Psychological stress and hypnosis in ulcerative colitis.

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PSYCHOLOGICAL STRESS AND HYPNOSIS IN ULCERATIVE COLITIS

By

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ABSTRACT

Previous studies suggest that life events and chronic stress increase the risk of relapse in inflammatory bowel disease. Furthermore, experimental stress has been shown to worsen inflammation in animal models of colitis. Hypnotherapy is effective for functional gastrointestinal (GI) disorders and claimed by some patients to improve ulcerative colitis (UC).

Two major hypotheses are tested in this thesis:

i) Psychological stress can worsen inflammation via its effects on various systemic and rectal mucosal inflammatory variables in quiescent UC.

ii) Relaxation achieved through hypnosis can reduce inflammation via its effects on various systemic and mucosal inflammatory variables in active UC.

Patients with UC and healthy controls underwent an experimental stress test, hypnotherapy session or control procedure. Various systemic and, in patients with UC, rectal mucosal inflammatory measures were assessed before and after each procedure.

The major findings are as follows:

i) In patients with inactive UC, acute experimental stress increased LPS-stimulated TNF-α and IL-6 production by whole blood. Stress also increased leukocyte count, Natural Killer (NK) cell count, platelet activation and platelet-leukocyte aggregeate (PLA) formation. At the mucosal level, stress increased TNF-α in peri-mucosal fluid, and mucosal ROM production; it reduced rectal mucosal blood flow (RMBF).

ii) In patients with active UC, one session of hypnotherapy reduced serum IL-6 concentration and caused a transient reduction in NK cell numbers. At the mucosal level, hypnotherapy caused a reduction in the concentration in peri-mucosal fluid of Substance P, histamine and IL-13 and reduced RMBF.

iii) Chronic stress, as assessed by psychometric questionnaires, did not affect the response to acute experimental stress.

iv) There was no difference between the responses of patients with UC and healthy volunteers to any protocol.

In conclusion, stress increased, whilst hypnotherapy reduced various inflammatory measures at both the systemic and mucosal level in patients with UC. These effects might contribute to the reported adverse effects of stress and therapeutic efficacy of hypnotherapy in UC.

STATEMENT OF ORIGINALITY

An initial protocol for the study was in existence before my appointment as Clinical Research Fellow. Initial ethical approval for the study had also been granted by the North-East London Strategic Health Authority Ethics Committee. I then developed this protocol, in conjunction with Professor Rampton, into the final version described in this thesis.

I recruited the patients and healthy volunteers to the study, and performed the stress and control protocols entirely by myself. Dr David Jenkins, a qualified hypnotherapist, performed the sessions of hypnosis with both patients and healthy volunteers (Chapters 3, 8 and 9) whilst I measured the physiological variables and collected the various pre and post-hypnosis samples.

I measured the serum cytokines levels by ELISA and processed the samples to assess cytokine release by LPS-stimulated whole blood. I performed the various flow cytometry experiments necessary to assess the samples for NK cell count, platelet activation and platelet-leucocyte aggregate formation. I also performed the experiments to measure the cytokine levels in peri-mucosal fluid, reactive oxygen metabolite production by mucosal biopsies and rectal mucosal blood flow.

Dr Roger Feakins supervised the conventional haematoxylin and eosin staining of the pre- and post-stress mucosal biopsies and scored the degree of inflammation present (Chapters 3 and 7).

Dr Neil Rayment performed the immunofluorescence studies for mast cell activation on the pre- and post-stress rectal mucosal biopsies. He also performed the fluorescent in situ hybridisation studies to assess the mucosa-associated bacterial flora using a technique already developed in his laboratory (Dept of Life Sciences, Kings College London, London, UK)(Chapter 3 and 7).

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

In this chapter I will first provide a very brief overview of inflammatory bowel disease (IBD). This will be followed by a more detailed description of the factors involved in the aetiology of IBD. Particular attention will be paid to the role of psychological stress in the pathogenesis of IBD, as this subject forms the basis for much of this thesis. I will also give a description of the pathogenesis of IBD with an emphasis placed on the cells and inflammatory mediators studied in this thesis.

1.1.1 INFLAMMATORY BOWEL DISEASE

Crohn's disease (CD) and ulcerative colitis (UC) are chronic, relapsing and remitting inflammatory diseases of the gastrointestinal (GI) tract. They are characterised by a marked temporal variation in mucosal inflammation, from near normal in remission to severe ulceration during relapse. Both diseases are life-long, usually requiring continuous medication and often surgery.

1.1.2 CROHN'S DISEASE

CD is characterised by chronic transmural granulomatous intestinal inflammation with a tendency to form strictures and fistulae. It can affect any part of the GI tract, and often does so in discontinuity to form "skip" lesions. Most commonly it affects terminal ileum and ascending colon (ileocolonic disease).

CD is classified according to the site, extent and behaviour of disease. These factors govern the clinical symptoms. Active CD characteristically produces the triad of abdominal pain, diarrhoea and weight loss, although the preponderance of each symptom varies within and between patients. Small bowel CD is often complicated by the development of luminal stenosis due to inflammatory or fibrotic strictures leading to obstructive symptoms; fistulation and local abscess formation are more common. Colonic CD is less often complicated by strictures and tends to cause more severe diarrhoea than small bowel CD. However, in contrast to UC, significant rectal bleeding is unusual.

CD can present at any age but the commonest age for onset is between 15-40 years.

1.1.3 ULCERATIVE COLITIS

The inflammation in UC involves only the mucosa of the colon and rectum, and is characterised by diffuse infiltration of the lamina propria by mixed acute and chronic inflammatory cells leading to epithelial damage and formation of crypt abscesses. It affects only the large bowel, although in rare cases there can be a "backwash" ileitis. It most commonly involves the rectum and extends proximally in continuity to a varying degree.

Bloody diarrhoea is the characteristic symptom of active UC. During acute attacks, systemic features such as anorexia, fever and malaise are also common.

The commonest age for initial presentation is 15-30 years and there is a second smaller peak at 50-70 years.

1.2 AETIOLOGY

The aetiology of both CD and UC involves a complex interaction between genes and environment.

1.2.1 GENETIC FACTORS

Ethnic and Racial Studies

Epidemiological data has shown that the incidence of CD and UC varies according to ethnic background. The incidences of CD and UC in the Caucasian population of Western Europe and North America are approximately 6 per 100,000 and 8 per 100, 000 respectively (1;2). The incidence of both diseases is lower among African Americans, Hispanics and Asians, although exact estimates vary (3). Among ethnic groups in the USA, Jews have the greatest risk for developing IBD, with an incidence rate 2 to 4-fold greater than non-Jewish Caucasians (4).

Family Studies

IBD has been shown to cluster in families. Between 10-20% of patients with IBD have a first degree relative who also has IBD (5;6). The empiric risk for developing IBD in siblings and offspring of a patient with CD is 10% and 16% respectively, and 3% and 6% for those of a patient with UC (7). The percentage of cases aggregating in families is higher in paediatric IBD, suggesting that genetic factors may play a stronger role in early onset disease. The average age of onset for familial CD is 22 years, compared with 27 years for sporadic cases (8). Similarly the average age of onset for familial UC is 23 years compared to 29 years for spontaneous onset cases (9). There is also good phenotypic concordance between family members with CD, indicating that the susceptibility gene may vary from one family to another.

Relatives of a patient with CD are more likely to develop CD than UC, but there is also a higher incidence of UC in such relatives than in the general population, suggesting that the two diseases are related at least at the level of genetic susceptibility.

The expression of perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) and antibodies to the cell wall mannan polysaccharide Saccharomyces cerevisiae (ASCA) has also been observed to be a familial trait in IBD (10). High levels of p-ANCA antibody are associated with UC whilst ASCA antibodies are associated with small bowel CD.

Twin Studies

Twin studies have helped to determine the relative contributions of genes and environment to disease aetiology. Tysk et al reported the relative concordance rates for monozygotic twins to be 63% for CD and 19% for UC, indicating that there is a greater genetic component to the aetiology of CD than UC (11).

Taken together, the evidence from ethnic and family studies of IBD is in keeping with a model of disease pathogenesis involving multiple susceptibility genes, with some common to both CD and UC, and some separately linked to one disease or the other.

Genetic Linkage Studies in Inflammatory Bowel disease

Genetic linkage studies use genome wide scans to type genetic markers in families containing more than one affected member for the purposes of identifying chromosomal regions shared in excess of statistical expectation. If the disease-susceptibility gene is located close enough to the marker it is less likely to be separated during meiosis and will therefore be co-inherited. Once linkage is identified, the identification of specific genes requires the use of genetic association studies. Numerous genome wide scans have identified several IBD susceptibility loci.

The IBD1 locus

The IBD1 locus is in the peri-centromeric region of chromosome 16 and is the best understood susceptibility gene. It shows positive linkage for CD but not UC (12). Linkage to this region is now recognised as being largely due to 3 major, relatively uncommon, amino acid polymorphisms within the NOD2/CARD15 gene (see below).

The IBD2 locus

The IBD2 locus is located on chromosome 12 and has a greater linkage to UC than CD (13). A number of candidate genes have been investigated in this region but thus far none has been found to be linked to UC or CD.

The IBD3 locus

The IBD3 locus is located on chromosome 6p and includes genes encoding the major histocompatability complex (14-16). It has been consistently shown to have linkage to both UC and CD. The HLA region has been shown to contribute 64%-100% for UC and 10-33% for CD to the genetic risk for this region (17;18). The IBD3 locus also contains the gene encoding tumour necrosis factor (TNF).

The IBD5 locus

A genome wide scan in 158 Canadian families identified a region on chromosome 5q as contributing to susceptibility for early onset CD (19). This region contains the genes encoding for a number of immunoregulatory cytokines including Interluekin (IL)-3, IL-4, IL-5 and IL-14, but thus far a specific gene has not been identified.

Genetic association studies in IBD

Genetic association studies test for differences in allelic frequency in patients compared with control individuals. In contrast to genetic linkage studies, in out-bred populations disease associations are observed typically over much more limited regions containing only a few functional candidate genes. Most candidate genes studied thus far have concentrated on those thought to play a role in immune regulation.

NOD2/Caspase-Activation Recruitment Domains 15

Genetic association studies have consistently shown that the association with CD at the IBD1 locus can be attributed to mutations in the NOD2/CARD15 gene (20;21). NOD2/CARD15 is expressed in peripheral blood monocytes and other mucosal cell types such as Paneth cells (22), and is related structurally to R proteins in plants. It is well conserved through many species of plants and animals and mediates host resistance to

microbial pathogens. The C-terminus contains a leucine-rich repeat (LLR) domain which functions to recognise broad types of microbial components such as bacterial lipopolysaccharides and peptidoglycan (21;23). Three major coding polymorphisms within or near the LRR of NOD2/CARD15 have been found to be highly associated with CD. In the European and North American Caucasian population having one altered copy of the gene increases the risk for CD by 2-4 fold whilst having two copies increases the risk by 20-40 fold. This indicates that NOD2/CARD15 functions to a large extent in an autosomal recessive fashion. 8-17% of patients with Crohn's disease in this population are recessive for one of the mutations. The frequency of alleles, however, is considerably lower among the African American population (24), and in the Japanese population no association between NOD2/CARD15 mutations and CD has been shown (25).

NOD2/CARD15 and genotype-phenotype correlations in CD

In multiple studies a significant association has been shown between ileal disease and one or more of the NOD2/CARD15 alleles (26;27). In particular, individuals who are homozygotes for NOD2/CARD15 mutations rarely develop colonic CD. Less well established is the relation of NOD2/CARD15 mutations to the behaviour of CD. This in part relates to the different systems used to classify CD and also that disease behaviour may vary over time. However, some studies have shown an association between NOD2/CARD15 mutations and a stricturing disease phenotype.

The function of NOD2

Quite how a mutation in NOD2 leads to a predisposition to CD is unknown. Transient transfection assays using the major Crohn's disease-associated NOD2 mutants have consistently shown defective cytokine responses to muramyl dipeptide, a component of peptidoglycan and a NOD2 ligand (28). In contrast, CD is associated with an increased production of these inflammatory cytokines.

Several theories have been proposed to explain this paradox. Kobayashi et al have proposed that a loss of NOD2 function in epithelial cells leads to a reduced production of defensins. Defensins are naturally occurring anti-bacterial proteins which are produced by Paneth cells. Kobayashi et al propose that it is this NOD2 mutation-

related reduction in defensin production which predisposes to the development of Crohn's disease (29).

Maeda et al found that bone marrow-derived macrophages from mice into which a NOD2 mutation had been introduced, in fact showed a greater response to muramyl dipeptide rather than a reduced repsonse. They suggest that macrophages of patients with a NOD2 mutation also show an enhanced responsiveness to bacterial peptidoglycan, and that this could underlie the predisposition to Crohn's disease (30).

A third proposal is that NOD2 normally functions to inhibit signals delivered via the stimulation of TLR2 by muramyl dipeptide. A loss of this inhibiting signal leads to enhanced cytokine responses by macrophages (or dendritic cells) to commensal bacteria and resultant inflammation (31).

Association studies and the HLA region (IBD3 locus)

The MHC genes included in the IBD3 region encode for HLA proteins which are responsible for presenting peptides to T-cells. HLA class I proteins are present on all cells whilst HLA class II molecules are expressed only on specialised immune cells. There is a high degree of polymorphism within these proteins in the region of the peptide-binding groove and, hence, individual differences in the capacity to bind and present antigenic peptides to the acquired arm of the immune system.

There is evidence that specific HLA class II associations contribute to overall IBD disease pathogenesis, particularly UC. A meta-analysis of 29 studies showed an association of UC with DR2, DR9 and DRB1*0103 and a negative association with DR4. DRB1*103 is present in 0.2% to 3.2% of the general population compared with 6 to 10% of patients with UC (32). DRB1*103 also affects UC phenotype as it is present in 16% of patients with extensive UC and is associated with increased rates of surgery (33). For CD, a positive association has been demonstrated with DR7, DRB3*301 and DQ4 and a negative association with DR2 and DR3 (34).

Association studies and the Tumour Necrosis Factor gene (IBD3 locus)

TNF-α is a major inflammatory cytokine which has been shown to be pivotal in the inflammatory process in both CD and UC (see section 1.3.10.1). Increased

concentrations are shown in the mucosa of patients with both CD and UC and anti-TNF-α therapy has been shown to be of benefit in both diseases (35;36).

TNF-α expression involves a variety of elements located in the gene's promoter region. Three promoter polymorphisms have been shown to be associated with an increased susceptibility to CD in a Japanese population, and one with an increased incidence of CD in a European population (37;38).

IL-23 receptor polypmorphisms and Crohn's disease

Recently an association has been found between CD and the IL-23 receptor region on chromosome 1p31 (39). IL-23 is a newly discovered member of the IL-12-related cytokine family, and is primarily involved in the differentiation of pathogenic T cells characterized by their production of IL-17 (40). IL-17 is a pro-inflammatory cytokine thought to be important in a number of autoimmune diseases. This IL-23/IL-17 inflammatory axis may be important in the pathogenesis of IBD. A coding variant of this region (rs11209026, c.1142G>A, p.Arg381Gln) has been shown to confer protection against Crohn's disease. Additional non-coding IL23R variants have also been found to be independently associated with CD (39).

1.2.2 ENVIRONMENTAL FACTORS

Even within the same ethnic group there is a geographical variation in the prevalence of IBD. The observed concordance rate for monozygotic twins is only 63% in CD and 19% in UC (41). These two observations together suggest that environmental factors make a considerable contribution to disease incidence and phenotype in Crohn's disease and even more so in UC. However, the environmental factors which both predispose to disease development and provoke disease relapse are poorly understood.

Table 1.1 Environmental factors which may be important in disease incidence and phenotype in IBD.

Environmental Factor	Crohn's disease	Ulcerative Colitis
Microbial Factors		
Mucosa associated flora	Increased E.coli	Increased E.coli
Mycobacteria	Possible causative agent	No association
Enteropathogenic organisms	Associated with relapse	Associated with relapse
Dietary Factors		
High carbohydrate diet	Increased incidence	No association
Sulphur intake	Possible causative agent	Possible causative agent
Toothpaste	Possible causative agent	Possible causative agent
Refrigerated food	Possible causative agent	Possible causative agent
Drugs		
NSAIDs	Associated with relapse	Associated with relapse
Oral contraceptive pill	Increased incidence	No association
Appendicectomy	No association	Decreased incidence
Smoking	Increased incidence	Decreased incidence
Psychological Stress	Associated with relapse	Associated with relapse

1.2.2.1 Microbial factors

Mucosa-associated flora

As already described it is likely that the inflammatory response in IBD represents an abnormal immune reaction to luminal bacteria. Evidence for this comes from the observation that IL-10 knockout mice, which normally develop a colitis, do not do so in sterile conditions. The identification of a specific causative bacterium has not yet been established; this in part reflects the complexity of the normal luminal flora. In the normal individual there are 500-1000 species of bacteria in human gut and only 20% of these have been successfully cultured. In contrast, some commensal flora, such as *bifidobacteria* and *Lactobacillus,* may have beneficial effects, either by modifying the actions of pathogenic bacteria, or by actions on host mucosal function (42;43).

Several studies have reported that mucosal associated *E.coli* are more common in biopsies taken from individuals with Crohn's disease (44-48). Two of these studies have also reported an increase in mucosal associated *E.coli* in individuals with UC (44;47). The correlation between the site of the bacteria and the presence of inflammation is poor suggesting that the *E.coli* may play a pathogenic role rather than merely colonising inflamed mucosa (44;46). There is also evidence that in patients with CD, *E.coli* are not just present in increased numbers on the mucosal surface but also invade the underlying lamina propria. *E.coli* can be cultured from tissue in patients with CD but not in healthy controls (49). These lamina propria *E.coli* are present not just in the stroma but also within macrophages where they may appear in clusters (50). In these sites they are likely to contribute further to disease activation through stimulation of production of chemokines and cytokines, such IL-8 and TNF-α (51), which are central to the pathogenesis of IBD. The mechanisms underlying increased adherence and penetration into the mucosa of *E coli* in IBD are unknown.

Work in our laboratory supports these observations. Using the technique of fluorescent in situ hybridisation (FISH) (see section 3.17), our unit has shown that there are greater numbers of mucosa surface-associated *E.coli* in mucosal biopsies from

patients with CD and active UC than in non-inflammatory controls (52). *E.coli* were also found as individual bacteria and in clusters within macrophages in the lamina propria in patients with CD and UC but not in controls. Epithelia-associated counts of bifidobacteria were reduced in both active and inactive UC compared to controls (52).

Mycobacteria

CD bears many histological similarities to intestinal tuberculosis and hence there has been considerable speculation as to whether CD might be due to a mycobacterial cause (53). Mycobacterium avium subspecies paratuberculosis (MAP) is an acid-fast bacillus capable of infecting the enteric tract in cattle mainly, leading to Johne's disease, an illness characterised by granulomatous inflammation similar to that seen in Crohn's.

Mycobacterium avium subspecies paratuberculosis (MAP) has been detected in tissue of patients with Crohn's disease by culture and other molecular methods (54;55). The importance of this finding is unknown as MAP is widespread in the environment and can also be detected in the tissues of many individuals who do not have IBD. Long term trials of treatment with conventional anti-tuberculous medication have not yielded promising results (56) with most recently a multicenter Australian study of triple anti-MAP therapy given over a two year period producing a negative outcome (WS Selby, verbal communication, British Society of Gastroenterology, Birmingham March 2005).

Enteropathogenic organisms in relapses of patients with IBD

Several studies have demonstrated an increased frequency of enteropathogenic organisms in patients with IBD who have relapsed, compared to the general population. The incidence and species of bacteria found varies according to study. A recent study in our own unit found the overall incidence of concomitant infection to be 11% in patients relapsing with IBD, with clostridium difficile being the commonest agent (57).

1.2.2.2 Dietary factors

Elemental diets are an effective treatment for active Crohn's disease, although probably less efficacious than corticosteroids (58). Many patients feel that dietary factors

are important in determining disease behaviour but proving a relationship between dietary factors and IBD has remained extremely difficult.

An excess of each of the major components of the diet has been advanced as being important for the development of IBD. Increased carbohydrate intake, and in particular increased intake of refined sugar, has been shown consistently to be associated with CD (59;60). Trials of diets low in unrefined carbohydrates, however, have not been shown to be of therapeutic benefit in maintaining remission or controlling flares of CD (61;62). Fat intake has been reported as having a positive association with UC, whilst fruit, vegetables and fibre decreased the risk of IBD (60).

Sulphur intake

Roediger et al have suggested that dietary intake and luminal metabolism of sulphur-containing compounds by sulphate-reducing bacteria are important in the aetiology of IBD (63). Over the last century the modern diet has incorporated increasing amounts of sulphur-containing compounds. Sulphur not recycled by an intestinal scavenging pump passes into the colon where it is metabolised by sulphate-reducing bacteria, a group of bacteria identified as being more common in individuals with UC and in particular in those with active disease (64). Metabolism of sulphur by these bacteria generates sulphoxide compounds which have been shown to be deleterious to colonic mucosa (65). These compounds also reduce the uptake of butyrate and other short chain fatty acids (SCFA) by colonocytes, which are important in maintaining mucosal integrity. However, treatment of UC with SCFA enemas has generally produced negative results (66).

Toothpaste and microparticles

A variety of substances contained in toothpaste have been demonstrated as being harmful to the GI tract in animal studies (67). In particular some of the particulate materials used as abrasives, such as tri-calcium phosphate and quartz, have been identified as capable of penetrating the epithelium and creating enteric lesions similar to those seen in CD. However, the possibility has not been rigorously studied and there is little supportive data in man.

The possibility of microparticles as a causative factor in IBD, and especially in Crohn's, has been taken further by some investigators. They suggest that microparticles have become an increasing component of a modern diet and this explains the increasing incidence of IBD. Powell et al have proposed that titanium, silicon and aluminium oxides are all ingested as food additives (68). These microparticles are taken up by specialised epithelial M-cells but are undegradable and accumulate in lymphoid tissue. They are proposed to act as adjuvants permitting the absorption of other antigens and preventing their appropriate disposition by the immune system, altering normal mucosal tolerance and stimulating an immune response. In an initial pilot randomised double-blind controlled trial, 20 patients were allocated to either a normal diet or to a diet low in microparticles. Adherence to the low microparticle diet for four months led to a fall in the Crohn's Disease Activity Index (69). There was little change in the control group. However, in a later study by the same group with larger numbers of patients, the addition of diets low in microparticles or calcium lead to no additional benefit beyond oral corticosteroids in patients with active CD (70). Also when Crohn's patients were compared with a control group for their intake of microparticlulates, no differences were observed (71).

Refrigerated Food

Another theory suggests that IBD results from chronic exposure to organisms which can survive at low temperatures, such as *Listeria monocytogenes, Yersinia enterocolitica, Clostridium botulinum,* and *Bacillus ceraus.* Exposure to these organisms has increased over the last century due to the increasing use of refrigeration in the preservation of food (72). In support of this theory, Liu et al found that 75% of intestinal and mesenteric lymph node specimens from 16 individuals with CD stained positively with antibodies to listeria. Macrophages and giant cells immunolabelled for this antigen were found in the samples underneath intestinal ulcers, along fissures, around abscesses,within the lamina propria and within the germinal centres of lymph nodes (50).

1.2.2.3 Drugs

Non-steroidal anti-inflammatory drugs

The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown in some studies, but not all, to be positively associated with a higher risk of relapse in quiescent IBD (73). The inconsistency between studies is likely to be due to small sample sizes and methodological problems. One study found that administration of naproxen or nabumetone for 9 days led to a 20% relapse rate in patients with IBD, with a corresponding increase in levels of faecal calprotectin (74). Taking diclofenac for 14 days has been shown to lead to the formation of small bowel pathology, as detected by wireless capsule endoscopy, in 70% of healthy volunteers, again with a corresponding increase in faecal calprotectin (75).

There is less data on the effects of selective COX-2 inhibitors in patients with IBD. One study has reported a higher incidence of disease relapse in patients with IBD who were taking rofecoxib (76). There is currently no data to suggest COX-2 inhibitors are less likely to cause relapse than conventional NSAIDs.

Some supporting evidence for a role for NSAIDs in provoking replapse in IBD is provided by animal models. IL-10 knockout mice develop a far more rapid and severe colitis when given NSAIDS (77). The mechanism may involve the blockade of the production of protective prostaglandins, such as PGE2, or the increased intestinal permeability observed with NSAID use (78).

The oral contraceptive pill

The relative risk of Crohn's disease has been found to be increased for women taking the oral contraceptive pill (OCP). A relative risk of 1.44 (95% CI 1.1-1.9) was calculated in a meta-analysis of OCP use in an IBD population (79). The mechanism by which this might occur is unknown, although it may involve the increased likelihood of thrombosis leading to multifocal mucosal micro-infarction (80).

1.2.2.4 Appendicectomy

The prevalence of UC is lower in patients who have developed appendicitis whilst under the age of 20 than those who have not (81). Patients with UC also have lower rates of appendicectomy than the general population. Performing appendicectomy electively can prevent the development of colitis in some mouse models, but has yet to be shown to be consistently efficacious in man (82).

1.2.2.5 Smoking

Tobacco exposure, and in particular cigarette smoking, is the most well established environmental factor which influences IBD. Smoking has been shown consistently to be associated with an increased incidence of, and have an adverse effect on, the clinical course of CD, but to have a protective effect against UC.

Smoking and Crohn's disease

Approximately 50% of patients with Crohn's disease smoke: this is considerably higher than the prevalence of cigarette smoking in the general population. In a large meta-analysis the odds ratio for Crohn's disease was 2.0 (95% CI 1.65-2.47) for smokers and 1.8 (95% CI 1.33-2.51) for ex-smokers compared to the general population (83).

Cigarette smoking has also been shown to have an adverse effect on clinical course. Patients with Crohn's colitis who smoke experience more relapses than those who do not (84). Cottone et al found smoking to be an independent risk factor for the development of clinical, surgical and endoscopic reccurrence in 182 patients who had undergone surgery for Crohn's disease (85). In contrast, smoking cessation has been shown to reduce the future requirement for steroids and the risk of operations for recurrent Crohn's disease (86). Non-smoking is also associated with a higher rate and longer duration of response to infliximab therapy (87). Smoking also affects disease phenotype and is associated with a higher incidence of ileal disease and a fistulizing pattern of disease behaviour (88).

Smoking and ulcerative colitis

In contrast to CD, the prevalence of smoking in patients with UC is 11-23%, lower than the prevalence of smoking in the general population. In a meta-analysis the pooled odds ratio of UC was 0.41 (95% CI 0.34-0.48) in smokers compared to non-smokers (83). There was also a dose-response relationship with heavier tobacco consumption increasing the protective effect.

Smoking also appears to alleviate the clinical course of UC. In a study of 30 intermittent smokers with UC, resuming smoking led to an improvement in symptoms in 50% of cases (89). Those who responded were on average heavier smokers than those who did not. Smoking has also been shown in some studies to be associated with lower rates of hospitalisation and colectomy (90;91).

Transdermal nicotine and nicotine enemas have been shown to have clinical benefit in active UC in a limited number of trials. Pullan et al treated 72 patients with active left-sided UC with either transdermal nicotine or placebo over six weeks (92). Mesalamine and steroids were also continued. The improvement in clinical and histological grades was greater in the group treated with nicotine. However, nicotine patches have not been found to be effective in preventing disease relapse in patients with quiescent UC (93).

In two small pilot trials in active distal UC, liquid nicotine enemas were effective. However the liquid enemas were difficult to retain, limiting their practical use (94;95).

Possible Pathogenic Mechanisms of Smoking on IBD

The active ingredient in tobacco and the exact mechanisms by which it affects IBD are unknown. Smoking is known to affect a wide range of both adaptive and innate immune functions which may have relevance to the pathogenesis of IBD. Carbon monoxide exposure reduced lipopolysaccharide-stimulated secretion of inflammatory cytokines by macrophages (96). Smoking led to a reduction in IL-1beta, IL-2, IL-8, IL-10 and TNF-α levels in peripheral blood mononuclear cells and of IL-1beta and IL-8 in colonic tissue from healthy controls and patients with IBD (97-100). Heavy smokers have reduced IgA levels in both saliva and intestinal secretions (101;102). Smokers also

show an increase in suppressor CD8+ T cells and a diminished ratio of CD4/CD8 T cells in peripheral blood (103).

Patients with UC are reported to have an increased rectal blood flow (104). Rectal blood flow has been reported to be reduced by smoking, and may be one of the mechanisms whereby smoking influences the course of UC (104).

Smoking increases the pro-coagulant activity of blood and promotes microvascular thrombosis (105;106). This may be of relevance to Crohn's disease if the theory of chronic mesenteric vasculitis as a pathogenic mechanism is correct (80).

It is also tempting to speculate that another mechanism by which smoking might affect UC is through its ameliorative effects on stress. Patients often claim that smoking reduces stress levels and it is possible that this reduction may lead to the beneficial effects of smoking seen in UC. However, there is as yet no data to support this theory directly.

Psychological stress

As the effects of stress and stress reduction therapy in UC forms the basis for this thesis, the role of stress in the aetiology of IBD will be discussed separately at the end of this chapter.

1.3 PATHOGENESIS

Although the exact mechanisms of the pathogenesis of IBD are unknown, it is generally accepted that CD and UC involve immune dysregulation, with a genetically determined abnormal mucosal response to the bacterial flora of the intestine (107). Normal immune tolerance to bacterial antigens appears to be lost and an inflammatory response develops.

The most important cell-types, cytokines and mediators in this process, and particularly those relevant to this thesis, will be discussed in turn.

1.3.1 INTESTINAL EPITHELIAL CELLS

The epithelial cell layer is in constant contact with both pathogenic and commensal bacteria and acts as a barrier, preventing invasion of the underlying mucosa. Epithelial cells are capable of a variety of immunological functions including the production of inflammatory cytokines such as IL-8 (108). Specialist epithelial cells, named M-cells, which overlie the Peyer's patches, continually sample the mucosal flora for potential antigens (109). These antigens are passed to dendritic cells which process and present the antigens to T-cells (see below). Paneth cells, another kind of specialised epithelial cell, produce a range of antimicrobial peptides and proteins such as defensins.

1.3.2 DENDRITIC CELLS

Dendritic cells are now recognised as the principal antigen-presenting cell of the gut. They process bacterial antigens, which have either been sampled directly from the gut lumen or received from M-cells, and present them via major histocompatibility class II (MHC) to T-cells (110). This interaction leads to the production of cytokines and chemokines and the development of an inflammatory response.

1.3.3 T-CELLS

CD4 positive T helper cells are regarded by many as the pivotal cell type in determining and driving the inflammatory response in IBD (111). They bear T-cell receptors which are capable of recognising foreign antigens when they are presented in

conjunction with major histocompatibility type II. This interaction, if accompanied by other co-stimulatory molecules, causes stimulation of the T-cell and the production of pro-inflammatory cytokines and chemokines. This leads to the recruitment of further T cells and other inflammatory cell types from blood vessels with the consequent development of acute mucosal inflammation.

1.3.4 MACROPHAGES

Macrophages play an important role in both the innate and adaptive immune responses of healthy individuals and are likely to be important in the pathogenesis of IBD. They are able to recognise micro-organisms and their products, and respond by phagocytosis, micṛobial killing and the secretion of cytokines such as IL-12 and IL-18 (112;113). Stimulation of toll-like receptor 4 (TLR-4) on the macrophage cell surface by bacterial LPS, leads to the expression of IL-1beta and TNF-α (114;115). Macrophages also present antigens to T-cells, affecting the adaptive immune response.

There are a large number of macrophages in the lamina propria of normal small and large bowel. In healthy individuals a number of the inflammatory responses of these macrophages, such as their ability to produce IL-1 beta and to respond to stimulation by LPS, seem to be down-regulated. However, in individuals with IBD this is not the case, with lamina propria macrophages capable of greater inflammatory responses (116;117). The ability of macrophages to respond to LPS is also affected by mutations in the NOD-2 gene (see above), supporting the theory that this interaction is important in IBD (118;119).

1.3.5 MAST CELLS

Mast cells arise from CD34-positive bone marrow precursors and circulate in an immature form, only maturing once in a tissue site. Two types of mast cells are recognised, those from connective tissue and those found in mucosae, whose activities are T-cell-dependent (120). Mast cell granules contain a range of inflammatory mediators which are released when the mast cell is activated. Some of these are relatively specific to mast cells such as histamine and tryptase, whilst others, such as TNF-α, are released by a range of inflammatory cells (121). There is increased

activation of mucosal mast cells in active UC (122) and increased luminal release of histamine and other mast cell mediators (123). Activated mast cells may be a source of the increased mucosal release of TNF-α seen in active IBD active IBD (121). Tryptase has a number of inflammatory actions which may contribute to the pathogenesis of IBD. It leads to increased expression of P-selectin (see section on platelets and platelet leukocyte aggregates below) and increased neutrophil recruitment to the lamina propria. Tryptase also activates the protease-activated receptor 2 (PAR2) intracellular signaling pathway (124), which by altering colonic epithelial intercellular tight junctions may increase mucosal permeability to bacteria (125). In this context, PAR2 expression is increased in UC (126), and has been shown to induce experimental colitis (127). Increased mast cell activation may be mediated at least partly through stimulation of their TLR4 receptors (128).

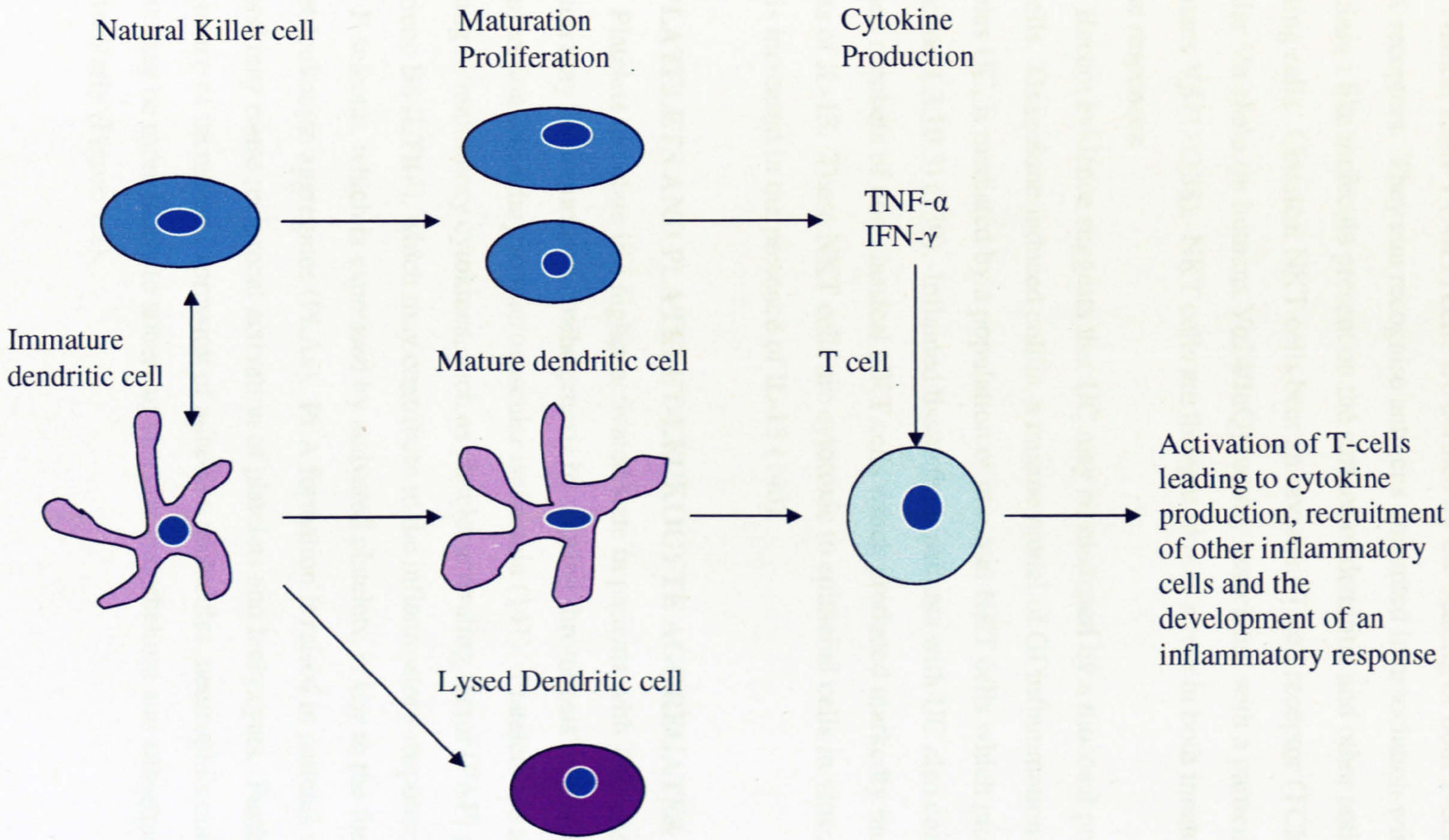
A pathogenic role for mast cells in IBD is suggested by open-label studies indicating that an inhibitor of the mast cell mediator, tryptase, may have benefit in mild-moderately active UC (129). The mast cell stabilizer, ketotifen, has also been shown to have benefit in a pilot open-labelled study of ten children with UC (130).

1.3.6 NATURAL KILLER CELLS (NK CELLS)

NK cells are traditionally considered a component of the innate immune system, killing tumour and virally infected cells via their interaction with MHC I (131). More recently they have also been shown to affect adaptive immunity via their interactions with dendritic cells (132). NK cells can localise to areas of inflammation and interact with immature dendritic cells causing maturation and proliferation of both the dendritic cell and NK cell. The mature dendritic cells interact with T-cells, determining the inflammatory response, whilst the stimulated NK cells are able to produce cytokines such as TNF-α and IFN-gamma (Figure 1.1) (133).

NK cells are not generally regarded as an important cell type in the pathogenesis of IBD although there are some reports of increases in both mucosal numbers and activation of NK cells in active IBD (134;135). Increases in NK cell number are one of the most consistent findings associated with acute stress and hence our evaluation of this variable in this study (136;137).

Figure 1.1 Dendritic cell and NK cell interactions.



1.3.7 NATURAL KILLER T CELLS (NKT CELLS)

Natural killer T (NKT) cells are defined by the bearing of both T-cell receptors and NK receptors. They can recognise antigens presented in association with CD1d, an MHC class-I like molecule present on the surface of dendritic and other antigen presenting cells. Classical NKT cells bear an invariant T-cell receptor (TCR) with a particular Vα chain (in humans Vα24/JαQ) usually associated with a particular Vβ chain (in humans Vβ11) (138). NKT cells are thought to play a role in both innate and acquired immune responses.

Recent evidence suggests that UC may be mediated by a mucosal population of NKT cells. Oxazolone-induced colitis, a murine model of GI inflammation which resembles UC, is mediated by a population of mucosal NKT cells which produce IL-13 (see section 1.3.10.3) (139). Inflamed tissue from patients with UC also contains increased numbers of non-classical NKT cells which produced markedly increased amounts of IL-13. These NKT cells are cytotoxic to epithelial cells in vitro, an effect which is increased in the presence of IL-13 (140).

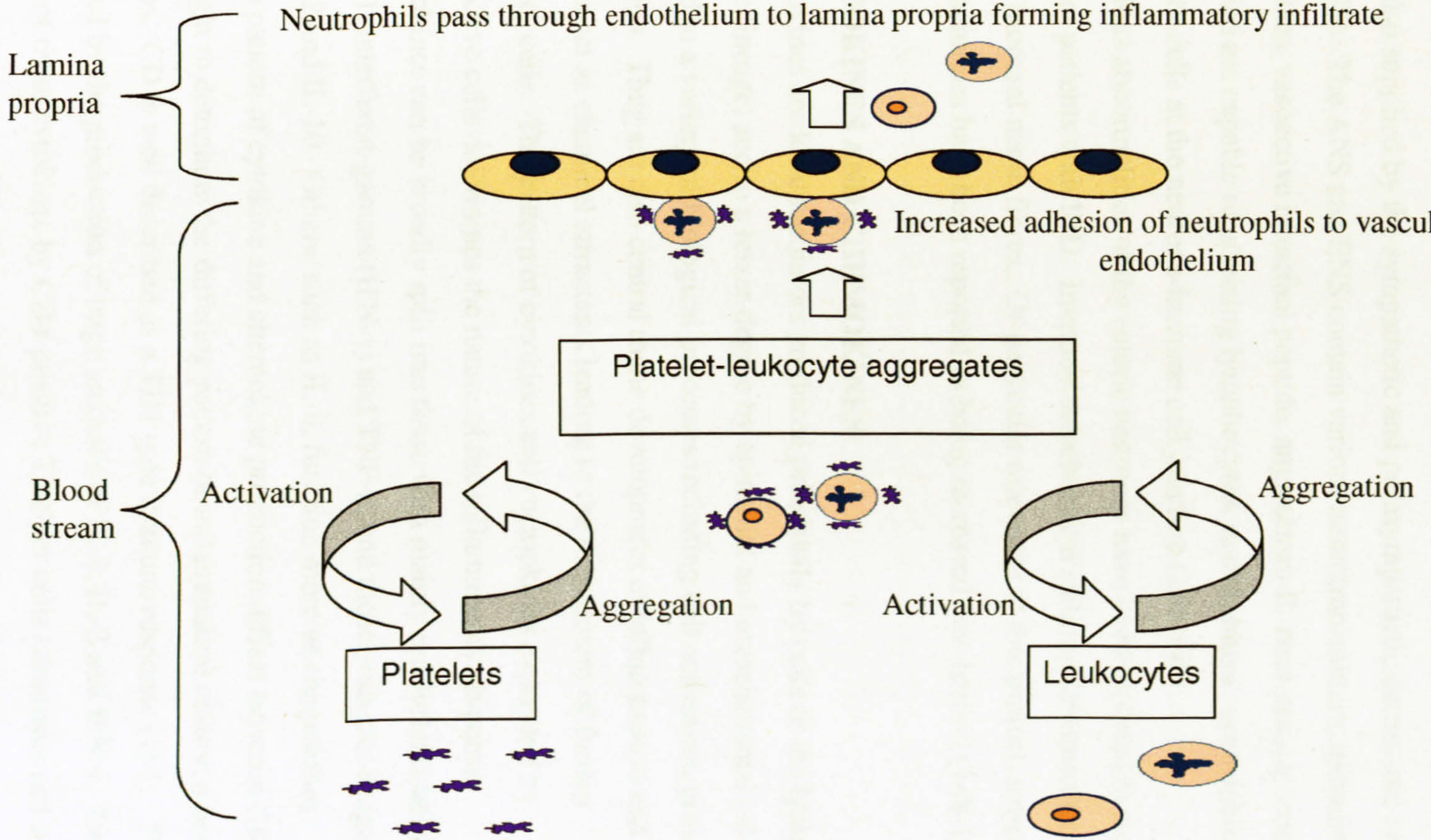
1.3.8 PLATELETS AND PLATELET-LEUKOCYTE AGGREGATES

Platelets circulate in a higher activated state in patients with IBD and platelet activation may be important in pathogenesis by causing thrombosis formation and microinfarction secondary to microvascular ischaemia (141). Platelets are also capable of producing inflammatory cytokines, such as platelet activating factor (PAF) and leukotriene B4 (LTB4), which may contribute to the inflammatory response (142).

P-selectin, which is expressed by activated platelets, is key to the formation of platlelet-leukocyte aggregates (PLAs). PLA formation is raised in patients with IBD (143) and may cause reciprocal activation of platelets and leukocytes. Furthermore as a consequence of increased expression of adhesion molecules, neutrophils complexed with platelets may be more likely to adhere to vascular endothelium and subsequently migrate extravascularly (Figure 1.2).

Figure 1.2

Inflammatory effects of platelet-leukocyte aggregate formation



1.3.9 ENTERIC NEURONES

The enteric nervous system (ENS) contains 100 million neurones and regulates the motility, exocrine and endocrine functions and microcirculation of the GI tract (144). The gut is also supplied by the sympathetic and parasympathetic autonomic nervous system (ANS). The ANS and ENS contain various neurotransmitters, including catecholamines, vasoactive intestinal peptide, angiotensin II, neurotensin, somatostatin and SP, which are capable of affecting lymphocytes, macrophages, neutrophils, and other inflammatory cells at the neurone-immune cell junction (145-147).

Various abnormalities of the enteric neurones have been reported in tissue samples from patients with IBD. Immunohistochemical staining demonstrates increased numbers of mucosal nerve fibres. Of particular relevance to this project, substance P-containing neurones have been reported as being increased (see below) (148-150).

1.3.10 CYTOKINES AND CHEMOKINES

Cytokines are local mediators produced principally by cells of the lymphoid and macrophage lineage, and to a lesser degree by epithelial and mesenchymal cells. They are involved in a variety of biological processes including cell activation, growth and differentiation. They are also central to the development of inflammation and immunity. Chemokines act as chemical attractants leading to the recruitment of further inflammatory cells. The pattern of cytokines and chemokines expressed by immunoreactive cells determines the nature of the inflammatory response.

Cytokines can be broadly split into those with mainly pro-inflammatory effects such as IL-1, interferon-gamma (IFN-γ) and TNF-α, and those with more regulatory roles such as IL-2 and IL-10. Others, such as IL-8, function more as chemokines.

The pattern of cytokine and chemokine production differs between CD and UC and is thought to determine the differing pattern of inflammation observed between the two diseases. CD is well described as a TH1 type immune response (151). This is characterised by the production of large amounts of IL-1, IL-2 and IFN-γ. The production of these cytokines by CD4-positive T helper cells stimulates cell mediated immunity, with increased T cell cytotoxicity characteristic of CD. Increased mucosal

production of IL-12 has also been demonstrated in CD and it has been suggested that IL-12 may play a key role in determining a TH1 type immune response (151-153). IL-23 shows close homology to IL-12 and recent genetic evidence suggests IL-23 may also be important in the pathogenesis of CD (39). Mucosal production of IL-18 is also increased in CD, and is thought to be involved in perpetuating the TH1 cell responses (112;154).

The immune response in UC is more difficult to classify than that of CD, but is more in keeping with a TH2 type immune response. There is the production of large amounts of IL-4, IL-5, IL-10 and IL-13 with reduced production of IFN-γ (155). These cytokines are more associated with the stimulation of B-cells and an antibody response. However, the division of CD and UC into the classic TH1 and TH2 paradigm is overly simplistic and it is likely that these pathways co-exist rather than being mutually exclusive.

In addition to affecting T and B cells, certain cytokines such as IL-1, TNF-α and IFN-γ lead to upregulation of adhesion molecules and chemokines such as IL-8. These mediate the recruitment of further blood-borne inflammatory cells via activated endothelium. This leads to amplification of the inflammatory cascade and secretion of more inflammatory mediators, destructive enzymes and free radicals leading to futher tissue injury.

Table 1.2

Cytokine changes in Crohn's disease and UC

	Crohn's disease	**Ulcerative Colitis**
Cytokine pattern	**TH1 type**	**TH2 type**
	Increased	*Increased*
	IL-1, IL-2, IL-12, IL-23, IL-18, IFN-γ, TNF-α	IL-4, IL-5, IL-10, IL-13, TNF- α
	Decreased	*Decreased*
	IL-10	IFN-γ

CYTOKINES OF PARTICULAR RELEVANCE TO THIS THESIS

1.3.10.1 Tumour necrosis factor-alpha

Tumour necrosis factor-α is a 17-kD pleiotropic cytokine produced by macrophages, monocytes, T cells and more pertinently to this study by NK and mast cells (121). Although regarded as a TH1 type cytokine, TNF-α production is increased in both CD and UC, and it is recognised as playing a key role in the pathogenesis of IBD (156;157).

TNF-α is released in large amounts by macrophages in response to stimulation by bacterial products such as LPS. TNF-α has a range of inflammatory actions including stimulating increased production of other inflammatory cytokines such as IL-1, IL-6 (see below) and IL-8 (158). One of the key actions of TNF-α in the pathogenesis of IBD appears to be to stimulate increased production of IFN-γ (158). TNF-α also stimulates the expression of adhesion molecules, proliferation of fibroblasts, as well as initiating cytotoxic, apoptotic and acute phase responses (158;159). Many of the pro-inflammatory actions of TNF-α are mediated via increases in the nuclear transcription factor NF-kappa B (160).

Animal studies have demonstrated prevention of the development of colitis in TNF-α knockout models (161). Correspondingly a lethal colitis develops in animals harbouring a TNF-α transgene (162).

Increased TNF-α producing cells and the spontaneous production of TNF-α have been demonstrated in studies of isolated lamina propria mononuclear cells (LPMCs) in inflamed and non-inflamed mucosa in patients with IBD (163;164). Several studies have reported that elevated serum levels of TNF-α correlate with clinical and laboratory indices of disease activity. Correspondingly, therapy with antibodies to TNF-α has been shown to be effective in both CD disease and more recently UC (35;36).

1.3.10.2 Interleukin-6

Interleukin-6 is a major inflammatory cytokine produced by many different immune cell types including monocytes, macrophages, T-cells, B-cells, eosinophils and mast cells (165;166). It is one of the major physiological mediators of the acute phase

reaction and is responsible for stimulating the production of C-reactive protein (CRP) by hepatocytes (167). It has a range of pro-inflammatory actions including both promoting maturation of B-cells and differentiation of mature and immature T-cells into cytotoxic T-cells.

IL-6 also stimulates the release of corticotrophin releasing factor (CRF) in the hypothalamus leading to adrenocorticotrophic hormone (ACTH) release by the pituitary and consequently increased glucorticoid production in the adrenal cortex (168;169). As the principal action of glucocorticoids is anti-inflammatory, this mechanism forms a negative feedback loop linking the immune and neuroendocrine systems which is relevant to this thesis.

Serum and saliva IL-6 concentrations have been found to be increased in patients with inflammatory bowel disease (170;171). The mucosal production and concentration of IL-6 has also been found to be increased in both CD and UC (172;173). IL-6 has also been found to be an important mediator in animal models of colitis (174). It has been suggested that one important action of IL-6 in the pathogenesis of IBD involves IL-6 binding to its soluble receptor (sIL-6R) forming a complex which interacts with gp130 on CD4 T-cells. This leads to an increased expression and nuclear translocation of STAT-3 causing the induction of anti-apoptotic genes. This is thought to lead to an augmented resistance of lamina propria T-cells to apoptosis with an ensuing T-cell expansion and the continuation of colonic inflammation (175;176).

In a pilot study involving 36 patients with active CD, a humanised monoclonal antibody to IL-6 receptor led to a greater clinical response rate than placebo. The drug was given as biweekly 8mg/kg infusions and was well tolerated. There was a normalisation of the acute phase response in all patients given the active treatment but there was no difference in terms of endoscopic or histological response compared to placebo (177).

1.3.10.3 Interleukin-13

As discussed above mucosal production of IL-13 has shown to be increased in oxazolone-induced colitis and the development of GI inflammation in this model can be prevented by the administration of an IL-13 inhibitor (178).

Lamina propria mononuclear cells (LPMCs) from patients with UC produce more IL-13 in response to stimulation with antibodies to CD2 and CD28 than LPMCs from individuals with CD (179). IL-13 also impaired epithelial barrier function in HT-29 cells in vitro, due to an increased incidence of apoptosis and an increased expression of the pore-forming tight junction protein claudin-2 (180).

1.3.11 REACTIVE OXYGEN SPECIES

In vitro studies have shown that rectal mucosal biopsies from patients with UC produce more reactive oxygen metabolites (ROMs) than rectal mucosal biopsies from healthy controls (181). ROMs are produced by a wide variety of inflammatory cells, in particular neutrophils as part of the oxidative burst. Many of these molecules are highly injurious to tissue. It is possible that much of the mucosal damage observed in inflammatory bowel disease is mediated by the acute inflammatory infiltrate of neutrophils and the production of ROMs (182).

While there have been no controlled studies to assess the efficacy of specific antioxidants in IBD, the beneficial effects of 5-ASAs are thought to be caused, at least in part, by their ROM scavenging properties. 5-ASAs have been shown attenuate the production of ROMs by stimulated polymorhponuclear leukocytes in vitro at concentrations comparable to clinical doses (183-185). They also protect cells in culture from the oxidative damage produced by activated neutrophils (186;187). Colonic epithelial cell loss secondary to ROMs is reduced by 5-ASAs in animal models of colitis (188).

1.3.12 SUBSTANCE P

Substance P is a peptide neurotransmitter, common throughout the GI tract. It is produced principally by neurones, but is also released by macrophages, eosinophils, lymphocytes and dendritic cells. In addition to its action as a neurotransmitter, SP also has pro-inflammatory actions: it enhances cytokine production and stimulates chemotaxis of inflammatory cells (189). It also increases the expression of leukocyte adhesion molecules on microvascular endothelium, and of CD11b on neutrophils, facilitating leukocyte adhesion at sites of inflammation (189).

Elevated levels of SP and increases in neurones containing SP have been reported, in some but not all studies, in colonic tissue from patients with IBD although its possible contribution to the pathogenesis of IBD is largely unproven. SP-containing neurones are found in close association with mast cells and incubation with SP of colonic biopsies from patients with IBD increases mast cell-mediated histamine release (190).

1.4 THE ROLE OF PSYCHOLOGICAL STRESS IN INFLAMMATORY BOWEL DISEASE

Psychological stress has long been anecdotally reported as increasing the likelihood of relapse in patients with IBD (191).

1.4.1 STRESS AND THE STRESS RESPONSE (Figure 1.3)

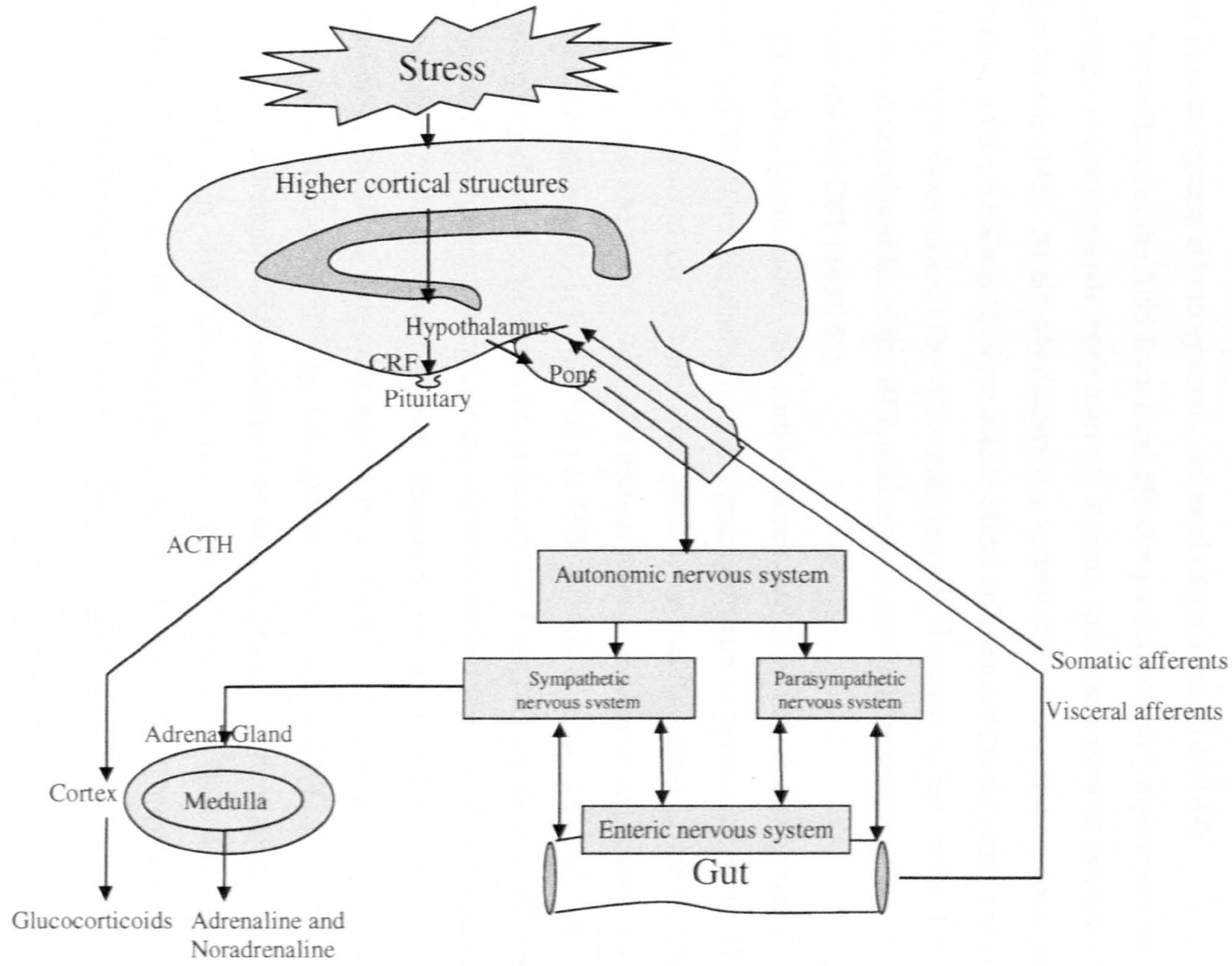
To maintain homeostasis, a living organism must constantly adapt at a molecular, cellular, physiological and behavioural level to environmental alterations. Stress can be defined as any threat to an organism's homeostasis (192). The function of the stress response is to maintain homeostasis and may involve both physiological and behavioural adaptations.

The stress response involves the complex integration of a series of interconnected regions within the brain, most notably the hypothalamus, amygdala and hippocampus (193). This network receives input from both visceral and somatic afferents, and from higher cortical structures. In turn, it governs the neuroendocrine stress response via two interconnected effector pathways: the HPA axis and the autonomic nervous system (ANS).

Stress stimulates the release of corticotrophin releasing factor (CRF) from the hypothalamus causing the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. This in turn stimulates the secretion of cortisol, the principal glucocorticoid, from the adrenal cortex.

Stress activates direct descending neural pathways from the hypothalamus to the pontomedullary nuclei which control the autonomic response. Stimulation of the sympathetic nervous system in response to stress causes the release of adrenaline and noradrenaline from the adrenal medulla. Neurones of the sympathetic ANS also supply the entire gut directly, whilst the vagus and sacral nerves provide parasympathetic input to the upper gut and to the distal colon and rectum, respectively (144). The efferent and afferent neurones of the sympathetic and parasympathetic ANS communicate with the gut's own rich nerve supply, the ENS, and this network has been termed the brain-gut axis (144).

Figure 1.3 Pathways mediating the effects of stress on the gastrointestinal tract



Abbreviations: CRF corticotropin releasing factor, ACTH adrenocorticotrophic hormone

1.4.2 PSYCHONEUROIMMUNOLOGY

It is increasingly recognised that the HPA axis, ANS and ENS can interact directly with the immune system. Psychoneuroimmunology is the study of the mechanisms by which behavioural factors and CNS function can influence inflammation and the immune system at both systemic and local tissue levels (145-147).

