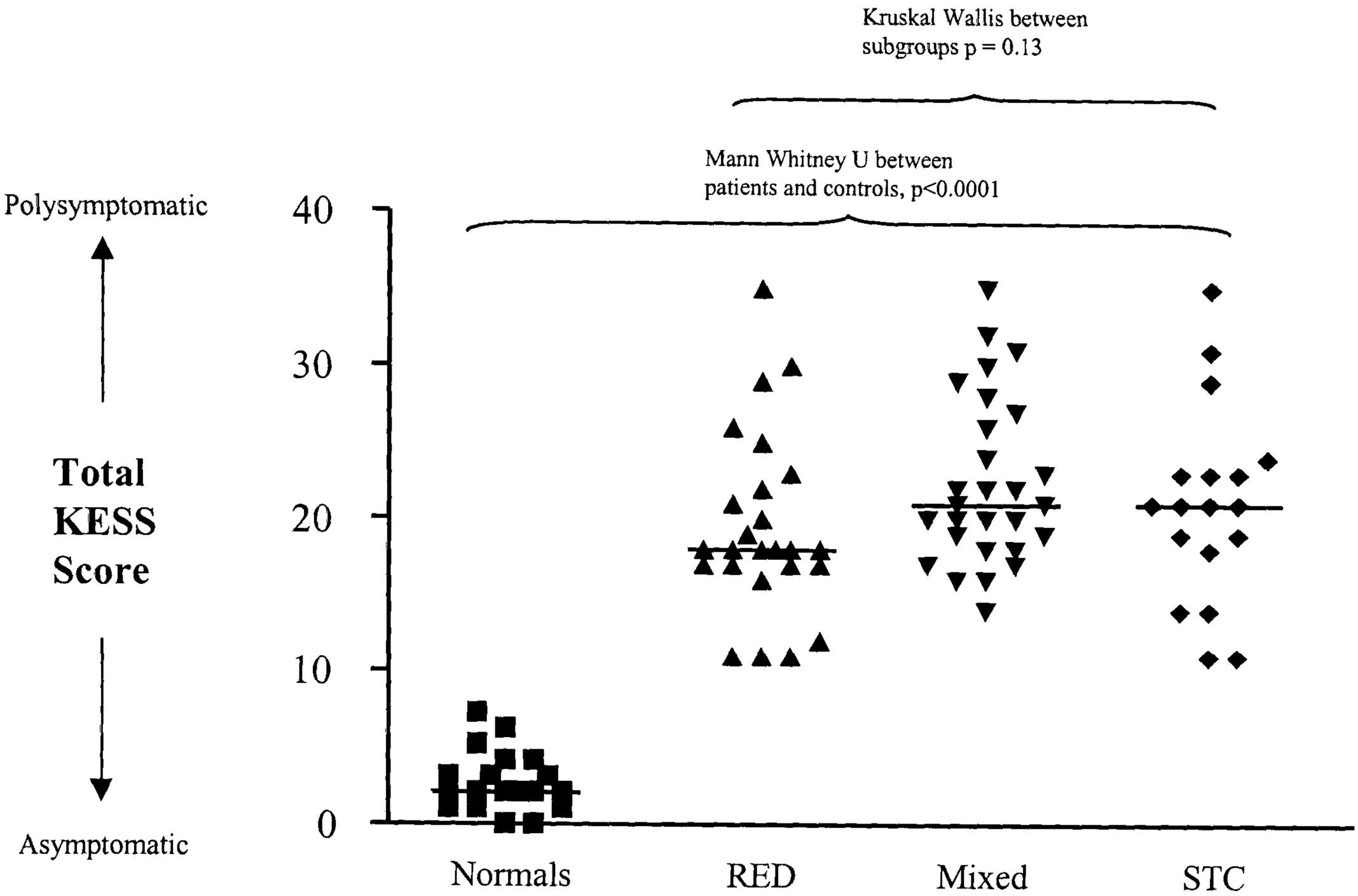


Fig 4.03. Scatter plot showing distribution and medians of total KESS score for controls and patients All test patients had a KESS total >= 10, whereas no control had a score > 6. There was a significant difference between patients and controls, Mann Whitney U-test, $p < 0.0001$, but not between patient sub-groups (Kruskal-Wallis, $p = 0.13$).

Independent physiological review

After review of all investigation results, the numbers of patients allocated to each of the 3 patho-physiological sub-groups were : STC, n = 18; RED, n = 25; and mixed, n = 28. Median marker retention in those patients with a transit abnormality (n = 46) was 96% at 100 hrs. RED was attributable to one or more of the following: intrarectal intussusception, n = 20; external rectal prolapse, n = 2; functional rectocele, n = 22; rectal hyposensation (abnormally reduced sensation to balloon distension combined with failure to evacuate due to loss of urge), n = 11; poor evacuatory effort, n = 9; failure of pelvic floor relaxation on attempted defecation, n = 3. No patient included

in the study was subsequently found to have entirely normal colorectal function on investigation.

There was no significant difference between the median total KESS scores for patients in each patho-physiological sub-group ($p = 0.13$), (Figure 4.03).

Discriminant analysis

Stepwise discriminant analysis of all symptoms identified five that were predictive of the patho-physiological sub-group to which each patient had been allocated as a result of investigation. These were, duration of constipation [dur], laxative assistance [lax], frequency of bowel movement [freq], abdominal pain [abdopn], and time in lavatory per evacuatory attempt [time] (see Table 4.01). Prior probabilities (required for such an analysis) were based on validated diagnoses for all patients referred to our clinic over the last 4 years, i.e. RED=0.39, Mixed=0.35, STC=0.26. The classification functions for each sub-group were:

RED = –5.813+(0.969*dur)–(0.005*lax)–(1.484*freq)+(1.899*abdopn)+(2.003*time)

Mixed = –6.233+(1.475*dur)+(0.882*lax)–(0.007*freq)+(1.035*abdopn)+(1.169*time)

STC = –6.566+(1.766*dur)+(0.666*lax)+(0.447*freq)+(1.039*abdopn)+(0.339*time)

A new patient would be classified by calculating the total for all three classification functions (by summing the constant and the products of the symptom scores and the coefficients, as shown in the equations above), and would be assigned into the group which yielded the highest score. Thus, for a patient with scores of 3, 2, 2, 3, 3 for the five symptoms, the function scores would be 4.338, 6.547 and 5.539 respectively, and the patient would be classified as belonging to the mixed sub-group. The performance of these functions in classifying the patients who were studied is shown in table 2a; the performance of these functions in classifying new patients was estimated by cross-validation and is shown in table 2b.

Table 4.01: Classification functions derived from the discriminant analysis.

Symptom	*STC*	*Mixed*	*RED*
Constant	-6.566	-6.233	-5.813
Dur	1.766	1.475	0.969
Lax	0.666	0.882	-0.005
Freq	0.447	-0.007	-1.484
Abdopn	1.039	1.035	1.899
Time	0.339	1.169	2.003

New patients are classified by comparing the total discriminant scores for the three sub-groups, calculated by summing the constant and the products of the symptom scores and the coefficients shown in the table. The patient is classified into the group which yields the highest discriminant score. **Key:** Dur = duration of constipation, Lax = laxative use, Freq = frequency of bowel movement, Abdopn = abdominal pain, Time = time taken to evacuate / attempt.

Table 4.02: Classification matrix.

Sub-group into which patient classified by classification functions	**Allocated (true) sub-group by physiology**		
	STC	Mixed	RED
STC	11	5	1
Mixed	7	15	4
RED	0	8	20
Number (%) correct by group	11/18 (61%)	15/28 (54%)	20/25 (80%)
Total number (%) percent correct		**46/71 (65%)**	

(a) The table shows the frequencies of patients in allocated patho-physiological sub-

groups that were assigned into each of the sub-groups by the classification functions.

Sub-group into which patient classified by classification functions	**Allocated (true) sub-group by physiology**		
	STC	Mixed	RED
STC	7	5	1
Mixed	11	15	4
RED	0	8	20
Percent correct (95% CI)	39% (17-64)	46% (26-66)	76% (55-91)
Total percent correct (95% CI)		**55% (43-67)**	

(b) Cross-validated estimates of the percentages (95% confidence intervals) of new patients in validated patho-physiology sub-groups that would be classified into each of the sub-groups by the classification functions.

4.4 DISCUSSION

Constipation is a poorly defined clinical symptom, not a definitive diagnosis. Investigations aim to identify underlying functional (physiological) abnormalities contributing to the disorder. Either delayed colonic transit or rectal dysfunction alone can cause many of the symptoms ascribed to constipation, yet the two abnormalities frequently co-exist (Keighley & Schouler, 1984; Nyam *et al.*, 1997; Koch *et al.*, 1997; Halverson & Orkin, 1998; Bernini *et al.*, 1998; Hasegawa *et al.*, 1999). The results of investigation are important in guiding management (Wexner *et al.*, 1991; Pemberton *et al.*, 1991; Piccirillo *et al.*, 1995; Pfeifer *et al.*, 1996; Lubowski *et al.*, 1996; Nyam *et al.*, 1997).

The KESS questionnaire is a modified version of that used in the CCS (Agachan *et al.*, 1996). Changes in questions were based upon our clinical impression of those

symptoms most likely to be discriminatory. Furthermore some questions were modified to improve ease of interview. For example, the question of unsuccessful evacuation attempts (per day in the CCS) was altered to allow this question to be answered by patients who have less than one evacuation (successful or unsuccessful) per day. Four of the KESS questions were based on the commonly quoted guidelines for diagnosing constipation (Whitehead *et al.*, 1991), with a score of one or more points signaling fulfillment of each criterion. Patients with severe chronic constipation have usually tried multiple remedies for their condition before seeking a specialist opinion. The KESS score takes account of this by asking patients to indicate whether long term laxatives have been used, and whether they continue to be effective. The questionnaire also asks patients about their bowel frequency and other symptoms while taking their current medication. The only question that may not be contemporary for all patients is the request for a description of stool consistency 'without laxatives'. The questionnaire also recognizes the possibility that severe constipation can have pan-enteric effects by assigning higher scores if bloating causes loss of appetite or nausea.

The KESS total score delineates patients with constipation clearly from normals. Although it should be noted that controls had not been subjected to physiological testing, they were selected on the basis of being asymptomatic. The new questionnaire was validated by comparison with the CCS (Agachan *et al.*, 1996). This had itself been validated as a tool for distinguishing constipated patients with a proven patho-physiological abnormality from those in whom physiological investigations were normal, predicting 96% of cases correctly. There is no known biological or physiological marker for the severity of constipation, so it is unclear what the significance of a total score is. The score may be used, however, to monitor the efficacy of treatment modalities, simply by omitting the score for duration of symptoms from the total.

Previous studies have attempted to divide constipated patients into RED, STC and mixed sub-groups on the basis of symptoms and investigation results. Karlbom *et al.*

(Karlbom *et al.*, 1995) assessed bowel frequency and evacuation symptoms, and found that these were closely related to physiological findings. However the study did not clearly describe the method of symptom analysis, and failed to indicate whether allocation to sub-groups was made before or after investigation. Koch *et al.* (Koch *et al.*, 1997) divided a series of patients into similar sub-groups on the basis of investigation, and then examined their perceived symptoms. They found that some symptoms were associated with STC, whilst others were associated with RED. A significantly different distribution of symptoms between pathophysiological groups was demonstrated using a chi-square analysis, but the specificity of single symptoms was low. Discriminant analysis showed no superiority of combinations of symptoms, but the methods and results of such analyses were not presented. Grotz *et al.* (Grotz *et al.*, 1994), who also included patients with normal transit constipation, sought to classify 184 patients into patho-physiological sub-groups based on psychological stress and colorectal symptoms. Although when matched for age and sex, some symptoms were independently discriminatory, the authors were unable to demonstrate significant correlation between symptoms and type of constipation when multiple logistic regression analysis was used. Recently, Mertz *et al.* (Mertz *et al.*, 1999) found clusters of symptoms from which they defined sub-groups of patients with constipation, using a method of factor analysis. The discriminatory capacity of this study for physiologically defined sub-groups is not clear.

Unlike previous symptom questionnaires, the KESS questionnaire appears to be able to discriminate between patho-physiological sub-groups for the majority of patients with constipation (55%, CI 43% to 67%). Discriminant scores predict patients with pure STC or RED better than patients with mixed abnormalities. Both the selection of variables for the discriminant analysis and the level of classification performance ideally need to be validated in a new sample of patients.

The relatively low level of discrimination could have been caused by adoption of sub-optimal questions. In addition, the diagnostic specificities of current "gold standard" physiological tests of transit and anorectal function are themselves not absolute.

Furthermore, it is possible that allocation of patients to one of three sub-groups may introduce error because of artificial "pigeonholing" of disorders that in reality may represent a more continuous spectrum of disease. We accept that the analysis presented for the prediction of patho-physiology in this study may be unwieldy for use in the routine clinical setting, but modern spreadsheet applications allow the equations of classification functions to be calculated easily.

Whilst this is the first study utilizing appropriate statistical methodology to demonstrate the discriminatory ability of multiple symptoms in constipation, the overall discriminatory power is insufficient for its immediate application in clinical practice. However, we have explored the ability of KESS scores to discriminate single pathologies from other groups. For example, in attempting to predict which patients have isolated RED, it proved possible to choose a criterion that had a sensitivity of 48.0% (95% CI = 28% to 69%) and a specificity of 100% (95% CI = 0% to 8%) (see ROC curve: Figure 4.04). In other words, this cut-off criterion should, on average identify almost half the RED only patients with high confidence that they do not have STC. However, the wide confidence intervals around the estimates of sensitivity and specificity, and the fact that the logistic model has not been validated in any way (neither by cross-validation nor using an independent patient sample), means that this finding should be viewed with some caution. As in the case of the discriminant analysis, it is also desirable to check the selection of variables for the logistic model in an independent patient sample.

Since the identification of patho-physiological sub-groups amongst constipated patients affects management and prognosis (Wexner *et al.*, 1991; Pemberton *et al.*, 1991; Piccirillo *et al.*, 1995; Pfeifer *et al.*, 1996; Lubowski *et al.*, 1996; Nyam *et al.*, 1997), extensive physiological testing of patients with intractable constipation remains the optimal method of evaluation when management strategies are being planned.

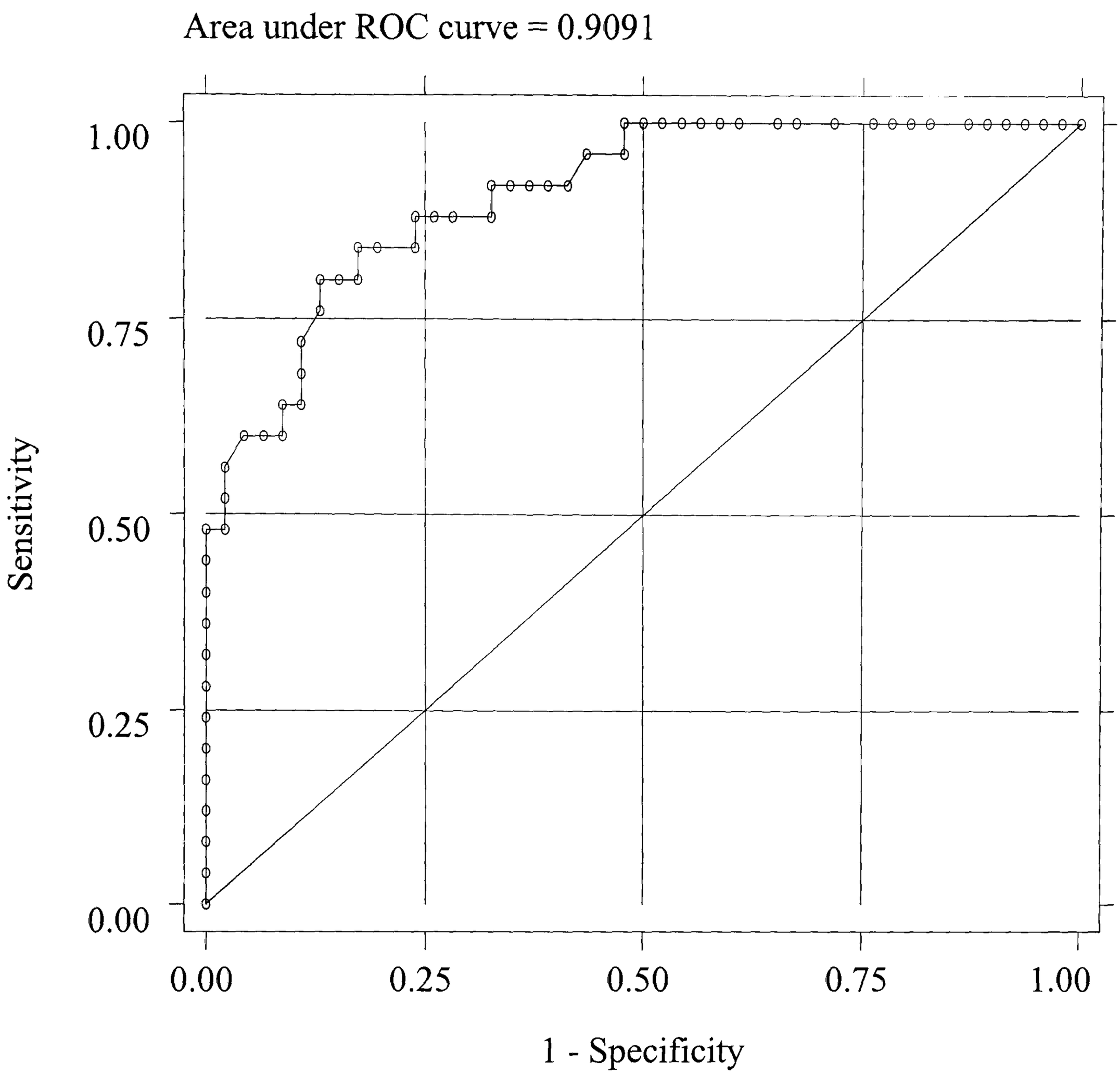


Fig 4.04: ROC curve for a logistic regression model to predict RED only from discriminant scores for RED, MIXED and STC groups. The figure shows the trade-off between sensitivity and specificity in discriminating RED only patients from MIXED and STC patients.

Whilst it is accepted that an experienced coloproctologist may be able to derive all pertinent information from history taking, the use of a questionnaire by others in the clinical assessment of patients with constipation may be helpful in prompting complete symptom assessment, monitoring treatment efficacy, and in the future, rationalizing further investigation. This is the first study utilizing appropriate statistical methodology to demonstrate a discriminatory ability of multiple symptoms in constipation.

5 SENSORY AND AUTONOMIC NEUROPATHY IN PATIENTS WITH SLOW TRANSIT CONSTIPATION

5.1 INTRODUCTION

The important role of extrinsic neuronal influences in the control of colonic motility has been discussed in the introduction. Autonomic dysfunction is an established cause of constipation in common conditions such as diabetes mellitus (Feldman *et al.*, 1983; Sharma *et al.*, 1995), and in rarer conditions associated with dysautonomia, such as the acute and subacute autonomic neuropathies (Khurana *et al.*, 1988). These may affect both sympathetic and parasympathetic systems (pandysautonomia), or be selective e.g. "pure" cholinergic dysautonomia, as is seen for instance in the Shy-Drager syndrome (Khurana *et al.*, 1980). Constipation may be the most common gastrointestinal manifestation of diabetes (Feldman *et al.*, 1983; Sharma *et al.*, 1995), and is associated with colonic motor abnormalities (Battle *et al.*, 1980), and prolongation of transit through all bowel segments (Battle *et al.*, 1980; Iber *et al.*, 1993; Maleki *et al.*, 1998). Gastrointestinal motor abnormalities in diabetes occur in both type I (insulin-dependent) and II (non-insulin-dependent) diabetes, and are associated with generalised autonomic neuropathy (cardiovascular and genitourinary dysfunction) (Low *et al.*, 1975; Werth *et al.*, 1992), and other diabetes-related complications such as sensorimotor peripheral neuropathy (Hollis *et al.*, 1977; Russell *et al.*, 1983). Anorectal sensorimotor abnormalities are also common in diabetics with or without faecal incontinence (Rogers *et al.*, 1988; Sun *et al.*, 1996).

A number of studies of patients with STC have indicated that dysmotility may exist in any of the extracolonic regions of the gastrointestinal tract: oesophagus (Watier *et al.*, 1983; Reynolds *et al.*, 1987; Redmond *et al.*, 1995; Ghosh *et al.*, 1996), stomach (Reynolds *et al.*, 1987; Van der Sijp *et al.*, 1993; Ghosh *et al.*, 1996; McDonald *et al.*, 1997), duodenum (Bassotti *et al.*, 1996; Glia & Lindberg, 1998), and ileum (Panagamuwa *et al.*, 1994). In addition, sensory or motor abnormalities of the urinary

tract have been demonstrated in some patients (Abdel-Rahman *et al.*, 1981; Watier *et al.*, 1983; Bannister *et al.*, 1988; Kerrigan *et al.*, 1989). The suggestion has therefore been made that STC might be a manifestation of a systemic, possibly autonomic disease process (Watier *et al.*, 1983; Waldron *et al.*, 1989).

Sweat regulation has been used as a test of autonomic function in diabetics since 1942 (Kahn & Rothman, 1942), and a number of methods are now available (Low *et al.*, 1983; Ewing *et al.*, 1988; Low, 1993). Such methods have subsequently been used to test a variety of patients with "functional gastrointestinal disorders". The first study by Camilleri and Fealey (Camilleri & Fealey, 1990) using the thermoregulatory test and a quantitative sudomotor axon reflex test to iontophoresed acetylcholine (Low *et al.*, 1983), showed in 8 patients with severe (principally upper) gastrointestinal problems that idiopathic autonomic denervation may be an aetiological factor in non-organic intestinal dysmotility. All these patients had patchy anhidrosis, and 2 had positive cardiovascular tests. A further study by the same group from the Mayo Clinic (Bharucha *et al.*, 1993) used this methodology to test 113 consecutive patients with a variety of gastrointestinal motility disorders, finding similar evidence of autonomic dysfunction in approximately 70% of diabetics and patients with neurological disease, and in approximately 30% of other motility disorders including IBS. Neither of these studies tested patients with STC.

Waldron *et al.* (Waldron *et al.*, 1989) used conventional cardiovascular autonomic tests described by Ewing and Clark (Ewing & Clarke, 1982) in a small number of patients with intractable constipation. The inconsistent results, may have reflected the poor sensitivity of the tests used (Fisher & Frier, 1989), or reflect the heterogeneity of the group studied. The same researchers (Altomare *et al.*, 1992) went on to test 23 patients with idiopathic slow transit constipation using the acetylcholine sweat spot test described by Ryder (Ryder *et al.*, 1988). They found patchy anhidrosis in 11, and concluded that the results indicated a reduction in post-ganglionic sympathetic sudomotor fibre function, which might reflect a similar disturbance of extrinsic sympathetic inhibitory fibre function in the gut. The acetylcholine sweat test cannot

however differentiate between intrinsic sweat gland dysfunction and that caused by denervation because acetylcholine can stimulate receptors both on the nerve and the gland itself (Jolliffe *et al.*, 1995). Raethjen *et al.* (Raethjen *et al.*, 1997) used a range of quantitative peripheral sensory and autonomic tests, including direct and axon-reflex sweat tests (described below in detail). Their finding of selective autonomic and sensory deficits in patients with STC was moderated by the small patient number.

The aim of this study was to test a large cohort of patients with STC with a range of standard neurophysiological and selective quantitative tests of sensory and autonomic function (Anand *et al.*, 1996), and to compare these findings with other factors, including familial association, prognosis and gastrointestinal physiological measurements. Normal controls and diabetics with gastrointestinal involvement were studied for comparison.

5.2 METHODS

5.2.1 PATIENTS

Forty-one patients were referred consecutively for study between October 1996 and February 1998. All had proven STC by the criteria previously described (Chapter 2). Thirty-nine women, and 2 men, median age 39 years, range 13-62 were studied. Notably, no patient had any evidence of any other disease process that might lead to peripheral neuropathy in the lower limb such as diabetes mellitus or spinal trauma. No patient had been previously studied (Altomare *et al.*, 1992; Raejthen *et al.*, 1997).

20 age and sex-matched healthy volunteers acted as controls. 12 diabetic patients (8 male, 4 female) with gastrointestinal involvement were studied as a positive control population.

5.2.2 METHODS (GENERAL)

The subjects underwent a number of tests to assess both small (autonomic and sensory) and large fibre function (sensory and motor) (see table 5.01).

Table 5.01: Autonomic and somatic fibre tests

Test		Fibre type
Autonomic function tests		
Sweat tests		Post-ganglionic cholinergic sympathetic
Cardiovascular tests		Mixed sympathetic / parasympathetic
Somatic function tests		
Thermal thresholds	Cool	Small myelinated sensory (Aδ)
	Warm	Small myelinated / unmyelinated sensory (Aδ / C)
	Heat pain	Unmyelinated sensory / nociceptive (C)
Axon reflex vasodilatation		Unmyelinated nociceptive (C)
Tactile threshold		Large myelinated sensory (Aβ)
Vibration threshold		Large myelinated sensory (Aβ)
Sural nerve conduction		Large myelinated sensory (Aβ)
Peroneal nerve conduction		Large motor (Aα)

All tests were carried out at a single visit in a quiet, air conditioned and thermostatically controlled room (21-23°C). Subjects lay supine for all tests, except when seated upright for thermal threshold and vibration tests. All sites tested for axon reflex vasodilatation or sweating were cleaned prior to injection with an alcohol wipe to remove dirt and grease, which may have affected recordings. Test solutions were drawn up into 28–guage

hypodermic syringes (Lo-dose, Beckton-Dickinson, 0.5ml capacity) and administered immediately. Details on the preparation of solutions used for injection are given in appendix 5.01.

5.2.3 SMALL FIBRE TESTS

Thermal perception thresholds (unmyelinated & small myelinated sensory fibres)

Thresholds for warm and cool sensation, and heat pain were measured using a Marstock stimulator (Thermotest, Somedic Ab, Stockholm, Sweden) (see Figure 5.01) as described by Guy *et al.* (Guy *et al.*, 1985). By means of the Peltier principle, accurate stimulator paddle temperatures are produced when pre-calculated electric currents pass through the bimetallic junction of dissimilar materials within the stimulator. The instep of the foot rested on the stimulator paddle (2.5 cm x 5 cm), which was placed on a firm surface. Ramp thermal stimuli were delivered with a standard rate of change of temperature of 1°C per second, rising from a neutral temperature (30.5°C) up to the temperature when the subject was aware of the desired sensation (method of limits).

Each subject was given instruction to press a button immediately when they became aware of a given sensation, which then returned the stimulator to the neutral starting point. Each sensation was tested 5 times in succession, and the threshold was taken as the average degree centigrade change from the neutral point calculated from the recording.

Sweat tests (cholinergic sympathetic sudomotor fibres)

The principle of direct and indirect sweat tests is shown in Figure 5.02. Nicotine (0.8 μg in 0.2 ml) was injected intradermally (Jolliffe *et al.*, 1995; Anand *et al.*, 1996) into the skin of the lateral calf to produce an indirect sweating response via a sudomotor axon reflex. Absolute peak sweat rate in $g/m^2/h$ was quantified locally in each case over an area of 1.13 cm^2 by an evaporimeter (Servomed Evaporimeter EP1 Ab Stockholm, Sweden) (Nilsson, 1977) (Figure 5.03). The peak usually occurred within about 5 minutes of injection, and rapidly declined thereafter (see figure 5.04).

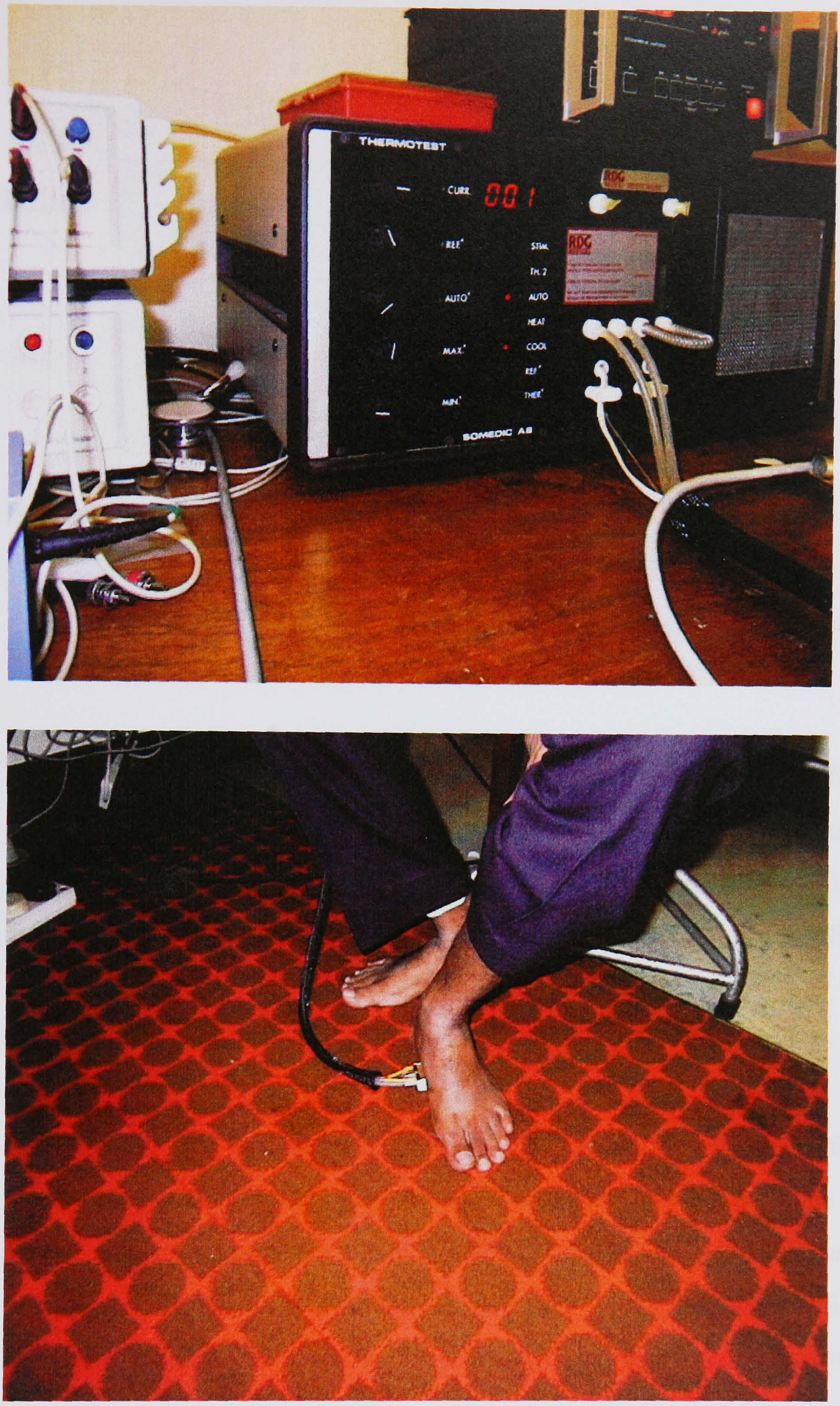


Figure 5.01: Thresholds for warm and cool sensation, and heat pain were measured using the Marstock stimulator (Thermotest, Somedic Ab, Stockholm, Sweden). The instep of the foot rested on the stimulator paddle (2.5 cm x 5 cm).

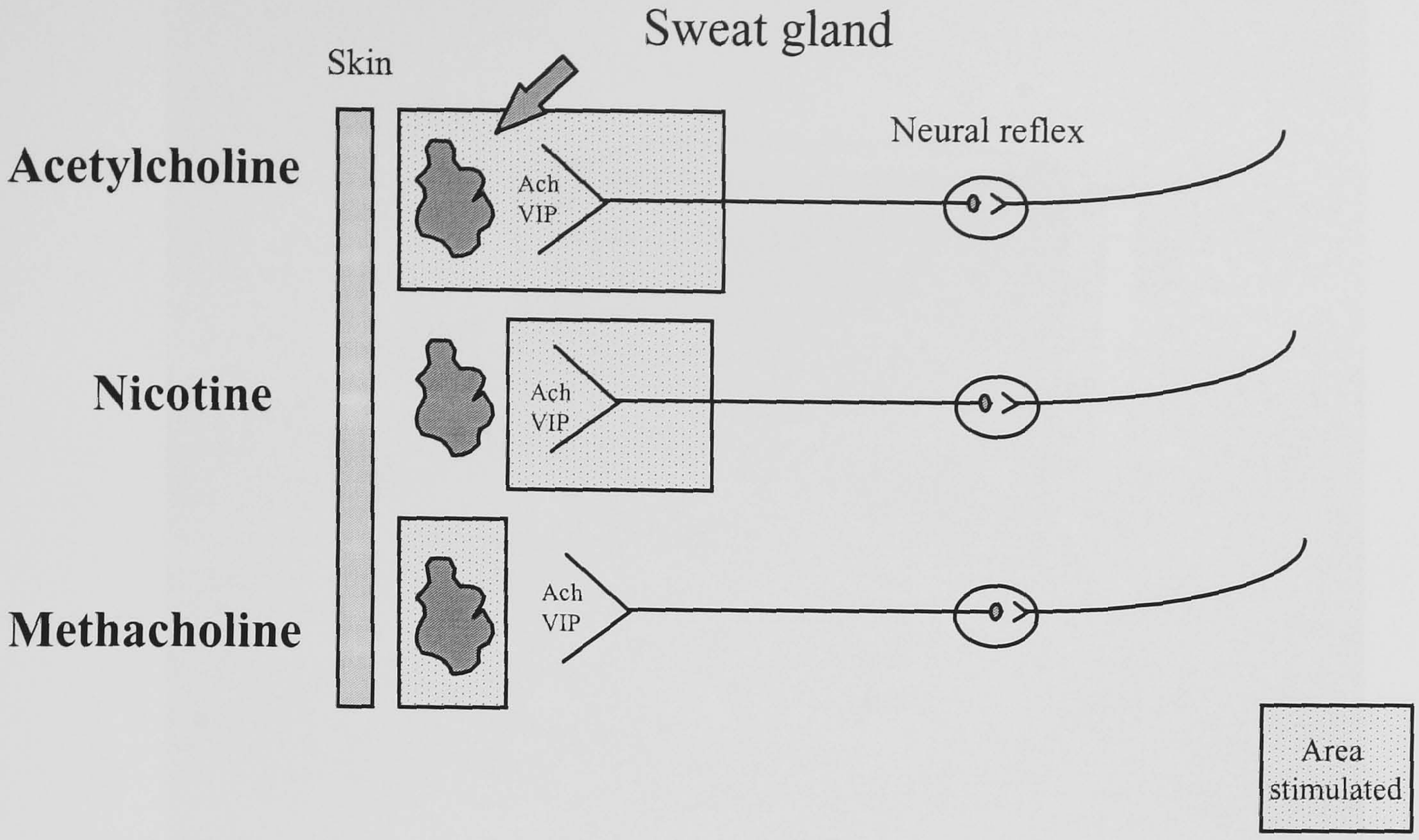


Figure 5.02: Principle of sweat tests. Acetylcholine, as used in previous studies stimulates both the sweat gland directly, and the neural reflex. in contrast, the use individually of both nicotine which stimulates the neural reflex, and methacholine which stimulates the sweat gland directly allows the site of any sweating abnormality to be defined.

When a poor indirect response was obtained, the test was repeated in the calf and volar forearm, and intradermal methacholine (4μg in 0.1ml) was injected at a nearby site to give a direct sudomotor response, also measured with the evaporimeter. In each case, care was taken to place the head of the evaporimeter (using surgical tape) over the bleb of the intradermal injection, avoiding the entry point of the injecting needle, where fluid may leak back.

Axon reflex vasodilatation (unmyelinated sensory fibres)

Axon reflex vasodilatation is the flare component of Lewis' triple response in skin. This was induced by intradermal capsaicin (0.05 μg in 10 μl), which selectively activates nociceptive fibres.

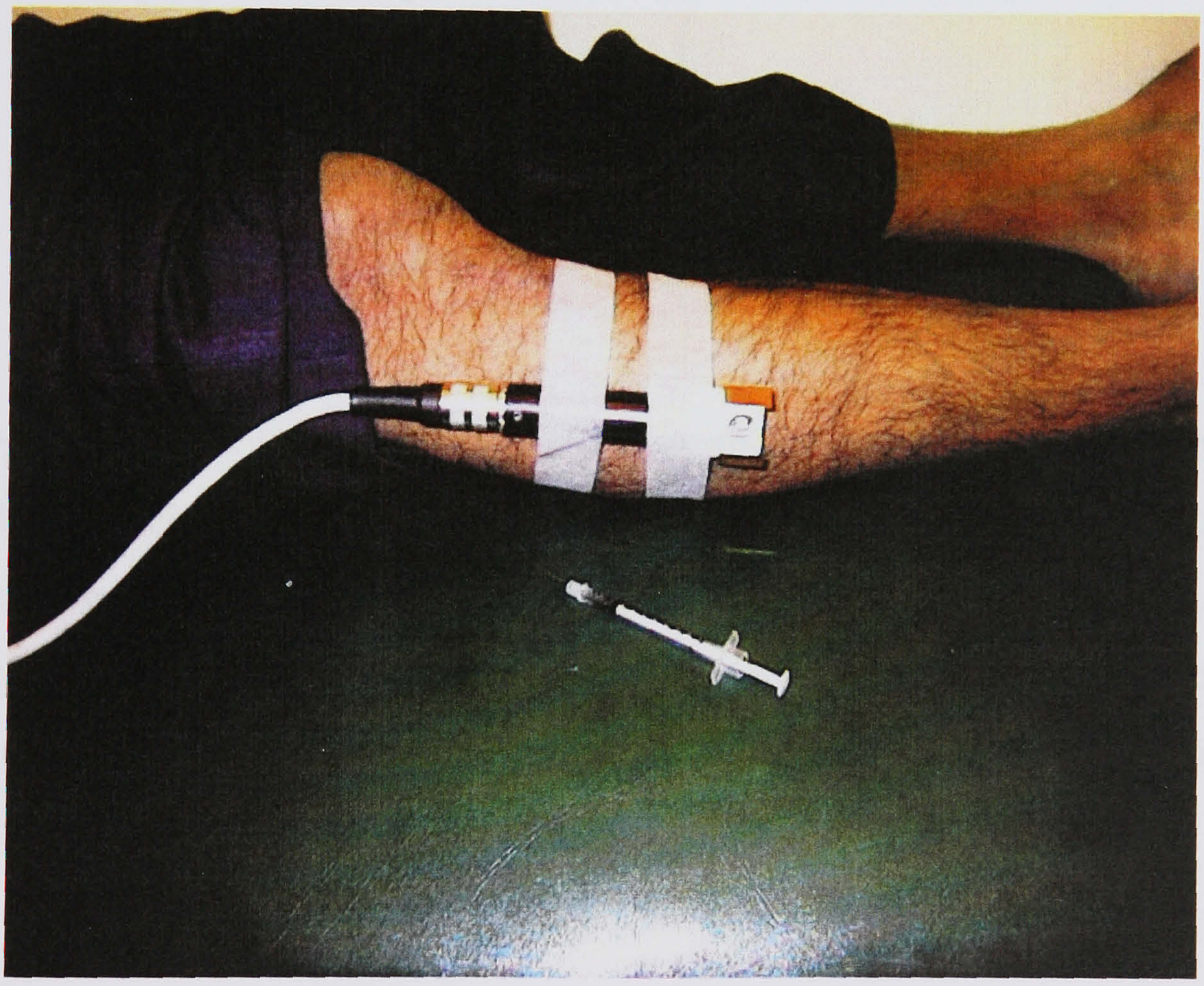


Fig 5.03: Sweat testing. Nicotine or methacholine are injected intrademally into the skin of the lateral calf to produce an indirect (axon-reflex) or direct sweating response respectively. Absolute peak sweat rate in $g/m^2/h$ was quantified locally in each case over an area of 1.13 cm^2 by an evaporimeter (Servomed Evaporimeter EP1 Ab Stockholm, Sweden).

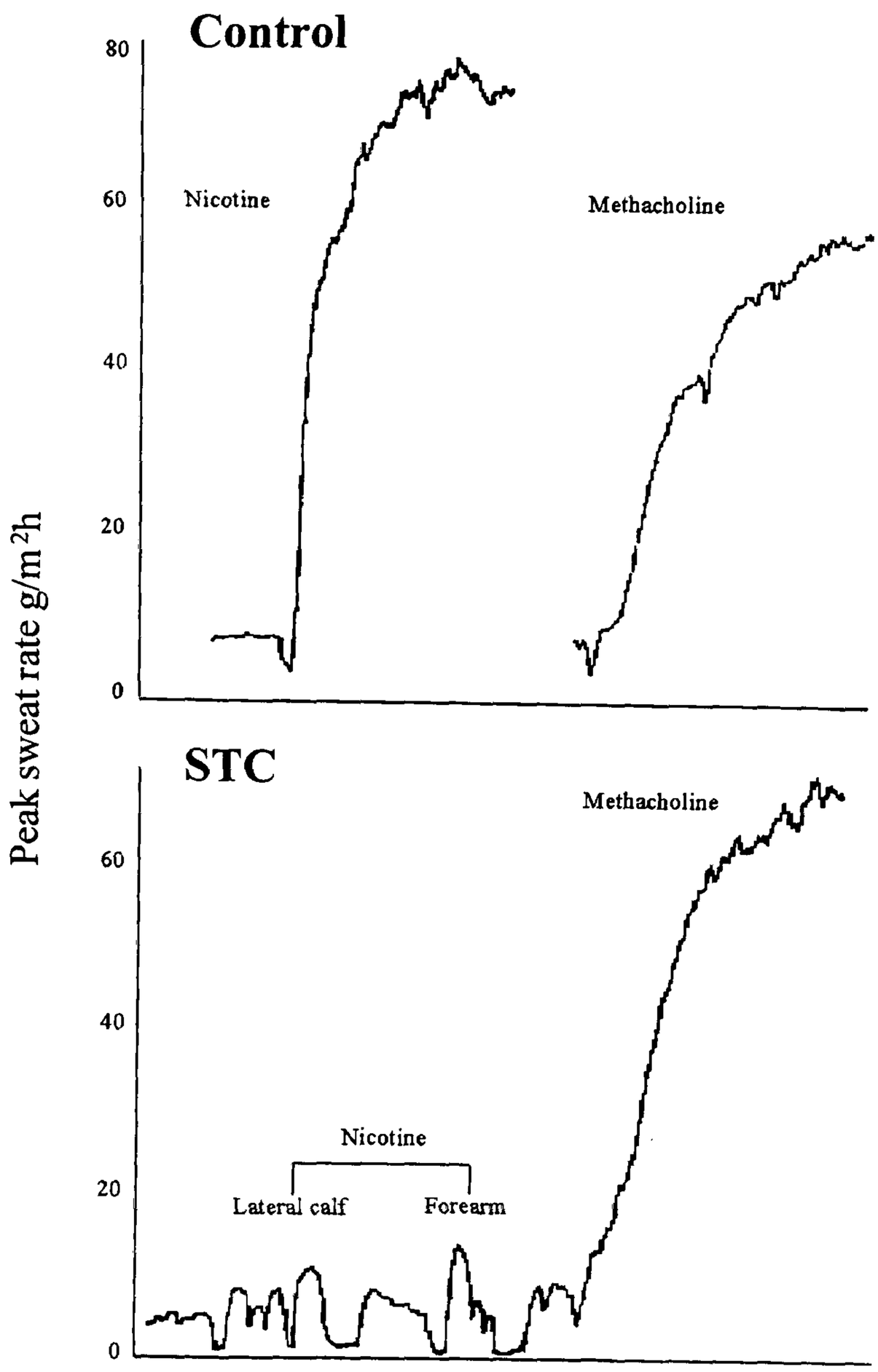


Figure 5.04: The figure shows 2 sweating responses from a normal control and a patient with STC. The upper trace demonstrates a normal response both to nicotine (axon-reflex sweating) and methacholine (direct gland stimulation). In contrast, the STC patient has a minimal axon-reflex response, both in the lateral calf and on repetition in the forearm, but a normal direct response when methacholine is used. Both subjects were females of the same age.

The immediate increased capillary flux as a result of vasodilatation was measured with a laser-Doppler (Jolliffe *et al.*, 1995; Anand *et al.*, 1996) (Perimed PF 4, Stockholm) (Figure 5.05a), and recorded in flux units. Laser (coherent) light is transmitted to the skin via one fibre of a standard probe, and the scatter of this light by moving red cells undergoes a Doppler frequency shift. Reflected light is transmitted back via a second

fibre, and converted to a voltage signal in mV by a sensitive photoelectric cell (expressed as "flux units"). The signal produced is related linearly to the flux of red cells, i.e. number of cells times their velocity (Nilsson *et al.*, 1980; Parkhouse & Le Quesne, 1988). The increase in capillary flux, calculated as the peak minus baseline was recorded (see Figure 5.05b). The probe was carefully placed on the skin of the lateral calf in a perspex holding device away from previously tested areas (above) (Figure 5.05a). A small bleb was created intradermally, and any leakage of blood or solution from the testing site was carefully wiped away to prevent false high recordings. Care was also taken to attach the probe holding device (using surgical tape) so as to avoid compression of the tissue (reducing blood flow) or formation of an air interface (reducing signal). The test was repeated once when abnormally low or high results were obtained [accurate figures for reproducibility are not available for this test, because of the discomfort of repetition].

5.2.4 LARGE FIBRE TESTS

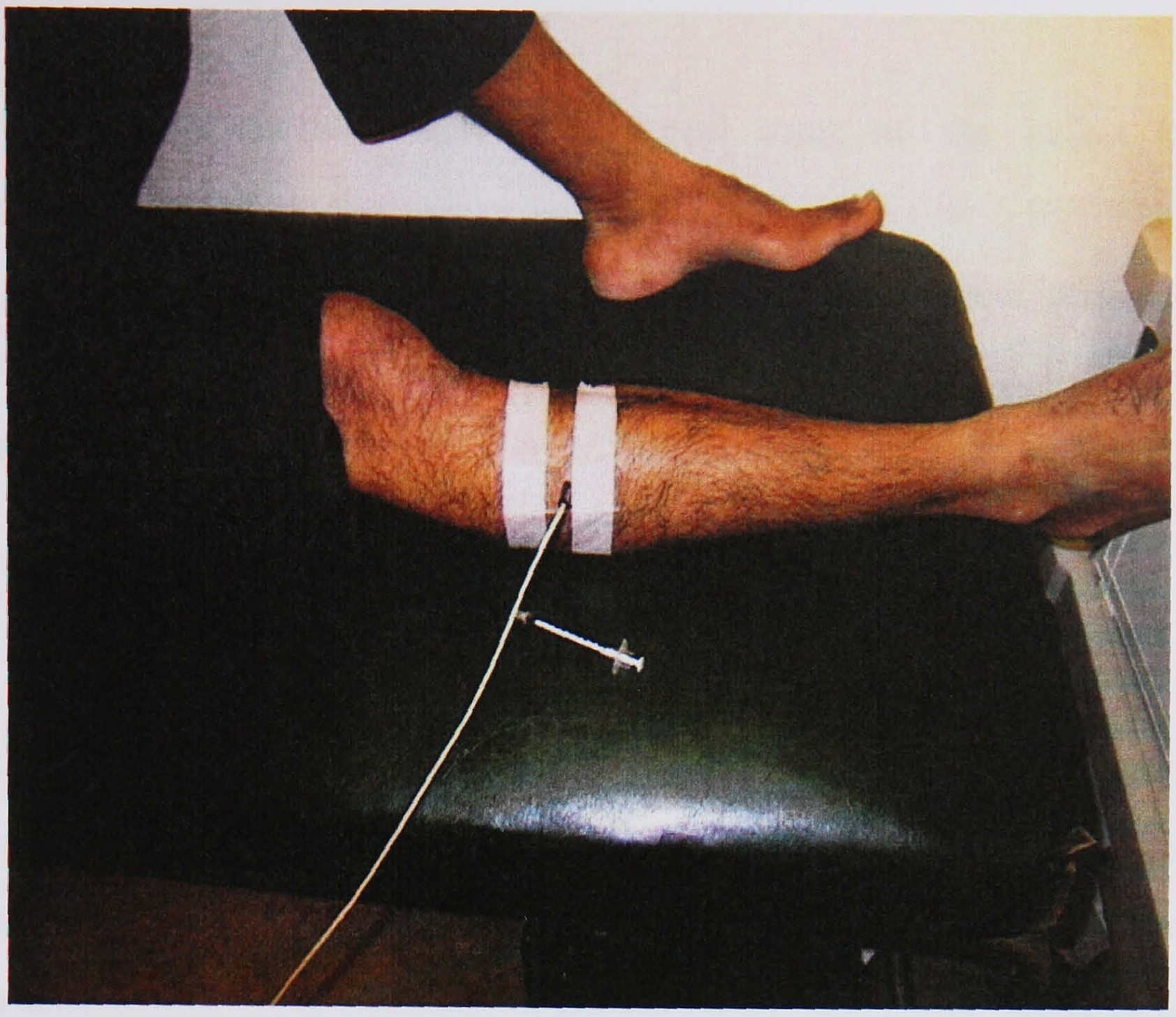
Nerve conduction studies

These were performed by a single consultant neurophysiologist using a Dantec Counterpoint (Denmark) by conventional methods. The sural nerve (sensory) and deep peroneal nerve (motor) were stimulated, and the sensory action potential (SAP), and motor action potential (MAP) delay and amplitude were recorded. Conduction velocity (in m/sec) was then obtained by calculation.

Mechanical and vibration thresholds

Both were tested using a method of limits, progressively larger stimuli being applied to the dorsal aspect of the great toe with the patients' eyes closed. Semmes-Weinstein monofilaments ("von Frey hairs") were used to produce a punctate stimulus on the dorsum of the great toe (Bell-Krotoski *et al.*, 1993) (Figure 5.06a). Hairs are numbered 1 to 20. The relationship between the hair number and $\log_{10}$ force in tenths of milligrams is linear, i.e. there is a curvilinear relationship between actual forces and hair numbers (Weinstein *et al.*, 1993).

(a)



(b)

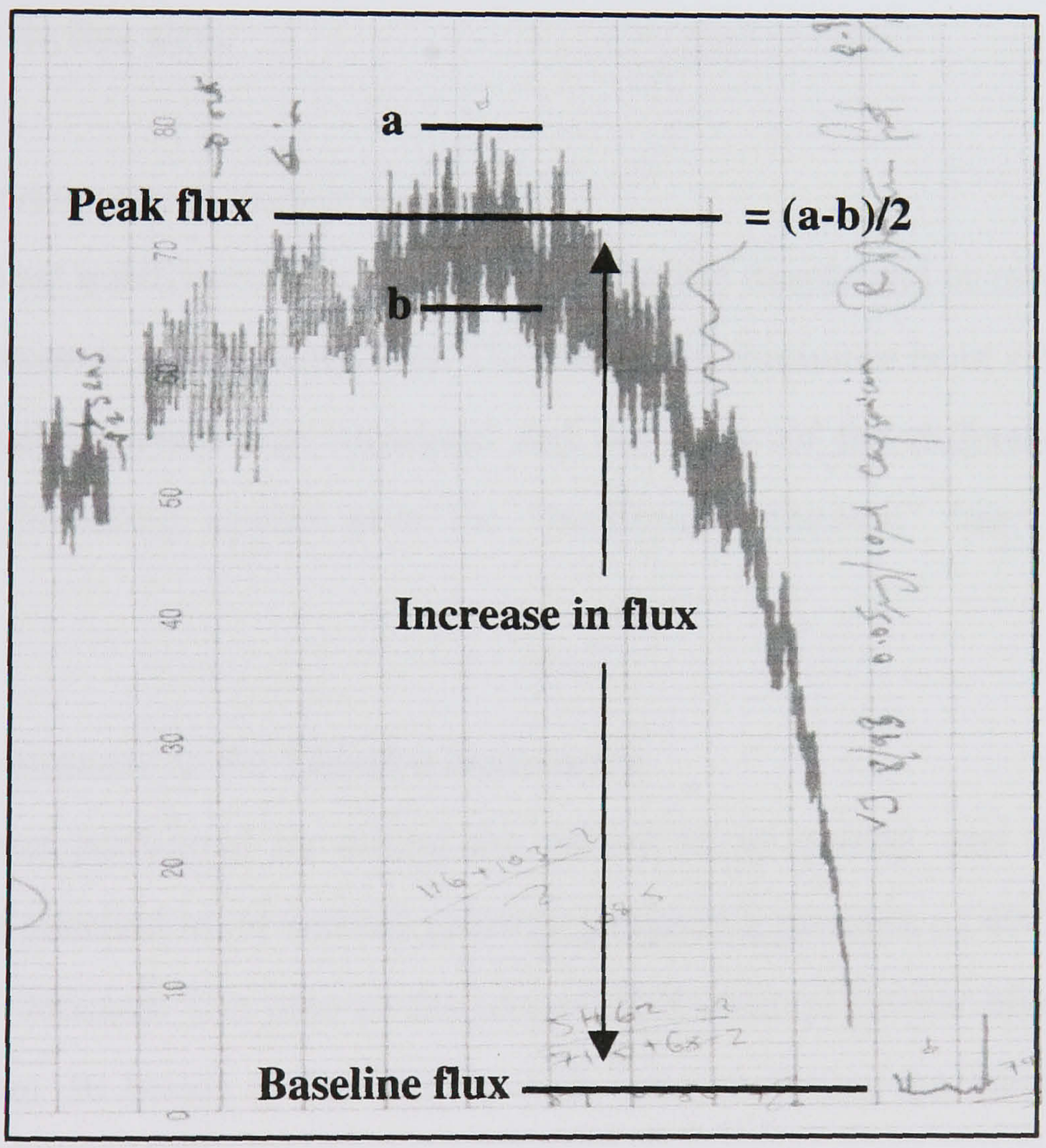


Figure 5.05: Axon-reflex vasodilatation. (a) This was induced by injection of intradermal capsaicin (0.05 μg in 10 μl), as this selectively activates nociceptive fibres. (b) The immediate increased capillary flux as a result of vasodilatation was measured with a laser-Doppler = [(a-b/2) - baseline flux].

Vibration was tested at the metatarsophalangeal joint of the hallux using a Biothesiometer (Bio-Medical Instrument Co., Ohio, USA) (Figure 5.06b). Increasing vibrameter relative amplitudes were applied starting at zero, the subject indicating when they were first aware of the stimulus (vibration, not pressure or touch). Reproducibility of the procedure has been validated (Guy *et al.*, 1985).

5.2.5 NON-INVASIVE CARDIOVASCULAR AUTONOMIC TESTS

Four standard tests were used: heart-rate responses to deep breathing, valsalva & standing up, and the blood pressure response to standing up. All have been previously validated, and control ranges established (Ewing *et al.*, 1988). All tests were performed using a portable digital ECG machine, employing only the 4 limb leads to obtain a continuous rhythm strip.

Heart-rate response to deep breathing.

The patient sat quietly and was then asked to breathe deeply and evenly at 6 breaths per minute (5 seconds in, 5 seconds out). The maximum-minimum heart rate during each 10 second breathing cycle was measured and the mean of the differences during three successive breathing cycles gave the "maximum-minimum" heart rate (beats per minute).

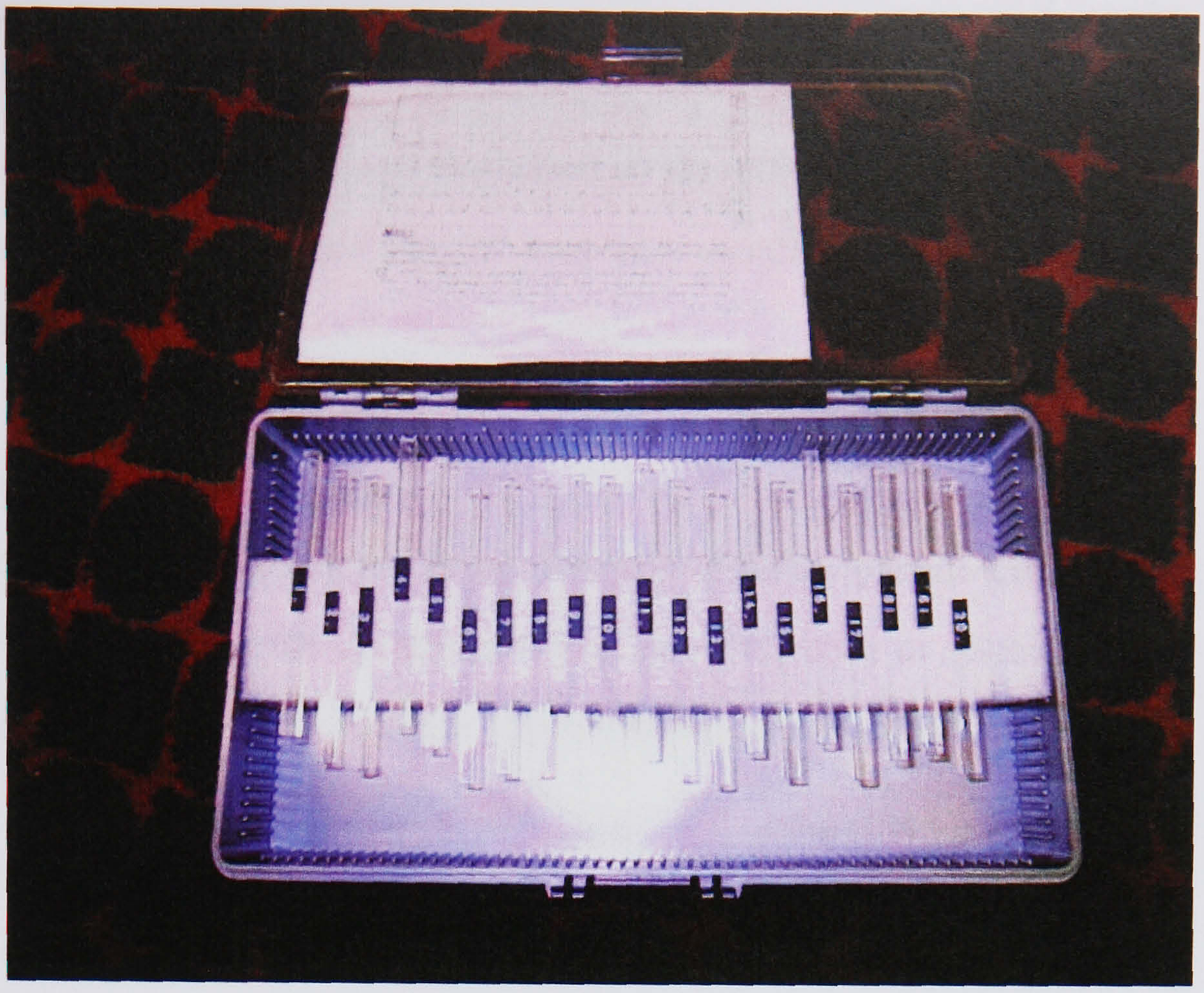
Heart rate response to the Valsalva manoeuvre

This test was performed by asking the patient to sit quietly, and then blow into a mouthpiece attached to an aneroid pressure gauge at a pressure of 40-50mmHg, and to hold for 15 seconds. The ratio of the longest R-R interval shortly after the manoeuvre (within about 20 beats) to the shortest R-R interval during the manoeuvre was then measured. The result was expressed as the Valsalva ratio.

Heart rate response to standing up.

The patient lied supine quietly on a mechanical tilt table, and was then tilted rapidly to an upright position.

(a)



(b)

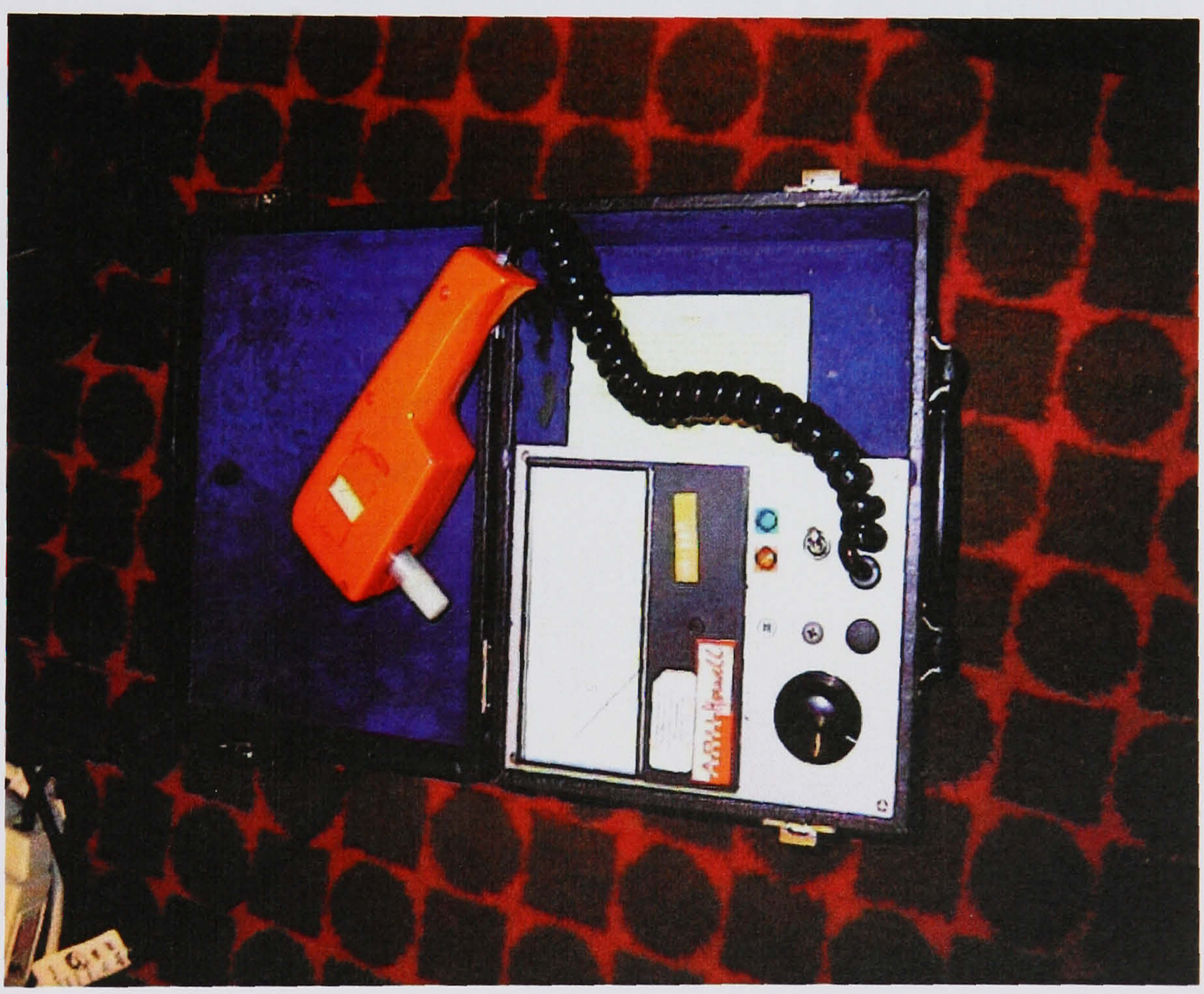


Figure 5.06: Mechanical and vibration thresholds. Both were tested using a method of limits, progressively larger stimuli being applied to the dorsal aspect of the great toe. **(a)** Semmes-Weinstein monofilaments ("von Frey hairs") were used to produce a punctate stimulus on the dorsum of the great toe. **(b)** Vibration was tested at the metatarsophalangeal joint of the hallux using a Biothesiometer.

The characteristic heart rate response was expressed as the 30: 15 ratio, which is the ratio of the longest R-R interval around the 30th beat after standing to the shortest R-R interval around the 15th beat.

Blood-pressure response to standing up.

This test was performed by measuring the blood pressure while the patient was supine, and then 1 minute after tilting to the upright position. The difference in systolic blood pressure (rise or fall) was taken as the measure of postural blood-pressure change.

5.2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using the Kruskal-Wallis one-way ANOVA for non parametric data. Individual groups were compared using Dunn's multiple comparison test. Control values were obtained for all tests except for the non-invasive cardiovascular autonomic tests, and abnormal results were defined as lying outside two standard deviations from the control population mean, allowing for age. For the cardiovascular tests, the presence of single test abnormalities, and of autonomic neuropathy and its classification by severity (according to the degree and number of abnormalities) were defined from published series (Ewing *et al.*, 1988).

5.3 RESULTS

5.3.1 CLINICAL AND PHYSIOLOGICAL DATA

STC patients

Range of duration of constipation in STC patients was 1.5 to 52 years. 32 / 41 (78%) arose *de-novo* in childhood or teenage (CIST). A further 3 patients had symptoms arising in childhood, but worsened by hysterectomy (C/PIST). A total of 4 patients (1 teenage onset, and 3 adult onset) gave a history of pelvic events associated with onset of symptoms (PIST). Two of these patients' symptoms arose following normal

vaginal deliveries (both primagravidae without complicated labour other than first degree tears), 1 arose after oophorectomy and 1 after hysterectomy, both without perioperative complications. A further two patients had *de-novo* adult onset of symptoms (AOIST). Nine patients had undergone colectomy (5 subtotal, 4 segmental resection) prior to the study. Taken as a group, the median bowel habit was once every 14 days. Patients typically complained of other defaecatory symptoms, and abdominal pain and bloating.

Eleven patients also gave a history of urinary symptoms especially frequency and urgency, 2 each of decreased sweating and dizziness on standing. Peripheral neuropathic symptoms, including a feeling parasthesiae, numbness or pain were uncommon, occurring in only 4 patients. Three STC patients had a history of psychiatric illness (depression), and 2 others used anti-anxiolytics. Thirty-four continued to regularly use laxatives.

Median radioopaque marker retention (performed in all 41 patients) was 98% (range 20-100% at 100 hours). Delayed transit, which was confirmed in 28 patients by a radioisotope transit study showed a generalised delay in 22 and segmental delay in 6. Twenty patients had rectal hyposensation to distension for one or more parameter compared to normal ranges (Jameson *et al.*, 1994) (Chapter 3). Sixteen patients (from 34 who underwent proctography) had disordered rectal evacuation secondary to a mechanical obstruction to defaecation or failure to strain by any cause (Chapter 3).

A history of significant symptomatic constipation in one or more family members was elicited in 25 / 41 (61%) of STC patients. In addition, 4 index patients had a family history of Hirschsprung's disease, and 1 patient had an affected identical twin.

Diabetic patients

Eight insulin dependent (IDDM), and 4 non-insulin dependent diabetics were studied. Duration of disease since diagnosis ranged from 2 to 25 years, and duration of

gastrointestinal symptoms from 1 to 17 years. Frequency of defaecation ranged from every 4 days (constipated) to approximately 10 times per day (diarrhoea), with or without incontinence. Five patients had marked urinary problems, and 5 / 8 males had erectile dysfunction. 10 / 12 patients had a diagnosis of peripheral neuropathy, 5 of retinopathy, 3 of nephropathy, and 2 of a Horner's syndrome. Some gastrointestinal physiological studies had been performed for 5 diabetics included in the study. Marked anorectal abnormalities were present in all 3 tested (reduced sphincter function, prolonged pudendal nerve motor latencies, rectal hyposensation). Both patients undergoing small intestinal manometry had neuropathic changes consistent with diabetic enteropathy (interpreted and reported by D.L.Wingate, GISRU).

Clinical neurological examination

There were no significant abnormalities in the STC group on neurological clinical examination, apart from one patient with a healed right-sided malleolar ulcer who had clinically reduced pin-prick sensation below the ankle. 10 of 12 diabetics had clinical signs of peripheral neuropathy (sensory, motor or both).

5.3.2 CONVENTIONAL NEUROPHYSIOLOGY AND AUTONOMIC TESTS

The full results of these tests for all groups are listed in appendix 5.02

Nerve conduction studies

STC patients had no abnormalities of motor-sensory nerve conduction. In the diabetic patients, abnormalities of nerve conduction were found in 10/12 patients (sensory and motor abnormalities in 6 and sensory only in 4), with mixed features of axonal loss and demyelination.

Non-invasive cardiovascular autonomic tests

Two patients with STC had one abnormality each, and 1 had a borderline abnormality, indicating that cardiovascular evidence of significant autonomic neuropathy was not

present in any patient (early involvement possible). In contrast, 5 diabetics (from 9 tested) had "definite" or "severe" autonomic involvement by their disease (Ewing *et al.*, 1988).

5.3.3 QUANTITATIVE PERIPHERAL TESTS

The full results for all groups are shown in appendix 5.03

Control values and cut-offs of normality

The numeric limits of normality for each quantitative test were taken from the control values (n = 20), and calculated from the control mean +/- 2 S.D. (Table 5.02).

Table 5.02: Quantitative peripheral sensory and autonomic tests: control values and cut-offs for normality.

Test	*Normal range*	*Mean +/-2 S.D. (cut-off)*
Light touch (Log^{10} force)	1 – 7	≥ 8
Vibration (Biothesiometer units)	5 – 11	≥ 12
Cool threshold (°C)	1 – 3.5	≥ 3.3
Warm threshold (°C)	1.4 – 5.1	≥ 5.4
HeatPain threshold (°C)	7.7 – 16.1	≥ 17.0
Axon-reflex sweating (g/m^2h)	32 – 84	≤ 22
Axon-reflex vasodilatation (flux)	44 – 140	≤ 37

Small sensory fibre tests

STC patients showed a significant elevation of warm sensory threshold (n = 41, median 4.2 ^{0}C, range 2 - 12.1) compared to controls (n = 20, median 3.2 ^{0}C, range 1.4 – 5.1, $p <$ 0.05) (Figure 5.07).

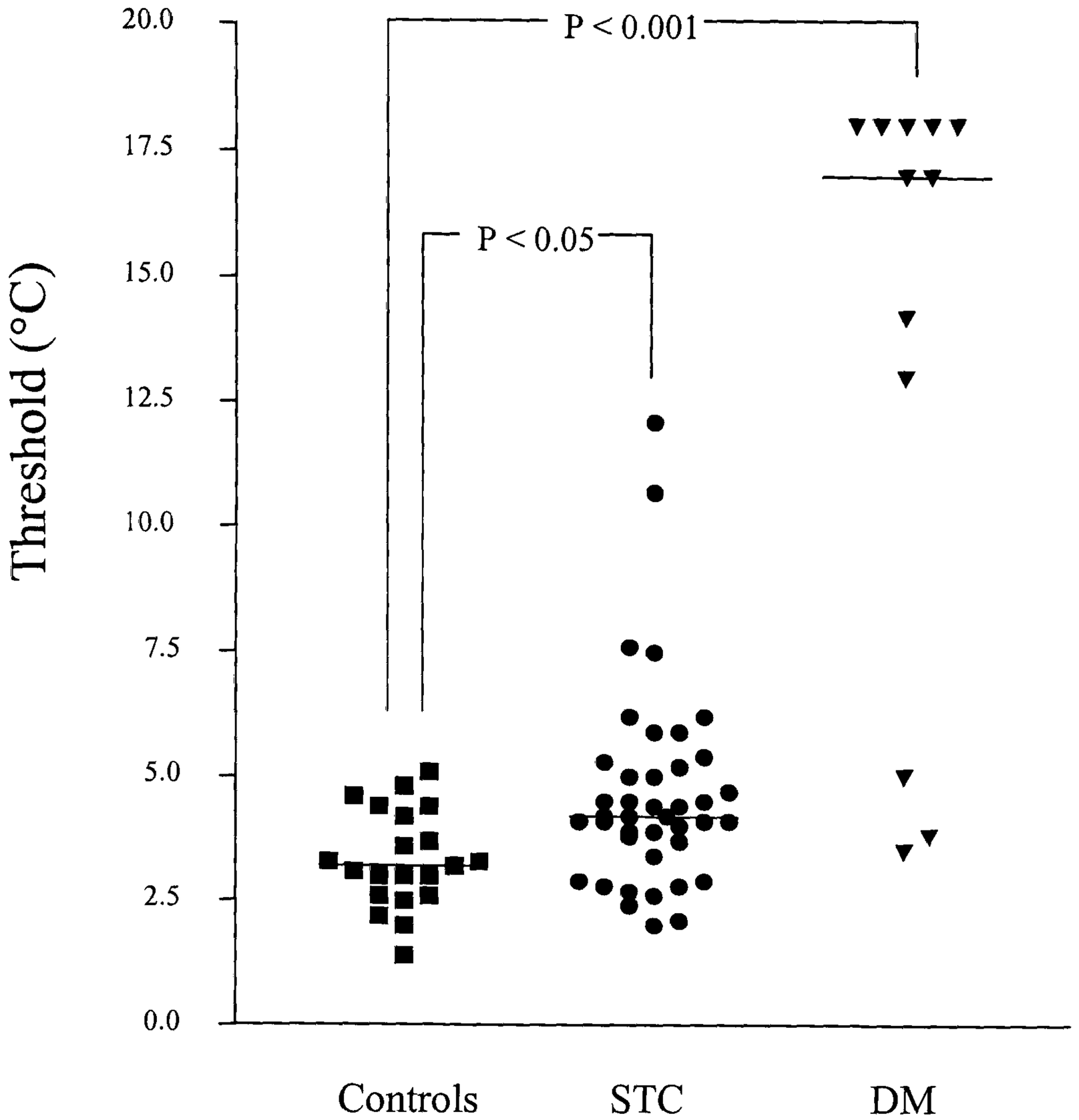


Figure 5.07: Scatter plot of warm thresholds for STC patients *vs.* controls and diabetics. Some STC patients, and most diabetics had raised thresholds leading to significant differences between the groups (Kruskal-Wallis one-way ANOVA).

Cool thresholds were elevated, but not significantly (n = 41, median 2.1 ^{0}C, range 0.9 - 5.6) compared to controls (n = 20, median 1.5 ^{0}C, range 1 - 3.5, $p < 0.05$). Both these abnormalities were more marked in the diabetics ($p < 0.001$ for both warm and cool sensory thresholds). Three STC patients had an increased threshold to heat pain, and 2 of these patients had significantly reduced axon-reflex vasodilatation (both tests of unmyelinated nociceptor fibres). These 3 patients had marked abnormalities on other small sensory fibre tests. 10 diabetic patients had no threshold to heat pain within the safety limits of the study, and 7 of these had a significant reduction of axon-reflex vasodilatation.

Sweat tests

Nicotine-induced axon-reflex sweating was significantly decreased in the STC patients (n = 39, median 39 g/m^{2}h, range 7-74) compared to controls (n = 20, median 47 g/m^{2}h, range 32-84, $p < 0.04$) (Figure 5.08). STC patients, like the diabetics, appeared to fall into two subgroups, with an apparently distinct subgroup lying below 2 SD of the control mean (STC subgroup: n = 12, median 13 g/m^{2}h, compared to controls: $p <$ 0.0001, Mann Whitney U-test). Notably, on directly stimulating the sweat glands with methacholine, there was no significant difference between the controls (median 62 g/m^{2}h, range 42-92) and STC patients (median 62 g/m^{2}h, range 33-87) (Figures 5.03 and 5.08).

Large sensory fibre tests

Vibration threshold was not significantly different between patients (n = 41, median 8.5, range 3-20) and controls (n = 17, median 7, range 5-11, $p > 0.05$); however, 9 STC patients had slightly elevated thresholds with respect to the defined cut-off of normality. Most diabetics had markedly elevated vibration thresholds (n = 12, median 30, range 14-50, $p < 0.001$). The range of tactile threshold (monofilaments) was similar between controls and constipated patients: only 2 STC patients, but 6 diabetics had a raised thresholds.

5.3.4 SUMMARY OF RESULTS

Twenty STC patients, i.e. approximately 50% of the total, shared a total of 38 abnormal test results (range 1 - 5 per patient). Eight patients had sensory fibre abnormalities only, 3 patients had an abnormality of sympathetic function only, and 9 patients had both. Of the patients with sensory abnormalities, over half had small sensory fibre deficits. There was no overall correlation of test abnormalities with age or mode of onset, or laxative use. Of the 8 STC patients with a previous colectomy, 7 had abnormalities on quantitative tests. There was no difference in symptom profiles e.g. infrequency of bowel habit etc. between patients with or without abnormalities. All the 4 patients with peripheral neuropathic symptoms on questioning had abnormalities, but there was no

correlation with urinary symptoms.