Nerve fibres of the ANS form close effector junctions with lymphocytes and macrophages in lymph glands, bone marrow, thymus, spleen and mucosa-associated lymphoid tissue (145). As already discussed, a variety of neurotransmitters contained in the neurones of the ANS and ENS are able to affect inflammatory cells within the gut (145-147). Lymphocytes and other inflammatory cells also carry receptors for the hormones and neuropeptides of the HPA axis such as growth hormone, ACTH, corticosteroids and CRF (145;146).

At high concentrations glucocorticoids, released from the adrenal cortex in response to ACTH from the pituitary, have a mainly immunosuppressive action. They increase the synthesis of anti-inflammatory proteins such as lipocortin-1, interleukin-1 receptor antagonist and IL-10 (194). The transcription of several inflammatory cytokines and chemokines such as IL-1, IL-6 (195) and TNF-α (196), is reduced by glucocorticoids via an inhibitory effect on the transcription factors AP-1 and NF-kappa-beta (197;198). Glucocorticoids also promote apoptosis in inflammatory cell types which include T cells and eosinophils (199). However, at lower concentrations cortisol exhibits an immunostimulatory effect. For example, pre-treatment with low dose cortisol increases the production of IL-6 and TNF-α by macrophages in response to LPS (200).

Similarly adrenaline and noradrenaline have mixed effects on immune and inflammatory function. Adrenaline infusion causes a rise in serum IL-6, and increases in LPS-induced IL-8 and IL-10 synthesis by whole blood (201-203). It also increases the number of circulating cytotoxic T-cells and NK cells, but decreases TNF-α production by monocytes (204;205).

1.4.3 THE EFFECTS OF STRESS ON THE SYSTEMIC IMMUNE SYSTEM IN HUMANS (Table 1.3)

The effects of psychological stress on the systemic immune and inflammatory system are complex, and depend on both the duration and intensity of the stressor (206). Both chronic stress and acute stress are associated with alterations in systemic immune and inflammatory function which may have relevance to the pathogenesis of IBD.

1.4.3.1 Chronic psychological stress, adverse life-events and systemic immune and inflammatory function (Table 1.3)

Chronic sustained stress, such as that due to adverse life events, causes a prolonged increase in cortisol over several days which is characteristically associated with immunosupression (207). Bereavement, depression and marital separation have all been shown to reduce the numbers of CD8+ lymphocytes, natural killer (NK) cells and macrophages found in blood (208-212).

However, in addition to immunosuppression, chronic psychological stress has also been shown to be associated with sub-clinical increases in inflammation. Patients with depression, and middle-aged and elderly patients with reduced heart rate variability, a measure of increased sympathetic tone and chronic stress, have both been found to have a raised serum CRP (213;214). The inflammatory theory of depression suggests that depression can be considered a psychoneuroimmunological disorder with an increase in inflammatory cytokines within the brain (215). Antidepressants of several classes decrease the production of several inflammatory cytokines within the brain such as TNF-alpha and IFN-gamma in animal models (216;217). In humans, tricylcic antidepressants have been found to reduce the production of various inflammatory cytokines by peripheral blood mononuclear cells (218).

1.4.3.2 Acute psychological stress, experimental stress and systemic immune and inflammatory function (Table 1.3)

Examining the *in vivo* effects of psychological stress is difficult as stress is a subjective experience which is hard to define objectively and to simulate in a controlled, experimental environment. Placing patients under sustained psychological stress would

also be unethical. Despite these limitations, various experimental models have been designed which are capable of inducing mild acute psychological stress as assessed by the ANS and HPA response (see Section 3.5) (219;220).

The dichotomous listening test, where different auditory inputs are played into the subject's ears, has been one of the commonest techniques used to assess the effects of psychological stress on human GI physiology (221). Difficult mental arithmetic tests (MAT), which include negative feedback and varying degrees of auditory distraction, are also used and are easily reproducible (137;222). The Trier Social Stress Test (TSST) involves an oral presentation in front of a critical audience (220;223;224) whilst the Stroop word-colour interference test involves identifying the colour of ink used to write a word which is different to the meaning of the word itself; for example the word blue written in red ink (225).

Both real-life acute stress and experimental stress tests cause acute stimulation of the sympathetic nervous system, with an almost immediate rise in adrenaline and noradrenaline (226). This is followed by a rise in cortisol but both changes are maintained over only a few hours. Stimulation of the stress axes in this way is associated with an immune enhancement (227). There is an increase in the serum levels and the production by whole blood of inflammatory cytokines known to be important in the pathogenesis of IBD. Whole blood from medical students taken the day before an examination produced more TNF-α, IL-6 and IFN-γ when stimulated with lipopolysaccharide (LPS) than when taken several weeks later (228). Similarly blood stimulated with LPS for 24 hours produced more IL-6 and IFN-γ after an acute stressor than before (229-233), and serum levels of IL-6 and IL-2 receptor antagonist were increased two hours after a stressful behavioural task (234). Another study reported reduced IL-10 production by peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagluttinin (PHA) after an acute stressor compared to before.

Acute stress has also been shown to cause a leucocytosis in healthy subjects with a rapid redistribution of the lymphocyte population. There is a rise in the percentages of CD8+ cytotoxic T-cells and NK cells, and a corresponding increase in their cytolytic activity as assessed by chromium release assays (136;219;235;236).

Platelet activation, as assessed by aggregation and production of inflammatory mediators and platelet-dependent thrombin generation, has been shown to be increased by experimental stress in healthy subjects (237-239). As aforementioned platelets circulate in a higher activated state in patients with IBD (143) and platelet activation may be important in pathogenesis by causing thrombosis formation and microinfarction secondary to microvascular ischaemia (240). The stress-induced activation of platelets could be inhibited by beta-blockers rather than aspirin, suggesting that sympathetic stimulation is key in the process (241). Platelet-leukocyte aggregate formation is also increased by acute experimental psychological stress (242); this variable is also raised in patients with IBD (143).

An acute stressor has also been reported as causing a transient increase in neutrophil activation as assessed by the oxidative capacity of whole blood to reduce nitro-blue tetrazolium (243).

1.4.3.3 The response to acute stress in the presence of chronic stress (Table 1.3)

The physiological and immune response to acute experimental stress is exaggerated by the presence of chronic psychological stress. Individuals with high chronic stress levels, such as those caring for a long-term dependent or women with a strong family history of breast cancer, showed greater and more prolonged increases in sympathetic activation in response to acute stressors than controls (137;223). This was associated with a greater increase in NK cell numbers, albeit with an attenuated increase in their activity, in the chronically stressed subjects than in controls (137). Fatigued breast cancer survivors also show a greater inflammatory response to an experimental stressor, with increased IL-1beta and IL-6 production by LPS-stimulated whole blood than controls (231).

Antidepressant treatment with citralopram has been reported in one study to normalise the cardiovascular response to acute stress in depressed individuals (244).

1.4.3.4 The response to acute experimental stress in the presence of chronic inflammatory disease (Table 1.3)

The immune response to acute experimental stress may also altered by the presence of chronic inflammatory disease. Patients with systemic lupus erythematosus (SLE) showed an increase in IL-4-producing peripheral blood mononuclear cells, as assessed by staining of intracellular cytokines, in response to the Trier Social Stress Test, where as control subjects did not (233). The stress-induced redistribution of the leukocyte subsets was also altered in patients with SLE, with an attenuated increase in the number of NK cells and their cytolytic activity in comparison to healthy controls (245). However another study failed to find any differences between patients with chronic psoriasis and healthy individuals in response to an acute experimental stressor (230).

Table 1.3 Summary of the effects of adverse life events and acute experimental stress on systemic immune and inflammatory function in man.

Stress	Number of subjects	Effect	Reference
Stressful Life Events			
Bereavement	20000 widows and widowers	Increased mortality	Lusyne et al (2001)(246)
Bereavement	37 widows	Decreased NK and cytotoxic T-cell activity	Irwin et al (1997)(208)
Major depression	36 HV	Reduced NK cell number	Frank et al (2002)(209)
Major depression	46 depressed patients and 46 HV	Reduced NK cell activity	Chu et al (2002)(210)
Geriatric depression	166 depressed patients	Reduced CD4 and CD8 lymphocytes	Fortes et al (2003)(211)
Adolescent depression	36 depressed and 36 HV	Reduced NK cell number	Schleifer et al (2002)(212)
Academic examinations	64 adolescents	Reduced lymphocyte proliferation and neutrophil production of superoxides	Kang et al (1996)(247)
Academic examinations	38 HV	Increased TNF-α, IFN-gamma, IL-6 production by LPS stimulated whole blood	Maes et al (1998)(228)

Stress	Number of subjects	Effect	Reference
Acute Experimental Stress			
TSST	32 HV	Increased IL-6 production by LPS stimulated whole blood	Goebel et al (2000)(229)
TSST	25 HV	Increased IL-1beta and TNF-α production by LPS stimulated whole blood	Ackerman et al (1998)(232)
TSST	15 HV	Increased IFN-gamma and IL-10 production by PBMC	Jacobs et al (2001)(233)
TSST	15 breast cancer survivors	Increased IL-1 beta and IL-6 production by whole blood	Bower et al (2007)(231)
TSST	23 psoriasis patients and 23 HV	Increased CD3, CD8 and NK cells. Increased IFN-γ and decreased IL-10 production	Buske-Kirschbaum (2007) (230)
Stroop	13 HV	Increased serum IL-6 levels	Steptoe et al (2001)(234)
TSST	30 HV	Increased CD8 lymphocytes and NK cells	Marsland et al (1995)(219)
TSST, MAT	15 atopic dermatitis, 15HV	Increased CD8 and NK cells	Schmid-Ott et al (2001)(236)
TSST	15 HV	Increased NK cells	Pawlak et al (1999)(245)
MAT	23 HV	Increased NK cell number and lytic activity	Pike et al (1997)(137)

TSST	39 female HV	Increased NK cells	Wright et al (2007) (235)
Stroop	11 HV under 35	Increased thrombin-induced platelet fibrinognen binding	Wallen et al (1999)(238)
MAT	12 HV	Increased platelet dependent thrombin generation	Kawano et al (2000)(241)
Stroop	40 HV	Increased platelet dependent ATP secretion	Malkoff et al (1993)(239)
Stroop	8 HV	Increased platelet dependent beta-thromboglobulin and serotonin secretion	Naesh et al (1993)(237)
Stroop	37 HV	Increased PLA formation	Steptoe et al. (2003)(242)
TSST	17 HV	Transient increased neutrophil activation	Ellard et al (2001) (243)

TSST Trier social stress test, MAT mental arithmetic test, Stroop word-colour interference test, HV healthy volunteers, NK natural killer, LPS lipopoysaccharide, PLA platelet-leukocyte aggregate, PBMC peripheral blood mononuclear cell

1.4.4 PSYCHOLOGICAL STRESS AND GASTROINTESTINAL IMMUNE AND INFLAMMATORY FUNCTION IN MAN

1.4.4.1 Psychiatric disease and IBD (Table 1.4)

In the 1950s, IBD was classified as a psychosomatic disorder (191;248) with many early studies finding an association between IBD and psychiatric diagnoses (249-251). However, a review in 1990 of 138 such studies found most to have serious flaws, whilst in the seven which did not, there was no association between psychiatric disease and UC (252). In contrast, in a study published in 2004, Mittermaier et al reported that

patients with inactive UC had a significantly increased chance of relapse over the next eighteen months if their baseline score on the Beck's Depression Inventory was raised (253).

1.4.4.2 Stress and the development of IBD (Table 1.4)

Although there have been may studies examining whether stress can provoke relapse in established IBD there have been few analyses of stress as an initiating factor. In the 1960s it was observed that Bedouin Arabs developed an increased prevalence of colitis when moved from nomadic communities into government housing (254). It was proposed that it was the stress of relocation which caused the increased risk of developing IBD. However, studies on the same population since have suggested that in fact exposure to new environmental factors such as hygiene or food may be more important (255). Li et al used retrospective population data to examine whether a stressful life event could predispose to IBD (256). They found the incidence of IBD to be the same in the group of 21,000 individuals who had lost a child as in the general population. In a case-control study, Lerebours et al found there to be a greater incidence of adverse life events in the 6 months prior to the onset of disease in 167 patients with CD and 84 patients with UC. However, once adjusted for depression, anxiety scores and lifestyle differences in a multivariate analysis, the association was no longer significant (257).

1.4.4.3 Stress and Relapse in IBD (Table 1.4)

Surveys consistently show that between 50 and 75% of patients with IBD believe that stress plays a role in causing relapse of their disease (249;258). Placebo response rates in many therapeutic trials of IBD remain as high as 30-40% (259). This response rate relates not only to subjective measures such as patients' feelings of well being, but also to objective measures such as the degree of mucosal inflammation seen at endoscopy, and provides further evidence to suggest that changes in psychological state can affect disease activity (260).

Over the years there have been many case studies which have suggested that adverse life events can be a causal factor in relapse in IBD. However, although suggestive, these retrospective studies are frequently flawed by recall bias. Well-

designed, prospective investigations of stress as causative factor for relapse in IBD are difficult to perform. They require a long study period to allow a sufficient number of relapses to occur to test for correlation, and a high degree of patient compliance for the collection of detailed diary records of life events and GI symptoms. There are often confounding changes in medication during the study period. Lastly, the definition of what constitutes a stressful life-event and what constitutes relapse is variable. In the latter context many of the clinical scoring systems commonly used to assess disease activity in IBD such as the CDAI (261) and SCCAI (262) contain variables, such as stool frequency, which are known to be affected adversely by stress but which do not necessarily reflect a worsening of inflammation.

Table 1.4 Summary of studies assessing association between stress and IBD

Variable	Patients	Finding	Reference
DEVELOPMENT OF IBD			
Retrospective analysis following relocation into government housing	Bedouin arabs	Increased incidence of colitis	Salem SN (1967)(254)
Retrospective 16 year analysis of prevalence of IBD in patients who lost a child and controls	21,062 parents who lost a child	No increased prevalence of IBD	Li et al (2004) (256)
Retrospective case control study of life events and IBD onset	167 CD 74 UC	No association	Lerebours (2007) (257)
RELAPSE IN ESTABLISHED IBD			
I. Chronic Stress			
Adverse Life-events			
Retrospective analysis of life events preceding disease onset	60 patients with UC	Positive association	Fava GA (1976)(263)
Retrospective 2 year analysis of life events preceding disease relapse	70 patients with CD, 44 patients with UC	Positive correlation with relapse in CD but not UC	Paar et al (1988)(264)
Analysis of life events preceding relapse	30 patients with UC	Positive association	Bach et al (1990)(265)
Prospective 6 months of life events preceding exacerbation	124 patients with IBD	Positive association with no lag time	Duffy et al (1991)(266)

Prospective 2 year analysis of life events preceding disease relapse	32 patients with IBD	No association	North et al (1991)(267)
Prospective 1 year analysis of adverse life events and disease relapse	108 patients with IBD	No association	Von Wietersheim et al (1992)(268)
Retrospective analysis of incidence of adverse life events in preceding 12 months in patients with UC and healthy controls	122 patients with UC	Greater incidence of life events in preceding 12 months in patients with UC	Tocchi et al (1997) (269)
1 year prospective analysis of life events and disease relapse	60 patients with UC	Positive association of life events and relapse in the following month	Bitton et al (2003) (270)
Prospective 2 year analysis of depression and life events and disease relapse	18 patients with CD	Positive association of life events and depression with disease relapse simultaneously and at 8-12 weeks	Mardini et al (2004) (271)
Prospective study 11 month analyses of life events and disease relapse	163 patients with IBD	No association	Vidal (2006) (272)
Chonic Perceived Stress			
Prospective 2 year analysis of PSQ score and disease	62 patients with UC	Increased risk of exacerbation with	Levenstein et al (2000) (273)

exacerbation		increased PSQ score	
Analysis of mucosal endoscopic appearance and PSQ score	46 patients with asymptomatic UC	Increased PSQ score in patients with mucosa abnormalities	Levenstein et al (1994) (274)
II. Acute Stress			
Daily Stress			
Prospective 28 days of daily stress with self-rated disease severity	10 patients with CD	Positive association	Garrett et al (1991)(275)
Prospective 1 year analysis of stress with disease exacerbation	11 patients with IBD	Positive association	Greene et al (1994) (276)

CD Crohn's Disease, UC UC, IBD inflammatory bowel disease, PSQ perceived stress questionnaire

1.4.4.4 Adverse life events and relapse in IBD (Table 1.4)

Most of the prospective analyses of the role of stress in IBD are studies of the relationship between adverse life events and disease relapse. Probably for the reasons cited above, these studies have produced mixed results (Table 1.4). Two more recent analyses, in which meticulous attempts were made to address these methodological problems, both found life events to be associated with a higher risk of subsequent relapse. Bitton et al found the number of stressful life events in the preceding month to be a risk factor for relapse in a 1 year prospective study of sixty patients with UC (270). Similarly, Mardini et al found depression and life events to be predictors of relapse in a two year prospective study of eighteen patients with Crohn's disease (271). However, in an analysis of 163 patients with both types of IBD enrolled in remission, Vidal et al found no association between adverse life events and the likelihood of relapse in the following month (272).

One possible explanation for the inconsistent findings in these studies is that stress affects disease activity in only certain individuals with IBD. In a study of 148 patients with UC, Maunder et al found that disease activity correlated with measures of psychological distress only in p-ANCA negative individuals. In p-ANCA positive subjects there was no correlation (277).

1.4.4.5 Chronic perceived stress and relapse in IBD (Table 1.3)

It is now also recognised that an individual's stress response depends on their perception of the significance of the stressor, a factor which is not taken into account in a standard record of life events. The Perceived Stress Questionnaire (PSQ) was developed to overcome this limitation. In a prospective cohort study of 62 patients with UC, a score in the upper tertile of the PSQ over the previous two years significantly increased the actuarial risk of an exacerbation and was also predictive of mucosal abnormalities in patients with UC who reported no symptoms (273;274). Ninety percent of patients who scored in the upper tertile of the PSQ experienced a relapse during the study as compared with 44% in the lower tertile. In these studies a chronic state of heightened stress appeared important in predicting relapse.

1.4.4.6 Acute daily stress and relapse in IBD (Table 1.4)

Only two studies have examined whether daily stress can affect disease activity in IBD. In the first study involving 10 patients with CD, it was found that in certain individuals daily stress was strongly correlated with self-rated disease activity (275). In. the second, a study of 11 patients with IBD, a positive concurrent relationship was found between both daily and current monthly psychosocial stress and IBD activity (276). However, levels of stress in the month before were found to be inversely correlated with IBD activity. Both studies involved only small numbers of patients with relatively short periods of follow up. Therefore on the basis of this evidence it is hard to be sure whether daily stress pre-disposes to relapse in IBD.

1.4.4.7 Acute experimental stress and gastrointestinal immune and inflammatory function

There have been few examinations of the effects of experimental stress on gastrointestinal inflammation in man. One study found that physical stress, induced by immersion of the hand in iced water, increased the luminal jejunal concentration of the mast cell mediators tryptase and histamine in healthy volunteers and even more so in those with food allergies (278). A second study found that repeated administration of the same stress over several days lead to an increase in the proportion of degranulating and activated mucosal mast cells seen at electron microscopy in mucosal biopsies from both patients with CD and UC (279).

There is also limited anecdotal evidence that artificially induced alterations in neural function can affect gastrointestinal inflammation. One reported example is of a man whose previously refractory UC went into complete remission following a Brown-Sequard paralysis at the level of C5 (280). Kemler described a patient who suffered recurrent flares of his UC in association with spinal cord stimulation given for post-traumatic pain in his arm (281). Neuromodulatory drugs such as lidocaine (282), which decreases neuronal release of Substance P, clonidine (283) and nicotine (284) have been claimed for many years to have benefit in UC in clinical trials and remain potential therapeutic agents in IBD (285).

1.4.4.8 Functioning of the stress axes in IBD

Several studies have suggested that the functioning of the HPA axis may be altered in patients with IBD and this may be important in relation to stress-induced increases in disease activity. Usually, the release of CRF within the pituitary, and hence the serum concentration of cortisol, is increased by inflammatory cytokines, particularly IL-6. As the prevailing action of cortisol is anti-inflammatory in this context it provides a negative feedback which reduces inflammation. However, in a study of 64 patients with UC, serum concentrations of cortisol were found to bear no relation to serum IL-6 levels or to scores of disease activity (286).

In a second study, Straub et al examined the correlation between sympathetic tone, as indicated by serum levels of neuropeptide Y, and HPA axis stimulation, as

measured by serum cortisol, in healthy controls and patients with IBD (168). In healthy volunteers, the two variables were positively correlated. However, in patients with IBD, there was no such correlation, with a higher level of neuropeptide Y and a lower level of cortisol. Although in this study the concomitant use of oral steroids may have affected results, the authors suggest that in patients with IBD there may be uncoupling of the sympathetic nervous system and HPA axis. Chronically raised levels of inflammatory cytokines in the blood due to active IBD may thus blunt the response of the HPA axis to both inflammation and acute stress, as has been found in other chronic inflammatory diseases.

The function of the ANS may also be altered in patients with IBD as some authors have reported marked autonomic nervous hyperreflexia in patients with UC and CD (287). Maunder et al found an atypical reduced pattern of ANS activation in response to stress to be associated with reduced disease activity months later (288).

1.4.4.9 Acute psychological stress and gastrointestinal motility and water and ion secretion (Fig 1.4)

Acute psychological stress has effects on gastrointestinal motility and water and ion secretion. Thus, in healthy human volunteers acute short term stress in the form of painful stimuli, dichotomous listening tests and stressful interviews enhanced colonic motility (289). Dichotomous listening tests and cold pain stress have also been shown to increase jejunal water and sodium and chloride ion secretion (278;290;291). Although these are non-inflammatory changes, they could contribute to stress-induced increases in symptomatology in patients with IBD.

1.4.4.10 Psychological stress and pain processing

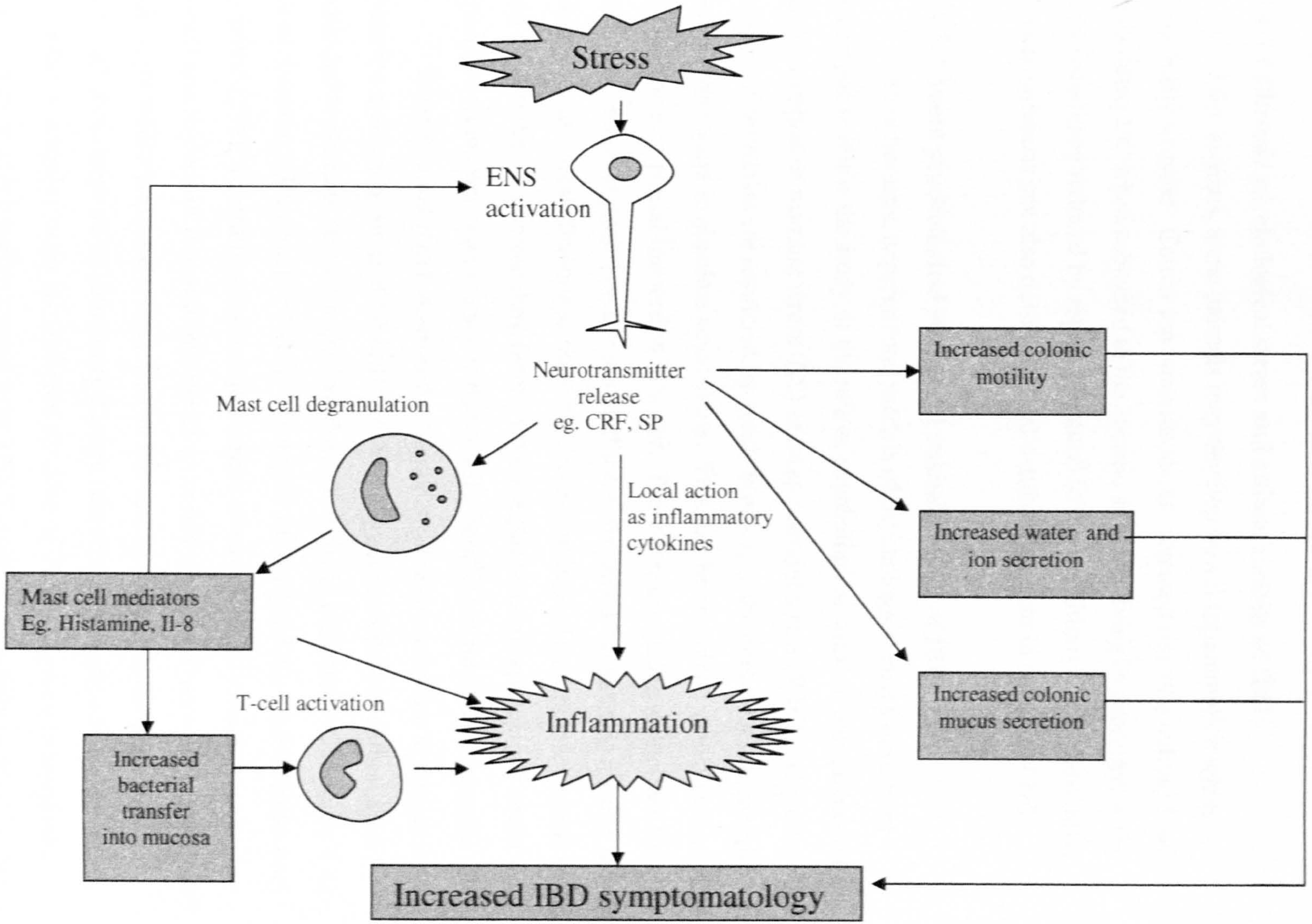
Acute psychological stress has been shown to decrease thresholds for the perception of pain. Dichotomous listening tests decreased the threshold for the perception of pain in response to rectal distension in both patients with IBS and healthy volunteers (292;293). Although this experiment has not been repeated with patients with IBD, if stress does lower pain thresholds in these patients it may, in part, explain how acute stress can worsen IBD symptomatology.

The central release of Substance P (SP) from afferent neurones has been shown to be important in mediating stress-induced gastrointestinal hyperalgesia. The central administration of a SP antagonist prevented restraint stress-induced hypersensitivity to rectal distension in the guinea pig. However, the SP antagonist had no effect on rectal sensitivity in animals which had not been sensitised with restraint stress (294).

1.4.4.11 The role of peripheral substance P (Fig 1.4)

In addition to its central effects, the peripheral release of SP from the ENS may have a role in stress-induced increases in mucosal inflammation. Although there is no published data to show an increase in mucosal SP in response to stress, SP-containing neurones are found in close association with mast cells, a cell type implicated as central in mediating stress-induced permeability changes (see Section 1.4.5.4). SP has been shown to increase histamine release from mucosal mast cells in patients with IBD (190). Lastly, SP can act not only as a neurotransmitter but also an inflammatory cytokine in its own right, enhancing cytokine production and stimulating chemotaxis of inflammatory cells (189). It also increases the expression of leukocyte adhesion molecules on microvascular endothelium, and of CD11b on neutrophils, facilitating leukocyte adhesion at sites of inflammation (189).

Figure 1.4 Pathways by which the ENS is likely to mediate stress-induced increases in IBD symptomatology and disease activity



1.4.5 PSYCHOLOGICAL STRESS AND GASTROINTESTINAL IMMUNE AND INFLAMMATORY FUNCTION IN ANIMALS

1.4.5.1 Chronic psychological stress and animal models of IBD

Like humans, some animals may develop bowel inflammation when chronically stressed. Cotton top tamarins are at increased risk of a colitis closely resembling UC when subjected to the chronic stress of living in captivity, with remission being induced by return to natural living conditions (295). Similarly, Siamese gibbons can also develop a fatal colitis when held in captivity (296).

1.4.5.2 Acute psychological stress and animal models of IBD

As in humans, experimental models of psychological stress have been developed to allow the study of the role of psychological stress in animal models of IBD. A period of restraint stress (RS) or wrap restraint stress (WRS), where an animal's movements are restricted by gentle binding, is the commonest technique used in the rodent to simulate acute stress. This can be combined with either a cold environment or partial immersion in water. The water avoidance stress (WAS), where an animal is placed on a small platform surrounded by water, is another model used for acute stress. Prolonged maternal separation is used to simulate chronic stress and depression. A confounding factor, which requires use of appropriate control groups in all animal experiments, is that routine handling is inherently stressful (297).

Experimental psychological stress appears able to contribute to both the initiation and reactivation of GI inflammation in animal models of colitis. Rats subjected to restraint stress for the four days prior to the induction of colitis by 2,4,6-trinitrobenzenesulfonic acid (TNBS) developed an increased mucosal inflammatory response (298). Restraint stress in the absence of any other co-stimuli caused a partial reactivation of mucosal inflammation in rats which had recovered from TNBS colitis six weeks previously; there was an increase in colonic myeloperoxidase, although there were no inflammatory changes detectable by light microscopy (299). A period of restraint stress also lowered the dose of TNBS required to reactivate colitis in mice which had recovered from TNBS colitis eight weeks previously (300).

This susceptibility was transferable between mice by a population of CD4-rich lymphocytes taken from spleen and mesenteric lymph nodes, suggesting that stress induced-reactivation is dependent on the presence of key immune cells. The recipient mice did not develop colitis immediately, but required a lower dose of TNBS in combination with restraint stress to cause mucosal ulceration than did controls. Stress induced reactivation of colitis in healed mice also appears to be dependent on noradrenergic and cholinergic pathways as it can be blocked by treating the animals with hexamethonium (a nicotinic cholinergic ganglion blocking agent) or the co-administration of atropine (a muscarinic antagonist) and bretylium (a noradrenergic ganglion blocking agent) (301).

Chronic stress appears to render an animal with colitis more vulnerable to the effects of acute stress. Adult rats which underwent previous prolonged maternal separation and which were then subjected to a series of inescapable foot shocks, experienced a greater severity of DSS-induced colitis than did controls (297). This sensitivity to the colitis induced by DSS and acute stress, in chronically stressed mice, was abolished by the administration of antidepressants (302).

1.4.5.3 Functioning of the stress axes in animal models of IBD

There have been no studies to assess the functioning of the stress axes in animal models with IBD. However, it has been shown that reduced HPA axis function renders rodents susceptible to stress-induced increases in GI inflammation. If, as discussed above, HPA axis function is reduced in patients with IBD this observation may be relevant to stress-induced increases in disease activity. LEW/N rats have a reduced CRF content of the hypothalamus and paraventricular nucleus compared to controls. They have a markedly decreased plasma ACTH and corticosterone response to stressful stimuli, and have long been recognised as being more susceptible to inflammatory disorders such as the arthritis induced by intra-articular injections of streptococcal cell wall (303). LEW/N rats also show an increased susceptibility to TNBS-induced colitis, an effect which was found to be dependent on co-existing stress (304). Thus, although stress-naive animals developed a similar degree of intestinal inflammation to control rats at 7 days after TNBS

administration, intestinal inflammation was greater in LEW/N rats than in appropriate control animals if they were restrained for 6 days prior to being given TNBS.

1.4.5.4 Psychological stress and intestinal barrier function and bacterial-host interactions (Fig 1.4)

Experimental stress in animals increases intestinal mucosal permeability and also alters bacterial-host interactions. These changes are likely to contribute to mucosal inflammation, and fit with the hypothesis that such inflammation is driven by mucosal flora (107).

Restraint stress in rats has been shown to increase jejunal and colonic permeability to inert marker molecules, such as mannitol and Cr-EDTA, and to antigenic proteins such as horse radish peroxidase (HRP) (305). Under normal conditions, HRP passes through the epithelial cells via pinocytosis but, after chronic restraint stress, it also passed through the epithelial layer paracellularly. Although the gut mucosa appeared normal to examination under the light microscope, electron microscopy demonstrated increased HRP both within intracellular endosomes and in inter-epithelial cell tight junctions.(306) Acute stress reduced the expression of tight junctional proteins integral to the maintenance of GI barrier function (307). The effects of acute stress on permeability can be enhanced by the co-administration of a chemical irritant. Rats subjected to foot shock stress and then given a dose of DSS too low to cause a colitis in isolation, show a greater increase in colonic permeability to EDTA than rats given stress or DSS alone (308). Again, chronic stress appears to enhance the effect of acute stress, since prior maternal separation augmented restraint stress-induced increases in permeability (309).

The increase in permeability caused by stress appears to be dependent on cholinergic innervation, as it was blocked with atropine and was more marked in cholinesterase-deficient Wistar-Kyoto rats (310). Mast cells are also important in mediating permeability changes, since mast cell-deficient rats exposed to repeated restraint stress lost weight to a similar extent to wild-type rats but without showing changes in intestinal permeability (311). Restraint stress increased the histamine content of colonic mucosal mast cells in rats, an effect which appeared dependent on

both central CRF and IL-1 (312). Increased colonic permeability caused by stress was also mimicked by the injection of peripheral CRF (306).

In addition, it has recently been shown that bacterial-mucosal interactions are also affected by experimental stress in animal models. One hour sessions of water-avoidance stress for ten consecutive days increased the phagocytic uptake of killed *E.coli* into follicle-associated epithelium in mice (313). Follicle-associated epithelium overlies Peyer's patches and contains M cells which are in close association with dendritic cells, the principal antigen-presenting cell of the gut. These changes were not found in villi-associated epithelium and did not occur in mast cell-deficient rats (313;314). Prolonged neonatal separation in rats has been shown to lead to increased bacterial adherence and penetration into colonic epithelium compared to control animals (315). Further evidence that stress may increase GI uptake of bacteria is provided by the finding that psychological stress facilitates the translocation of indigenous bacteria into a host animal. Bailey et al found that both chronic restraint stress and repeated social disruption lead to an increase in the number of animals with detectable levels of bacteria in their inguinal and mesenteric lymph node compared to controls (316).

Catecholamines also appear to have a role in altering bacterial adherence and uptake by gut mucosa. Chen et al reported that both dopamine and noradrenaline increased the adherence of *E. coli 0157* to murine caecal mucosa (317), whilst Green at al found that pre-treatment of isolated porcine jejunal Peyer's patches with noradrenaline led to increased internalisation of *Salmonella choleraesuis* and *E.coli 0157* but not of non-pathogenic *E. coli* (318).

It is possible that mast cells, stimulated by neurotransmitters such as CRF and substance P released by enteric neurones in response to stressful stimuli, increase bacterial adherence and uptake via the release of mast cell mediators. This in turn could lead to the sensitisation of T-cells and the production of IFN-γ and TNF-α which may both initiate inflammation and cause a secondary increase in permeability. This theory is consistent with the recent finding that increases in intestinal permeability to the inert marker Cr-EDTA, in response to mixed restraint and acoustic stress, were dependent on the presence of T-cells and IFN-γ (319).

Application of IFN-γ to gastrointestinal epithelial cells in culture increased both paracellular permeability and internalization of *Shigella sonnei* (320). Expression of mRNA for TNF-α in mucosa correlates with endosomal uptake of horseradish peroxidase (HRP) in resected ileal mucosa mounted in Ussing chambers (321).

1.4.5.5 Psychological stress and gastrointestinal motility and water and ion secretion (Fig 1.4)

As in humans, acute stress in the form of restraint stress, loud noise, inescapable foot shock or water avoidance all increase colonic motility and defecation in the rodent (322). The mechanisms for these changes involve CRF and its receptors (see below) (323).

Similar alterations in ion and water transport to those seen in humans are also well described in animals in response to psychological stress. Increases in GI water and chloride ion secretion occur in response to restraint stress in the rat (324). It is now recognised that, as with changes in intestinal permeability, this secretory response is related to both cholinergic nerves and mast cells as it was increased in cholinesterase-deficient Wistar-Kyoto rats, blocked by pre-treatment with atropine and absent in mast-cell deficient rats (310;311). Restraint stress also increased colonic mucus secretion in ex-vivo colonic segments and in vivo as measured by histological goblet cell depletion (325-327). This effect could also be reproduced by peripheral CRF administration and inhibited by mast cell stabilisers (328).

1.4.5.6 The role of CRF in mediating stress-related gastrointestinal changes in animal models (Fig 1.4)

CRF is a 41 amino acid neuropeptide which exerts its effects via two adenylate cyclase coupled receptors, CRF-R1 and CRF-R2 (329). It has a pivotal role in mediating the effects of stress on the gastrointestinal tract in animal models, some of which may relevant to stress-induced increases in IBD activity in humans.

Central injection of CRF in the rat induces behaviour normally seen in response to stressful stimuli (330). It also reproduces the motility changes usually seen in response to stress, with decreased gastric emptying and increased colonic

motility. The use of selective CRF-receptor antagonists has proven that central CRF increases colonic motility via stimulation of CRF-R1 and delays gastric emptying via stimulation of CRF-R2 (323).

More recent evidence has also implicated a role for peripheral CRF in mediating stress-induced motility changes. The peripheral administration of CRF antagonists has been shown to abolish restraint stress-induced increases in colonic motility and decreases in gastric motility (331). As described above, both peripheral and central CRF are important in mediating stress-induced increases in gastrointestinal permeability. Furthermore, CRF appears to have a secondary peripheral action as an inflammatory cytokine. It stimulates IL-1, IL-2, and IL-6 secretion and IL-2 receptor expression by peripheral blood mononuclear cells (332-334), T and B lymphocyte proliferation and enhancement of NK cytotoxicity (335). It exerts a mixed effect on cytokine production by endothelial cells, with both a pro-inflammatory action via CRF-2 receptors, and an anti-inflammatory action via CRF-1 receptors (336). More recent evidence for a functional role for CRF in animal models of GI inflammation comes from the finding that pre-treatment with a CRF antagonist reduced the inflammation caused by the injection of C.difficile toxin into rat terminal ileum (337).

CRF levels are increased in caecal biopsies from rats with experimental colitis induced by the intra-mural injection of peptidoglycan-polysaccaride polymers (338). In the chronically inflamed caecum, abundant immunoreactive CRF was found in inflammatory cells, mesenchymal cells and myenteric plexi; in contrast, in non-inflamed caecum only minimal CRF-containing cells were found. Whether CRF has a role in mediating stress-related gastrointestinal changes in humans with IBD is unknown. However there is some evidence of a functional role for CRF in the pathogenesis of IBD as CRF levels are increased in lamina propria mononuclear cells (LPMC) in colonic biopsies from patients with active UC (339;340).

1.4.5 STRESS-REDUCTION THERAPY IN IBD (Table 1.5)

If psychological stress is indeed a pathogenic factor in IBD, then stress reduction therapy may have therapeutic benefit. However, despite recent advances in

our understanding of the relationship between psychological stress and IBD, most stress reduction therapy remains unformalised, and studies of its efficacy in patients with IBD are few. There are a wide variety of psychotherapeutic interventions which could be assessed making standardisation difficult. Due to the nature of the intervention performing the trials in a blinded controlled manner is also difficult and as already discussed, with placebo rates of up to 40% (259;260), genuine therapeutic effects can be hard to detect.

The results of the few trials which have been reported are mixed (Table 1.5). Milne et al found that a stress management course in addition to conventional treatment significantly reduced the CDAI score over the follow up period of one year in a randomized trial of 80 patients with CD (341). However, the groups were not equivalent in CDAI score pre-treatment, with more patients in the non-treatment group having a CDAI score <150 at baseline. In another study, self-guided stress management was found to reduce the symptoms reported by a group of 15 patients with CD compared to those given conventional treatment. However the symptom scores used were not validated scores of disease activity (342).

Schwarz and Blanchard found that combined complementary medical treatment, including cognitive behavioural therapy, muscle relaxation techniques and patient education was successful in reducing stress but did not improve IBD symptoms (343). However, the treatment groups were not comparable, with 70% of patients in the treatment group having CD compared with only 30% in the non-treatment group. In a well-designed study, Jantschek et al failed to find that the addition of short-term psychodynamic therapy and relaxation training led to a reduction in the number, duration or severity of relapses in a study of 108 patients with CD (344). Langhorst found that a comprehensive life-style modification program given to 60 patients with quiescent UC lead to an improvement in quality of life scores at 3 months but had no effect on disease activity (345). Further work is required before a view can be taken as to whether stress reduction therapy might have therapeutic benefit in IBD.

Table 1.5 Summary of trials of stress reduction therapy in IBD

Intervention	Patients	Outcome	Reference
Cognitive behavioural therapy, muscle relaxation	11 patients with IBD	No improvement in IBD symptoms	Schwarz (343)
Psychotherapy	108 patients with CD	No reduction in number or severity of relapses over two years	Jantschek (344)
Stress management programme	80 patients with CD	Reduced CDAI over next year	Milne (341)
Stress management programme	45 patients with CD	Reduced IBD symptoms over next year	Garcia-Vega (346)
Lifestyle modification including stress reduction	60 patients with UC	No effect on disease activity	Langhorst (345)

1.5 HYPNOSIS AND HYPNOTHERAPY

1.5.1 TRANCE (347)

The attainment of a "trance state" is central to the practice of hypnosis. However, despite hypnosis's long history, the trance state remains poorly defined. In essence it refers to a state of mind in which the individual is more receptive to the suggestions of the therapist. A trance state can also be described as a waking state in which the person's attention is focused away from his or her surroundings and absorbed by inner experiences such as feelings, cognitions and imagery. In this way the hypnotic trance is similar to the natural state of daydreaming or meditation but the subject's thoughts can be guided by the therapist. It has also been proposed that in this state the conscious mind can be bypassed and suggestions placed directly in the unconscious. In essence, the trance state is a receptive state in which to place positive suggestions and perform psychotherapy.

1.5.2 FURTHER PROPERTIES OF THE TRANCE STATE

Further properties of trance include the following which, although not guaranteeing trance, nor being exclusive to trance, do have an increased probability of occurrence in the trance state.

1. Temporal distortion. Alteration in the experience of the passage of time with more often a loss of recollected time
2. Some amnesia for events which clearly occurred as the subject responded at the time. This property is similar to that of "highway trance" where the subject has no memory of some part of the journey but was clearly awake at the time.
3. Attenuation and increased tolerance to ongoing discomfort or pain.
4. With regular practice an alleviation of the effects of stress.

1.5.3 SUGGESTION AND HYPNOTHERAPY

Suggestion is, in conjunction with trance, the other key component of hypnosis and hypnotherapy. A suggestion involves a communication conveyed

verbally by the hypnotherapist that directs the subject's imagination in such a way as to elicit intended alterations in sensations, perceptions, feelings, thoughts and behaviour. A post-hypnotic suggestion is intended to cause a delayed rather than immediate response, usually after the hypnosis session has been concluded: "You will feel increasingly confident over the next few days and weeks." They are the commonest form of suggestion used in hypnosis as the desired goal of therapy is usually a long term change in feelings or behaviour.

1.5.4 HYPNOSIS AND THE IMMUNE SYSTEM

There have been relatively few investigations of the effects of hypnosis on the immune system. The majority of studies have examined whether hypnotherapy can ameliorate the immune changes induced by chronic stress. Naito et al sampled blood from 48 medical students during a non-stressed period and also immediately prior to medical exams (348). In the time period between the two samples being taken the students were randomised to one of three groups taught either self-hypnosis, Johrei (a Japanese relaxation technique) or a control relaxation procedure. In the control group it was found that NK cell count and NK cell activity decreased whilst CD3(+)CD4(+) cells increased prior to exams. However, in the hypnosis group NK cell levels and CD3(+)CD4(+) cell levels were maintained. Similarly Gruzelier et al found reduced levels of NK cells and CD8(+) lymphocytes in blood samples taken from students prior to exams (349). As before, in a group of students who had been taught and practised self –hypnosis these changes were reduced. In contrast to Naito et al, Keicolt-Glaser et al reported reduced levels of CD3(+) and CD4(+) leukocytes in blood samples taken from medical students prior to examinations (350). However, as with the other studies discussed, these changes were abolished in a group of students who had been taught self-hypnosis.

Fewer studies have examined the direct effects of hypnotherapy on the immune system. Zachariae et al found that hypnosis, both with and without suggestions to enhance immune function, lead to a brief reduction in NK-cell activity and lymphocyte proliferative responses to mitogens (351). However, they did not observe any sustained immune changes in either group. Wood et al reported that the

proportion of T-cells expressing IFN-γ and IL-2 was reduced in blood samples taken from seven highly hypnotizable healthy volunteers immediately after hypnosis (352).

The apparent contradiction in the effects of hypnosis on NK cells in the studies discussed above may well be due to the differing effects seen with single and repeated sessions. In this regard it is similar to the effects of stress on NK cells. Medical exams represent a chronic stress exerted over several weeks. This classically leads to a fall in NK cell numbers which is opposed by repeated sessions of self-hypnosis. Acute stress usually causes an acute increase in NK cells, whereas a single session of hypnotherapy has the opposite effect.

1.5.5 HYPNOTHERAPY IN PSYCHOSOMATIC DISEASE

A disease with a psychosomatic component is one in which the physical symptoms can be induced or worsened by psychological factors including stress. Hypnosis and other psychological treatments, such as cognitive behavioural therapy, have been shown to be effective in many of these disorders.

Friedman et al found hypnosis to be an effective treatment for essential hypertension in a randomised study comparing hypnotherapy, cognitive behavioural therapy and a control procedure (353). Hypnosis has been used with positive results to treat the dermatological complaints of eczema, psoriasis and urticaria (354;355).

There is some evidence to suggest that hypnosis may be effective in asthma, an inflammatory disease with a psychosomatic component. Maher-Loughann et al found hypnosis to be superior to breathing exercises in the treatment of asthma and that the maximum improvement occurred between sessions 7 and 12 of a weekly treatment regime (356). Ewer et al found a 75% improvement in the degree of bronchial hyper-responsiveness to a standardised metacholine challenge test in 12 highly hypnotisable adults with moderate asthma after a six week course of hypnotherapy (357). Langewitz et al reported significant improvements in visual analogue scale (VAS) scores of symptoms and well-being in a group of 79 patients with hay-fever taught either self-hypnosis or a control procedure (358).

1.5.6 HYPNOSIS IN GASTROINTESTINAL DISEASE

There is a substantial volume of good scientific evidence to support the use of hypnotherapy in functional gastrointestinal disease.

In 1984 Whorwell et al published a small but well-designed placebo-controlled trial of hypnosis as a treatment of irritable bowel syndrome (359). 30 patients with severe refractory irritable bowel syndrome (IBS) were randomised to receive either seven sessions of hypnotherapy or psychotherapy and placebo pills. Hypnosis, in contrast to placebo, substantially improved the IBS symptoms of all patients. Since this first study the same group have published several other larger studies with longer follow-up (360). While the 100% response rate of the first trial has not been replicated, all the studies have shown a significant improvement in the hypnotherapy-treated group. In a prospective study of 204 patients with refractory IBS treated with 12 sessions of hypnotherapy over a 3 month period, there was an initial improvement rate of 71% and this was maintained in 81% of these patients at 5 years (58% improvement rate overall) (360). Although, in the initial phase, hypnotherapy is labour intensive, self-hypnosis is easily taught although its practice requires patient compliance. Whilst Whorwell's group remains the best established in the use of hypnotherapy in GI disease, several other groups both from within and outside the UK have also demonstrated the benefit of hypnotherapy in IBS.

The mechanism or mechanisms by which hypnotherapy improves the symptoms of IBS remain largely unknown. Hypnosis does seem to have effects on GI physiology which may have relevance. The hypnotic state increases oro-caecal transit time and reduces colonic motility (361). Hypnotherapy has also been shown to increase pain thresholds to rectal distension in patients with IBS who have abnormally low baseline pain thresholds (362). However, it is equally possible that the mechanism may involve cognitive changes in perception of the disease and symptoms.

Whorwell's group have extended the use of hypnotherapy to the treatment of upper GI tract disease. In a study of 126 patients with functional dyspepsia, 16 sessions of weekly hypnotherapy lead to an improvement in short-term symptom scores in 59% of patients compared to 41% in the placebo group (363). Furthermore

this continued to improve after the treatment to an average of 73% at the one year follow-up point. A reduced need for alternative medication and an improved quality of life were also noted in the hypnosis treatment group. In an earlier study the same group assessed the efficacy of hypnotherapy in preventing relapse of duodenal ulceration (364). Thirty patients were successfully treated for a duodenal ulcer with ranitidine and then randomised to a further ten weeks of either drug treatment and hypnotherapy, or drug treatment alone. At one year, 8 of the hypnotherapy group (51%) and 15 (100%) of the control group had relapsed.

1.5.7 HYPNOSIS IN INFLAMMATORY BOWEL DISEASE

There are several anecdotal reports from patients to suggest that hypnotherapy may have benefit in IBD. However, there has been only one non-blinded study; in this, a trend to improvement in the IBDQ score of 12 patients with active Crohn's disease and UC given six weeks of hypnotherapy did not reach statistical significance (p=0.08) (365).

1.6 SUMMARY

The aetiology of IBD is complex and represents an interaction of multiple genetic and environmental factors. Although there has been significant recent progress in identifying the genetic factors important in the aetiology of IBD, the environmental factors remain incompletely understood.

Although studies of the relationship between adverse life events and disease relapse in IBD remain inconclusive, with the development of the concept of the brain-gut axis there is an increasing awareness that psychological factors can affect GI inflammation. This is strongly supported by data from animal models of colitis which suggest that both acute and chronic stress can contribute to the development and relapse of GI inflammation. Alterations in intestinal permeability and changes in mucosal-bacterial interactions may both be important in effecting these changes and which also seem dependent on mast cells and corticotrophin releasing factor. Studies from humans with IBD hint that alterations in the balance between the HPA and ANS

may also be important. However, there is little data on the effects of acute stress in humans with IBD.

Currently, analyses of the role of stress reduction therapy in IBD are limited and have produced conflicting results. Hypnotherapy has been shown to be effective in functional gut disease and reported anecdotally as beneficial in IBD. The mechanisms by which hypnosis might act in IBD have not been investigated.

CHAPTER 2

HYPOTHESIS AND AIMS

2.1 INTRODUCTION

Ulcerative Colitis is a chronic inflammatory condition of the bowel of unknown aetiology. It is characterised by periods of remission and relapse. As discussed in chapter 1, there is increasing evidence that both adverse life-events and chronic psychological stress and can increase the incidence of relapse in IBD and in particular UC. There is also supporting evidence from animal models of colitis that acute stress can contribute to both the initiation and reactivation of GI inflammation. Stress is thought to act through as yet incompletely defined neuroimmunomodulatory pathways. There have been few studies of the effects of acute psychological stress on GI inflammation in patients with IBD.

There have been only a limited number of studies examining the therapeutic potential of stress-reduction therapy in IBD. Hypnotherapy is a formalised treatment approach using the induction of trance to treat specific problems with relaxation, suggestion and imagery. It is an effective treatment in irritable bowel syndrome, non-ulcer dyspepsia and relapsing duodenal ulcer disease and has been reported anecdotally to be of benefit in IBD. The effects of hypnotherapy in patients with IBD and the mechanisms by which it might act have not yet been described.

2.2 HYPOTHESES

Two main hypotheses are tested in this thesis:

1. Inflammation in quiescent UC can be increased by acute psychological stress
2. Inflammation in active UC can be reduced by the relaxation achieved through hypnosis

There are two other subsidiary hypotheses tested in this thesis:

1. An individual's autonomic and inflammatory response to acute stress is affected by levels of chronic stress
2. The response of individuals with UC to acute psychological stress and hypnosis is different to that of healthy volunteers

2.3 AIMS

1. To investigate the effects of acute psychological stress on various measures of systemic inflammation and physiology in healthy volunteers and systemic and rectal mucosal inflammation and physiology in patients with quiescent UC
2. To investigate the effects of a single session of hypnotherapy on the on the same measures in healthy volunteers and in patients with active UC
3. To compare the effects of stress and hypnosis with those of a control procedure
4. To assess the relationship between chronic stress and the response to acute stress and hypnosis in both healthy volunteers and patients with UC

2.4 METHODS (Chapter 3)

The measures used to assess the responses to stress and hypnosis had either been previously implicated in the response to stress or hypnosis (histamine (278), substance P (SP) (190), NK cells (219;348;349), rectal mucosal blood flow (366)), are thought to play a pathogenic role in IBD (IL-13 (367), TNF-α (368)) or appear to be involved in both the stress response and IBD (IL-6 (369), platelet activation (141), platelet-leukocyte aggregate formation (143), reactive oxygen metabolite production (181)).

ASSESSMENT OF SYSTEMIC AND RECTAL PHYSIOLOGICAL RESPONSE

The systemic and rectal physiological response to stress, hypnotherapy and the control procedure was assessed by the methods described below

1. *Systemic autonomic response.* Pulse and blood pressure were measured before, at 15 minute intervals during and after each session of stress, hypnotherapy or control procedure
2. *Rectal autonomic response.* Rectal mucosal blood flow was recorded *in vivo* using laser doppler flowmetry before and after stress, hypnosis or control protocols.

ASSESSMENT OF SYSTEMIC INFLAMMATORY RESPONSE

The following systemic inflammatory measures were tested before and after psychological stress, hypnosis and control protocol:

1. *Serum cytokines.* Concentrations of the inflammatory cytokines IL-6 and IL-13 were measured by ELISA
2. *Whole blood cytokine production in vitro.* After incubation of whole blood for 24 hours with lipopolysacharide, serum TNF-α and IL-6 concentrations were measured by ELISA.
3. *Total leukocyte count.* The circulating total leucoyte count was assessed using a standard automated coulter counter.
4. *Natural Killer (NK) cell numbers.* Circulating NK cell numbers, as a percentage of leukocytes in peripheral venous blood samples, were measured using flow cytometry.
5. *Platelet activation.* Platelet activation was assessed in peripheral blood samples by measuring the percentage of platelets expressing p-selectin using flow cytometry.
6. *Platelet-leukocyte aggregate formation.* PLA formation in peripheral blood was assessed using flow cytometry.

ASSESSMENT OF RECTAL MUCOSAL INFLAMMATORY REPSONSE

The following measures of rectal mucosal inflammation were tested before and after psychological stress, hypnosis and the control procedure:

1. *Release of cytokines and other inflammatory mediators by rectal mucosa.* A piece of filter paper was placed on the rectal mucosa and allowed to adsorb peri-mucosal fluid. The filter paper was then incubated in buffer for 24 hours. Cytokine (TNF-α, IL-13), mast cell mediator (histamine) and neurotransmitter (Substance P) concentrations were then measured by ELISA.
2. *Production of reactive oxygen metabolites.* Rectal biopsies were analysed for their production of reactive oxygen metabolites *in vitro* by incubating them with luminol and measuring chemiluminescence.
3. *Histology of rectal mucosal biopsies.* Conventional haemotoxalin and eosin histological staining was performed on rectal biopsies and inflammation scored by a blinded histopathologist.
4. *Mast cell numbers and degranulation.* Immunofluorescence was performed on snap frozen rectal biopsies using antibodies to the mast cell mediator tryptase. The number and percentage of mast cells degranulating was scored by a blinded histopathologist.
5. *Mucosa-associated bacteria.* Fluorescent in-situ hybridisation (FISH) studies were performed on snap frozen rectal biopsies. The numbers of *E.coli* and total bacteria adherent to mucosa and in the lamina propria were scored by a blinded histopathologist.

ASSESSMENT OF CHRONIC STRESS LEVELS

To assess the relationship between the response to acute stress, hypnotherapy or the control procedure and chronic stress levels, subjects completed several well-validated psychometric tests of chronic stress: the Hospital Anxiety and Depression Score (HADS), the State-Trait Anxiety Inventory (STAXI), the Perceived Stress Questionnaire (PSQ) and the Bradford Somatic Inventory (BSI).

CHAPTER 3

PATIENTS AND METHODS

3.1 PATIENTS AND HEALTHY VOLUNTEERS

Patients with inactive and active UC were recruited from the outpatient clinics at the Royal London Hospital. Healthy volunteers comprised students and staff from the Royal London Hospital and Queen Mary's School of Medicine and Dentistry. As described in Chapter 2, exam-related stress has been shown to alter the immune response of medical students to experimental stress protocols. The medical students recruited were not undertaking any examinations at the time of their participation in the study.

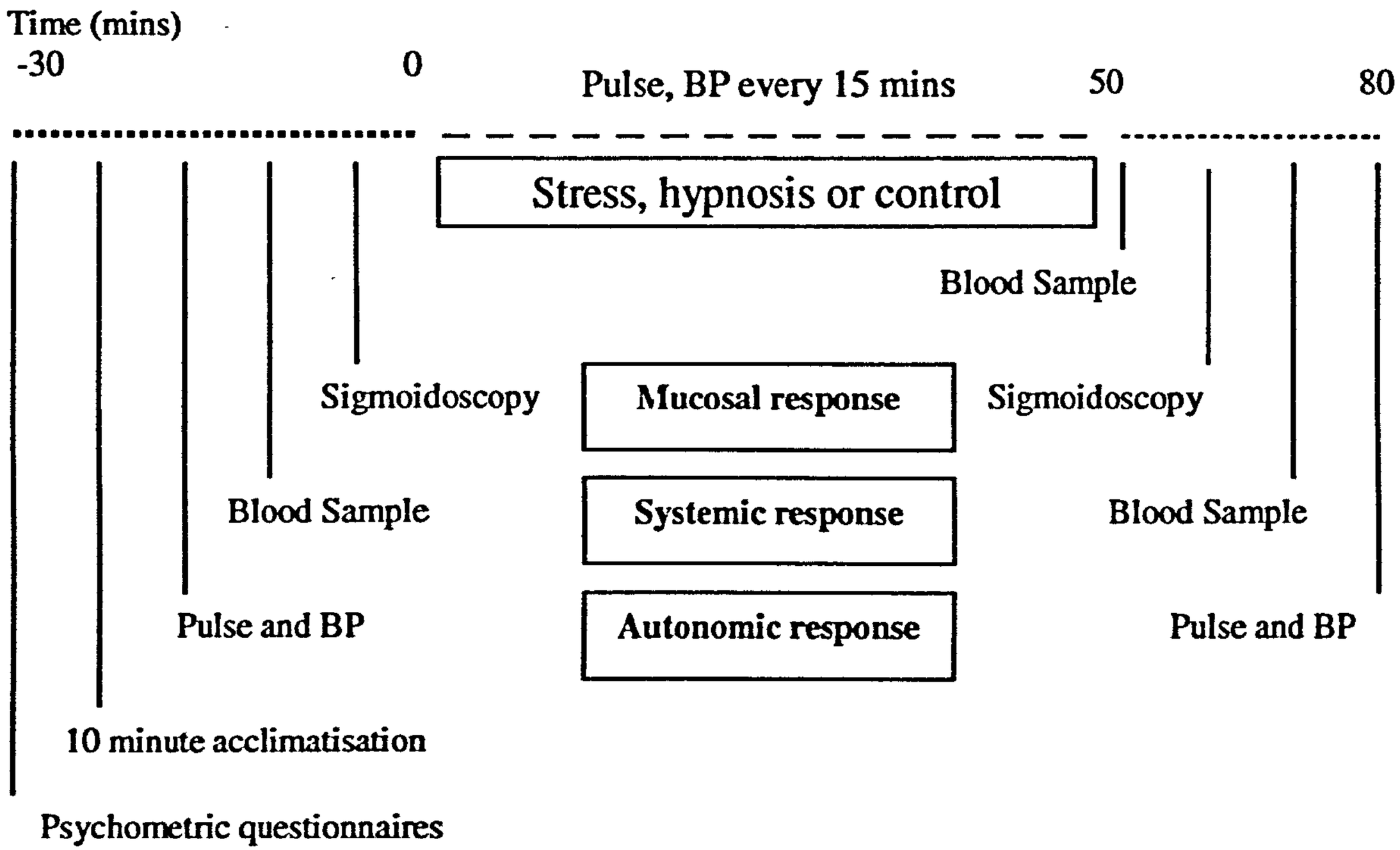
3.2 ETHICS

Ethical approval for these studies was granted by the North-East London Strategic Health Authority Ethics Committee. Each patient gave written informed consent before participation.