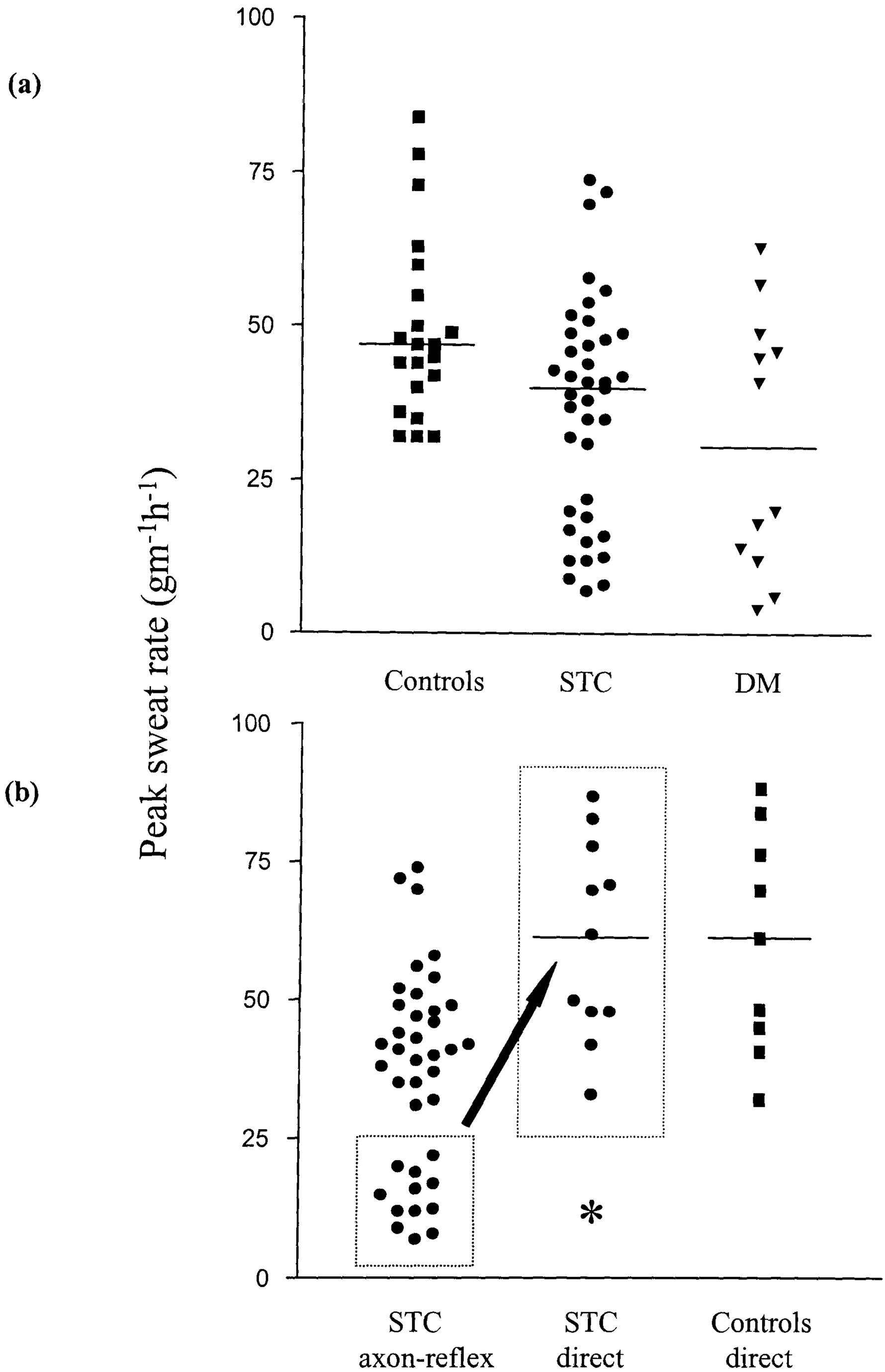


Figure 5.08: (a) Nicotine-induced axon-reflex sweating is significantly decreased in the STC patients vs. controls. STC patients, like the diabetics, appeared to fall into two subgroups, with a distinct subgroup (n = 12) lying below 2 SD of the control

mean. **(b)** On directly stimulating the sweat glands with methacholine, there was no difference between controls and STC patients. **KEY**: * - one patient refused a further injection, therefore n = 11 (*vs*. n = 12 for axon-reflex)

Of the 20 patients with decreased rectal sensation for distension, 11 had small fibre abnormalities compared with 5 of the 16 patients who had normal rectal sensation. There was however no association between peripheral neuronal dysfunction and rectal hyposensation by contingency calculation (Fisher's exact test, $p = 0.5$). Likewise, no association was observed between abnormal peripheral small fibre test results and anal sphincter function, radio-opaque marker retention, pattern of transit abnormality on isotope scintigraphy, defaecatory abnormalities on evacuation proctography or small intestinal manometry.

5.4 DISCUSSION

The results demonstrate that in a proportion of patients with STC there is evidence of autonomic and sensory dysfunction in the lower limbs. The decreased axon-reflex sweating indicates neural dysfunction, confirmed by the normal response of the gland itself when stimulated directly. The overlap with abnormal thermal thresholds and axon-reflex vasodilatation suggests a generalised small fibre neuropathy in some patients. Unlike diabetics, where abnormalities on neurological clinical examination or standard neurophysiological tests were present, these quantitative functional tests may be the only indicators of an underlying small fibre neuropathy in patients with STC, although the degree of abnormality was in general less than that for diabetics with gastrointestinal involvement.

Possible confounding variables

Age has been shown to effect both autonomic tests (Foster *et al*., 1976), and small fibre sensory function (Meh & Denislic, 1994). However, significant changes are normally only observed after the age of 70 years, and the oldest patient in this study was only 62

years of age. In addition, the group with abnormalities did not differ in age significantly from those without, or from controls. Whilst prolonged neurotoxic laxative use could theoretically influence the findings of this study, the lack of correlation between prolonged use and the presence of abnormalities does not support this hypothesis. Indeed, only one patient included in the study could have potentially used for a significant period an outdated laxative which may have been systemically neurotoxic e.g. podophyllin, and we have no evidence that this was the case. No other drug was consistently used by a significant proportion of patients in the study to account for neurotoxicity. A high proportion (7 / 8) of the patients undergoing surgery for constipation prior to the study had significant abnormalities on testing. There is similarly no clear explanation for such neural dysfunction arising as a sequel to abdominal surgery. Because of the nature of consent, which was performed by the author, it was not possible at the time to perform the tests in a blinded fashion, and this may have introduced some observer bias. However, towards the end of the study, some patients were referred before the results of their transit studies were known.

Pathophysiological implications

The implications of the findings of the study to the understanding of the aetiology of STC are not entirely clear. The results would support, like previous studies (Altomare *et al.*, 1992, Raethjen *et al.*, 1997) a common, possibly selective disease process of autonomic neurones, or of small sensory fibres in patients with STC. This could explain the observations of systemic (generalised intestinal) physiological abnormalities and extragastrointestinal e.g. bladder abnormalities in patients with STC (see introduction), supporting the view that the presence of abnormalities such as rectal hyposensation may represent a primary neuropathic disorder, rather than one occurring secondary to prolonged constipation (Kamm & Lennard-Jones, 1990). Abnormalities of rectal sensation were most marked for defaecatory desire and maximum tolerable volume, which may reflect dysfunction of small unmyelinated afferents in the rectum.

More problematic is the explanation of how dysfunction of certain peripheral fibre types could translate, were it to occur systemically, into a loss of propulsive activity in the

colon observed in STC. For instance, the consequence of sudomotor dysfunction, indicative of a postganglionic sympathetic impairment, would seem, at least on the basis of experimental studies of sympathetic nerve section or stimulation, to result in a disinhibition of colonic motility (see 1.5.4). Two explanations might explain this incongruence. Firstly, increased motility does not necessarily result in increased transit. Constipating drugs such as opioids increase motility while inhibiting propulsive activity (1.6.5), and abnormal hypermotility patterns have been reported to occur in the constipation predominant irritable bowel syndrome (Kumar & Wingate, 1985). Secondly, the decrease in sudomotor, and indeed small fibre sensory function may relate to alterations in the expression of certain neurotransmitters with a role in gut motility, rather than a loss of one specific functional subset of autonomic neurons. Sudomotor and visceral efferents share acetylcholine and vasoactive intestinal polypeptide (VIP) as neuroeffector agents, and visceral and somatic small fibre afferents the peptides substance P (SP), and calcitonin gene-related peptide (CGRP) which mediate axon-reflex vasodilatation (Goyal & Hirano, 1996). These nerve fibres and agents have been implicated in diabetic sensory and autonomic polyneuropathy, and are attributed to changes in specific neurotrophic factors (Anand *et al.*, 1996). The abnormal findings in the limbs in some STC patients may therefore indicate a common mechanism contributory to enteric nerve dysfunction, leading to this form of constipation. In support, whilst the high variability of the studies must be noted (see 1.5.3), decreased levels of gut SP (Goldin *et al.*, 1989; Tzavella *et al.*, 1996; Porter *et al.*, 1998) and VIP-immunoreactivity (Romanska *et al.*, 1996), and *in-vitro* stimulated ACh release (Burleigh, 1988) have been associated with STC. Cholinergic deficits, occurring systemically are indeed associated with constipation (Inamdar *et al.*, 1982; Vassallo *et al.*, 1991).

This study, whilst demonstrating that peripheral sympathetic cholinergic and small fibre sensory function abnormalities are present in some patients with STC, still does not prove that such deficits are the cause of constipation. Whilst it is unclear how this might be directly proven, quantitative studies of unmyelinated fibres in sural nerve biopsies would at least allow morphological proof of peripheral fibre deficits in these patients.

Furthermore, studies of neurotrophic factors and their receptors in affected colon and sural nerve, may help answer whether a common mechanism exists to explain changes in both the skin and gastrointestinal tract.

Familial and genetic implications

This study revealed a history of constipation in close family relatives in approximately 60% of STC patients. This is in accord with a previous study, which reported a family history in 55% of females with idiopathic constipation (Chaussade *et al.*, 1986), and is greater than published population prevalence ranges (2-34%) (Whitehead *et al.*, 1991). Whilst this finding may support genetic aetiology, social or dietary factors may also be responsible. This study also demonstrated four STC patients with one or more family relative with Hirschsprung's disease. A number of mutations have now been described in Hirschsprung's disease, including *RET* and its principle ligand, Glial cell-derived neurotrophic factor (GDNF). A potential role in the pathogenesis of STC for these factors is tested by screening of these patients for mutations in chapter 6.

6 SCREENING OF PATIENTS WITH IDIOPATHIC SLOW TRANSIT CONSTIPATION FOR MUTATIONS OF THE RET PROTO-ONCOGENE AND GDNF

6.1 INTRODUCTION

Chapter 1 introduced the hypothesis that STC patients with early-onset symptoms (chronic idiopathic STC), might, like some other gastrointestinal phenotypes which arise in infancy or childhood, be the result of a primary neuronal abnormality caused by mutations of genes that are known to be important during ontogeny of the ENS (Lyonnet *et al.*, 1993; Aurrichio *et al.*, 1996). Interest in this hypothesis was strengthened by the findings of the neurophysiological study, described in the last chapter, in which a history of constipation was observed in one or more family members in approximately 60% of patients studied, and 4 index patients with idiopathic STC had one or more relative with Hirschsprung's disease (HSCR). A history of constipation in close family relatives of patients with STC had been elicited in about half the patients included in a previous publication (Chaussade *et al.*, 1989), but the observation of a possible association between STC and HSCR was novel. The background to the genetics of HSCR relevant to this chapter is discussed below.

6.1.1 Hirschsprung's disease

Hirschsprung's disease (HSCR) is characterised by the congenital absence of ganglion cells of the enteric nerve plexus in all or part of the hind gut, and in a few, the midgut. It occurs in approximately 1 in 5000 live births in both sporadic (80%) and familial (20%) forms (Passarge, 1967). In familial cases, the pattern of inheritance may be autosomal dominant, recessive or polygenic (Badner *et al.*, 1990). Mutations of a number of genes have now been described for HSCR including those encoding the *RET* proto-oncogene (Lyonnet *et al.*, 1993; Angrist *et al.*, 1993; Edery *et al.*, 1994a; Romeo *et al.*, 1994), its

ligand glial cell line-derived neurotrophic factor (GDNF) (Angrist *et al.*, 1996; Saloman *et al.*, 1996), the G protein-coupled endothelin-B receptor (Puffenburger *et al.*, 1996), its ligand, endothelin 3 (Hofstra *et al.*, 1996), and more recently SOX10 (Pingault *et al.*, 1998) and neurturin (Doray *et al.*, 1998).

6.1.2 The *RET* proto-oncogene

The human *RET* proto-oncogene (*C-RET*) (**RE**arranged during **T**ransfection) was discovered as an oncogene activated by rearrangement with a foreign sequence in a fibroblast focus-forming assay (Takashi & Cooper 1987; Takahashi *et al.*, 1988). The gene has been mapped to human chromosome 10 at q11.2, and encodes a member of the receptor tyrosine kinase (RTK) family, RET protein. The key structural features of RET include extracellular cadherin-like and cysteine-rich domains, a transmembrane domain and an intracellular domain with tyrosine kinase function (Eng, 1999). As with other RTKs, RET spans the cellular membrane and is activated by the binding of certain ligands, including GDNF (see below) to novel glycosylphosphoinositol-linked extracellular proteins (Jing *et al.*, 1996, Durbec *et al.*, 1996a) which reside in association with RET on the cell membrane. These co-receptors have now been assigned the terms GFRα-1-4 (Trupp *et al.*, 1998; Thompson *et al.*, 1998). The result is the formation of a multimeric complex between the activated GFR-α receptor and RET, with subsequent autophosphorylation, and phosphorylation of other intracellular substrates (Figure 6.01). The RET gene comprises at least 20 exons (Pasini *et al.*, 1995a), and undergoes alternative 5' and 3' splices predicted to result in up to 10 different protein isoforms (Kwok *et al.*, 1993; Lorenzo *et al.*, 1995).

RET is expressed in tissues of neural crest origin, and in tumours of neural crest derivatives such as medullary thyroid carcinoma (MTC) and phaeochromocytoma. *RET* plays a crucial role in kidney morphogenesis and in the survival and differentiation of several sub-populations of neurones in the peripheral and central nervous systems (Schuchardt *et al.*, 1994; Durbec *et al.*, 1996a; Sanchez *et al.*, 1996). It has now been recognised as the susceptibility locus for several inherited disorders of neural crest

development including: multiple endocrine neoplasia type 2 (MEN-2) (Mulligan *et al.*, 1993; Hofstra *et al.*, 1994) and HSCR (Lyonnet *et al.*, 1993; Angrist *et al.*, 1993; Edery *et al.*, 1994a; Hofstra *et al.*, 1994; Romeo *et al.*, 1994). A large number of germline mutations have been identified for both conditions, the majority of which are distinct to each disease, and have also been demonstrated somatically in phaeochromocytomas, and MTC *(see recent reviews)* (Eng & Mulligan, 1997; Eng, 1999).

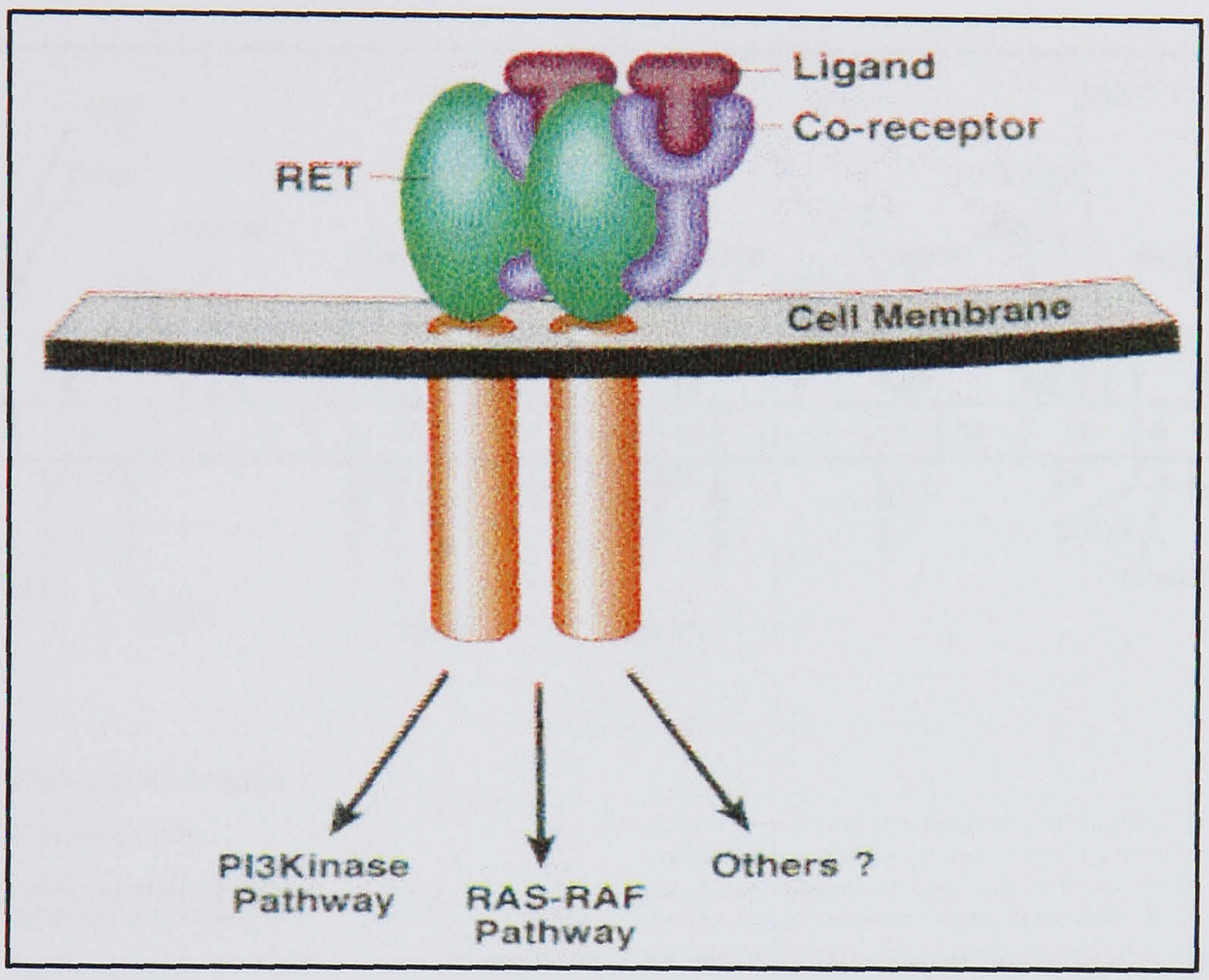


Figure 6.01: The RET receptor tyrosine kinase is positioned in the cell membrane. It is activated when its ligand binds a co-receptor and the complex in turn interacts with RET. Reproduced with permission: Eng C. RET proto-oncogene in the development of human cancer. *J Clin Oncol* 1999; **17**: 381.

Mutations of *RET* have been recognised in both familial autosomal dominant, and sporadic HSCR cases (Lyonnet *et al.*, 1993, Angrist *et al.*, 1993). Whereas rare microscopic and submicroscopic deletions of chromosome 10q11.2 result in the loss of a single *RET* allele (Luo *et al.*, 1993), the majority of cases result from one of a range of point mutations scattered throughout the extracellular domain and intracellular tyrosine kinase domain (Edery *et al.*, 1994a, Romeo *et al.*, 1994; Angrist *et al.*, 1995).

Frameshift and nonsense mutations are predicted to result in RET protein truncations, but missense point mutations also occur throughout the gene (see Figure 6.02). Recent evidence suggests that *RET* mutations may lead to severe impairment of RET tyrosine kinase activity (Pasini *et al.*, 1995b) by a number of intracellular mechanisms, including failure of transport of the RET protein to the cell membrane, or lack of its maturation (Iwashita *et al.*, 1996; Carlomagno *et al.*, 1996; Ito *et al.*, 1997).

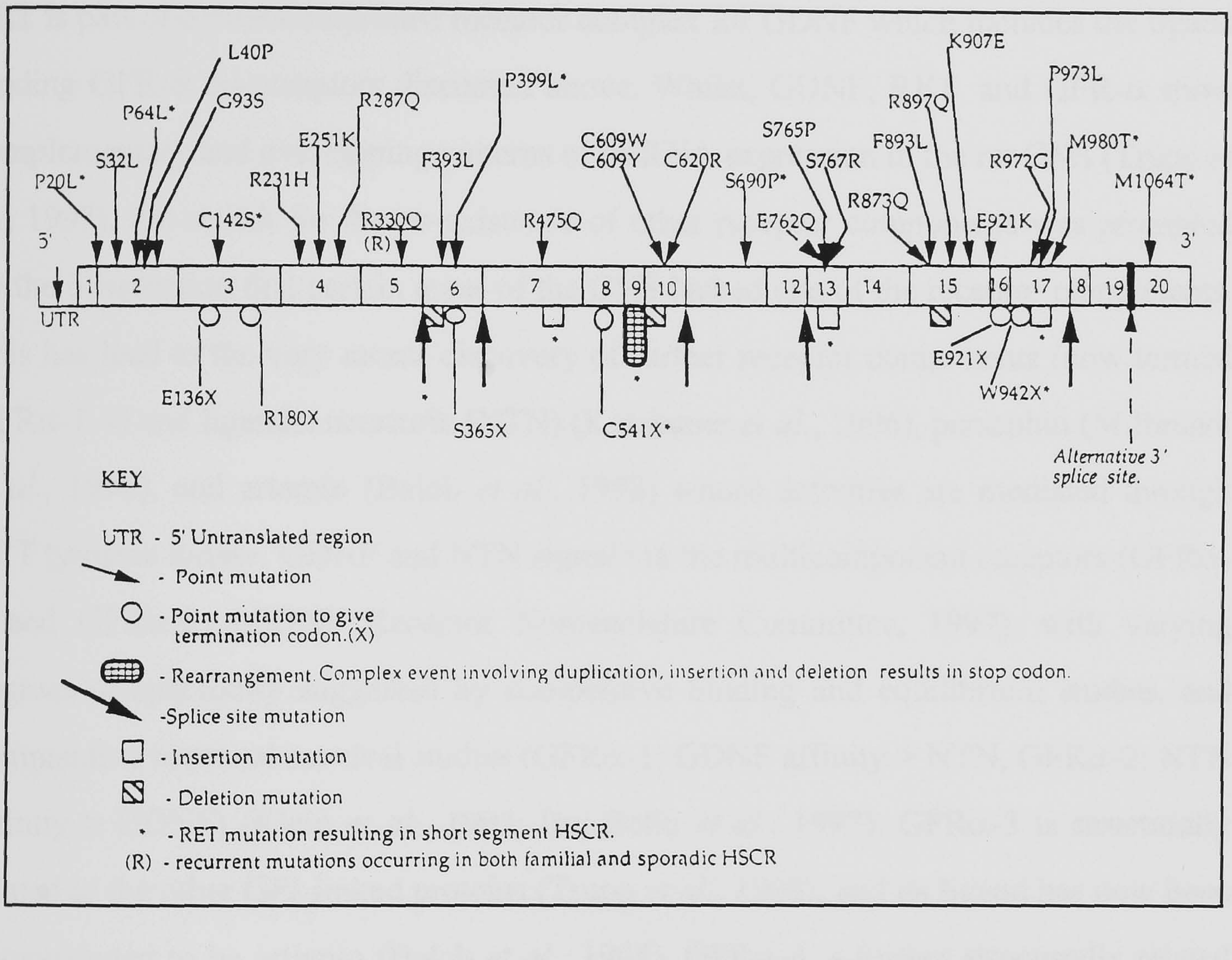


Figure 6.02. Whereas rare microscopic and submicroscopic deletions of chromosome 10q11.2 result in the loss of a single *RET* allele, the majority of cases result from one of a range of point mutations scattered throughout the regions encoding the extracellular domain and intracellular tyrosine kinase domain of RET. Reproduced with permission: Eng *et al. Hum Mutat* 1997; **9**: 97-109.

6.1.3 Glial cell line-derived Neurotrophic Factor (GDNF)

A major step toward understanding the biological actions of RET was the identification

of one of its ligands, GDNF (Trupp *et al.*, 1995). GDNF is a polypeptide, structurally related to members of the transforming growth factor-β (TGF-β) superfamily (Attisano *et al.*, 1994). GDNF was purified from the conditioned medium of a glial cell line on the basis of its ability to stimulate dopamine uptake in primary cultures of embryonic rat ventral midbrain neurons (Lin *et al.*, 1993). GDNF promotes the survival and phenotype of several populations of peripheral and central neurons, including sympathetic, sensory and motor neurones (Henderson *et al.*, 1994; Trupp *et al.*, 1995; Buj-Bello *et al.*, 1995). RET is part of a multicomponent receptor complex for GDNF which includes the ligand binding GFR-α co-receptors discussed above. Whilst, GDNF, RET, and GFR-α show complementary and overlapping patterns of m-RNA expression in the rat CNS (Trupp *et al.*, 1997), the search for the co-existence of other receptor components was prompted by the observation that certain areas of the CNS lacked one of the receptor components. This has lead to the very recent discovery of further receptor components (now termed GFRα-1-4) and ligands: neurturin (NTN) (Kotzbauer *et al.*, 1996), persephin (Milbrandt *et al.*, 1998), and artemin (Baloh *et al.*, 1998) whose activities are mediated through RET tyrosine kinase. GDNF and NTN signal via the multicomponent receptors (GFRα-1 and GFRα-2) (GDNF Receptor Nomenclature Committee, 1997), with varying degrees of specificity suggested by competitive binding and equilibrium studies, and comparative neuronal survival studies (GFRα-1: GDNF affinity > NTN, GFRα-2: NTN affinity > GDNF) (Klein *et al.*, 1997; Buj-Bello *et al.*, 1997). GFRα-3 is structurally related to the other GPI-linked proteins (Trupp *et al.*, 1998), and its ligand has now been demonstrated to be artemin (Baloh *et al.*, 1998). GFRα-4, a further structurally related protein (Thompson *et al.*, 1998) is now known to be the receptor for the ligand persephin (Enokido *et al.*, 1998). The gene for GDNF has been mapped to human chromosome 5 at p12-p13.1 (Schindelhauer *et al.*, 1995), has 2 exons, and gives rise to two alternatively-spliced forms that code for prepropeptides (Springer *et al.*, 1995).

6.1.4 RET / GDNF and chronic idiopathic constipation

Analysis of the *RET* gene in HSCR by Edery *et al.* (Edery *et al.*, 1994b) identified germline mutations in three of nine subjects with severe constipation starting in

childhood. These were relatives of patients with proven HSCR in the study, and 8 of the 9 were female. Whilst some of these had radiological evidence of megacolon, no physiological or histopathological data was available. Similarly, a further study screening for *GDNF* mutations in HSCR, demonstrated a single kindred with mutations of both *GDNF* and *RET* in which some female family members had "chronic constipation starting in childhood" (Saloman *et al.*, 1996) but again, physiological or histological features were not discussed. Considerable diversity of phenotypes, even cosegregating to one allelic alteration has been demonstrated with *RET* (Mulligan *et al.*, 1994; Amiel *et al.*, 1998) and *GDNF* mutations (Amiel *et al.*, 1998), and patients with one such phenotype, MEN-2B, commonly first present with severe constipation. It is therefore possible that STC could represent a further diverse phenotype i.e. a variant of HSCR, possibly as a result of modifying gene activity.

6.1.5 Aims

The aim of this study was to test the hypothesis that *RET* / *GDNF* mutations are responsible for the functional abnormalities observed in STC. This was tested in 4 families in which idiopathic STC was associated with HSCR, and a series of patients with the fully characterised chronic idiopathic slow transit phenotype and with a family history of constipation in one or more relative, but without a family history of HSCR. The selection of these 2 genes rather than others known to be involved in the pathogenesis of HSCR was based on the evidence above, and on the grounds that *RET* remains the commonest and best accepted gene implicated in HSCR. In addition, the methodology for performing studies of these genes was available in the UK at the time of the study (Cancer Research Council, Human Cancer Genetics Group, University of Cambridge).

6.2 MUTATION SCREENING: BACKGROUND TO METHODOLOGY

A number of methods have now been developed to faciliatate DNA mutation screening. Regardless of the exact methods utilised, there are a number of laboratory-based steps

common to this process. DNA must be extracted and purified (6.2.1), amplified to produce adequate amounts for subsequent use (section 6.2.2 – polymerase chain reaction), and then mutations detected by the various available methods (6.2.3).

6.2.1 PREPARATION OF DNA

A number of techniques can be used for the extraction and purification of DNA including phenol extraction, caesium chloride density purification and salt precipitation procedures. The latter are generally more popular because of the low toxicity of the reagents used (Miller *et al.*, 1988). There are a number of common steps to salt precipitation techniques: white cell isolation and lysis, deproteination, RNAase treatment, DNA recovery (salt and alcohol to precipitate DNA, centrifuge to collect), and finally DNA rehydration. Some modern techniques use pre-prepared reagents in kits, and have high yields with acceptable purity. Following DNA preparation, the purity and yield can be assessed using optical density measurements (spectrophotometry).

6.2.2 THE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is still a relatively new technique. In the short time since its invention it has revolutionised many aspects of molecular biology, in particular the analysis of small specific sequences for single base pair alterations. PCR provides a simplified alternative to scoring restriction fragment length polymorphisms, and is less laborious and time consuming than standard Southern blotting techniques.

PCR is an *in vitro* technique designed to isolate and amplify small, specific segments of DNA between 10^5 and 10^8 fold from insignificant quantities of template (less than 1μg) in a short space of time (Saiki *et al.*, 1985). In principal, two synthetic oligonucleotide primers are designed, which are typically 20-25 nucleotides long, and flank the DNA segment to be amplified. These primers, orientated 5' to 3', are hybridised to opposite strands of the target sequence and extended using DNA polymerase until the region between the two primers is completely replicated. Initial hybridisation of the two oligonucleotides requires heat

denaturation of the double-stranded DNA template. The temperature is then lowered to an optimum at which primers anneal to their complementary sequences. Finally, polymerase elongation, again requiring an optimal temperature, completes the synthesis which effectively results in doubling the concentration of the target DNA segment. This cycle of events involving denaturation followed by annealing followed by elongation is repeated for 25-35 rounds to obtain an enriched concentrate of specific DNA. Since it was initially reported, advances in PCR technology have greatly increased its efficiency while generally simplifying and speeding up the technique. Initially, the DNA polymerase used was derived from *Esherichia coli*, and was thermally unstable at the high temperatures used in the denaturation step, requiring it to be replaced during each round of amplification (Saiki *et al.*, 1985). In contrast the thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus* (*Taq*) was found to have a higher optimum temperature (70-75°C) giving it greater specificity and yield, and also retained its activity after heat denaturation (Saiki *et al.*, 1988). Commercially available thermal cyclers specifically designed for PCR and with the ability to accurately control the temperatures required for each stage of the PCR reaction, have simplified and improved the speed and efficiency of the technique as a whole.

6.2.3 DETECTION OF SINGLE BASE CHANGES IN DNA

The ability to detect single base changes in genomic DNA is of fundamental importance to the study of genetic diseases by identifying causal mutations. In addition, the identification of DNA polymorphisms provide invaluable genetic markers for linkage studies either in the localisation of particular genes or diagnostically through determining the segregation of genetic diseases within families (Gusella *et al.*, 1982). Where target sequences are known, Southern analysis using radioactively labelled allele-specific oligonucleotide probes may be used to test for the presence or absence of a mutation by its ability or inability to hybridise to the target sequence, and has been used in clinical diagnostic testing (Shuber *et al.*, 1993).

However, circumstances often require the detection of mutations when the precise nature of the alteration is not known. Direct DNA sequencing may be used to scan a gene for unknown mutations but is laborious, costly and very time consuming with present

technology. Restriction fragment length polymorphism (RFLP) analysis using Southern blotting with probe hybridisation can detect mutations which alter cutting sites of restriction enzymes, and in its own right has been employed successfully at the level of routine diagnosis (Chang and Kan, 1982; Orkin *et al.,* 1982). However, palindromic sequences of the type recognised by restriction enzymes are uncommon and the chances of detecting single base mutations on this basis are consequently low. For these reasons further methods have been developed for the detection of subtle genetic alterations with a high degree of speed and efficiency. Single-strand conformation polymorphism analysis is an example of these methods and can detect the presence or absence of a mutation, whilst relying subsequently on direct sequencing to characterise the change.

6.2.3.1 Single-strand conformation polymorphism analysis (SSCP)

Single-strand conformation polymorphism analysis (SSCP) was first developed by Orita *et al.* (Orita *et al.*, 1989). The basic principle on which SSCP relies is that under non-denaturing conditions single-stranded DNA molecules take on a folded secondary structure which is stabilised by weak intramolecular interactions. In general these interactions are base pairing hydrogen bonds and so, as a consequence, two strands differing by only a single base will have different conformations which potentially manifest themselves in their electrophoretic mobility (Orita *et al.*, 1989).

After heat denaturation with formamide to retain a single-stranded state, DNA fragments are electrophoresed through a non-denaturing polyacrylamide gel matrix. The two complementary strands will usually resolve as two SSCP conformers of distinct mobility, although under various conditions a single strand can adopt more than one conformation. A mutation resulting in heterozygosity will be characterised by the presence of extra bands in addition to those produced by the normal homozygote, base-pair changes being identified as variants among DNAs of a sample set. Radioactively labelled DNA fragments generated by PCR are visualised after autoradiography (Sheffield *et al.,* 1993), or alternatively non-radioisotopic methods for detection, such as silver staining, may be used (Ainsworth *et al.,* 1990).

After denaturation to generate single-stranded DNA a certain amount of reannealing inevitably occurs leading to heteroduplex formation between mutant and wild-type fragments. When electrophoresed through a non-denaturing polyacrylamide gel these heteroduplex mismatches can been seen to run significantly slower than either mutant or wild-type homoduplexes. This feature has favourably increased the informativeness of the SSCP technique and is particularly useful for detecting small insertions and deletions e.g. Familial Adenomatous Polyposis (Cottrell *et al.*, 1992). The estimated efficiency of SSCP appears to vary between studies but the general indications are that between 75 and 90% of single base mutations are detectable in DNA fragments ranging from 100-450bp, with an optimal size of approximately 200bp in length (Sheffield *et al.,* 1993; Condie *et al.,* 1993). Various parameters can be modified to increase the sensitivity of the technique, in particular the running conditions (e.g. temperature during electrophoresis; concentration of running buffer) and gel content (Orita *et al.,* 1989). SSCP has been used to detect mutations in a number of human genes involved in disease including *RET* (Groden *et al.*, 1993; Ceccherini *et al.*, 1994; Gayther *et al.*, 1995).

6.2.3.2 Direct DNA sequencing

DNA sequencing can be performed using chemical or enzymatic methods. Both methods involve four base-specific reactions that remain incomplete, yielding a heterogeneous population of molecules that end in one of the four nucleotides in each of four different reactions. The enzymatic sequencing method as first described by Sanger (Sanger *et al.*, 1977) has now essentially replaced the chemical method (Maxam & Gilbert, 1977), and uses dideoxynucleotides (ddNTPs) which differ from normal deoxynucleotides (dNTPs) in that they cannot be extended by DNA polymerase. These populations of molecules are then resolved on a denaturing polyacrylamide gel. Modern enzymatic sequencing uses a thermal-stable DNA polymerase with thermal cycling to allow single tube reactions (one per sample), very small amounts of DNA template, and analysis of large constructs. The enzyme and fluorescent dye-labelled terminators are included in a single premix (kit) to prepare samples for automated sequencing. The

fluorescent dye-labelled terminator ddNTPs, when incorporated, allow laser-detection of bands on the gel, and computer led data acquisition and analysis. Modern enzymes compared with the original DNA polymerases have reduced discrimination for ddNTPS, and can use much lower initial levels of dye-labelled terminators, leading to reduced final levels of unincorporated dye-labelled terminators in each reaction.

6.3 MATERIALS AND METHODS

6.3.1 PATIENTS

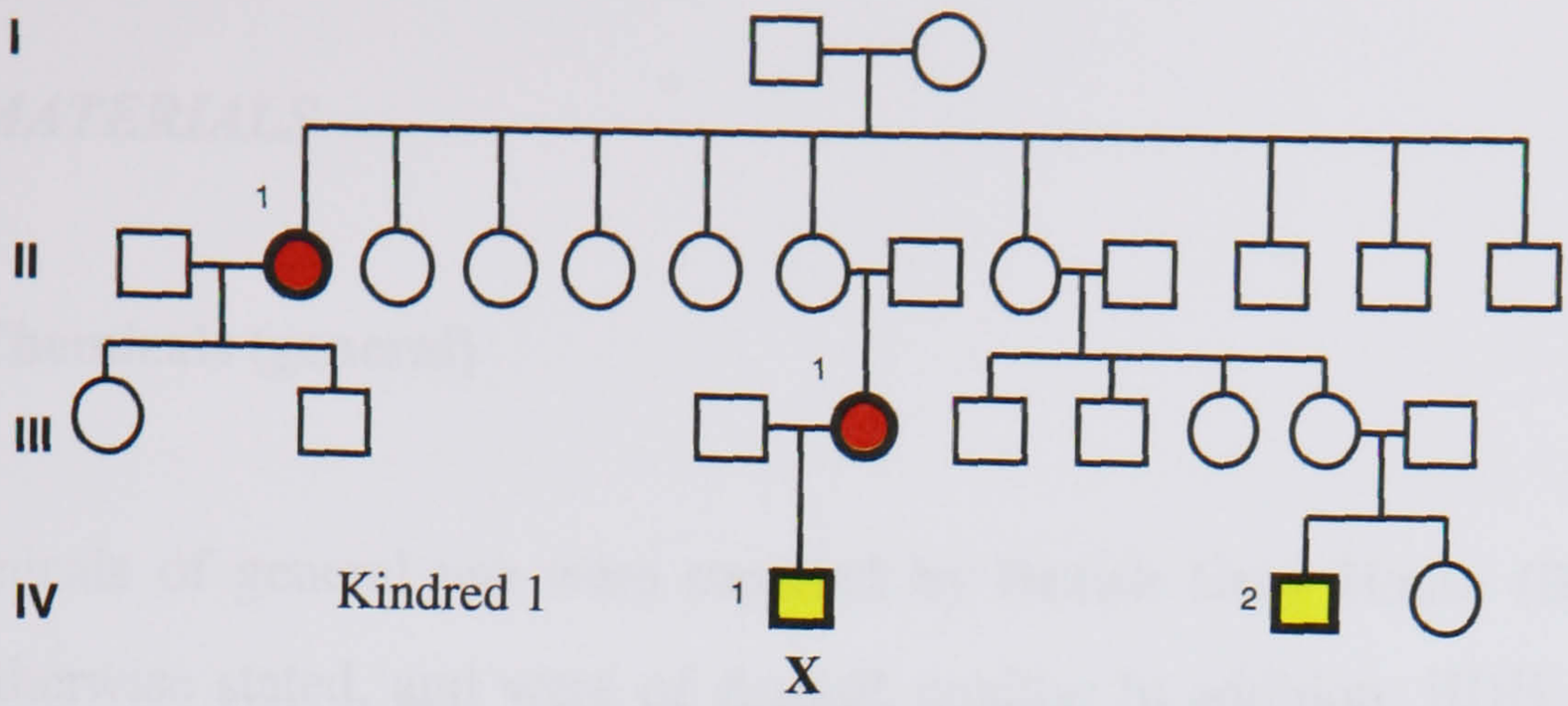
We screened 4 STC patients and affected relatives with Hirschsprung's disease from 4 families with both conditions (8 individuals in total), and 12 STC patients with a family history of constipation in one or more relative, including one patient with an affected identical twin, but with no family history of Hirschsprung's disease. The family trees of the patients with HSCR relatives are shown in figure 6.03.