3.3 EXPERIMENTAL PROTOCOL

The stress, hypnotherapy and control sessions followed the same general experimental protocol. An overview of the general protocol will be given first, followed by a separate more detailed discussion of the stress, hypnosis and control protocol. Equipment and reagents are listed separately for each section. All equipment and reagents were from Sigma-Aldrich UK (Gillingham, UK) unless otherwise stated.

Figure 3.1 Overall experimental protocol for stress, hypnotherapy and control procedure



3.3.1 OVERVIEW OF STRESS, HYPNOTHERAPY AND CONTROL SESSIONS

Each protocol involved a 50 minute session of stress, hypnotherapy or control procedure, preceded by psychometric questionnaires, a baseline sigmoidoscopy and blood test, and followed by a second sigmoidoscopy and a further two blood tests (Figure 3.1).

Timing and preparation

In order to avoid the potential influence of diurnal variation due to changes in endogenous cortisol production, the stress, hypnotherapy and control protocols were all performed between 9am and 12 noon. As caffeine and nicotine are stimulants which can alter the stress response, patients were asked to have a light breakfast but to abstain from coffee, tea or cigarettes on the morning of the test.

Previous work has shown that rectal blood flow varies in pre-menopausal females with the menstrual cycle. Rectal blood flow is raised and much more variable in the luteal phase of the cycle. Therefore in pre-menopausal female subjects measurements of rectal blood flow were performed only during the follicular phase of their menstrual cycle.(370)

Assessment of clinical activity (Table 3.1)

Disease activity in subjects with UC was assessed using the simple clinical disease activity index (SCCAI) (262). This is a simple five-point questionnaire which has been validated for use in assessing disease activity in UC. Answers to each question are given a score and scores are added to give an overall total. A total score of greater than three was taken as indicative of active disease.

Table 3.1. The Simple Clinical Colitis Activity Index (262)

Symptom	Score
Bowel Frequency (day)	
1-3	0
4-6	1
7-9	2
>9	3
Bowel frequency (night)	
1-3	1
4-6	2
Urgency of defacation	
Hurry	1
Immediately	2
Incontinence	3
Blood in stool	
Trace	1
Occasionally frank	2
Usually frank	3
General well being	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extracolonic features	1 per manifestation

Psychometric Questionnaires

On arrival, patients completed several psychometric tests: the Hospital Anxiety Depression Score (HADS),(371) the State-Trait Anxiety Index (STAXI),(372) the Perceived Stress Questionnaire (PSQ) (see Appendix I),(373) and the Bradford Somatic inventory (BSI)(374) (see appendix I and Section 3.3.5 for further details).

10 minute period of acclimatisation

The patients were then asked to lie on an examination couch in a quiet room and all experiments were performed thereafter in this position. Subjects were first given 10 minutes, without disturbance, to acclimatise to their surroundings.

Baseline Visual Analogue scale

Subjects undergoing the stress or control sessions were asked to record on a 10 point visual analogue scale (0 very relaxed, 10 very stressed) how they felt at that time. This was not part of protocol for subjects undergoing the hypnotherapy session as depth perception was used instead to assess the effectiveness of this procedure.

Baseline Pulse and blood pressure

Following the period of acclimatisation a baseline blood pressure (BP) and pulse reading was taken using a Dynamap blood pressure monitor.

Baseline blood test

An initial 20 ml blood sample was then taken from the antecubital vein using an 18G needle. 6ml of blood was transferred to an empty vacutainer for later use in the assessment of serum cytokine levels. 6ml was transferred to a tube containing lithium heparin for culture with LPS and 2ml to a vacutainer containing potassium ethylenediaminetetraacetic acid (KEDTA) for measurement of total leukocyte count. Finally, 4ml was transferred to a vacutainer containing KEDTA, and then a second vacutainer containing citrate, theophylline, adenosine, dipyridamole (CTAD). The KEDTA/CTAD sample was stored on ice as previous work has shown minimal ex-

vivo activation of platelets and minimal platelet-leukocyte aggregate formation in blood anti-coagulated with this combination and stored at 2-8°C. (143;375) (The CTAD tubes were stored in light-protected boxes and removed immediately before use.)

Baseline Sigmoidoscopy

In the patients with UC, a rigid sigmoidoscopy was then performed and mucosal appearance assessed using Baron's score (Table 3.2) (376). A score of greater than 1 was taken as indicating active disease. Rectal Mucosal Blood flow was measured using a MoorLAB laser Doppler flowmeter and an MP6a endoscopic probe (see section 3.3.13).(370) After this, a sample of perimucosal fluid was collected using the filter paper technique for later assessment of mediator and cytokine levels (see section 3.3.10).(377) Finally, a rectal biopsy was taken and placed in 1ml of pre-oxygenated Tyrode's solution for measurement of reactive oxygen metabolite (ROM) production (see section 3.3.11) (181) and conventional histology (378) (see section 3.3.12) and/or in liquid nitrogen for assessment of mast cell degranulation by immunohistochemistry (see section 3.3.14) and mucosa associated bacteria by FISH (see section 3.3.15).

Table 3.2 Baron's score of mucosal appearance in ulcerative colitis (376)

Score	Mucosal Appearance
0	Normal
1	Loss of vascular pattern (oedema) but no bleeding
2	Friable, bleeding to light touch
3	Ulcerated and or spontaneously haemorrhagic

Stress, hypnotherapy and control protocol

For details of stress, hypnotherapy and control sessions see sections 3.3.2, 3.3.3 and 3.3.4 respectively. Throughout each of the 50 minute sessions a pulse and blood pressure reading were taken every 15 minutes.

Immediate post-protocol blood test

Immediately after completion of the test, a second 20ml blood sample was taken and processed like the first blood sample.

Post-protocol sigmoidoscopy

A second sigmoidoscopy was then performed with assessment of rectal mucosal blood flow, collection of peri-mucosal fluid and rectal biopsy as before.

30 minute post-protocol blood test

Thirty minutes after the completion of the test a third 20ml blood sample was taken and processed like the first two samples.

Post-protocol visual analogue scale

Subjects who had undergone the stress or control protocols were asked to mark once more on the same scale the maximum stress they had felt during the stress or control session.

Recovery pulse and blood pressure reading

A final recovery pulse and BP reading was taken.

Healthy volunteers underwent the same stress and control protocols as patients with UC except that rigid sigmoidoscopy together with assessment of rectal mucosal blood flow, collection of peri-mucosal fluid and rectal biopsy was not performed. The reason for this was that we believed that requesting healthy volunteers to undergo rigid sigmoidoscopy with rectal biopsy twice would have reduced volunteer

recruitment and prejudiced ethical approval for the study. We also felt that, whilst patients with long standing UC, many of whom undergo such examination relatively regularly, do not find rigid sigmoidoscopy stressful, healthy volunteers would. Inclusion of this procedure could therefore have increased the stress induced by both the control and stress protocols in healthy volunteers compared to patients with UC, limiting the validity of comparisons between groups.

3.3.2 STRESS PROTOCOL

Equipment

IQ tests from Measure your own IQ. (379)

2 portable CD players with removal of one earpiece from each set of headphones (Dixon's Superstore, Mile End, London, UK)

Dynamap blood pressure machine (Critikon, Florida, USA)

Methods

As described in Chapter 1, simulating the effects of real-life stress with an acute experimental stress test is extremely difficult. However, several techniques have been developed and used previously in both psychological and gastroenterological research which seem capable of consistently inducing mild, acute, psychological stress. It has been repeatedly demonstrated that these techniques are capable of inducing both a cardiovascular response, with a rise in pulse and blood pressure and also an immune response. As described in chapter 1, leucoyte count and distribution, cytokine production by whole blood, platelet activation and platelet-leucocyte aggregate formation have all been found to be altered in humans by these various methods. There is no data to suggest that one technique produces a different physiological or immune response to another. Nor is there any data to demonstrate the level of stress which must be induced to cause an immune response. Therefore an assumption had to be made that if a stress protocol was capable of inducing a consistent cardiovascular response it was also likely that it would be capable of inducing an immune response.

Clearly the stress induced by these studies is by its nature artificial and therefore extremely difficult to comare with the stress of real life. However, in one study the effects of one of these techniques, the dichotomous listening test, was found to have similar stress-induced effects on GI motility to the real-life stress of driving in London traffic for several hours (221).

The experimental techniques developed to incuce stress in an experimental situation include:

1. Dichotomous listening tests

This technique has been used in several studies in the field of gastroenterology to examine the effects of psychological stress on GI physiology. Several variations of the technique have been used.

i. Conflicting musical inputs - In perhaps the simplest version contrasting types of music are played into each of the subject's ears. Folk and rock music are the commonest genres of music used.(380;381) Subjects are asked to concentrate on one of the auditory inputs.

ii. Spoken word dichotomous listening test - In a second version an extra requirement for mental concentration is added. The subject is played two spoken word inputs into either ear. They are asked to switch their focus from one input to the other at the sound of a bell which is rung at random. They are asked to repeat out loud every third word.(221;382)

iii. Auditory delay dichotomous listening test - A more complex technique involves reading aloud from a text whilst the subject's own voice is replayed into their ears after an added time delay. The time delay can be varied but is usually approximately 0.1ms. This usually causes the subject to stutter in their reading. Additional pressure is exerted by informing the subject before commencing the test that they should stop as infrequently as possible. Each time a pause does occur the investigator reminds the subject to begin once more as quickly as possible.(221)

2. Trier Social Stress Test (TSST)

The TSST has been used widely in the field of psychology for investigating

. the effects of psychological stress. The subject is requested to prepare and give a short oral presentation. The presentation is usually based around an imaginary stressful situation such as making a defence in a court case for a speeding fine. The number of presentations can be varied depending on the length of test required. The presence of an audience is vital to increase the

pressure. The audience is asked to give no positive feedback but to appear critical instead.(220;223;224)

3. Mental arithmetic tests

Subjects can be asked to perform difficult mental arithmetic as a test of mental concentration.(137;222) The length and difficulty of the test can be varied as required. It can be performed in front of an audience who can be asked to give critical feedback. Alternatively an intrusive sound such as a buzzer can be added each time an incorrect answer is given. Auditory distraction can be added in the form of a simple dichotomous listening test.

4. Stroop word colour interference test

The Stroop colour word interference involves identifying the meaning of a colour word when it is written in ink of a different colour. For example, identifying the meaning of the word blue when it is written in a red ink.(225) The subject can be asked to give each answer in time to a metronome, limiting the time available and increasing stress.

5. Immersion of the hand in iced water

This is a well accepted procedure to induce a degree of physical discomfort and hence a stress response (279). However, the procedure cannot be sustained for 50 minutes due to the level of discomfort. It also represents a physical rather than psychological stress.

It is possible to identify the following common features of these stress tests:

1. A requirement for mental concentration
2. Often there is an element of multi-tasking
3. Critical feedback
4. Time pressure
5. Additional auditory distraction

Development of the stress protocol

For the purposes of our experimental protocol the stress test was required to fulfil the following requirements:

1. The stress test should cause an autonomic response with an increase in pulse rate and blood pressure (and therefore also assumed to be capable of inducing an immune response)
2. The stress test should be subjectively stressful as rated on a visual analogue ten point scale
3. The stress test should exert a sustained effect over 50 minutes. (This length of time was thought to be the optimum for a session of hypnotherapy. Thus by ensuring a stress test of the same time period the stress and hypnotherapy protocols were similar.)

Two volunteers performed several of the different stress tests. They rated their subjective experience of the test on a 10 point visual analogue scale and had their pulse and blood pressure measured every fifteen minutes. They also gave subjective general feedback as to their experience:

1 Dichotomous listening test

   a. Conflicting musical inputs - Subjects did not find a simple dichotomous listening test, where different types of music are played in either ear, subjectively stressful. After the initial sensation they rapidly adjusted to the test and were able to block out the conflicting sounds. There were also no changes in pulse or blood pressure. This test was also not felt to be sustainable for 50 minutes.
   b. Spoken word dichotomous listening test - Switching attention between different spoken inputs and repeating aloud the third word was felt subjectively to be stressful initially. However, it was impossible for the investigator to assess whether the correct word was being given as

the auditory inputs were given via headphones. The subjects also lost interest long before 50 minutes.

c. Auditory delay dichotomous listening test - Although initially rated as stressful, subjects were rapidly able to adjust to this form of auditory feedback. The test was therefore felt to be unsustainable for fifty minutes.

2 Mental arithmetic tests – Subjects found the performance of mental arithmetic stressful. However there was no standardisation as to the difficulty of the test or the amount of time which should be allowed for the subject to complete the test.

3 The Trier Social Stress Test was unsuitable for this protocol as it required the presence of an audience.

4 The Stroop Colour Word interference test might have been suitable but the software for this test is restricted.

Final Stress Test Protocol

It was felt that the best approach lay in modifying the mental arithmetic test and combining this with added auditory distraction of conflicting types of music being played simultaneously in either ear.

After discussion, it was decided that an IQ test might be a suitable test requiring mental concentration. IQ tests have the advantage of having been standardised to the population. They are supposed to be of a level of difficulty which should allow all to attempt the test. The time taken to complete the IQ test has also been standardised. By giving the subjects less than the recommended time to complete the test, it was possible to make completing the test very difficult thereby increasing the stress level.

Final Stress Test Protocol

1. Prior to the test the patient was informed that they would be performing a test which would measure intelligence and its relationship to stress and UC. Subjects were told that it was important that they completed the test to the best of their ability and a particular emphasis was placed on the importance of completing the test.
2. The test was begun and the volume of the two CD players set at a level deemed to be sufficiently loud to disrupt concentration.
3. After 5 minutes the test was stopped temporarily. The first questions were worked through with the subject and the correct answers given. This ensured that those subjects who had no prior experience of an IQ test could see the type of thought process required to answer the questions. It also ensured that the subjects were aware that there were correct answers to each of the questions. (One subject stated that he thought that there might be no correct answers to any of the questions and that the whole test was a "trick".) As all subjects gave at least one incorrect answer in the pilot run this also provided an opportunity for negative feedback.
4. The test was then re-started.

5. After 15 minutes the first pulse and blood pressure reading was taken.
6. After 25 minutes the subjects were informed that half of the allotted time had passed and that they should be half way through the test. In all cases the subjects were still on the first IQ test and so were encouraged to increase their speed in order to complete the test.
7. After 30 minutes a second pulse and blood pressure reading was taken
8. At 35 minutes subjects were informed that there were only 15 minutes left and again encouraged to increase their speed of answer in order to complete the test.
9. At 45 minutes subjects were informed that there were only five minutes remaining to complete the test. A third pulse and blood pressure reading was also taken.
10. At 50 minutes the test was concluded.

3.3.3 HYPNOTHERAPY PROTOCOL (SEE APPENDIX II FOR DETAILED DESCRIPTION OF HYPNOTHERAPY SESSION)

Equipment

Dynamap blood pressure monitor (Critikon, Tampa, Florida, USA)

Methods

Subjects underwent a 50 minute session of hypnotherapy focussed on achieving general relaxation and relieving specific GI symptoms. All sessions were performed by Dr D Jenkins, a medically qualified hypnotherapist and ex-president of the Medical Society of Hypnosis. Prior to the hypnotherapy session, a 20 minute interview was conducted to create rapport between subject and therapist and to provide information on which to base the session. Speigel's score of hypnotisability was also measured for each subject prior to beginning the hypnotherapy session (383). Trance was induced using a standard relaxation induction and deepening techniques. Hypnotherapy sessions combined both stress reduction and modification of their concept of inflammation in UC using visualisation.

Throughout the hypnotherapy session subjective depth was assessed every 15 minutes by asking the patient to rate on a scale of 0 to 10 (0 no trance, 10 very deep) how deep they felt themselves to be at that time. Pulse and blood pressure were recorded every 15 min during the session. After the session all subjects were questioned as to whether they had experienced time distortion.

3.3.4 CONTROL PROTOCOL

Equipment

CD Walkman (Dixon's superstore, Mile End, London, UK)

Dynamap blood pressure machine (Critikon, Florida, USA)

Methods

Subjects were asked to listen to relaxing music of their choice for 50 minutes. Subjects were offered a choice of the following or allowed to listen to their own choice of relaxing music.

1. Brahms – Piano concerto no 2.
2. The Carpenters - Greatest Hits

3.3.5 PSYCHOMETRIC QUESTIONNAIRES (SEE APPENDIX I)

Equipment

Hospital Anxiety Depression Scale (HADS) (384)

State Trait Anxiety Inventory (STAI) (385)

Perceived Stress Questionnaire (PSQ) (373)

Bradford Somatic Inventory was used by kind permission of Dr. D. Mumford, Dept of Psychiatry, University of Bristol, UK (374)

Methods

As described, subjects were asked to complete four commonly used psychometric questionnaires prior to beginning the experimental protocol. These four questionnaires are designed to measure stress over various periods of time ranging from the last few days to the last few years.

1. The Hospital Anxiety and Depression Scale (386)

The Hospital Anxiety Depression Scale (HADS) is a self-assessment questionnaire designed to measure the states of both anxiety and depression. It consists of a simple questionnaire of 14 questions which are divided into two subscales; one measuring anxiety (A-scale) and the other depression (D-scale). Each is scored separately. The HADS is a present-state instrument as subjects are asked to state how they have felt over "the last few days."

The anxiety scale measures general anxiety and is not focused upon a specific situation. The questions cover the concepts off restlessness and anxious thoughts. The depression scale is largely focused upon loss of interest and diminished pleasure response. This state, the lowering of hedonic tone, has been recognised as a reliable guide to the type of mood disorder of biological origin.

The questionnaire is simple to complete and usually can be done within five minutes. The Anxiety and Depression subscales scores are determined by adding the numbers in the A and D columns respectively. Interpretation is then via the scoring bands below.

Table 3.3 Interpretation of the Hospital Anxiety and Depression Scale (scores apply to both scales) (387).

Score	Interpretation
0-7	No anxiety or depresssion
8-10	Mild anxiety or depression
11-14	Moderate anxiety or depression
15-21	Severe anxiety or depression

2. The State-Trait Anxiety Index (388)

The State-Trait anxiety index is a designed to measure both acute anxiety and also a tendency to anxiety. It consists of two twenty point questionnaires. The first relates to the current state of anxiety or how the subject is feeling "right now", the second relates to how the subject generally feels or their anxiety trait. Each point consists of a statement of mood related to anxiety and the subject is asked how this relates to how they feel on a four point scale. Scores for each question are totalled to give both a state and anxiety trait score. Whilst there is no definition of what constitutes a normal or abnormal score on either scale, individual scores can be compared with the published values of a normal population.

Table 3.4. State-Trait Anxiety Index scores for working adults (mean and standard deviation shown) (389).

	Male	Female
S-anxiety score	35.7 ± 10.4	35.2±10.6
T-anxiety score	34.9 ± 9.2	34.8 ± 9.2

3. The Perceived Stress Questionnaire (373) (See Appendix I)

The Perceived Stress Questionnaire (PSQ) is a research tool designed to measure stress in the context of clinical psychosomatic research. It consists of a thirty point questionnaire. Each question takes the form of a statement describing mood. The subject is asked to describe how often they believe each statement applies to them on a four point scale. A score of 1-4 is given for each question and the values totalled to give an overall score. The PSQ is a measure of long-term perceived stress as questions relate to how the subject has felt "in the long run, over the last year or two". No data is available on normal scores for the PSQ.

4. The Bradford Somatic Inventory (374) (see Appendix I)

The Bradford Somatic Inventory (BSI) is a tool used to identify somatic symptoms associated with anxiety and depression. It consists of a forty point questionnaire. Each question relates to whether a subject has experienced a particular symptom during the preceding month. The question is answered as a yes/no and the yes answers are added to give a total out of 40. The BSI was first developed for use with Pakistani patients and indigenous patients attending a UK hospital. It has, however, since been validated for use in many other languages and ethnic groups. A score of greater than seventeen is taken as being a possible indicator of a depressive or anxiety-related disorder.

3.3.6 SERUM CYTOKINES

Equipment

Vacutainers (BD Biosciences, Cowley, Oxford, UK)

Reagents

IL-6 ELISAs (R+D systems, Abingdon, Oxford, UK)

IL-13 ELISAs (Immunodiagnostic systems, Tyne and Wear, UK)

Methods

Serum samples were collected and kept in frozen aliquots at -80°C until later analysis of IL-6 and IL-13 levels by ELISA used according to the manufacturer's instructions. The coefficients of variation (CV) for duplicate estimates of serum IL-6 and IL-13 measurement was 3.1 (n=8) and 8.9% (n=8) respectively. Serum TNF-α levels were below the minimum sensitivity of the ELISA in the majority of subjects and so were not assessed.

3.3.7 CYTOKINE PRODUCTION DURING CULTURE OF WHOLE BLOOD WITH LIPOPOLYSACCARRIDE

Equipment

Lithium-containing vacutainers (green top) (BD Biosciences, Cowley, Oxford, UK)

Reagents

Lipopolysaccharide

24 well culture plates (Falcon plastics, Cannock, Saffs, UK)

RPMI culture medium

IL-6 and TNF-α ELISAs (R+D systems, Abingdon, Oxford, UK)

Methods

Lipopolysaccaride (LPS) is a gram-negative bacterial endotoxin which directly triggers monocyte activation via toll like receptor 4 (TLR-4). This leads to the release of large amounts of monocyte-produced cytokines such as IL-6, IL-12 and TNF-α. The culture of blood with LPS forms a model of whole blood monokine release and has been employed to characterise the inflammatory response of various patient groups such as those with multiple sclerosis (390) and rheumatoid arthritis (391). The method has also proved useful for ex-vivo monitoring of immunmodulatory treatments for example in patients treated with granulocyte colony stimulating factor (GCSF) (392). Whilst this technique is suitable for assessing the release of cytokines produced by monocytes such as TNF-α and IL-6, it is not suitable for assessing the release of IL-13, which is principally produced by lymphocytes (393).

Published methodology for the culture of blood with LPS varies (229;232;390). Blood can be cultured with varying concentrations of LPS and can be diluted in various ratios with culture medium. Therefore, preliminary experiments were performed to determine the optimum concentration of LPS required to stimulate

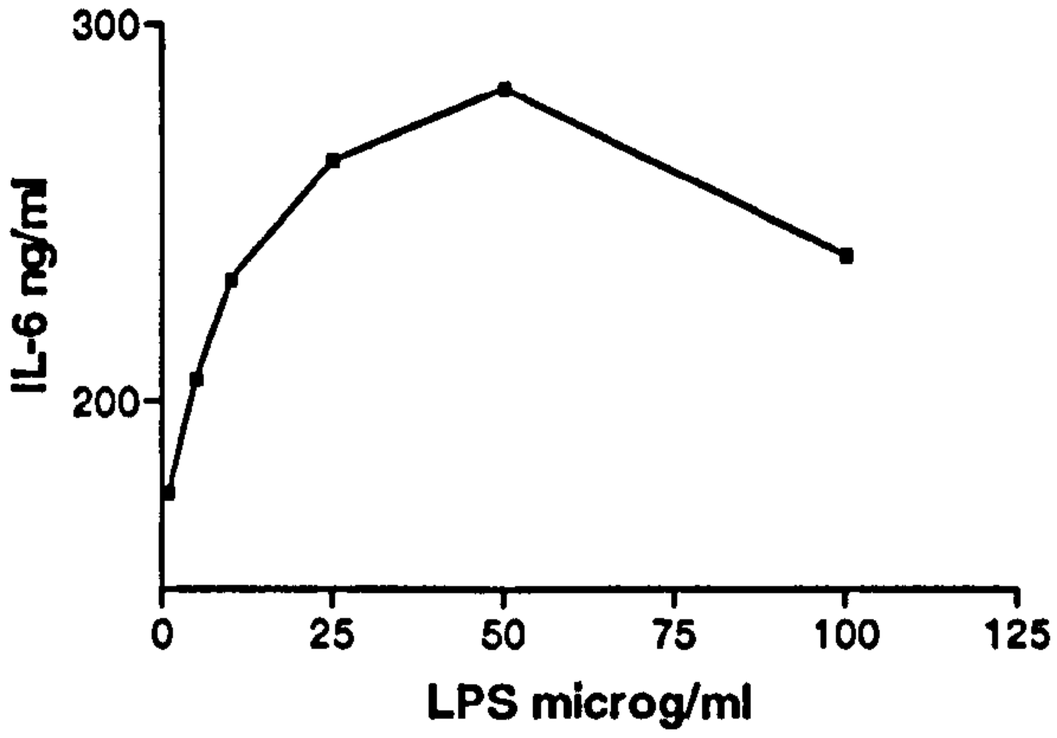
a cytokine response and whether dilution with culture medium was required. IL-6 production was used as the prototypical cytokine to assess the model.

Blood was collected from a healthy volunteer into a vacutainer containing lithium heparin. For the culture of whole blood without dilution in culture medium, 1ml of blood was placed in each well of a 24 well culture plate. Varying amounts of LPS were added to each well to give a range of final concentrations (1-100mcg/ml). The culture plate was then placed in an incubator at 37°C in an atmosphere of 5%CO2 for 24 hours. After 24 hours the samples were collected and centrifuged at 6000rpm for 8 minutes. The supernatant was then collected and stored at -80°C for subsequent analysis in duplicate by ELISA for IL-6 concentration.

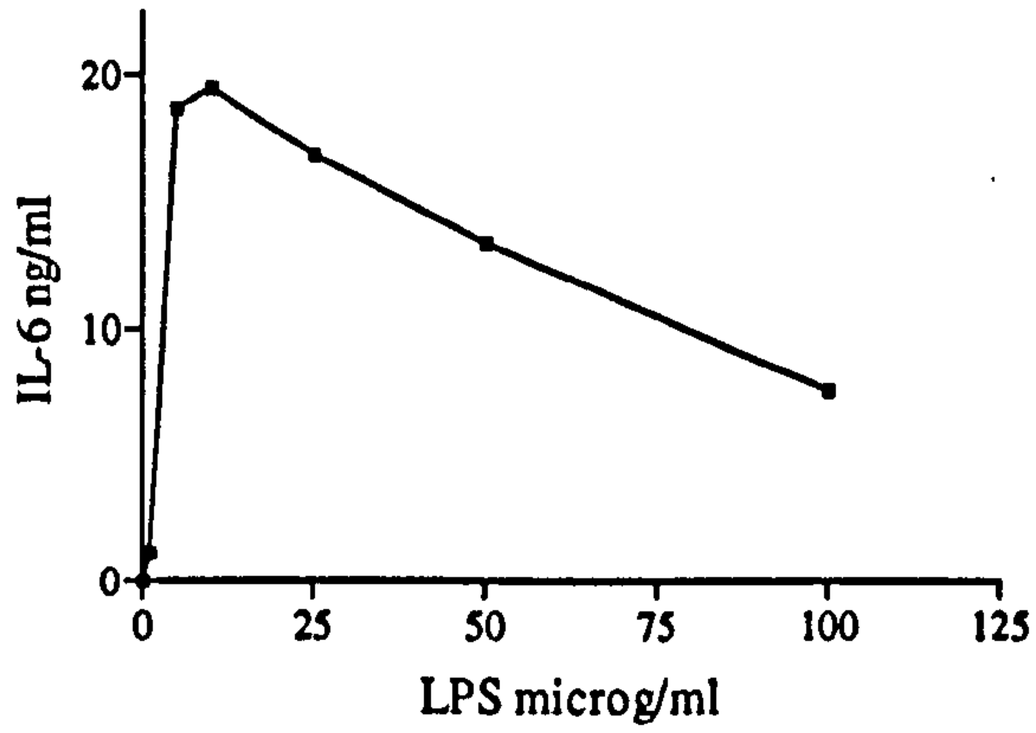
For the culture of blood with 1:10 dilution in culture medium 100mcl of blood was placed in each well of a 24 well culture plate and 1ml of pre-warmed RPMI culture medium added. LPS was then added and culture and analysis performed as before. Culture with each concentration of LPS was performed in duplicate.

Figure 3.2 The production of IL-6 by neat blood and blood diluted 1:10 with RPMI and stimulated with varying concentrations of LPS. Data points are the mean of 2 duplicates. Note the inverted U-shaped dose response curve in each instance.

A. Neat Blood



B. Blood diluted 1:10 with RPMI culture medium



It was decided that the culture of whole blood without dilution in culture medium gave the best graduated dose response curve. A sub-maximal stimulating concentration of LPS was chosen for future experiments as it was thought that this would allow the hypothesised increase in IL-6 release to be shown in response to stress and the hypothesised decrease to be shown in response to hypnotherapy. 25ncg/ml of LPS stimulated IL-6 release in the region of midpoint of the dose response curve and therefore future experiments were performed with1ml of whole blood cultured neat with 25mcg/ml of LPS.

3.3.8 WHITE CELL COUNT

Equipment

Vacutainer containing EDTA (Beckman Coulter, High Wycombe, Bucks, UK)

Coulter LLH 750 machine (Beckman Coulter, High Wycombe, Bucks, UK)

Methods

Total leukocyte count (WBC) was measured in blood samples anti-coagulated with EDTA using a standard Coulter LLH 750 machine (BD Biosciences, Cowley, Oxford, UK) in the Barts and London NHS Trust Haematology laboratory (Royal London Hospital).

3.3.9 FLOW CYTOMETRY OF NATURAL KILLER CELLS, PLATELET ACTIVATION AND PLATELET-LEUKOCYTE AGGREGATE FORMATION

Flow Cytometry

Flow cytometry can rapidly measure specific characteristics of a large number of individual cells. Cells are labelled with an antibody which also carries a fluorochrome which is known to fluoresce with a specific frequency of light. The labelled cells are suspended in solution and then passed individually through the flow chamber of the flow cytometer at a rate of 1000 to 10000 cells per minute.

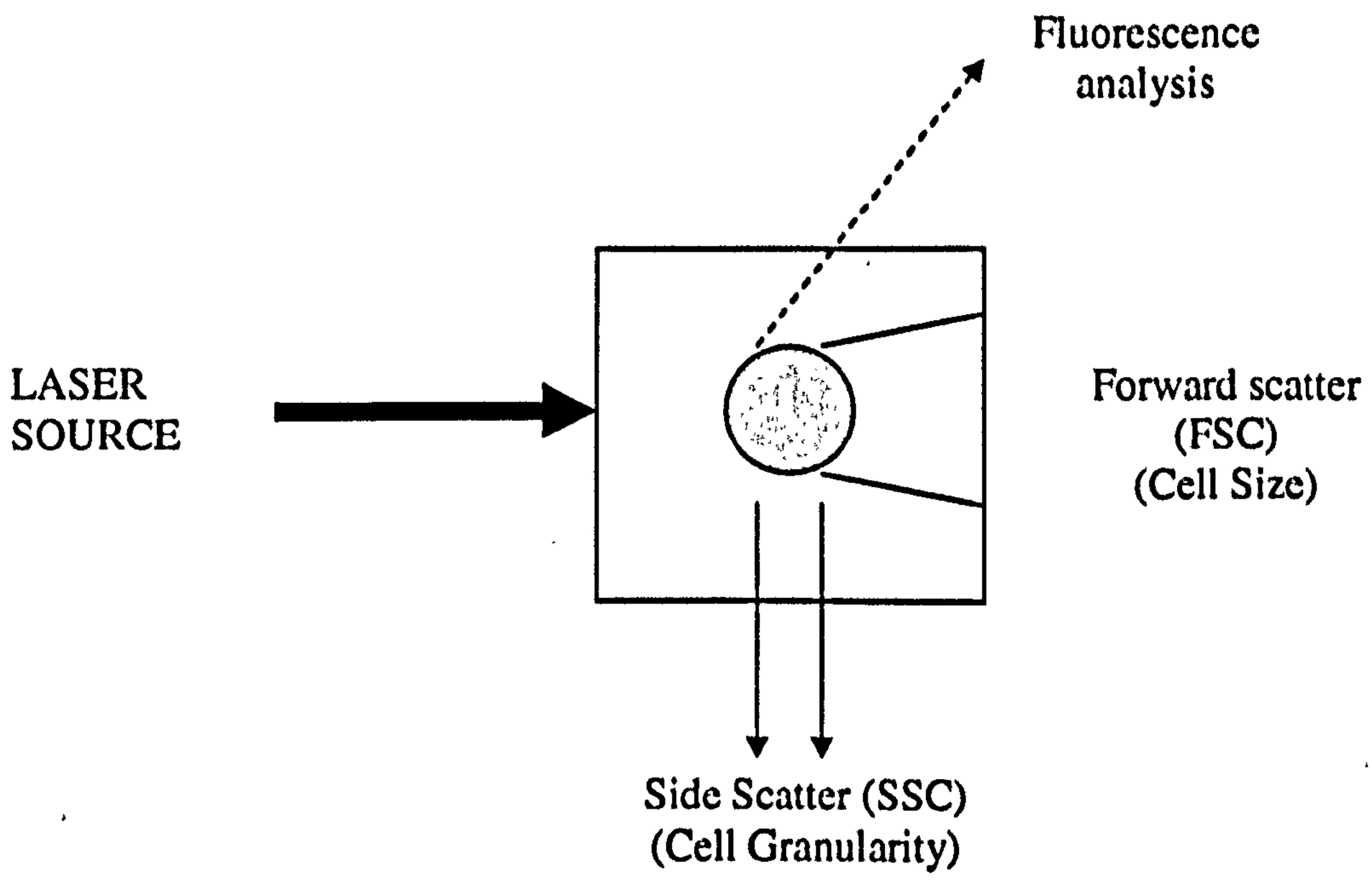
Whilst being passed through the flow chamber the cells are also passed through a focussed beam of a laser light. Exposure of labelled cells to light at the correct excitation wavelength causes them to fluoresce. The emitted light is detected and processed and varies according to the forward (FSC) and side scattering (SSC) properties of the cell. By using antibodies labelled with fluorochromes which emit light with non-overlapping frequencies more than one molecule can be labelled.

The flow cytometer was calibrated and standardised before use with fluorochrome-labelled beads (Dako, Ely UK).

All samples processed for flow cytometry were anticoagulated using ethylenediaminetetraacetic acid (EDTA) and citrate, theophylline, adenosine, dipyridamole (CTAD) (see section 3.3.1) (375).

Figure 3.3 Schematic representation of flow cytometric analysis

Cells pass in single file in fromt of a laser beam. "Forward scatter" is proportional to the cell surface area and "side scatter" to cell granularity. Each cell can be characterised in terms of its size, granularity and fluorescence properties.



3.3.9.1 Natural Killer cells

Natural killer cells were defined as lymphocytes with dual positivity for the cell markers CD16 and CD56.

Equipment

FACScan equipped with CellQuest® software (BD Biosciences, Cowley, Oxford, UK)

Reagents

Antibodies

Mouse fluorescein isothiocyanate (FITC)-conjugated anti-human CD16 (CD62P) (Coulter, Luton, UK)

Mouse phycoerythretin (PE)-conjugated anti-human CD56 (Coulter, Luton, UK).

Mouse IgG1-FITC and mouse IgG1-PE isotype control antibodies (Coulter, Luton, UK).

CD16

CD16 antibodies recognise a 50 to 65 kilodalton (kd) antigen present on human natural killer (NK) lymphocytes which is the IgG Fc receptor III.(394)

The CD16 antigen is expressed on approximately 15% of peripheral blood lymphocytes and is present on virtually all resting NK lymphocytes (395). The CD16 antigen can also be expressed on CD3+ T lymphocytes in certain individuals and may be present on some neutrophils. Co-expression of CD16 and CD56 is present only on NK cells (395).

CD56

CD56 is an antigen which is the 140-kilodalton (kDa) isoform of the neural cell adhesion molecule (NCAM). The 140kDa core protein is extensively

glycosylated to give a mature antigen. The CD56 antigen is present on approximately 10% to 25% of peripheral blood lymphocytes (395). It is present on essentially all resting and activated $CD16^+$NK lymphocytes and approximately 5% of $CD3^+$ peripheral blood lymphocytes (395).

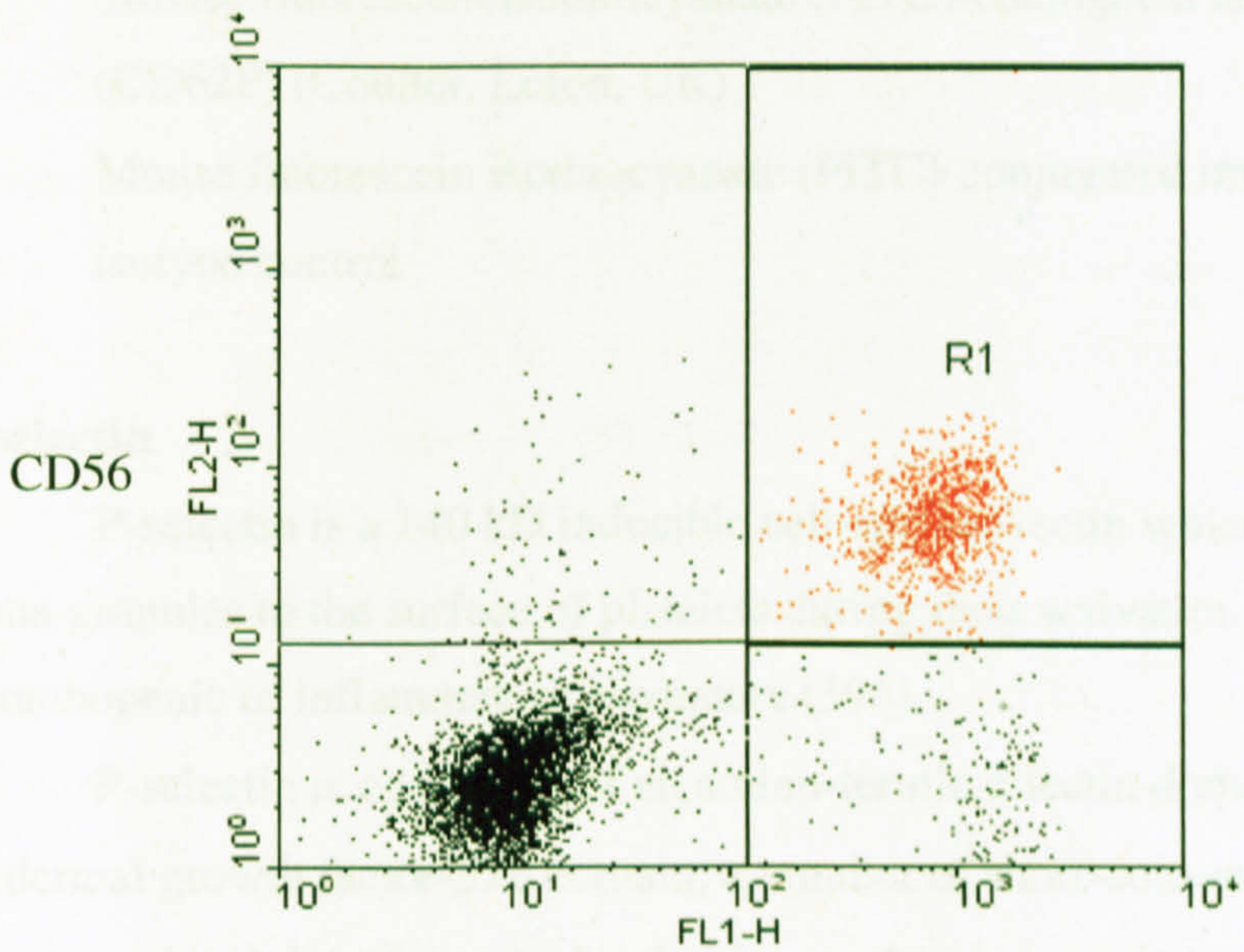
Methods

100 microlitres of blood was incubated with 10 microlitres of each of the appropriate antibodies for 5 minutes on ice. The red cells were then haemolysed by processing the sample through a Coulter TQ Preparation machine. The sample was then centrifuged at 2200 rpm for 5 minutes and the supernatant discarded. The pellet of cells remaining was resuspended in 1ml of Tyrode's solution and analysed by flow cytometry at a rate of 12 microlitres per minute.

The monocytes and lymphocytes were first identified on a plot of FSC against SSC. These were then taken to a second plot of FITC positivity (FH1) against PE positivity (FH2). NK cells were identified as a discrete population of cells positive for both CD16 (FITC) and CD56 (PE). This was expressed as a percentage of the total number of lymphocytes and monocytes. Non-specific binding was assessed by incubating the sample with FITC-IgG1 and PE-IgG1 isotype control antibodies. The CV of duplicate measurements for NK cells was 1.5% (n=8).

Figure 3.4 Flow cytometric analysis of NK cell number as a percentage of lymphocytes and monocytes.

Leukocytes are first identified on a plot of forward scatter (FSC) against side scatter (SSC). They are then taken to a second plot of CD16 versus CD56 positivity as shown below. CD16 and CD56 positivity are plotted on the x and y axis on log scale. The cells highlighted as red within the region R1 show both CD16 and CD56 positivity and represent NK cells. These are expressed as a percentage of the total number of lymphocytes and monocytes in the plot.



3.3.9.2 Platelet activation

P-Selectin, a cell surface membrane glycoprotein, was used to assess platelet activation.

Equipment

FACScan equipped with CellQuest® software (BD Biosciences, Cowley, Oxford, UK)

Reagents

Antibodies

Mouse fluorescein isothiocyanate (FITC)-conjugated anti-human P-selectin (CD62P) (Coulter, Luton, UK)

Mouse fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G1 isotype control

P-selectin

P-selectin is a 140 kD inducible cell-surface lectin which is transported from alpha-granules to the surface of platelets during their activation in response to thrombogenic or inflammatory mediators (396).

P-selectin is composed of an amino-terminal lectin domain followed by an epidermal growth factor-like domain, a number of short-consensus complement receptor related domains, a hydrophobic membrane-spanning region and a cytoplasmic domain. The storage sites of inactive P-selectin are the alpha-granules of platelets and Weibel-Palade bodies of endothelial cells. Within minutes of activation by a thrombogenic or inflammatory stimulus, P-selectin is mobilised to the cell surface where it acts as an adhesion molecule (396). Inducing agents include thrombin, histamine, complement fragments, ROMs and cytokines.

P-selectin mediates the interactions between platelets, the endothelium and inflammatory cells including neutrophils (395). Specific sialylated carbohydrate

moieties on neutrophils interact with P-selectin on the surface of platelets, an interaction mediated by the lectin domain of the P-selectin (143).

P-selectin not only mediates the adhesion of platelets to inflammatory cells, but also primes these cells for subsequent responses that augment inflammation. Increased platelet P-selectin expression has been demonstrated in inflammatory processes in animals, as well as in man in various disease states including IBD (141).

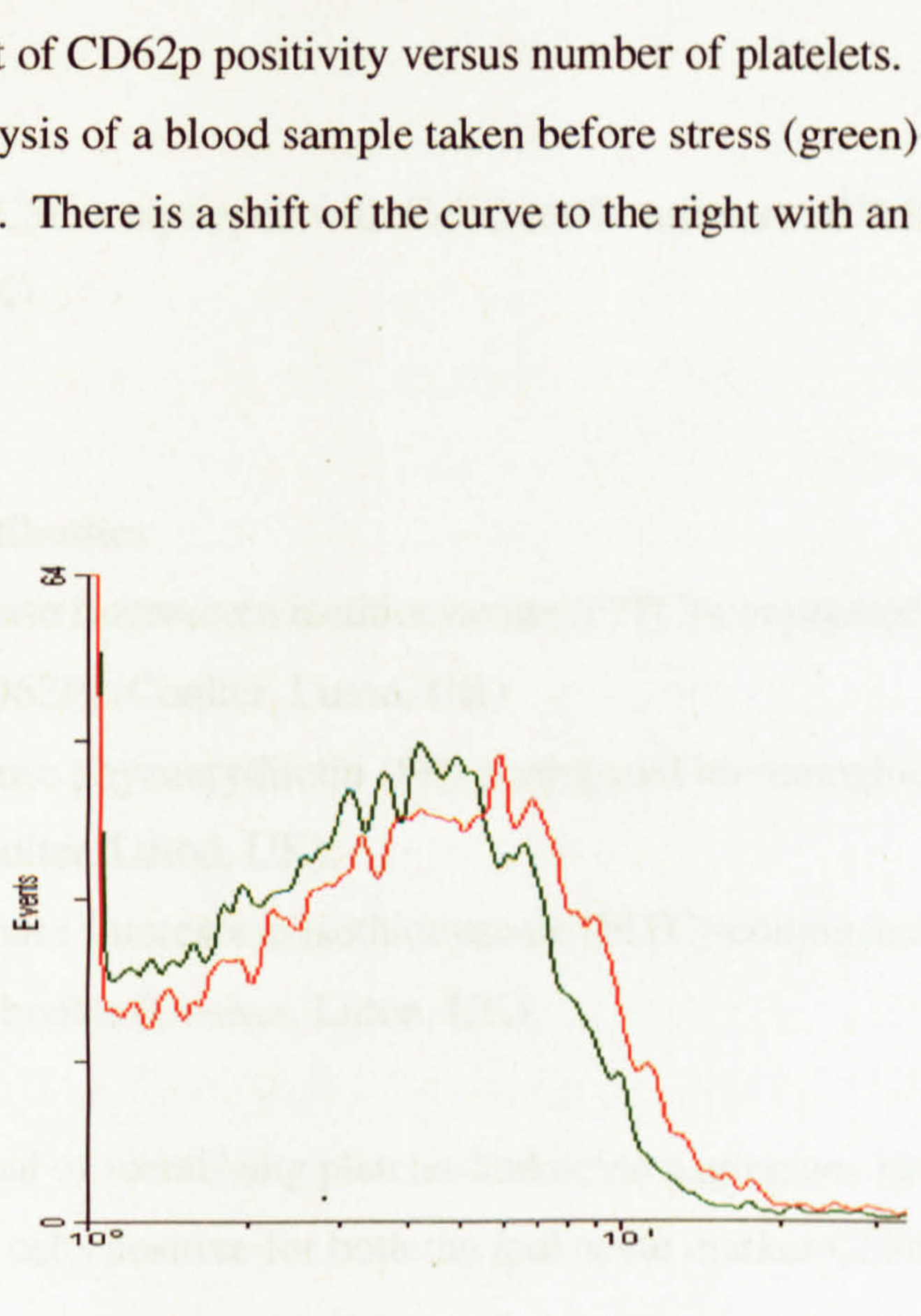
Methods

The use of flow cytometry to assess platelet activation allows analysis of samples of whole blood, minimising the manipulation of samples and thus preventing in vitro platelet activation and potential loss of sub-populations. 5 microlitres of blood was pipetted into a tube containing 5 microlitres of the appropriate FITC-labelled antibody. 90 microlitres of Tyrode's solution was added and the tube incubated for 5 minutes on ice. After this period the contents of the tube were diluted out by the addition of a further 0.9ml of Tyrodes solution. The sample was then analysed in the flow cytometer at a rate of 12 microlitres/minute using methods developed in our laboratory.(141)

The platelets were first identified on a plot of forward scatter (FSC) against side scatter (SSC). The level of non-specific binding was assessed by incubating the blood with FITC-labeled isotype mouse antibody (IgG1). The platelets were taken to a plot of frequency of events against FITC fluorescence intensity. The threshhold of fluorescence required for the event to be regarded FITC positive was set so that <1% of platelets stained positive with the control antibody. CD62P positivity was assessed by incubating the blood with an FITC-labelled CD62P antibody. Changes in P-selectin expression were then recorded on the gated platelets. The CV of duplicate measurements of P-selectin expression was 7.2% (n=8).

Figure 3.5 Flow cytometric analysis of platelet activation as determined by p-selectin expression

Platelets are first identified on a plot of FSC against SSC. They are then taken to a second plot of CD62p positivity versus number of platelets. The example below shows analysis of a blood sample taken before stress (green) and a sample taken after stress (red). There is a shift of the curve to the right with an increase in CD62p positivity.



3.3.9.3 Platelet-leukocyte aggregate formation

Platelet-leukocyte aggregates were identified using the leukocyte marker CD45 and the platelet marker CD42a.

Equipment

FACScan equipped with CellQuest® software (BD Biosciences, Cowley, Oxford, UK)

Reagents

Antibodies

Mouse fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 (CD62P) (Coulter, Luton, UK)

Mouse phycoerythretin (PE)-conjugated immunoglobulin anti-human CD42a (Coulter, Luton, UK).

Mouse fluorescein isothiocyanate (FITC)-conjugated IgG2a isotype control antibodies (Coulter, Luton, UK).

The principal of identifying platelet-leukocyte aggregates by flow cytometry involved identifying cells positive for both the leukocyte marker CD45, and the platelet marker CD42a.

CD45

CD45 is a protein tyrosine phosphatase (PTP) which is located in all hematopoietic cells except erythrocytes and platelets (395). CD45 is also called the common leukocyte antigen. CD45 has several isoforms and hematopoietic cells express one or more of the isoforms. CD45 is uniformly distributed in the plasma membrane of T cells and B cells (395). CD45 consists of a long single chain transmembrane protein with approximately 1100-1300 amino acids. It is a protein tyrosine phosphatase that functions to regulate kinases required for T and B cell receptor-signal transduction (395).

CD42a

CD42a is a 17-22 kilodalton (kd) single-chain platelet membrane glycoprotein also known as gp IX. Gp IX is a member of the leucine-rich glycoprotein (LRG) family of proteins, each of which is encoded by a separate gene(397). Gp IX is present on resting and activated platelets and on megakaryocytes and is essential for normal platelet adhesion and activation. It forms a complex with two other membrane bound glycoproteins gp Ib and gp V (397). The gp-Ib-IX-V complex contains a binding site for Von Willebrand factor (vWF) which mediates the activation-independent shear-dependent adhesion of platelets to the exposed vascular subendothelium. The Ib-IX-V complex functions as an attachment site anchoring the plasma membrane to its subjacent skeleton, thereby stabilising the membrane and maintaining platelet shape and vWF function (397).

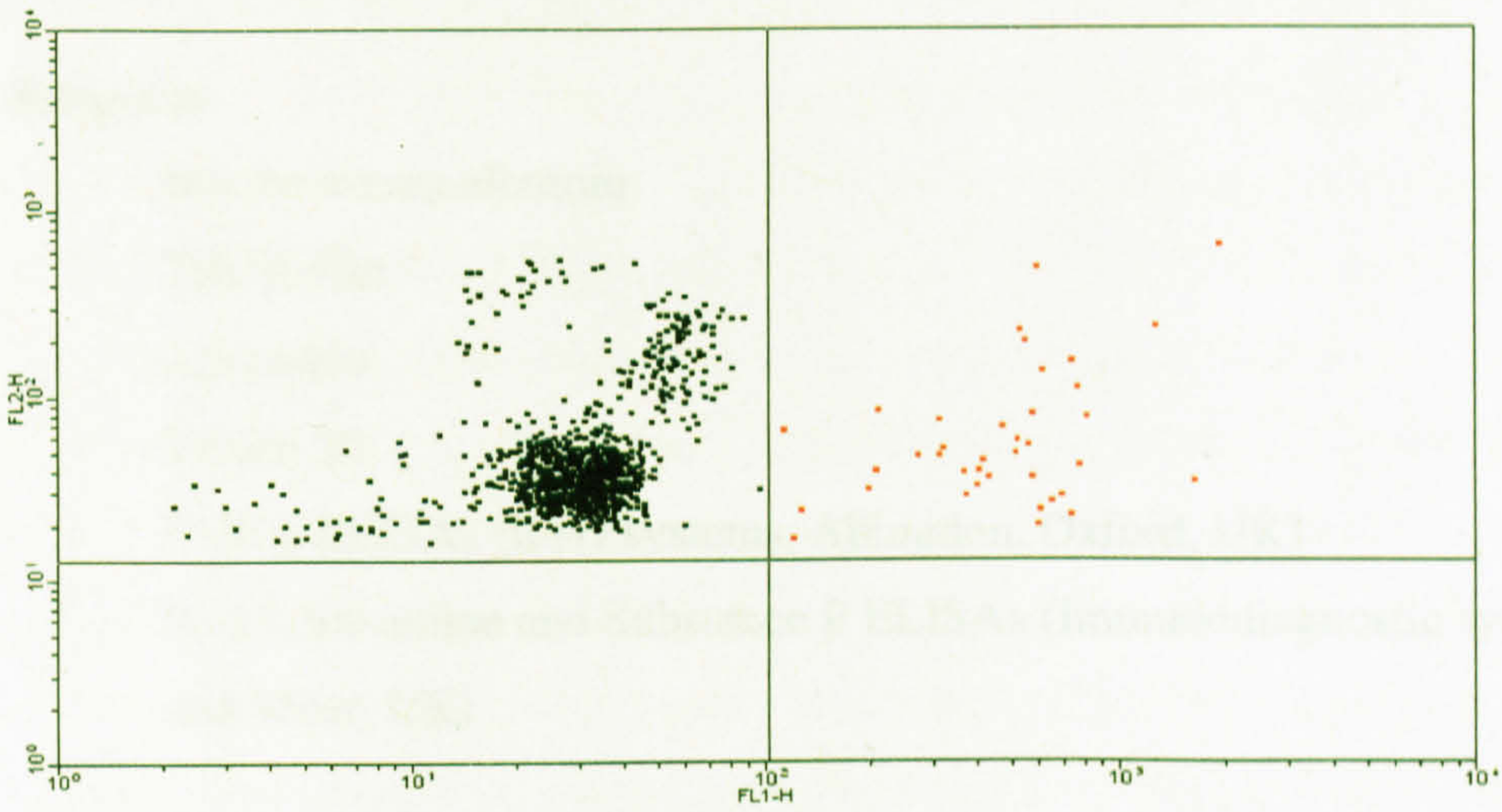
Methods

Using methods already in use in our unit (141), five microlitres of blood was pipetted into a tube containing 5 microlitres of PE-labelled CD56 and either FITC-labelled CD16 or FITC-labelled IgG2a. 90 microlitres of Tyrode's solution was added and the tube incubated for 5 minutes on ice. After this period the contents of the tube were diluted by the addition of a further 0.9ml of Tyrodes solution. The sample was then analysed in the flow cytometer at a rate of 12 microlitres/minute.(143)

Leukocytes were first identified on a plot of FFSC against SSSC. These were then taken to a plot of FITC against PE positivity. CD42a and CD45 positive events were recorded as a percentage of 1,000 gated leukocytes. Background non-specific antibody binding was assessed by measuring the proportion of IgG2a and CD45 positive events.

Figure 3.6 Flow cytometric analysis of platelet-leukocyte aggregate formation

Leukocytes are first identified on a plot of FSC versus SSC. They are taken to a plot of CD42a positivity versus CD45 positivity as shown below. Cells positive for both CD42a and CD45 represent platelet-leukocyte aggregates (shown in red) and are recorded as a percentage of the total number of leukocytes.



3.3.10 MEASUREMENT OF IN VIVO RECTAL MUCOSAL CYTOKINE AND MEDIATOR PRODUCTION IN ULCERATIVE COLITIS USING A FILTER PAPER TECHNIQUE

Equipment

Rigid sigmoidoscope (Falcon plastics, Staffordshire UK)

Rigid biopsy forceps

Filter paper Whatmann no 42

Reagents

Bovine serum albumin

Tris Buffer

Aprotonin

Tween 20

TNF-α ELISAs (R+D systems, Abingdon, Oxford, UK)

IL-13, histamine and Substance P ELISAs (Immunodiagnostic systems, Tyne and Wear, UK)

Methods

Measuring the in-vivo mucosal production of inflammatory cytokines is methodologically difficult. In vitro biopsy methods using tissue obtained by endoscopic colorectal biopsy have demonstrated increased concentrations of prostaglandins (398), thromboxane (399), leukotrienes (400), platelet activating factor(401) and proinflammatory cytokines (402) in active UC. However, the trauma of biopsy collection may alter the profile of mediators generated by the mucosa (377).

Rectal dialysis has been used to quantify mucosal production of eicosanoids (403), histamine (123) and interleukin-8.(404) Although safe for patients, the procedure is lengthy, taking up to four hours to complete. The technique is also limited to molecules which are small enough to pass through the dialysis membrane.

In 1996, Hendal and colleagues described a method of assessing rectal mucosal production of interleukin-1β and IL-1 receptor by direct application, via a

sigmoidoscope, of a filter paper to inflamed rectal mucosa for a period of up to one minute.(405) Carty et al in our unit then extended this technique to show that it could also be used to measure the in vivo production of TNF-α, thromboxane B2 (TXB2) and prostaglandin E2 (PGE2). Mucosal release of IL-1β, TNF-α, TXB2 and PGE2 was increased in active UC and was shown to be correlated directly with disease activity.(377)

No bowel cleansing preparation was used prior to the study. Through a standard rigid sigmoidoscope, a piece of filter paper (Whatmann No 42, 7mm x 30mm) was apposed to the rectal mucosa until visibly soaked with luminal secretion (up to 60 seconds). The filter paper was then removed and placed in a vial containing 1ml of Tris buffer 0.1M pH 7.4 in normal saline with human serum albumin (0.3%), sodium azide (0.1%) and Tween (0.002%). To inhibit degradation of peptides *in vitro* 1KU of the protease inhibitor aproprotinin was added immediately prior to the addition of the filter paper. The vial was placed on a rocker and agitated gently for 24 hours at 4C. Contaminating particles were then removed by centrifugation and the solution stored at -70C until analysis.

Concentrations of Substance P, histamine, TNF-α and IL-13 in the buffer were then measured using commercially available ELISAs. Samples were analysed in duplicate or triplicate as recommended by the manufacturers' instructions.

Recovery experiments

For each mediator assessed by the filter paper method, *in vitro* recovery experiments were performed. 50 or 100μL of a known concentration of each mediator was pipetted onto a strip of filter paper. This was then incubated in the buffer for 24 hours using the above protocol. The resultant concentration of the mediator in the buffer was then measured by ELISA and the percentage of the mediator recovered calculated. These experiments were important given earlier data indicating that some cytokines, such as interferon-γ, appeared to remain adherent to the filter paper and therefore give very low recoveries.(377)

Results

Table 3.5 Recovery of inflammatory mediators and cytokines using the filter paper method. Median and IQR shown.

Mediator	N	Recovery %
Substance P	4	62 (72-82)
Histamine	4	81 (69-103)
IL-13	4	85 (76-97)
TNF-α	4	89 (79-99)
IL-6	8	0 (0-0.5)

As shown above, preliminary experiments revealed that IL-6 did not dissociate from the filter paper into the buffer so that concentrations of IL-6 in peri-mucosal fluid could not be assessed using this method.