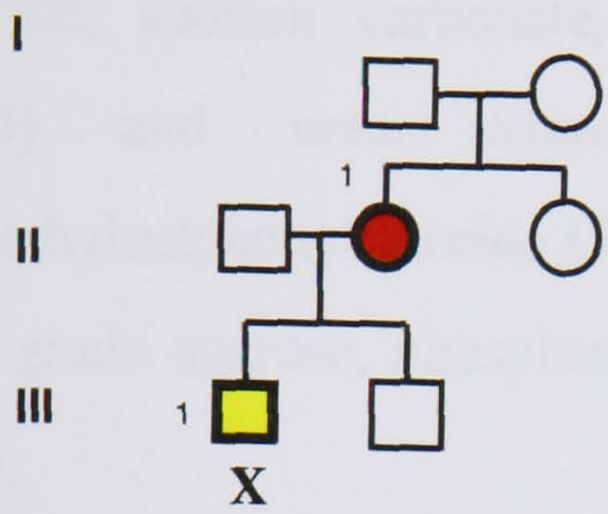
STC patients (clinical and physiological features)

The 16 cases studied were all female, median age 40 years, range 26-63. Chronic idiopathic STC was defined by criteria used in chapters 2 and 3. Notably, no patient had radiological evidence of idiopathic megabowel, and all had an intact rectoanal inhibitory reflex. Six patients had undergone segmental or subtotal colonic resection for the condition. No patient had evidence of aganglionosis or other neuronal abnormality on routine histological examination of tissue from resection or from rectal biopsy.

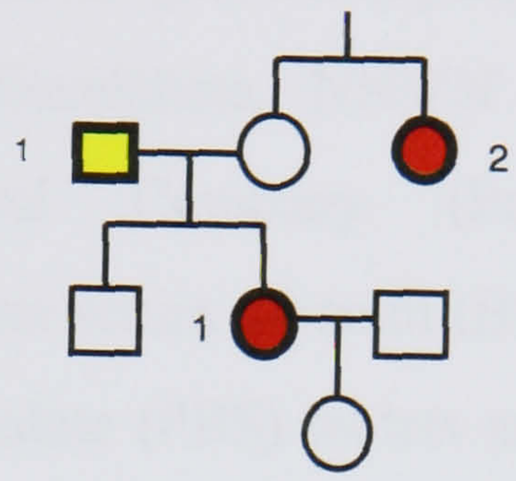
HSCR cases

Material from 4 / 6 relatives of STC cases with HSCR, from 3 of the 4 families was available for analysis (see Figure 6.03). The final family HSCR relative was unavailable for venepuncture (emigrant to Australia). Patients studied included 3 with short segment disease, and one with total colonic aganglionosis and sensorineural deafness, but no evidence of pigmentary abnormalities. All cases had previously undergone a pull through procedure, and had HSCR based on histological examination of affected gut.

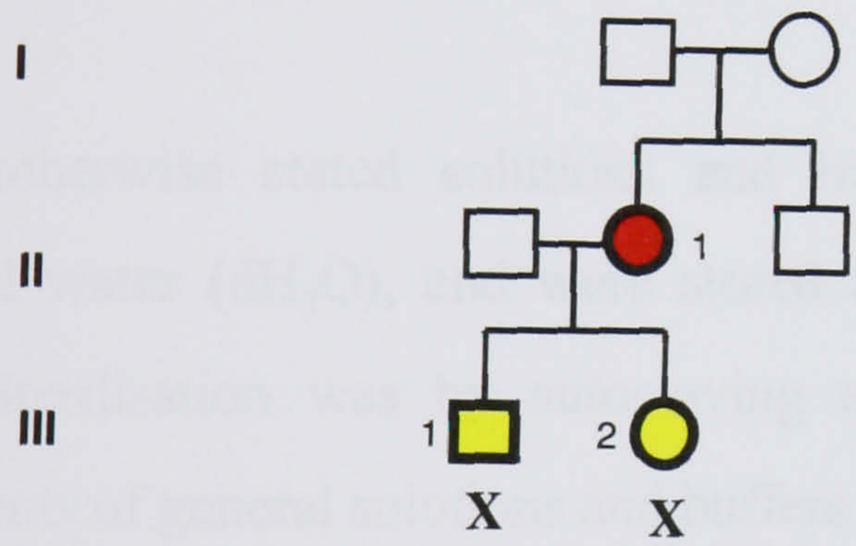




Kindred 2



Kindred 3



Kindred 4

KEY = HSCR

= STC

X = DNA screened

Figure 6.03. Familial association of slow transit constipation (STC) with Hirschsprung's disease (HSCR) in four families.

kindred 1	II.1	STC, onset in childhood, worsenened by childbirth
	III.1	Idiopathic constipation
	IV.1	Anorectal HSCR
	IV.2	Anorectal HSCR
kindred 2	II.1	STC from teenage
	III.1	Total colonic aganglionosis and sensorineural deafness
kindred 3	I.1	Anorectal HSCR
	I.2	Intractable constipation since childhood
	II.1	STC from childhood, worsened by childbirth
kindred 4	II.1	STC from childhood
	III.1	Anorectal HSCR
	III.2	Anorectal HSCR

6.3.2 MATERIALS

6.3.2.1 Chemicals (general)

All chemicals of general use were supplied by British Drug House (BDH, Poole, UK) unless otherwise stated, and were of AnalaR quality. In addition, BDH also supplied the specific reagents: trichloroacetic acid, glycerine, gluteraldehyde, silver nitrate, sodium borohydride, sodium carbonate, sodium thiosulphate, NNN'N' tetramethylenediamide (TEMED) and urea. Sigma Chemical Company (Poole, UK) supplied phenylmethylsulphonylfluoride (PMSF), bovine serum albumin (BSA), ethidium bromide, standard grade agarose, phosphate buffered saline (PBS) tablets and dimethyl sulphoxide (DMSO).

6.3.2.2 Solutions and buffers

Unless otherwise stated solutions and buffers were prepared using distilled and deionised water (dH_2O), and were stored at room temperature (i.e. between 15 and 25°C). Sterilisation was by autoclaving at 15lbs psi 121°C for 30 minutes. The components of general solutions and buffers are presented in appendix 6.01.

6.3.3 METHODS: MUTATION ANALYSIS

6.3.3.1 DNA isolation and purification

Peripheral venous blood samples (10ml) were taken from a single antecubital fossa venepuncture, and stored in heparin ETDA containing vacutainer (Becton Dickinson & Co., NJ, USA) bottles at - 30°C until use. Genomic DNA was isolated using a salt precipitation kit (Puregene, Gentra systems Inc., USA) (Appendix 6.02). Following rehydration, yield was assessed using spectrophotometry (compared to 100ng/ml calf thymus DNA standard) (Appendix 6.03), and samples were further diluted to give a final DNA concentration of approximately 50ng/ml.

6.3.3.2. DNA amplification

Oligonucleotides.

Primer sequences were initially taken from Ceccherini *et al.* (Ceccherini *et al.*, 1995), an erratum to previously published material (Ceccherini *et al.*, 1994). Oligonucleotide primers were purchased from Operon Technologies Inc, Alameda, USA / MWG-biotech, Milton Keynes UK, and were supplied lyophilized with the molar yield stated. Primers were diluted in dH_2O to 20μM concentrations. Melting temperatures of the oligonucleotides had been calculated using the following formula, which takes into account the G and C content of the primer:

$$T_m = 4(G+C)\ 2(A+T)$$

However, most primers were initially assayed between 52° and 66°C to assess their optimal annealing temperature.

Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were prepared under sterile conditions using sterile Gilson pipette tips to minimise DNA contamination. The 20 exons of the *RET* gene, and 2 exons of *GDNF* were amplified in 30μl reactions containing DNA (25-100 ng), 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5-2.0 mM $MgCl_2$, 200μM dNTPs and 0.06 μl Red hot Taq polymerase (Advanced Biotechnologies Ltd., UK) with 0.006 μM (3μl of 20mM) of each appropriate primer. PCR reactions were performed in a Hybaid Omnigene thermal-cycler (Hybaid Ltd., Ashford, UK). Thirty-five amplification cycles were performed with a preceding touchdown protocol (Appendix 6.04) in 96 well plates with foil heat-sealed covers. PCR primers and annealing temperatures are shown in appendix 6.05.

Agarose gel electrophoresis

Amplified DNA was size fractionated by agarose gel electrophoresis to confirm the optimal annealing temperatures of oligonucleotides. Gels were prepared to a concentration of 1.5% by heat dissolving (microwave oven) 7.5g of agarose in 500ml of

1 x TBE. Molten agarose was premixed with ethidium bromide to a concentration of 1mg/ml, and poured into midi-gel moulds (Scotlab, Coatbridge, UK) with 20-well gel-slot-formers and left to set at room temperature. Prior to electrophoresis, 10µl PCR products were combined with a one-tenth volume of loading buffer and loaded into well-slots submerged under running buffer (1 x TBE). Electrophoresis was performed at 90V for approximately 1 hour. Estimation of the size of fractionated DNA was achieved by similarly electrophoresing a 1kb ladder (Advanced Biotechnologies Ltd., UK). Ethidium bromide stained gels were viewed under ultra violet trans-illumination.

6.3.3.3 SSCP/HA methods

DNA preparation

Reaction products (5µl) were diluted 1:1 with a denaturing buffer (formamide 95% with 0.025% xylene cyanol and 0.025% bromophenol blue), heated to 99°C for 10min and quenched on ice, prior to loading onto 0.8% MDE gels (see below).

Gel preparation and electrophoresis

The SSCP gel matrix used was MDE (Mutation Detection Enhancement matrix), an acrylamide substitute, and was run at 2 gel conditions: with and without 10% glycerol. The composition of each of these gels is shown in appendix 6.06. Gels were poured between glass plates measuring 20cm x 20 cm x 1 mm with 25-well gel-slot-formers. Electrophoresis was performed using a Bio-Rad Protean II gel system (Bio-Rad Laboratories, CA, USA) and 4 gels could be run per apparatus. This allowed a total of 96 samples to be analysed with 4 ladders per apparatus. Gels were run at 200 / 400V for 12-18 hours at 10°C and stained with silver nitrate (Appendix 6.07) (Gayther *et al.*, 1995).

6.3.3.4 Direct DNA sequencing

Samples

Samples that showed a variant by SSCP and / or heteroduplex analysis at one or more

condition were sequenced to characterise the nucleotide change. In addition, 10 patients with HSCR, MEN type 2A or Familial Medullary Thyroid Cancer in which mutations of exons 10 or 11 of RET had previously been identified were used as controls. In 10 of the 20 cases (including all HSCR / STC families) and all controls, the entire RET (except for exon 1) and *GDNF* coding sequences including intron-exon boundaries were sequenced in entirety. It was not possible to amplify exon 1 consistently, so as a result this exon was not screened for mutations, however all sites of mutations (Figure 6.02) (Eng, 1997), and polymorphisms (Table 6.01) (Ceccherini *et al.*, 1995, Eng, 1997) that have been previously reported in HSCR were sequenced. [Sequencing of exon 1: six attempts were made, and included the use of additional published, and computer-designed primers. These difficulties, principally as a result of the high G-C content of this exon, are well recognised (Charis Eng, Harvard, personal correspondence)]

Table 6.01 Sequence polymorphisms in the RET proto-oncogene

Exon No	***Codon No***	***Polymorphism***	***Amino acid alteration***	***Published frequency***	***Observed frequency***
2	45	GCG to GCA	Ala	0.29	0.25
3	125	GTC to GTA	Val	0.02	0.0
7	432	GCG to GCA	Ala	0.71	0.56
11	691	GGT to AGT	Gly to Ser	0.21	0.17
13	769	CTT to CTG	Leu	0.26	0.27
15	899	GTT to TTT	Val	0.79	0.48
17	975	AAC to AAT	Asn	NK	0.0
18	982	CGC to TGC	Arg to Cys	NK	0.0

Methods

In all cases, both strands were sequenced using the fluorescent dideoxy terminator method, using the TAQ SS sequencing kit (Perkin-Elmer, Alameda, USA). Sequence

reactions were performed in 20µl reactions containing 8µl of supplied terminator ready mix, 4µl DNA (PCR product), 1.6µl of diluted (2µM) oligonucleotide and 6.4µl H_2O, and using a Hybaid thermal cycler (25 cycles of 96°C for 30 secs, 50°C for 15 secs and 60°C for 4 mins).

Sequencing reaction products were then rehydrated, precipitated and washed as per protocol (Appendix 6.08). 5µl of loading dye was added (Blue dextran / formamide (1:5), and the samples denatured at 90°C for 2 mins, and placed on wet ice before loading 2µl of each sample into the gel. Polyacrylamide sequencing gels were poured between glass plates measuring 50cm x 30 cm x 0.5 mm with 36-well gel-slot-formers. The steps in gel preparation are listed in appendix 6.09.

Fluorometric sequences were analysed using the Applied Biosystems 373 automated DNA sequencer®, according to the manufacturer's protocol (Perkin-Elmer, Alameda, USA). Additional primer sequences were used for sequencing for exons 2 and 4 of *RET*, and exon 1 of *GDNF* (Appendix 6.05).

6.4 RESULTS

6.4.1 SSCP / HA

In patients, single strand mobility variants were seen on analysis of exons 2, 13, 15, and 17 of *RET* under one or both gel conditions. Nine out of ten positive controls demonstrated a clear mobility shift of the single or double stranded DNA conformation for the mutant exon under both conditions (Figure 6.04 a). No variants in *GDNF* were detected.

6.4.2 DIRECT DNA SEQUENCING

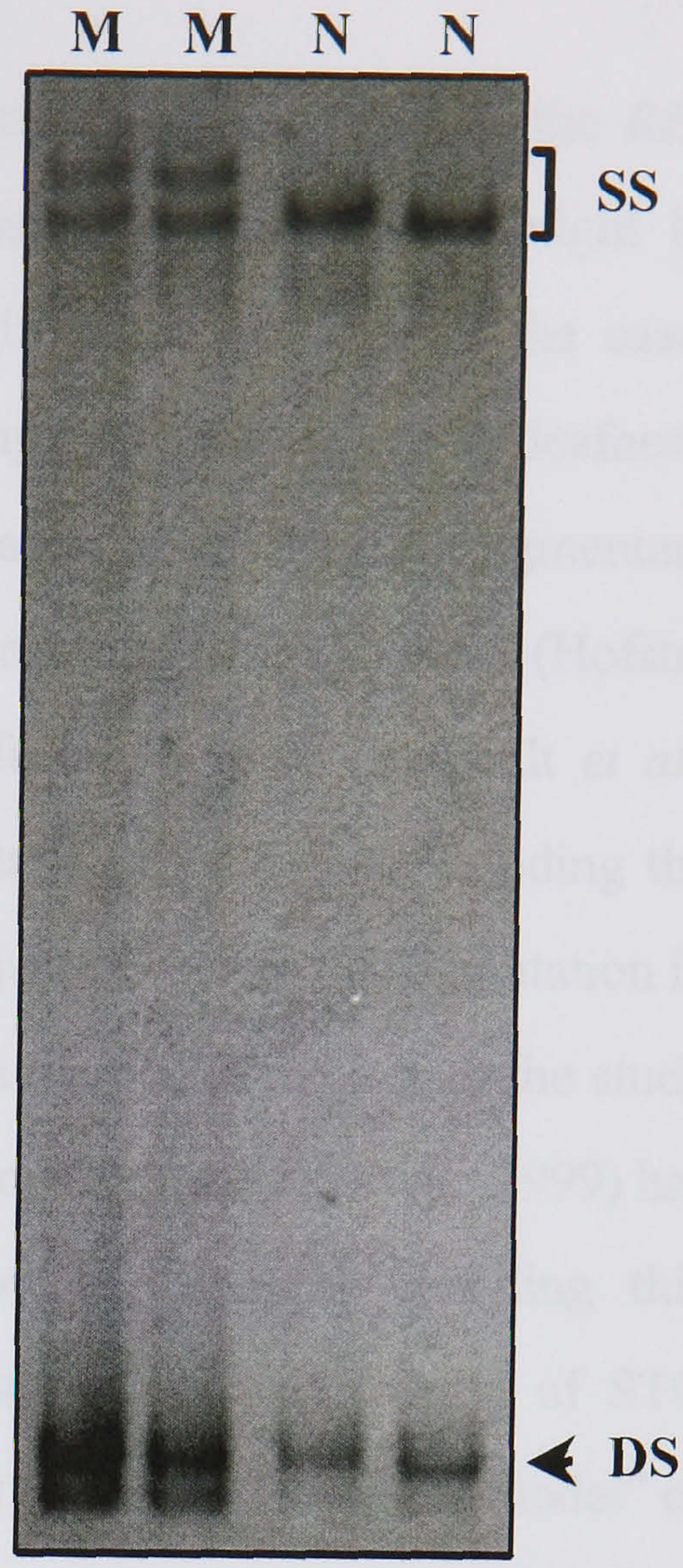
Published sequence polymorphisms were demonstrated for exon 2 (GCG-GCA, Ala 45), exon 7 (GCG-GCA, Ala 432), exon 11 (GGT-AGT, Gly 691- Ser), exon 13, CTT-CTG,

Leu 769), and exon 15 (TCC-TCG, Ser 899), exon 17 (AAC-AAT, Asn 975). The rarer exon 3 and 18 polymorphisms were not found. The allelic frequencies and PIC (polymorphism information content) values of these polymorphisms did not differ significantly from previously reported frequencies (Ceccherini *et al.*, 1995) (Table 6.01). No published or new mutation was seen in any of the exons of *RET* or *GDNF*. The predisposing MEN-2A, FMTC or HSCR mutations (missense changes in one of 4 highly conserved Cys residues in *RET* exon 10 at codons 609, 611, 618 or 620) were detected by sequence alterations in all 10 positive control subjects (Figure 6.04 b).

6.5 DISCUSSION

The study failed to demonstrate germline mutations or rare polymorphisms of *RET* or *GDNF* in any of the HSCR or ISTC cases studied. The validity of the methodology was demonstrated by the study of positive control samples (sensitivity of SSCP/HA and direct DNA sequencing: 90 / 100% respectively), and by detection of common polymorphic nucleotide changes with a comparable frequency to published data (Ceccherini *et al.*, 1995). It can be concluded that in the subjects studied, mutations of *GDNF* or *RET* are not the cause of either the chronic idiopathic STC or HSCR phenotype. The finding of a normal frequency of common polymorphisms, and absence of rare sequence polymorphisms suggests that coding region variants of *RET* or *GDNF* were also unlikely to have caused these conditions by a modifier effect on other genetic loci. Although exon 1 was not successfully sequenced, no mutation has been previously reported in this exon in any of the conditions associated with *RET*. Germline mutations of *RET* or *GDNF* therefore appear to have no role in the aetiology of the patients with chronic idiopathic STC that were included in this study. However, because the relatives with HSCR in the families studied also had no mutations, we cannot comment on whether such mutations might have a role in the aetiology of STC in other families containing mutation-positive HSCR patients. In addition, in view of the relatively low number of subjects studied overall, we can only conclude that such mutations are unlikely to be a common cause of STC.

Figure 6.04



(a) SSCP / Heteroduplex analysis of RET exon 10 PCR products on 0.8% MDE gel: 2 / 4 samples with known mutations in exon 10 demonstrate clear mobility variance.

(**Key:** M = mutant, N = normal, SS = single strand, DS = double strand)

(b) Direct DNA sequencing. The positive control sample shows a missense change in the highly conserved Cys residues in RET exon 10 at codon 618: (TGC to GGC), Cys to Gly. KEY: M = mutant, N = normal

This study only screened 2 genes implicated in the pathogenesis of HSCR: the *RET* proto-oncogene and *GDNF*. It therefore remains possible that mutations might be present in other (unstudied) HSCR candidate genes. In particular, one of the cases screened in this study had HSCR associated with congenital sensorineural deafness. This case, whilst lacking other features of Shah-Waardenburg disease (pigmentary abnormalities), may implicate involvement of the gene encoding Endothelin-3 (Hofstra *et al.*, 1996; Edery *et al.*, 1996) or the transcription factor, SOX10 (Pingault *et al.*, 1998). Furthermore, since the time of this study, a mutation of the gene encoding the RET receptor ligand neurturin has been identified in conjunction with a *RET* mutation in one large kindred of HSCR (Doray *et al.*, 1998). This is of special interest to the study of patients with STC, because subsequent, very recent work (Heukeroth *et al.*, 1999) has shown that transgenic mice with a targeted deletion of the gene encoding this neurotrophin have an almost identical clinical and tissue phenotype to that of STC (Chapter 1). Such mice, which actually represent the first animal "aetiological model" of constipation also had parasympathetic deficits, whose possible importance in STC has similarly been discussed in chapter 1.

The role of the other ligands, persephin (Milbrandt *et al.*, 1998), and artemin (Baloh *et al.*, 1998), as well as the family of RET coreceptors (GFR-α1-4) (Thompson *et al.*, 1998) in enteric neurogenesis is not known, although GFR-α1 mutations were not found by the recent screening of a large panel of HSCR cases (Angrist *et al.*, 1998). Alternatively, STC and other "adult" functional bowel diseases may arise as a result of alterations in the local tissue expression of GDNF / RET, or other neurotrophic factors and their receptor components. Many such factors, their receptors, and associated neuropoietic cytokines are known to have an important role in the pre-natal development of the ENS (Durbec *et al.*; 1996b, Gershon *et al.*, 1997; Chalonizitis *et al.*, 1998), and have been shown to be expressed in post-natal gut (Hoehner *et al.*, 1996; Bär *et al.*, 1997; Wartiovaara *et al.*, 1998) where their role is not known. Future studies are required to address these hypotheses, and as such, enhance the understanding of ontogeny of lifelong motility disorders at genetic, molecular and cellular levels.

7 DETECTION OF AUTOANTIBODIES TO EXPRESSED NEURONAL CHANNELS IN SLOW TRANSIT CONSTIPATION

7.1 INTRODUCTION

7.11 GENERAL: CHANNELOPATHIES

Disorders of ion channels (channelopathies) are increasingly being identified and represent a rapidly expanding area of neurology, with a diverse range of neurological diseases having been found to be thus caused (Rose, 1998 [review]).

Ion channel dysfunction would be an attractive hypothesis for the causation of STC because:

1. The motility of the gut is dependent on nerve-muscle interaction (1.4), and the function of nerves and muscle is clearly dependent on ion channel function. Studies of colonic contractile activity in STC suggest neuronal or myopathic dysfunction (1.5.1). Channel dysfunction could be consistent with either the relative paucity of specific morphological findings in STC, especially on routine histological examination (1.5.3), or with the low grade ganglionitis accompanied by intraepithelial lymphocytosis, which has been observed in one recent study (Lindberg *et al.*, 1999).

2. Channelopathies can result from inherited genetic mutations of genes encoding channel components (Browne *et al.*, 1994; Ophoff *et al.*, 1996) or from autoantibody-mediated channel effects (Lang & Vincent, 1996). For instance, congenital myokymia may result from mutation of genes encoding voltage-gated potassium channel (VGKC) components (Browne *et al.*, 1995), and from

antibodies to VGKCs in the acquired form (Shilito *et al.*, 1995; Hart *et al.*, 1997). The different mode and time of onset of STC within defined clinical sub-groups (Chapter 3) is consistent with the concept of distinct aetiological strategies, convergent on a singular clinical phenotype. In particular, a major sub-group of patients with STC present in later life, some of whom have no obvious trigger for their complaint (Waldron *et al.*, 1988).

7.1.2 AUTOIMMUNE CHANNELOPATHIES

Antibody-mediated autoimmunity underlies a diverse range of disorders, particularly in the nervous system where the extracellular domains of ion channels and receptors are especially vulnerable targets. Autoimmunity to ion channels can occur as a paraneoplastic effect or in the absence of neoplasia. Antibodies have been demonstrated to ion channels in several nervous system disorders (Table 7.01)

The following indirect evidence exists for an autoimmune aetiology in conditions of gut dysmotility:

1. Visceral involvement occurs in a proportion of patients with known autoimmune diseases e.g. connective tissue disorders (Sjogren, 1994), and can occur in the absence of other manifestations e.g. scleroderma (Rodnan & Fennell, 1961; Nojima *et al.*, 1996). In such patients with predominantly gut involvement, serum antibodies have been demonstrated to neurone-specific RNA/DNA-binding proteins (Nojima *et al.*, 1996), and antibodies to myenteric neurofilaments (Howe *et al.*, 1997) have been shown to cause alterations of intestinal myoelectric activity on murine passive transfer experiments (Eaker *et al.*, 1999). Slow colonic transit is an accepted finding in some patients with scleroderma (Basilisco *et al.*, 1993).

Table: 7.01: Main autoimmune channelopathies

Clinical condition	*Autoantibody*	*References*
The Lambert-Eaton myasthenic syndrome *(LEMS)* (Lambert *et al.*, 1956)	anti-voltage-gated calcium channel antibodies (anti-VGCC)	Lang *et al.*, 1981; Prior *et al.*, 1985; Roberts *et al.*, 1985; Lennon *et al.*, 1995; Meriney *et al.*, 1996
Neuromyotonia (Isaacs' syndrome) (Isaacs, 1961)	anti-voltage-gated potassium channel antibodies (anti-VGKC)	Sinha *et al.*, 1991; Newsom- Davis and Mills, 1993; Shilito *et al.*, 1995; Hart *et al.*, 1997
Myasthenia gravis (MG)	muscle acetylcholine receptor, ion channel receptor antibodies (Anti-AChR: α1 subunit)	Vincent & Newsom-Davis, 1985; Vincent, 1994
Subacute idiopathic or secondary autonomic neuropathy (Suarez *et al.*, 1994)	ganglionic acetylcholine receptor ion channel receptor antibodies (Anti-AChR: α3 subunit)	Vernino *et al.*, 1998

2. Antibodies directed to non-channel neuronal antigens have been described in paraneoplastic disorders of the ENS (Lennon *et al.*, 1991a, Condom *et al.*, 1993). Intestinal pseudo-obstruction has long been recognised as a paraneoplastic accompaniment of small cell carcinoma of the lung (SCLC) (Lhermitte *et al.*, 1980, Schuffler at al, 1983), but the association of antibodies with pseudoobstruction (Lennon *et al.*, 1991a), and a broader spectrum of dysmotility disorders (Lennon *et al.*, 1991b) was recognised for the first time in 1991. The

finding of antineuronal nuclear antibodies (ANNA-1 also known as "anti-Hu") and pseudo-obstruction was confirmed in 1993 (Condom *et al.*, 1993). Severe gut dysmotility is in fact the presenting complaint of 12% of patients with SCLC paraneoplasia, and can focally affect any level of the gut (Lucchinetti *et al.*, 1998). Anti-neuronal antibodies have also been detected in association with other neoplasms (Schobinger-Clément *et al.*, 1999; Gerl *et al.*, 1992) and in small numbers of patients with severe gut dysmotility without neoplasia (Smith *et al.*, 1997).

3. Autonomic symptoms are found in some patients with LEMS (Rubenstein *et al.*, 1979), and anti-VGCC autoantibodies have been reported in patients with autonomic dysfunction and LEMS (O'Suilleabhain *et al.*, 1998). Experimentally, LEMS sera can impair Ca^{2+} channel-dependent components of mouse postganglionic sympathetic neurotransmission (Waterman *et al.*, 1997). Recently, antibodies to the neuronal nicotinic acetylcholine (ACh) receptor (AChR), which is structurally related to the motor endplate AChR affected in MG (Lindstrom, 1996), have been described in patients with idiopathic subacute autonomic neuropathy i.e. without neoplasia (Vernino *et al.*, 1998), and gastrointestinal dysmotility was a prominent and initial symptom in approximately one third of patients (Vernino *et al.*, 1998). Gastrointestinal symptoms, including severe constipation occur in approximately 75% of patients with idiopathic autonomic neuropathy (Suarez *et al.*, 1994), and conversely, autonomic abnormalities have been reported in STC and other conditions of severe gut dysmotility in previous studies (Camilleri & Fealey, 1990; Altomare *et al.*, 1992; Camilleri *et al.*, 1993; Bharucha *et al.*, 1993; Raethjen *et al.*, 1997), and this thesis (Chapter 5).

7.1.3 BACKGROUND TO METHODS

A full discussion of the topological and recently elucidated crystallographic structure (Doyle *et al.*, 1998) of ion channels and channel-neurotoxin interactions is beyond the scope of this thesis. It is sufficient to write that there is vast, and ever increasing spectrum of different channels which have different ion conductances, are gated by

different mechanisms, are found in different tissues, and which subserve different functions. Such pharmacological and functional diversity has a molecular basis; channel structure being dependent on the assembly of several protein sub-units which themselves are individually expressed by families of genes (Pongs, 1993; Wang *et al.*, 1993; Rettig *et al.*, 1994; Lindstrom, 1996).

Over the past 30 years, a number of specific neurotoxins from a variety of vertebrate and invertebrate species have been discovered and structurally characterised. In accord with their own amino acid sequence (Gasparini *et al.*, 1998), such toxins have differential affinities to channel types and subtypes. These toxins have been an invaluable tool in the pharmacological classification of ion channels (Llinas *et al.*, 1989; Moczydlowski *et al.*, 1988; Castle *et al.*, 1989). In autoimmune neurological disease, the detection of circulating antibodies has largely depended on the availability of such target-specific neurotoxins e.g. ∂-bungarotoxin in MG (Vincent & Newsom-Davis, 1985), which are used in immunoprecipitation assays. The principle of immunoprecipitation assays is shown in figure 7.01.

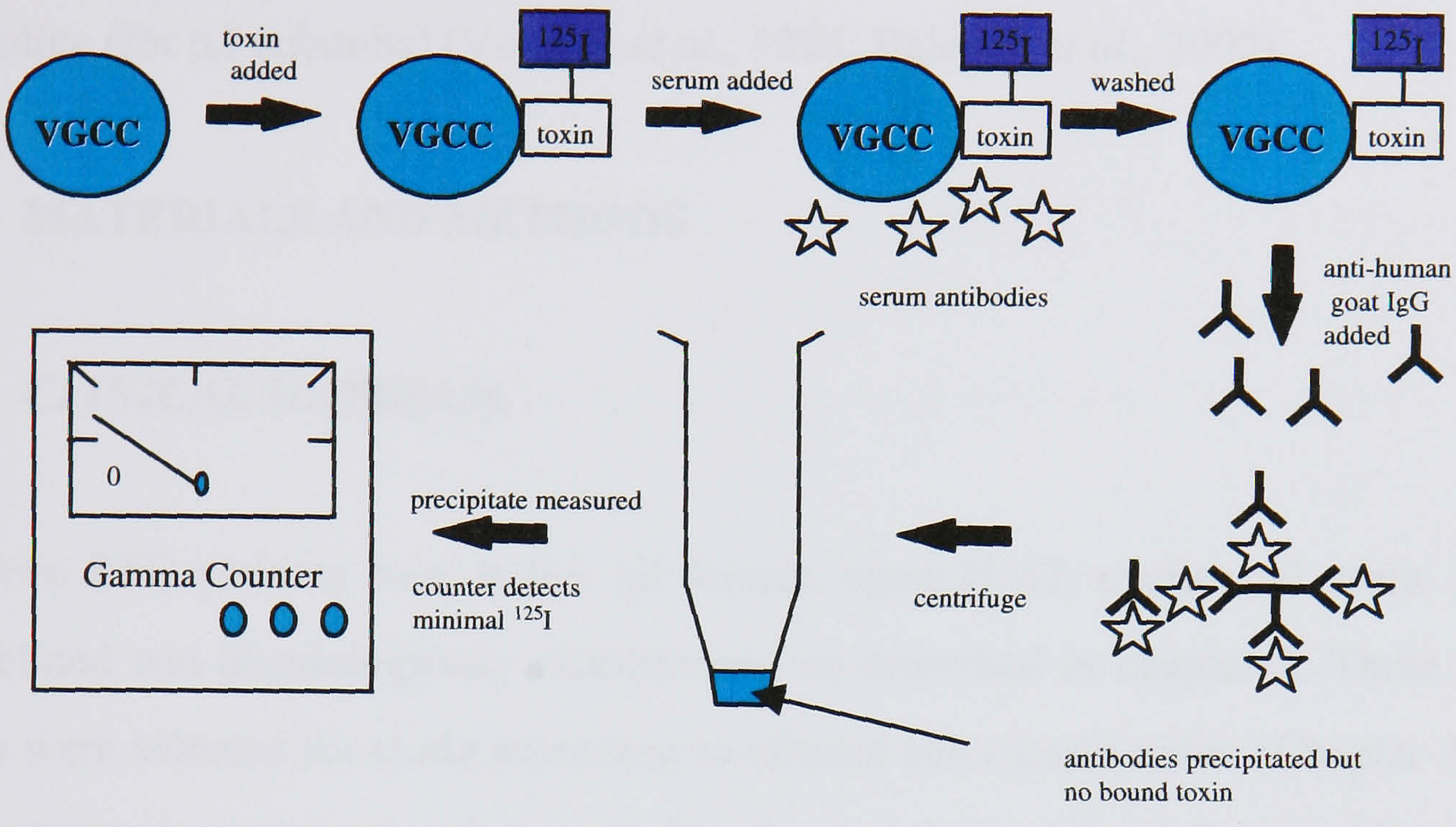
7.1.4 AIMS

In this study we used previously validated immunoprecipitation assays to:

1. Detect antibodies to neuronal *Shaker*-related voltage-gated potassium channels (VGKCs) using radio-labelled α-dendrotoxin, a neurotoxin from the elapid (eastern green mamba) snake (Dolly *et al.*, 1984; Castle *et al.*, 1989; Hart *et al.*, 1997).
2. Detect antibodies to voltage-gated calcium channels (VGCCs) using radio-labelled ω-conotoxins (MVIIC & GVIA), neurotoxins from the cone snail (Llinas *et al.*, 1989), which bind to P/Q-type and N-type VGCCs respectively (Lennon *et al.*, 1995; Motomura *et al.*, 1995; Motomura *et al.*, 1997).
3. Detect antibodies to muscle-specific AChR ion channel α1 subunits using the well-characterised toxin derived from the Taiwan banded krait snake, ∂-

bungarotoxin. This assay has been in use for more than 20 years and is similar to that originally described by Lindstrom *et al*. (Lindstrom *et al*., 1976; Vincent & Newsom-Davis, 1985).

(a) immunoprecipitation assay (1)



(b) immunoprecipitation assay (2)

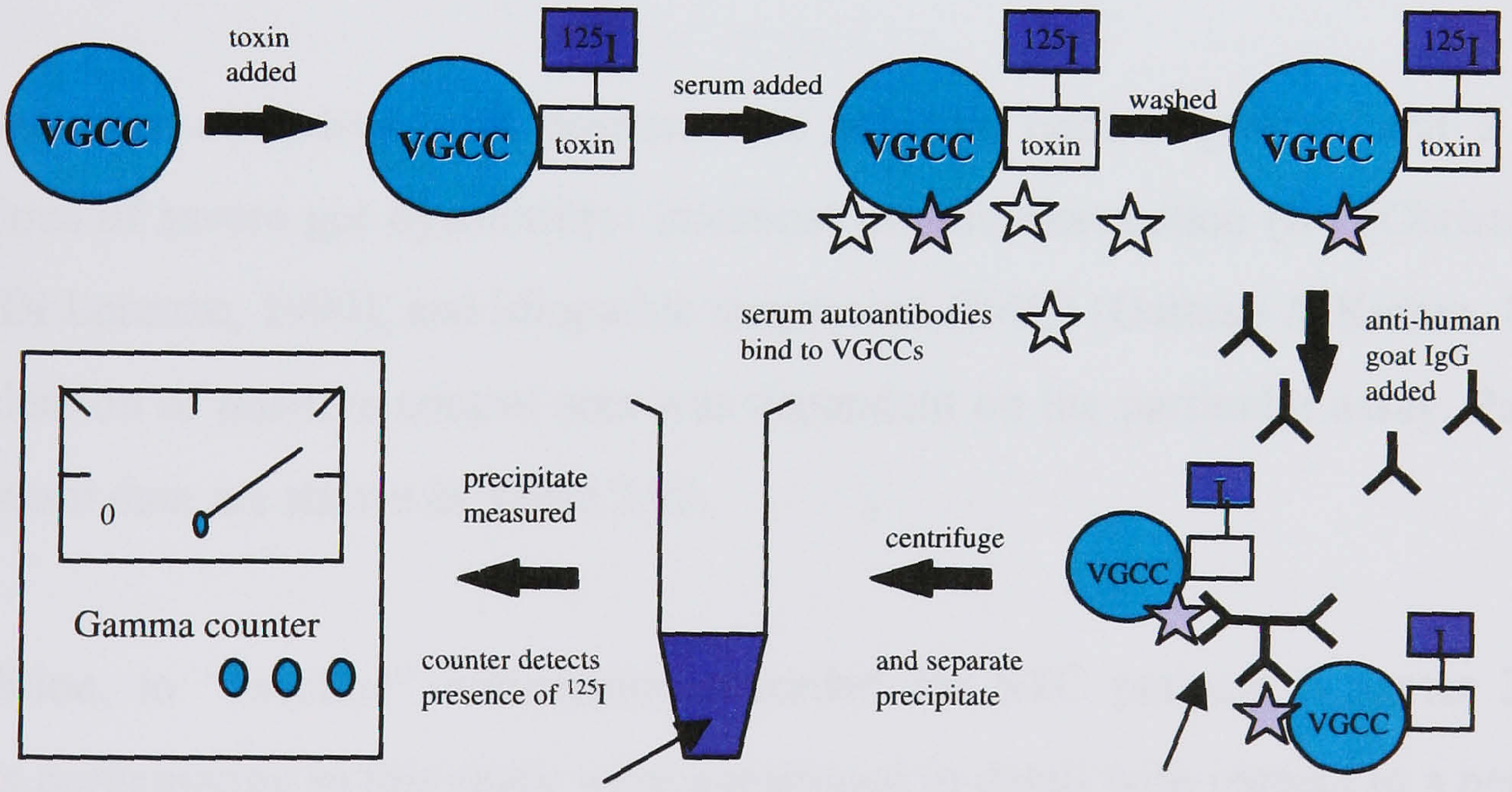


Fig 7.01: The diagrams show schematically the principle behind radio-immunoprecipitation assays. Radiolabelled toxin bound to extracted channel preparation is precipitated by anti-human IgG in the presence of antibody (**b**), but remains in solution when autoantibodies are absent (**a**).

Additional samples of sera were sent to C.Gotti, CNR Cellular and Molecular Pharmacology Center, Dept. of Pharmacology, University of Milan, Italy for immunoprecipitation assays to detect autoantibodies to specific neuronal subunits of AChR ion channels using either α-bungarotoxin (for α7 subunits) (Orr-Urtrreger *et al.*, 1997; Balestra *et al.*, 2000), or the toxin derived from the Equadorian poison frog, epibatidine (for α3 subunits) (Vernino *et al.*, 1998; Balestra *et al.*, 2000).

7.2 MATERIALS AND METHODS

7.2.1 CLINICAL MATERIAL

Forty-two STC patients were tested, all female, aged 25-62, median 40 years. STC was defined and physiologically characterised as described in chapter 2. Three sub-groups were selected for study according to clinical sub-classification (Chapter 3). A deliberate emphasis was placed on including those patients with adult-onset idiopathic STC (AOIST).

Findings were compared with positive and negative control groups, and 2 other conditions of severe gut dysmotility: intestinal pseudo-obstruction (IP) (Christensen, 1990; Di Lorenzo, 1999), and idiopathic megacolon (IMC) (Gattuso & Kamm, 1997). The selection of positive control sera was dependent on the particular assay. Patients and control data are shown in Table 7.02.

In addition, to "baseline" information recorded for STC patients (Chapter 2), all patients participating in this study were questioned in detail with respect to a personal or family history, or symptoms suggestive of autoimmune, neurological or neoplastic disease. These data and the main demographic data for test-groups are shown in appendix 7.01. No STC patient had MG or thymoma, lung carcinoma, SCLC or haemoptysis, or clinical evidence of myokymia or pseudomyotonia.

Table 7.02: Patients included in immunoprecipitation studies

Study group	*Description*	*N*
Chronic idiopathic STC (CIST)	*de-novo* before the age of 20 years (< 5 years, n = 4; < 10 years, n = 15; > 10 years, n = 4)	22
Post pelvic intervention STC (PIST)	temporally related to childbirth (n = 5) or pelvic surgery (n = 5)	10
Adult-onset idiopathic STC (AOIST)	onset *de-novo* after the age of 20 years	10
Total STC		***42***
Healthy subjects	negative controls	6
Neuromyotonia	positive controls: KGKC assay	8
LEMS	positive controls: KGCC assay	4
MG	positive controls: AChR-α1 subunit assay	6
Intestinal pseudo-obstruction	(comparison group)	10
Idiopathic megacolon	(comparison group)	4
Total (all included)	***VGCC assays***	***66***
	VGKC assay	***70***
	AChR-α1 subunit assay	***68***

7.2.2 ANTI-CHANNEL ANTIBODY IMMUNOPRECIPITATION ASSAYS

Twenty millilitres of venous whole blood was taken by antecubital fossa venepuncture and centrifuged for 10 min at 3,500rpm. Serum was removed and stored at -70°C in Eppendorf aliquots (≅ 4ml / patient) until use. Assay reagents and buffers are listed in appendices 7.02 and 7.03 respectively.

All assays were performed independently by 2 or more study participants who were blinded to the underlying clinical diagnosis by numerical coding of the sera. Positive sera were re-tested a further 1–2 times (i.e. 3-4 times in total).

7.2.2.1 Preparation of channel extracts

Preparation of voltage-gated channel extracts (VGCs)

Rabbit brain was used for the preparation of VGKC and VGCC channels (see appendix 7.04 for methods). [Results have previously been shown to be comparable using human and rabbit cerebellar extracts as an antigenic source for potassium (unpublished data), and calcium channels (Motomura *et al.*, 1997)] For use in the assay, the membrane preparation was diluted 1:5 in 2% digitonin solubilisation buffer, incubated and centrifuged. The supernatants were removed, and used as the source of VGCs in the assay (see appendix 7.05).

AChR α1 subunit channel extracts

Previous studies required muscle membranes for the preparation of AChRs, using muscle obtained fresh from human amputated limbs. AChRs can now produced by recombinant techniques, and were purchased from RSR Ltd., Cardiff, UK. Receptor extracts generated from 2 commercial *in-vitro* cell lines were mixed in equal volumes and diluted in bovine serum (additive) before toxin labelling and subsequent use in the assay.

7.2.2.2. Immunoprecipitation assays

Detailed methods are shown in the appendices:

1. Sera were tested for antibodies to rabbit brain P/Q and N-type VGCCs using immunoprecipitation of VGCCs extracted from rabbit cerebellum and labelled with ^{125}I-labelled-ω-conotoxin MVIIC (^{125}I-ω-CmTx) or ^{125}I-labelled-ω-conotoxin GVIA (^{125}I-ω-CgTx) (Appendix 7.06).

2. Sera were tested for antibodies to rabbit brain VGKCs using immunoprecipitation of VGKCs extracted from rabbit cerebellum and labelled with ^{125}I-labelled-α-dendrotoxin (^{125}I-α-DnTx) (Appendix 7.07).
3. Sera were tested for antibodies to muscle ACh receptors using immunoprecipitation of human recombinant AChRs labelled with ^{125}I-alpha-bungarotoxin (^{125}I-∂-BuTx) (Appendix 7.08).

In brief, defrosted aliquots of extract were labelled with a saturating (5fmol) concentration of radiolabelled toxin and incubated with diluted sera overnight at 4°C. Excess sheep anti-human IgG serum was then added to precipitate serum antibodies and incubated for 1 hour at 4°C. The precipitates were pelleted, washed and counted. Results were expressed as picomoles of labelled toxin binding sites precipitated per litre of serum after subtraction of non-specific binding (calculated from control mean) (Appendix 7.09 for calculations).

7.2.3 NEUROPHYSIOLOGICAL ASSESSMENT

Patients with anti-VGKC antibodies, detected by immunoprecipitation underwent detailed neurophysiological assessment to exclude neuromyotonia. All studies were performed by a consultant neurophysiologist (Dr David Ingram, Department of Neurophysiology, St Bartholomew's Hospital, London).

7.2.4 STATISTICS

Agreements between repeated assays were expressed as the mean and 95% CI of the differences, with the Wilcoxon signed rank test used to test the effectiveness of pairing. (proportional agreements by log transformation of data were also calculated). The final result was calculated as the mean of the results from the 2 independently performed assays (3-4 assays for positive results). Antibody titres were considered positive if more than the mean plus 3 S.D. for the control sera. For groups, data were expressed as medians with ranges. Contingencies for the distribution of positive sera

to multiple groups were calculated using the chi-square test.

7.3 RESULTS

7.3.1 ANTI-CHANNEL ANTIBODY IMMUNOPRECIPITATION ASSAYS

Voltage-gated calcium channels (VGCCs)

Assays performed twice independently showed reasonable agreement between results: P/Q-type: mean difference between assays = -26 pM (95% CI – 9 to – 60) (proportional agreement 41-52%), p = 0.0003, N-type: mean difference between assays = + 15 pM (95% CI + 9 to + 50) (proportional agreement 123-157%), p = 0.001. All positive and negative control sera were positive or negative respectively in both assays. All positive controls (LEMS) had strongly positive sera in the P/Q-type VGCC assay, and 50% were also positive in the N-type VGCC assay. In the VGCC P/Q-type assay, 4 / 10 patients with IP showed some precipitation of the complex (whole group median = 31, range –60 to 142pM). Although these fell outside the mean + 3 S.D. of the control sera (> 60 pM of ω-CmTx binding sites), they were not of an order of magnitude which was comparable to the LEMS positive control sera (median 866, range 267 – 967pM) (Figure 7.03a). No STC or IMC patient had a positive serum in this assay, and the contingency of such a distribution of 4 positives to this one group was highly significant ($p < 0.0001$, chi-square test). In the N-type assay, one patient with STC showed a consistently strong precipitation of the labelled complex in a range comparable to LEMS positive controls (whole STC group: median 7, range –58 to 325pM). [LEMS median: 120, range -30 to 475pM; negative control mean: 0, range –9 to 31; mean plus 3 S.D. = 60pM] (Figure 7.03b). This patient is discussed below ('patient 1'). A further 3 STC patients had borderline results.

Voltage-gated potassium channels (VGKCs)

The 2 assays performed independently showed very high agreement of results: mean difference between assays = + 16 pM (95% CI + 6 to + 38), (proportional agreement 106 – 138%), $p < 0.0001$.

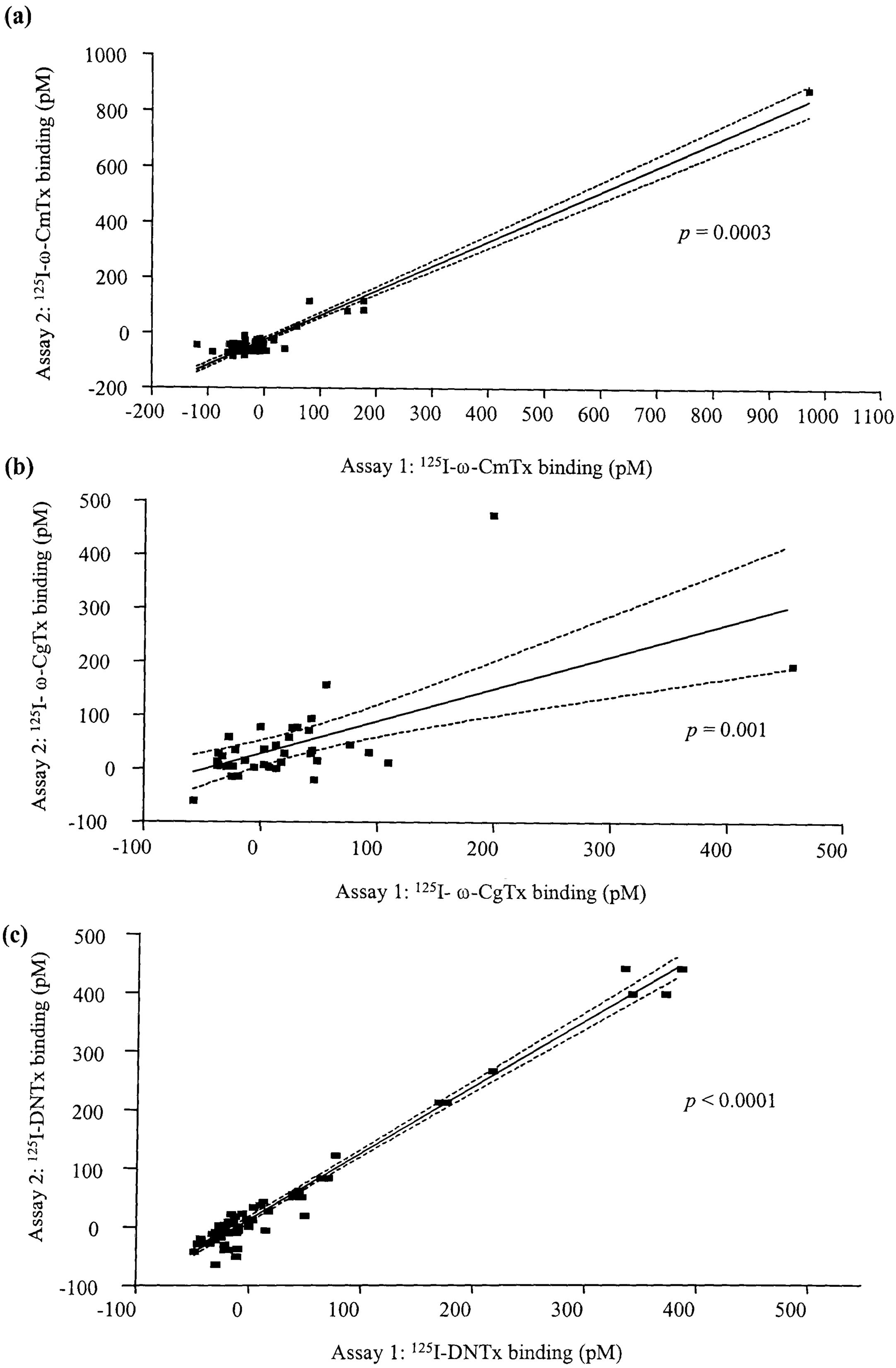


Fig 7.02: Correlations between independently repeated assays. **(a)** VGCC: P/Q-type, **(b)** VGCC:N-type, and **(c)** VGKC (see text). Effectiveness of pairing is shown by *p*-values (Wilcoxon test).

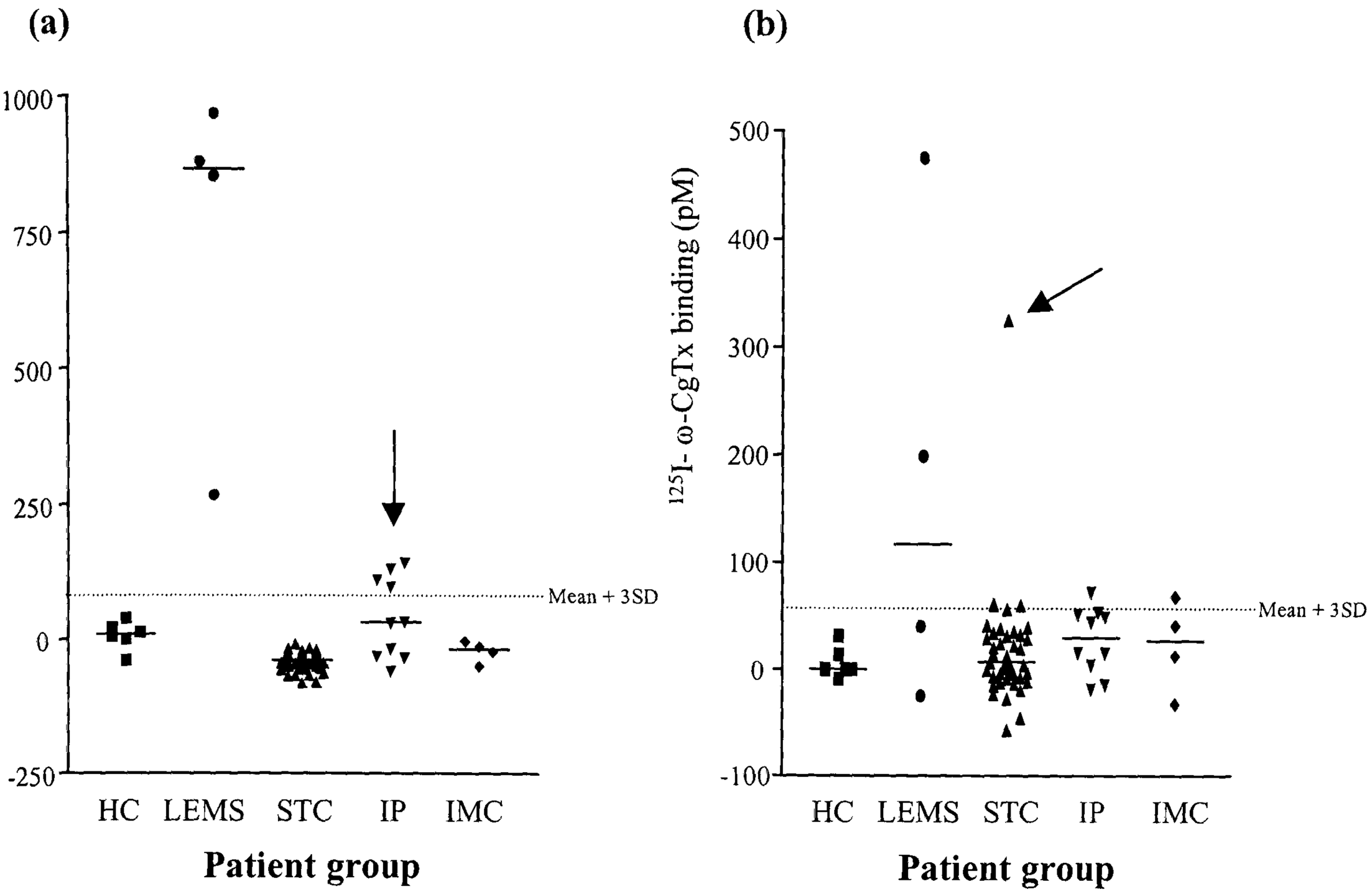


Fig 7.03: Scatter plots of results from anti-VGCC immunoprecipitation assays: anti-VGCC P/Q-type **(a)**, and anti-VGCC N-type **(b)**. Four IP sera are weakly positive for anti-P/Q-type antibodies (arrowed); the contingency of the distribution of these 4 positives to this single test group was highly significant, and unlikely to have occurred as a result of chance alone ($p < 0.0001$). A single STC sera is strongly positive sera for anti-N-type antibodies (arrowed). **KEY:** HC = healthy controls, LEMS = Lambert-Eaton myasthenic syndrome, STC = slow transit constipation, IP = intestinal pseudo-obstruction, IMC = idiopathic megacolon.

Two of the 42 sera from patients with STC showed precipitation of the labelled complex (Figure 7.04a) that was greater than the mean plus 3 S.D. for control sera (> 110 pmol of α-DnTx binding sites / litre of serum) (whole STC group: median -7, range –45 to 645). These 2 patients both fell in the AOIST sub-group of STC and are described in detail below ('patients 2 and 3'). The contingency of distribution of these 2 patients to this one smaller sub-group alone was significant i.e. unlikely to be the result of chance alone ($p < 0.04$, chi-square test) (Figure 7.04b).

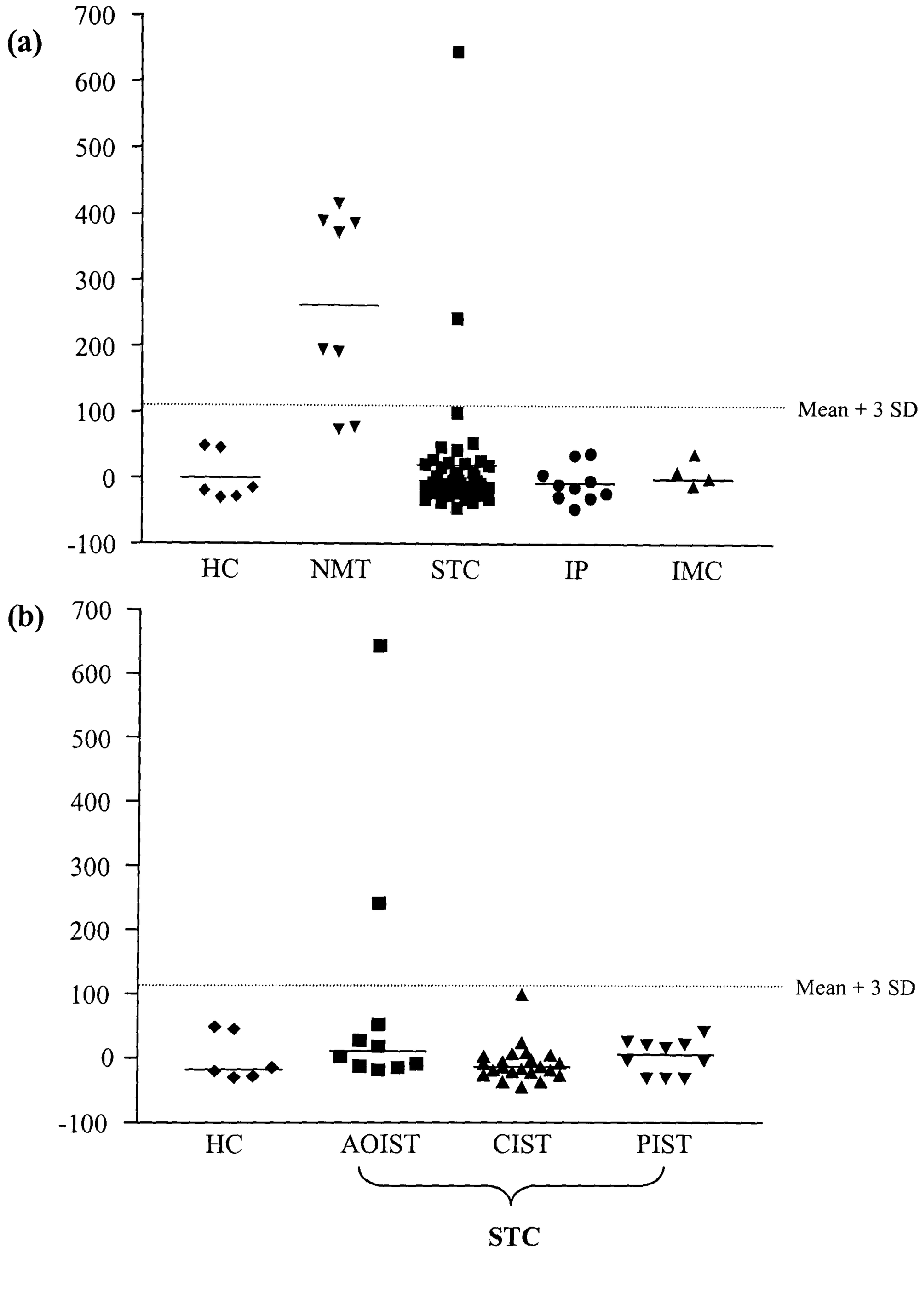


Fig 7.04: Scatter plots of results from anti-VGKC immunoprecipitation assay: **(a)** all groups studied, and **(b)** STC by sub-group *vs.* healthy controls only. Two patients with STC have clearly positive sera with titres comparable to sera from the positive control neuromyotonia patients (NMT). Both these patients had symptoms which arose *de-novo* in adulthood i.e. adult-onset idiopathic STC (AOIST). **KEY**: CIST = chronic idiopathic STC, PIST = STC arising following pelvic surgery or childbirth.

Six of the 8 patients with NMT, included as positive controls, had positive sera (whole group: median 283, range 74 to 415pM), but none of the 6 healthy controls (whole group: median -17, range –30 to 49pM), 10 patients with IP (whole group: median –12, range –46 to 37pM), or 4 patients with idiopathic megacolon (range –12 to 48pM). The results for both positive and negative controls were comparable with those from a previously published series (Hart *et al.*, 1997).

Muscle acetylcholine receptor: alpha-1 subunit

No values for labelled channel binding were noted that differed from the normal range, regardless of group (Mean 0 ± 0.3, range – 4 to 4 pM) [positive sera ≅ 500-1000pM].

Neuronal acetylcholine receptor: alpha-3 and alpha-7 subunits

Provisional results awaited from Italy indicate no STC positives. One positive sera from an IP patient requires further confirmation.

7.3.2 PATIENT CASE REPORTS

Patient 1. A 31-year-old woman with no significant medical or surgical history presented in 1998 with a 7 year history of severe defaecatory symptoms: infrequent passage of hard stool (bowels open every 2-3 weeks), and straining with unsuccessful or incomplete evacuation. She complained of some bloating and occasional abdominal pain, and was dependent on oral laxative ingestion. Her problems started after the birth of her 3rd child by normal (uncomplicated) vaginal delivery. Isotope scintigraphy demonstrated a left-sided pattern of delay.

She had reduced external anal sphincteric function, raised thresholds to rectal balloon distension, and was able to evacuate only 50% of the contrast at defaecography because of a functioning large rectocele / small anterior intussusception. Upper gastrointestinal motility studies demonstrated minor abnormalities of oesophageal and

small bowel motility, but normal scintigraphic gastric emptying. A left hemicolectomy was performed in 1999 with adequate surgical outcome (bowels open 1-3 times per day with symptomatic improvement). Routine histology demonstrated melanosis coli, but no other abnormality. Immunostaining with a variety of antibodies to cytoskeletal, neural, and other cellular proteins failed to demonstrate myopathic or neuropathic abnormalities.

Patient 2. A 34-year-old woman with no significant medical or surgical history presented in 1996 with a 6 year history of severe defaecatory symptoms: infrequent passage of hard pellet stool (bowels open every 2-3 weeks), and straining with unsuccessful, painful and incomplete evacuation. In addition she complained of bloating, abdominal pain and occasional nausea. Prior to this time she opened her bowels without straining every day. She had tried a variety of laxatives, and enemata and suppositories without sustained success. She had 2 children by normal vaginal delivery, and no gynaecological history. She denied any precipitating or temporally associated events with the onset of symptoms. Transit studies, including isotope scintigraphy confirmed generalised STC. She had normal sphincteric function, normal defaecography with no proctographic abnormality, but had raised thresholds to rectal balloon distension. Upper gastrointestinal motility studies were not performed. A total colectomy with ileorectal anastomosis was performed in 1997 with poor surgical outcome (bowels open 2-3 times per day, but continued severe abdominal pain, bloating and nausea and vomiting). Routine histology, and immunostaining with a variety of antibodies to cytoskeletal, neural, and other cellular proteins failed to demonstrate myopathic or neuropathic abnormalities. Subsequent chest radiograph and upper and lower GI endoscopy were normal.

Patient 3 A 62-year-old woman with no significant medical or surgical history presented in 1998 with a 1 year history of defaecatory symptoms: infrequent passage of hard stool (bowels open every 2-3 days with laxatives), and straining with unsuccessful, painful and incomplete evacuation. In addition she complained of some bloating and abdominal pain. Prior to this time she opened her bowels without

straining or laxatives daily. She was dependent on a variety of laxatives, and enemata / suppositories. She had 2 children by vaginal delivery, one requiring forceps assistance, but no gynaecological history. She denied any precipitating or temporally associated events with the onset of symptoms. Transit studies, including isotope scintigraphy confirmed generalised STC. She had normal sphincteric function but had markedly raised thresholds to rectal balloon distension and was able to evacuate only 50% of the contrast at defaecography because of verbally-reported loss of urge. Upper gastrointestinal motility studies, demonstrated abnormal oesophageal and small bowel manometry, but normal gastric emptying. She has been managed medically (without surgery) to date, and has been referred for biofeedback.

All 3 patients had a normal barium enema, and blood tests had excluded endocrine or metabolic disease. No patient had urinary or neurological symptoms, or a personal or family history of neurological, autoimmune or neoplastic disease. All patients were non-smokers. Clinical examination and neurophysiological tests, including nerve conduction studies and electromyography (EMG) were normal in both patients with anti-VGKC antibodies, excluding neuromyotonia.

7.4 DISCUSSION

Summary of results

The study shows 3 of 42 patients with STC who have high serum levels of either anti-"native" VGKC or N-type VGCC antibodies. The 2 patients with anti-VGKC antibodies were both from the smaller adult-onset idiopathic clinical sub-group, i.e. patients whose symptoms arose *de-novo* in adulthood, without a temporally related pelvic event, or other precipitating factor (Waldron *et al.*, 1988). The single patient with anti-VGCC was from the larger post-pelvic intervention sub-group, having symptoms which arose following normal childbirth. In addition, 4 patients with intestinal pseudo-obstruction (IP) had low levels of anti-P/Q-type VGCC autoantibodies. All these findings were reproducible in blinded independent assays.

Significance and interpretations

There are several possible interpretations of these results. Firstly, the observed high titres of antibodies might be a false finding reflecting a methodological error. This seems unlikely given the blinded repeatability of the results, and the consistent positive and negative control values which were comparable with published series, and historical data from the laboratory.

Secondly, antibodies whilst present may be of no pathogenic potential. In general, a much higher frequency of positive sera, such as that observed for anti-P/Q-VGCC in LEMS i.e. 90% (Lennon *et al.*, 1995) is considered to indicate a high probability of disease causation. The low frequency of patients with anti-channel autoantibodies in this study would therefore not generally support a pathogenic role in these syndromes. The possibility of finding non-pathogenic autoantibodies has been previously suggested with respect to VGCCs (Lennon, 1994). It is possible that intracytoplasmic components of ion channels which are normally inaccessible to antibodies might be rendered accessible *in vitro* by solubilisation, but not be candidates for antigenicity *in vivo*. If this were the case, we might however expect to find a proportion of all patient groups, including negative controls with similar positive results.

The serum levels of anti-VGKC autoantibodies in the 2 positive STC patients were comparable with those in acquired neuromyotonia (NMT) (Shilito *et al.*, 1995; Hart *et al.*, 1997), a condition of the peripheral nervous system characterised by hyperexcitability of motor nerves that results in continuous muscle fibre activity (myokymia), muscle cramps and weakness (Isaacs, 1961). Antibodies to VGKCs have been detected in over 50% of cases, using immunoprecipitation of VGKCs extracted from the human brain, and labelled, as in this study with ^{125}I-α-dendrotoxin (Shilito *et al.*, 1995; Hart *et al.*, 1997). In these assays, no healthy control had a comparable positive result. It is also of interest that both positive patients were from the same, smaller AOIST sub-group with a very similar gastrointestinal physiological phenotype (rectal hyposensation, upper GI problems / dysmotility), and that positives

were not observed in other STC patient sub-groups. It has been noted that such a distribution would be unlikely to occur by chance alone.

The single VGCC N-type positive serum had a titre of over 200pM, a level comparable only with LEMS patients in a previous large study (Lennon *et al.*, 1995). In this seminal work, 47 healthy controls all had serum levels of antibody <20pM (Lennon *et al.*, 1995), only 1 / 28 scleroderma patients had a positive serum at a level of only 45pM, and 90 patients with SCLC without paraneoplasia had approximately 20% positivity, but only at a median level of 67pM. Such a high level of antibody has therefore only been reported in disease states where it has been assumed that such antibodies are of pathogenic significance. Low positive titres of anti-P/Q-type VGCC antibodies (e.g. approx. 100pM), similar to those observed in the 4 / 10 IP patients in this study, have been demonstrated in patients with a variety of other paraneoplastic syndromes. For instance, levels were comparable with 70 patients with paraneoplastic encephalomyeloneuropathies in a study where only a single healthy control had a positive serum of 59pM (Lennon *et al.*, 1995). These autoantibody levels, whilst lower than LEMS serum levels, might therefore still be of significance. The distribution of all 4 positives to this group would be extremely unlikely by chance alone.

Finally, pathogenic antibodies whilst present, might be secondary to as yet undiagnosed neoplasia. On the basis of the ages, general medical health, non-smoking status, and relatively long follow-up of the patients in this study, this hypothesis seems unlikely.

If antibodies to ion channels detected in this study are a real finding of pathogenic significance, how might they lead to the observed phenotypes (STC and IP)? With respect to the VGKC autoantibodies, it is of interest that neither STC patient had CNS or PNS symptoms or signs, even on detailed testing. Similarly, gastrointestinal symptoms are not a recognised feature of neuromyotonia. VGKCs are the most heterogeneous of the family of voltage-gated cation channels (Pongs, 1993). At least

six *shaker*-related VGKC α-subunits have been isolated from human brain, each encoded by a different gene (Pongs, 1993). A functional VGKC consists of 4 transmembrane α-subunits that combine as homomultimeric or heteromultimeric tetramers (Wang *et al.*, 1993) to interact with intracellular B-subunits, which are thought to form a tetramer (Rettig *et al.*, 1994). Different toxins from a variety of species block particular types of K^+ channel (Castle *et al.*, 1989). α-dendrotoxin has a selective action on VGKCs, but like other toxins can bind a number of subtypes of VGKC. It is therefore possible that the antibodies demonstrated in STC and neuromyotonia have different VGKC subtype binding properties and therefore clinical effects, although the receptors themselves all bind to labelled α-dendrotoxin.

Mice injected with plasma or IgG from patients with neuromyotonia show increased quantal release of neurotransmitter at the neuromuscular junction, consistent with the idea that antibodies against VGKCs lead to increased nerve excitability and consequent prolongation of the action potential at the nerve terminal (Shilito *et al.*, 1995). Such excitability, was it to occur in the gut, might produce a reinforcement of colonic contractions but a decrease in "purposeful" propulsive activity leading to an overall decrease in transit. This would thus mimic the putative constipating physiological effects of opiates (Frantzides *et al.*, 1992), which lead to STC (Kaufman *et al.*, 1988) (see 1.6.5).

Approximately 30-50% of patients with LEMS have anti-N-type VGCC antibodies, compared to the majority who have anti-P/Q-type (Lennon *et al.*, 1995). Although the majority of patients with paraneoplastic syndromes who have anti-N-type also have anti-P/Q-type antibodies, some patients have an isolated high-titre autoantibody to N-type VGCCs (Lennon *et al.*, 1995). At one time, it was thought that N-type VGCCs might preferentially mediate postganglionic autonomic rather than somatic transmission (Herning *et al.*, 1988). However, more recent correlative clinical evidence (O'Suilleabhain *et al.*, 1998), and the results of experimental serum transfer experiments (Waterman *et al.*, 1997) do not now support this hypothesis. The significance of the anti-N-type autoantibody in the single STC patient, like the role of

N-type channels in LEMS, remains unclear. Anti-P/Q VGCC autoantibodies are thought to be of greater significance in disruption of postganglionic autonomic transmission (Waterman *et al.*, 1997), and such autoimmunity therefore remains a possible mechanism for the pathogenesis of the IP phenotype in the 4 patients with positive sera. It has been noted that autonomic deficits are a common finding in such patients (Camilleri & Fealey, 1990; Bharucha *et al.*, 1993; Camilleri *et al.*, 1993).

The origin of antibodies, in the absence of neoplasia, in the patients described has not been addressed by this study. Antibodies to antigens in SCLC and other paraneoplastic syndromes are thought to be due to an immune reaction to abherrantly expressed proteins on the malignant cell surface (Dalmau *et al.*, 1995). However, only about 20% of cases of NMT have an associated SCLC or thymoma, and the majority (80%) of cases occur without evidence of neoplasia (Newsom-Davis & Mills, 1993). Similarly, approximately 40% of LEMS have no detectable neoplasm (O'Neill *et al.*, 1988). There is now a considerable body of experimental work suggesting that immunomodulation of enteric neuromuscular function, possibly as a consequence of clinical or subclinical enteric infection, may have an aetiological role in intestinal dysmotility (see review: Collins, 1996). Similarly, there is human epidemiological evidence that cases of adult-onset dysmotility (especially IBS) can follow enteric infection (Lindberg *et al.*, 1999). Recent histological studies suggest that inflammation affecting the myenteric plexus, although generally less marked than in established cases of autoantibody-mediated plexitis (Smith *et al.*, 1997), may be present in these patients (Lindberg *et al.*, 1999). Chagas' disease is an example of an infection leading to a humoral autoimmune response directed against enteric neurones which results in functional intestinal obstruction (Goin *et al.*, 1999). As for viruses (Debinski *et al.*, 1997), further evidence for infectious agents in the aetiopathogenesis of severe idiopathic human dysmotility should continue to be sought.

Conclusions

Whatever the origin, this study suggests that anti-channel antibodies may have a role in some acquired gastrointestinal motility disorders. Whilst this study has demonstrated the presence of antibodies to VGCs, it has not proved their causal association with STC. The epitopes recognised by the antibodies require characterisation, and the distribution of target epitopes in the gut require demonstration. Passive serum transfer experimentation in a murine model could attempt to reproduce of the STC or IP phenotype. If acquired cases do indeed have an autoimmune channel-related aetiology, it will be of considerable interest to perform molecular genetic studies to test whether early-onset cases have a developmental, possibly congenital abnormality of channel protein morphology.

Many gastroenterologists typically associate ion channels with the generation and propagation of action potentials in cardiac myocytes or in neurones of the central and peripheral nervous systems, but it is important to note that such channels play an equally critical role in the functional biology of the gastrointestinal tract. Within the near future, channel blockers will be likely to be introduced as treatments for a variety of gut disorders (Lencer & Alper, 1999), including gastric and intestinal motility disorders (Hong *et al.*, 1997), and it is expected that their role in disease causation, like that of the PNS and CNS channelopathies will soon be elucidated.

8 SMOOTH MUSCLE DEGENERATION WITH INCLUSION BODIES IN SLOW TRANSIT CONSTIPATION

8.1 INTRODUCTION

Smooth muscle is the final effector of gastrointestinal motor activity, and loss of co-ordinated smooth muscular function may result in altered colonic motility. Recent advances have led to the recognition of some (albeit rare) underlying structural and morphological abnormalities of the smooth muscle of the gastrointestinal tract which may be primary or secondary. Primary myopathies are due to an innate abnormality in enteric muscle and can be congenital, or of early or late onset (see reviews by Smith & Milla, 1997; Martin *et al.*, 1999) (Table 8.01). Secondary muscle disease can occur as part of a multisystem disease such as scleroderma, but can also complicate abdominal or pelvic radiotherapy. Unlike the secondary myopathies, the aetiology of most primary myopathies has not been elucidated, and the possibility that developmental morphogenic abnormalities could result from defective genetic control of the complex sequential events in gut development has been discussed in the introduction (1.6.1.2).

Most reported primary and secondary myopathies are characterised by intestinal dilatation e.g. chronic idiopathic intestinal pseudoobstruction (CIIP), though some of these patients may present with constipation. Consistent myopathic findings have not been demonstrated by light microscopy in patients with STC (Park *et al.*, 1995, Benson *et al.*, 1992), and *in-vitro* physiological studies have had similarly conflicting results (Hoyle *et al.*, 1992, Slater *et al.*, 1997, Mitolo-Chieppa *et al.*, 1999).

Whilst one ultrastructural study has observed morphological changes in patients with STC (Benson *et al.*, 1992), unlike cases of childhood pseudoobstruction, detailed ultrastructural studies of smooth muscle have generally not been performed.

Table 8.01: Classification of enteric myopathies

PRIMARY MYOPATHIES	
Congenital / Early-onset	***Abnormal developmental (morphogenic) phenotypes***
	Focal absence of enteric muscle coats
	Segmental fusion of enteric muscle coats
	Presence of additional muscle coats
	Other phenotypes
	Myopathy with autophagic activity
	Pink blush myopathy with nuclear crowding
	Contractile protein abnormality
	Myopathy with atrophy and fibrosis
Late-onset	***Myopathies with atrophy and fibrosis***
	Hollow visceral myopathies: sporadic and familial
	Degenerative leiomyopathy
	Autoimmune myopathy
	Inclusion body myopathies
	Polyglucosan body myopathy
	Mitochondrial leiomyopathy
SECONDARY MYOPATHIES	
Systemic disorders	Desmin myopathy
	Muscular dystrophies
	Mitochondrial cytopathies
	Metabolic storage disorders
	Amyloidosis
	Progressive systemic sclerosis
	Other collagen vascular disorders
Local disorders	Obstructive / post-irradiation muscle failure

[Modified with permission from Martin *et al*., 1999]

Morphological changes seen in intestinal myopathies include varying degrees of fibrosis, hypertrophy or atrophy of muscle fibres, vacuolation of myocytes (Schuffler *et al.*, 1977, Martin *et al.*, 1990, Smith & Milla, 1997), alteration in the immunohistochemical staining pattern of myocyte contractile proteins (Smith *et al.*, 1992), abnormal layering of the muscle (Smith & Milla, 1997), and the presence of intracellular inclusion bodies (Martin *et al.*, 1990).