3.3.11 REACTIVE OXYGEN METABOLITE PRODUCTION BY MUCOSAL BIOPSIES

Equipment

Scintillation vials (Falcon Plastics, Cannock, Staffs, UK)

Bertholdt LB953 chemiluminometer (EG + G, Berthold, Bad Wildbad, Germany)

Reagents

Luminol

Dimethyl sulfoxide (DMSO)

Tyrode's solution

Methods

Chemiluminescence is a non-specific but sensitive method of detecting oxidizing species. The luminol used reacts with O_2- and H_2O_2 to form 3-aminophthalate and N-methylacridone. The excited electrons in these compounds revert to their ground state with the emission of energy as light which is detected by the photomultiplier tubes of the scintillation counter. O_2- and H_2O_2 are produced via a myeloperoxidase-catalysed reaction, the principal source for which is the neutrophil.(181) The amount of ROM production detected by this method has been shown to correlate well with disease activity in IBD assessed both macroscopically and microscopically in previous studies here and elsewhere (181;406).

On the day of use luminol was dissolved in dimethyl sulfoxide (DMSO) at 50mg/ml and then diluted to 300μM in Tyrode's solution. 1ml 300μM luminol was placed in a scintillation vial and a background count was performed for 4 minutes. Biopsies were transferred from the pre-oxygenated Tyrode's solution to the scintillation vials. Luminescence from each sample was immediately counted for 4 mins in the Bertholdt LB953 luminometer. Samples were then blot dried and weighed. Chemiluminescence was expressed as counts/min/mg tissue weight after subtraction of background. The CV for ROM production by paired biopsies assessed by this method is 47% (181).

Preliminary experiments on the effects of biopsy storage time on ROM production by mucosal biopsies

As the experimental protocol dictated that each stress, hypnotherapy or control session lasted 50 minutes, the initial mucosal biopsy had to spend at least one hour in the pre-oxygenated Tyrode's solution before being analysed for ROM production. Preliminary experiments were therefore performed to assess how this would affect ROM production. Paired biopsies were collected at colonoscopy from 2 patients with active UC (both Baron's endoscopic grade II), 2 patients with inactive UC (both Baron's endoscopic Grade 0) and 1 control patient with a normal colonoscopy. The first biopsy from each pair was processed immediately in the manner described above and the second after 2 hours.

Results

Table 3.6 Reactive oxygen metabolite production by paired mucosal biopsies immediately and 2 hours after being taken

Sample	Initial chemiluminescence (photons/mcg/min)	Chemiluminescence after 2 hours (photons/mcg/min)
Active UC 1	313	458
Active UC 2	776	689
Inactive UC 1	82	129
Inactive UC 2	46	38
Control	24	29

It was concluded from this result that storing the biopsies for up to two hours in oxygenated Tyrode's solution had no significant effect on ROM production

3.3.12 HAEMATOXYLIN AND EOSIN HISTOLOGY OF RECTAL MUCOSAL BIOPSIES

After analysis of ROM production, rectal mucosal biopsies were coded and then transferred to formalin solution and stored. Pre- and post-stress rectal biopsies were later stained with a standard haematoxylin and eosin protocol. The degree of inflammation present in each biopsy was scored by a single histologist (Dr Roger Feakins, Department of Histopathology, Royal London Hospital), blinded to the origin of the samples.(378)

Table 3.7 Saverymuttu Scoring System for Rectal Mucosal Biopsies (378)

Lamina propria mononuclear cells		**Lamina propria neutrophils**	
Normal	0	Normal	0
Slight increase	1	Slight increase	1
Moderate increase	2	Moderate increase	2
Marked increase	3	Marked increase	3
Enterocytes		**Crypts**	
Normal	0	Normal	0
Loss of single cells	1	Single inflammatory cells	1
Loss of groups of cells	2	Cryptitis	2
Frank ulceration	3	Crypt abscesses	3

3.3.13 RECTAL MUCOSAL BLOOD FLOW

Equipment

Rigid Sigmoidoscope (Falcon Plastics, Staffordshire, UK)

Moorlab laser Doppler flowmeter (Moorlab, Axeminster, Devon, UK)

Mp6a endoscopic probe (Moorlab, Axeminster, Devon, UK)

Moorlab software (Moorlab, Axeminster, Devon, UK)

Laser Doppler Flow measurement of blood flow

Laser Doppler flowmetry measures the frequency shift in light which occurs during reflection of laser light from a moving object. In tissue, blood cells account for most of the moving structures and the speed of their movement determines the frequency of light which is reflected. The strength of the reflected signal and the degree of shift can be combined to give a value for flux which represents the volume of blood flow. The technique has found a wide variety of clinical applications within medicine including vascular disorders such as Raynaud's phenomenon and skin grafting. More recently the technique has also been adapted to allow measurement of rectal mucosal blood flow.(370)

Laser Doppler Flow measurement of rectal blood flow

Previous work has shown that, in healthy individuals, rectal blood flow, as assessed by laser Doppler flowmetry, remains reasonably constant over time.(370) The exception is in pre-menopausal women, where blood flow is greater and less reproducible during the luteal than the follicular phase of the menstrual cycle. Mean rectal mucosal blood is also increased after a standard meal; it decreases after smoking but returns to baseline after 30 minutes.(370) Hence as mentioned above, in pre-menopausal female subjects measurements of rectal blood flow were performed only during the follicular phase of their menstrual cycle and all patients were asked to have only a light breakfast and to abstain from cigarettes on the morning of the test.

In healthy individuals, laser Doppler blood flow has also been shown to be a measure of extrinsic autonomic innervation to the gut.(407) Decreased sympathetic

tone, such as that induced by oral beta blockers, caused an increase in rectal blood flow.(370)

Rectal blood flow in patients with ulcerative colitis

In areas of inflamed mucosa or skin, such as in patients with UC, capillary blood flow reflects not just extrinsic innervation but also the local action of inflammatory mediators. Many inflammatory mediators have a vasodilatory effect and vasodilatation is one of the classic inflammatory features of calor, rubor, tumor and dolor. In a study of sensitisation of the gut to food antigens, rectal blood flow, visible erythema and histological submucosal oedema were shown to be positively correlated.(408)

Reports of rectal blood flow in UC are mixed. Srivasta et al reported that rectal mucosal blood flow was increased in patients with inactive UC compared to controls.(104) However, Guslandi et al found rectal blood flow to be decreased in patients with both inactive and active UC.(409) Some of these differences are likely to relate to differences in technical equipment with varying depths of penetration of the mucosa by the laser and different analysis software.

The MOORlab Laser Doppler Flowmeter and rectal probe

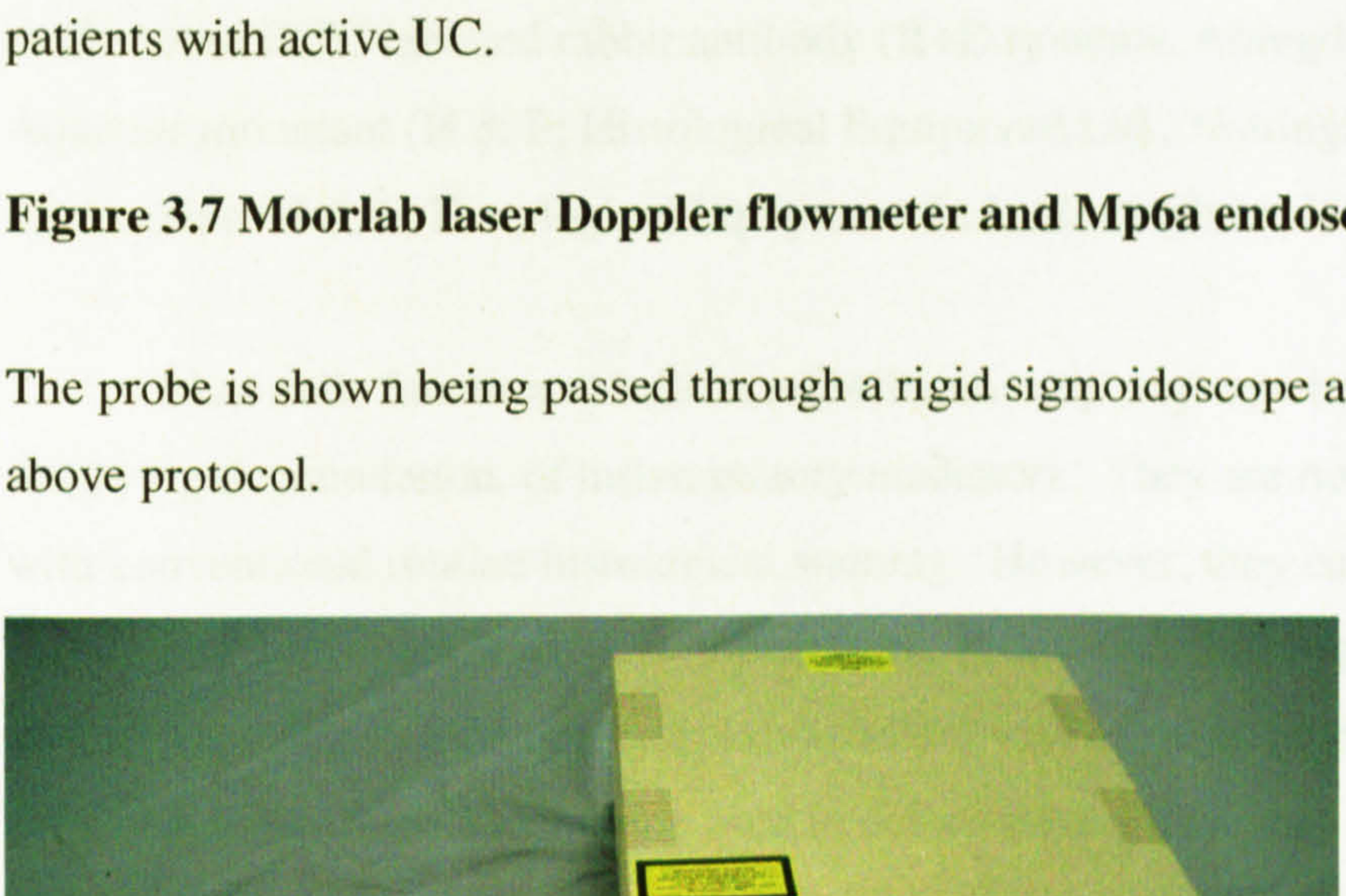
The Moorlab laser Doppler flowmeter produces a low intensity beam of monochromatic coherent 780nm light generated by an infrared laser diode source and delivered via a fibreoptic probe. Reflected light is detected by a photocell and the signal processed to determine frequency shift. The approximate area of measurement is $1mm^2$ at a depth of up to 1mm from the probe. The flux signal is fed to a monitoring computer where the data is analysed by Moorlab software to form a trace of flux against time. From this trace the average flux can be calculated for any given time period. Movement artefact is also eliminated by the software which averages recorded values over 0.1 millliseconds.

Methods

As stated in the overall protocol rigid sigmoidoscopy with assessment of rectal blood flow was performed before and after the stress, hypnosis and control protocols. The patients were examined in the left lateral position with no prior bowel preparation. The rigid sigmoidoscope was inserted with minimal air insufflation. The laser Doppler probe was then placed next to the rectal mucosa through the rigid sigmoidoscope 10cm above the anal margin under direct visualisation. The blood flow reading was allowed to stabilise over 30 seconds. Four quadrantic thirty second readings of blood flow were then taken. The mean of these four readings was calculated and taken as the mucosal blood flow. The CV for quadrantic sets of rectal mucosal blood flow measurements was 17% in patients with inactive UC and 31% in patients with active UC.

Figure 3.7 Moorlab laser Doppler flowmeter and Mp6a endoscopic probe.

The probe is shown being passed through a rigid sigmoidoscope as described in the above protocol.



3.3.14 IMMUNOFLUORESCENCE OF MAST CELL DEGRANULATION

Equipment

Cryostat vials

OCT mountant (H & E; Histological Equipment Ltd., Nottingham, UK)

Microtome (Bal-Tec, Cheshire, UK)

Vectabond 4 spot slides (Vector Laboratories, Burlingame, California, USA)

Acetone

Phosphate buffered saline (PBS)

2% Rabbit serum (R+D systems, Abingdon, Oxford, UK)

Anti-human tryptase mouse antibody (R+D systems, Abingdon, Oxford, UK)

Anti-mouse FITC labelled rabbit antibody (R+D systems, Abingdon, Oxford, UK)

Aqueous mountant (H & E; Histological Equipment Ltd., Nottingham, UK)

Cover-slips (H & E; Histological Equipment Ltd., Nottingham, UK)

Mast cells function to initiate an inflammatory response by the release, following degranulation, of inflammatory mediators. They are not readily identifiable with conventional routine histological staining. However, they can be stained with certain blue basic dies, such as toluidine blue, with which they appear characteristically as having a cytoplasm packed with large granules (410).

A more reliable technique used to detect mast cells is that of immunofluorescent staining of biopsies. This technique involves first staining the specimens with an antibody which binds to the mast cell granules. Antibodies to tryptase, a molecule stored in mast cell granules but released upon activation, are the most commonly used (411;412). This primary antibody is then labelled with a secondary antibody to which a fluorochrome is attached. Using this technique both resting and activated mast cells can be identified (see Figure). In this protocol, immunofluorescent staining of rectal biopsies for the mast cell mediator tryptase was used.

Mast cells can also been seen well with electron microscopy. Both resting and degranulating mast cells can be identified with this technique (279).

The scientist performing the procedure (Dr Neil Rayment) was blinded to the source of each biopsy sample.

Experimental Protocol

Immediately after being taken, rectal biopsies were placed in cryostat vials and snap frozen in liquid nitrogen. Samples were stored at -80°C until future use. On the day of processing, the specimens were removed and orientated onto OCT mountant. 5µm cryostat sections were cut using a microtome and mounted onto washed Vectabond 4 spot slides. The slides were stored at -20°C until use, as per manufacturer's recommendations. Sections were thawed and fixed in cold acetone for 15 mins before being washed briefly in PBS. In order to reduce non-specific background staining, slides were treated with a blocking antibody. In this case, sections were incubated in 2% normal rabbit serum in PBS for 20 mins at 21°C. Sections were then washed three times in PBS at 21°C for 5 minutes each wash. Next, the sections were incubated with the primary antibody. In this protocol, slides were incubated in a 1/100 dilution of anti-human tryptase mouse antibody for 60 mins at 21°C. Sections were washed a further three times in PBS at 21°C for 5 minutes each wash. A 1/50 dilution of anti-mouse FITC-labelled rabbit antibody was used as the secondary antibody and the slides were incubated in this solution for 45 mins at 21°C. Sections were then washed a further three times in PBS at 21°C for 5 minutes each wash and mounted in aqueous mountant (containing anti-fade). A cover-slip placed over the sample and a Zeiss Axiophot MOT microscope with conventional epi-fluorescence used to view them. An Axiocam HR digital camera attached to Axiocam software was used to photograph the slides.

The total number of mast cells and the percentage degranulating were counted for each specimen.

Figure 3.8 Resting mast cell stained with anti-human tryptase antibody and anti-mouse FITC-labelled rabbit antibody.

Mast cell is the fluorescent red cell in the middle of the figure. Tryptase is stored in granules within the mast cell cytoplasm.

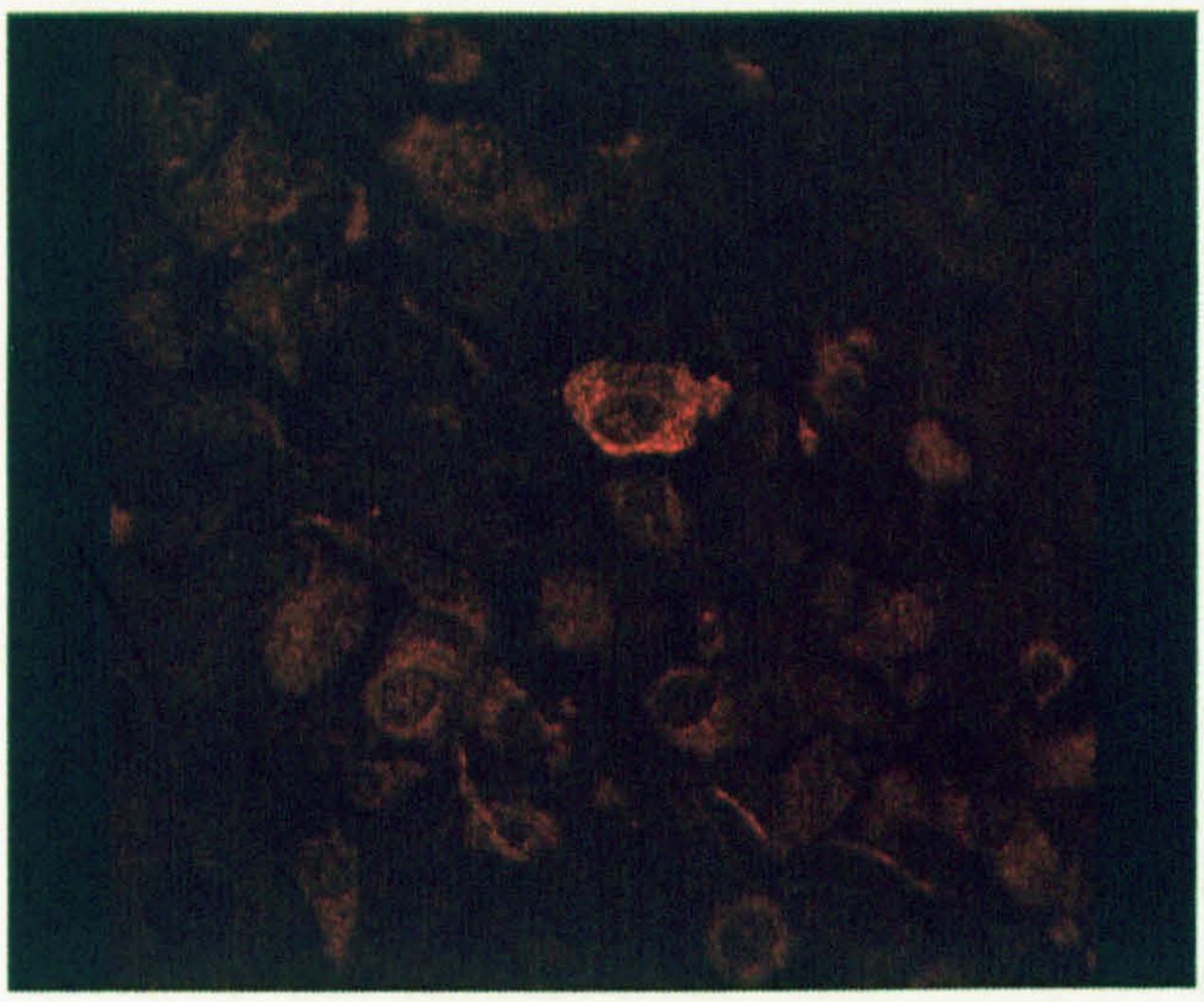
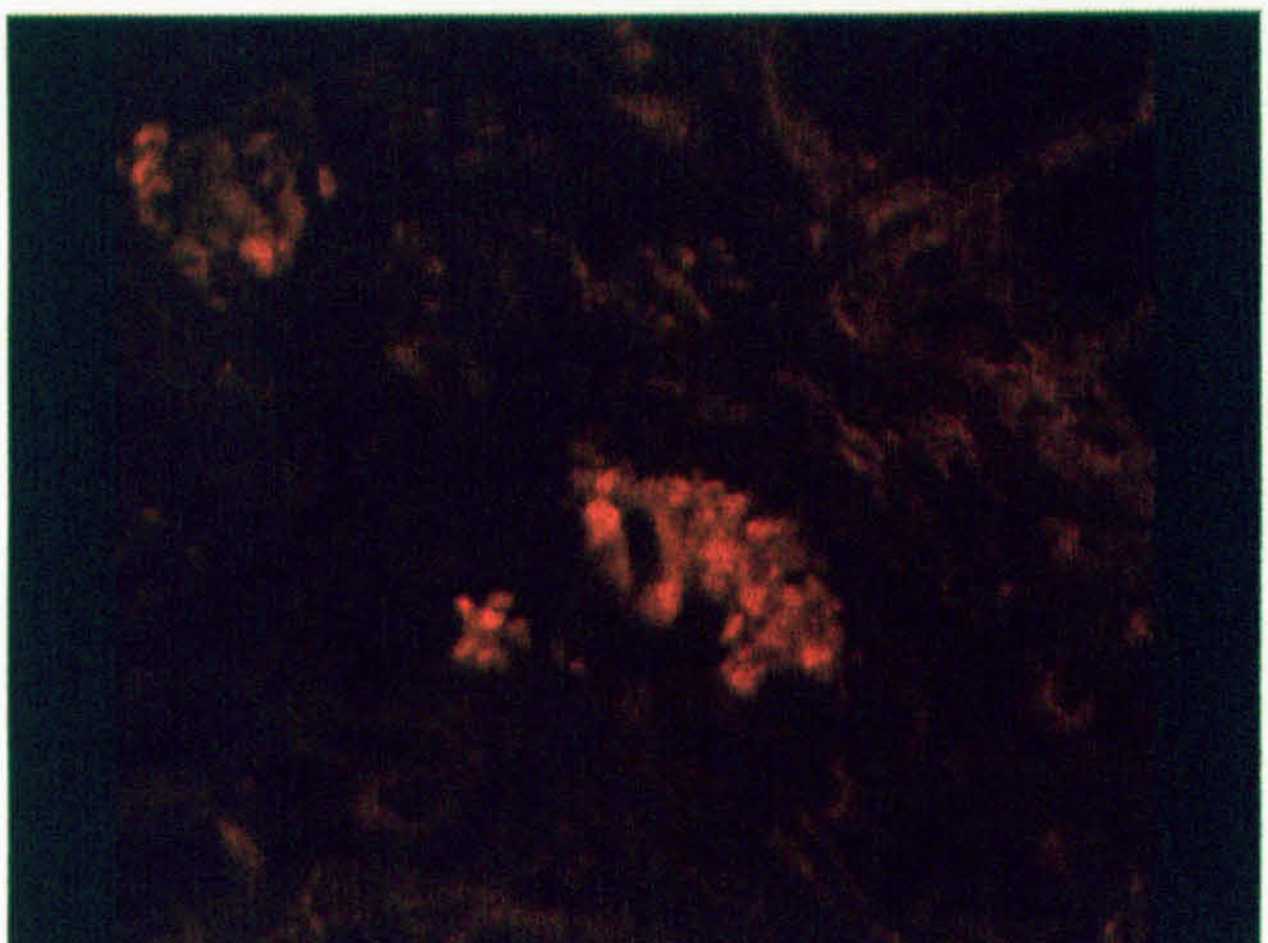


Figure 3.9 Degranulating (activated) mast cell labelled with anti-human tryptase mouse antibody and anti-mouse FITC labelled antibody.

The mast cell is the fluorescent cell within the middle of the figure. The tryptase can be seen being released with degranulation.



3.3.15 FLUORESCENCE IN-SITU HYBRIDISATION OF MUCOSAL ASSOCIATED BACTERIA

Equipment

Cryostat vials

OCT mountant (H & E; Histological Equipment Ltd., Nottingham, UK)

Microtome (Bal-Tec, Cheshire, UK)

Vectabond 4 spot slides (Vector Laboratories, Burlingame, California, USA)

Phosphate buffered saline (PBS)

Paraformaldehyde

Triton

Tris-hydochloride

Sodium Chloride

Probes (a gift from Professor G Gibson, School of Food Biosciences, Reading University and synthesised by Microsynth, Lustenau, Austria)

The pathogenesis of IBD is likely to involve an abnormal immune reaction to colonic mucosal bacteria. Specifically adherent and invading strains of E.coli may have a pro-inflammatory role (413). Most studies assessing mucosal flora have used the relatively non-specific technique of culture of biopsies obtained at colonoscopy. Others studies have relied on polymerase chain reaction-based techniques or electron microscopic examination of mucosal biopsies.

Our own laboratory has developed a fluorescent in situ hybridisation (FISH) technique to identify mucosa-associated bacterial flora (64). The technique uses FISH probes and has the advantages of being quantitative, genera-specific and allowing direct visualisation of both bacteria adherent to the mucosa and bacteria in the lamina propria. A universal probe (EUB) allows quantification of the total number of bacteria.

Table 3.6 Species-specific oligonucleotide probes used for fluorescent in situ-hybridisation. Probes are labeled at the 5' end with the fluorescent label cy3.

Bacterial species	Clone	Sequence
E. coli	1531	5`-CAC-CGT-AGT-GCC-TCG-TCA-TCA-3`
Total bacteria (EUB)	146	5`-TAC-GGA-TTT-CAC-TCC-T-3`

Experimental protocol (64)

As with the immnofluorescent staining technique used for assessing mast cells, specimens were snap frozen in liquid nitrogen and stored at -80°C until future use. On the day of processing, specimens were removed and orientated onto OCT mountant. 5µm cryostat sections were cut using a microtome and mounted onto washed Vectabond 4 spot slides. The slides were stored at -20°C until use. Sections were thawed briefly and fixed in 4% paraformaldehyde/PBS for 20 mins at 21°C. After a brief wash in PBS, sections were permeabilised by treating with 2% Triton x 100/PBS for 20 mins at 21°C. This solution was removed by washing the sections three times for five minute each wash in PBS at 21°C. The slides were then incubated in the hybridisation buffer (40Mm Tris-HCl, 1.8M NaCl, 0.5%SDS) for 60 mins at required hybridisation temperature. The hybridisation buffer was "tapped off" and replaced with a hybridisation buffer containing 200ng of the relevant probe; E.coli or total bacteria. A cover-slip was placed over each slide and the sections sealed in a moist chamber. This was then placed in a hybridisation oven at 50°C for 16 hrs.

Following removal from the oven, cover-slips were removed by dipping the slides briefly in PBS. The hybridization/probe solution was replaced with fresh hybridisation solution but without SDS and the slides incubated for 60mins at 21°C. Slides were then washed once in PBS for 15mins at 21°C. Slides were mounted in aqueous mountant (containing anti-fade) and a cover-slip placed over each sample. A Leica SP2 confocal microscope was used to view the slides. The number of *E.coli* and total bacteria/mm^2 of epithelial surface and the number of *E.coli* and total bacteria/mm^2 of lamina propria were counted.

Figure 3.10 E.coli adherent to mucosa as shown by FISH technique

E.coli can be seen as fluorecscent red objects on the surface of the mucosa.

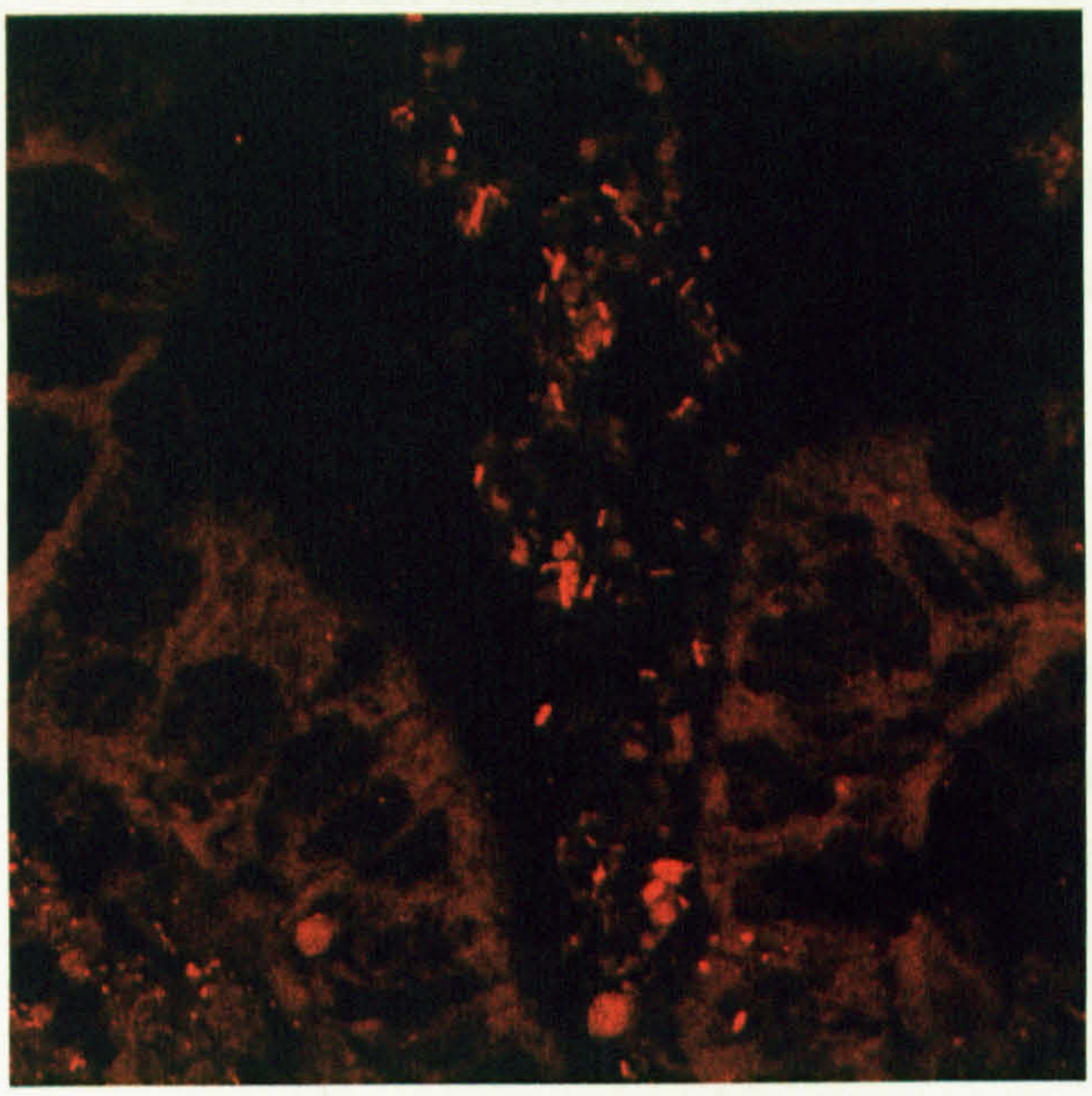
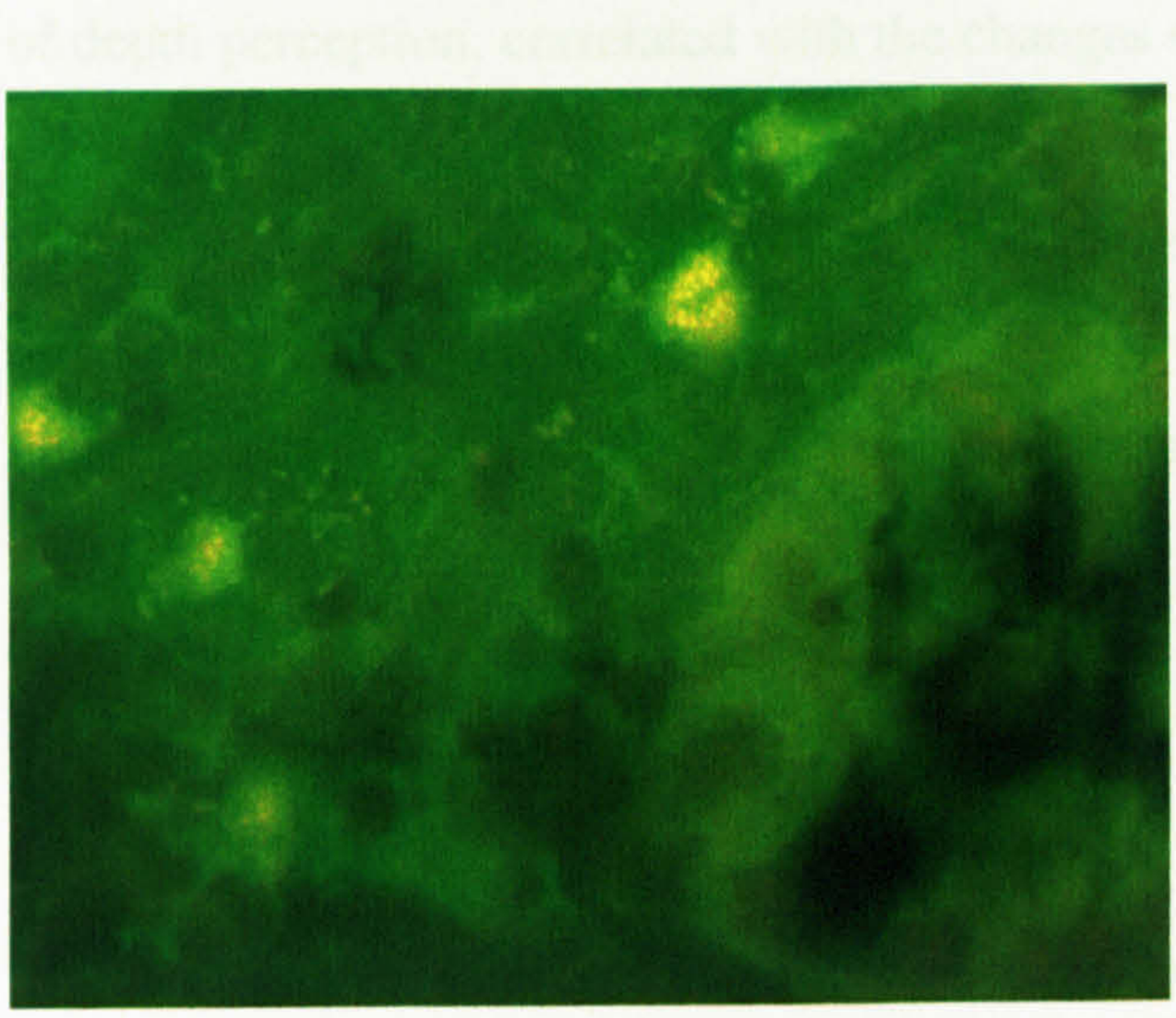


Figure 3.11 E.coli present in the lamina propria as shown by FISH technique

E.coli can be seen as fluorescent orange objects within a CD68 positive macrophage in the lamina propria



3.4 CALCULATIONS AND STATISTICS

Results are expressed as median and inter-quartile range (IQR) unless otherwise stated. A Kruskal Wallis test for non-paired non-parametric data was initially used to compare the baseline values for the three different groups: healthy volunteers, patients with inactive UC and patients with active UC. If the Kruskal-Wallis test was significant, a Mann Whitney U test was then used to compare individual groups.

A Friedman test for paired non-parametric data was initially performed to compare the three time points before, immediately and 30 mins after the stress, hypnotherapy and control protocols. If the Friedman test was significant, individual time points were then compared by the Wilcoxon signed rank test. The Mann-Whitney U test for non-paired non-parametric data was used to compare the changes recorded in the variables in response to stress and hypnotherapy between patients with UC and healthy controls. Spearman's non-parametric rank test was used to assess whether levels of chronic perceived stress correlated with changes seen in response to the stress and hypnotherapy protocols. Spearman's rank test was also used to assess whether Spiegel's score of hypnotisability (383), or the self-rated score of depth perception, correlated with the changes seen in response to the hypnotherapy protocols.

In each instance, 2-tailed $P<0.05$ was taken as statistically significant. The Bonferroni adjustment for multiple comparisons was not used as there was an a priori hypothesis of the effects of stress and hypnotherapy on the inflammatory measures assessed.(414)

3.5 POWER AND SAMPLE SIZE

At the time of the design of the experimental protocol, there was no data available on the effects of acute psychological stress or hypnotherapy on the autonomic and inflammatory response in patients with UC. Therefore it was difficult to perform formal power calculations.

However, to detect an autonomic response to the stress test and hypnotherapy protocol, we calculated that with a study sample size of 36 patients with a 2:1 randomisation to the stress or hypnotherapy and control protocols, we would be able to detect a change in pulse rate of 5 bpm with a power of 80% and a significance level of 0.05 (2 tailed) (the standard deviation of baseline heart rate being 5.8 bpm) (415). Similarly, a study sample size of 24 patients with UC and 12 healthy controls, would allow detection of a difference in pulse rate of 5 bpm in response to the stress or hypnotherapy protocol between the two patient groups with a power of 80%, and a significance level of 0.05 (2 tailed).

CHAPTER 4

BASELINE VALUES FOR SYSTEMIC MEASURES IN HEALTHY VOLUNTEERS AND PATIENTS WITH ACTIVE AND INACTIVE ULCERATIVE COLITIS

4.1 SUMMARY

Aims: To compare the systemic autonomic and inflammatory measures described in Chapter 3, in healthy volunteers and patients with active and in active UC.

Methods: As part of the stress and hypnotherapy protocols a range of systemic autonomic and inflammatory variables were assessed in the above three subject groups.

Results: The principal findings in this chapter are:

1. Of the autonomic measures, median pulse rate was 5bpm greater in patients with active UC than patients with inactive UC ($p=0.01$) and healthy volunteers ($p=0.02$). Median systolic BP was 10mmHg greater in patients with active UC and 7mmHG greater in patients with inactive UC than healthy volunteers ($p=0.002$ and $p=0.03$ respectively). The median diastolic BP of patients with active UC was 8mmHg greater than that of healthy volunteers ($p=0.02$).

2. Of the systemic inflammatory parameters assessed, serum IL-6 concentration was greater in patients with active UC than patients with inactive UC ($p=0.005$) and healthy volunteers ($p<0.0001$). Serum IL-6 concentrations were also greater in patients with inactive UC than healthy volunteers ($p=0.0004$). Serum IL-13 concentrations were greater in patients with active UC than patients with inactive UC ($p=0.0003$) and healthy volunteers ($p<0.0002$). LPS-stimulated TNF-α production by whole blood was greater in patients with active UC than both patients with inactive UC ($p<0.0001$) and healthy volunteers ($p<0.0001$). Leukocyte count was greater in patients with active UC and inactive UC than healthy volunteers ($p<0.0001$ and $p=0.01$ respectively). Platelet activation was greater in active than inactive disease ($p=0.04$).

3. With regards to the psychometric questionnaires, patients with active UC scored higher than patients with inactive UC and healthy volunteers on the Hospital Anxiety and Depression Scale (HADS), the State Trait Anxiety Inventory (STAI), the Perceived Stress Questionnaire (PSQ) and Bradford Somatic inventory ($P<0.05$ in each case). Patients with inactive UC scored higher than healthy volunteers on the HADS, PSQ and BSI ($P<0.05$ in each case).

Conclusions: The majority of the inflammatory measures assessed were greater in patients with active disease, suggesting that their levels reflect disease activity. Chronic stress levels as assessed by psychometric questionnaire were greater in patients with active UC than healthy volunteers and patients with inactive UC.

4.2 INTRODUCTION

The experimental protocols used in this thesis (Chapter 3) involved performing stress, hypnotherapy and control sessions in patients with active and inactive UC and in healthy volunteers.

In this chapter we will compare the pre-protocol baseline values of both the systemic autonomic and inflammatory measures in these three groups: patients with active UC, patients with inactive UC and healthy volunteers. Several of these inflammatory measures have been assessed in these groups previously: serum IL-6 (170;171), platelet activation (141) and PLA formation (143) but the others have not. For the measures which have not been previously assessed, it is important to demonstrate whether they are raised in active UC, since this would support their use as relevant measures of inflammation. An appreciation of the magnitude of the differences between quiescent and active disease would put into perspective the changes observed in response to stress and hypnotherapy which are discussed in chapters 6 and 8.

Psychometric questionnaires were used in the protocol to relate the effects of chronic stress to the response to acute stress. Baseline scores on these psychometric questionnaires are also described in this chapter.

4.3 PATIENT DEMOGRAPHICS

32 healthy volunteers, 35 patients with inactive UC and 25 patients with active UC partcipated in the studies involving the stress, hypnotherapy and control protocols.

Table 4.1 Sex, age, disease extent, treatment, Baron's sigmoidoscopic score and Simple Colitis Activity Index (SCCAI) for patients with inactive UC, active UC and healthy volunteers. Median (IQR) are shown except for age where median (range) is given.

Patients	N	
Inactive UC	35	
Age		45 (23-65)
Sex		16 Male
Disease extent		43% total
		23% left-sided
		34% distal
Treatment		80% 5-ASA
		11% thiopurines
		3% oral methotrexate
		0% corticosteroids
		14% on topical therapy
Baron's score		0 (0-1)
SSCAI		1 (0-2)
Active UC	25	
Age		40 (23-64)
Sex		15 Male
Disease extent		60% total
		16% left-sided
		24% distal
Treatment		96% 5-ASA
		32% thiopurines
		4% oral ciclosporin
		0% corticosteroids
		20% Topical
Baron's score		2 (2-3)

	SSCAI	6 (5.5-8)
Healthy Volunteers	32	
	Age	31 (23-59)
	Sex	15 Male

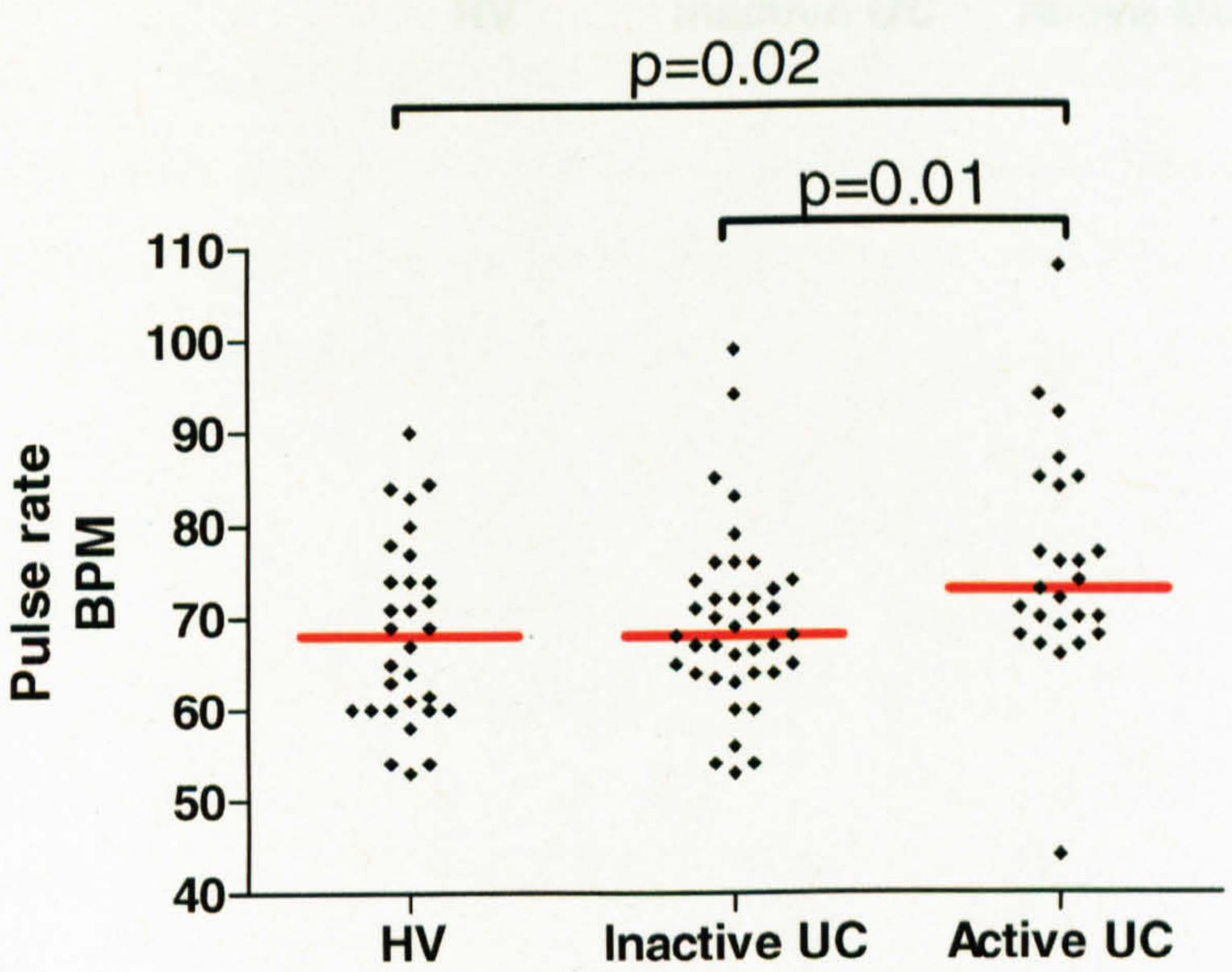
4.4 RESULTS

4.4.1 AUTONOMIC MEASURES

4.4.1.1 Pulse Rate (Figure 4.1 and Table 4.2)

The median pulse rate of the patients with active UC was 5bpm greater than the median pulse rate of patients with inactive UC and 5bpm greater than the median pulse rate of healthy volunteers.

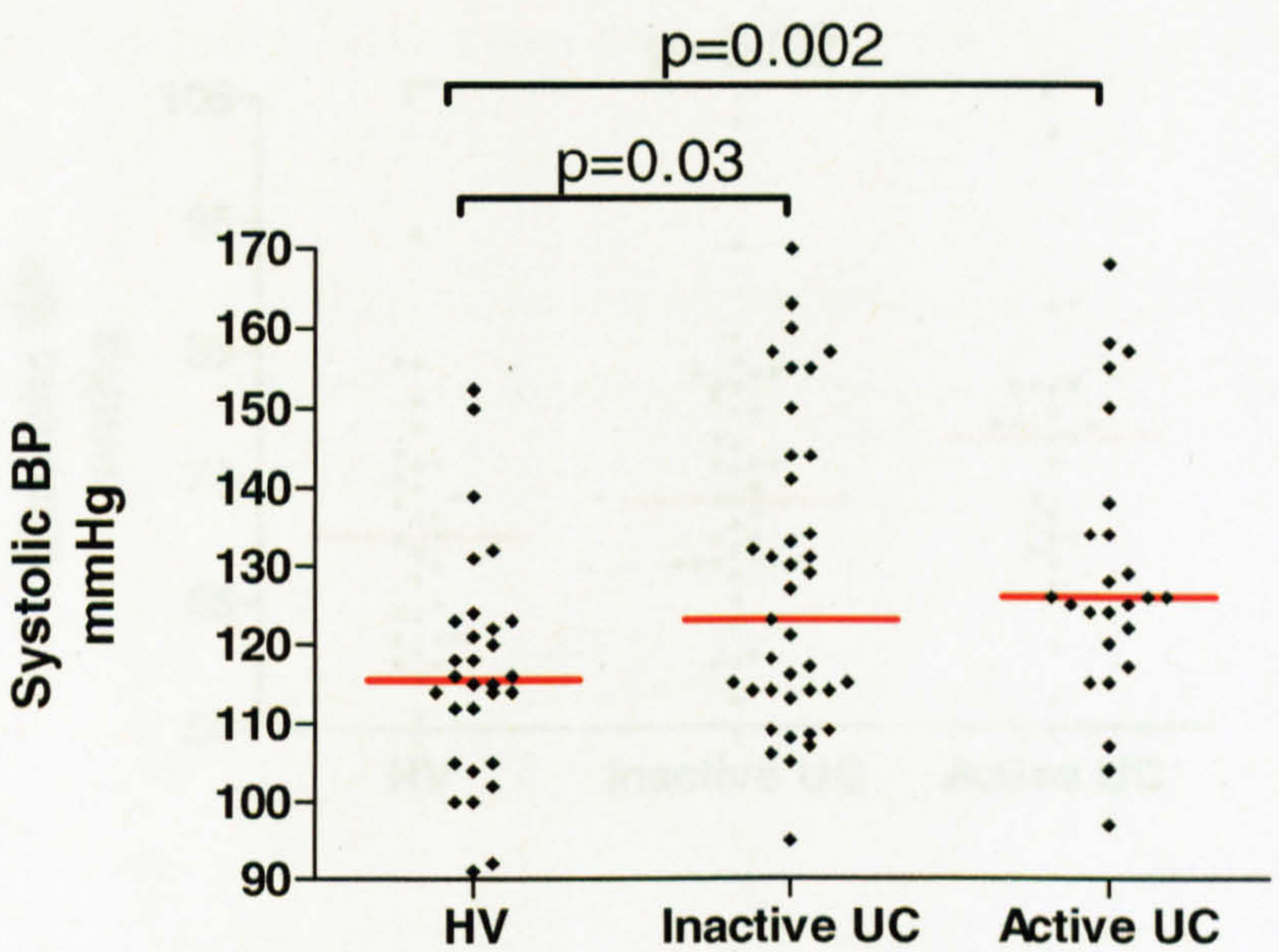
Fig 4.1 Baseline pulse rate (BPM) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). The median value is shown as the red bar.



4.4.1.2 Systolic BP (Figure 4.3 and Table 4.2)

The systolic BP of the healthy volunteers was a median of 7mmHg lower than the patients with inactive UC and 10mmHg lower than patients with active UC.

Fig 4.2 Baseline systolic BP (mmHg) for healthy volunteers (n=32) (HV) and patients with inactive (n=35) and active UC (n=25). Median value is shown as the red bar.



4.4.1.3 Diastolic BP (Figure 4.3 and Table 4.3)

The diastolic BP of patients with active UC was a median of 8mmHg greater than that of healthy volunteers.

Fig 4.3 Baseline diastolic BP (mmHg) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). Median value is shown as the red bar.

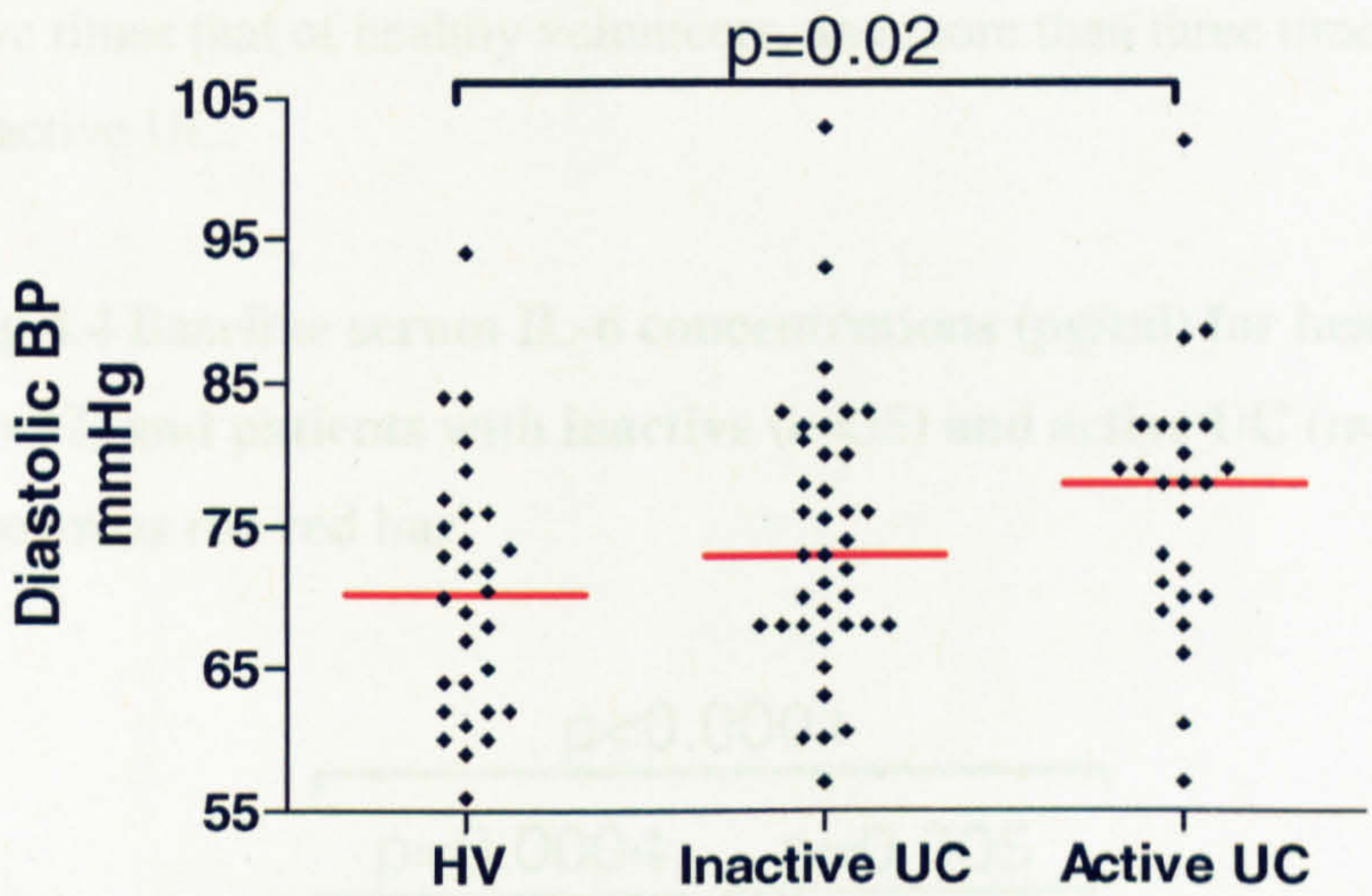


Table 4.2 Baseline pulse rate (bpm), systolic and diastolic blood pressure (mmHg) for healthy volunteers and patients with active and inactive UC. Median and IQR shown. *p<0.05 from healthy volunteer baseline. †p<0.05 from patients with inactive UC baseline.

	Healthy Volunteers	Patients with inactive UC	Patients with active UC
Pulse Rate	68 (60-76)	68 (64-74)	73 (69-85) *†
Systolic BP	116 (105-123)	123 (114-144)*	126 (119-136) *
Diastolic BP	70 (62-76)	73 (68-81)	78 (70-82)*

4.4.2 SYSTEMIC INFLAMMATORY VARIABLES

4.4.2.1 Serum cytokine concentrations (Figure 4.4 and 4.5, and Table4.3)

The median serum IL-6 concentration in patients with inactive UC was twice that of healthy volunteers. The median serum IL-6 concentration in patients with active UC was nearly four-fold greater than that of healthy volunteers and double that of patients with inactive UC.

The median serum IL-13 concentration in patients with active UC was over five times that of healthy volunteers, and more than three time that of patients with inactive UC.

Fig 4.4 Baseline serum IL-6 concentrations (pg/ml) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). Median value is shown as the red bar.

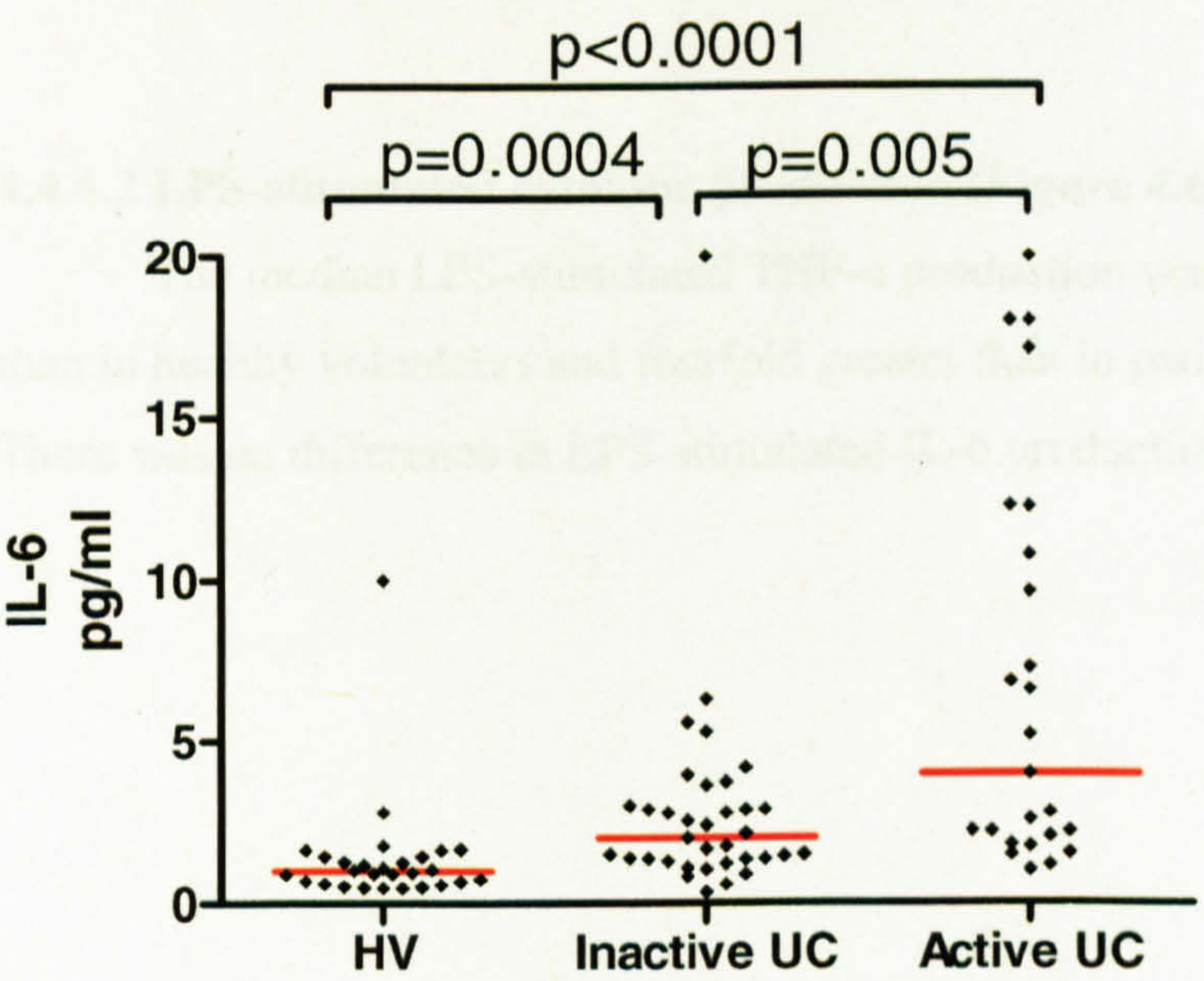
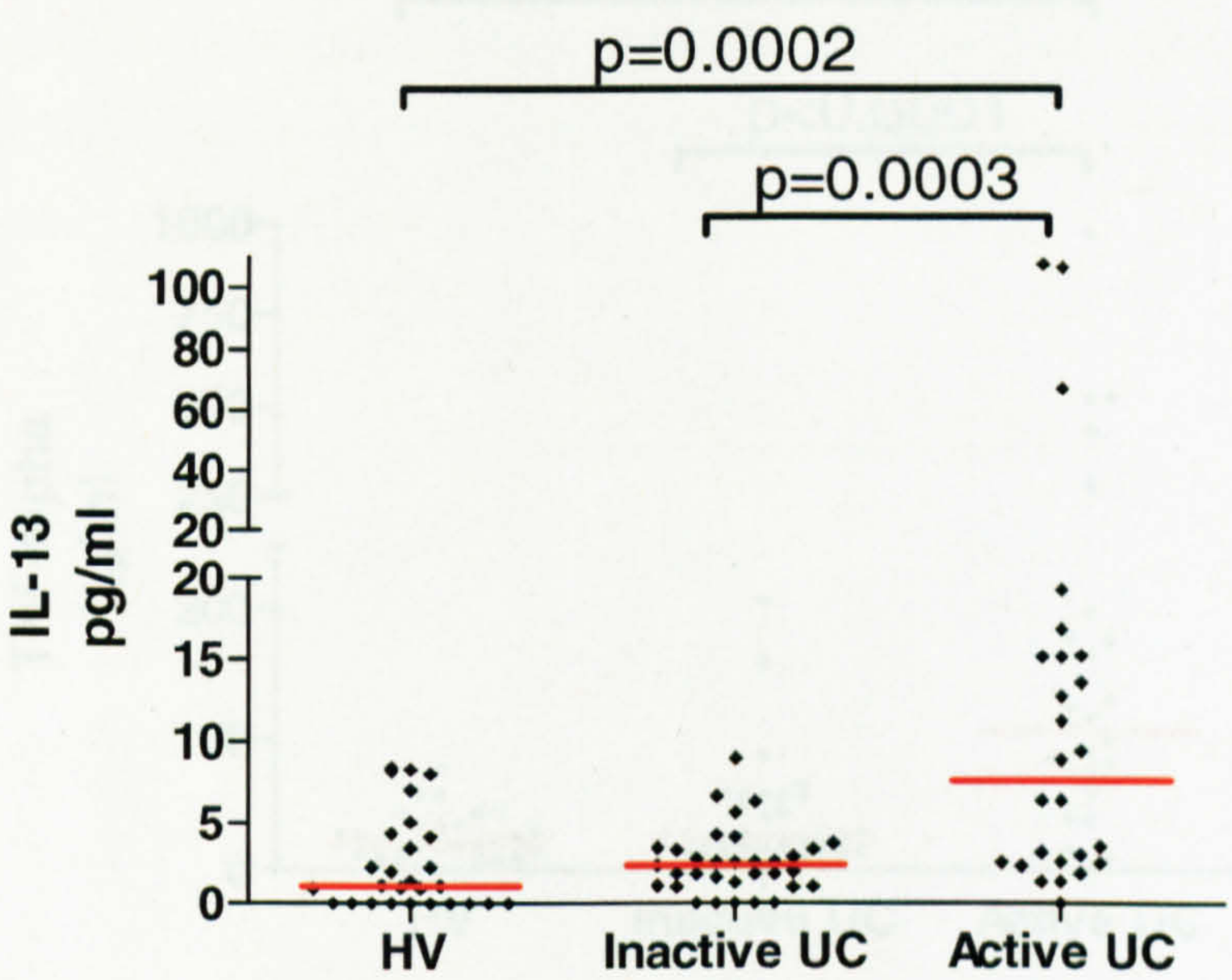


Fig 4.5 Baseline Serum IL-13 concentrations (pg/ml) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). Median value is shown as the red bar.



4.4.4.2 LPS-stimulated cytokine production (Figure 4.6 and Table 4.3)

The median LPS-stimulated TNF-α production was over five times greater than in healthy volunteers and fourfold greater than in patients with inactive UC. There was no difference in LPS-stimulated IL-6 production between the three groups.

Fig 4.6 Baseline LPS-stimulated TNF-α production (ng/ml) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). Median value is shown as the red bar.

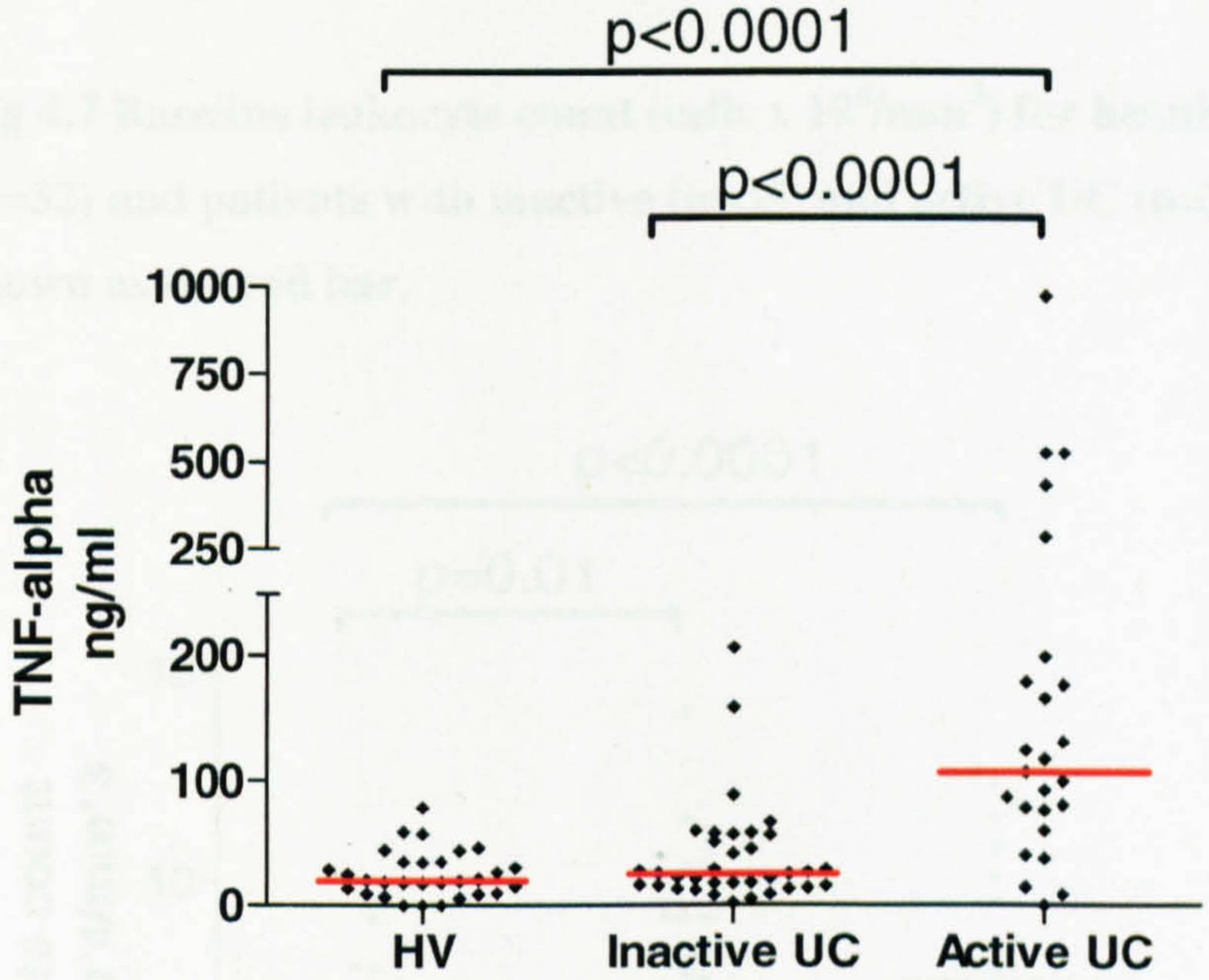


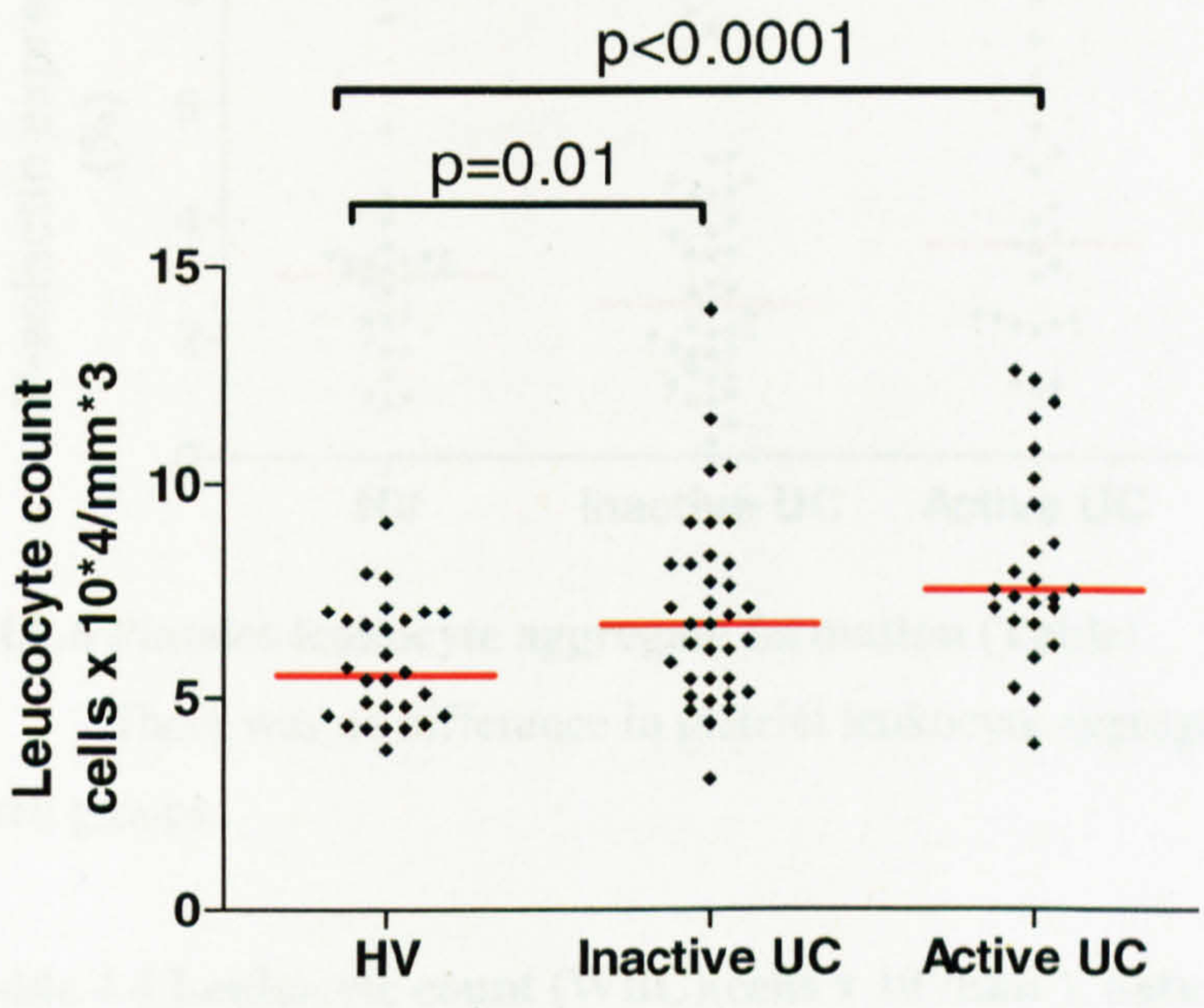
Table 4.3 Serum IL-6 and IL-13 concentrations (pg/ml) and LPS-stimulated TNF-α and IL-6 production (ng/ml) by whole blood in healthy volunteers and patients with inactive and active UC. *p<0.05 from baseline value for healthy volunteers. †p<0.05 from baseline for patients with inactive UC.

	Healthy Volunteers	Patients with inactive UC	Patients with active UC
Serum IL-6	1.0 (0.6-1.5)	2.0 (1.4-3.0)*	4.0 (1.9-11.5)*†
Serum IL-13	1.5 (0-4.3)	2.4 (1.1-3.4)	7.6 (2.5-15.2)*†
LPS TNF-α	20 (10-35)	25 (15-57)	106 (68-189)*†
LPS IL-6	208 (148-289)	233 (182-436)	262 (183-446)

4.4.2.3 Leukocyte count (Figure 4.7 and Table 4.4)

Median leukocyte count was over a third greater in patients with active UC and over 20% greater in patients with inactive UC than in healthy volunteers.

Fig 4.7 Baseline leukocyte count (cells x $10^4/mm^3$) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). Median value shown as the red bar.



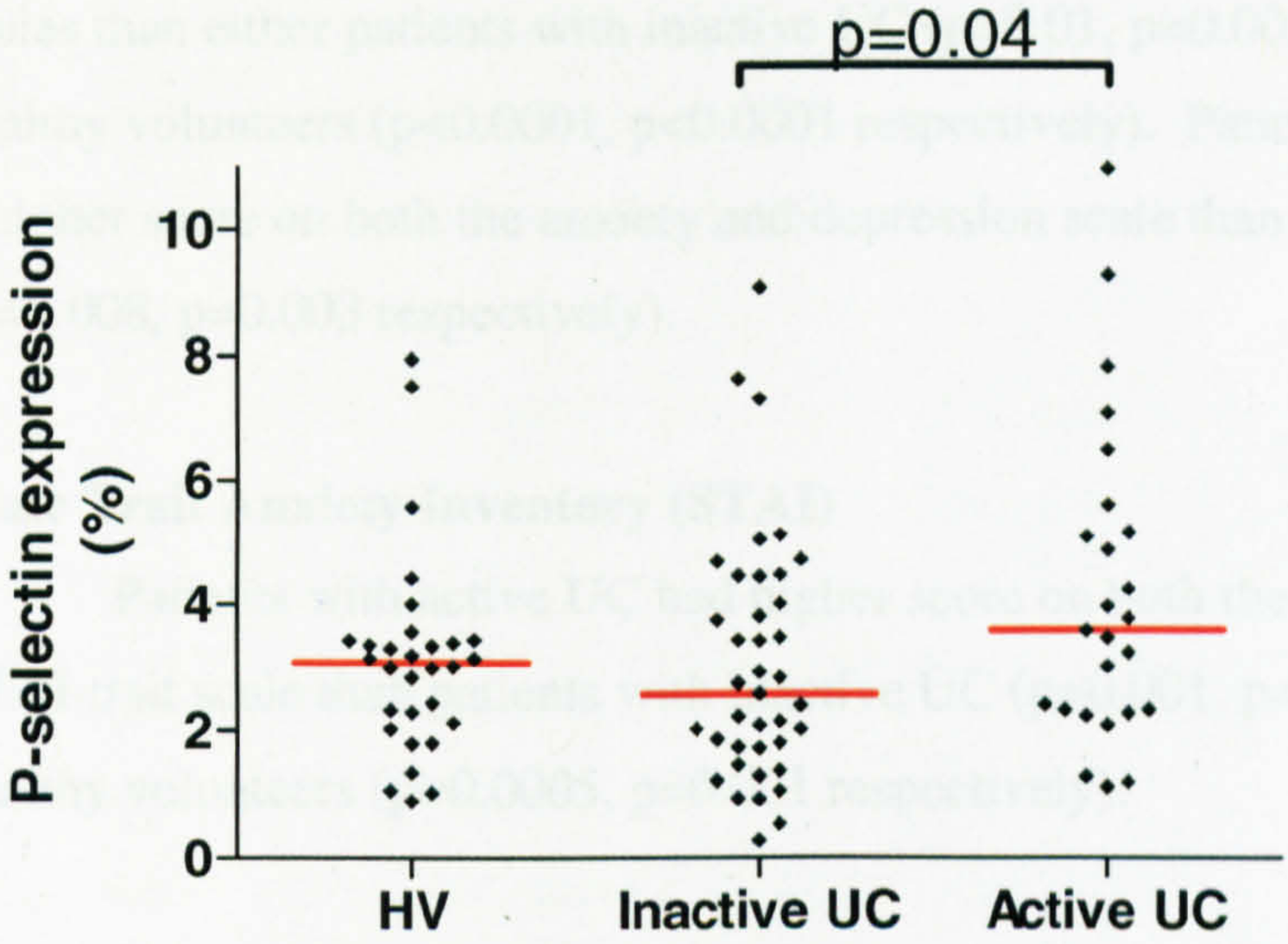
4.4.2.4 Natural Killer Cell count (Table 4.4)

There was no difference in the baseline NK cell count between groups.

4.4.2.5 Platelet activation (P-selectin expression) (Figure 4.8 and Table 4.4)

Median platelet activation as measured by P-selectin expression was 40% greater in patients with active UC than patients with inactive UC.

Fig 4.8 Baseline platelet activation (P-selectin expression (%)) for healthy volunteers (HV) (n=32) and patients with inactive (n=35) and active UC (n=25). Median is shown as red bar.



4.4.2.6 Platelet-leukocyte aggregate formation (Table)

There was no difference in platelet leukocyte aggregate formation between the three groups.

Table 4.4 Leukocyte count (WBC)(cells x 10^4/mm^3), natural killer cell count (%), platelet activation (p-selectin expression (%)) and platelet-leukocyte aggregrate (PLA) formation (%) in healthy volunteers and patients with active and inactive UC. *p<0.05 from baseline value for healthy volunteers. †p<0.05 from baseline value for patients with inactive UC.

	Healthy Volunteers	Patients with inactive UC	Patients with active UC
WBC	5.5 (4.6-6.9)	6.7 (5.4-8.1)*	7.5 (6.7-9.8)*
NK count	6.8 (5.3-9.3)	6.8 (4.4-9.7)	6.8 (4.8-9.1)
P-selectin	3.1 (2.0-3.4)	2.6 (1.7-4.5)	3.6 (2.3-5.4)†
PLA	2.7 (2.0-3.8)	2.4 (1.7-3.2)	2.9(2.2-3.5)

4.4.3 PSYCHOMETRIC QUESTIONNAIRES (Table 4.5)

Hospital Anxiety and Depression Scale (HADS)

Patients with active UC had higher scores on both the anxiety and depression scales than either patients with inactive UC (p=0.01, p=0.003 respectively) and healthy volunteers (p<0.0001, p<0.0001 respectively). Patients with inactive UC had a higher score on both the anxiety and depression scale than healthy volunteers (p=0.008, p=0.003 respectively).

State Trait Anxiety Inventory (STAI)

Patients with active UC had higher score on both the STAI-state scale and the STAI-trait scale than patients with inactive UC (p=0.001, p=0.003 respectively) and healthy volunteers (p=0.0005, p=0.001 respectively).

Perceived Stress Questionnaire (PSQ)

Patients with active UC had higher score on the Perceived Stress Questionnaire than patients with inactive UC (p=0.02) and healthy volunteers (p<0.0001). Patients with inactive UC had a higher score on the Perceived Stress Questionnaire than healthy volunteers (p=0.01).

Bradford Somatic Inventory (BSI)

Patients with active UC had higher scores on the Bradford Somatic Inventory than patients with inactive UC (p=0.01) and healthy volunteers (p<0.0001). Patients with inactive UC had a higher score on the Bradford Somatic Inventory scale than healthy volunteers (p=0.001).

Table 4.5 Hospital Anxiety and Depression Scale-Anxiety (HADS-A), Hospital Anxiety and Depression Scale-Depression (HADS-D), State Trait Anxiety Inventory-state (STAI-state), State Trait Anxiety Inventory-trait (STAI-trait), Perceived Stress Questionnaire (PSQ) and Bradford Somatic Inventory (BSI) scores for healthy volunteers (n=32) and patients with inactive (n=35) and active UC (n=25).*p<0.05 from baseline value for healthy volunteers. †p<0.05 from baseline value for patients with inactive UC.

	Healthy Volunteers	Patients with inactive UC	Patients with active UC
N	32	35	25
HADS-A	4 (3-7)	7 (5-10)*	11 (7.5-13)*†
HADS-D	1 (1-3)	3 (2-4)*	5.5 (4-8.5)*†
STAI-state	31 (26-36)	33 (25-40)	43 (34-50)*†
STAI-trait	24 (28-44)	36 (33-43)	46 (38-52)*†
PSQ	52 (47-62)	62 (53-70)*	68 (62-85)*†
BSI	5 (2-8.5)	9 (5-15)*	15 (11-19)*†

4.5 DISCUSSION

In this chapter, we have compared the baseline values for the physiological and systemic inflammatory measures which were assessed as part of the experimental protocol (chapter 3) in the three subject groups. We found the majority of systemic inflammatory variables to be raised in patients with acute UC compared to patients with quiescent UC and healthy controls. Each will be discussed in turn.

AUTONOMIC MEASURES

Pulse rate was greater in patients with active UC than patients with inactive UC and healthy volunteers. This is not an unexpected finding as the presence of a tachycardia is well recognized as being a clinical marker of severely active UC. Systolic BP was greater in patients with active UC and inactive UC than HVs.

Diastolic BP was greater in patients with active UC than HVs. This is likely to be due to the differences in ages between the groups. The subjects with active and inactive UC were older than the HVs. Systolic and diastolic BP correlated with age in both patients with active (R=0.56, P=0.02 and R=0.58, P=0.02 respectively) and inactive UC (R=0.58, P=0.001 and R=0.41, P=0.03 respectively). The range of ages in the healthy volunteers was too narrow to allow assessment for a correlation in this group. The differences in pulse rate are unlikely to be explained by the differences in age as there was no correlation between age and pulse rate in any of the patient groups.

SERUM CYTOKINES

Serum IL-6 concentrations were greater in patients with active UC than patients with inactive and HVs. The serum IL-6 concentration was also greater in patients with inactive UC than HVs. IL-6 is a pleiotropic inflammatory cytokine with many inflammatory actions (see chapter 1). Its saliva and serum concentrations have previously been found to be raised both in CD and also in UC (170;171;416;417); indeed an anti-IL6 antibody has also been shown to be of benefit in CD (177). The role of IL-6 in stimulating the hypothalamus to initiate the stress response also makes it a useful inflammatory measure in this study (168).

Serum IL-13 concentrations were greater in patients with active UC than patients with inactive UC and HVs. There was no difference between the serum concentrations of IL-13 in patients with inactive UC and HVs.

Recent evidence suggests that IL-13 may be important in the pathophysiology of UC. Mucosal production of IL-13 is increased in oxazolone-induced colitis, a murine model of GI inflammation which closely resembles UC (418). In human studies, stimulated lamina propria mononuclear cells (LPMCs) from resected colonic specimens from patients with UC have been shown to produce more IL-13 than LPMCs from colonic specimens from patients with CD or non-inflammatory controls (419). IL-13 has also been shown to impair the epithelial barrier function of colonic cells in vitro via increased apoptosis and expression of the pore-forming tight junction protein claudin-2.(420) Although IL-13 production by LPMCs has been shown to be increased in active UC (421), as yet there appears to have been no data published

regarding the circulating levels of IL-13 in patients with UC. However, further work is needed to examine whether serum IL-13 concentration might prove a useful marker of disease activity in UC.

LPS-STIMULATED CYTOKINE PRODUCTION BY WHOLE BLOOD

TNF-α production by whole blood was greater in patients with active UC than in patients with inactive UC and than in healthy volunteers. This is unlikely to be explained simply by the increased leukocyte count found in patients with active UC for two reasons. Firstly, there was no difference between the leukocyte counts in patients with active and inactive UC, and secondly TNF-α production by whole blood did not correlate with leukocyte count in any patient group.

The finding that TNF-α production is raised in active UC supports the use of this variable as a measure of the systemic inflammatory response in this study. The production of inflammatory cytokines by macrophages in response to bacterial products such as LPS may well be an important process in the pathophysiology of IBD (51). Therefore changes in TNF-α production by LPS-stimulated blood in response to stress or hypnosis may be important in the explaining related changes in disease activity.

LEUKOCYTE COUNT

Total leukocyte count was greater in patients with active and inactive UC than healthy volunteers. Leukocyte count is generally regarded as a poor marker of disease activity in UC compared to other laboratory measures such as CRP (422). This observation was not explained by the different ages of the three subject groups as age did not correlate with leukocyte count within any of these cohorts. None of the patients was taking, or had recently been on an oral corticosteroid, so steroid use can also not explain these findings.

NK CELL COUNT

Rises in NK cell count are consistently reported in response to acute experimental stress (137;235;236;245). Its inclusion as a systemic inflammatory

measure in this study provided a means to assess whether the stress protocol was sufficiently stressful as to cause an inflammatory response. The recently recognized interactions between dendritic cells and NK cells also makes assessment of NK count pertinent to this study (see chapter 1) (423). However, our failure to find a difference between NK cell counts in the three patient groups would suggest that although a rise in NK cell count might act as an initiator for inflammation in IBD it is unlikely to have a longer term role.

PLATELET ACTIVATION AND PLATELET-LEUKOCYTE AGGREGATE FORMATION

Platelet activation and PLA formation have both been reported as being raised in patients with IBD (141;143). However, in this study we did not find there to be a difference in either measure between patients with UC and HVs. The cause for this discrepancy is not immediately apparent. The healthy volunteers were younger than the patients with UC. However, p-selectin expression and PLA formation are not recognised as being inversely associated with age (141;143). Neither, in this study, did age correlate with p-selectin expression or PLA formation in healthy volunteers or patients with active or inactive UC.

One possible explanation might be the use of concomitant drug therapy. Both clopidogrel and HMG-CoA reductase inhibitors have been shown to be associated with reduced p-selectin expression and PLA formation (424;425). As these drugs are usually prescribed to reduce risks of cardiovascular events in a middle aged or elderly population, it is possible that more of the patients with UC than the healthy volunteers were taking them. However, unfortunately this data is not available.

We did find platelet activation to be greater in patients with active than inactive UC. This was not found in previous studies performed in our unit but supports its use as a systemic inflammatory measure in this study.

PSYCHOMETRIC QUESTIONNAIRES

Patients with active UC had higher levels of short and long term stress, as assessed by psychometric questionnaires, than patients with inactive disease and

healthy volunteers. This is in keeping with the assumption that the presence of active disease is inherently stressful for patients. Similar results have been found by others (273;274;373). Patients with inactive UC scored higher than healthy volunteers on the majority of the psychometric questionnaires used to assess stress. This finding would suggest that the presence of inactive disease is also stressful. These observations are not explained by age differences between the HVs and patients with active and inactive disease as age did not correlate with the scores on any of the questionnaires.

4.6 CONCLUSION

Most of the inflammatory measures used to assess systemic inflammation were found to be raised in patients with active UC compared to patients with inactive UC and healthy volunteers. Of particular note serum IL-13 was greater in patients active UC than those with inactive disease and healthy volunteers, and greater in patients with inactive UC than healthy volunteers. Further work is needed to assess if serum IL-13 might be a useful measure of disease activity in UC.

CHAPTER 5

BASELINE VALUES FOR MUCOSAL MEASURES IN PATIENTS WITH ACTIVE AND INACTIVE ULCERATIVE COLITIS

5.1 SUMMARY

Aims: To compare the rectal mucosal measures of inflammation described in Chapter 3 and rectal mucosal blood flow in patients with inactive and active UC.

Methods: As part of the stress and hypnotherapy protocols, several rectal mucosal inflammatory variables were assessed in patients with active and inactive disease.

Results: The principal findings in this chapter are:

1. Substance P ($p=0.05$), histamine ($p=0.008$), IL-13 ($p=0.002$) and TNF-α concentrations ($p<0.0001$) in rectal peri-mucosal fluid were higher in active than inactive UC.
2. Reactive oxygen metabolite (ROM) production by mucosal biopsies ($p=0.0002$) was greater in patients with active than inactive UC.
3. Rectal mucosal blood flow (RMBF) ($p=0.009$) was higher in patients with active disease.

Conclusion: All the measures assessed were increased in active compared with inactive UC, suggesting that their levels reflect disease activity and supporting the view that they may be important in disease pathogenesis.

5.2 INTRODUCTION

In addition to the measurement of systemic inflammatory variables, as part of the experimental protocol several measures of mucosal inflammation were made before and after the stress, hypnotherapy and control sessions. In this chapter we will discuss the baseline pre-protocol levels of these measures in patients with active and inactive UC. Several of the inflammatory parameters assessed have not been analysed with these methods in active and inactive UC previously. As indicated in Chapter 3, we did not undertake sigmoidoscopy in healthy volunteers, so this group is not available for comparison.

Although assessing the concentration of inflammatory mediators in rectal peri-mucosal fluid by the use of the filter paper technique is an established methodology (426), it has not been used previously to assess some of the mediators

described in this experimental protocol. Rectal fluid TNF-α concentrations have been shown to be raised in active UC with this technique (377), but SP, histamine and IL-13 concentrations have not been previously assessed. ROM production by mucosal biopsies assessed using chemiluminescence has been shown previously to be greater in active UC than in quiescent disease (181). Rectal mucosal blood flow in UC has been assessed using laser Doppler flowmetry and was found to be increased in active disease, but the MOORlab equipment used in this protocol has not been used previously for this purpose (104).

As before, demonstration that these measures are raised in active compared to inactive UC gives validity to their use as measures of inflammation; furthermore an appreciation of the magnitude of the differences between active and inactive disease is useful in assessing the responses to stress and hypnotherapy described in Chapters 7 and 9.

5.3 PATIENT DEMOGRAPHICS

35 patients with inactive UC and 25 patients with active UC took part in the studies involving the stress, hypnotherapy and control protocols. Their demographics are described in Chapter 4 (Table 4.1)

5.4 RESULTS

Peri-mucosal fluid samples were available from 33 patients with inactive UC as in 2 patients with inactive UC the samples were defrosted by accident during storage and could not therefore be used. ROM production was assessed on 27 pairs of rectal mucosal biopsies from patients with inactive UC. Eight pairs of biopsies from patients with inactive UC were used for mast cell and mucosa-associated bacteria studies. ROM production and rectal mucosal blood flow were assessed in 23 patients with active UC. In 2 patients with active UC these variables could not be assessed due to equipment failure.

5.4.1 CYTOKINE AND MEDIATOR CONCENTRATIONS IN RECTAL PERI-MUCOSAL FLUID (Figure 5.1, 5.2, 5.3 and 5.4 and Table 5.1)

In rectal peri-mucosal fluid from patients with active UC, the median substance P concentration was over two and a half times greater, the median histamine concentration was over threefold greater, the median IL-13 concentration over twofold greater and the median TNF-α concentration over eight times greater than in peri-mucosal fluid from patients with inactive UC.

Fig 5.1 Baseline substance P concentrations (pg/ml) in rectal peri-mucosal fluid for patients with inactive (n=33) and active UC (n=25). Median value shown as red bar.

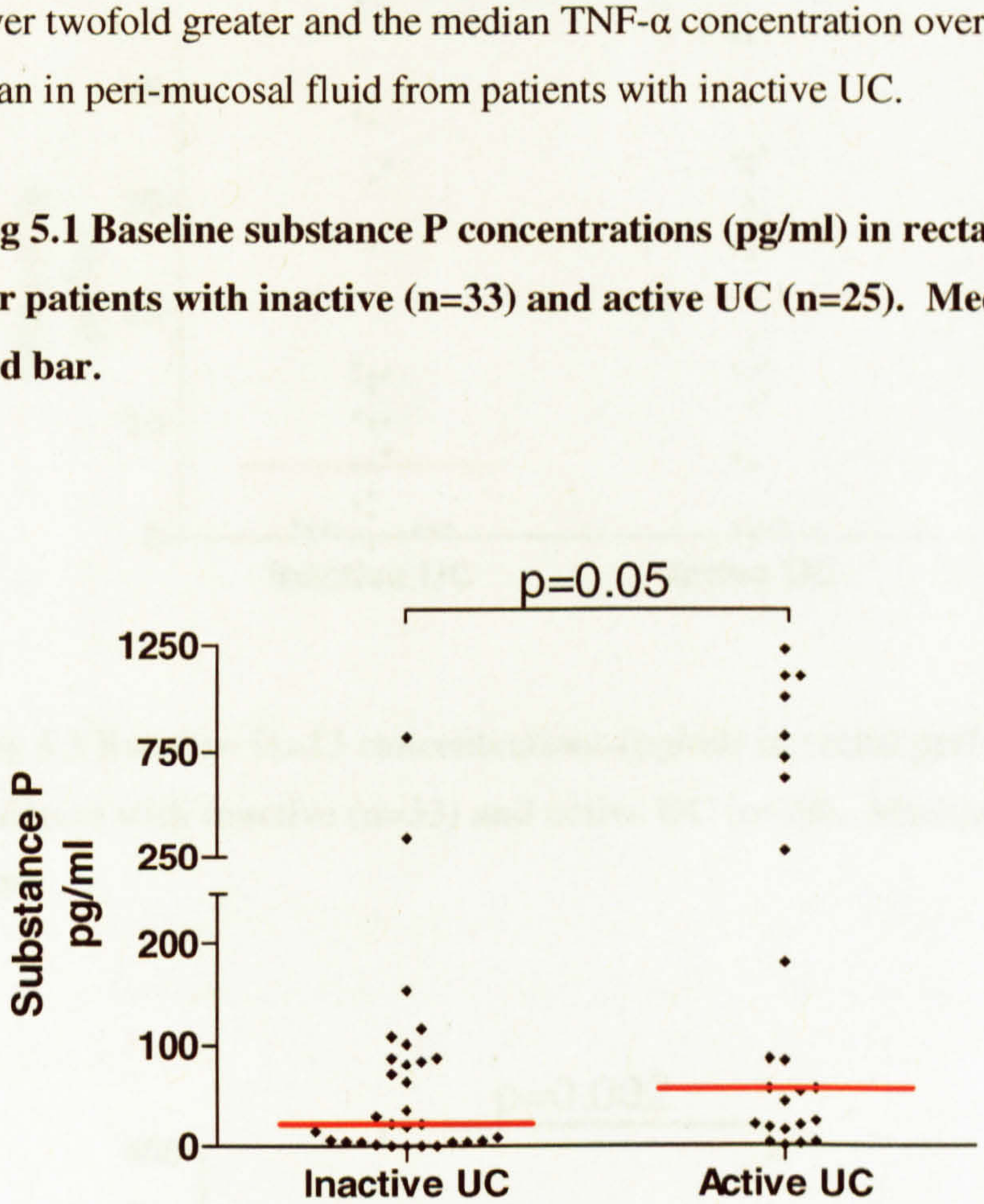


Fig 5.2 Baseline histamine concentrations (pg/ml) in rectal peri-mucosal fluid for patients with inactive (n=33) and active UC (n=25). Median value shown as red bar.

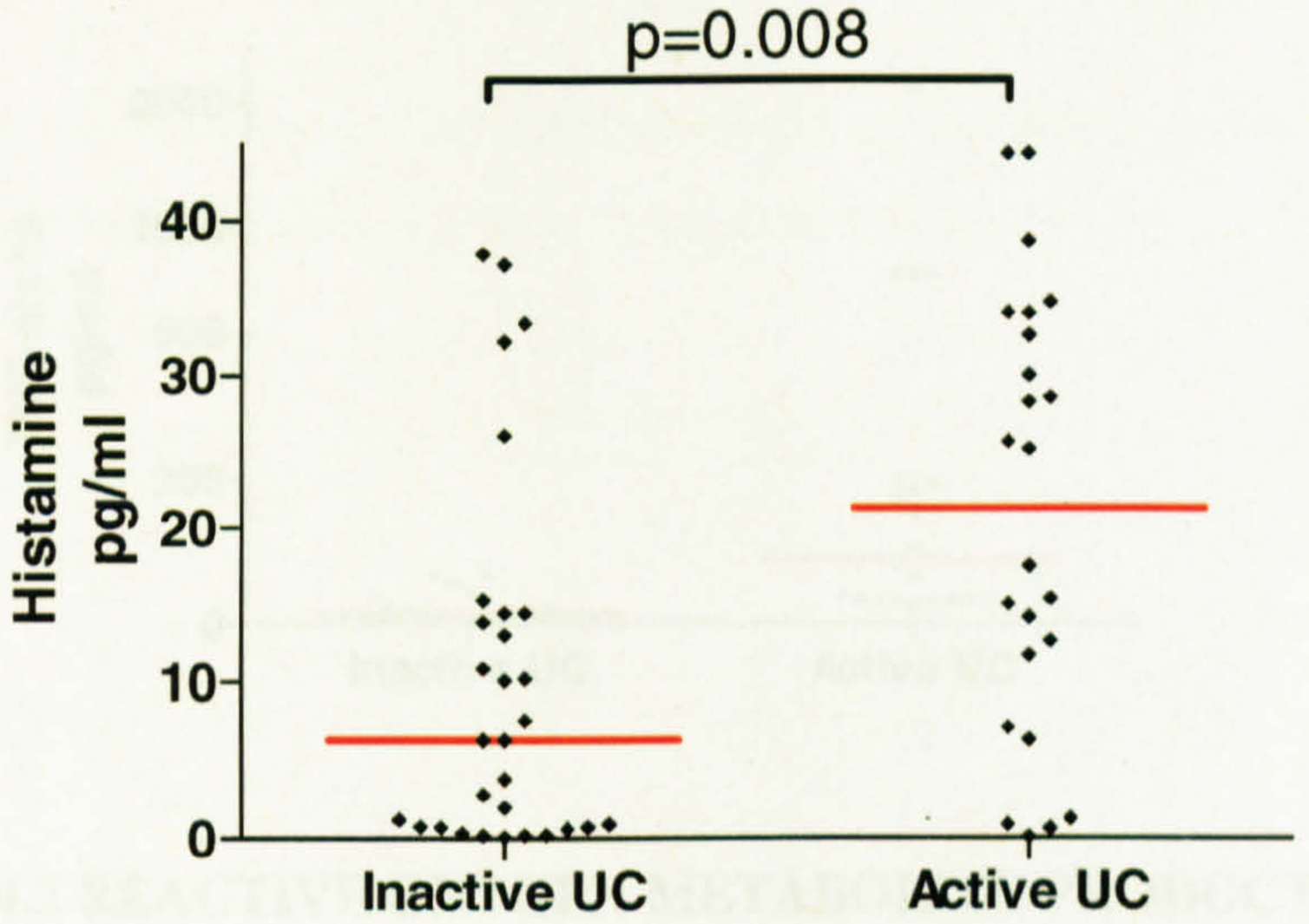


Fig 5.3 Baseline IL-13 concentrations (pg/ml) in rectal peri-mucosal fluid for patients with inactive (n=33) and active UC (n=25). Median value shown as red bar.

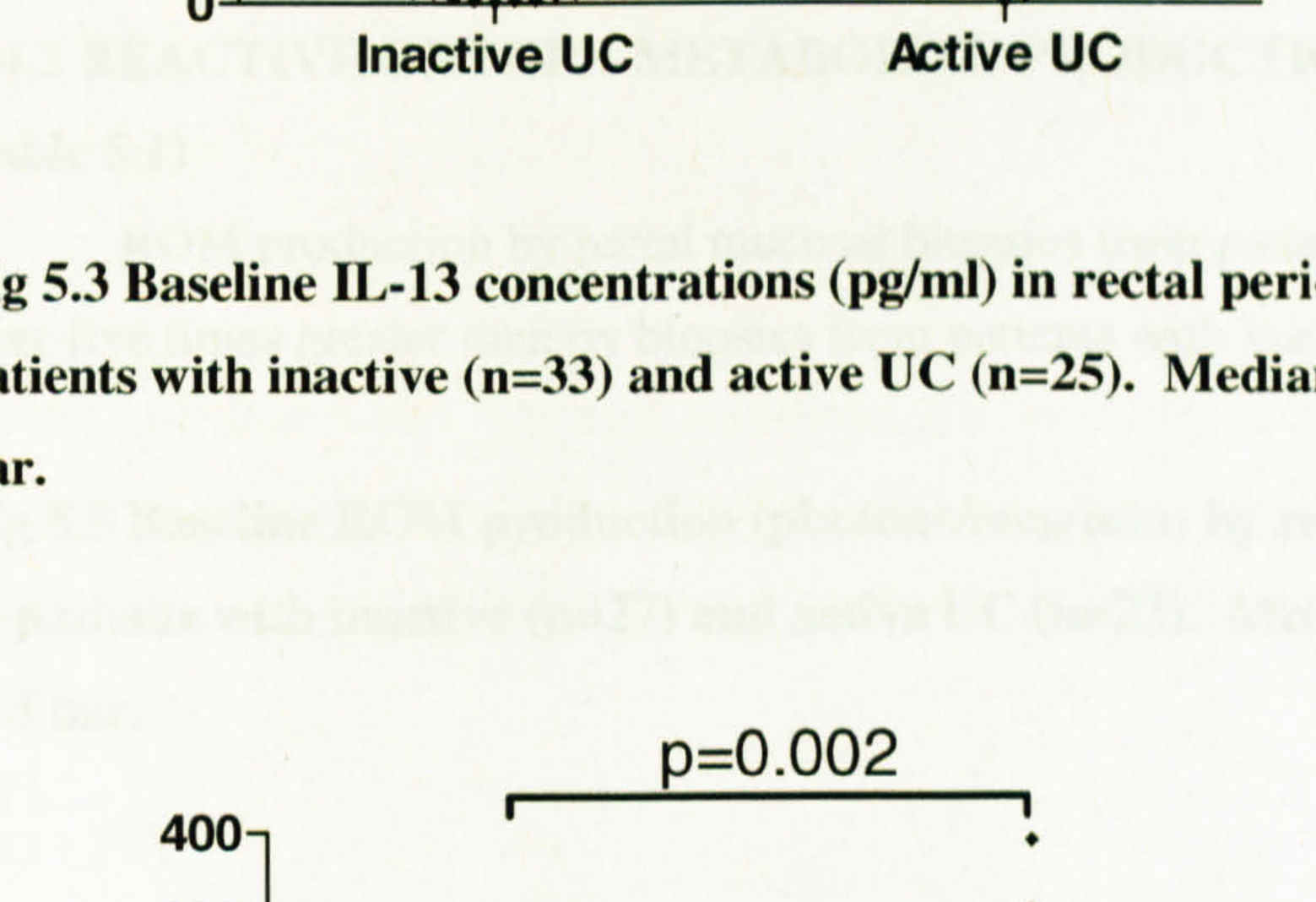
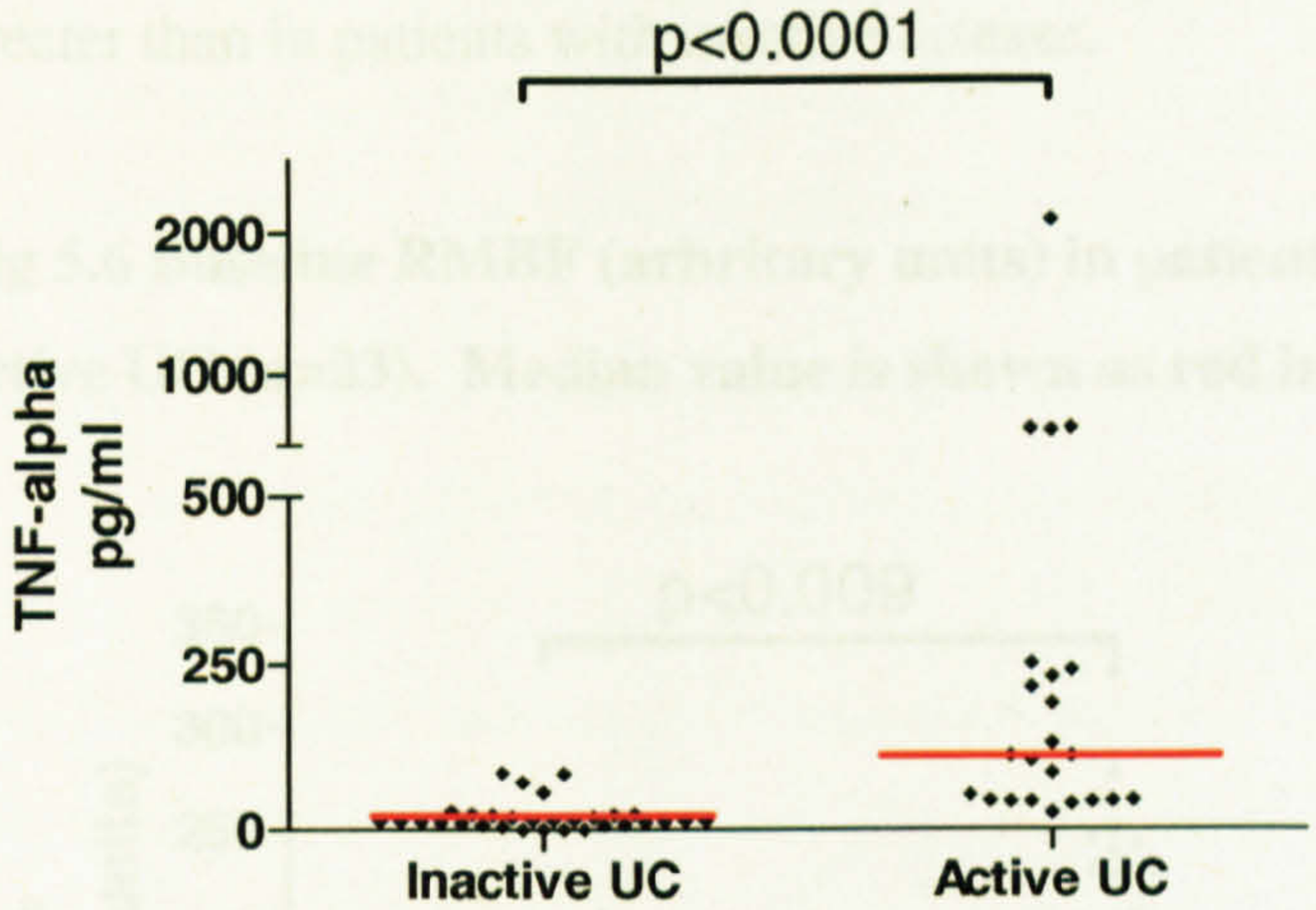


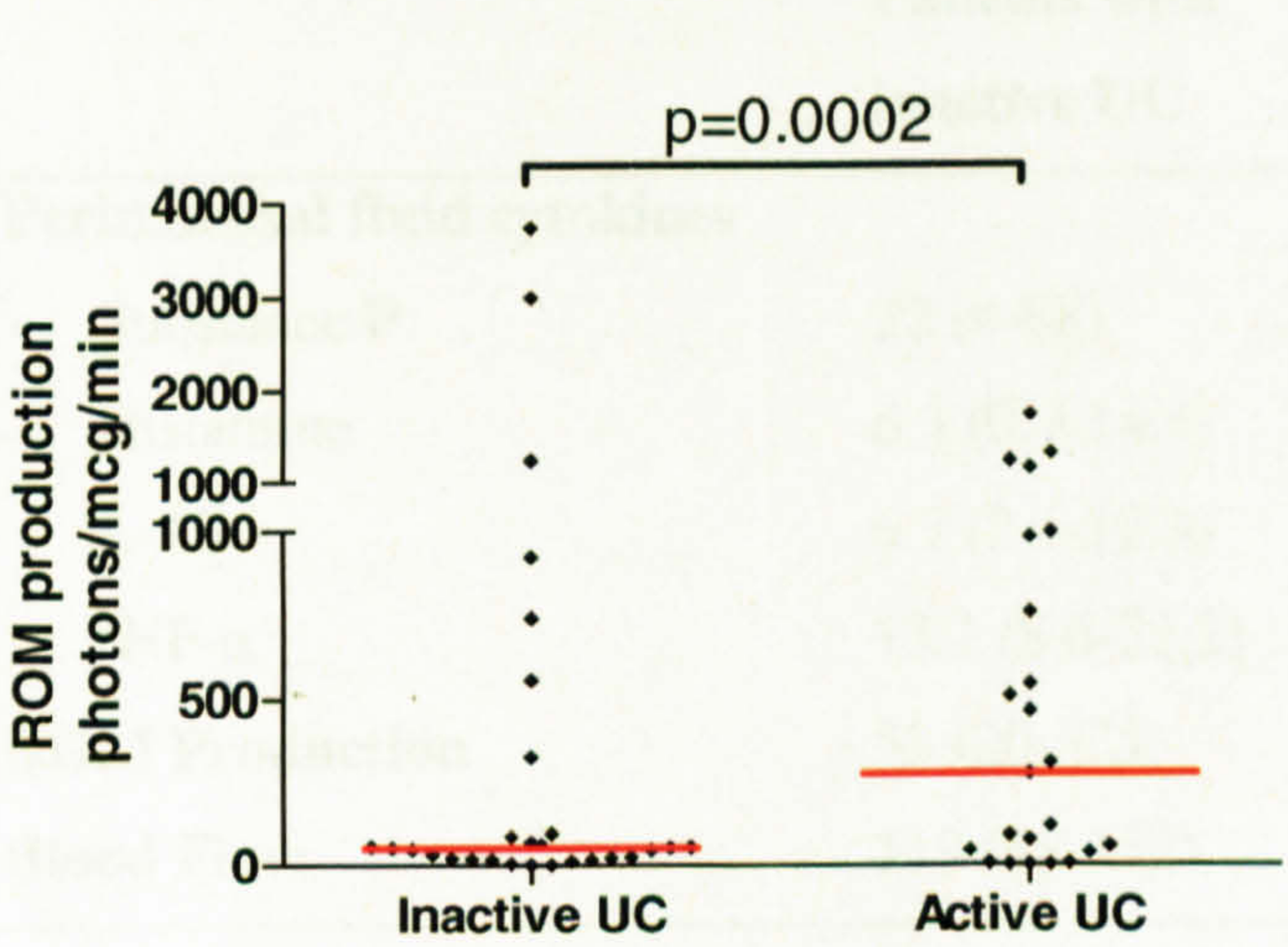
Fig 5.4 Baseline TNF-α concentrations (pg/ml) in rectal peri-mucosal fluid for patients with inactive (n=33) and active UC (n=25). Median shown as red bar.



5.4.2 REACTIVE OXYGEN METABOLITE PRODUCTION (Figure 5.5 and Table 5.1)

ROM production by rectal mucosal biopsies from patients with active UC was over five times greater than by biopsies from patients with inactive UC.

Fig 5.5 Baseline ROM production (photons/mcg/min) by rectal mucosal biopsies in patients with inactive (n=27) and active UC (n=23). Median value shown as red bar.



5.4.3 RECTAL MUCOSAL BLOOD FLOW (RMBF) (Figure 5.6 and Table 5.1)

Rectal mucosal blood flow in patients with active UC was a median of 75% greater than in patients with inactive disease.

Fig 5.6 Baseline RMBF (arbritary units) in patients with inactive (n=35) and active UC (n=23). Median value is shown as red bar.

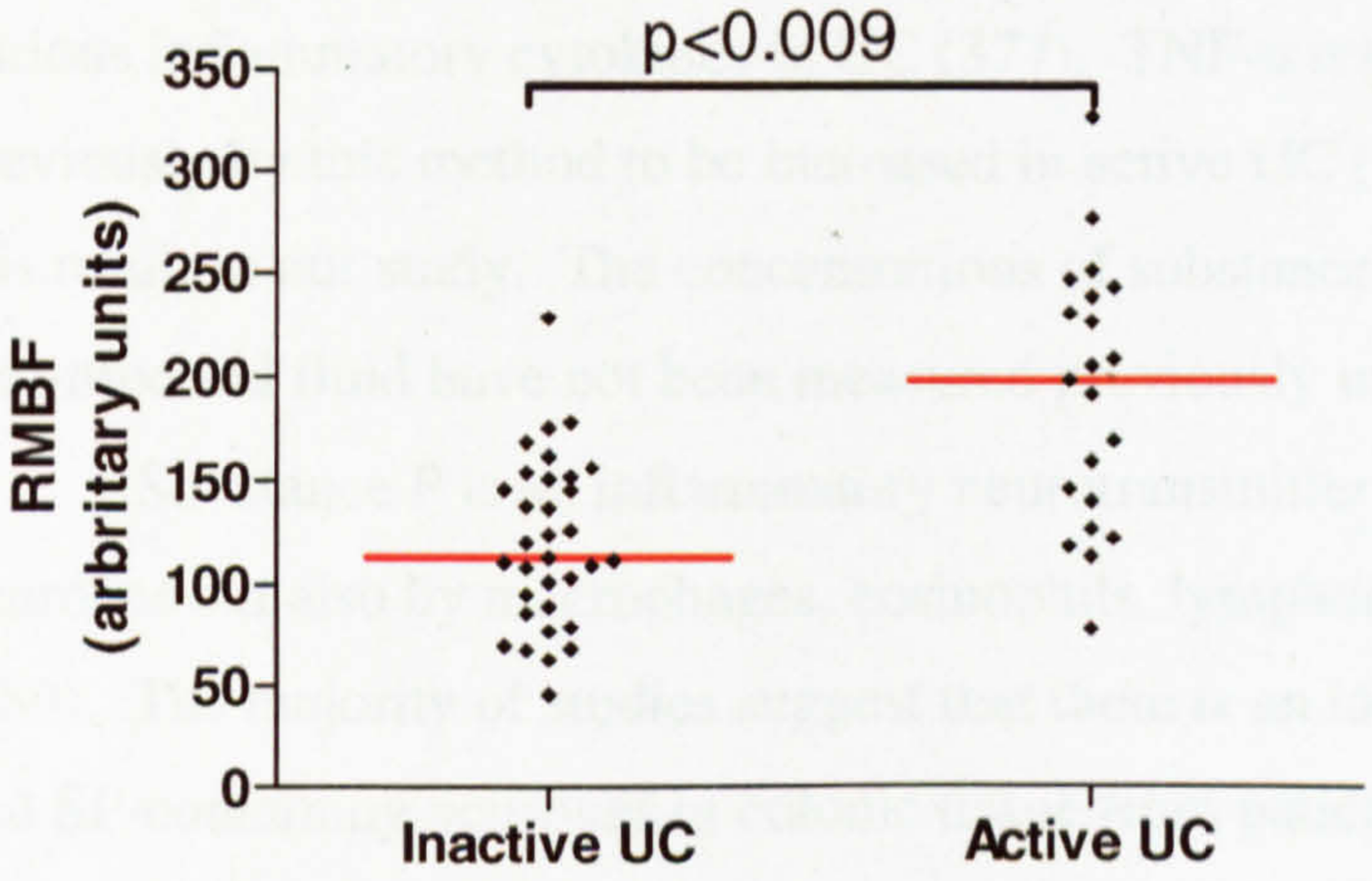


Table 5.1 Substance P (pg/ml), histamine (pg/ml), IL-13 (pg/ml) and TNF-α (pg/ml) concentrations in peri-mucosal fluid, ROM production (photons/mcg/min) and RMBF (arbitrary units) in patients with inactive and active UC.

	Patients with inactive UC	Patients with active UC
Perimucosal fluid cytokines		
· Substance P	22 (4-88)	57 (18-718)*
Histamine	6.3 (0.7-14.4)	21.4 (9.4-33.3)*
IL-13	6.7 (2.1-15.3)	18.7 (5.4-46.9)*
TNF-α	13.1 (8.6-22.2)	111.1 (43.1-241.3)*
ROM Production	53 (20-325)	275 (38-986)*
Blood Flow	113 (88-152)	198 (133-241)*

5.5 DISCUSSION

As with the comparison of systemic inflammatory measures, the rectal inflammatory measures assessed were found to be greater in patients with active UC than inactive UC. Each will be discussed in turn.

RECTAL PERI-MUCOSAL FLUID CYTOKINE LEVELS

The filter paper technique has been used previously to assess the release of various inflammatory cytokines in UC (377). TNF-α release has been shown previously by this method to be increased in active UC (377), and we have replicated this result in our study. The concentrations of substance P, histamine and IL-13 in peri-mucosal fluid have not been measured previously using this technique.

Substance P is an inflammatory neurotransmitter released principally by neurones but also by macrophages, eosinophils, lymphocytes and dendritic cells (189). The majority of studies suggest that there is an increase in SP concentrations and SP-containing neurones in colonic tissue from patients with active IBD (189), although there is not complete agreement. In this study we have shown for the first time that there is increased mucosal release of SP *in vivo* in active UC. The pro-inflammatory effects of stress in animal models of colitis seem dependent on the degranulation of mast cells (278;314;327). The stimulus for mast cell degranulation in response to stress is likely to involve the release of key neurotransmitters from the ENS, and SP is a possible candidate. Indeed as already discussed, incubation with SP of colonic biopsies from patients with IBD increases mast cell-mediated histamine release (190).

As mast cell degranulation appears a key step in mediating stress-related changes in UC (278;314;327), the assessment of a mast cell mediator, histamine, is of particular relevance to this study. Histamine release has been shown to be increased in active UC using the technique of rectal dialysis (123). In this study, we have found that rectal histamine release is increased as assessed by the filter paper technique also.

A mucosal population of IL-13-secreting NKT cells has recently been implicated in the pathogenesis of UC. LPMCs from patients with UC have been

shown to produce more IL-13 in response to stimulation in vitro than LPMCs from healthy controls (427). In this study we have demonstrated that there is increased IL-13 release *in vivo* as assessed by the filter paper technique. There is no data on the role that IL-13 release might play in the stress or relaxation-related changes in disease activity in UC (see Chapter 1).

REACTIVE OXYGEN METABOLITE PRODUCTION BY RECTAL BIOPSIES

Previous work has shown that ROM production by rectal mucosal biopsies is markedly increased in patients with active colitis (181;406). We have replicated this result in our own study. The increased ROM production is thought to derive largely from the presence of acute inflammatory neutrophils in the lamina propria (182). It must be noted, however, that there is large both inter and intra-individual variability with this technique (see Chapter 3). This might affect the sensitivity of this technique for detecting small changes in response to psychological stress and hypnotherapy.

RECTAL MUCOSAL BLOOD FLOW

As discussed in chapter 3, rectal mucosal blood flow in the healthy individual reflects the autonomic tone in the rectum at that time; it increases with increasing parasympathetic tone and decreases with increasing sympathetic tone (407). However, there is some evidence to suggest that rectal mucosal blood flow also increases in response to inflammation. In a study of the effects of mucosal injection of food antigens, RMBF was found to correlate with the development of sub-mucosal oedema and visible erythema (408).

In UC there are mixed reports on the effects of an active colitis on rectal mucosal blood flow. Srivasta et al found RMBF to be increased in quiescent UC compared to healthy controls (104). In contrast, Guslandi found rectal blood flow to be decreased in both active and inactive UC (409). In this study we have found rectal mucosal blood flow to be increased in active UC compared to inactive disease. We also found RMBF to be more variable in active colitis than inactive UC. There was a greater coefficient of variation between the four quadrantic regions in active UC than

in inactive UC (31% vs 17%) and a greater IQR in active disease than inactive disease (133-241 vs 88-152 respectively). This increased variability may explain the discrepancies described between studies on the effects of active and inactive UC on rectal mucosal blood flow. The other factor which may be relevant is that different equipment was used to measure rectal mucosal blood flow in the various published studies.

With regards to measuring RMBF in this study, the effects of each protocol may be complex and depend on two differing effects. If the stress protocol increases sympathetic innervation, as would be expected (137), then acting on autonomic function alone stress would be expected to decrease RMBF. However, if stress has a pro-inflammatory effect and, as this comparison suggests, RMBF is increased by active colitis, then RMBF should be increased. The opposite should apply to hypnosis which might be expected to increase rectal parasympathetic tone and have a possible anti-inflammatory effect. The results we obtained are described in Chapters 7 and 9.

5.6 CONCLUSION

The rectal mucosal release of SP, histamine, IL-13 and TNF-α, as assessed by the filter paper method, were all more than doubled in patients with active UC compared to inactive disease. ROM was over five times greater in active UC and RMBF more than 50% greater in active compared to inactive disease. These findings support the use of these variables as inflammatory measures in this study.

CHAPTER 6

THE SYSTEMIC RESPONSE TO STRESS IN HEALTHY VOLUNTEERS AND PATIENTS WITH INACTIVE ULCERATIVE COLITIS

6.1 SUMMARY

Aims: As per protocol (Chapter 3), to assess the effects of acute psychological stress at the systemic autonomic and inflammatory level.