Inclusion body myopathies

In intestinal smooth muscle cells, inclusion bodies have been described in a variety of disorders including viral infection, adult polysaccharidosis (Peress *et al.*, 1979), Lafora's disease (Harriman & Millar, 1955, Gambetti *et al.*, 1971), and desmin myopathy (Ariza *et al.*, 1995). Many of these disorders are systemic and the consequences of gut smooth muscle dysfunction are usually less clinically significant than failure of other involved organs. Specific to the gastrointestinal tract, a familial myopathy affecting the smooth muscle of the internal anal sphincter has been reported in a number of women who presented with proctalgia fugax, and hypertrophy of the internal sphincter (Kamm *et al.*, 1991; Martin *et al.*, 1992). The characteristic pathological features of this disorder were evident on periodic acid-Schiff (PAS) staining, when ovoid inclusion bodies, 2-30 μm in length, were demonstrated. These inclusion bodies resisted diastase predigestion, and had a similar staining profile (Diezel, 1956), and ultrastructural appearance (Ramsey, 1965) to the polyglucosan structures of corpora amylacea, seen as a usual feature in the ageing brain. Structurally similar polyglucosan bodies have also been described in intestinal smooth muscle cells of aged dogs, without apparent associated muscle dysfunction (Kamiya *et al.*, 1983). These were clearly evident on light microscopy with PAS staining, were 5-20 μm in diameter, and were variably stained with other methods. Ultrastructural study revealed a filamentous composition, which was identical to previously reported Lafora bodies found in the CNS (Harriman & Millar, 1955). Such inclusions increased in number with age, and were found throughout the intestinal tract, although predominantly in the ileum and large intestine. In the human gut polyglucosan inclusions have been noted in small numbers of patients with intestinal

pseudoobstruction (Venislos *et al.*, 1988; Fogel *et al.*, 1993).

Eosinophilic inclusion bodies evident on conventional staining have been described in the intestinal tract of diabetics with severe autonomic neuropathy (Duchen *et al.*, 1980; Moscoso *et al.*, 1986). We had previously demonstrated similar inclusion bodies in a small number of patients with STC (Martin *et al.*, 1999). These may result from smooth muscle cell injury, occurring as a secondary phenomenon to other processes such as denervation or faecal stasis, or may reflect a new primary pathology which is specific to STC.

The aim of this study was to further explore the hypothesis that STC may be associated with the finding of inclusion bodies in gastrointestinal tissue. By the use of selected control populations with blinded analysis, we aimed to clarify whether these inclusions were a specific unique finding to idiopathic STC, reflecting a primary myopathic aetiology, or were a secondary response of smooth muscle cells to either denervation or other factors such as faecal stasis or laxative use.

8.2 MATERIALS AND METHODS

8.2.1 PATIENTS

Patients and controls were placed into one of six defined groups for inclusion in the study (Table 8.02).

8.2.1.1 Slow transit constipation sub-groups

Patients were defined as having STC by criteria discussed in chapter 2. These patients were further subdivided on the basis of clinical classification into those with idiopathic STC (chronic idiopathic and adult onset idiopathic STC i.e. CIST + AOIST), and those with post pelvic intervention STC (PIST) (see chapter 3). Other groups, i.e. C/PIST and NIST were excluded on the basis of the small number of

tissues available.

Table 8.02: Subjects included in study by group

Group	*Reason for inclusion ("model")*	*N*	*Age:med. (range)*	*Sex F / M*
Idiopathic STC	Test patients	25	42 (24 – 73)	25 / 0
Post pelvic injury STC	Extrinsic pelvic denervation	11	37 (28-54)	10 / 1
Rectal evacuation disorder	Faecal stasis / laxative use	5	42 (25-47)	5 / 0
Chagas' disease	Acquired intrinsic denervation	6	64 (57-69)	2 / 4
Hirschsprung's disease	Failure of innervation	10	0.5 (0.2–1.0)	3 / 7
Normal controls	Ageing	80	70 (20-92)	mixed

Idiopathic STC patients (CIST and AOIST)

Twenty five patients with idiopathic STC were studied. All were female, age 24 – 73, median 42 years. In all patients, constipation had arisen *de-novo* in childhood (n = 15), adolescence (n = 6), or after the age of 20 (n = 4), and patients denied any effect of subsequent pelvic surgery or childbirth.

Post pelvic intervention STC patients (PIST)

Eleven patients (10 female) with onset of constipation after pelvic intervention were studied; age 28 – 54, median 37 years. Constipation had followed pelvic surgery (n = 8, hysterectomy in 6), obstetric episode (n = 2), and blunt pelvic trauma (n = 1, male patient). In all cases, surgery had not directly affected the continuity of the bowel itself, and patients had had no preceding gastrointestinal symptoms.

8.2.1.2 Specific control groups

Rectal evacuation disorders (RED)

Patients with severe rectal evacuation disorders were included to control for the possible effects of chronic laxative use, and faecal stasis. All patients had normal transit studies. Five patients, all female were studied, age 25 – 47, median 42 years. The main diagnosis was prolapse in two, prolapse and rectocele in two, and non-relaxing pelvic floor in one.

Chagas' disease

These tissues were included as a control for the effects of acquired intrinsic denervation (Wood *et al.*, 1982; Brandt de Oliveira *et al.*, 1998). Six patients were studied, 2 female, age 57-69, median 64 years. Three patients had undergone colonic resection for megacolon, and 3 were obtained fresh at post-mortem examination of patients with fatal cardiomyopathy. Tissues for study were taken from a region of macroscopically undilated sigmoid colon in all cases.

Hirschsprung's disease (HSCR)

Tissue sections of mid-colon were taken from a series of patients with total colonic aganglionosis. These were included to control for the effects of congenital failure of innervation. Ten patients, 7 male, 3 female were included, age 0.2 – 1.0, median 0.5 years.

Normal controls

Tissues included normal ileal and colonic tissue removed for the treatment of colorectal cancer (n = 75) and ileal and colonic tissue from patients with familial adenomatous polyposis (FAP) (n =2). The latter, and a small number of ascending colonic tissues from patients with ileal Crohn's disease (n = 3) were included to help provide a comprehensive age range of normal control tissue. These tissues were only selected if the region of the colon was macroscopically normal, and the muscularis propria microscopically unaffected by the disease process. Ages ranged from 20 – 92

years, and were mixed equally for sex.

8.2.2 METHODS

8.2.2.1 Tissue collection

For all STC and RED patients, once the colon was removed, specimens were removed from the antimesenteric aspect of the colonic and / or ileal wall from inter-taenial portions. In patients undergoing subtotal colectomy, tissue specimens were taken from the terminal ileum (proximal resection margin), ascending colon (approximately 5 cm distal to the caecum), and sigmoid colon (approximately 5 cm proximal to the distal resection margin). When right or left hemicolectomy had been performed, similarly situated specimens were removed from the ileum and ascending colon or sigmoid colon respectively. Tissues from patients with RED were obtained at sigmoid colectomy or by biopsy from the site of sigmoid colonic conduit formation (Williams *et al.*, 1994). Normal control (ileal, ascending and sigmoid colonic) tissue, and Chagas' disease (sigmoid colonic) tissue was obtained from the archive of the Royal London Hospital, Dept. of Morbid Anatomy, and from Dr Ricardo Brandt de Oliveira (Ribeiro Preto, Brasil) respectively. The total number of tissues obtained from different sites by different procedures for each group is shown in Table 8.03.

8.2.2.2 Histological methods

All solutions and reagents used are shown in appendix 8.01. A summary of the processing of STC tissues is shown in appendix 8.02. The specimens used for this study were fixed for 24-36 hours in 10% formalin solution, and mounted in paraffin blocks by standard methodology.

8.2.2.3 Light microscopy

5 μm sections were cut in a longitudinal axis, collected on chrome-alum coated glass

slides, and stained with haematoxylin and eosin (H&E) (Appendix 8.03). Periodic acid-Schiff (PAS) preparations with and without diastase predigestion (stains for glycogen / glycoproteins) were made for all tissues (Appendix 8.03).

Table 8.03: Tissues included in study by group

Group	*Terminal Ileum*	*Ascending colon*	*Sigmoid colon*	*Obtained from*
Idiopathic STC	11	18	8	SC / R & LHC
Post pelvic injury STC	4	4	7	SC / R & LHC
Rectal evacuation disorder	0	0	5	LHC / conduit
Chagas' disease	0	0	6	CU, PM
Hirschsprung's disease	0	0	10	Pull throughs
Normal controls	40	50	20	R & LHC, AR, PC

Key: SC = subtotal colectomy, R & LHC = right and left hemicolectomy, CU = colonic resection unspecified, PM = post-mortem, AR = anterior resection, PC = proctocolectomy.

8.2.2.4 Blinding

All slides were labelled with a department code, and were then distributed randomly to slide containers by someone not involved in the analysis.

8.2.2.5 Qualitative and quantitative analysis

Whole H&E and PAS sections were systematically analysed at a magnification of x125 or x250 by 2 observers using a double-headed Olympus BH2 microscope. The

presence, distribution and number of inclusion bodies were noted. Validation of findings between observers was assessed by further independently viewing 20 sections that had been picked at random, and comparing the findings. The area of the muscularis propria of each section was calculated using a Magiscan M2® imaging system (Applied Imaging International, Sunderland, UK) by a further observer who was not aware of the histological findings. The assessment of muscle area was validated by repetition, and a second observer with subsequent intra and inter-observer studies of 10 sections. The number of inclusions per mm^2 of muscularis externa was then calculated.

8.2.2.6 Analysis of composition

A panel of sections in which inclusion bodies were detected were used to establish their possible composition using further sections, routine stains, and immunohistochemistry (Table 8.03). Serial 4 μm sections were taken through 10 inclusion bodies to further establish the 3 dimensional morphology on H&E staining. Additional routine histological stains included a range of trichrome stains to demonstrate various connective tissue elements: Massons trichrome, PTAH, Picro-Mallory and Martius yellow crystal brilliant scarlet. Other stains were used to detect collagen: elastic van Gieson; myelin: Luxol fast blue; amyloid: Alcian blue and Congo red; and lipofuschins: Sudan black and long Ziehl-Neelson; (Appendix 8.03).

Immunostaining was performed using a kit-based (Dako, Ely, Cambs, UK) indirect immunoperoxidase method (Appendix 8.04), with a variety of different antibodies to cytoskeletal and other cellular proteins (Table 8.04). Thermal or enzymatic antigen retrieval was used for some epitopes. The antibodies used had specificities against epitopes of actin, smooth muscle actin, vimentin, desmin (Smith & Milla, 1997), β-tubulin, amyloid precursor protein (APP) and ubiquitin (Mather *et al.*, 1993). Immunostaining of neural elements was performed using antibodies to neurone

specific enolase (NSE), neurofilament 2F11, S100, and PGP 9.5 as described previously (Martin *et al.*, 1990).

Because no nuclear material was detected in the inclusions on serial sectioning, immunostaining using a cytoplasmic apoptotic marker (Annexin V, Chemicon Int.) (Koopman *et al.*, 1994) was subsequently performed on a further panel of sections. Annexin V is a calcium-dependent phospholipid binding protein that detects the apoptosis-associated translocation of phosphatidylserine from the cytosol to the cell membrane, and had been previously used immunohistochemically to detect apoptosis of neuronal and smooth muscle cells (Giambanco *et al.*, 1991).

8.2.2.7 Statistical analysis

Intra and inter observer limits of agreement were expressed as the mean and 95% CI of the differences, with the Wilcoxon signed rank test used to test the significance of effectiveness of pairing. The frequency of occurrence of inclusions was compared between STC and controls using Fisher's exact contingency calculation. The number of inclusions per unit area was compared between STC and controls using the Mann Whitney U-test. Because of the marked age effect observed, a further age-matched sub-group of controls (range 20 – 73 years, median 46) were selected (all patients in age range) from the total for comparison with the STC groups.

8.3 RESULTS

8.3.1 VALIDATION OF METHODOLOGY

There was a high level of agreement between the 2 observers for the quantitation of inclusion bodies (mean difference = 0 inclusions, 95% CI: -1 to +1 inclusion, $p < 0.0001$). Likewise, there was minimal intra-observer (mean difference = 1mm^2, 95% CI: -2 to +4 mm^2, $p < 0.0001$), or inter-observer error (mean difference = 0 mm^2, 95% CI: -2 to +2 mm^2, $p < 0.0001$) with r espect to the

estimation of area of muscularis propria by image analysis.

Table 8.04: Routine and immunostaining.

ROUTINE STAINS

----------All tissues----------

Haematoxylin and eosin (H&E)

PAS with and without diastase predigestion (PAS / D-PAS)

----------Panel of tissues with inclusions----------

Alcian blue	Martius yellow crystal brilliant scarlet (MSB)
Congo red	Picro-Mallory
Elastic van gieson	Phosphotungstic acid haematoxylin (PTAH)
Luxol fast blue	Sudan Black
Long Ziehl-Neelson	Toluidine Blue
Masson Trichrome	

IMMUNOSTAINS

Antibody	Manufacturer	Type	Raised in	Raised against	Retrieval methods
Vimentin	Dako	monoclonal	mouse	swine	none
Desmin	Dako	monoclonal	mouse	human	none
Actin	Dako	monoclonal	mouse	human	none
Smooth muscle actin	Dako	monoclonal	mouse	human	none
β-Ubiquitin	Dako	polyclonal	rabbit	ubiquitin*	trypsin
β-Tubulin	Roche	monoclonal	mouse	human	none
α-amyloid precursor protein A4	Roche	monoclonal	mouse	human	none
NSE	Dako	monoclonal	mouse	human	none
PGP 9.5	Novocastra	monoclonal	mouse	human	micowave

S100	Dako	polyclonal	rabbit	cow	trypsin
Neurofilament 2F11	Dako	monoclonal	mouse	human	none
Annexin V	Chemicon	monoclonal	mouse	human	citrate

* affinity purified antibody (Mather *et al.*, 1993)

8.3.2 QUALITATIVE FINDINGS

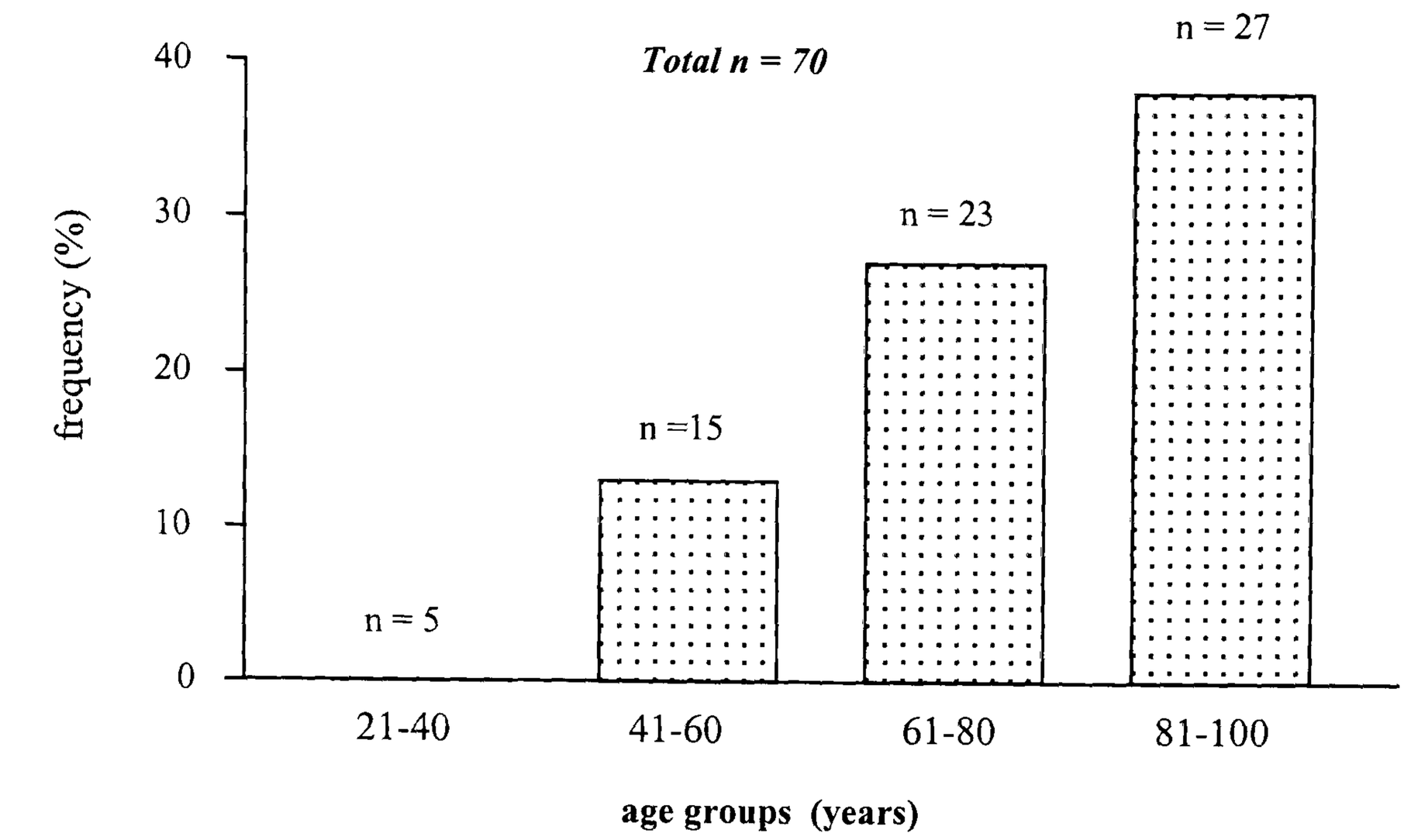
Inclusion bodies

Inclusion bodies (described 8.3.4) were detected in ascending colonic, sigmoid colonic and ileal specimens from normal subjects. In both the colon and ileum, the frequency of patients with inclusions increased with age (Figure 8.01a) . In patients classified as having idiopathic STC, inclusions were a more frequent finding compared with selected age matched normal subjects in the ileum (33% *vs.* 9%, n.s), the ascending colon (50% *vs.* 19%, $p < 0.05$, Fisher's exact test), and sigmoid colon (43% *vs.* 20%, n.s) (Figure 8.02 a-c). In patients classified as having post pelvic intervention STC, inclusions were observed with a similar frequency to idiopathic STC patients within the ileum (25%) and ascending colon (50%) (Figure 8.02 a,b). They were however, a very frequent finding in the sigmoid colon (71%) (Figure 8.02 c). In the RED group, inclusions were seen in the sigmoid colon, but only with a frequency similar to age matched controls. (Figure 8.02 c). All of the small number of sigmoid colonic tissues studied from patients with Chagas' disease had inclusions (Figure 8.02 c). No inclusion bodies were observed in the HSCR colonic tissues.

Other morphological findings

Morphological abnormalities of smooth muscle were observed in 4 idiopathic STC patients (fibrosis: 1, apoptosis: 2, degeneration of muscle cells: 2), and 2 post pelvic intervention STC patients (degenerating fibres, fibrosis). Chagas' tissues all had microscopic evidence of myenteric plexus damage, and 4 / 7 had mild to severe muscular degeneration, with multiple apoptotic and degenerate smooth muscle cells, and occasional lymphocytic infiltrates.

a.



b.

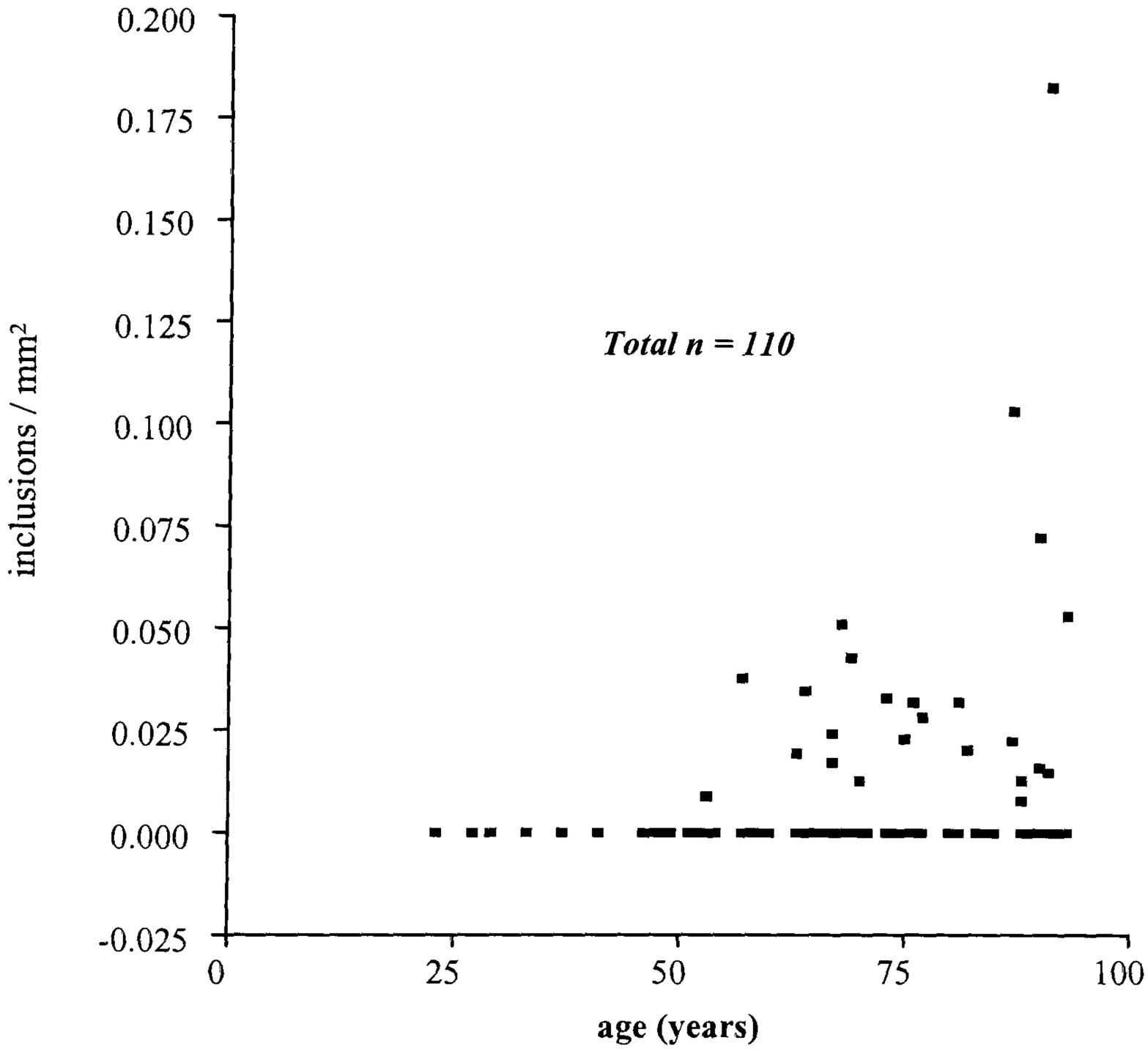
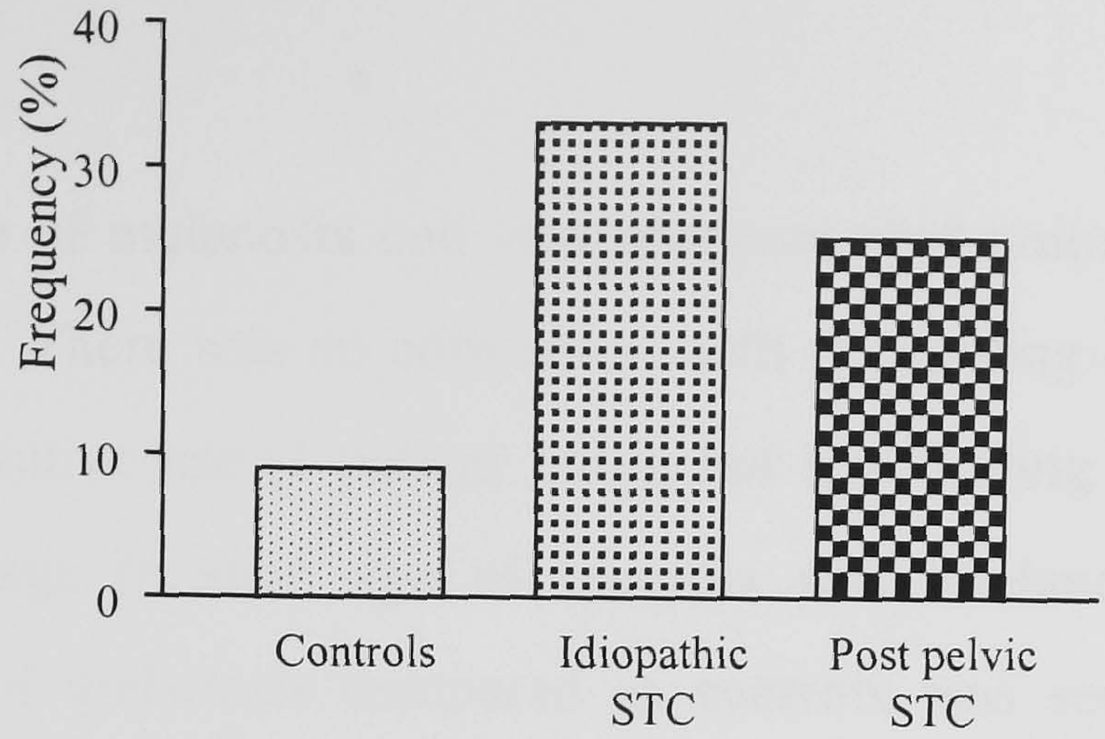
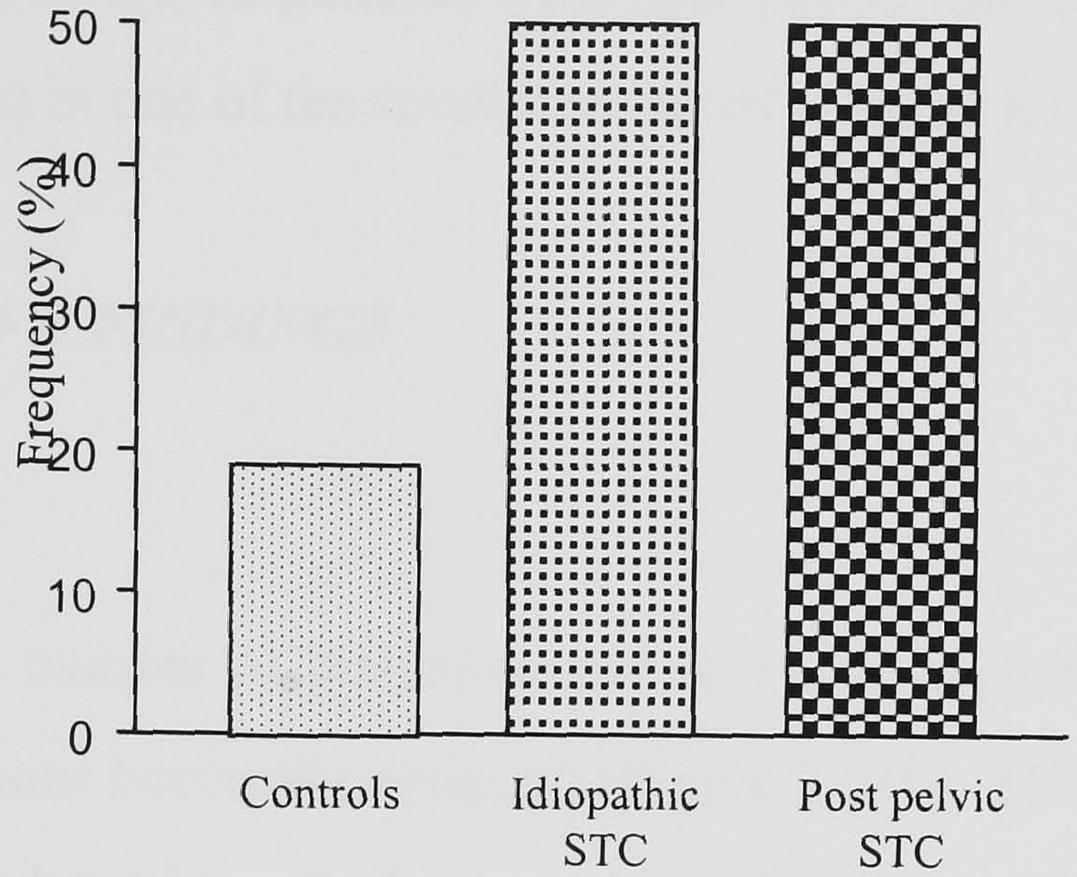


Figure 8.01: (a) Frequency of normal control subjects with inclusion bodies in the ascending (n = 50), and sigmoid colon (n = 20) by age group. The bar chart shows an almost linear increase in frequency with increasing age. **(b)** Density of inclusions in colonic (n = 70) and ileal (n = 40) specimens from normal control subjects by age. With advancing age, the data appears to separate into 2 sub-populations.

a. Ileum



b. ascending colon



c. Sigmoid colon

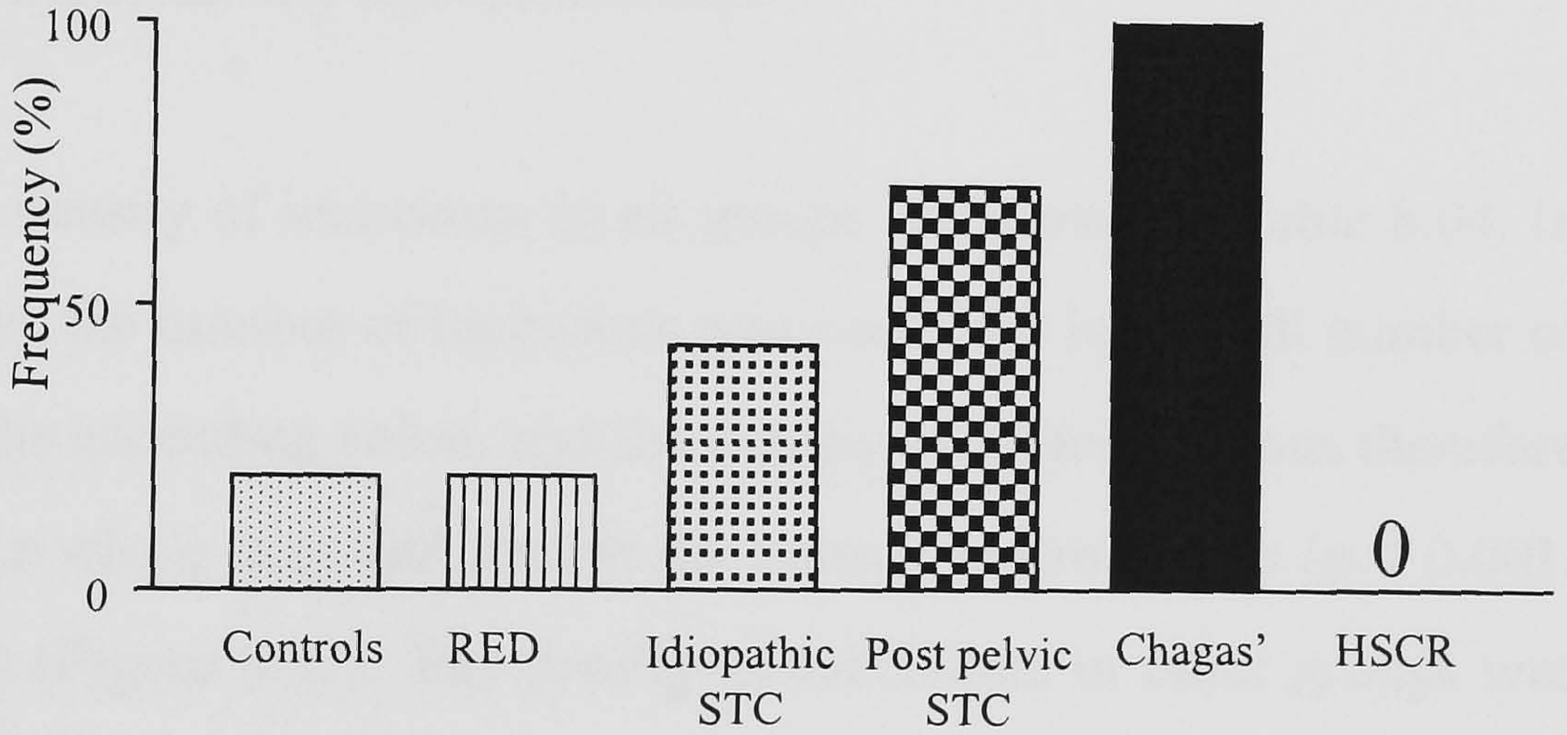


Figure 8.02: Frequency of patients with inclusion bodies in study groups *vs.* age matched controls for **(a)** ileal, **(b)** ascending colonic, and **(c)** sigmoid colonic tissue. An increased frequency of STC patients with inclusions was seen for all three tissues studied. The frequency was similar for idiopathic, and post pelvic intervention patients in the ileum and ascending colon, but was increased more for the latter group in the sigmoid colon. 100% of Chagas' disease patients had sigmoid inclusions, but no inclusions were seen in HSCR tissues, and no increase of inclusions over control frequency was seen in the RED group.

Microscopic evidence of melanosis coli was observed in 6 colonic specimens (9%) from normal subjects. There was no correlation with the finding of inclusion bodies, or with prolonged laxative use (1 patient only), but this finding may however have reflected advancing age (median age of controls with melanosis 88 years). An increased incidence of melanosis compared to controls was seen in patients with idiopathic STC (n = 4, 16%), in patients with post pelvic intervention STC (n = 3, 30%), and was observed in one of the small number of isolated RED patients (20%).

8.3.3 QUANTITATIVE FINDINGS

Density

In normal subjects, the number of inclusions per unit area appeared to increase with age with 2 sub-populations becoming apparent (Figure 8.01b). This distribution of the data set may reflect, at least in part, the increasing frequency of older patients with inclusions, rather than a true density-age relationship.

The ranges, and mean density of inclusions in all groups are shown in Table 8.04. In idiopathic STC patients, the number of inclusions was very high in a small number of patients especially in the ascending colon, and the density of inclusions was therefore significantly higher as a whole than that seen in the normal control group ($p < 0.001$, Mann Whitney U-test) (Figure 8.03). The density of inclusions in other groups was not statistically greater than that of controls.

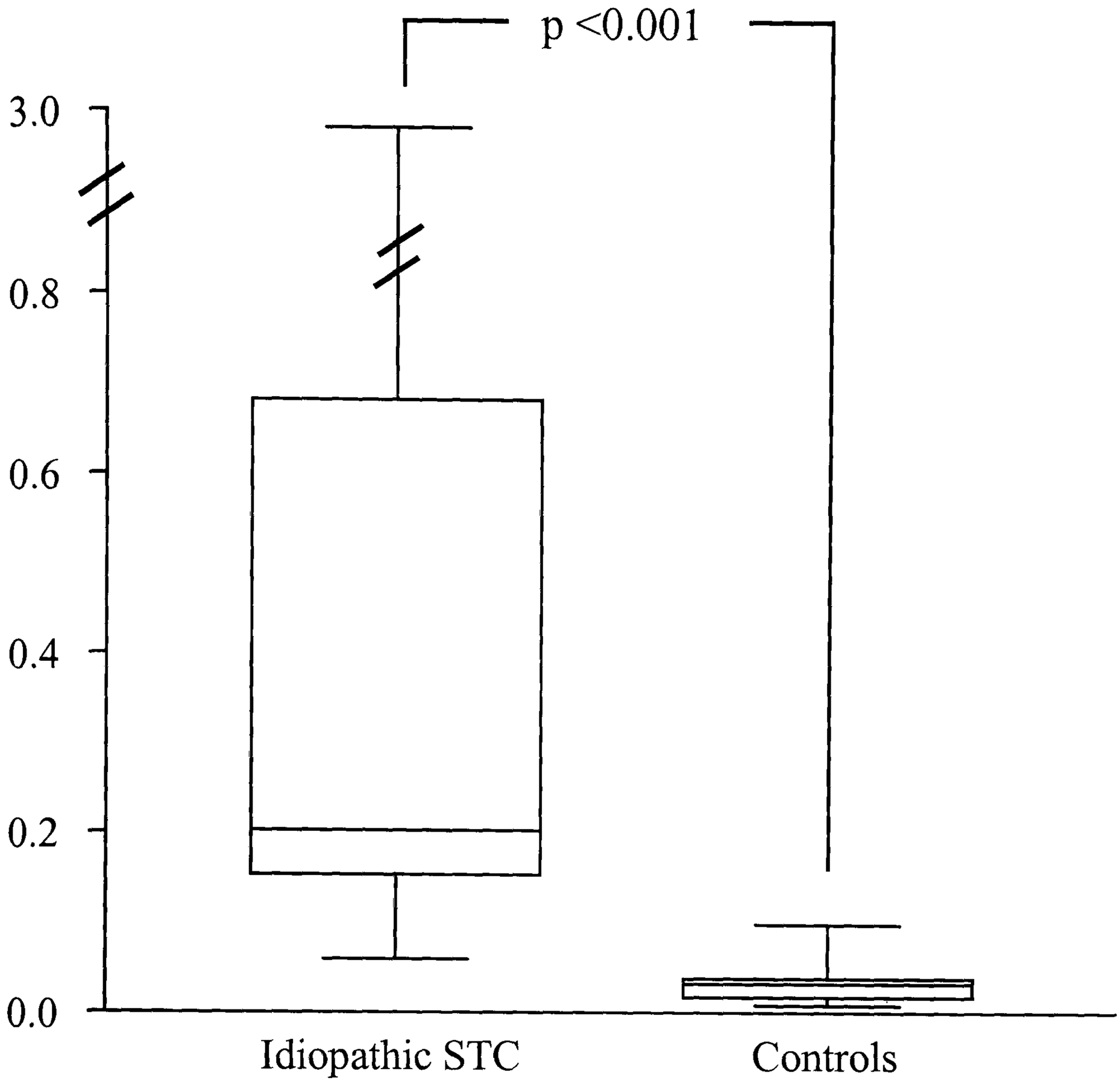


Figure 8.03: Number of inclusions per unit area "density" in the ascending colon of idiopathic STC patients, and controls. There was a significant difference between groups, $p < 0.001$, Mann Whitney U-test.

Distribution

In normal subjects, 58% of inclusions were found in the longitudinal muscle layer, 29% in the circular layer and 13% in the intermuscular plane adjacent to neural elements. In contrast, 192 (96%) of inclusions in idiopathic STC patients were found in the longitudinal muscle layer, with the remainder (only 4%) in the circular layer. In post pelvic intervention STC, 73% of inclusions were in the longitudinal muscle layer, with the remainder (27%) found adjacent to neuronal elements in the intermuscular

plane. A similar predominance of longitudinal layer inclusions (84%) was seen in the Chagas' disease patients.