Methods: As described in Chapter 3, patients with inactive UC and healthy volunteers underwent the stress and control protocols. Autonomic and systemic inflammatory measures were assessed before and after each protocol in both groups.

Results: The following results were found:

1. Subjects found the stress protocol subjectively stressful, with an increase of 2.5 units on the visual analogue scale (VAS) in patients with UC ($p=0.003$) and 2.5 units in healthy volunteers (HVs) ($p=0.008$).
2. The stress test induced an autonomic response in both patients with UC and HVs. In patients with UC, stress increased pulse rate by 7 bpm ($p=0.0002$), systolic BP by 12mmHg ($p<0.0001$) and diastolic BP by 7mmHg ($p<0.0001$). In HVs stress increased pulse by 11bpm ($p=0.02$) and systolic BP by 9 mmHg ($p=0.03$).
3. Stress increased LPS-stimulated TNF-α and IL-6 production by 54% ($p=0.004$) and 11% ($p=0.04$) respectively in patients with UC, and TNF-α production by 94% in HVs ($p=0.03$).
4. Leukocyte count increased by 16% in patients with UC ($p=0.01$) and by 17% in HVs ($p=0.04$) in response to stress.
5. NK cell count increased by 18% ($p=0.008$) in patients with UC in response to stress.
6. Stress increased platelet activation by 65% in patients with UC ($p<0.0001$) and by 64% in HVs ($p=0.001$). PLA formation was increased by 25% in patients with UC ($p=0.004$) and by 6% in HVs ($p=0.03$) in response to stress.
7. There were no differences between the responses of the patients with UC and HV.
8. The control protocol did not change any of the variables measured in either the patients with UC or healthy volunteers.
9. Chronic stress, as assessed by psychometric questionnaires, did not affect the autonomic or inflammatory response of patients with UC or HVs to the acute stress test.

Conclusion: It is possible that some of these systemic pro-inflammatory changes may contribute to the adverse effects of stress in UC.

6.2 INTRODUCTION

As described in Chapter 1, adverse life-events and chronic psychological stress have been found to be associated with an increased incidence of disease relapse in patients with quiescent UC (270;273). Acute stress has been shown to contribute to the activation and reactivation of experimental colitis in animal models (299;300). There have, however, been few studies which have examined the effects of stress on inflammation in patients with IBD (279). The mechanisms by which stress might worsen UC remain unknown.

In this chapter we will describe the systemic responses of both patients with quiescent UC and healthy volunteers to the stress protocol described in chapter 3. The hypothesis which underlies this work is that stress might lead to an increased rate of relapse in UC via its pro-inflammatory effects on various systemic inflammatory measures.

The inflammatory response to stress was subsequently related to psychometric assessments of chronic perceived stress levels of each of the participants.

For ease of reading the mucosal responses of patients with UC to stress will be described separately in Chapter 7.

6.3 DEMOGRAPHICS OF PATIENTS AND HEALTHY VOLUNTEERS

25 patients with inactive UC, as defined by a Baron's score of less than 2, and 11 healthy volunteers underwent the stress protocol. 10 patients with quiescent UC and 11 healthy volunteers underwent the control protocol.

Table 6.1 Sex, age, disease extent, treatment, Baron's score and Simple Colitis Activity Index (SCCAI) for patients with inactive UC and healthy volunteers undergoing the stress and control protocols.

Protocol	Stress	Control
UC (n=25)		**UC** (n=10)
Age	44 (28-64)	52 (23-65)
Sex	13 Male	3 male
Disease extent	48% total	30% total
	20% left-sided	30% left-sided
	32% distal	40% distal
Treatment	76% on 5-ASA	90% 5-ASA
	12% thiopurines	10% thiopurines
	4% methotrexate	0% corticosteroids
	0% corticosteroids	
Baron's score	0 (0-1)	0 (0-1)
SSCAI	1 (0-2)	1 (0-2)
HV (n=11)		**HV** (n=11)
Age	27 (23-56)	36 (27-57)
Sex	4 male	6 male

6.4 RESULTS

6.4.1 SUBJECTIVE RESPONSE TO STRESS PROTOCOL (Figure 6.1 and 6.2)

As assessed by visual analogue scale (VAS), patients with UC and healthy volunteers (HV) rated the stress protocol as stressful with a median increase of 2.5 units in UC (3(1-5) vs. 5.5(4-8), $p<0.0001$) and 2.5 units in HV (2(2-3) vs. 4.5 (3.5-6.0), $p=0.008$). The one patient with UC and the two HV who scored the stress procedure non-stressful on VAS showed no autonomic response or inflammatory response in the majority of variables measured. These three subjects were included in the analysis of changes elicited in response to the stress protocol.

Figure 6.1 Effects of stress protocol on visual analogue scale (VAS) stress score in patients with inactive UC (n=25). Median is shown in red.

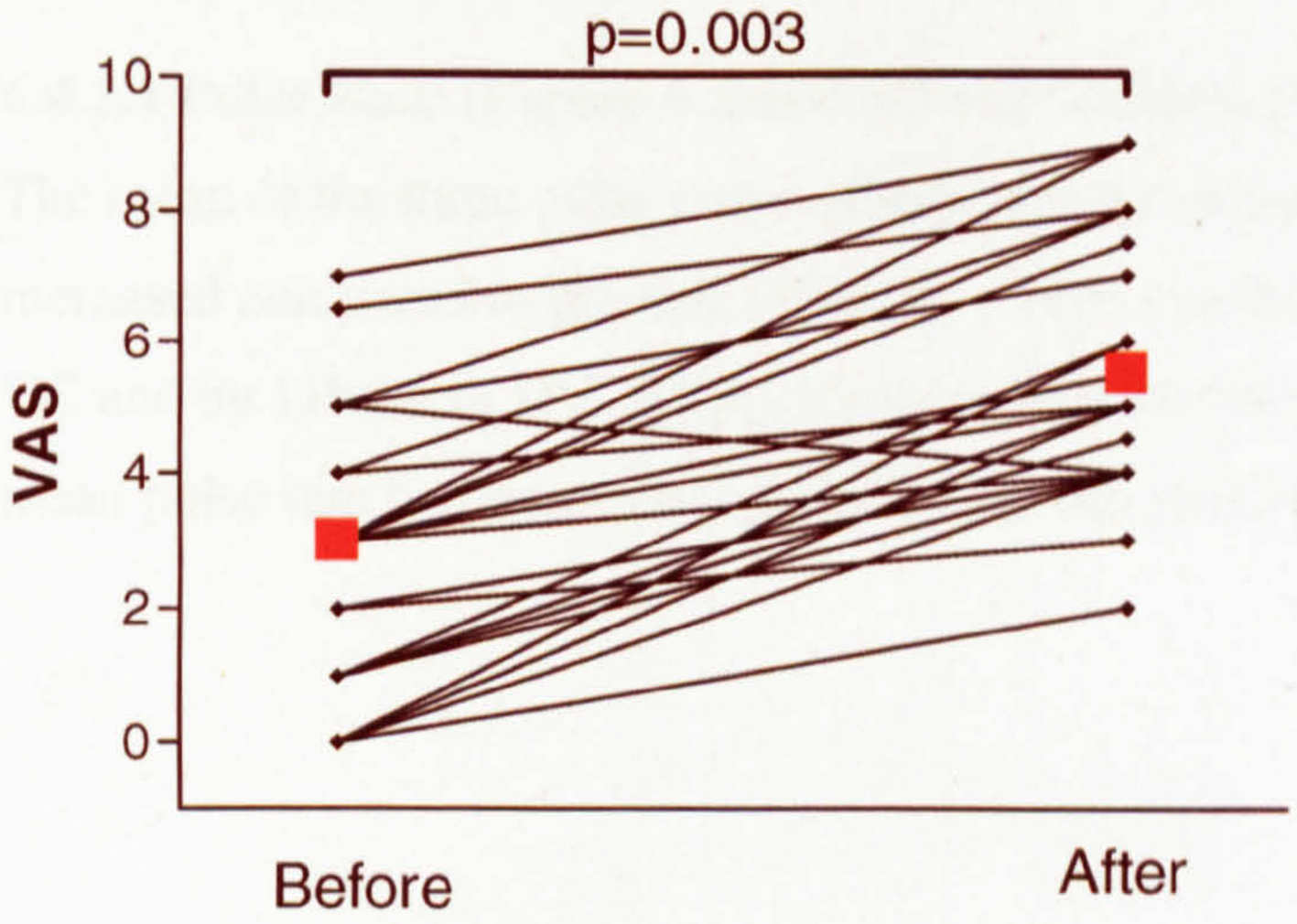
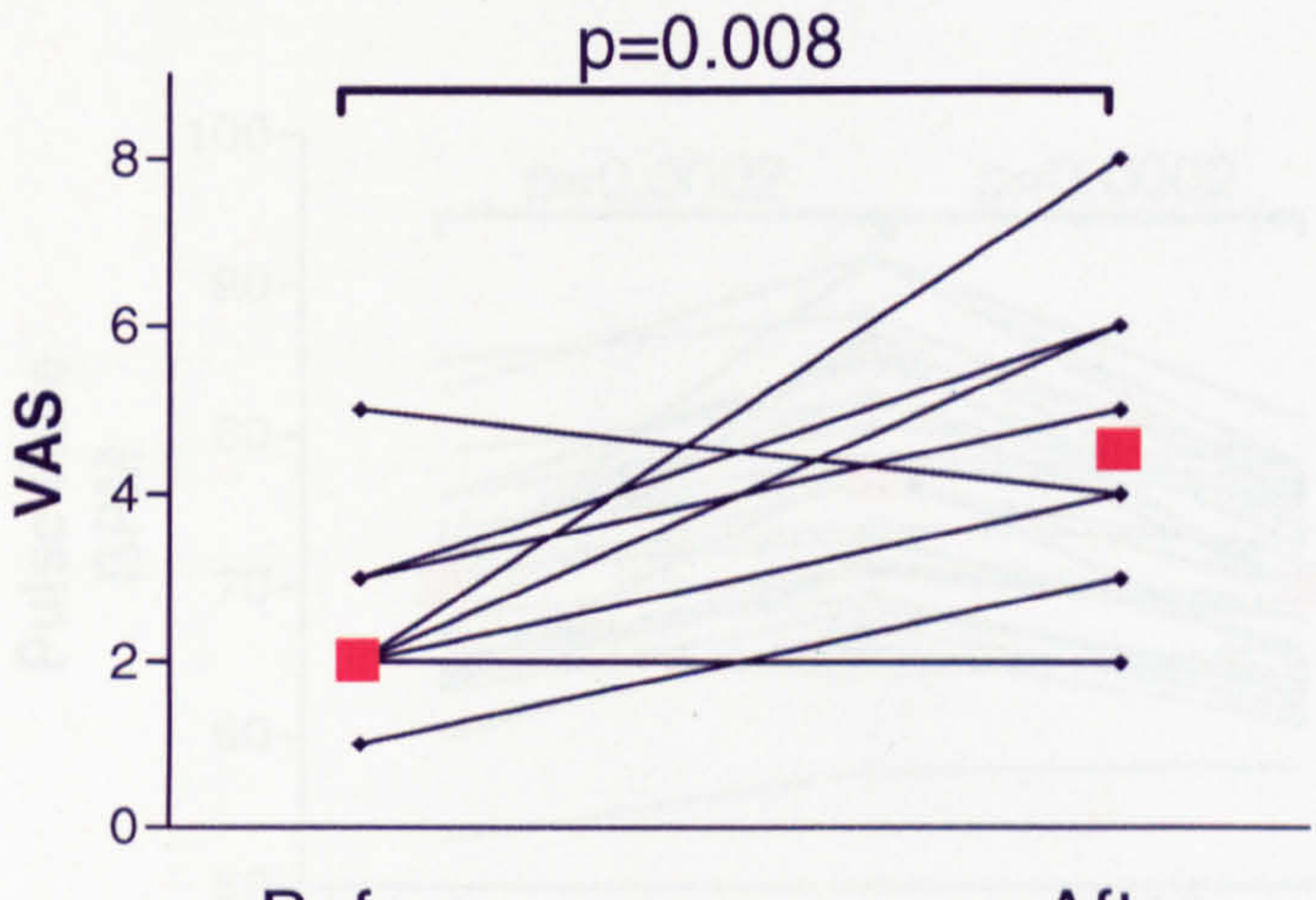


Figure 6.2 Effects of stress protocol on visual analogue score (VAS) stress score in healthy volunteers (n=11). Median is shown in red.



6.4.2 AUTONOMIC RESPONSE

6.4.2.1 Pulse Rate (Figure 6.2 and 6.3 and Table 6.2)

The mean of the three pulse rate readings measured during the stress protocol was increased compared to pre-test values by 7 bpm (median) in patients with quiescent UC and by 11bpm in HV. Thirty minutes after the end of the stress protocol, the mean pulse rate had returned to baseline in both groups.

Figure 6.3 Effects of stress protocol on pulse rate (bpm) in patients with inactive UC (n=25). The points shown as "During" are the mean of the three readings taken during the stress test. Median is shown in red.

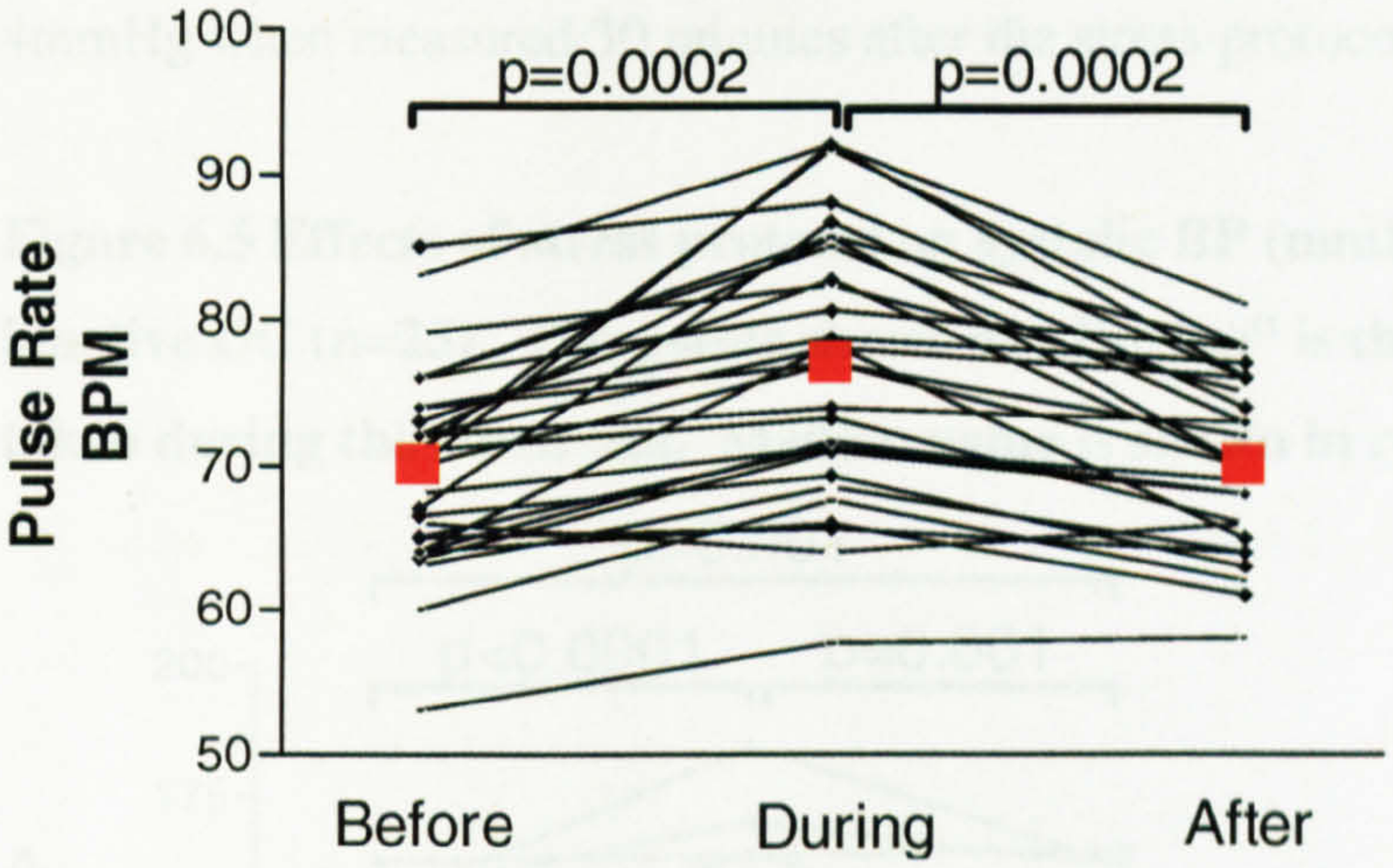
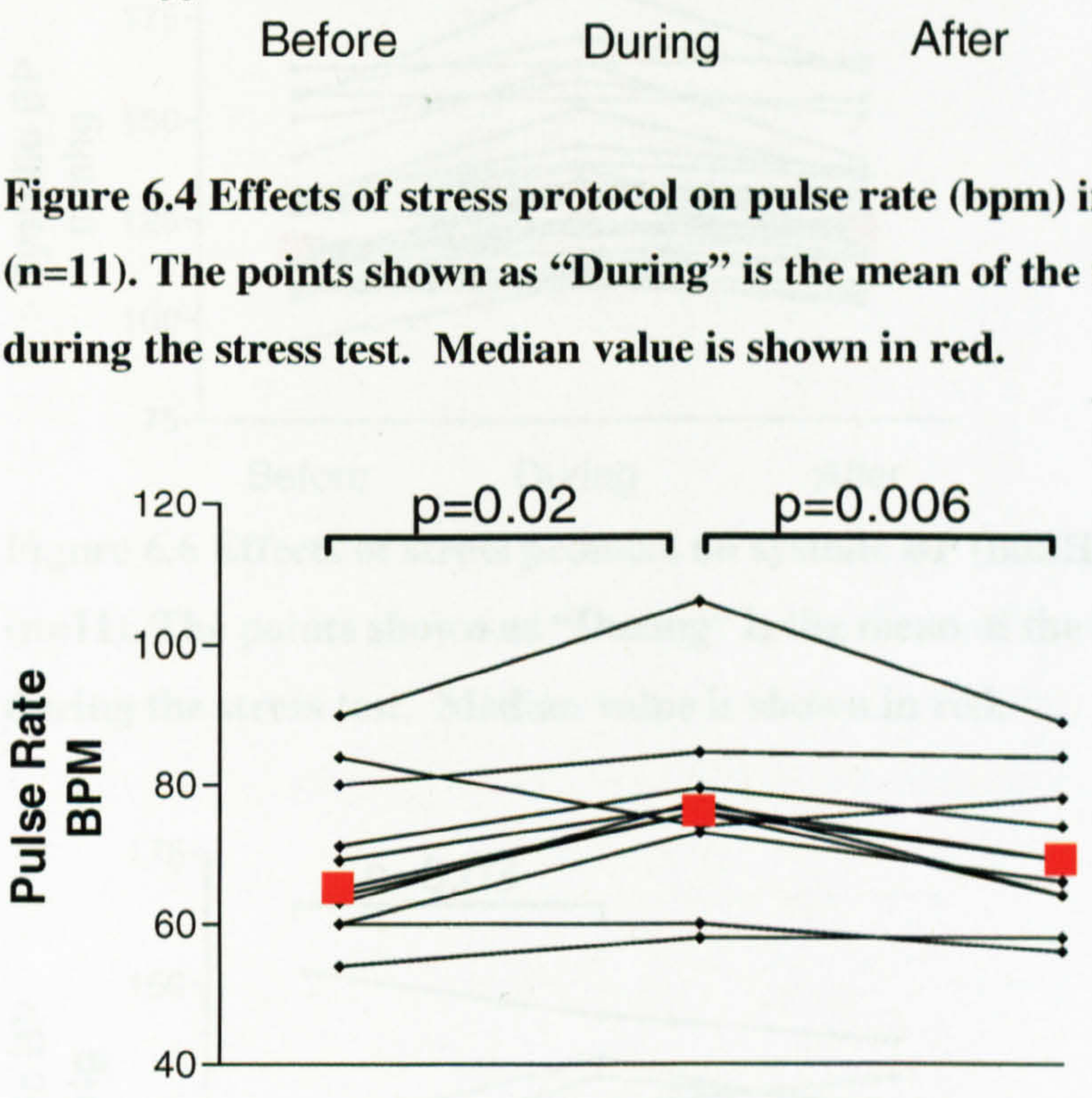


Figure 6.4 Effects of stress protocol on pulse rate (bpm) in healthy volunteers (n=11). The points shown as "During" is the mean of the three readings taken during the stress test. Median value is shown in red.



6.4.4.2 Systolic Blood Pressure (Figure 6.5 and 6.6 and Table 6.2)

Mean systolic blood pressure increased during the stress protocol by a median of 12 mmHg in patients with UC and by 9 mmHg in HV. In patients with UC, the systolic BP fell, but remained elevated compared to pre-test levels, by median 4mmHg when measured 30 minutes after the stress protocol.

Figure 6.5 Effects of stress protocol on systolic BP (mmHg) in patients with inactive UC (n=25). The points shown as "During" is the mean of three readings taken during the stress test. Median value is shown in red.

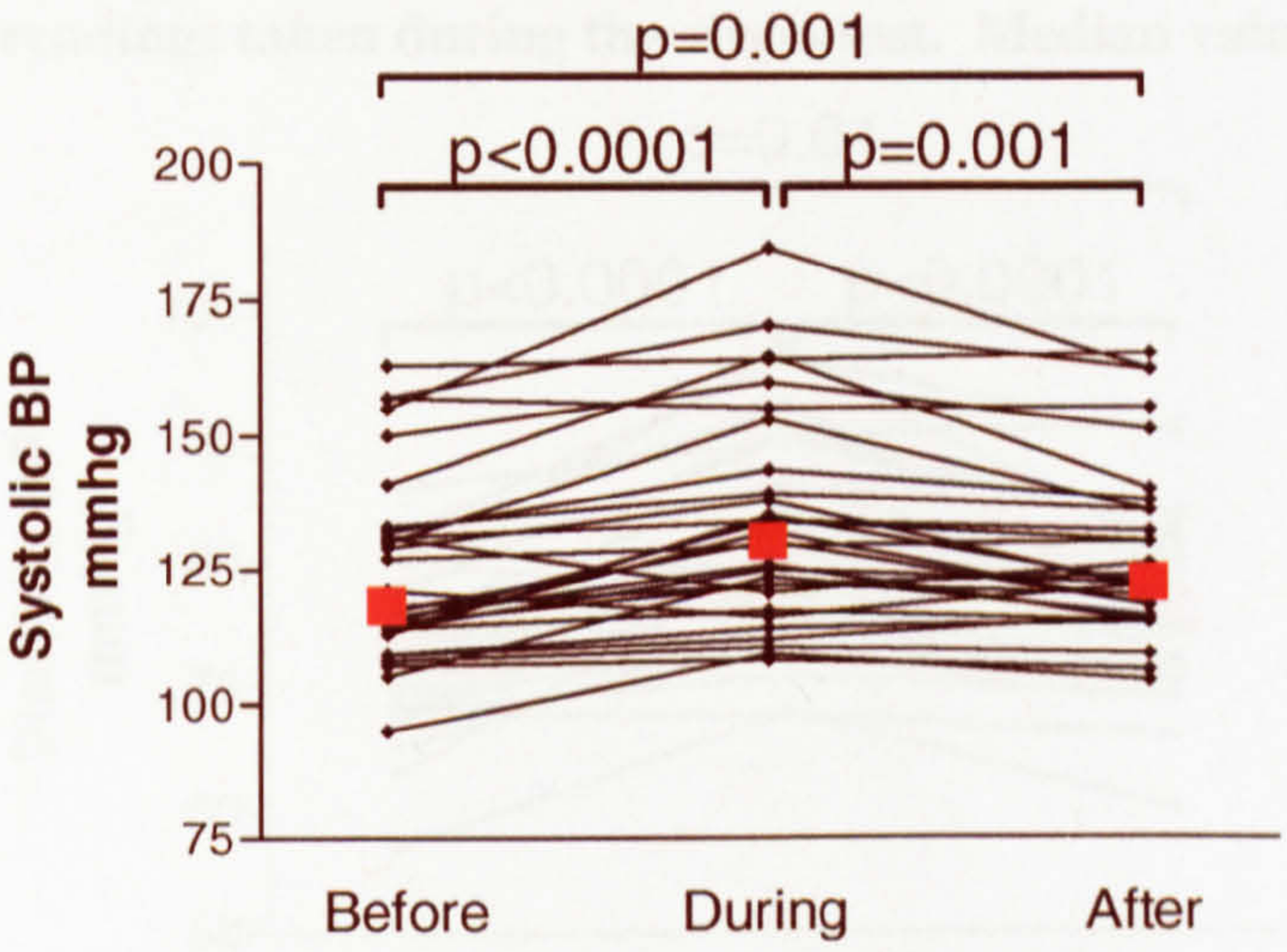
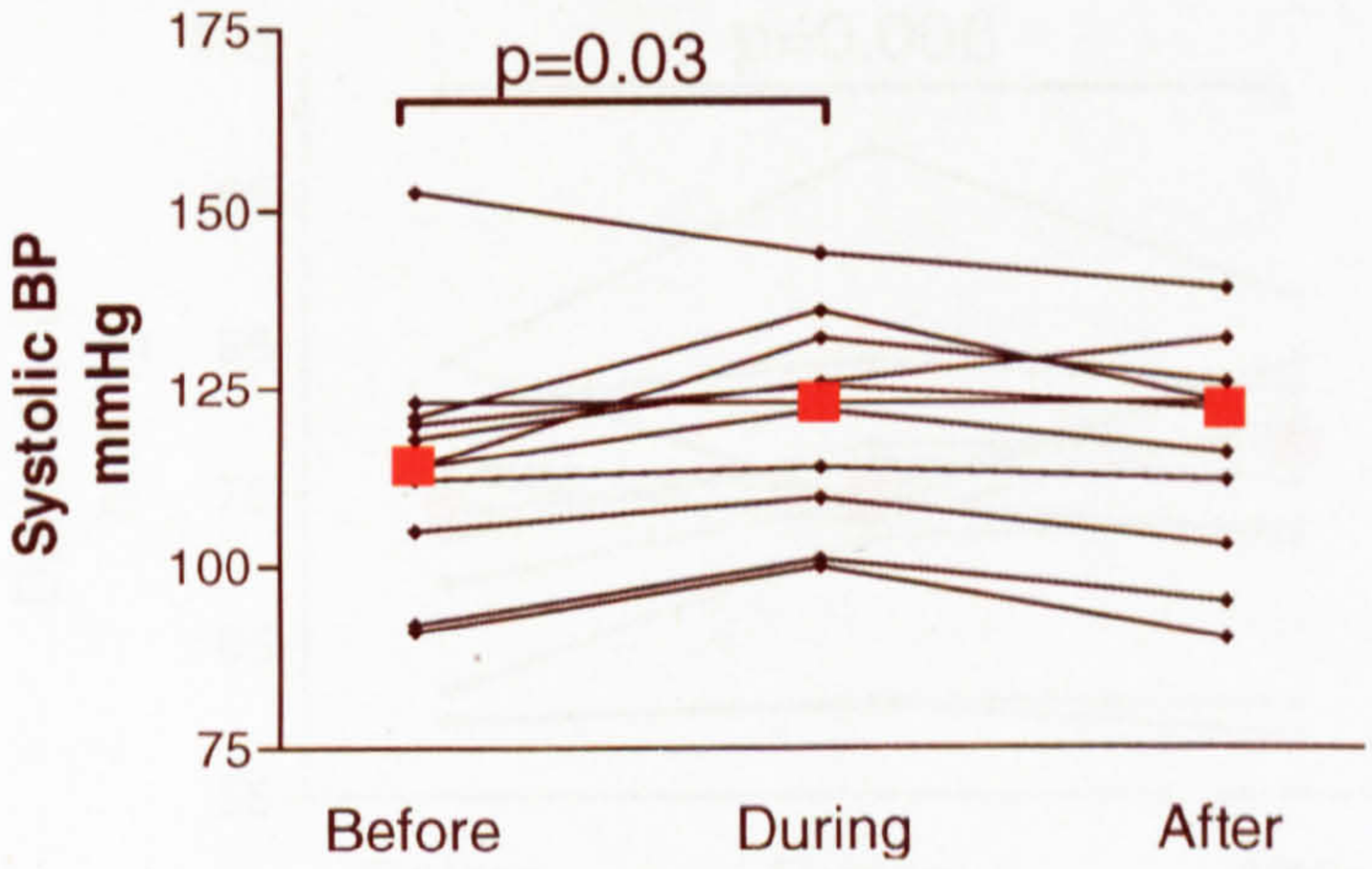


Figure 6.6 Effects of stress protocol on systolic BP (mmHg) in healthy volunteers (n=11). The points shown as "During" is the mean of the three reading taken during the stress test. Median value is shown in red.



6.4.2.3 Diastolic Blood Pressure (Figure 7.7 and 6.8 and Table 6.2)

In patients with UC, mean diastolic BP increased by 7mmHg during stress ($p<0.0001$). As with systolic BP, in patients with UC the diastolic BP fell when measured 30 minutes later, but remained elevated by median 4mmHg compared to baseline ($p=0.01$). Diastolic BP was also elevated in HV 30 minutes after the stress protocol ($p=0.006$).

Figure 6.7 Effects of stress protocol on diastolic BP (mmHg) in patients with inactive UC (n=25). The points shown as "During" is the mean of the three readings taken during the stress test. Median value is shown in red.

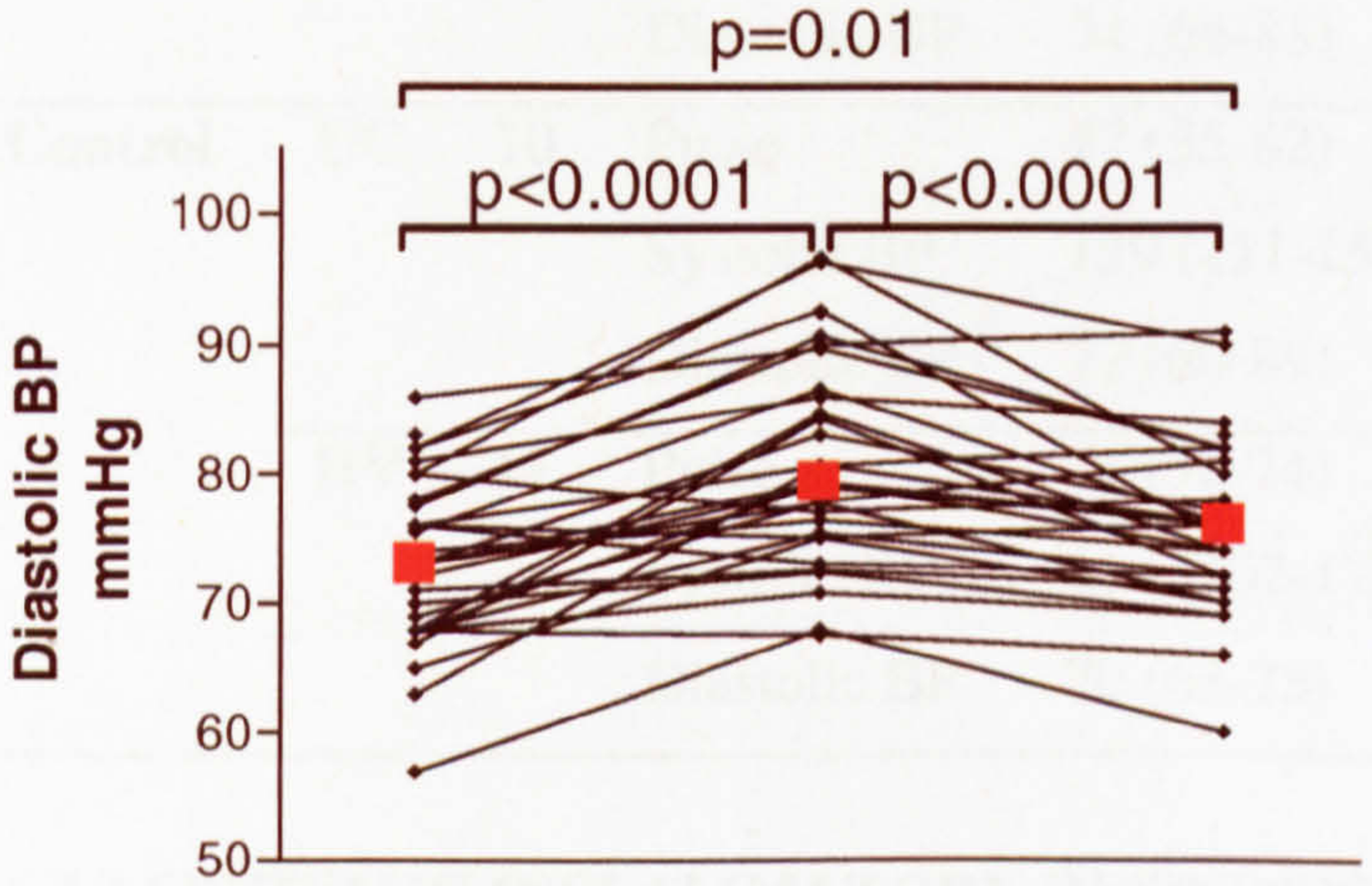


Figure 6.8 Effects of stress protocol on diastolic BP (mmHg) in healthy volunteers (n=11). The points shown as "During" is the mean of the three readings taken during the stress test. Median value is shown in red.

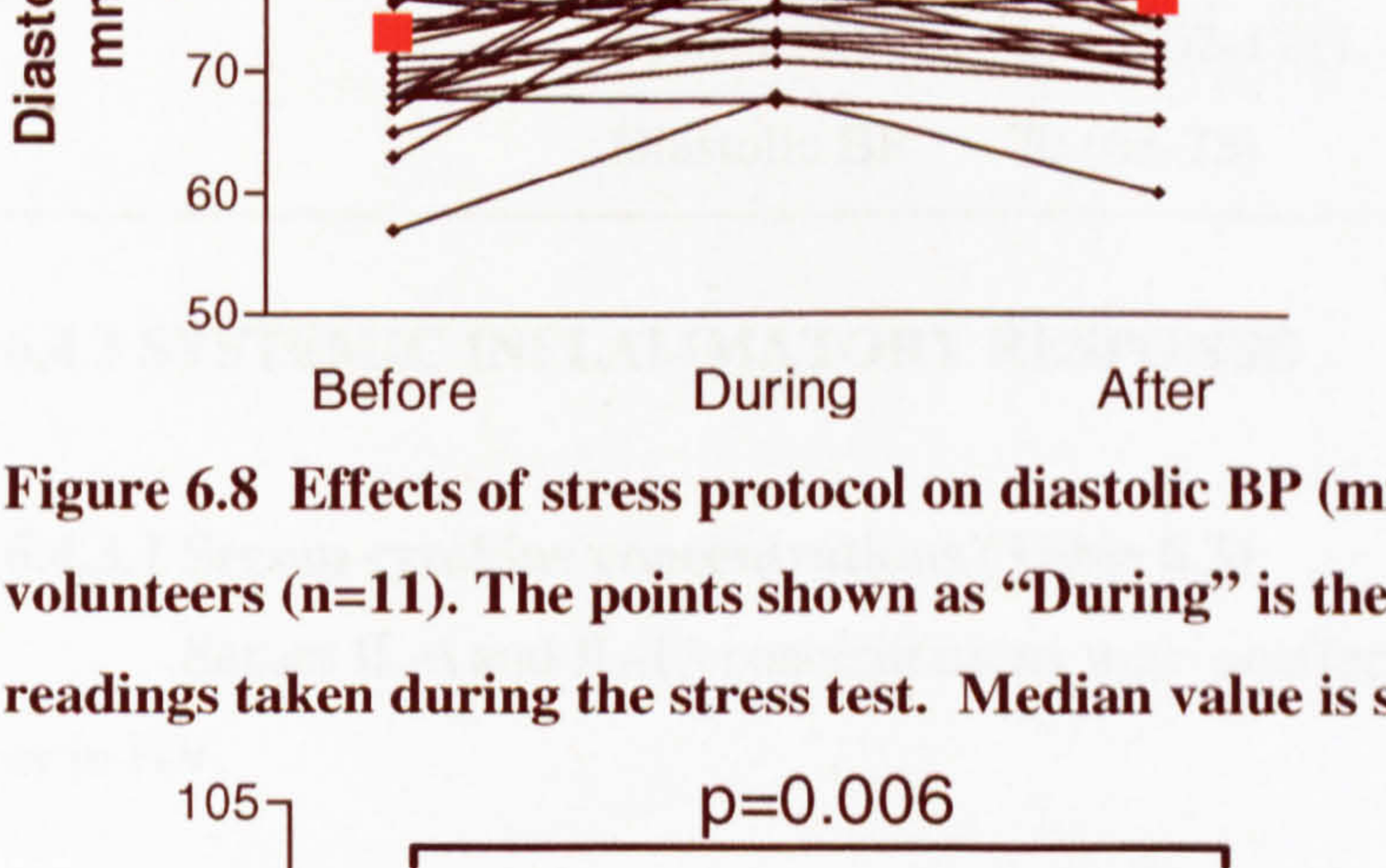


Table 6.2 Pulse (bpm), systolic and diastolic BP (mmHg) in response to stress and control protocols in patients with UC and healthy volunteers (HV). * p<0.05 from pre-procedure value.

Protocol		N		Before	During/After	30 mins after
Stress	UC	25	Pulse	70 (65-74)	77 (70-85)*	70 (66-76)
			Systolic BP	118 (113-133)	130 (119-148)*	122 (116-137)*
			Diastolic BP	72 (68-79)	79 (75-88)*	76 (72-82)*
	HV	11	Pulse	65 (62-82)	76 (74-82)*	69 (64-81)
			Systolic BP	114 (109-122)	123 (112-134)*	122 (108-129)
			Diastolic BP	74 (66-83)	75 (73-82)	78 (73-84)*
Control	UC	10	Pulse	67 (55-82)	66 (56-79)	67 (57-78)
			Systolic BP	139 (111-158)	130 (108-150)	133 (113-150)
			Diastolic BP	77 (60-89)	75 (61-88)	76 (60-87)
	HV	11	Pulse	71 (56-74)	66 (61-74)	68 (60-73)
			Systolic BP	114 (102-121)	110 (102-122)	112 (104-124)
			Diastolic BP	70 (63-75)	69 (62-75)	72 (63-77)

6.4.3 SYSTEMIC INFLAMMATORY RESPONSE

6.4.3.1 Serum cytokine concentrations (Table 6.3)

Serum IL-6 and IL-13 concentrations were unaffected by stress in either UC or in HV.

6.4.3.2 LPS-stimulated cytokine production (Figure 6.9, 6.10 and 6.11 and Table 6.3)

The production of TNF-α by LPS-stimulated whole blood was increased in the sample taken 30 minutes after stress by over 50% in patients with UC (p=0.004) and nearly doubled in HVs (p=0.03). Median production of IL-6 by LPS-stimulated whole blood rose in the sample taken 30 minutes after the stress protocol by 11% in patients with UC (p=0.04).

Figure 6.9 The effects of stress on TNF-α LPS stimulated production by whole blood from patients with ulcerative colitis (n=25). Median value is shown in red.

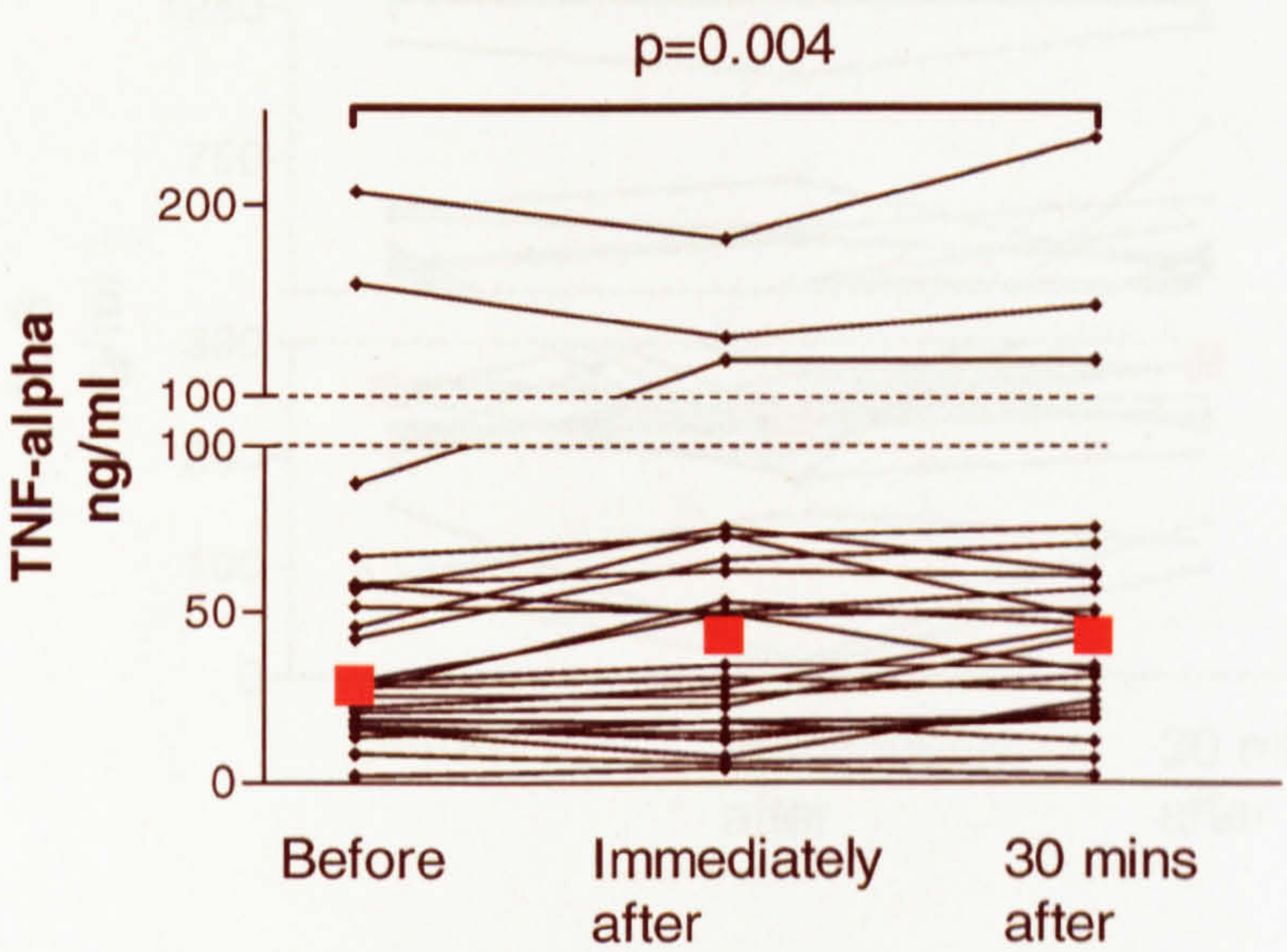


Figure 6.10 The effects of stress on LPS stimulated TNF-α production by blood from healthy volunteers (n=11). Median value is shown in red.

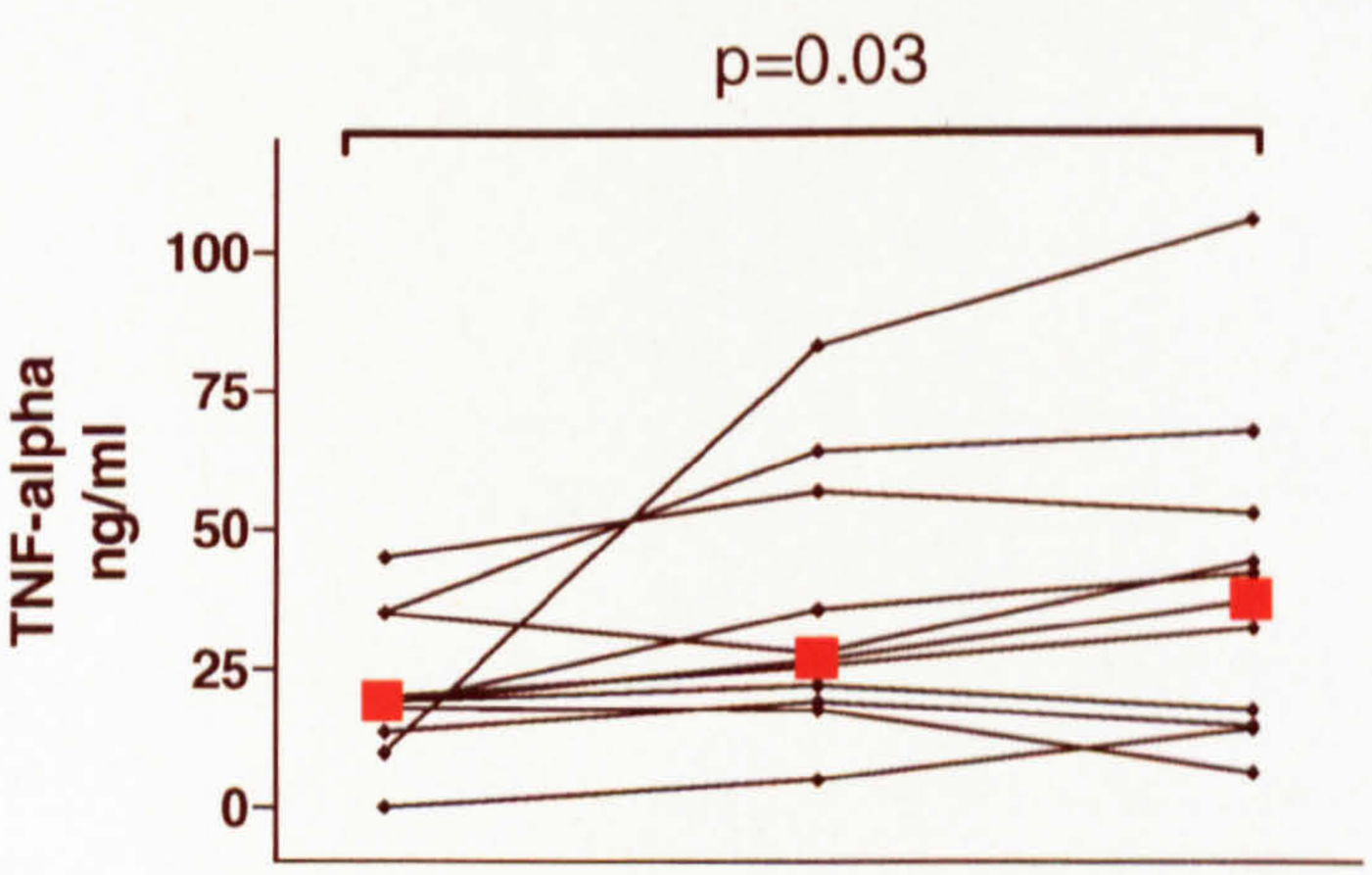


Figure 6.11 The effects of stress on IL-6 production by LPS-stimulated whole blood in patients with UC (n=25). Median value is shown in red.

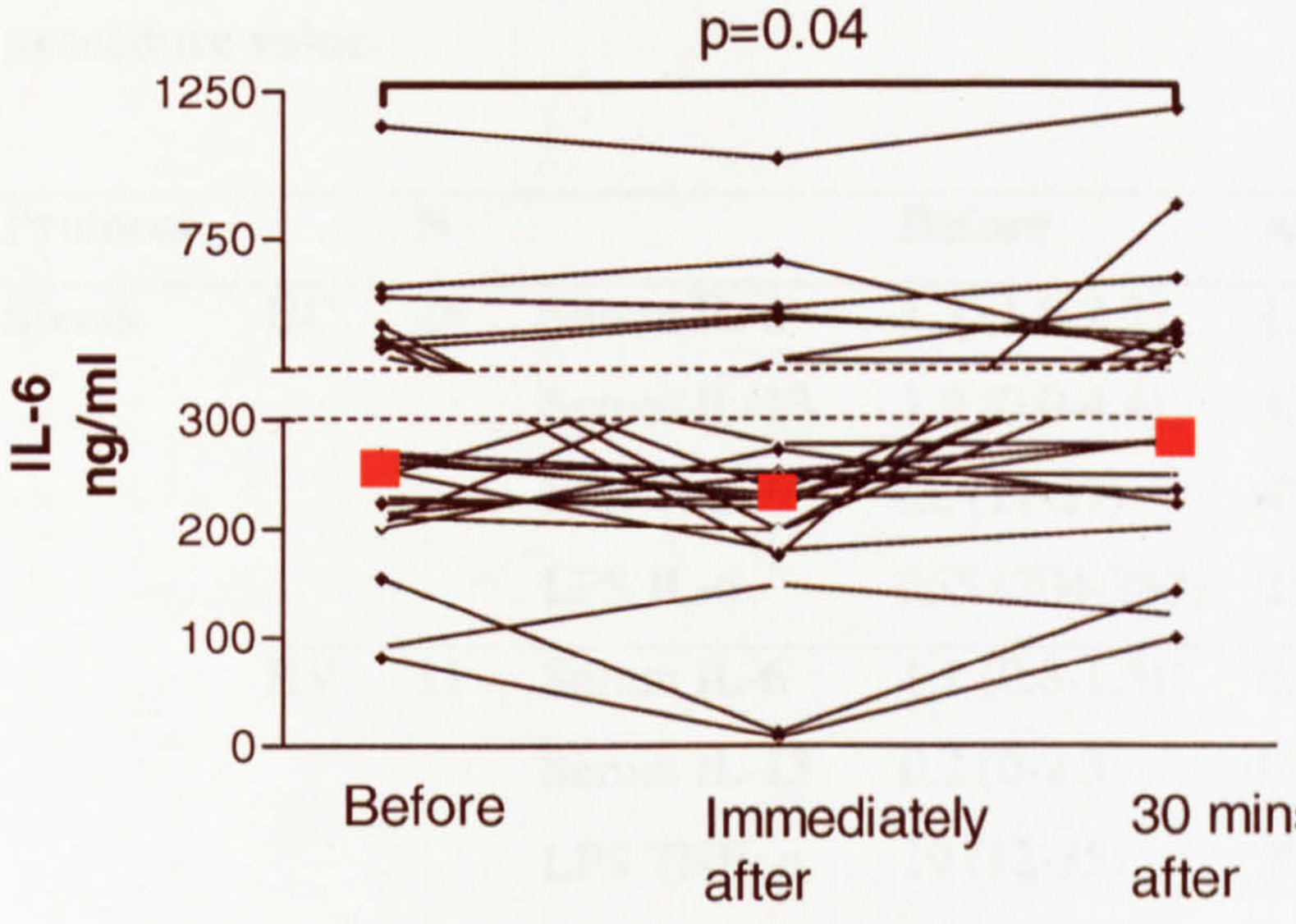


Table 6.3 Serum IL-6 and IL-13 concentrations (pg/ml), and LPS-stimulated IL-6 and TNF- production (ng/ml) by whole blood, in response to stress and control protocols in patients with UC and healthy volunteers (HV). * p<0.05 from pre-procedure value.

Protocol		N		Before	After	30 mins after
Stress	UC	25	Serum IL-6	1.3 (1.0-2.2)	1.4 (1.0-2.2)	1.5 (1.0-1.9)
			Serum IL-13	1.9 (0.0-4.4)	1.7 (0.0-4.5)	1.7 (0.1-4.8)
			LPS TNF-α	28 (17-57)	43 (17-69)	43 (20-62)*
			LPS IL-6	255 (204-387)	232 (179-306)	284 (214-411)*
	HV	11	Serum IL-6	1.1 (0.8-1.5)	0.84 (0.5-1.3)	1.2 (0.6-1.4)
			Serum IL-13	0.2 (0-2.3)	0.4 (0-3.4)	0.8 (0-2.5)
			LPS TNF- α	19 (12-35)	27 (19-60)	37 (14-60)*
			LPS IL-6	212 (156-293)	220 (187-305)	245 (211-382)
Control	UC	10	Serum IL-6	2.8 (2.4-4.4)	2.6 (2.4-3.4)	2.7 (2.1-3.7)
			Serum IL-13	1.3 (0.8-2.2)	1.2 (0.9-2.3)	1.6 (0.9-2.1)
			LPS TNF- α	18 (13-59)	19 (12-60)	20 (16-72)
			LPS IL-6	223 (169-537)	218 (143-427)	254 (173-527)
	HV	11	Serum IL-6	0.6 (0.5-0.9)	0.6 (0.4-1.3)	0.7 (0.4-1.5)
			Serum IL-13	0.8 (0-2.1)	1.0 (0-2.4)	0.7 (0-1.8)
			LPS TNF- α	18 (8-30)	19 (11-26)	20 (11-32)
			LPS IL-6	223 (164-302)	245 (203-345)	232 (195-315)

6.4.3.3 Leukocyte Count (Figure 6.12 and 6.13 and Table 6.4)

Total white cell count (WBC) increased compared to baseline in the sample taken 30 minutes after stress by 16% (median) in patients with UC (p=0.01) and 17% in HV (p=0.04).

Figure 6.12 The effects of stress on leukocyte count in patients with inactive UC (n=25). Median value shown in red.

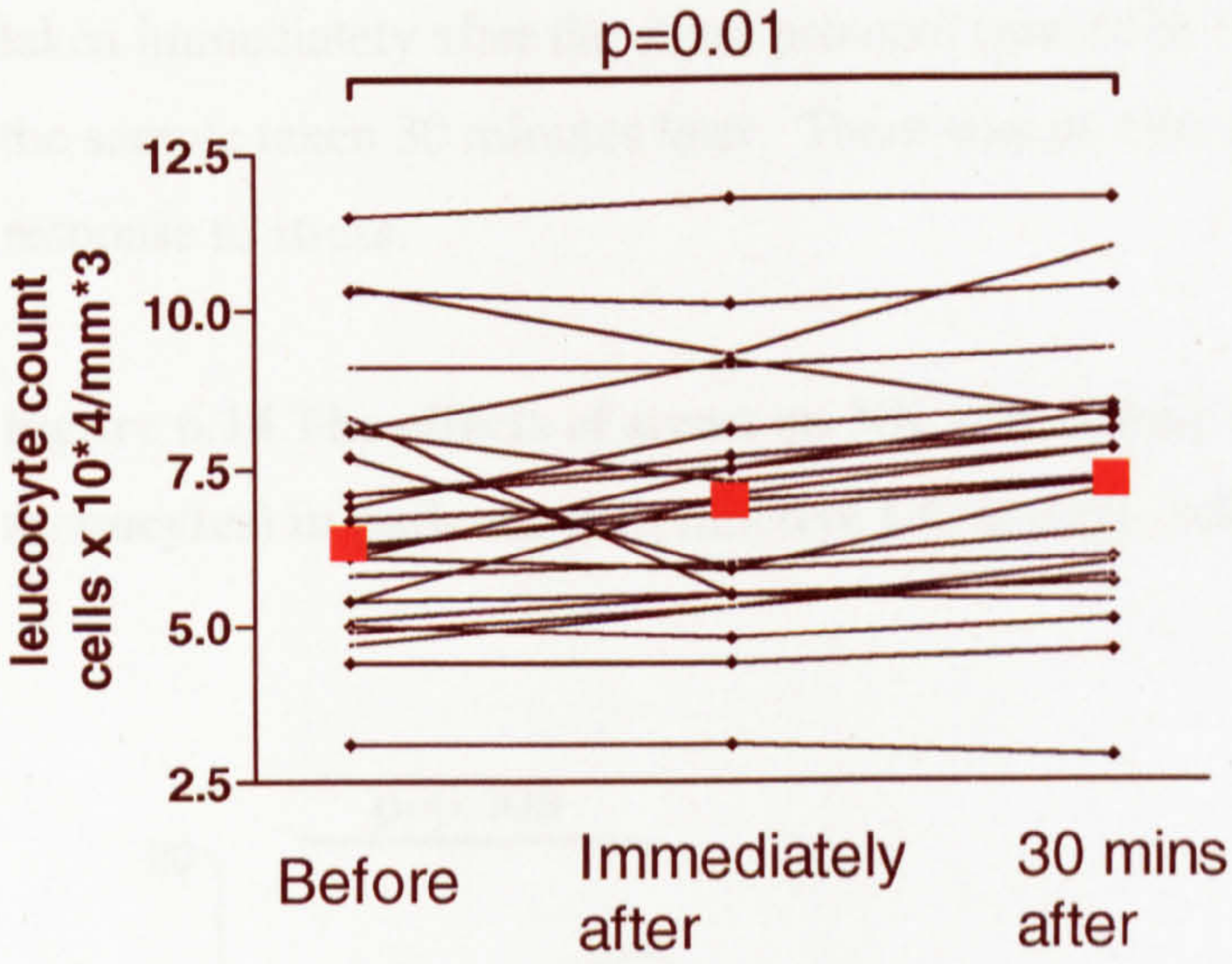
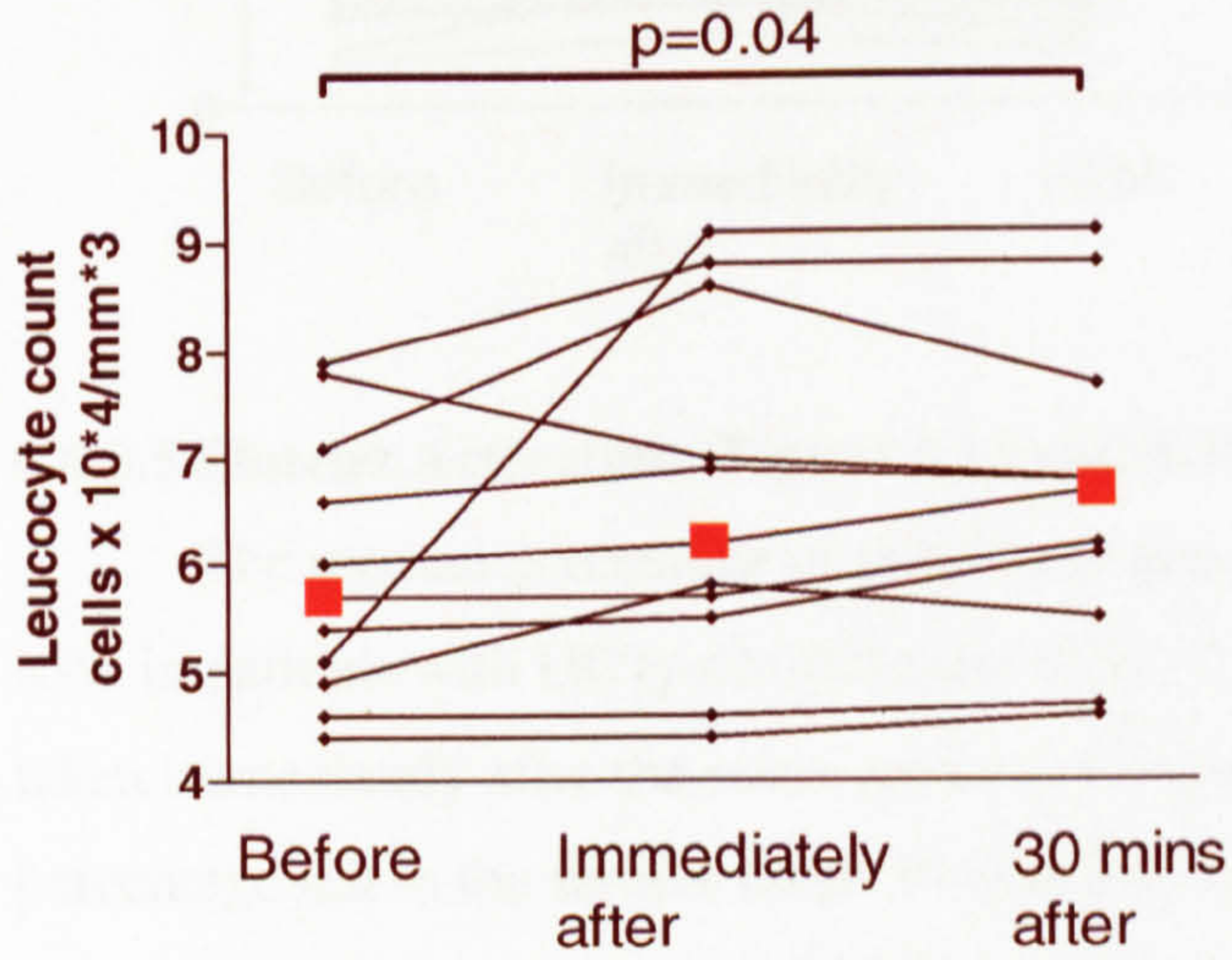


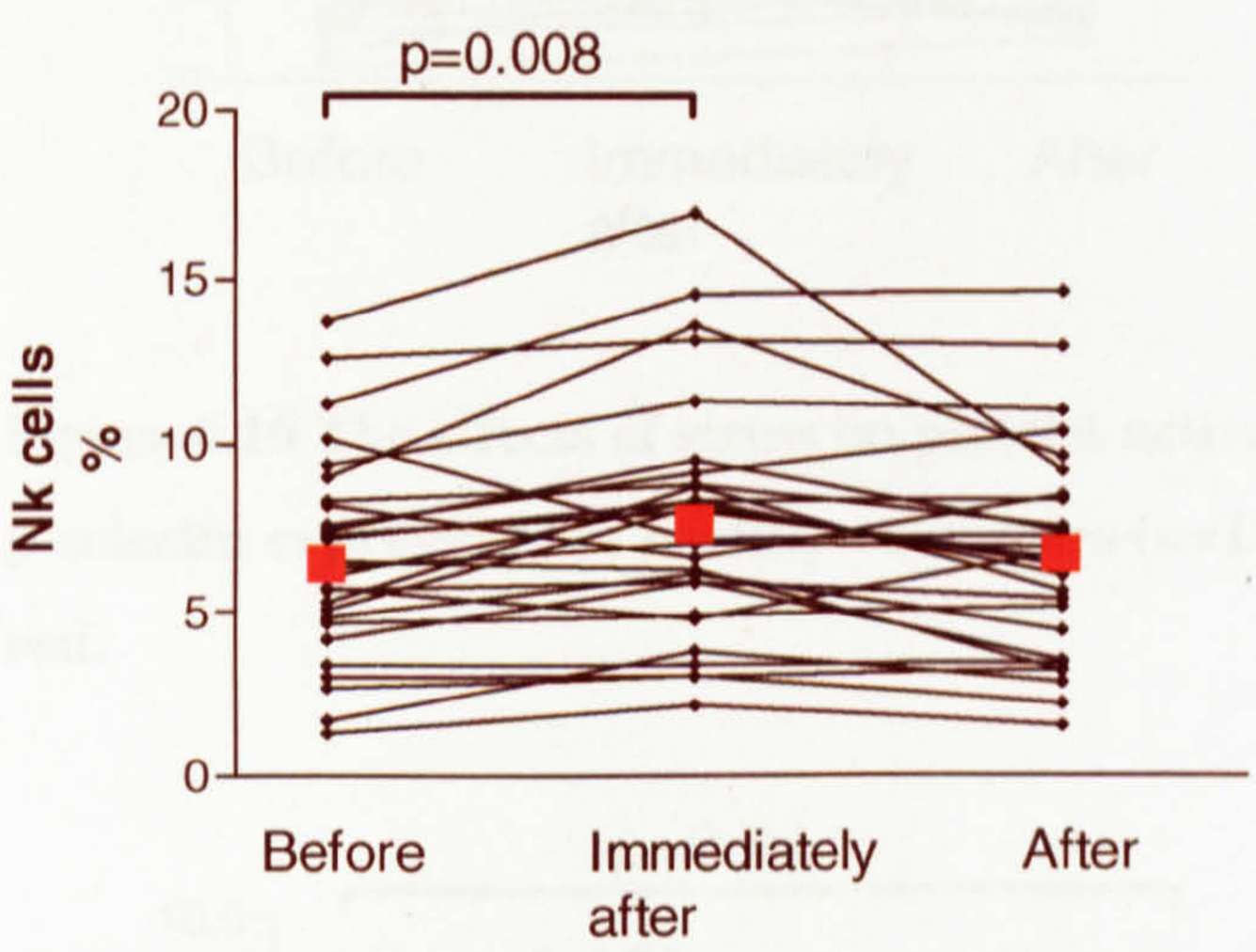
Figure 6.13 The effects of stress on leukocyte count in healthy volunteers (n=11). Median value is shown in red.



6.4.3.4 Natural Killer (NK) cell numbers (Figure 6.14 and Table 6.4)

In patients with quiescent UC, median NK cell numbers, expressed as a percentage of lymphocytes and monocytes, were increased by 18% in the sample taken immediately after the stress protocol (p=0.008) but had returned to base line in the sample taken 30 minutes later. There was no change in NK cell count in HVs in response to stress.

Figure 6.14 The effects of stress on NK cell count (% of lymphocytes and monocytes) in patients with inactive UC (n=25). Median value is shown in red.



6.4.3.5 Platelet Activation (Figure 6.15 and 6.16 and Table 6.4)

The median percentage of platelets expressing p-selectin was increased by 65% in patients with UC (p<0.0001) and by 61% in HV (p=0.04) in the blood sample taken immediately after the stress protocol compared to the pre-test sample. This percentage fell in the sample taken 30 minutes later but remained elevated compared to baseline (29% in patients with UC (p=0.001) and 22% in HV (p=0.001)).

Figure 6.15 The effects of stress on platelet activation (p-selection expression (% of platelets positive for p-selectin)) in patients with inactive UC (n=25). Median value is shown in red.

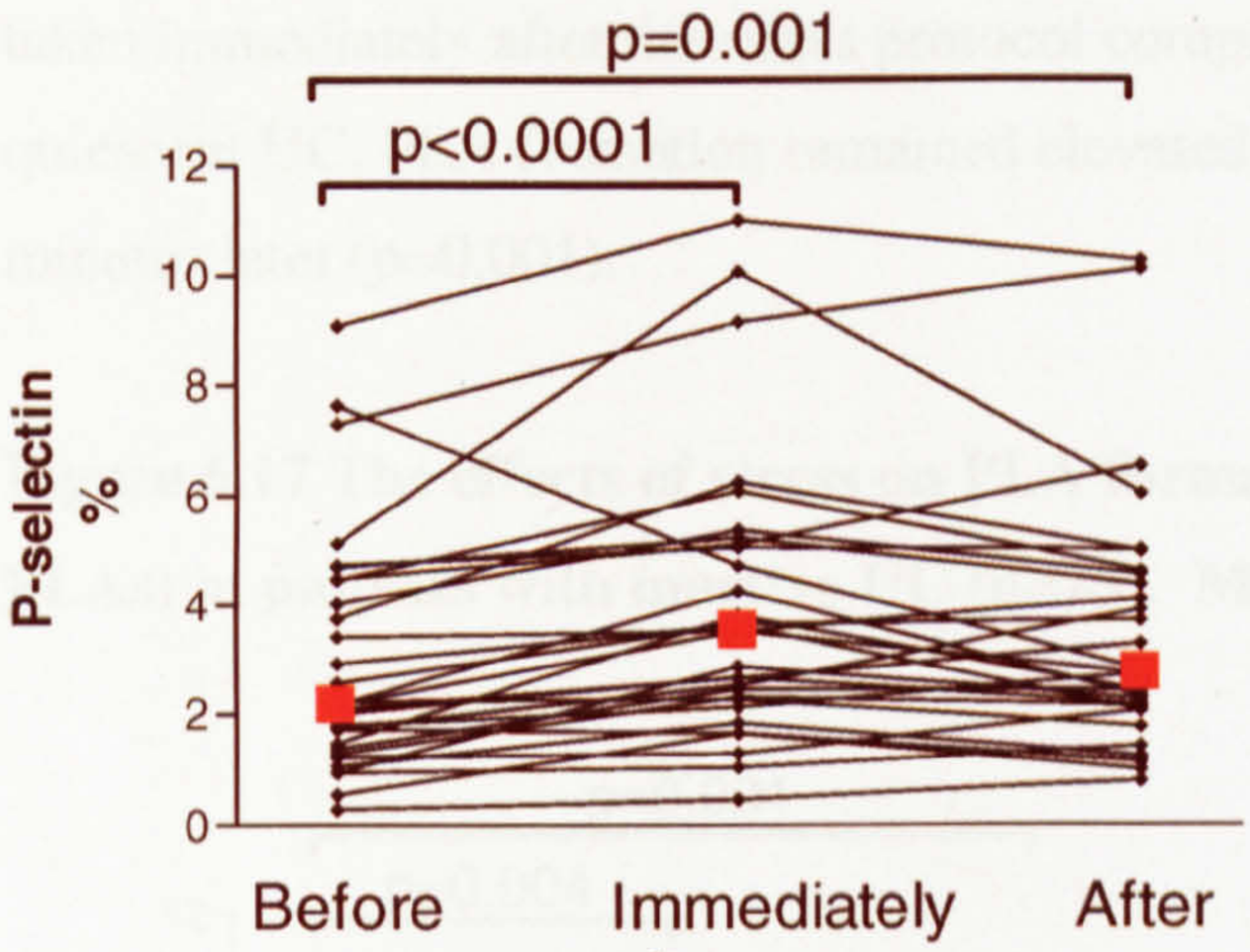
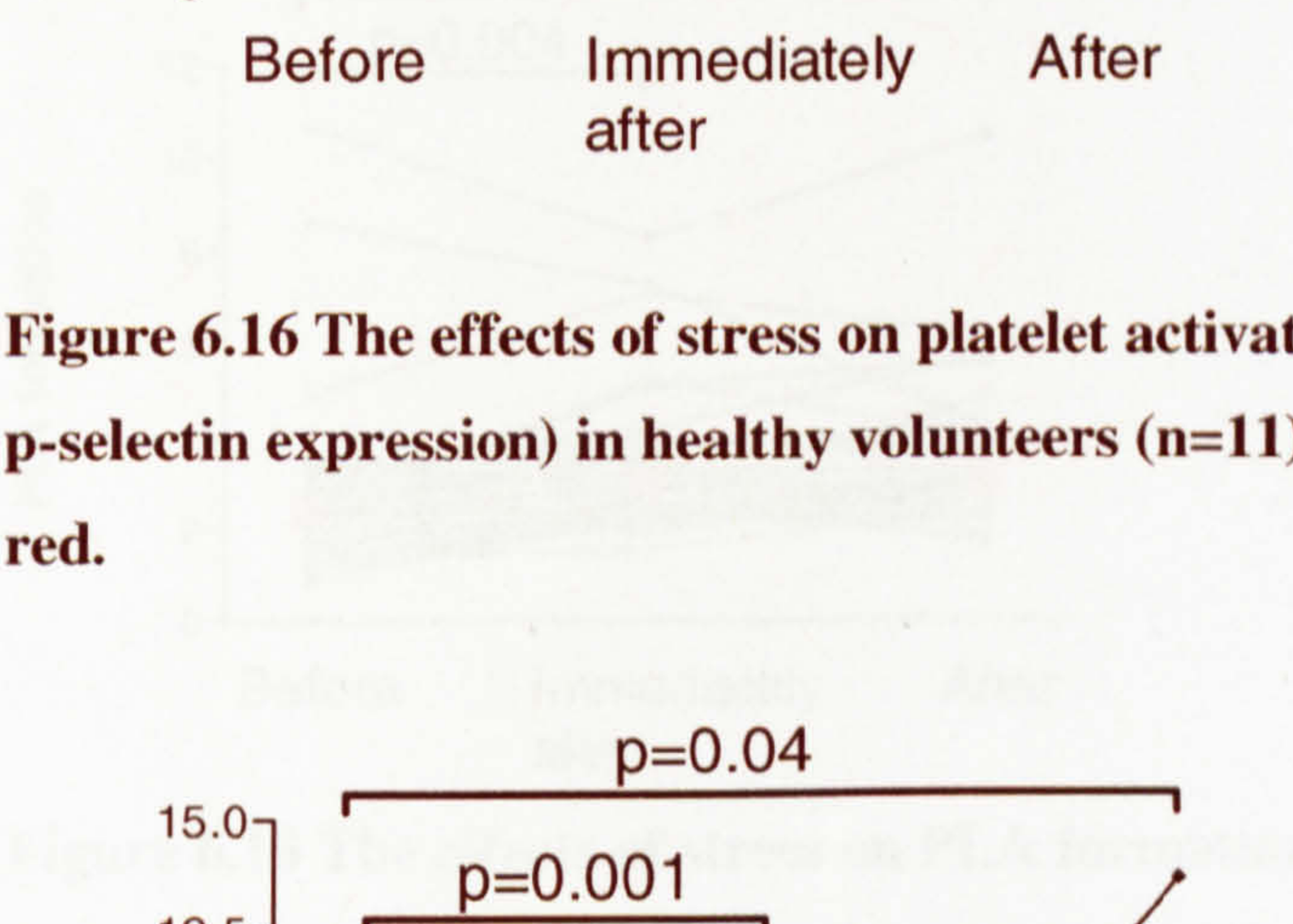


Figure 6.16 The effects of stress on platelet activation (% of platelets positive for p-selectin expression) in healthy volunteers (n=11). Median value is shown in red.



6.4.3.6 Platelet-leukocyte aggregate (PLA) formation (Figure 6.17 and 6.18 and Table 6.4)

The median percentage of leukocytes forming PLAs was increased by 25% in patients with quiescent UC (p=0.004) and by 6% in HV (p=0.03) in the blood sample taken immediately after the stress protocol compared to base-line. In patients with quiescent UC, PLA formation remained elevated by 25% in the sample taken 30 minutes later (p=0.001).

Figure 6.17 The effects of stress on PLA formation (% of leukocytes forming PLAs) in patients with inactive UC (n=25). Median value is shown in red.

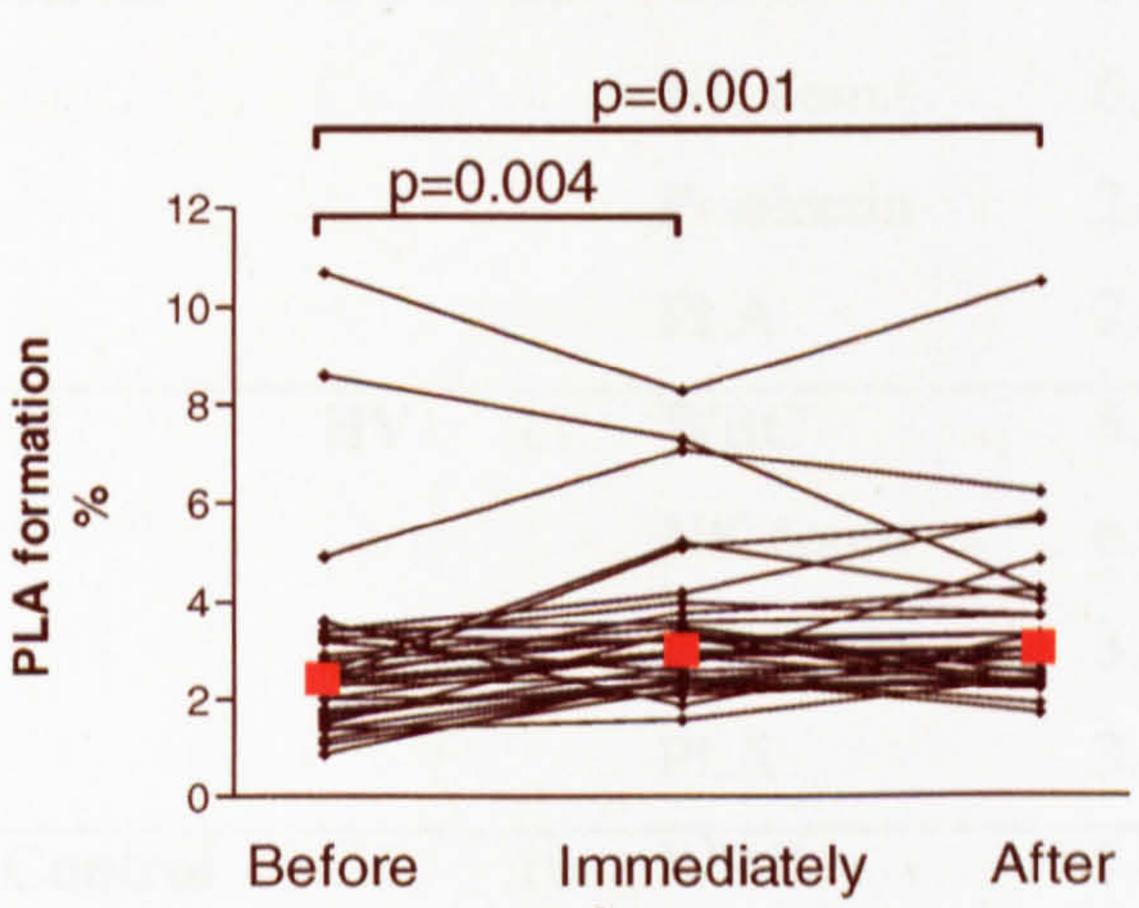


Figure 6.18 The effects of stress on PLA formation (% of leukocytes forming PLAs) in healthy volunteers (n=11). Median value is shown in red.

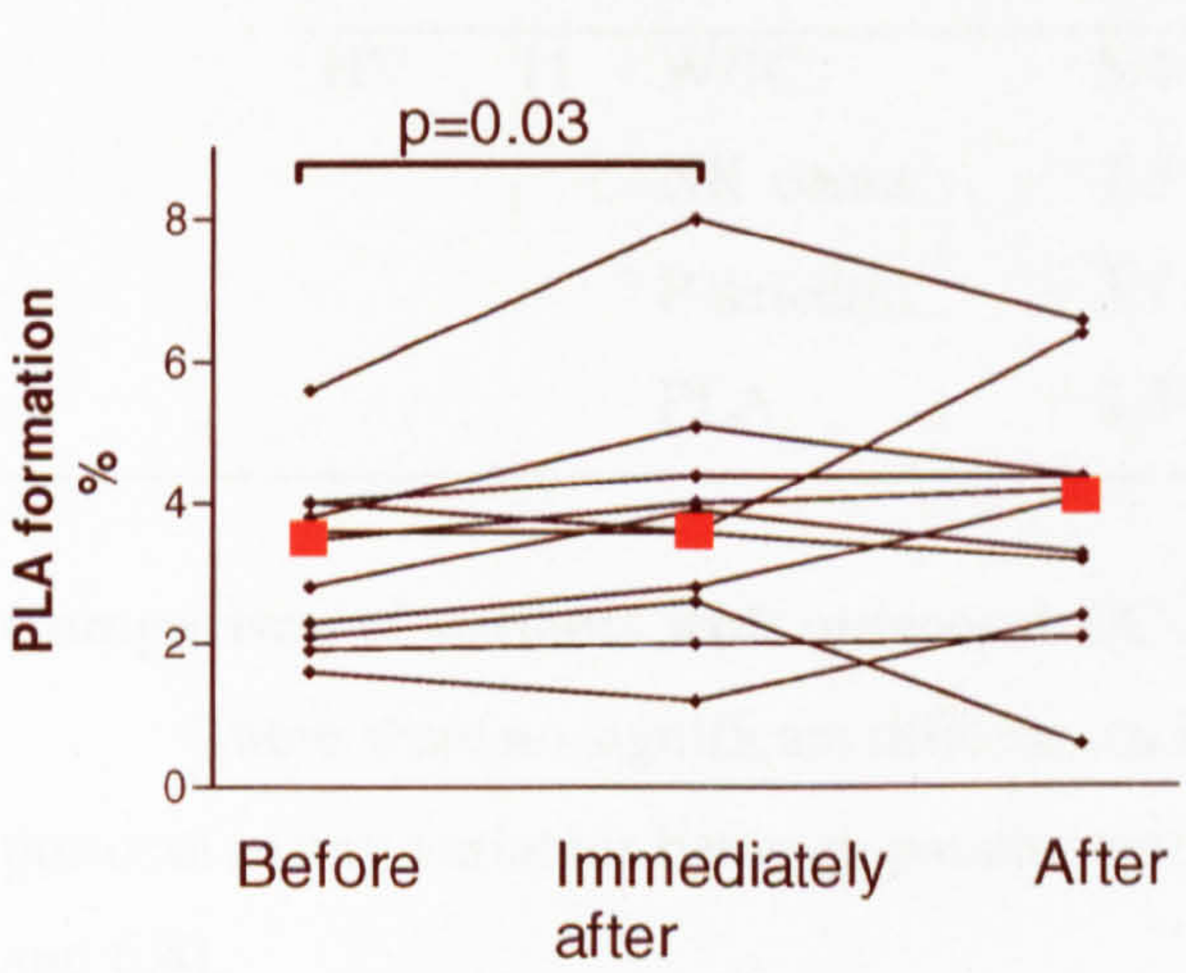


Table 6.4 Total leukocyte count (WBC) (cells x $10^{x4}/mm^3$), natural killer (NK) cell number (% of monocytes and lymphocytes), platelet activation (% of platelets positive for p-selectin) and platelet-leukocyte aggregate (PLA) formation (% of leukocytes forming PLAs) in response to stress and control protocols in patients with UC and healthy volunteers (HV). * p<0.05 from pre-procedure value.

Protocol		N		Before	Immediately after	30 mins after
Stress	UC	25	WBC	6.3 (5.3-8.1)	7.0 (5.5-7.9)	7.3 (5.8-8.4)*
			NK count	6.4 (4.8-8.7)	7.6 (5.4-9.3)*	6.7 (4.0-8.8)
			P-selectin	2.2 (1.3-4.5)	3.5 (2.2-5.2)*	2.8 (2.1-4.6)*
			PLA	2.4 (1.7-3.3)	3.0 (2.4-3.9)*	3.0 (2.4-4.1)*
	HV	11	WBC	5.7 (5-7.5)	6.2 (5.6-8.7)	6.7 (5.8-8.3)*
			NK count	6.2 (5.1-8.6)	7.4 (5.8-9.1)	5.8 (5.1-7.0)
			P-selectin	3.1 (2.4-3.7)	5.0 (2.5-6.3)*	3.8 (3.0-6.7)*
			PLA	3.4 (2.2-4.0)	3.6 (2.7-4.8)*	4.1 (2.8-5.4)
Control	UC	10	WBC	7.4 (5.2-9.1)	7.7 (5.5-8.6)	7.8 (5.7-9.2)
			NK count	7.1 (3.2-9.4)	5.9 (3.7-9.0)	6.9 (4.0-8.0)
			P-selectin	3.1 (2.2-4.1)	3.5 (2.4-4.2)	3.0 (2.1-3.7)
			PLA	2.3 (1.7-3.2)	2.4 (1.8-3.3)	2.1 (1.8-2.9)
	HV	11	WBC	5.4 (4.6-8)	5 (4.4-6.6)	4.9 (4.7-6.5)
			NK count	7.3 (6.1-10.1)	8.8 (6.0-10.6)	8.2 (5.6-9.9)
			P-selectin	3.1 (2.3-3.5)	2.9 (2.5-4.0)	3.2 (2.5-4.8)
			PLA	2.5 (2.2-3.8)	2.4 (2.0-3.4)	2.5 (2.0-4.2)

Comparison of patients with quiescent UC and healthy volunteers

There were no significant differences in the changes elicited by the stress protocol in any variables between patients with inactive UC and HV (Tables 6.2, 6.3 and 6.4).

Relationship between age and responses to stress protocol

Although the patients with UC had a higher median age than the HV, age did not correlate with any of the changes seen in response to the stress protocol in either group.

Effect of immunosuppressant medication on response to stress protocol

Four of the patients with UC undergoing the stress protocol were taking either thiopurines or methotrexate. This is too small a number to allow comparison of the response to stress with that of patients who were not taking immunosuppressants. Furthermore removal of these four patients from the analysis did not significantly alter any of the results reported above.

Psychometric questionnaires

None of the measures of long term stress (STAI-T, HADS, PSQ or BSI) correlated with the changes in any of the variables measured in response to the stress protocol. However, scores on the STAI-S scale did correlate with changes in NK cells levels (R=+0.56, p=0.004) and PLA formation (R=+0.43, p=0.01). There were no differences in scores of any of the psychometric questionnaires between patients with UC undergoing the stress protocol and patients with UC undergoing the control protocol.

Effects of control protocol

The control protocol caused no changes in any of the variables assessed in either patients with inactive UC or HV (Tables 6.2, 6.3 and 6.4).

6.5 DISCUSSION

Stress was found to increase a range of systemic inflammatory variables each of which will be discussed in turn.

LPS-STIMULATED CYTOKINE PRODUCTION.

The LPS-stimulated production of TNF-α and IL-6 by whole blood was increased in the samples taken after stress. This is unlikely to be explained simply by the stress-induced increase in leukocyte count as the percentage increases in TNF-α and IL-6 production by whole blood did not correlate with the percentage increases in leukocyte count in any patient group.

Other studies have shown that stress alters cytokine production by whole blood in healthy volunteers. Blood taken from students before an exam produced more TNF-α and IL-6 when stimulated with LPS than blood taken after (228). Although the mechanism by which this occurs is unknown, adrenaline infusion has been shown to increase LPS-stimulated production of IL-8 and IL-10 by whole blood, and it is likely that sympathetic activation is important in mediating this effect (202;203). TNF-α is a pivotal cytokine in the pathogenesis of IBD (428) and the therapeutic benefit of anti-TNF-α antibodies has been shown in both Crohn's disease (429) and UC (430). IL-6 is also a potentially important inflammatory cytokine in IBD (431) and is the stimulus for C-reactive protein production (432). It is at least conceivable that psychological stress could worsen UC by increasing the production of TNF-α and IL-6 by leukocytes subsequently stimulated by exposure to bacterial products such as LPS.

NK CELL NUMBERS.

Increases in circulating NK cell number are consistently found in association with acute stress (219). Although traditionally considered a component of the innate immune system, more recently NK cells have also been shown to affect adaptive immunity via interactions with dendritic cells (433). NK cells can localise to areas of inflammation and interact with immature dendritic cells to stimulate their maturation and proliferation, which in turn influences their interactions with T-cells. However, the increases in NK cell number in this study were short-lived, having resolved within 30 minutes of the stress test. It therefore seems unlikely that changes in NK cell number play a major role in mediating stress-induced increases in IBD activity.

PLATELET ACTIVATION AND PLATELET-LEUKOCYTE AGGREGATE FORMATION.

Platelet activation and PLA formation have been shown to be increased by experimental stress in healthy subjects (241;242) We have found stress to have similar effects in quiescent UC. Beta-adrenergic stimulation may underlie this effect since exercise-induced platelet activation can be prevented by beta-blockers (241). Platelets circulate in a more activated state in patients with IBD and platelet activation may contribute to pathogenesis through direct pro-inflammatory effects and by causing mucosal thrombosis and microinfarction as a result of microvascular ischaemia(141). PLA formation is also increased in IBD and may facilitate extravasation of leukocytes to sites of mucosal inflammation(143). Increases in both platelet activation and PLA formation had not resolved in blood taken 30 minutes after stress and it is possible that increases in these variables could contribute to stress-induced increases in IBD activity.

COMPARISON OF PATIENTS WITH UC AND HEALTHY CONTROLS.

Previous work has suggested that patients with IBD may have a relative imbalance of HPA axis and autonomic function, with autonomic hypereflexia, and that this may be important in driving mucosal inflammation.(168;287) However, in our study there were no differences observed between patients with UC and healthy volunteers in both their autonomic and systemic inflammatory responses to the stress protocol. This would suggest that whilst stress may act as a trigger for exacerbations of UC, an exaggerated autonomic and inflammatory response to stress is not a primary aetiological factor in UC.

PSYCHOMETRIC QUESTIONNAIRES.

In animal studies, maternal deprivation in infancy, a model of chronic stress, rendered the adult rat more susceptible to the effects of acute restraint stress in augmenting dextran sulphate-induced colitis (297). Levenstein et al found high scores on the Perceived Stress Questionnaire to be predictive of relapse in patients with inactive UC (273). In our study, acute anxiety scores in patients with UC, as

measured by the STAI-S scale, directly correlated with stress-induced NK cell numbers and PLA formation. However, while patients with inactive UC reported higher chronic stress levels (PSQ score), were more anxious (HADS-A), and somatised (BSI) more than healthy volunteers, scores in these indices did not correlate with the changes observed in any of the inflammatory variables in response to the stress protocol.

6.6 CONCLUSION

The acute stress test stimulated an autonomic response in the majority of subjects with UC and HVs studied, and led to an increase in a range of inflammatory variables at the systemic level. Some of these changes may be important in mediating stress-related worsening of disease activity in UC.

CHAPTER 7

THE MUCOSAL RESPONSE TO STRESS IN PATIENTS WITH INACTIVE ULCERATIVE COLITIS

7.1 SUMMARY

Aim: To assess the effects of acute psychological stress on several inflammatory measures at the rectal mucosal level.

Methods: Individuals with quiescent UC underwent the stress test or control procedure and the rectal mucosal release of inflammatory cytokines, mucosal blood flow, reactive oxygen metabolite production and conventional histology were assessed before and after.

Results: The following results were found:

1. Stress more then doubled mucosal TNF-α release (p=0.03).
2. Stress increased mucosal ROM production by over fivefold (p= 0.001).
3. Rectal mucosal blood flow (RMBF) was reduced by 22% in response to stress (p=0.05).
4. There was no overall change in histological score (n=17) but in all five cases where there was a degree of inflammation present in the pre-stress biopsy (assessed by Saverymuttu score), there was an increase in histological score in the post-stress sample. These five patients also demonstrated a greater percentage increase in rectal mucosal TNF-α release than did the other twelve (p=0.04).
5. There was a trend towards increased mast cell degranulation detected by immunofluorescence (p=0.07) in the patients in whom this variable was assessed.
6. There was a trend towards increased numbers of *E.coli* present in the lamina propria as assessed by fluorescent-in-situ hybridisation in rectal biopsies taken after stress.
7. Chronic stress, as assessed by psychometric questionnaires, did not affect the mucosal response to the acute stress test.
8. The control protocol did not change any of the mucosal inflammatory measures assessed.

Conlusion: Stress increased rectal mucosal release of TNF-α and reactive oxygen metabolite production by rectal mucosal biopsies. Conventional histological examination suggests that the effects of stress may be increased in individuals with a pre-existing mucosal of inflammation.

7.2 INTRODUCTION

Acute physical stress has been shown to increase the release of mast cell mediators in the small bowel in healthy individuals (278). Repeated administration of a similar stress also caused increased mast cell degranulation at electron microscopy in mucosal biopsies in both healthy individuals and patients with IBD (279). Several of the stress-related pro-inflammatory changes seen in animal models of colitis also seem dependent on the degranulation of mast cells. The mechanism by which this might occur is unknown.

In this chapter we will describe the mucosal responses of patients with quiescent UC to the stress protocol described in chapter 3. The hypothesis underlying this work is that inflammatory changes occurring at the mucosal level might contribute to the reported stress-related increase in disease activity in UC. The variables assessed include pro-inflammatory neurotransmitters which may be important in stimulating mast cell degranulation (substance P (190)), measures of mast cell degranulation (histamine (123) and immunofluorescence), cytokines thought to be important in the pathogenesis of UC (IL-13 (180), TNF-α (36)), inflammatory free radicals (reactive oxygen metabolite production (181)), mucosal-associated bacteria (434) and rectal autonomic measures (rectal mucosal blood flow(407)) and conventional histology (378).

7.3 DEMOGRAPHICS OF PATIENTS AND HEALTHY VOLUNTEERS (Table 6.1)

The demographics of the patients with quiescent UC and healthy volunteers undergoing the stress and control protocols has already been described in Chapter 6.

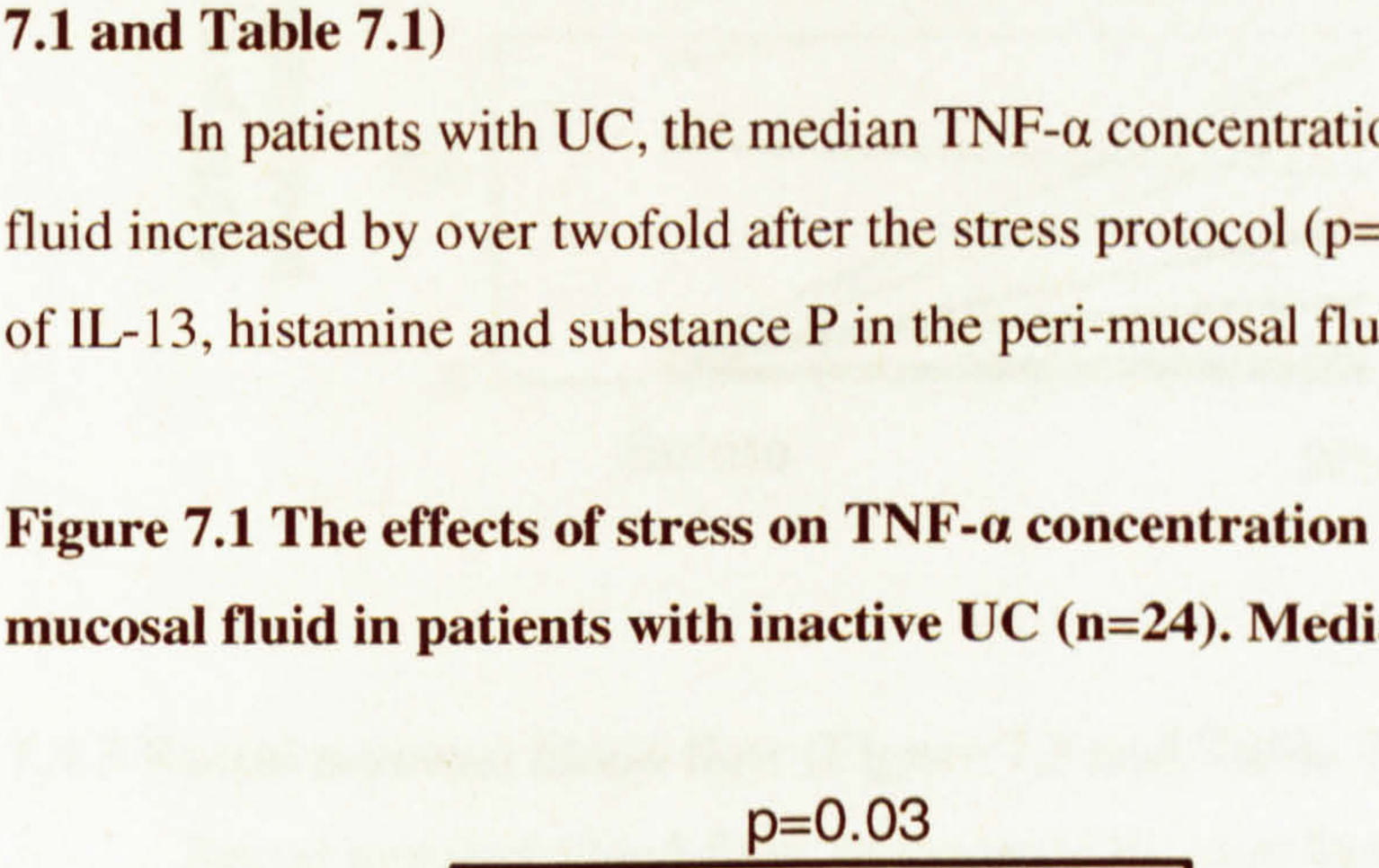
7.4 RESULTS

Perimucosal fluid samples were available from 24 of 25 patients undergoing the stress protocol and 9 of 10 patients undergoing the control protocol. In these two patients the samples were accidentally defrosted and could therefore not be used. ROM and conventional histology could be performed on paired pre- and post-stress biopsies in 17 of the 25 patients undergoing the stress protocol. Biopsies from the other eight patients were used for studies of mast cell degranulation and mucosal associated bacteria.

7.4.1 Cytokine and mediator concentrations in rectal peri-mucosal fluid (Figure 7.1 and Table 7.1)

In patients with UC, the median TNF-α concentration in rectal peri-mucosal fluid increased by over twofold after the stress protocol (p=0.03); the concentrations of IL-13, histamine and substance P in the peri-mucosal fluid did not change.

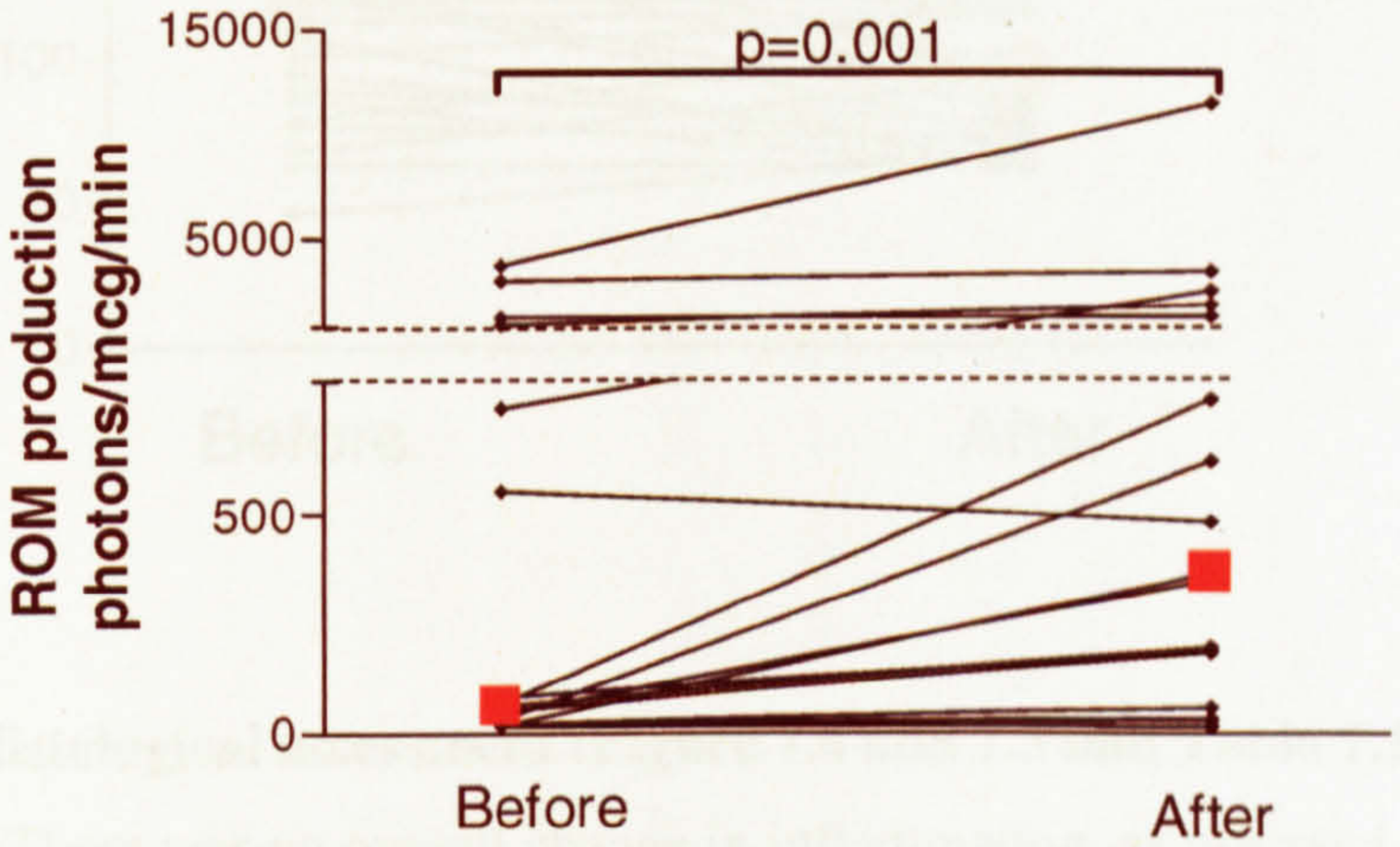
Figure 7.1 The effects of stress on TNF-α concentration (pg/ml) in rectal peri-mucosal fluid in patients with inactive UC (n=24). Median is shown in red.



7.4.2 Reactive oxygen metabolite production (Figure 7.2 and Table 7.1)

The stress protocol increased median mucosal production of ROMs by over fivefold (p=0.001).

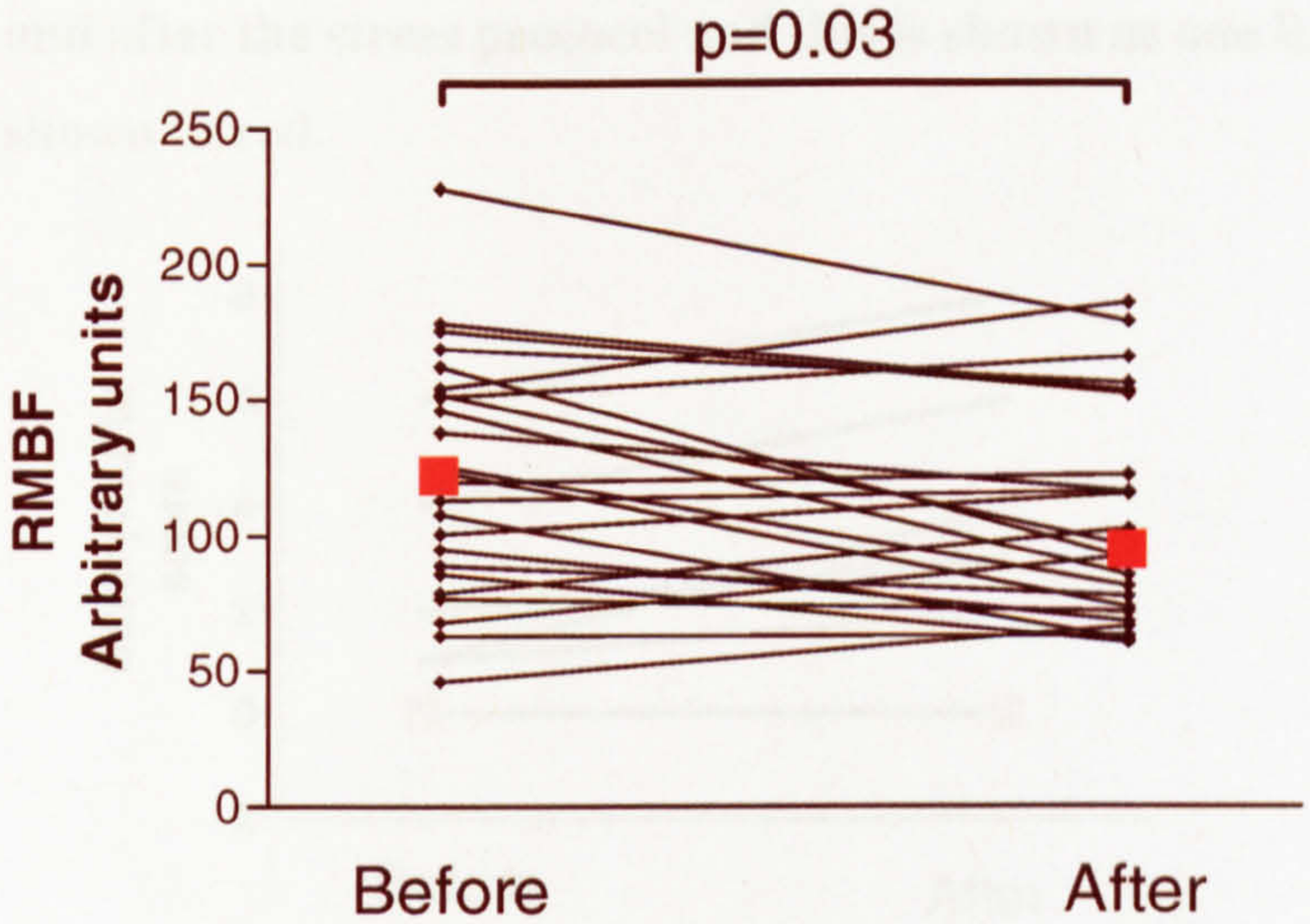
Figure 7.2 The effects of stress on ROM production (photons/mcg/min) by rectal mucosal biopsies from patients with inactive UC (n=17). Median value shown in red.



7.4.3 Rectal mucosal blood flow (Figure 7.3 and Table 7.1)

Rectal mucosal blood flow, as assessed by laser Doppler flowmetry, fell by 22% (median) after stress (p=0.05).

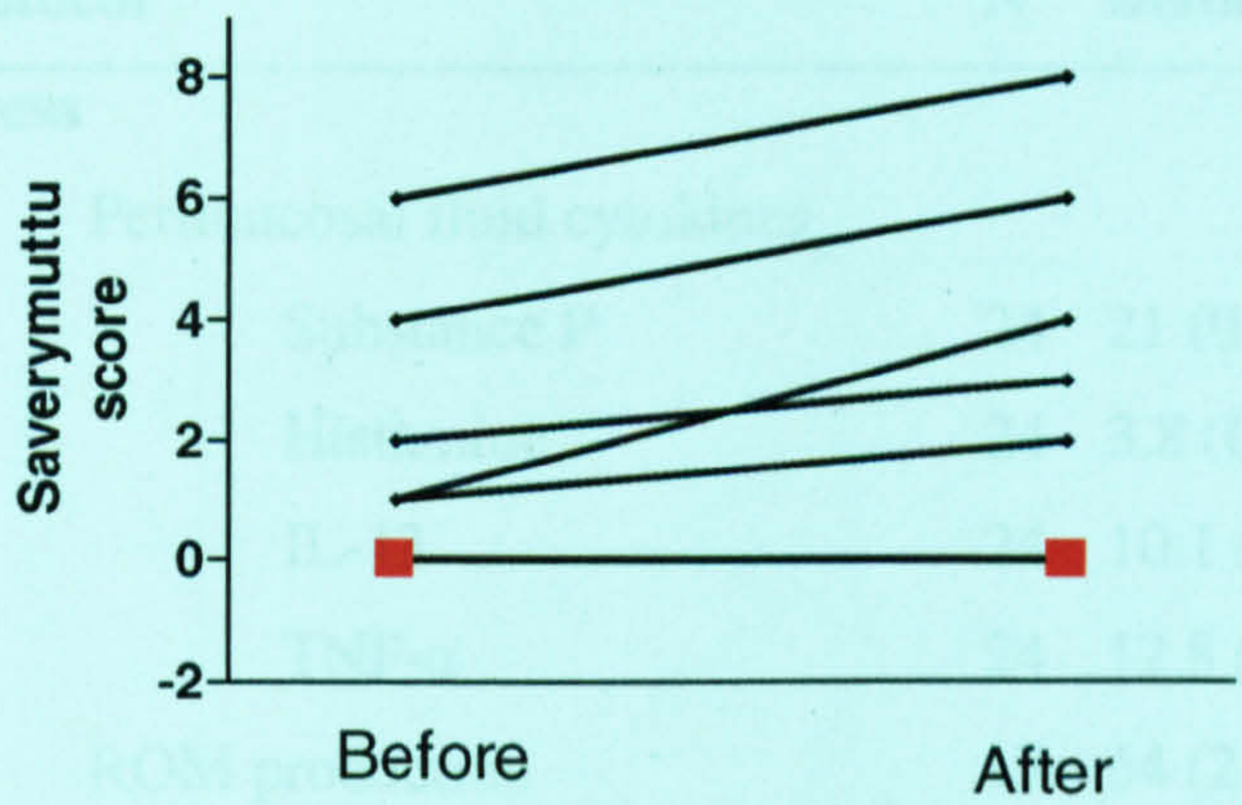
Figure 7.3 Effects of stress on RMBF in patients with inactive UC (n=25). Median value shown in red.



7.4.4 Histological assessment (Figure 7.4 and 7.5 and Table 7.1)

There was no overall change in inflammation, as assessed by Saverymuttu score (378), in the seventeen pairs of biopsies available for assessment, with 12 pairs scoring zero both before and after the stress protocol. However, in all five patients where there was a degree of inflammation present in the pre-stress biopsy (scores 1-6), there was an increase in histological score in the post-stress sample.

Figure 7.4 The effects of stress on histological score in mucosal biopsies in patients with inactive UC (n=17). Only five lines are shown as in 12 samples the histological score was zero, as assessed by the Saverymuttu score (378), before and after the stress protocol and this is shown as one line. Median value is shown in red.



The five patients whose mucosa demonstrated an increase in histological score also showed a greater percentage increase in TNF-α concentration in peri-mucosal fluid than the 12 cases where there was no change in histological score.

Figure 7.5 Percentage increase in mucosal TNF-α production in patients with and without a change in histological score in response to stress. Median value is shown in red.

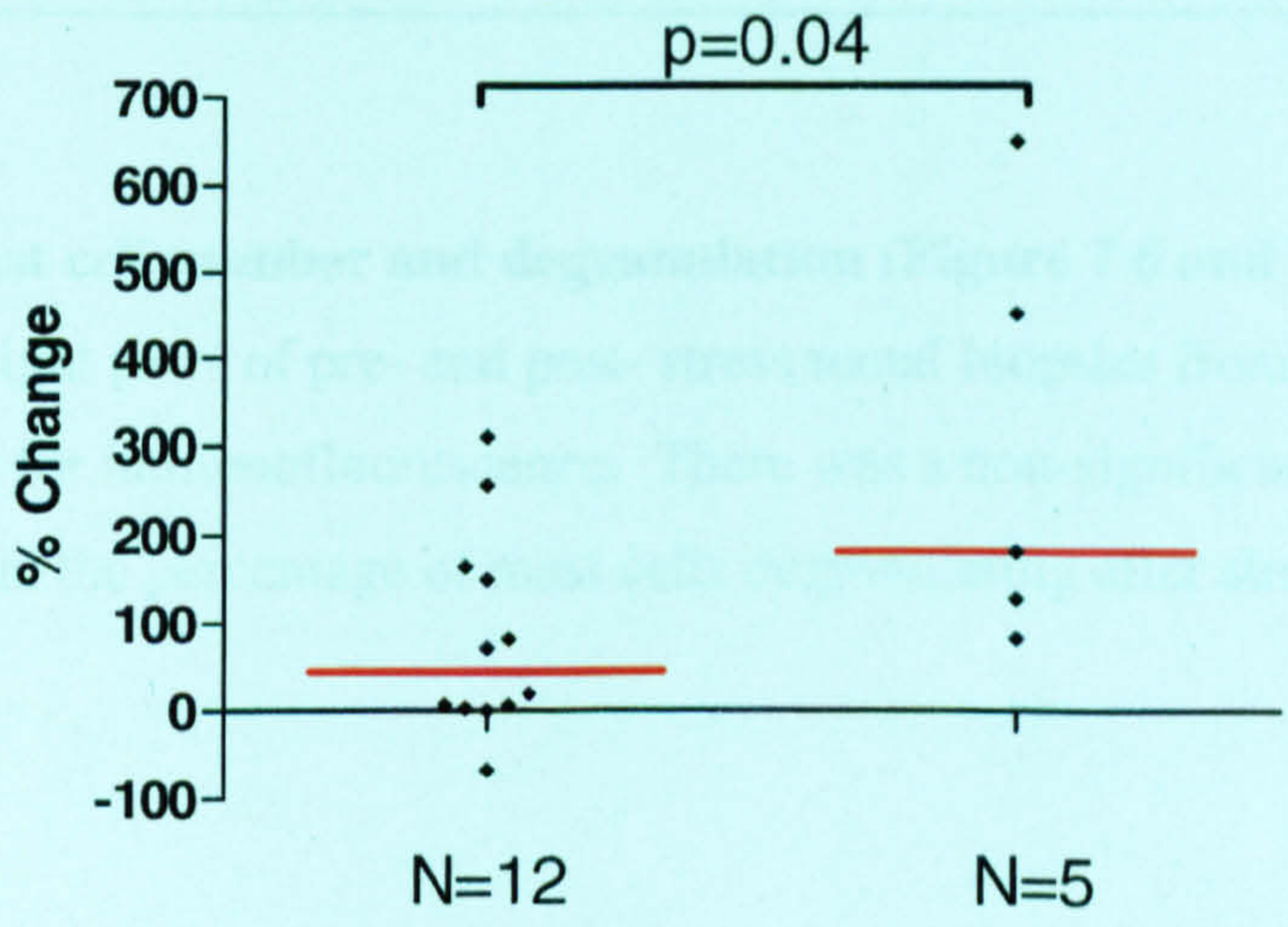


Table 7.1 Peri-mucosal fluid cytokine levels (pg/ml), reactive oxygen metabolite (ROM) production by mucosal biopsies (photons/mcg/min), rectal blood flow (arbitrary units) and histological score in response to stress and control protocol in patients with quiescent UC. * P<0.05 from pre-procedure value.

Protocol	N	Before	After
Stress			
Perimucosal fluid cytokines			
Substance P	24	21 (0-96)	15 (0-24)
Histamine	24	3.8 (0.7-14.5)	3.1 (1.4-21.9)
IL-13	24	10.1 (4.5-16.8)	10 (4.3-28.5)
TNF-α	24	12.8 (8.6-20.2)	25.8 (12.7-41.5)*
ROM production	17	64 (24-833)	368 (123-1569)*
Blood flow	25	122 (87-158)	95 (72-153)*
Histological Score	17	0 (0-0.5)	0 (0-2)
Control			
Perimucosal fluid cytokines			
Substance P	9	32 (0-94)	0 (0-5.2)
Histamine	9	2.0 (0.1-12.2)	2.1 (0.6-14.7)
IL-13	9	14.5 (8.5-20.1)	16.8 (9.2-25.2)
TNF-α	9	8.9 (0-15.5)	5.8 (0.6-12.2)
ROM production	10	31 (5-56)	25 (4-158)
Blood flow	10	111 (106-141)	111 (71-142)
Histological Score	10	0 (0-1)	0 (0-1)

7.4.5 Mast cell number and degranulation (Figure 7.6 and Table 7.2)

Eight pairs of pre- and post- stress rectal biopsies from patients with UC were available for immunofluorescence. There was a non-significant trend towards an increase in the percentage of mast cells degranulating after stress (p=0.07). There

was no obvious trend towards a change in total mast cell number. There were no control biopsies available for comparison.

Figure 7.6 The effects of stress on the percentage of mast cells degranulating in rectal biopsies from patients with inactive UC (n=8). Median value is shown in red.

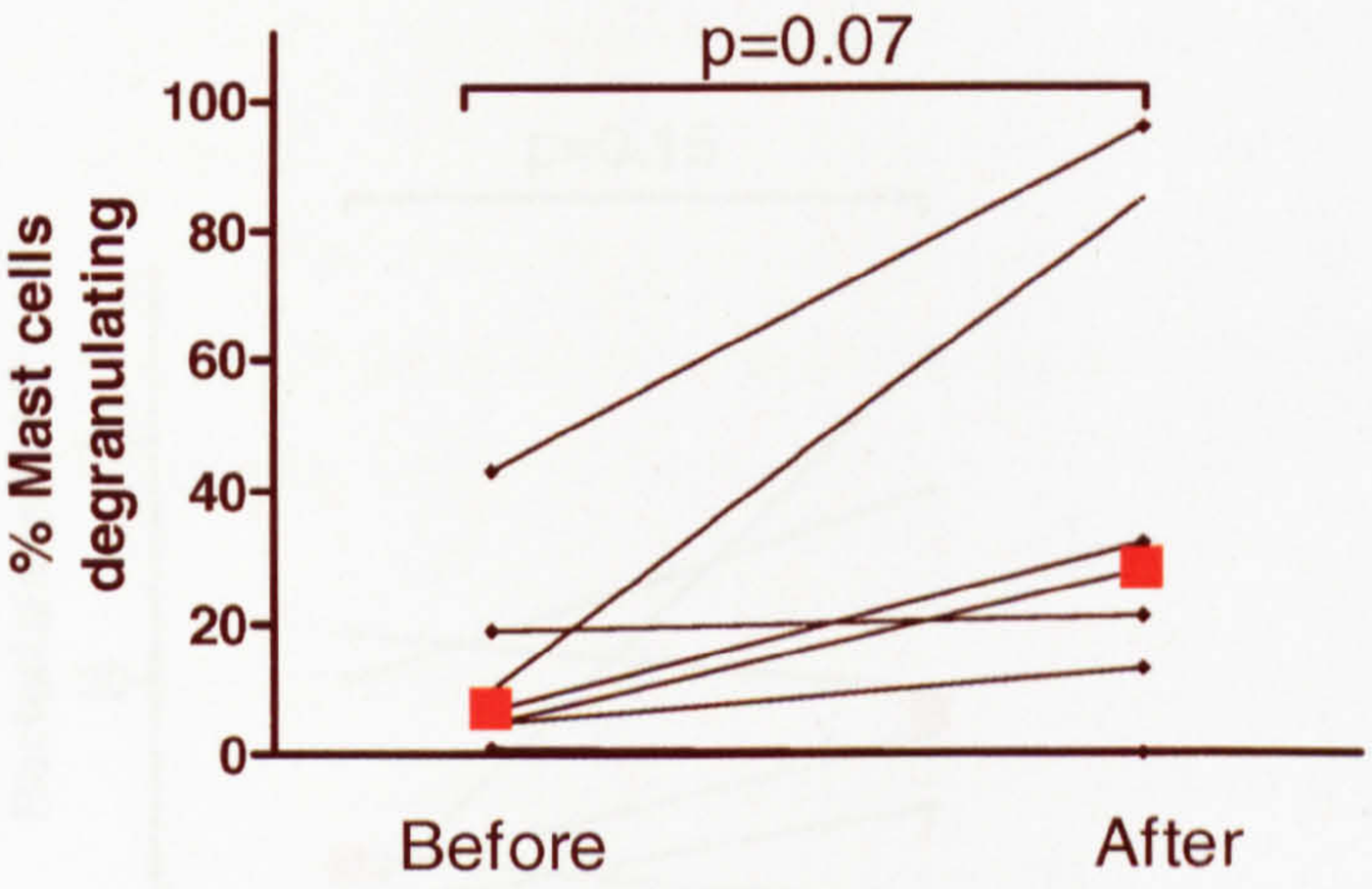


Table 7.2 Mast cells numbers, as a percentage of total cells, and percentage of mast cells degranulating in pre and post stress biopsies in inactive UC (n=8).