Table 8.05: Inclusions per total area of tissue studied (density) by group

Tissue	*Group*	*Total number of inclusions*	*Number in longitudinal layer*	*Total area (mm^2)*	*Density / mm^2*
Ileum	Idiopathic STC	8	8	503.8	0.016
	Post pelvic STC	6	5	116.5	0.051
	Controls	13	6	2229.7	0.006
Ascending colon	Idiopathic STC	181	173	811.9	0.223
	Post pelvic STC	3	3	166.8	0.018
	Controls	25	15*	2258.6	0.011
Sigmoid colon	Idiopathic STC	12	11	322.7	0.037
	Post pelvic STC	13	8†	377.6	0.034
	Controls	6	5‡	991.1	0.006
	RED	3	3	235.3	0.013
	Chagas disease	19	16	561.4	0.034
	HSCR	0	0	251.6	0

* perineural / im plane = 4, circular muscle = 6

† perineural / im plane = 5, circular muscle = 0

‡ perineural = 1, circular muscle = 0

No formal study of serial sections was undertaken to establish whether inclusions were distributed continuously throughout the colon and ileum. However, in STC patients where tissues from more than one site were studied (n = 9), the majority (n = 7, 78%) had inclusions at all sites studied, with those having high densities of inclusions at one site, having similarly large numbers of inclusions at others.

8.3.4 ANALYSIS OF COMPOSITION

On routine H&E staining, the inclusion bodies appeared amphophilic, being round or ovoid, approximately 4-22μm in diameter (Figure 8.04). Serial sections through inclusions revealed a homogeneous hyaline appearance without evidence of nuclear material. The inclusions were not stained with any of the tinctorial (except a faint pink stain with PTAH) or immunohistochemical methods used, despite the use of enthusiastic unmasking strategies. Unfortunately, the methodology used for the detection of apoptosis (Annexin V) (Giambanco et al, 1991) was not effective in demonstrating any staining, despite the use of unmasking strategies including those suggested by the manufacturer. Following enquiries with other groups, similar difficulties have been encountered, and the failure is likely to represent problems with the efficacy of the reagents rather than the technique used. It should however be noted that no specific positive controls were used for this immunostain.

8.3.5 CLINICAL AND PHYSIOLOGICAL CHARACTERISTICS OF STC PATIENTS WITH INCLUSION BODIES

Clinical and physiological data for STC patients with or without inclusion bodies were reviewed to see if differences existed between these patients. Table 8.05 shows some of the main parameters that were reviewed. Differences existed in reported pre-operative bowel frequency, with patients having inclusions tending to less frequent bowel actions than patients without inclusions. However, this difference was not statistically significant overall (range 3-48, median 15 days *vs*. range 2-30, median 7 days, $p = 0.08$, Mann Whitney U-test). Patients with inclusions were significantly more likely to have disordered rectal evacuation on proctographic study ($p = 0.02$, Fisher's exact test). Whilst the outcome from colectomy appeared to be worse in the inclusion-positive group (increased incidence of recurrent constipation, diarrhoea or incontinence requiring subsequent ileostomy formation), further direct comparison was not made because of the heterogeneity of surgical procedures performed, variable follow-up, and lack of comparable prospective outcome data between patients.

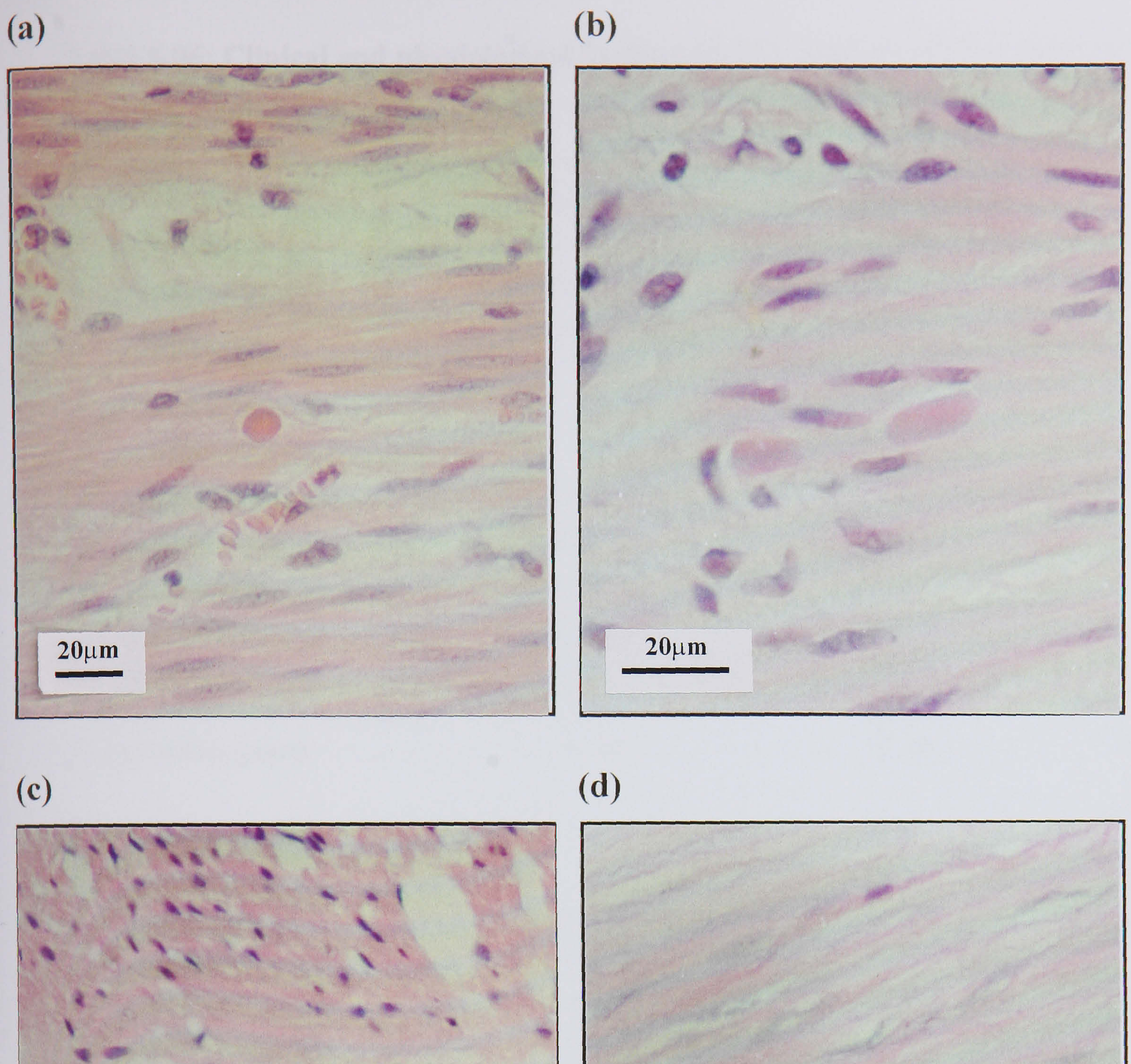


Figure 8.04: H&E staining of inclusion bodies in **(a)** ascending colon of normal control subject, aged 90 years (x100), **(b)** the ascending colon of idiopathic STC patient, aged 43 years (x160), **(c)** the sigmoid colon (perineural) of a patient with post-pelvic intervention STC, aged 45 years (x100), and **(d)** the sigmoid colon of a patient with Chagas' disease (x100).

Table 8.06: Clinical and physiological findings in STC patients with or without inclusion bodies

	No inclusions	*With inclusions*
Total number of patients	19	17
Age (years)	24-73 (median 46)	22-62 (median 40)
Clinical findings		
Pre-op bowel freq. (days)	2-30 (median 7)	3-48 (median 15)*
Upper GI symptoms	64%	69%
Urinary symptoms	63%	44%
Psychiatric illness	27%	45%
Laxatives	94%	83%
Successful outcome from surgery	75%	38%
Length history (years)	3 – 50	3 - 48
Physiological findings		
Anorectal manometry	No differences in sphincter function	
Rectal sensory abnormalities	45%	40%
defaecatory problems on evacuation proctography	14%	58% †
Transit: isotope scintigraphy	No differences in pattern of transit delay	
Abnormal small bowel motility	50%	67%

* $p = 0.08$, Mann Whitney U-test

† $p < 0.02$ chi square test

8.4 DISCUSSION

A high proportion of adults with STC had inclusion bodies present in both layers of the muscularis propria, particularly in the longitudinal layer, in the absence of other significant abnormality. Inclusions with an identical appearance were also seen in normal subjects with ageing, in all the small number of patients studied with Chagas' disease, but never in patients with HSCR. Inclusions were more frequent in STC

patients than in age-matched controls, and were seen in STC patients whose symptoms had arisen either *de-novo* or following pelvic intervention. The large number of patients studied, blinded design and validated reproducibility of the findings suggest that these are a real finding which to our knowledge have not previously been reported in these patient groups.

Duchen *et al.* (Duchen *et al.*, 1980) reported a series of post mortem findings in diabetic patients with autonomic neuropathy. These findings included the presence of eosinophilic rounded or club-shaped inclusion bodies within smooth muscle cells of the gastrointestinal and urinary tract which were evident on conventional staining. Moscoso *et al.* (Moscoso *et al.*, 1986) further demonstrated a form of necrobiosis and atrophy of smooth muscle in diabetic autonomic gastropathy, defining "M" bodies which were thought to represent individual "transformed" smooth muscle cells. These bodies had a similar eosinophilic hyaline appearance to those visualised by Duchen, and measured 4-25 μm in diameter.

The inclusion bodies visualised in our study were of an almost identical size range, had similar staining characteristics, and appeared morphologically identical to those demonstrated in these papers (Moscoso *et al.*, 1986, Duchen *et al.*, 1980). Taken together with our findings in the colon from patients with Chagas' disease, and in normal subjects with advancing age, it is possible that denervation may be the underlying cause of such smooth muscle changes. Degeneration, especially of certain subgroups of enteric neurones, has been shown with ageing in man and experimental animals (Gomes *et al.*, 1997, Gabella *et al.*, 1989), and is known to be a feature of Chagas' disease (Wood *et al.*, 1982; Brandt de Oliveira *et al.*, 1998; Goin *et al.*, 1999). This hypothesis is further supported by the finding of inclusions with a high frequency in patients with STC following pelvic surgery, injury or obstetric trauma (Roe *et al.*, 1988; MacDonald *et al.*, 1997). In such patients, it is generally believed, though not anatomically proven that the motility problem arises from extrinsic parasympathetic denervation as a result of injury to the nervi erigentes (Devroede *et al.*, 1979; Smith *et al.*, 1990; Varma 1992; MacDonald *et al.*, 1993) (1.5.4.4.1). The

finding of a decreasing frequency of inclusion bodies from caudad to cephalad (sigmoid > ascending colon > ileum) in the small number of these patients studied might reflect the distribution (Christensen, 1991) of the ascending colonic nerves. The finding of inclusion bodies in the ileum of idiopathic patients may concur with the upper gastrointestinal motor abnormalities, known to exist in these patients (Bassotti *et al.*, 1996; Glia & Lindberg, 1997; Mollen *et al.*, 1999), and which have similarly been demonstrated by studies included in this thesis (3.3.3.2).

It is therefore possible that the inclusions seen in the *de-novo* (idiopathic) STC group similarly occur as a result of some form of denervation process. This hypothesis would concur with a large number of studies which have indicated that neuronal degeneration occurs in such patients (Krishnamurthy *et al.*, 1985; Lincoln *et al.*, 1992; Shouten *et al.*, 1993; Porter *et al.*, 1998) (1.5.3). Since the STC group were not of an age range where inclusions occurred with a high frequency in normal subjects, it is possible, though no direct evidence currently exists, that such patients might have a similar, but "accelerated" degenerative process to that seen with ageing. Although ageing alone does not appear to affect transit times (Towers *et al.*, 1994), it may explain why constipation is a potential accompaniment of ageing (Whitehead *et al.*, 1989; Towers *et al.*, 1994).

It remains possible, although there is no published evidence to this effect, that inclusion bodies may represent a response to other mechanisms of smooth muscle injury, for instance laxative use or prolonged distension by faecal stasis. This hypothesis was not confirmed by our study, in which an albeit small number of RED patients who had a similarly chronic history of severe defaecatory symptoms and laxative use, did not have an increased incidence of inclusions compared to age-matched controls. In addition, there was no significant difference in laxative use between STC patients with or without inclusion bodies. There was some co-existence of inclusions with melanosis coli in the small number of STC patients with this additional finding, but not in controls. Our study, like recent others (Byers *et al.*, 1997) however failed to demonstrate that such mucosal pigmentation is specific to

patients using laxatives, since only one record of prolonged laxative use was seen in the 9% of normal control subjects with this finding. Since this group were generally of a markedly higher age than controls without melanosis, it may reflect age related colonic epithelial apoptosis as has been previously suggested (Byers *et al.*, 1997). It has been noted that the commonly held belief that currently used laxatives damage the bowel is not supported by scientific evidence (1.6.6).

It is not possible to say from this study whether the inclusions demonstrated are lying adjacent to, or replacing smooth muscle cells. The inclusions, like those shown previously (Duchen *et al.*, 1980, Moscoso *et al.*, 1986) had a homogeneous hyaline appearance, and serial sectioning did not reveal nuclear or other components by light microscopy. In addition, it was not possible to stain the inclusions with a wide range of conventional stains including PAS (although there was some faint pink staining with PTAH), and they were non-immunoreactive for a wide range of structural elements, including a range of anti-cytoskeletal and stress protein antibodies. This is not unprecedented in the field of inclusion body pathology. Ubiquitinated inclusions in anterior horn cells in motor neurone disease are not demonstrated by any method other than anti-ubiquitin immunohistochemistry and the core constituent protein remains elusive (Mather *et al.*, 1993). In this study, we failed to test whether such inclusions might represent some form of apoptotic degeneration of smooth muscle cells, and this remains a possibility. A simple origin therefore, from recognised cellular elements of the intracellular inclusion material in the colonic muscle has not been ascertained by this study, and necessary ultrastructural studies are in progress.

Interestingly, we did not observe inclusion bodies of polyglucosan composition which have been elegantly and clearly demonstrated in aged dogs by Kamiya *et al.* (Kamiya *et al.*, 1986). This group used dogs selected at random for autopsy. The genetic background of dogs tends to be less diverse than that of humans due to selective breeding. Such practices lead to a relatively high incidence of genetic disorders in inbred strains (Leighton, 1997, Wood & Lakhani, 1997). The differences in our findings in humans may relate to an inherited predisposition in dogs, albeit without an

accompanying motility dysfunction, although it is also conceivable that dietary differences may have a role.

The significance of the very high proportion of inclusions in longitudinal muscle layer of idiopathic STC patients, in contrast to the more balanced distribution in controls is not clear, although this observation may reflect the greater ease of visualising inclusions in longitudinally cut fibres. It is known, however that electrical control activities exhibited by the longitudinal muscle layer are responsible for control of long duration, propagating contractions (Sarna, 1991; Sarna, 1993), and that in STC, propulsive contractions are reduced (Bassotti *et al.*, 1988). The role of the longitudinal muscle layer in relation to spontaneous electrical activity is becoming increasingly established (Stevens *et al.*, 1999).

The clinical significance of the finding of inclusions in patients with STC cannot be judged from the design of this study. Comparison of patients with or without inclusions may suggest that patients with inclusion bodies are more likely to have a generalised intestinal disorder (GID), which is known to adversely effect outcome (Redmond *et al.*, 1996). If this were the case, it might be desirable to ascertain this diagnosis prior to consideration for colectomy. Certainly the outcome of the patients with inclusions in this study was very poor, with a high rate of stoma formation for recurrent or continuing symptoms, or intractable diarrhoea. Laparoscopic biopsy allows full thickness colonic tissue to be studied, and has been performed safely at our institution for a small number of these patients *(unpublished data of the authors),* and in children with chronic constipation (Hutson *et al.*, 1996). In one study patient, the finding of inclusion bodies on the laparoscopic biopsy of the sigmoid colon was confirmed by subsequent colectomy. The distribution throughout the bowel, and therefore positive predictive value of such a procedure has clearly not been addressed by this study. A positive impact of the finding of inclusions may be the relief felt by some patients to be given a defined structural abnormality as a potential explanation for their symptoms, which have often been attributed solely to a psychological disturbance.

9 SUMMARY AND CONCLUSIONS

9.1 CLINICAL AND PHYSIOLOGICAL HETEROGENEITY OF SLOW TRANSIT CONSTIPATION: DEFINING THE PROBLEM

9.1.1 SUMMARY

In order to perform further clinical, neurophysiological and aetiological studies of STC, a population of patients was defined. In total, 130 patients were included in the thesis for which extensive clinical and physiological data were collected and analysed. Distinct clinical sub-groups could be defined, based on age and mode of onset. These included some previously described sub-groups, such as chronic idiopathic STC (CIST) and STC following pelvic surgery or childbirth (PIST), but also 4 other sub-groups which included patients with other precipitating factors such as spinal injury. Physiological comparison of patients placed in such sub-groups revealed some similarities and differences. In particular, comparison of the 2 main sub-groups (CIST and PIST) demonstrated 3 important findings which were in contrast to previously published reports of smaller patient numbers. The pattern of transit is in fact similar in both groups i.e. predominantly (70%) generalised, the incidence of rectal evacuation disorder is significantly higher in patients with PIST (68% *vs.* 34%, $p = 0.04$), and small bowel motility disturbances occur in both groups (overall, 48%).

Such heterogeneity has implications for the perceived pathogenesis of STC and interpretation of variation in morphological and physiological studies. In addition, regardless of the effect of physiological differences, it is unclear what independent effect a variation in pathogenesis of STC might have on treatment outcome. The response to surgery might be expected to be different for diseases with diverse pathogenesis. Indeed, wide variation exists in the outcome of such patients following surgery. The classification system presented was used to select and clinically clarify

heterogeneity within patients selected for further studies in the thesis.

9.1.2 CONCLUSIONS

1. Whilst the clinical end-point i.e. that of severe intractable constipation with slow colonic transit may be indistinguishable between patients with STC, the group is clinically heterogeneous.
2. Some significant physiological differences are detectable between clinically defined sub-groups.
3. The generally accepted supposition that STC patients with symptoms arising after pelvic surgery or childbirth have an isolated disturbance of rectosigmoid motility is incorrect.

9.2 LINEAR DISCRIMINANT ANALYSIS OF SYMPTOMS IN PATIENTS WITH CHRONIC CONSTIPATION: VALIDATION OF A NEW SCORING SYSTEM (KESS)

9.2.1 SUMMARY

We assessed the ability of a new symptom scoring system (KESS) to assist in diagnosing constipation and to discriminate between the main patho-physiological sub-groups of patients, including those with STC. A structured symptom scoring questionnaire (11 questions) was completed by 71 chronically constipated patients, and by 20 asymptomatic controls. Linear discriminant analysis was used to assess the ability of different questionnaire symptoms to discriminate between these subgroups. The KESS total score delineated patients with constipation clearly from normals. Unlike previous studies utilising symptom questionnaires, the KESS questionnaire appeared to be able to discriminate between patho-physiological sub-groups for the majority of patients with constipation (55%, C.I. 43% to 67%). Discriminant scores predicted patients with pure STC or RED better than patients with mixed

abnormalities.

9.2.2 CONCLUSIONS

1. This new scoring system is a valid technique to assist in the diagnosis of constipation.
2. This is the first study utilising appropriate statistical methodology to demonstrate a discriminatory ability of multiple symptoms in constipation.
3. At present, symptom analysis does not adequately differentiate major patho-physiological sub-groups for use in clinical practice.

9.3 SENSORY AND AUTONOMIC NEUROPATHY IN PATIENTS WITH SLOW TRANSIT CONSTIPATION

9.3.1 SUMMARY

Previous studies have suggested that some patients with STC have autonomic and sensory dysfunction, detectable by peripheral testing. These studies have utilized relatively small patient numbers, and limited types of tests. We performed a range of standard neurophysiological and selective quantitative tests of sensory and autonomic function in 41 STC patients, compared to positive and negative control populations. The results demonstrated that approximately half of patients had evidence of autonomic and / or small sensory fibre function, which was detectable in the lower limbs by quantitative testing, but not evident on clinical neurological examination. Whether such changes are causative in terms of the observed gut phenotype requires further investigation. The aetiology of neural dysfunction remains unclear.

9.3.2 CONCLUSIONS

1. Quantitative tests provide evidence of a small fibre neuropathy in a proportion of patients with STC.

9.4 SCREENING OF PATIENTS WITH IDIOPATHIC SLOW TRANSIT CONSTIPATION FOR MUTATIONS OF THE *RET* PROTO-ONCOGENE AND *GDNF*

9.4.1 SUMMARY

The frequent onset in early childhood and history of constipation or Hirschsprung's disease (HSCR) in close family relatives (observed in the previous study) suggest that idiopathic STC could have a genetic basis. A number of germline mutations have been described in HSCR, including mutations of *RET*, and the gene encoding its ligand glial cell line-derived neurotrophic factor (GDNF). We screened a panel of chronic idiopathic STC patients, including 4 families in which there were relatives with HSCR, for *RET* and *GDNF* mutations previously identified in HSCR. The methodology was clearly validated by the use of positive controls, but although common sequence polymorphisms were demonstrated with comparable frequency to published data, no published or new mutation was seen in any of the exons of *RET* or *GDNF*.

9.4.2 CONCLUSIONS

1. Unlike in Hirschsprung's disease, mutation of *RET* or GDNF is not a frequent cause of idiopathic STC.

9.5 DETECTION OF AUTOANTIBODIES TO EXPRESSED NEURONAL ION CHANNELS IN SLOW TRANSIT CONSTIPATION

9.5.1 SUMMARY

Disorders of ion channels (channelopathies) are being increasingly identified, and have been implicated in several acquired disorders of gut motility with or without

dysautonomia. We screened sera from 42 patients with STC for autoantibodies to a panel of neuronal ion channels using previously validated immunoprecipitation studies. Three patients had high serum levels of either anti-"native" voltage gated calcium (VGCC) or potassium channel (VGKC) autoantibodies. The 2 patients with anti-VGKC autoantibodies both had severe symptoms that arose *de-novo* in adulthood. In addition, 4 / 10 patients with intestinal pseudo-obstruction, included as a comparison group, had anti-VGCC autoantibodies. The significance of these findings, which were reproducible in blinded, independently performed assays, is unclear, especially since autoantibodies were only found in a small proportion of patients. In such patients, if autoantibodies are of pathogenic significance, it is possible that selective effects on enteric neurones might lead to the observed phenotype in STC. Future studies may demonstrate a causative mechanism for such autoantibodies.

9.5.2 CONCLUSIONS

1. Anti-neuronal ion channel autoantibodies may have an as yet unrecognised role in the development of some acquired gastrointestinal motility disorders in a small proportion of patients.

9.6 SMOOTH MUSCLE DEGENERATION WITH INCLUSION BODIES IN SLOW TRANSIT CONSTIPATION

9.6.1 SUMMARY

Myopathies, including those characterised by the finding of inclusion bodies, have been described in enteric disorders. We identified inclusions present in STC patients, and tested whether these were a primary or secondary finding using a systematic, blinded, dual observer qualitative and quantitative analysis of colonic and ileal tissue from patients with STC (n = 36) compared with selected control populations (n = 101). Round or ovoid (4-22μm diameter) amphophilic inclusions increased in normals with ageing ($p < 0.02$). Inclusions were a more frequent finding in patients with

idiopathic STC compared with age matched controls or patients with rectal evacuation disorders: ileum (33% *vs.* 9%), ascending (50% *vs.* 19%, $p < 0.05$), and sigmoid colon (43% *vs.* 20%), and were very frequent in the sigmoid (71%) of patients with STC arising after pelvic surgery. The number of inclusions per unit area was significantly higher in patients with STC ($p < 0.001$). Inclusions were found in all Chagas' patients, but not with aganglionosis. The inclusion bodies visualised in our study were of an almost identical size range, had similar staining characteristics, and appeared morphologically identical to those demonstrated in previous studies of gastrointestinal tissues from patients with severe diabetic autonomic neuropathy. It was not possible to determine inclusion body composition with a wide range of conventional or immunostains.

9.6.2 CONCLUSIONS

1. An inclusion body myopathy is identifiable in patients with STC, and may arise secondary to denervation.
2. The composition of such inclusion bodies remains elusive.

9.7 CONCLUDING REMARKS

The aetiology of STC remains elusive. This thesis supports the view that the key to its understanding lies with knowledge of the interrelationships of nerve and muscle. There will continue to be much controversy over the nature of the primary deficit, nerve or muscle failure, but it seems likely that both the functional and structural integrity of gut smooth muscle is dependent on neural (chemical and electrical) trophic support, and similarly, that the maintenance of nerve function is likely to be dependent on end organ neurotrophic support. The balance of form and function, support and dependence holds

the key to this disorder, and further studies could be directed to understanding of this balance in health in the gut, and its possible aberration in disorders of motility. Such further knowledge of the pathogenesis of the condition may allow more directed therapies aimed at the reversal of the disease process or processes themselves.

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APPENDICES

2.01 CONTROL AND COMPARISON GROUPS

Type	*Chapter*	*Study type*	*Study subjects*
Negative controls	4	Symptom analysis	Healthy volunteers
	5	Neurophysiology	Healthy volunteers
	7	Autoantibody assays	Healthy volunteers
	8	Inclusion study	Neoplastic disease
Positive controls	5	Neurophysiology	Diabetes mellitus
	6	Mutation screening	Multiple endocrine neoplasia and HSCR
	7	Autoantibody assays	Neuromyotonia (Isaac's' syndrome)
			Lambert-Eaton myasthenic syndrome (LEMS)
			Myasthenia gravis (MG)
Specific comparison / control groups	7	Autoantibody assays	Intestinal pseudoobstruction
			Idiopathic megacolon
	8	Inclusion study	Rectal evacuation disorder
			Chagas' disease
			Hirschsprung's disease

2.02 DEMOGRAPHIC DATA

Age at presentation	Median 39, range 13-79 years
Sex	Female 126, Male 4 (ratio 32 : 1) (male = 3%)
Race	Caucasian 121, Asian 4, Afro-Caribbean 2, NK 3
Geographical address	Greater London 70, Essex 27, Other 18, NK 15

2.03 AGES OF SYMPTOM ONSET

Range	*Number of patients*	
Age 1 – 5	14	
Age 6 – 10	37	
Age 11 – 15	12	
Age 16 – 20	13	***Median 12 years (range 1 – 68)***
Age > 20	41	
Not known	2	
Total	***119****	* 11 patients excluded (see text)

2.04 MODE OF ONSET / PRECIPITATING EVENTS

Mode of onset	*Number*	*% total*
Idiopathic (no clear precipitating events)	84	64.6
Sexual or physical abuse	*2*	
Gastroenteritis	*2*	
Viral infection	*1*	
Minor anal trauma	*1*	
Foreign travel	*1*	
Obstetric episode	12	9.2
Gynaecological or other pelvic surgery	11	8.5
Idiopathic + subsequent pelvic event	11	8.5
Neurological injury / disease	7	5.4
Other (miscellaneous)	5	3.8

2.05 PRESENTING SYMPTOMS

Frequency of bowel action	median = 8 days
(number of days between successive bowel	range = 1 – 49 days
actions based on mean of ranges given)	not known = 15

Defaecatory symptoms	***Number of patients***	***Total known***	***% total***
Straining	102	112	89.3
Time spent per attempt at defaecation > 5 min	67	83	80.7
Incomplete evacuation	97	102	95.1
Unsuccessful evacuation	77	89	86.5
Painful evacuation	62	75	82.7
Stool consistency (hard / pellets)	88	99	88.9
Incontinence	13	64	20.3
Rectal prolapse (feeling of)	11	69	14.5
Digitation: rectal / vaginal	26	108	24.1
Bleeding per rectum	65	100	65.0
Other gastrointestinal symptoms			
Abdominal pain	113	119	95.0
Abdominal bloating	109	112	97.3
Nausea / vomiting	49	93	52.7

2.06 MANAGEMENT

Medical

Agent used	*Number of patients*	*Total known*	*% Total*
Laxatives	108	120	90.0
Suppositories	40	102	39.2
Enemas	29	102	28.4
Prokinetics	8	120	6.7

Surgical

Anorectal procedures		*Number of patients (from total = 130)*
Anal stretch		3
Lateral sphincterotomy		1
Rectal biopsy		4
Rectocele repair		2
Anterior and posterior repair		1
Rectopexy	abdominal	5
	Delormes	1

Colonic surgery	
Subtotal colectomy and ileorectal anastomosis	15 (12%)
Right hemicolectomy	5
Left hemicolectomy	6 (one with rectal reduction)
Sigmoid colectomy	3
Colonic conduit formation	6

Stoma formation

Ileostomy	primary	4
	Secondary	4
Colostomy	primary	2

Other management

Botulinum injections		2
Behavioural therapy	(Biofeedback)	2

2.07 PERSONAL HISTORY

Surgical history (NK = 15; total known = 115)	***Number***
Appendicectomy	13 (11%)
Cholecystectomy	5
Hernia repair (s)	4
Gastric surgery	2
Other	3
None	91

Obstetric history (126 female, NK = 12; total known = 114)

		Total	***Instrumentation***	***Caesarean section***
Parity	0	36	-	-
	1	14	1	1
	2	37	5	4
	3	14	0	4
	4	7	0	0
	>4	6	2	2
	total	***114***	***8***	***11***

Gynaecological history (126 female, NK = 13; total known = 113)

Disorders	***Number of patients***	***Surgery***	***Number of patients***
Endometriosis	5	Laparoscopy gynaecological surgery	15
Pelvic inflammatory disease	6		
Ovarian cystic disease	5	Dilatation and curettage	3
Cervical carcinoma or CIN III	3	Pelvic floor repair*	11
Ectopic pregnancy	2	Ovarian surgery	5
Uterine or bladder prolapse	5	Hysterectomy	38 (34%)
Fibroids	14	Cervical surgery	3
		Infertility treatment	2
Total	37 (33%)		64 (57%)

Psychiatric disorders	(NK = 30; total known = 100)
Depressive illness (unspec.)*	13 (13%)
Schizophrenia	0
Anxiety disorders	2
Eating disorders (anorexia nervosa)	1
Behavioural problems (unspec.)	2
Psychoactive substance dependence	2
Other	3
None	77 (77%)

* includes: unipolar and bipolar (n = 1) illness, suicide; n = 1, parasuicidal behaviour; n = 2

Past Medical History		(NK = 17; total known = 113)
Metabolic / Endocrine	Thyroid disease (treated)	5
	Diabetes Mellitus	0
	Hypercalcaemia	0
	Obesity	1
	Other	0
Cardiorespiratory	Asthma	2
	COPD	3
	Hypertension	5
	Cardiovascular other	4
Neoplastic disease	Benign breast disease	2
	Malignant melanoma (eye)	1*
	Pulmonary carcinoid	1*
Neurological disease	Epilepsy	3
	Migraine	2
	Multiple sclerosis	0
	CVA	0
Spinal disease	Spinal surgery (all)	9
	Vertebral fracture (all)	3
	Disc disease (all)	8
	Neoplasia (benign)	1
	Abscess	2†
	Spina Bifida	1

* = treated successfully, in remission at time of presentation

† = Pott's disease (TB spine); n = 1, pyogenic abscess; n = 1.

2.08 DRUG HISTORY

(excluding laxatives; NK = 34; total known = 96) *

Analgesia (all)	21 (22%)
Opiate containing medications †	11 (11%)
Non steroidal analgesia	3
Antidepressants (all)	7
Antianxiolytics	4
HRT	8
Inhalers for pulmonary disease	5
Nil	52

* only commonest used medications shown

† both analgesic and anti-diarrhoeal use included

2.09 FAMILY HISTORY

(NK = 39; total known = 91)

Constipation (any)	39 (43%)
Constipation (medical referral)	17 (19%)
Hirschsprung's disease	4
Nil	50 (55%)
Autoimmune & rheumatological	(see CHAPTER 7)

2.10 SYSTEMATIC ENQUIRY

Urological symptoms	(NK = 51; total known = 79)
Frequency	19 (24%)
Dysuria	4
Urgency	9
Nocturia	6
Incontinence (any)	15 (19%)
Total	31 (39%)

None	48 (61%)
Neurological symptoms	**(see CHAPTER 4)**
Rheumatological symptoms	**(see CHAPTER 7)**

5.01 SOLUTIONS FOR INJECTION:

Capsaicin was supplied (Sigma-Aldrich, Poole, Dorset, UK) as a colloidal mixture of 0.05µg/10µl in Tween-80 and saline as described by Simone *et al.*, (1989).

Nicotine hydrogen tartrate (BDH Chemicals Ltd) was supplied via Guys Hospital Pharmacy in 2ml glass vials at a final concentration of 10µg/ml.

Methacholine chloride solution was supplied in 2ml glass vials by Guys Hospital Pharmacy at a concentration of 100µg/ml.

All solutions were stored at 4°C, and expiry dates checked before use.

5.02 RESULTS OF CONVENTIONAL NEUROPHYSIOLOGICAL TESTS AND NON-INVASIVE AUTONOMIC TESTS

Routine neurophysiology

	STC		DM		Controls *
	Median	range	Median	range	Normal range
SNCV	46.7	38.6 - 70.0	34.8	0.0† - 47	> 42. 5
SNSAP	13.72.	4.8 – 26.8	2.0	0.0 – 13.4	> 7.8
PNCV	49.3	40.7 – 58.6	34.0	0.0 – 43.9	> 44.0
PNCAP	4.24	2.42 – 10.6	4.08	0.0 – 6.3	> 2.5
PNDML	4.5	1.6 – 10.9	2.7	0.0 – 7.8	< 6.0

Key:
- SNCV — Sural nerve conduction velocity (m / sec)
- SNSAP — Sural nerve sensory action potential (μV)
- PNCV — Peroneal nerve conduction velocity (m / sec)
- PNCAP — Peroneal nerve compound action potential (μV)
- PNDML — Peroneal nerve distal motor latency (msec)
- * — Dept. of Neurophysiology, Royal London Hospital control ranges for ages 18-50 years (c/o Dr Peter Misra).
- † — No response elicitable: value given as 0.0

Non-invasive autonomic tests

	Median	range	Median	range	Abnormal*
RR	32	8.4 – 66	11.0	2.5 – 26.0	< 10
VAL	1.38	1.10 – 2.04	1.11	1.03 – 1.15	< 1.2
BP tilt †	+ 4	0 – +18	+ 4	-30 – + 15	> -30
RR tilt	1.15	1.05 – 1.50	1.16	1.04 – 1.16	< 1.00

Key:
- RR — Heart-rate response to deep breathing (bpm difference)
- VAL — Valsalva ratio (ratio)
- BP tilt — Blood pressure response to standing using tilt-table (ratio)
- RR tilt — Heart-rate response to standing using tilt-table (ratio)
- * — Control ranges from literature: (Ewing, 1988)

†		Values may be positive (increase in blood pressure), or negative (drop in blood pressure i.e. postural hypotension)

5.03 FULL RESULTS OF QUANTITATIVE PERIPHERAL SENSORY AND AUTONOMIC TESTS

	STC		DM		Controls	
	Median	range	Median	range	Median	range
LT	4	1 - 9	9	4 –20	2	1 - 7
VIB	8	3 – 20	31	14 – 50	7	5 - 11
Cool	2.1	0.9 – 5.6	11.5	1.6 – 22.0	1.5	1.0 – 3.5
Warm	4.2	2.0 – 12.1	17.0	3.5 - >18.0	3.2	1.4 – 5.1
HeatPain	12.4	7.8 - >18 *	> 18	13.0 - > 18	12.0	7.7 – 16.1
ARS	40	7 – 74	31	4 – 63	47	32 - 84
DS	62	33 – 87	NP	NP	62	35 - 87
ARV	75	24 – 240	15	6 – 97	77	44 - 140

Key:

LT	Light touch threshold (no units)
VIB	Vibration threshold (Biosthesiometer units)
Cool	Cool threshold (°C)
Warm	Warm threshold (°C)
HeatPain	Heat as pain threshold (°C)
ARS	Axon reflex sweating ($gm^{-2}h^{-1}$)
DS	Direct sweating ($gm^{-2}h^{-1}$)
ARV	Axon-reflex vasodilatation (flux units)
NP	Not performed
*	No response within safety limit of test (>18°C)

6.01 SOLUTIONS AND BUFFERS

TBE: 90mM Tris-HCl, pH 8.0,
90mM boric acid,
2mM EDTA- made up as a 10X concentrate.

TNE (10X): 100mM Tris buffer,
10mM EDTA,
2M NaCl.

Formamide stop solution: 98% deionised formamide,
10mM EDTA, pH 8.0,
0.025% bromophenol blue,
0.025% xylene sulphate.

Loading buffer for agarose gels (10X): 10% sucrose,
0.025% bromophenol blue,
0.025% xylene cyanol.

Mutation detection enhancement (MDE)
Provided as gel solution (FMC bioproducts, Rockland, ME)

6.02 PUREGENE ® DNA ISOLATION

Cell lysis

1. 6 ml of whole blood added to 50ml tube containing 18 ml of RBC lysis buffer (provided in kit). Invert to mix and incubate for 10 mins at room temperature.
2. Centrifuge for 10 mins at 2000g. Remove supernatant leaving behind visible white pellet and small quantity of residual fluid.
3. Vortex the tube to resuspend WBC in residual supernatant.
4. Add 6 ml of cell lysis solution (in kit) to the tube, and pipette up and down to lyse cells. If cell clumps persist, incubate at 37°C until solution is homogenous.

RNAase treatment

1. Add 30µl RNAase A solution (in kit) to the cell lysate.
2. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15 mins.

Protein precipitation

1. Cool sample to room temperature
2. Add 2 ml of protein precipitation solution (in kit) to the cell lysate.
3. Vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate.
4. Centrifuge at 2000g for 10 mins until the precipitated proteins form a tight dark brown pellet.

DNA precipitation

1. Pour the supernatant containing the DNA into a clean 50 ml tube containing 6 ml 100% isopropanol.
2. Mix the sample by inverting gently 50 times until the white threads of DNA form a visible clump.
3. Centrifuge at 2000g for 3 mins.
4. Pour off supernatant and drain tube. Add 6 ml of 70% ethanol. Invert the tube several times to wash the DNA pellet.
5. Centrifuge at 2000g for 1 min. Carefully pour off the ethanol. Drain the tube and allow to air dry.

DNA hydration

1. Add 250µl DNA hydration solution (in kit).
2. Allow to rehydrate overnight at room temperature.
3. Store at - 4°C.

6.03 DNA SPECTOMETRY

1. Make 100ml of 1 x TNE in dH_2O (from 10 x TNE stock).
2. Add 10µl Hoechst dye stock (Pharmacia Biotech Inc., UK), shake to mix.
3. Add 2 ml of diluted dye to a 2 ml clean optical cuvette, dry exterior, and place in spectrometer and calibrate to zero.
4. With a 2µl pipette, add 2µl of DNA standard (calf thymus DNA, 100ng/ml) (Pharmacia Biotech Inc., UK) to the cuvette, mix and dry exterior.
5. Calibrate spectrometer to read 100 units.
6. Repeat zero and 100 unit calibrations until stable.
7. Repeat with 2µl test DNA and record reading 'x'.
8. Calculate DNA concentration 'y'in ng / ml by: y = x / 100.

6.04 TOUCHDOWN PCR PROTOCOLS

Temperatures (℃) & durations						*No of cycles*	
94	2 min					1	(denaturing)
94	10 sec	x + 4	10 sec	72	10sec	3	
94	10 sec	x + 2	10 sec	72	10sec	3	
94	40 sec	x	1 min	72	1 min	35	
72	10 min					1	(final elongation)

x = annealing temperature (below)

6.05 RET *and* GDNF *PRIMER SEQUENCES AND CONDITIONS*

Gene	**Exon**	**Primer**	**Annealing temp (°C)**
RET			
1	F	GCACCCGCCATCCAGACCC	60
	R	GAGCCGGCGGCCGGCAGAAC	
2	F	CCATATTCTCACCATCCCTC	50
	R	AGTGTCAGCGGCTGTGATAA	
3	F	GCCGATGCCCCCACAGACCT	56
	R	AAGACCAGCAGTAGCAGGCA	
4	F	CCCTGTCTGCTTGGTGCG	56
	R	GGACACTAAACCGACCGAG	
5	F	ACTGACCAACGCCCTCTG	56
	R	GCACCTCATTTCCTGGGGG	
6	F	ATTGTTGTGCCCCTACCTG	56
	R	CCCCAGACAGGCAATAGGTA	
7	F	TCTACCCTCAGGCCATTACAG	56
	R	GCTCCCAGACCCCGACCCT	
8	F	TGGTGCTGTTCCCTGTCC	56
	R	CCACCGGTGCCATCGCCCCT	
9	F	AGTCTGCTGTGTGTCCTGTG	56
	R	CCATGCCCTGATTAAACCCT	
10	F	GGAGGCTGAGTGGGCTACG	56

	R	CTGGGAGGTGGTGGTGGTC	
11	F	CCTCTGCGGTGCCAAGCCTC	56
	R	CCTCGTCTGCCCAGCGTTG	
12	F	GCCTTCTTCCTCCCCTGTCAT	50
	R	GAGACTCCCCCAGGGGCACTGT	
13	F	CTCTCTGTCTGAACTTGGGC	56
	R	TCACCCTGCAGCTGGCCTTA	
14	F	AAGACCCAAGCTGCCTGAC	56
	R	GTGGTGGGTCAGGGTGTGG	
15	F	GACTCGTGCTATTTTTCCTC	56
	R	TATCTTTCCTAGGCTTCCCA	
16	F	GTCTTTATTCCATCTTCTCT	56
	R	TCTGTAACCTCCACCCCAAG	
17	F	CACTGGTCCTTTCACTCTCT	56
	R	GGGAGGGAATGCACACAGAT	
18	F	TGTGGTGGGCTGTCCTTCTG	60
	R	CTGGGGTGAGGCTGGAGTCT	
19	F	TAGTTGTGGCACATGGCTTG	56
	R	GAGAGGAAGGATAGTGCAGA	
20	F	AGTTTTGGTTCTTCAGTGC	56
	R	GACTTTCCATTCTCAGCAT	

Additional for sequencing

2	R	TAAGGGCGGCTTGAGGAAGG	
4	F	TGTCTGCTTGGTGCGCAGGT	
	R	AGCACGCGCGGACAAGCAC	

GDNF

1	F	AAGTTATGGGATGTCGTGGC	56
	R	AGTCACTGCTCAGCGCGAA	
2	F	CAAATATGCCAGAGGATTATC	52
	R	TCAGATACATCCACACCTTTT	

Additional for sequencing

1	F	TGGGATGTCGTGGCTGT
	R	AGTCACTGCTCAGCGCGAAGG

6.06 SSCP GEL CONSTITUENTS (2 CONDITIONS)

1. 0.8% MDE (for 150 ml total)

60 ml 2 x MDE,

12 ml 10 x TBE,

78 ml H_20,

800µl 10% ammonium persulphate (APS),

80µl TEMED (reaction initiation).

2. 0.8% MDE / 10% glycerol (for 150 ml total)

60 ml 2 x MDE,

12 ml 10 x TBE,

63 ml H_20,

15ml glycerol,

800µl 10% ammonium persulphate (APS),

80µl TEMED (reaction initiation).

6.07 SILVER STAINING PROTOCOL FOR MDE GELS

1. Fix gels in a solution of 10% absolute ethanol / 0.5% acetic acid for 10 mins at room temperature.
2. Stain with 0.1% silver nitrate for 10 mins at room temperature.
3. Rinse gels with distilled water to remove excess silver solution.
4. Develop with a solution 1.5% NaOH / 0.01% $NaBH_4$ (sodium borohydride) / 0.048% formaldehyde for 20 mins at room temperature.
5. Fix stain with a solution of 0.75% $NaCO_3$ for 10 mins at room temperature.
6. Treat with 10% glycerol solution for 10-15 mins prior to drying gels.
7. Dry under vacuum for 90 mins at 80°C.

6.08 PREPARATION OF DNA FOR SEQUENCING

1. Add 80µl dH_2O to each reaction tube.
2. Precipitate DNA by vortexing with a solution of 15µl 2M sodium acetate, pH 4.6, 1µl glycogen and 250µl ethanol.
3. Store samples at -20°C for 60 mins.
4. Centrifuge at maximum speed for 20 mins.
5. Aspirate ethanol.
6. Wash in 70% ethanol.
7. Air dry DNA pellet.

6.09 PREPARATION OF ACRYLAMIDE SEQUENCING GELS

1. To 40g of urea, add 25 ml of δH_2O, 12 ml of acrylamide, and 1 g of amberlite (deionising) pellets. Stir until dissolved in a heated water bath.
2. Filter 8 ml of TBE followed by the gel mix and allow to degas.
3. Make up 10% APS.
4. To gel mix, add 400µl of 10% APS and 45µl of TEMED (reaction initiation).

7.01 PATIENT DETAILS

Main historical data for STC patients

Conditions	N	Description		N
Autoimmune disease	11	Personal history	Ankylosing spondylitis	1
			Psoriasis	2
		Family history only	IDDM	5
			Hypothyroidism	3
			Hyperthyroidism	1
			Rheumatoid arthritis	2
			Ankylosing spondylitis	1
Neurological disease	6	Personal history	Traumatic or degenerative spinal conditions	5
			Epilepsy	1
			Peripheral sensory neuropathy	1
Neoplastic disease	2	Personal history	Carcinoid (treated)	1
			Malignant melanoma (treated)	1

Symptoms	N	Notes	
Limb myalgia	5	pains in limb or limb girdle musculature	
Limb muscle weakness	3	investigations negative	
Limb arthralgia / arthritis	3	2 secondary to psoriatic arthropathy (hands only)	
		1 all rheumatological investigations: negative	
Distal parasthesias / numbness	6	4 hands only, 2 lower limb	
Urinary symptoms	11	frequency	7
		nocturia	2
		dysuria	2
		incontinence	6
		urgency	3
		retention	1

Main demographic data for patients included in assay studies

Group	*N*	*Sex F / M*	*age median (range) / years*	*duration of symptoms median (range) / years*
CIST	22	23 / 0	41 (25 – 62)	29 (17 – 47)
PIST	10	10 / 0	30 (26 – 55)	6 (1 – 32)
AOIST	10	10 / 0	38 (26 – 52)	6 (1 –12)
IP	10	3	52 (27 – 64)	10 (3 – 31)
IMC	4	1 / 3	32 (29 – 39)	28 (27 – 34)

7.02 ASSAY REAGENTS

All reagents were supplied by Sigma-Aldrich, Poole, Dorset, UK (Sigma), unless otherwise stated.

Protease inhibitors

Pepstatin A	P4265
Leupeptin	L2884
Phenylmethylsulphonylflouride (PMSF)	P7626

Detergents

Triton X100 (t-octylphenoxypolyethoxyethanol)	(9002.93-1)
Digitonin (50% pure)	Calbiochem-Novabiochem Corp (San Diego, California, USA)

Acetylcholine receptor (AChR) extract from TE671 and CN21 cell lines and AChR additive (bovine serum)

Both purchased from RSR Ltd., Cardiff, U.K.

Sheep anti-human IgG

Purchased from Dr Bernard Rees-Smith, Cardiff, UK

Unlabelled toxins

Calcium channels

Synthetic ω-conotoxin GVIA (4161-v)

Synthetic ω-conotoxin MVIIC (4283-v)

[Both purchased from Peptide Institute (European distributors, Scientific marketing, 189/191 High St, Barnet, Herts EN5 5SU). The toxins are received as lyophilised solids. They are reconstituted with distilled water at a concentration of 1mg/ml and stored at -70°C until use. Each toxin is used at a concentration of 1μM (mol wt. synthetic ω-conotoxin GVIA = 3161, synthetic ω-conotoxin MVIIC= 2750)].

Potassium channels

Synthetic α-dendrotoxin (α-DnTx) purchased from Latoxan (Valence, France)

Muscle AChR-α-1 channels

δ-Bungarotoxin (T3019)

Labelled toxins

Calcium channels

^{125}I-ω-conotoxin GVIA (^{125}I-ω-CgTx) purchased from Amersham International (IM217, 10μCi, S.A. > 2000 Ci/mmol).

^{125}I-ω-conotoxin MVIIC (^{125}I-ω-CmTx) purchased from New England Nuclear Ltd, Mass, USA (5μCi, S.A. > 2000 Ci/mmol).

Potassium channels

^{125}I-α-dendrotoxin (^{125}I-α-DnTx) purchased from Amersham International

Muscle AChR-α-1 channels

^{125}I-α-Bungarotoxin low specific activity (^{125}I-α-BnTx) purchased from Amersham International (IM109)

[The toxins are received as lyophilised powder. They are reconstituted with distilled water at a concentration of 0.05μCi/5μl and aliquoted into 5μCi amounts (0.5ml) and stored at -70°C until use].

7.03 ASSAY BUFFERS

1. Solubilisation buffer for brain membrane preparation and VGCC assay (SB)

pH 7.4, stored at -20°C until use:

25mM Tris-HCl	2.15g	
5mM HEPES	1.19g	for 1 litre
0.32M Sucrose	0.32g	

Just before use, add protease inhibitors:

Pepstatin	final concentration 1μM
Leupeptin	final concentration 2μM
PMSF	final concentration 0.1μM

2. 4% digitonin in SB

Digitonin purchased is 50% pure. Therefore the solution is made up to 8%: 1.6g digitonin in 20ml SB. This "solution" is left overnight at 4°C before use. There is a lot of insoluble material remaining; before use in the assay an aliquot must be microfuged: 13,000rpm / 5 min and clear supernatant removed for subsequent use.

3. 1M NaCl in SB 5.84g NaCl in 100ml SB

4. 2% Digitonin in solubilisation buffer for VGKC extraction only (DTX)

20mM Tris

100mM NaCl

5mM KCl

Natural pH ≅ 10.0, therefore add HCl to pH 7.12

1. To 2ml (will be 2.5 ml when 500µl membranes added), add digitonin 0.05g (50%) to give final concentration of 2% (active concentration: 1%).
2. Incubate in waterbath at 37°C for 15 min.
3. Just before use, add protease inhibitor: PMSF: 5µl of 0.1M in propan-2-ol (final concentration 0.2mM).

5. Incubation buffer (IB) (for VGCC assay only)

0.1% digitonin in 20mM phosphate buffer pH 7.4, stored at 4°C

500µl digitonin 4%

17.5 ml dH_20

2ml 0.2M Phosphate

6. Washing buffer (WB syn. PTX)

0.1% Triton X100 in 20mM phosphate buffer pH 7.4, stored at 4°C

7.04 BRAIN-MEMBRANE PREPARATION

The protocol is identical whether using human (frontal or cerebellar tissue) or rabbit tissue, and is a common step to both calcium and potassium channel assays. To prepare membranes from rabbit brain, 2 – 3 cm^3 sections of cerebellum were added immediately post-mortem to 20 ml of ice-cold buffer (25mM Tris-hydrochloric acid [HCl], 5mM HEPES, containing 0.32M sucrose, 2µM leupeptin, 1µM pepstatin, 0.1mM phenylmethylsulfonylfluoride [PMSF]). The sections were homogenised and centrifuged. The supernatants were removed, recentrifuged, and the pellets resuspended at a final protein concentration of 20 to 25 mg/ml and stored at -70°C.

1. Tissue removed as soon as possible post-mortem, cut into small pieces and dropped into liquid nitrogen. Then stored at -70°C until use.
2. Take 'x' gram of cerebellum, and make up with ice cold SB (with protease inhibitors added immediately before use) to 6-8 x volume of 'x'.

3. Homogenise using a Polytron Homogeniser (PT10-35, Kinematica GmbH, Littau, Switzerland). Full speed 3 x 10 sec, resting on ice in-between.
4. Spin 1000rpm (50g) / 10 min (Beckman J2-21 centrifuge).
5. Harvest supernatant.
6. Spin 13,000rpm (10,000g) / 10 min (Beckman J2-21 centrifuge).
7. Resuspend pellet in a volume of SB equivalent to original brain tissue weight.
8. Aliquot in 500µl amounts. Then store at -70°C until use.

7.05 PREPARATION OF CHANNEL EXTRACTS

Voltage-gated calcium channel (VGCC) extract preparation

(made in batches for storage before subsequent use)

1. Defrost aliquots of brain-membrane preparation.
2. Add 700µl SB (with fresh protease inhibitors), vortex, spin 13,000rpm / 5min (bench-top Eppendorf centrifuge), discard supernatant.
3. Add 1 ml SB, vortex, spin 13,000rpm / 5 min, discard supernatant.
4. To the pellet (approx. 200µl), add:

1M NaCl in SB	100µl
SB	200µl
4% digitonin in SB	500µl

therefore the final solution will be 2% digitonin, 0.1M NaCl

5. Rotate 1h, 4°C.
6. Spin 13,000rpm / 15 min.
7. Remove supernatant as source of VGCs; store at -70°C.

Voltage-gated potassium channel (VGKC) extract preparation

(made fresh for immediate use)

1. Defrost aliquots of brain-membrane preparation.
2. Add stepwise (2 x 250µl) to 2ml DTX (with fresh protease inhibitor), mix gently with pipette (1 : 5 dilution) (total volume 2.5ml: see above).
3. Incubate 37°C for 15 min.
4. Spin 13,000rpm / 15min (bench-top Eppendorf centrifuge).
5. Harvest the supernatant (VGKC extract) and store on wet ice until use.

7.06 VGCC RADIOIMMUNOASSAYS USING ^{125}I-ω-CONOTOXIN MVIIC (^{125}I-ω-CmTx) TO LABEL CEREBELLAR EXTRACTS OR ^{125}I-ω-CONOTOXIN GVIA (^{125}I-ω-CgTx) TO LABEL FRONTAL CORTICAL EXTRACTS

1. Defrost extracts on wet ice.
2. If not performed previously, carry out a total (labelled toxin only) and a non specific binding study (in the presence of excess unlabelled "cold" toxin). See appendix 7.11
3. In a 7ml bijou, add 25µl IB to 5µl extract (^{125}I-ω-CmTx: cerebellar extract) or 20µl IB to 10µl extract (^{125}I-ω-CgTx: frontal extract), i.e. to a total of 30µl per sample, multiplied by the total number of samples to be tested in assay.

i.e.	^{125}I-ω-CmTx:	for 50 samples = 250µl extract + 1.25ml IB
	^{125}I-ω-CgTx	for 50 samples = 500µl extract + 1.00ml IB

4. Defrost 1.0µCi aliquots of either ^{125}I-ω-conotoxin, make up each aliquot with 500µl IB, spin 13,000rpm / 5 min.
5. Add 5 fmol of either ^{125}I-ω-conotoxin per sample, usually 6-10 µl / sample: see appendix 7.12 for calculation.
6. Make up total volume / sample to 50µl with IB. i.e. 30µl + 6-10µl (above) + 20-

(6-10)µl. This is the labelled extract preparation.

7. Incubate 2h at 4°C on wet ice.
8. Dilute 10µl test sera 1 / 10 with IB, vortex, spin 13,000rpm / 5 min.
9. Add 25µl of supernatant from each sample of diluted serum to 50µl of labelled extract, vortex, and incubate overnight at 4°C.
10. Add 125µl of 1 / 5 diluted sheep anti-human IgG (diluted in PTX) to each sample (not microfuged)
11. Incubate 1h at 4°C.
12. Add 1ml PTX, mix gently, spin at 13,000rpm / 5 min (Bench-top Eppendorf centrifuge).
13. Remove supernatant by suction, wash pellet twice in 1ml PTX.

[N.B if large number of samples, perform steps 12 and 13 stepwise in sets to prevent time dependent dissociation of complexes into PTX].

14. Remove excess liquid. Count pellet on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).
15. Calculate final binding of labelled extract in pM / l using algorithm (Appendix 7.09)

7.07 VGKC RADIOIMMUNOASSAY USING ^{125}I-α-DENDROTOXIN (^{125}I-α-DNTX) TO LABEL CEREBELLAR EXTRACTS

1. Label freshly made VGKC extract (above). Calculate total amount required (50µl x number of samples), and mix gently with pipette:

   x µl VGKC extract

   x µl PTX

   x / 10 ^{125}I-α-DnTx

2. Incubate at room temp for 15 min.
3. Transfer to Eppendorfs, and microfuge 13,000rpm / 5 min before use.
4. Dilute 25µl patient sera 1 / 10 with PTX, vortex, spin 13,000rpm / 5 min.

5. Add 50µl of supernatant from each sample of diluted serum to 50µl of labelled extract, vortex, and incubate overnight at 4°C.
6. Add 250µl of 1 / 6 diluted sheep anti-human IgG (diluted in PTX) to each sample (not microfuged)
7. Incubate 1h at room temp.
8. Add 1ml PTX, vortex, spin at 13,000rpm / 5 min (Bench-top Eppendorf centrifuge).
9. Remove supernatant by suction, wash pellet twice in 1ml PTX.

[N.B if large number of samples, perform steps 12 and 13 stepwise in sets to prevent time dependent dissociation of complexes into PTX].

10. Remove excess liquid. Count pellet on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).

7.08 MUSCLE AChR RECEPTOR ASSAY USING ^{125}I-∂BUNGAROTOXIN (^{125}I-∂BuTx)

1. Add one part RSR CN21 extract to one part RSR TE671 extract and note the volume.
2. To the known volume add the RSR AChR "additive" at the current dilution and mix. (e.g. lot RE2 uses 165µl per ml of undiluted extract).
3. Leave at 4°C for 30 min.
4. Centrifuge 10,000 rpm at 4°C for 30 min.
5. Collect and measure the supernatant.
6. Dilute the supernatant 1:6 with PTX.
7. Dilute the 125-∂-bungarotoxin (BuTx) 1:10 with PTX in a sarstedt tube.
8. To two eppendorf tubes pipette 10µl of diluted BuTx each.
9. Count on gamma camera.
10. Calculate the required volume of diluted BuTx to label the extract at 1 x 10^6 counts per ml AChR extract. Divide the mean counts from the 10µl counted tubes by 10. Then divide 1 x 10^6 by this figure. This will give the volume in µl of

diluted BuTx needed per ml of AChR extract.

11. Add the required volume of BuTx, mix and leave for a minimum of 10 min (ideally more than 1h) before use.
11. Dilute 25µl patient sera 1 / 10 with PTX, vortex, spin 13,000rpm / 5 min.
12. Add 50µl / 10µl of the serum dilution to 2 further tubes for each sample (i.e. 2 rows).
13. Add 50µl labelled extract to both rows of tubes, mix, and incubate overnight at 4°C.
14. Add 250µl diluted sheep anti-human IgG (1:8 in PTX) to all tubes containing 50µl diluted sera and 75µl to those tubes containing 10µl diluted sera.
15. Leave for 1h at room temp.
16. Add 800µl / 400µl of PTX, mix gently, spin at 13,000rpm / 5 min (Bench-top Eppendorf centrifuge).
17. Remove supernatant by suction, wash pellet twice in further PTX.
18. Remove excess liquid. Count pellet on automatic gamma counter for 1 minute (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).

7.09 CONVERSION OF OBSERVED COUNTS TO CONCENTRATIONS OF ANTIBODY

Final concentrations of antibody are expressed as pmol / l serum (i.e. pM).

1. VGCC assays (P/Q and N-type)

Conversion is dependent on isotope decay. The conversion factor (κ) may be calculated from a further variable (λ) which is date dependent, and taken from the decay table (below), and the volume of serum used (2.5µl for all assays), where:

$\kappa = \lambda$ (fmol cpm-1) / 2.5µl

The final concentrations of antibody are then calculated, where:

[ab] = (observed cpm – mean control cpm) . κ

DECAY TABLE (see appendix 7.09)

	S.A.(Ci/mmol	1 cpm -fmol	5 fmol-cpm	DAYS	S.A.(Ci/mmol	1 cpm-fmol	5 fmol-cpm
-29	2572	0.000219	22839	21	1567	0.000359	13915
-28	2556	0.000220	22697	22	1548	0.000364	13746
-27	2539	0.000222	22546	23	1530	0.000368	13586
-26	2522	0.000223	22395	24	1512	0.000372	13427
-25	2505	0.000225	22244	25	1495	0.000377	13276
-24	2488	0.000226	22093	26	1478	0.000381	13125
-23	2473	0.000228	21960	27	1461	0.000385	12974
-22	2458	0.000229	21827	28	1444	0.000390	12823
-21	2433	0.000231	21605	29	1428	0.000394	12681
-20	2414	0.000233	21436	30	1412	0.000399	12539
-19	2396	0.000235	21276	31	1395	0.000404	12388
-18	2378	0.000237	21117	32	1378	0.000409	12237
-17	2359	0.000239	20948	33	1362	0.000413	12095
-16	2340	0.000241	20779	34	1346	0.000418	11952
-15	2320	0.000243	20602	35	1331	0.000423	11819
-14	2300	0.000245	20424	36	1316	0.000428	11686
-13	2280	0.000247	20246	37	1301	0.000433	11553
-12	2260	0.000249	20069	38	1286	0.000438	11420
-11	2240	0.000251	19891	39	1271	0.000443	11286
-10	2220	0.000254	19714	40	1256	0.000448	11153
-9	2199	0.000256	19527	41	1242	0.000453	11029
-8	2178	0.000259	19341	42	1228	0.004590	10905
-7	2156	0.000261	19145	43	1214	0.000464	10780
-6	2134	0.000264	18950	44	1200	0.000469	10656
-5	2112	0.000267	18755	45	1186	0.000475	10532
-4	2090	0.000269	18559	46	1172	0.000480	10407
-3	2068	0.000272	18364	47	1158	0.000486	10283
-2	2046	0.000275	18168	48	1144	0.000492	10159
-1	2023	0.000278	17964	49	1131	0.000498	10043
0	2000	0.000281	17760	50	1118	0.000504	9928
1	1977	0.000285	17556	51	1105	0.000510	9812
2	1954	0.000288	17352	52	1092	0.000516	9697
3	1932	0.000291	17156	53	1080	0.000521	9590
4	1910	0.000295	16961	54	1068	0.000527	9484
5	1888	0.000298	16765	55	1055	0.000534	9368
6	1866	0.000302	16570	56	1042	0.000540	9253
7	1844	0.000305	16375	57	1030	0.000547	9146
8	1822	0.000309	16179	58	1018	0.000553	9040
9	1801	0.000313	15993	59	1007	0.000559	8942
10	1780	0.000316	15806	60	996	0.000565	8844
11	1760	0.000320	15629	61	984	0.000572	8738
12	1740	0.000324	15451	62	972	0.000579	8631
13	1720	0.000327	15274	63	961	0.000586	8534
14	1700	0.000331	15096	64	950	0.000593	8436
15	1680	0.000335	14918	65	939	0.000600	8338
16	1660	0.000339	14741	66	928	0.000607	8241
17	1641	0.000343	14572	67	918	0.000613	8152
18	1622	0.000347	14403	68	908	0.000620	8063
19	1604	0.000351	14244	69	897	0.000628	7965
20	1586	0.000355	14084	70	886	0.000636	7868

e.g. on day –15

$\kappa = 0.000243$ (fmol cpm^{-1}) / 2.5µl

$\kappa = 0.243$ (pmol cpm^{-1}) / 2.5 l

$\kappa = 0.0972$ (pmol $cpm^{-1}\ l^{-1}$)

and: [ab] = (observed cpm – mean control cpm) . 0.0972

2. VGKC and AChR assays

Conversion factor = 0.1 (not isotope date dependent). Therefore final concentration = (observed cpm – mean control cpm) * 0.1

7.11 TOTAL AND NON-SPECIFIC BINDING CURVES FOR VGCC RADIOIMMUNOASSAY.

1. Perform this every 2 months.
2. Calculate a quantitative precipitation curve for known strongly positive (LEMS) and known negative (healthy control) sera. Total binding is calculated by using increasing serum concentrations in the presence of labelled toxin only, and non-specific binding, by repeating the assay at each serum concentration in the presence of excess unlabelled toxin. The specific binding is calculated at each point by subtracting the non-specific binding from the total binding.

7.12 CALCULATION OF VOLUME EQUIVALENT OF 5fMOL ^{125}I-ω-CONOTOXINS

1. Count actual omission in cpm of 10µl of either diluted ^{125}I-ω-conotoxin.
2. Find "expected count" using decay table (see below) and knowledge of the time period since preparation by the manufacturer (Amersham, UK). N.B "minus" days are a nuance of the decay curve and manufacture.
3. Calculate the volume (x) equal to 5fmol by using the equation:

   actual cpm / 10 µl = expected cpm / x

   i.e. on day – 23, if the observed count for 10µl is 37,000 cpm, then:

   37,000 / 10 = 21,960 / x

   and x = 6 µl (1 s.f).

8.01 SOLUTIONS AND REAGENTS

All reagents were supplied by MERCK (BDH) Chemicals, Poole, Dorset, UK (not stated) or Sigma-Aldrich, Poole, Dorset, UK (Sigma). Stept-ABC kit was supplied by DAKO, Ely, Cambridge, UK (kit code K0377).

Ethanol is absolute unless otherwise stated. IMS = 100% industrial methylated spirits.

General

Acid Alcohol	300ml dH_2O, 700ml IMS, 10ml conc. hydrochloric acid. (added to 990ml of the above)
Scott's Tap Water Substitute	2g potassium hydrocarbonate, 20g magnesium sulphate, 1000ml dH_2O.
Fixative and differentiator	99ml 95% ethanol, 3ml glacial acetic acid.
Hotchkiss Periodic Acid	0.4g periodic acid, 5ml 0.2M sodium acetate, 10ml dH_2O, 35ml ethanol.
Hotchkiss Reducing Rinse	1g potassium iodide, 1g sodium thiosulphate, 20ml dH_2O, 30ml ethanol, 1ml 1M HCl.

Routine Dyes

Gill's Haematoxylin	1L dH_2O, 625ml ethylene glycol, 10g haematoxylin, 1g sodium iodate,

	176g aluminium sulphate, 50ml glacial acetic acid, 825ml dH_2O.
Weigert's haematoxylin	1g haematoxylin, 100ml ethanol, 4ml 30% ferric chloride, 100ml dH_2O, 1ml hydrochloric acid.
1% Eosin	1g eosin Y, 100ml dH_2O.
Schiff's Reagent	1g basic fuchsin, 50ml 1M hydrochloric acid, 1g anhydrous sodium metabisulphate, 200ml dH_2O.
Celestine blue	1g solochrome prune, 0.5ml conc. hydrochloric acid, 100 ml 2.5% iron alum, 14ml glycerol.
Alcian blue	pH 0.2 – 1% Alcian Blue in 10% sulphuric acid. pH 1.0 – 1% Alcian Blue in 0.1M hydrochloric acid. pH 2.5 – 1% Alcian Blue in 3% acetic acid.
Congo red	0.5g Congo red, 50ml ethanol, 50ml dH_2O.
Congo red differentiator	0.2g potassium hydroxide, 100ml 50% ethanol.
Van Gieson solution	100ml saturated aqueous picric acid, 10 ml 1% aqueous acid fuchsin.
Ponceau fuchsin	0.7g Ponceau 2R, 0.35g acid fuchsin, 1ml glacial acetic acid,

	100ml dH_2O.
Sudan black solution	Saturated Sudan black B (in 70% alcohol).
Toluidine blue	1g toluidine blue, 1g sodium tetraborate, 100ml dH_2O.

Immunohistochemistry

Endogenous peroxidase block (0.3%)	1ml 30% hydrogen peroxide, 99ml 100% methanol.
Antibody diluent	Sodium chloride 4.1g, Tris 0.3g, Bovine albumin 0.2g (Sigma), Sodium azide 0.2g, 1M HCl 2ml, Casein 50ul (Sigma), dH2O 500ml.

Shake well, and adjust to pH 7.6 with 0.1M sodium hydroxide

0.05M Tris buffered saline pH 7.6	Sodium chloride 8.76g, Tris 6.06g, 1M HCl 36ml, dH_20 800ml.

Leave to stand for 1 hour, and then adjust to pH 7.6 with 1M HCl. Make up to 1L with dH_20

Stept-avidin-biotin-complex Solution (Strept-ABC) (DAKO)	TBS 5ml, Solution A & B: 1 drop.
Diaminobenzidine tetrachloride (DAB) solution	0.05M Tris buffered saline pH 7.6: 10ml, DAB 6mg (Sigma), Fresh 3% hydrogen peroxide 0.1ml.

Antigen retrieval

Citrate buffer working solution	Citrate buffer stock 100ml (see below), dH_2O 900ml.

Place into the large straining trough and mix thoroughly with a magnetic stirrer. Whilst still on the stirrer, pH to exactly 6.0 with 1M sodium hydoxide.

Citrate buffer stock	Citric acid 7.56g, Trisodium citrate 47.56g, dH_2O 2000ml.

store at 4°C.

EDTA buffer working solution	EDTA buffer stock 100ml (see below), dH_2O 900ml..

Place into the large straining trough and mix thoroughly with a magnetic stirrer. Whilst stirring, pH to exactly 8.0 with 1M sodium hydoxide.

EDTA buffer stock	EDTA 3.7g, dH_2O 1000ml.

8.02 STC TISSUE PROCESSING PROTOCOL

For freshly obtained colonic or ileal tissues:

1. Serosa and pericolic fat +/- any faecal residue removed.
2. Full thickness sections taken for fixation overnight in:
   a. 10% formal saline.
   b. Zamboni fixative.
   c. Bouin's fixative.
   d. 4% paraformaldehyde.
   e. Glutaraldehyde for EM.
3. Full thickness sections embedded fresh in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), using liquid nitrogen-cooled isopentane.
4. Full thickness section frozen fresh in liquid nitrogen.

5. Tissue layer separated into mucosa and muscularis propria components and:
   a. Small sections weighed and frozen fresh in liquid nitrogen
   b. Small sections extracted as protocol for future neurotrophin assay studies [details not included in thesis, although performed by author].

8.03 H&E AND PAS ROUTINE STAINING

H&E

1. Dewax section in xylene x 2.
2. Remove xylene with IMS x 2.
3. Rehydrate in dH_2O.
4. Stain in Gill's haematoxylin for 20 minutes.
5. Wash in tap water for 5 minutes (Scott's tap water substitute followed by a rinse in tap water may be used instead of washing in water.
6. Differentiate in 1% acid alcohol.
7. Wash in tap water for 5 minutes.
8. Stain in eosin for 3 minutes.
9. Differentiate in tap water.
10. Blot.
11. Dehydrate rapidly in IMS x 2.
12. Clear in xylene x 2.
13. Mount in Canada balsam.

PAS

1. Dewax section in xylene x 2.
2. Remove xylene with IMS x 2.
3. Treat with Hotchkiss periodic acid solution for 5 minutes.
4. Wash well in 70% ethanol.
5. Treat with Hotchkiss reducing rinse for 30 seconds.
6. Wash well in 70% ethanol.
7. Stain in Schiff's reagent for 10 – 30 minutes (dependent on the batch of Schiff's reagent in use).
8. Wash in tap water for 10 minutes.
9. Stain nuclei in celestine blue for 1 minute.
10. Wash in tap water for 5 minutes.
11. Differentiate if required in 1% acid alcohol
12. Wash in tap water for 5 minutes.
13. Rehydrate in IMS then xylene x 2.
14. Mount in Canada balsam

D-PAS

1. Dewax in xylene x 2.
2. Remove xylene with IMS x 2.
3. Wash with dH_2O.
4. Incubate with 0.1% aqueous malt diastase (BDH) at 37°C for 30 minutes.
5. Repeat as from step 2 of PAS protocol.

8.04 OTHER ROUTINE STAINING METHODS

ALCIAN BLUE

1. Dewax in xylene x 2.
2. Remove xylene with IMS x 2.
3. Wash with dH_2O.
4. Stain in Alcian blue for 30 minutes.
5. Wash in tap water for 10 minutes.
6. If required, lightly counterstain in filtered 1% aqueous neutral red for 1 minute.
7. Blot sections.
8. Rehydrate in IMS then xylene x 2.
9. Mount in Canada balsam.

CONGO RED

1. Dewax Sections in xylene x 2.
2. Remove xylene with IMS x 2.
3. Stain in Gill's haematoxylin for 15 minutes.
4. Wash in tap water for 5 minutes.
5. Differentiate in 1% acid alcohol.
6. Wash in tap water for 5 minutes.
7. Rinse in 70% alcohol.
8. Stain in filtered Congo red in a coplin jar for 15 minutes.
9. Blot.
10. Differentiate in Congo red differentiator.
11. Wash in tap water for 5 minutes.
12. Rehydrate in IMS then xylene x 2.
13. Mount in Canada Balsam.

ELASTIC VAN GIESON

1. Dewax section in xylene x 2.
2. Remove xylene with IMS x 2.
3. Rehydrate in dH_2O.
4. Stain in Weigert's iron haematoxylin solution for 30 minutes.
5. Wash in tap water for 5 minutes.
6. Differentiate in 1% acid alcohol.
7. Wash in tap water for 5 minutes.
8. Stain in van Gieson solution for 3 minutes.
9. Blot.
10. Rehydrate in IMS then xylene x 2.
11. Mount in Canada balsam.

MASSONS TRICHROME

1. Dewax sections in xylene x 2.
2. Remove xylene with IMS x 2.
3. Stain in Weigert's haematoxylin for 10 minutes.
4. Wash in tap water for 5 minutes.
5. Differentiate in 1% acid alcohol.
6. Wash in tap water for 5 minutes.
7. Stain in Ponceau fuchsin for 10 minutes.
8. Rinse in dH_2O.
9. Differentiate in 1% phosphotungstic acid.
10. Counterstain progressively in 2% light green.
11. Rinse in dH_2O.
12. Rehydrate in IMS then xylene x 2.
13. Mount in Canada balsam.

Phosphotungstic acid haematoxylin (PTAH)

1. Dewax section in xylene x 2.
2. Remove xylene with IMS x 2.
3. Treat with 0.25% aqueous potassium permanganate solution for 5 minutes.
4. Wash in tap water for 5 minutes.
5. Bleach with 5% aqueous oxalic acid solution.
6. Wash in tap water for 5 minutes.
7. Stain in PTAH solution for 12 – 24 hr at room temperature.
8. Wash in dH_2O.
9. Rehydrate in IMS then xylene x 2.
10. Mount in Canada balsam

SUDAN BLACK

1. Paraffin sections to 70% alcohol.
2. Stain overnight at room temperature in sudan black solution.
3. Wash in tap water for 5 minutes.
4. Wash out excess background in 70% alcohol.
5. Wash in tap water for 5 minutes.
6. Mount in aqueous mountant.

TOLUDINE BLUE

1. Fix in acetic acid for 1 minute.
2. Stain in toluidine blue for 1 minute.
3. Wash briefly in tap water.
4. Rinse in ethanol.
5. Differentiate in acetic alcohol.
6. Rinse in ethanol.
7. Clear in xylene
8. Mount in Canada balsam

8.04 IMMUNOHISTOCHEMICAL STAINING PROTOCOL USING DAKO STREPT-ABC

1. Dewax (xylene) and rehydrate sections in 2 changes 100% IMS.
2. Incubate endogenous peroxidase block for 15 minutes.
3. Wash sections in tap water for 2 minutes.
4. If antigen retrieval is required (below), go to appropriate procedure.
5. Transfer to tap water and wash for 2 minutes.
6. Soak sections in at least 2 changes of TBS for 3 minutes.
7. Dry carefully around the sections and encircle with PAP (DAKO) pen.
8. Apply blocking serum for 20 mins:
   - polyclonal antibodies: normal swine serum 1: 5 in antibody diluent.
   - monoclonal antibodies: normal rabbit serum 1: 5 in antibody diluent.
9. Drain blocking serum.
10. Apply primary antibody at the appropriate dilution in antibody diluent for 60 minutes (antibody diluent only to negative control sections).
11. Rinse section twice in TBS
12. Wipe around the sections, and apply secondary antibody for 30 minutes:

- polyclonal antibodies: biotinylated swine anti-rabbit 1: 200 in antibody diluent.
- monoclonal antibodies: biotinylated rabbit anti-mouse 1: 200 in antibody diluent.

13. Make up the Stept avidin-biotin complex solution (Strept-ABC). Vortex, and allow to complex for at least 30 minutes before use.
14. Rinse section twice in TBS
15. Apply Strept-ABC for 30 minutes
16. Rinse section twice in TBS
17. Apply filtered DAB solution for approximately 10 minutes (examine positive control slide for progress of development)
18. Rinse section twice in TBS
19. Counterstain (usually Gill's haematoxylin) if necessary
20. Dehydrate and mount.

8.05 ANTIGEN RETRIEVAL

Microwave retrieval

1. Place sections into black plastic staining rack leaving a gap between the slides.
2. Place rack into a large glass staining trough containing exactly 1000ml of the appropriate buffer.
3. Cover the trough with cling film and make several holes in the cling film.
4. Place the trough in the microwave and run at full power for 18 minutes.
5. With care, remove trough from microwave.
6. Carefully remove cling film.
7. Leave sections to stand in the hot buffer for 5 minutes.
8. Return to original technique (stage 5 of IHC protocol 8.04).

Trypsin retrieval

1. Place one empty coplin jar and one filled with dH_20 in a waterbath at 37°C.
2. Add 0.1g Trypsin to 100ml of 0.1% calcium chloride and mix.
3. Adjust to pH 7.8 with 0.1M sodium hydroxide.
4. Place sections in the preheated dH_20 at 37°C for 10mins.
5. Bring trypsin solution to 37°C using a hot plate stirrer (do not overheat).
6. Pour trypsin into the preheated coplin jar.

7. Place sections into the trypsin solution at 37°C for the appropriate time.
8. Return to original technique (stage 5 of IHC protocol 8.04).

Pronase retrieval

1. Microwave in citrate buffer (above).
2. Leave sections to stand in the hot buffer for 5 mins.
3. Wash briefly in dH_20.
4. Treat with Pronase (Protease XIV, Sigma) in PBS pH 7.4.
5. Return to original technique (stage 5 of IHC protocol 8.04).