	Pre-Stress	Post-Stress
Mast cell number (% total cells)	1.9 (1.3-2.3)	1.4 (1.0-1.9)
Degranulating mast cells (%)	7 (5-28)	28 (13-85)

7.4.6 Mucosa-associated flora (Figure 7.7 and Table 7.3)

Six pairs of pre- and post-stress biopsies were available for assessment of mucosa-associated flora using FISH. The total number of *E.coli*, and *E.coli* as a percentage of the total bacteria, were calculated for both bacteria adherent to the surface of the mucosa and bacteria found in the lamina propria. Only small numbers

of samples were available for analysis and none of the changes observed were statistically significant. However, there were non-significant trends to increased numbers of *E.coli* in the lamina propria (p=0.15). There were no control biopsies available for comparison.

Fig 7.7 The effects of stress on number of *E.coli* in the lamina propria in rectal biopsies in patients with inactive UC (n=6). Median value is shown in red.

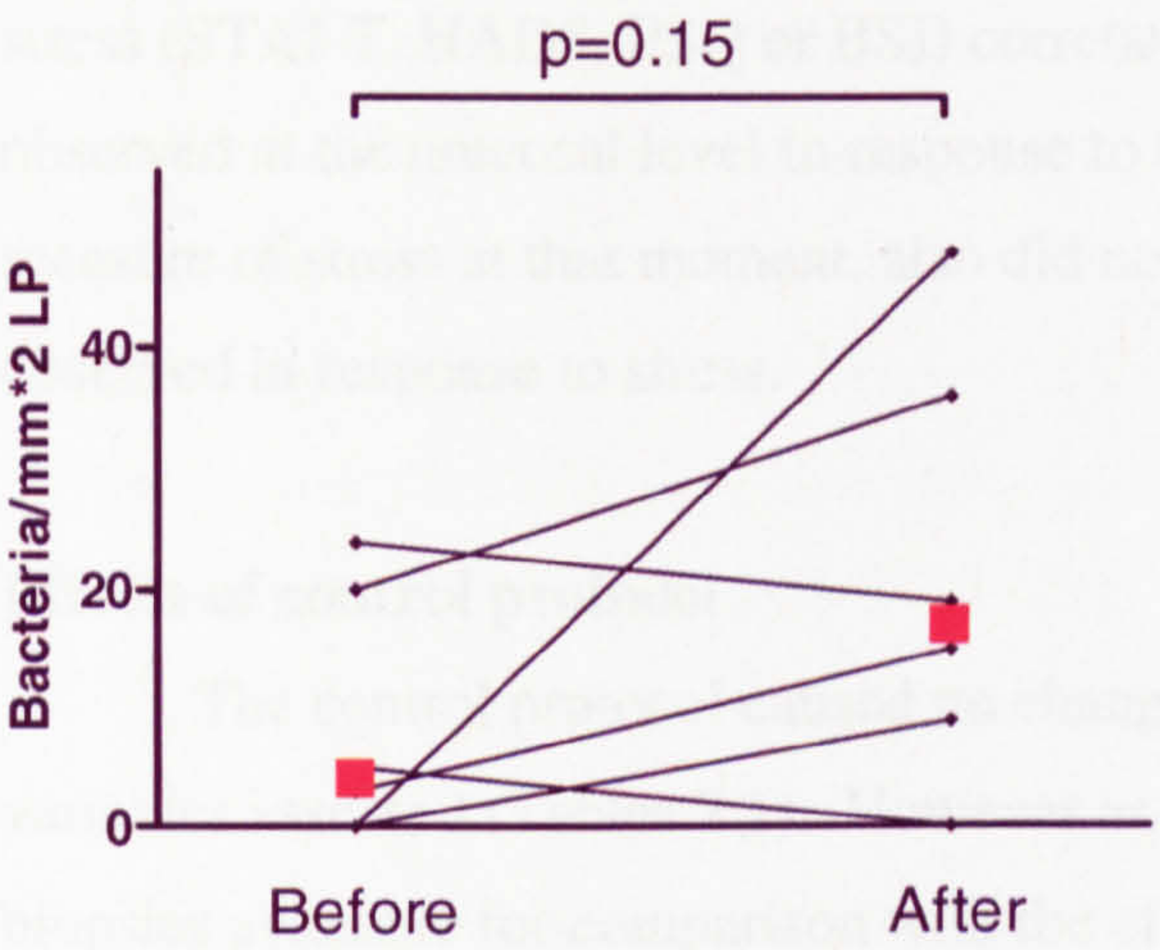


Table 7.3 Number *E.coli* and *E.coli* as a percentage of total bacteria adherent to mucosal surface and in the lamina propria in pre and post stress biopsies in inactive UC (n=6).

	Pre-Stress	**Post-Stress**
Surface adherent Bacteria		
E.coli /mm epithelial surface	90 (71-115)	112 (75-156)
Total Bacteria /mm epithelial surface	554 (464-683)	534 (302-629)
% E. coli	16 (14-18)	24 (14-61)
Bacteria in lamina propria		
E.coli /mm^2 lamina propria	4 (0-22)	17 (5-42)
Total Bacteria / mm^2 lamina propria	8 (2-24)	18 (6-44)
% E. coli	88 (0-98)	94 (41-98)

Effect of immunosuppressant medication on response to stress protocol

As with the systemic inflammatory reponse to stress, immunosuppressant therapy had no overt effect on the mucosal response to stress and the removal of the four patients taking thiopurines and methotrexate from the analysis did not significantly alter any of the results reported above.

Psychometric questionnaires

As with the systemic response to stress, none of the measures of long term stress (STAI-T, HADS, PSQ or BSI) correlated with the changes in the variables observed at the mucosal level in response to the stress protocol. The STAI-S, a measure of stress at that moment, also did not correlate with any of the changes observed in response to stress.

Effects of control protocol

The control protocol caused no changes at the mucosal level in any of the variables assessed (Tables 7.1). However as stated above, there were no control biopsies available for comparison with the effects of stress on mast cell numbers or mucosa-associated bacteria.

7.5 DISCUSSION

Experimental stress caused increases in several rectal mucosal inflammatory variables, which could theoretically contribute to stress-induced relapses in UC.

RECTAL PERI-MUCOSAL CYTOKINE AND MEDIATOR RELEASE.

It has been proposed that stress-induced increases in inflammation are mediated through increases in intestinal permeability, allowing exposure of the mucosal immune system to bacterial flora.

Restraint stress in rodents increased epithelial permeability to inert marker molecules such as EDTA and to antigenic proteins such as horseradish peroxidase

(305) and increased the phagocytic uptake of *E.coli* into follicular-associated epithelium (313).

The effects of restraint stress in these experiments did not occur in mast cell-depleted animals and it is likely that mucosal mast cell degranulation is an important step in mediating the pro-inflammatory effects of stress on the GI tract (311;314). Physical stress, caused by immersion of the hand in iced water, has been shown to increase the proportion of activated and degranulating mast cells seen on electron microscopy in healthy controls and even more so in patients with IBD (279). Mast cell granules contain a range of inflammatory cytokines including histamine and TNF-α (435). In vitro studies have shown that increases in ileal permeability are dependent on the production and release of TNF-α (436). In this study, we have shown an increase in the concentration of TNF-α, but not of histamine, in rectal peri-mucosal fluid after stress and this could conceivably lead to an increase in mucosal permeability and consequently inflammation.

The stimulus for mast cell degranulation after stress is likely to involve the release of specific neuropeptides from the enteric and autonomic nervous systems. One candidate neuropeptide, substance P (SP), has been shown to increase the release of mast cell mediators from colonic biopsies in patients with UC (190). SP also has direct pro-inflammatory effects, stimulating IL-8 secretion from epithelial cells (189). In our study stress did not increase the concentration of SP in rectal peri-mucosal fluid. This result may partly reflect the sensitivity of our method for assessing SP release, since in many samples the SP concentrations were below the limit of detection.

HISTOLOGICAL ASSESSMENT.

In most patients (12/17), there was no active histological inflammation on the Saverymuttu score (378) in the pre-stress biopsy: this could be either because the patients were in complete histological remission or because the Saverymuttu score is insufficiently sensitive to detect minor degrees of inflammation.

The failure to find an overall increase in inflammation in pre and post-stress rectal biopsies may be partly due to the short time course of the protocol and the

relatively mild nature of the stressor. However, we did observe an increase in histological score in all five pairs of biopsies in patients in whom there was a degree of inflammation (24) already present prior to the protocol. This observation might suggest that in individuals who are "primed", with a degree of inflammation already present, acute stress is able to able to worsen microscopic inflammation. Furthermore the patients who showed an increase in histological score also showed a greater percentage increase in rectal mucosal TNF-α release compared to those whose histological score remained unchanged. Given the importance of TNF-α as a key cytokine in the pathogenesis of IBD (36), this observation also supports the theory that patients who already have a mild degree of colonic inflammation present are more vulnerable to the pro-inflammatory effects of stress.

DEGRANULATING MAST CELLS

As described above, mast cell degranulation is likely to be an important step in mediating the pro-inflammatory effects of stress on the GI tract. Enhanced mast cell activation (122) and rectal mucosal histamine release (123) have reported previously in active UC. Furthermore, an inhibitor of the mast cell mediator, tryptase (129), and the mast cell stabiliser, ketotifen (130), have been shown to be of therapeutic benefit in small open-labelled trials in UC.

Although we did not find an increase in the mucosal release of histamine (see above) there was a non-significant trend to an increased percentage of activated mast cells as assessed by immunofluoresence in the post-stress biopsies of the patients studied. This is in keeping with the results of a previous study in which an increase in activated mast cells, as assessed by electron microscopy, occurred after repeated sessions of a physical stressor.(279) However, it must be stressed that in our work the number of samples available for analysis was small and there is no control data.

MUCOSA-ASSOCIATED BACTERIAL FLORA

The aetiology of IBD is thought to involve an abnormal immune response to luminal bacteria (107). Alteration in bacterial-mucosal interactions in response to stress could contribute to stress-related increases in intestinal inflammation in IBD.

Increased adherence of pathogenic bacteria to colonic mucosa has been shown to occur in response to long term stress in animal models (437). The stress hormone noradrenaline increases the adherence of *E. coli 0157* to murine caecal mucosa (317). As well as adherence, stress has been shown to increase uptake of bacteria into the gut mucosa in animal models. Chronic stress in mice increases the phagocytic uptake of killed *E.coli* into the follicle-associated epithelium which overlies Peyer's patches (313), and leads to increased numbers of animals with detectable levels of bacteria in their inguinal and mesenteric lymph nodes (316).

In this study we have observed a non-significant trend to increased numbers of *E.coli* found in the lamina propria in rectal biopsies taken after the stress protocol ($p=0.15$). However, the number of paired biopsies available for analysis was low and there were no control biopsies available for comparison. Previous work has shown the number of *E.coli* adherent to the rectal mucosa and the number of *E.coli* found in the lamina propria to be increased in patients with active UC compared to inactive UC and healthy controls (64).

RECTAL MUCOSAL REACTIVE OXYGEN METABOLITE (ROM) PRODUCTION.

Mucosal ROM production is increased in active compared with inactive UC (181) and may play a pathogenic role in IBD. Short-term mental stress has been shown to increase the oxidative activity of neutrophils in peripheral blood from healthy volunteers (438). In this study we have shown that psychological stress substantially increases rectal mucosal ROM production, an effect which could contribute to mucosal damage.

RECTAL MUCOSAL BLOOD FLOW.

Psychological stress reduced RMBF in patients with quiescent UC as it does in patients with irritable bowel syndrome and in healthy volunteers.(439) This is thought to be due to alterations in autonomic tone, with stress increasing sympathetic and reducing parasympathetic activation. Any cytokine induced increases in RMBF as a result of the pro-inflammatory effects of stress appear to be outweighed by its

autonomic effects. Indeed, it is conceivable that stress-induced reductions in RMBF could trigger relapse by causing mucosal ischaemia.

7.6 CONCLUSION

Stress appears able to increase a range of inflammatory variables at the mucosal level, an effect which is increased in individuals with pre-existing inflammation. Preliminary data suggests that stress may also increase mast cell degranulation and lead to an increased number of *E.coli* in the lamina propria.

CHAPTER 8

THE EFFECTS OF HYPNOTHERAPY ON SYTEMIC MEASURES IN PATIENTS WITH ACTIVE ULCERATIVE COLITIS AND HEALTHY VOLUNTEERS

8.1 SUMMARY

Aims: As per protocol (chapter 3), to assess the effects of one 50 minute session of gut-formalised hypnotherapy at the systemic autonomic and inflammatory levels.

Methods: As described in chapter 3, patients with active UC and healthy volunteers underwent the hypnotherapy and control protocols. Autonomic and systemic inflammatory measures were assessed before and after in both groups as described in Chapter 3.

Results: The principal findings described in this chapter are:

1. Trance was successfully induced in 16 of 17 (94%) of patients with active UC and 9 of 10 healthy volunteers (90%). There was no difference in hypnotisability score (383) or depth perception between patients with UC and healthy volunteers.
2. At the autonomic level, hypnotherapy reduced the pulse rate by 7 bpm in patients with UC (p=0.0009) and 11bpm in HVs (p=0.004). Systolic BP was reduced by 2mmHg in patients with UC (p=0.04) and 3mmHg in HVs (p=0.02).
3. Hypnotherapy reduced serum IL-6 concentration by 53% in patients with UC (p=0.001) and 23% in HVs (p=0.02). There was a transient fall in NK cells in response to hypnosis by 18% in patients with UC (p=0.01) and by 34% in HVs (p=0.04).
4. There was no difference between the responses of patients with UC and HVs to hypnosis.
5. Chronic stress, as assessed by psychometric questionnaires, hypnotisability, as assessed by Spiegel's score (383), and depth perception had no effect on the autonomic or inflammatory response to hypnosis.
5. The control protocol had no effect on any of the variables assessed.

Conclusion: Hypnotherapy altered autonomic balance temporarily in patients with active UC and HVs, with an increased para-sympathetic and reduced sympathetic tone. Hypnotherapy also reduced serum IL-6 levels, a cytokine which may be important in the pathogenesis of IBD.

8.2 INTRODUCTION

As previously described, hypnotherapy has been shown to be of therapeutic benefit in a range of inflammatory diseases which have a psychosomatic component. It is not known whether hypnotherapy works in these circumstances via a direct effect on the immune system or by altering symptom interpretation at a cognitive level. Few studies have examined the direct effects of hypnotherapy on the immune system. In one study hypnosis led to a brief reduction in NK-cell activity and lymphocyte proliferative responses to mitogens (351). In a second study, the proportion of T-cells expressing IFN-γ and IL-2 was reduced in blood samples taken from seven highly hypnotizable healthy volunteers immediately after hypnosis (352).

In this chapter we will describe the systemic responses of patients with active UC and healthy volunteers to the hypnotherapy protocol. The hypothesis underlying this work is that relaxation achieved through hypnosis is capable of reducing inflammation at the systemic level. The effects of hypnosis on the systemic inflammatory measures assessed in this protocol have not been previously examined. There is also limited data available on the effects of hypnotherapy in patients with UC (365).

The response to hypnotherapy was subsequently related to scores of hypnotisability (383) and to psychometric assessments of chronic perceived stress levels.

For ease of reading the mucosal responses of patients with UC to stress will be described separately in Chapter 9.

8.3 DEMOGRAPHICS OF PATIENTS AND HEALTHY VOLUNTEERS

17 patients with active UC, as defined by a Baron's sigmoidoscopic score of >1, and 10 healthy volunteers (HV) underwent the hypnotherapy protocol. 8 patients with active UC and 11 healthy volunteers underwent the control protocol.

Table 8.1 Sex, age, disease extent, treatment, Baron's score and Simple Colitis Activity Index for patients with active UC and healthy volunteers undergoing the hypnotherapy and control protocols.

Protocol		
Hypnosis		**Control**
UC (n=17)		**UC (n=8)**
Age	40 (23-63)	43 (25-60)
Sex	11 male	4 male
Disease extent	65% total	50% total
	11% left-sided	12% left-sided
	24 % distal	38% distal
Treatment	94% on 5-ASA	100% on 5-ASA
	24% thiopurines	38% thiopurines
	7% oral ciclosporin	0% corticosteroids
	0% corticosteroids	25% topical therapy
	18% topical therapy	
Baron's score	2 (2-3)	2.5 (2-3)
SSCAI	6 (5-7.5)	6.5 (6-9)
HV (n=10)		**HV (n=11)**
Age	29 (23-42)	36 (27-57)
Sex	5 male	6 male

8.4 RESULTS

8.4.1 INDUCTION OF TRANCE

As assessed by subjective depth perception and the experience of time distortion, trance was successfully induced in 16 of 17 patients and 9 of 10 healthy volunteers. In the one patient with UC and one healthy volunteer where trance could not be induced, the subjects reported this to be due to an inability to relax sufficiently

to allow trance to occur. The median score of Spiegel's test of hypnotisability was 5 (IQR 4-6.5) for patients with active UC and 4.5 (IQR 3-6) for healthy volunteers (p=0.33). The median of the three self-rated scores of depth perception was 6 (IQR 3-7) for patients with active UC and 5 (IQR 4-7) for healthy volunteers (p=0.19)

8.4.2 AUTONOMIC RESPONSE TO HYPNOTHERAPY

8.4.2.1 Pulse rate (Figure 8.1 and 8.2 and Table 8.2)

The mean of the three pulse rate readings measured during hypnosis was lower than pre-test values by 7 bpm (median) in patients with active UC (p=0.0009) and by 11bpm in HV (p=0.004). Thirty minutes after the end of the hypnosis protocol, pulse rate had returned to baseline in both groups.

Figure 8.1 The effects of hypnotherapy on pulse rate (bpm) in patients with active UC (n=17). The points shown as "During" are the mean of the three readings taken during the stress test. Median is shown in red.

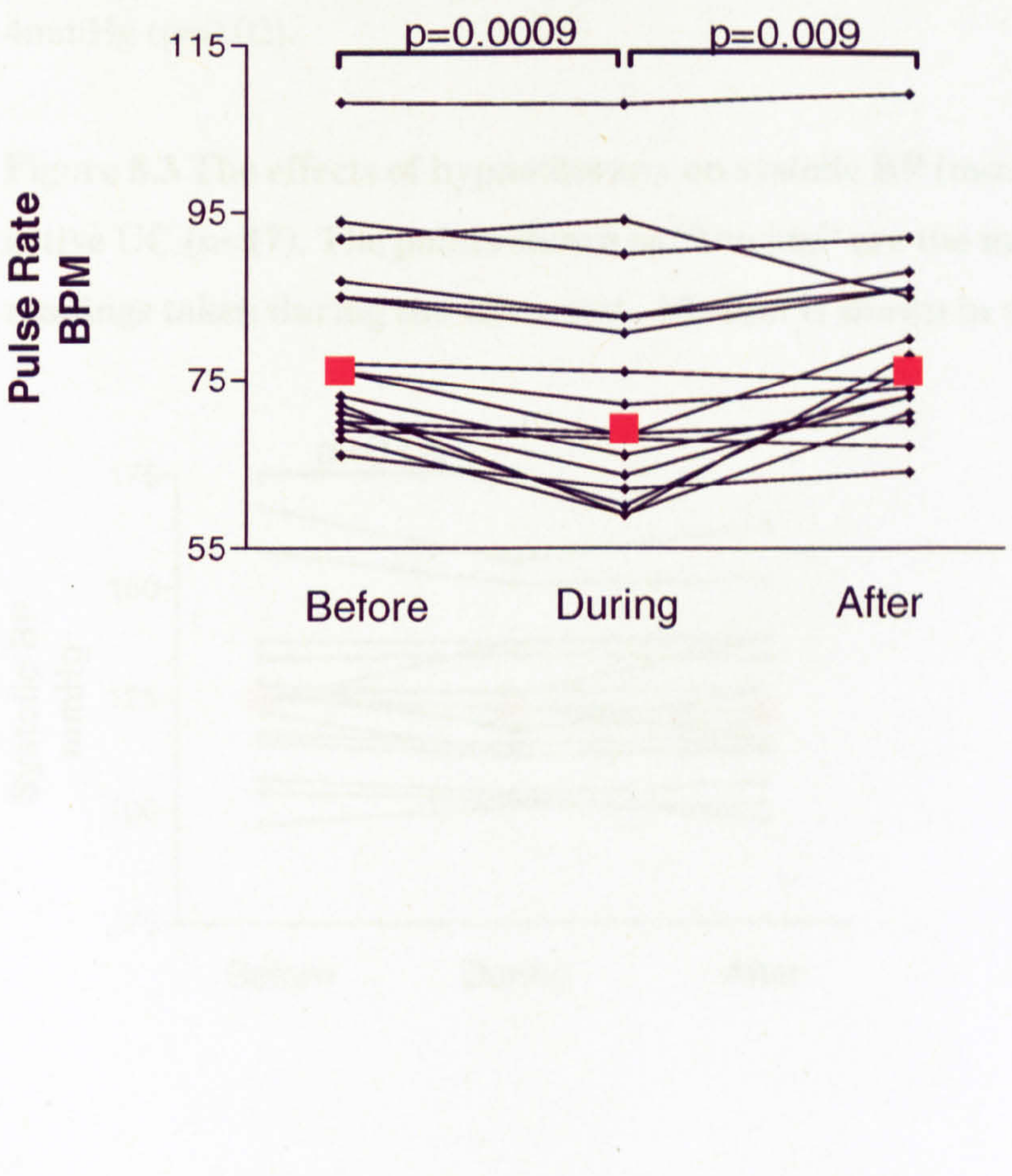
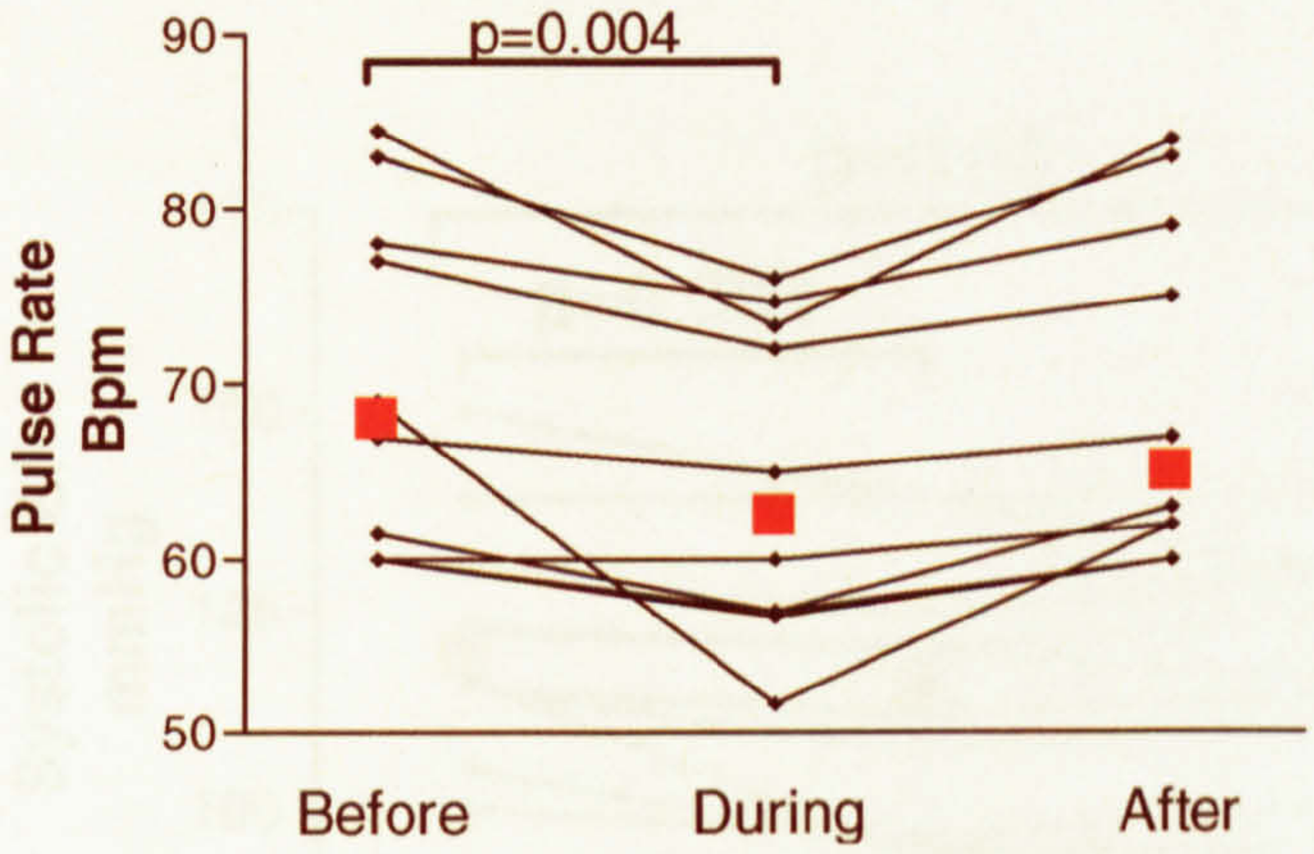


Figure 8.2 The effects of hypnotherapy on pulse rate (bpm) in healthy volunteers (n=10). The points shown as "During" are the mean of the three readings taken during the stress test. Median is shown in red.



8.4.2.2 Systolic blood pressure (Figure 8.3 and 8.4 and Table 8.2)

Mean systolic blood pressure decreased during hypnosis by 2 mmHg in patients with UC (p=0.04) and 3 mmHg in HV (p=0.004). In HV, when measured 30 minutes after the hypnotherapy protocol, the systolic BP remained lower than pre-test levels by 4mmHg (p=0.02).

Figure 8.3 The effects of hypnotherapy on systolic BP (mmHg) in patients with active UC (n=17). The points shown as "During" are the mean of the three readings taken during the stress test. Median is shown in red.

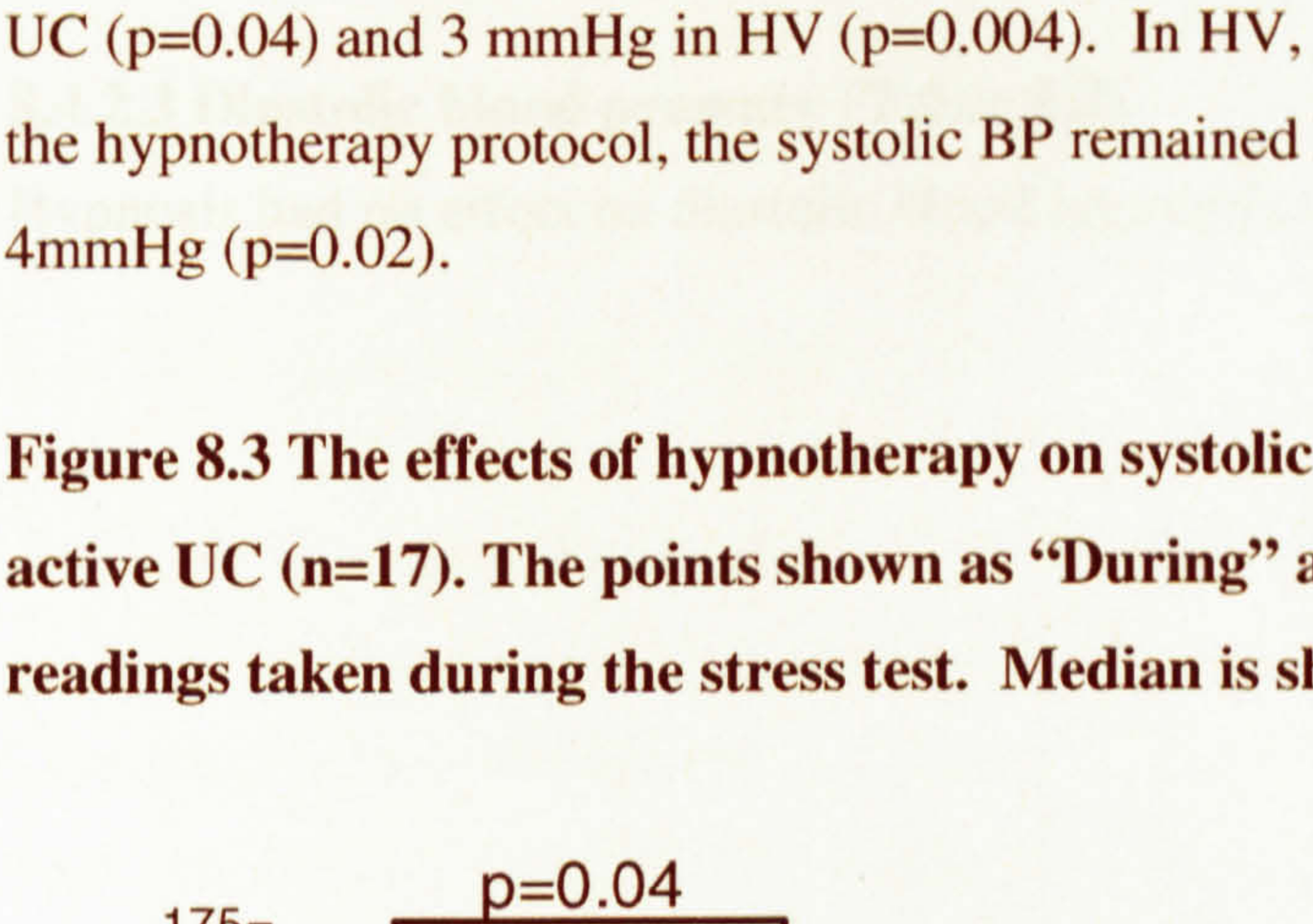
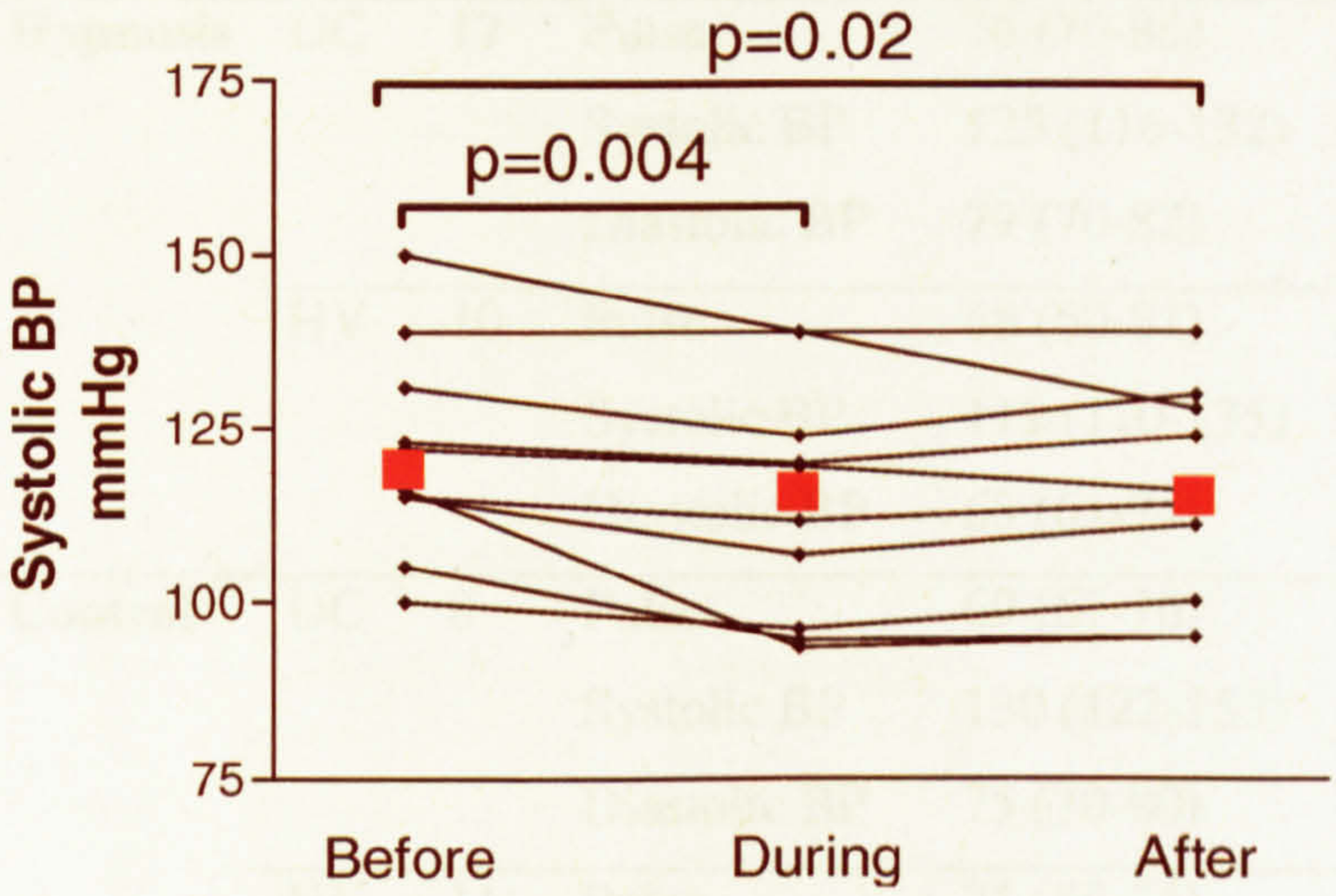


Figure 8.4 The effects of hypnotherapy on systolic BP (mmHg) in healthy volunteers (n=10). The points shown as "During" are the mean of the three readings taken during the stress test. Median is shown in red.



8.4.2.3 Diastolic blood pressure (Table 8.2)

Hypnosis had no effect on diastolic blood pressure in either group of subjects.

Table 8.2 Pulse (bpm), systolic and diastolic BP (mmHg) in response to hypnosis and control protocols in patients with UC and healthy volunteers (HV). Results are shown as median (IQR). * p<0.05 from pre-procedure value (paired data).

Protocol		N		Before	During/After	30 mins after
Hypnosis	UC	17	Pulse	76 (70-86)	69 (63-83)*	76 (72-86)
			Systolic BP	125 (116-132)	123 (112-137)*	122 (114-139)
			Diastolic BP	79 (70-82)	69 (75-90)	70 (75-81)
	HV	10	Pulse	68 (60-81)	57 (63-74)*	65 (61-81)
			Systolic BP	119 (110-135)	116 (95-132)*	115 (98-129)*
			Diastolic BP	68 (61-74)	68 (56-73)	69 (60-76)
Control	UC	8	Pulse	69 (67-76)	72 (64-75)	70 (67-74)
			Systolic BP	130 (122-153)	131 (117-148)	134 (116-147)
			Diastolic BP	75 (70-80)	75 (70-84)	75 (71-79)
	HV	11	Pulse	71 (56-74)	66 (61-74)	68 (60-73)
			Systolic BP	114 (102-121)	110 (102-122)	112 (104-124)
			Diastolic BP	70 (63-75)	69 (62-75)	72 (63-77)

8.4.3 SYSTEMIC INFLAMMATORY RESPONSE

8.4.3.1 Serum cytokine concentrations (Figure 8.5 and 8.6 and Table 8.3)

In patients with active UC, median serum IL-6 concentration in the blood sample taken immediately after hypnosis was decreased by over half compared to the pre-procedural value (p=0.0009) and had fallen slightly further in the sample taken thirty minutes later (p=0.01). In healthy volunteers, there was a more than 20% reduction in the median serum IL-6 concentration immediately after hypnosis (p=0.02) but none thirty minutes later. Serum IL-13 concentrations were unaltered after hypnotherapy in either UC or in HV.

Figure 8.5 The effects of hypnotherapy on serum IL-6 concentrations (pg/ml) in patients with active UC (n=17). Median is shown in red.

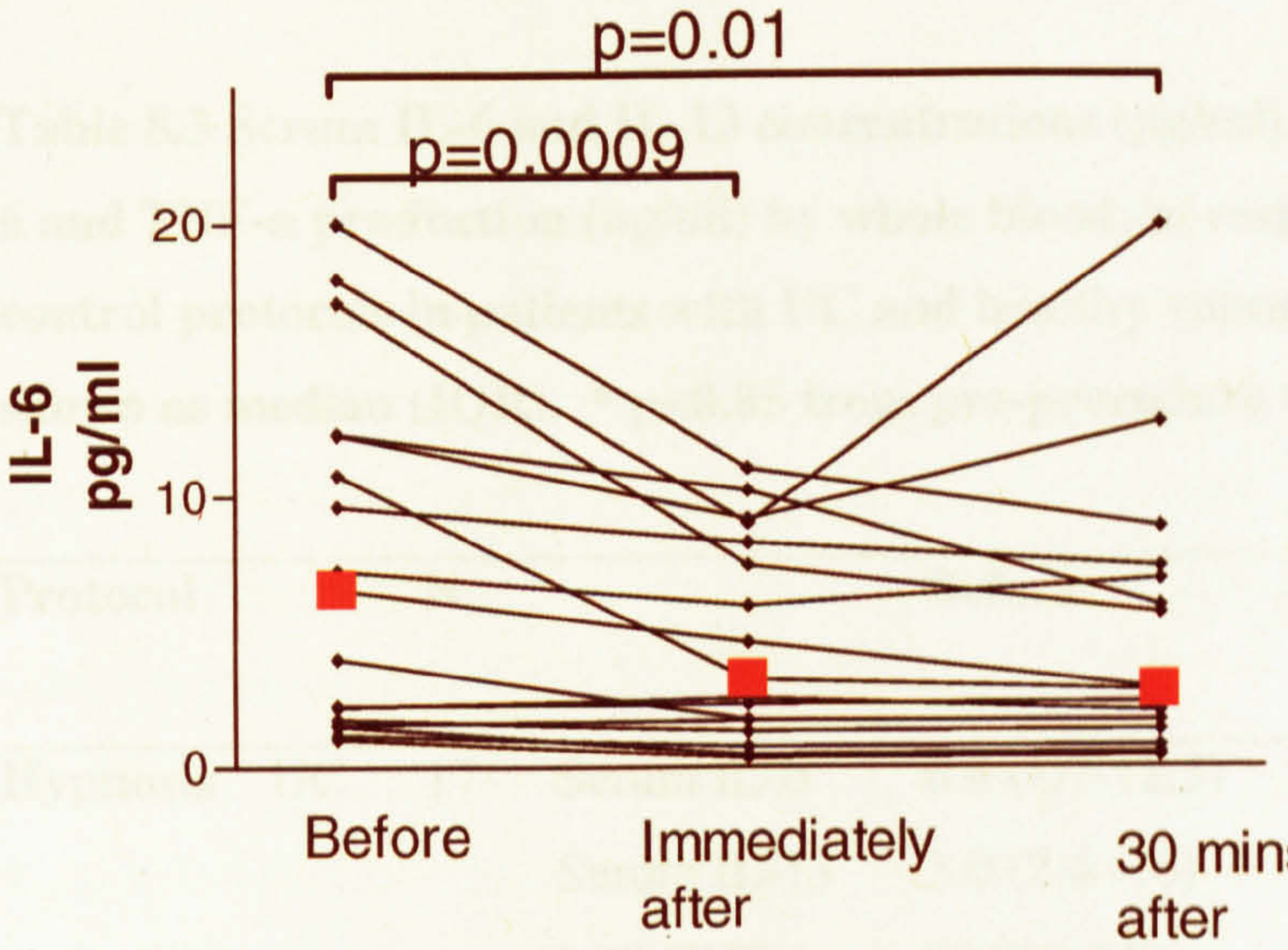
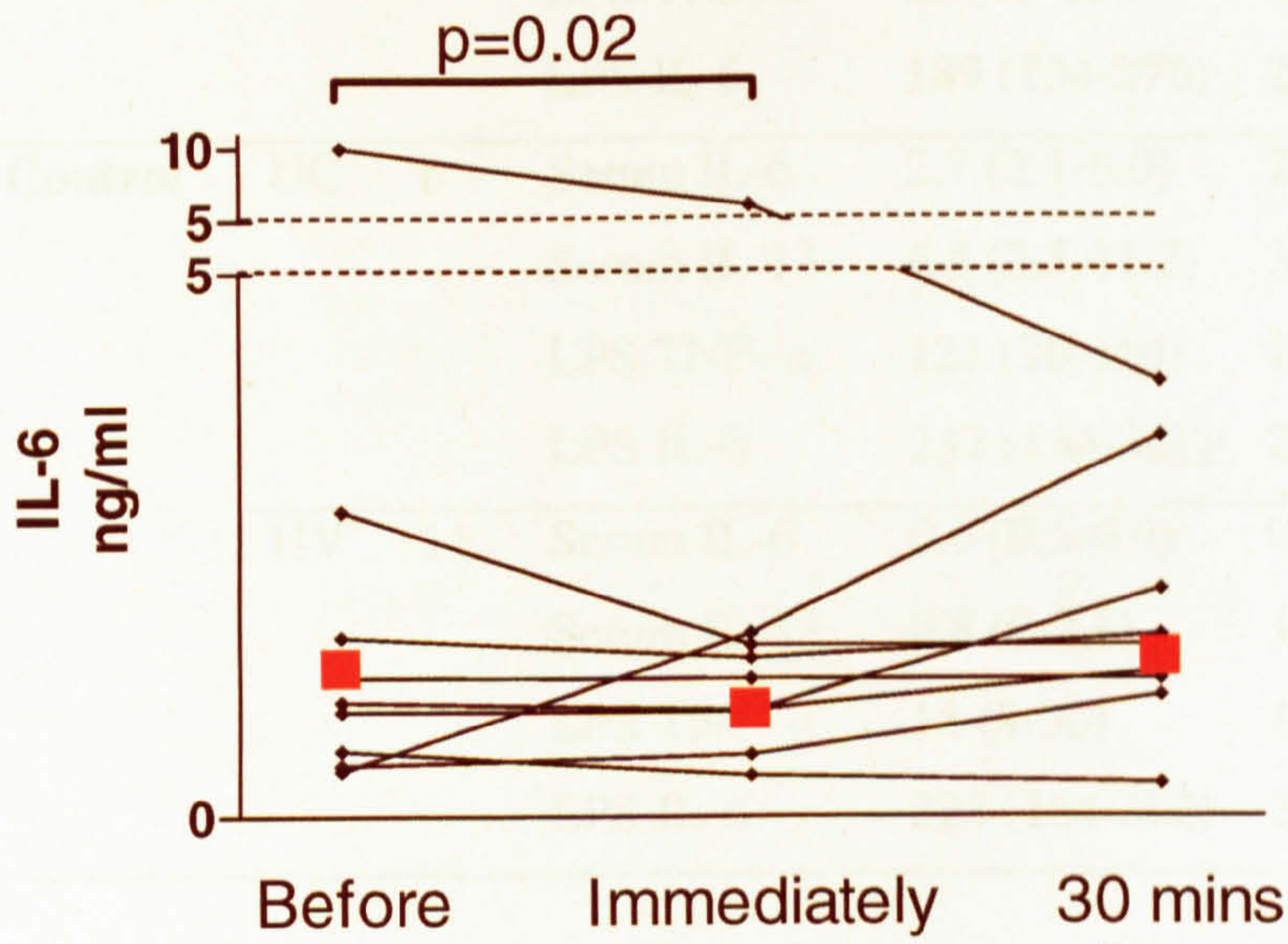


Figure 8.6 The effects of hypnotherapy on serum IL-6 concentrations (pg/ml) in healthy volunteers (n=10). Median is shown in red.



8.4.3.2 LPS-stimulated cytokine production (Table 8.3)

The production of TNF-α and IL-6 by LPS-stimulated whole blood was unchanged by hypnosis in patients with active UC or in healthy volunteers.

Table 8.3 Serum IL-6 and IL-13 concentrations (pg/ml), and LPS-stimulated IL-6 and TNF-α production (ng/ml) by whole blood, in response to hypnosis and control protocols in patients with UC and healthy volunteers (HV). Results are shown as median (IQR). * p<0.05 from pre-procedure value (paired data).

Protocol	N		Before	Immediately after	30 mins after
Hypnosis	UC	17	Serum IL-6	6.8 (1.7-12.3)	3.2 (1.5-8.7)*	2.8 (1.6-7.2)*
			Serum IL-13	5.0 (2.4 -16)	5.5 (2.5-14.1)	4.8 (2.7-14.7)
			LPS TNF-α	99 (59-243)	152 (32-436)	138 (26-459)
			LPS IL-6	285 (159-578)	254 (175-449)	262 (154-487)
	HV	10	Serum IL-6	1.3 (0.8-2.3)	1.0 (0.5-1.5)*	1.6 (1.2-2.8)
			Serum IL-13	1.0(0-5.2)	0.5(0-3.6)	0.4(0-3.9)
			LPS TNF- α	29 (18-52)	35 (30-83)	40 (15-56)
			LPS IL-6	189 (134-276)	211 (168-304)	176 (135-266)
Control	UC	8	Serum IL-6	2.7 (2.1-6.0)	2.8 (1.9-4.7)	3.2 (2.3-4.9)
			Serum IL-13	6.4 (2.5-11.2)	3.1 (7.0-12.3)	5.2 (2.1-13.7)
			LPS TNF- α	121 (70-154)	107 (63-247)	148 (75-255)
			LPS IL-6	237 (134-461)	255 (161-342)	264 (182-430)
	HV	11	Serum IL-6	0.6 (0.5-0.9)	0.6 (0.4-1.3)	0.7 (0.4-1.5)
			Serum IL-13	0.8 (0-2.1)	1.0 (0-2.4)	0.7 (0-1.8)
			LPS TNF- α	18 (8-30)	19 (11-26)	20 (11-32)
			LPS IL-6	223 (164-302)	245 (203-345)	232 (195-315)

8.4.3.3 Leukocyte count (Table 8.4)

Total white cell count (WBC) was unaffected by hypnotherapy in either group.

8.4.3.4 Natural Killer (NK) cell numbers (Figure 8.7 and 8.8 and Table 8.4)

In patients with active UC, median NK cell numbers, expressed as a percentage of lymphocytes and monocytes, were decreased by 18% in the sample taken immediately after the hypnotherapy protocol (p=0.01) but had returned to base line taken 30 minutes later. Similarly, in healthy volunteers NK cell numbers were decreased by 34% immediately after hypnosis (p=0.04) but had returned to baseline taken 30 minutes later.

Figure 8.7 The effects of hypnotherapy on NK cell count (%) in patients with active UC (n=17). Median value is shown in red.

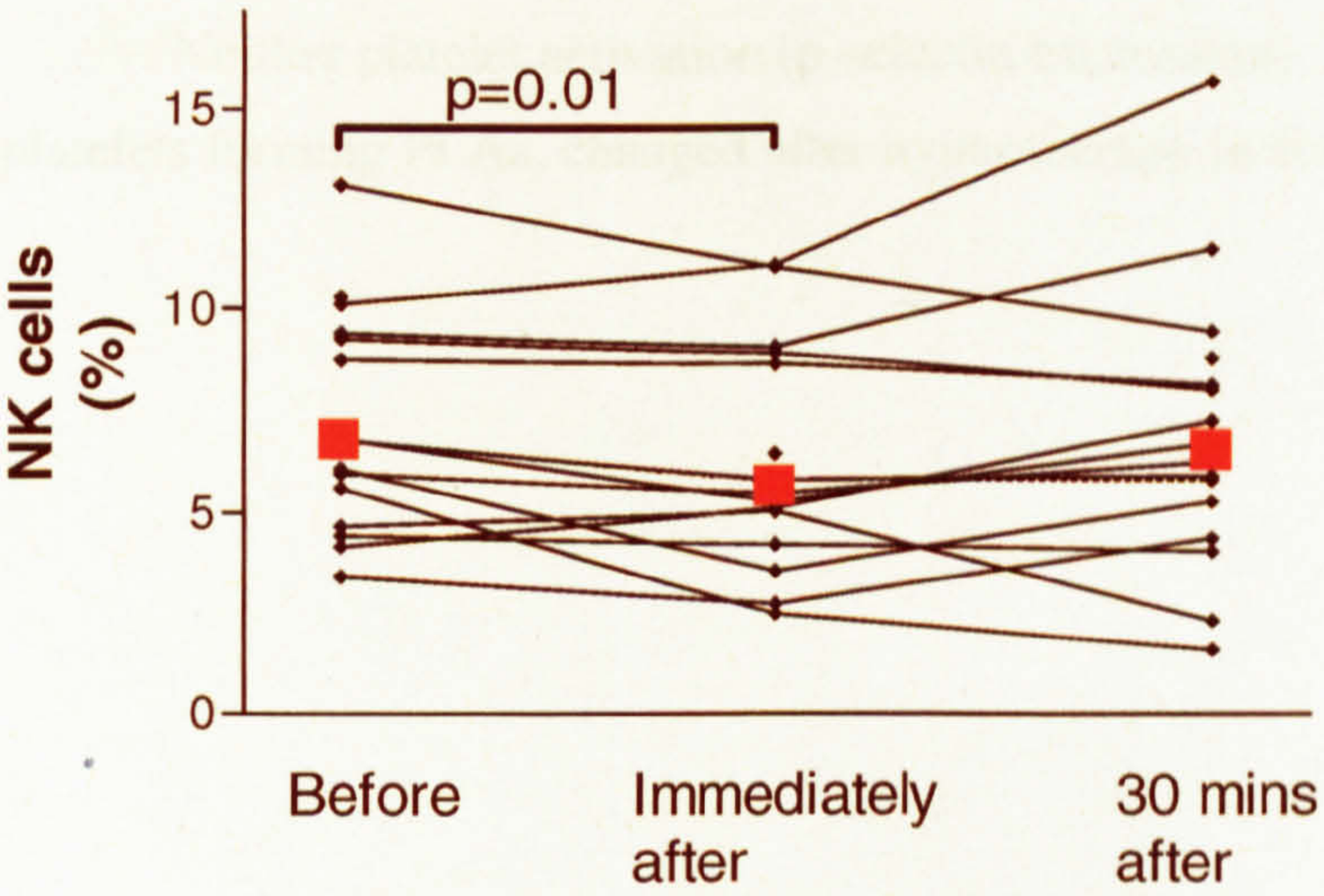
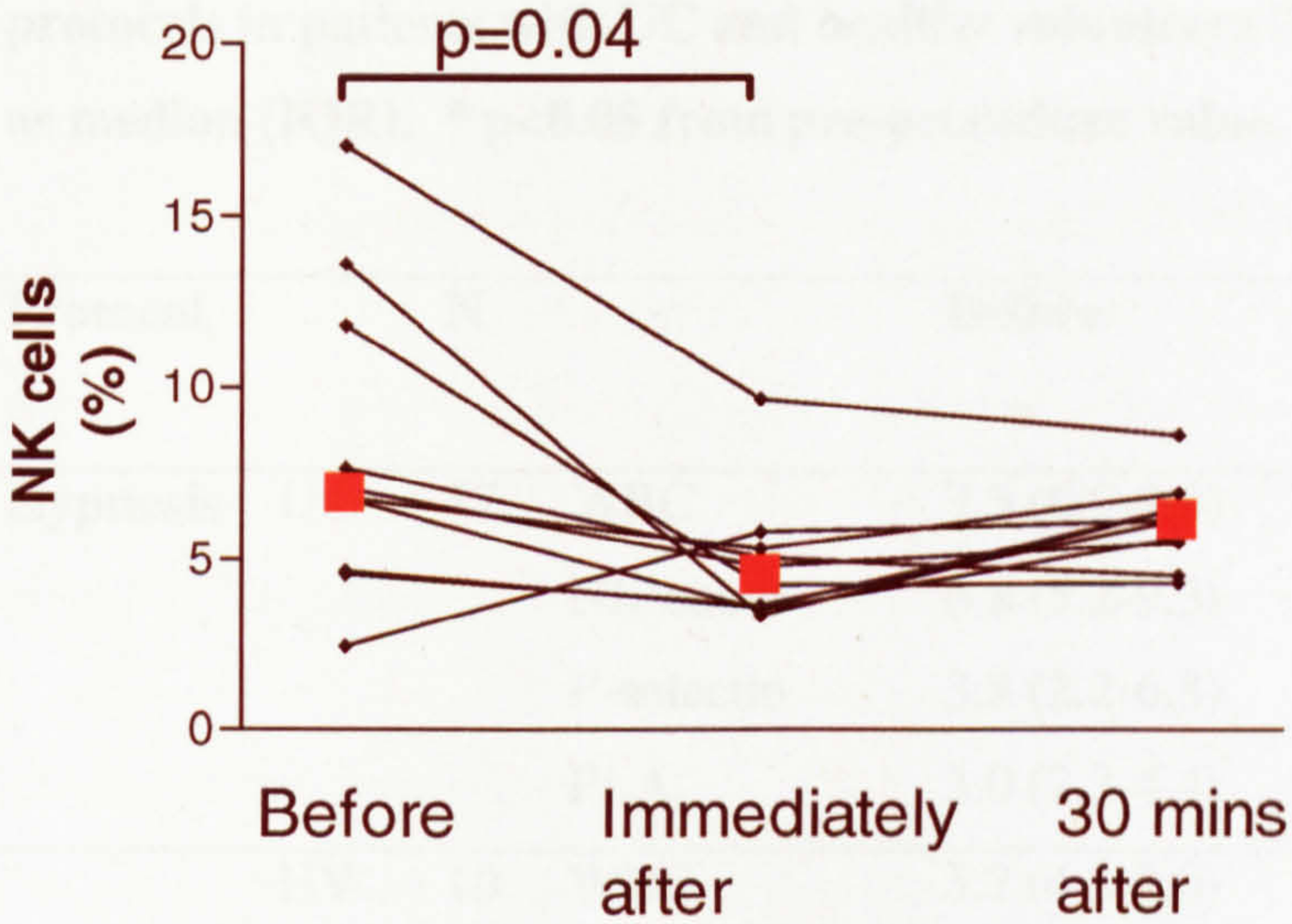


Figure 8.8 The effects of hypnotherapy on NK cell count (%) in healthy volunteers (n=10). Median value is shown in red.



8.4.3.5 Platelet activation and platelet-leukocyte aggregate (PLA) formation (Table 8.4)

Neither platelet activation (p-selectin expression), nor the percentage of platelets forming PLAs, changed after hypnotherapy in either group.

Table 8.4 Total leukocyte count (WBC) (cells x $10^{x4}/mm^3$), natural killer (NK) cell number (%), platelet activation (p-selectin expression (%)) and platelet-leukocyte aggregate (PLA) formation (%) in response to hypnosis and control protocols in patients with UC and healthy volunteers (HV). Results are shown as median (IQR). * p<0.05 from pre-procedure value.

Protocol		N		Before	Immediately after	30 mins after
Hypnosis	UC	17	WBC	7.5 (6.9-9.8)	7.5 (6.8-10.1)	7.9 (6.9-10.1)
			NK count	6.8 (5.2-9.3)	5.6 (4.6-8.8)*	6.5 (4.8-8.4)
			P-selectin	3.8 (2.2-6.8)	4.7 (1.9-7.0)	5.7 (3.9-6.8)
			PLA	3.0 (2.3-4.4)	3.0 (2.5-4.2)	3.4 (2.5-4.0)
	HV	10	WBC	5.2 (4.3-7.0)	5.4 (4.6-6.9)	5.3 (4.7-7.3)
			NK count	7.0 (4.6-12.7)	4.6 (3.6-5.6)*	6.2 (5.0-6.7)
			P-selectin	2.6 (1.9-3.9)	2.8 (2.0-6.2)	5.0 (2.0-7.3)
			PLA	2.6 (1.6-3.5)	2.3 (1.5-3.4)	3.6 (2.9-5.5)
Control	UC	8	WBC	7.2 (6.3-10.0)	7.8 (6.1-9.8)	8.0 (6.2-10.2)
			NK count	6.9 (3.8-8.1)	4.8 (3.4-7.6)	5.3 (3.9-7.9)
			P-selectin	2.0 (1.7-2.3)	2.1 (1.3-3.0)	2.0 (1.7-3.1)
			PLA	2.9 (2.0-3.3)	2.7 (2.6-2.8)	2.6 (2.1-3.0)
	HV	11	WBC	5.4 (4.6-8)	5 (4.4-6.6)	4.9 (4.7-6.5)
			NK count	7.3 (6.1-10.1)	8.8 (6.0-10.6)	8.2 (5.6-9.9)
			P-selectin	3.1 (2.3-3.5)	2.9 (2.5-4.0)	3.2 (2.5-4.8)
			PLA	2.5 (2.2-3.8)	2.4 (2.0-3.4)	2.5 (2.0-4.2)

Comparison of responses to hypnosis in patients with active UC and healthy volunteers

The absolute reductions in serum IL-6 concentrations in response to hypnosis were greater in patients with active UC than in HV (p=0.03, Table 8.3). However, as the baseline value was higher in the patients with UC, the percentage change in serum

IL-6 was similar in both groups. There were no other differences in the magnitude of the changes elicited by the hypnotherapy protocol in any of the autonomic or inflammatory variables assessed between patients with UC and HV (Tables 8.2, 8.3 and 8.4).

Hypnotisability, depth perception and response to hypnosis

There were no differences in Spiegel's score of hypnotisability or the mean of the three self-rated depth scores between patients with active UC and healthy volunteers. Neither did either of these measures correlate with the changes observed in any of the autonomic or inflammatory variables in response to hypnosis.

Psychometric questionnaires

None of the measures of long term stress (STAI-T, HADS, PSQ or BSI) correlated with the changes in any of the variables occurring in response to the hypnotherapy protocol. However, scores on the STAI-S scale did correlate directly with the reductions in pulse rate produced by hypnosis ($R=+0.54$, $p=0.03$).

Relationships of age to response to hypnotherapy protocol

The median age (40yrs) of the patients with UC was not quite significantly higher than that of the HV (23yrs, $p=0.07$) (Table 8.1). Age did not correlate with any of the changes seen in response to hypnosis in either group.

Effects of control protocol

The control protocol caused no changes in any of the variables assessed in either patients with active UC or HV (Tables 8.2, 8.3 and 8.4).

8.5 DISCUSSION

We have assessed the effects of hypnotherapy given to patients with active UC on a range of systemic inflammatory variables, the majority of which we have

described as being raised in active compared to inactive disease (Chapter 4). Hypnotherapy reduced some, but not all, of these variables, at the systemic level towards the values found in quiescent UC.

AUTONOMIC RESPONSE.

During hypnosis there was a reduction in pulse rate and systolic blood pressure. Although the changes were small, they are opposite to those observed in response to acute stress (Chapter 5), and indicate a change in the autonomic balance, with a decrease in sympathetic, and an increase in parasympathetic tone. Acute stimulation of the sympathetic nervous system, with the release of adrenaline and noradrenaline, has been shown to have mainly pro-inflammatory effects (201;204). Parasympathetic stimulation of the vagus nerve with release of acetylcholine reduces the production of certain inflammatory cytokines including TNF-α and has been shown recently to be a key regulator in several animal models of inflammatory disease (440). The changes in autonomic tone induced by hypnotherapy could thus be a mechanism by which the observed reductions in some of the inflammatory variables assessed occurred.

SERUM CYTOKINES

Perhaps the most important finding in this chapter was that hypnotherapy reduced serum concentrations of IL-6 by a median of 53% in patients with active UC and by 23% in healthy volunteers. The base-line median serum IL-6 concentration was over three times that previously found in patients with quiescent disease (Chapter 4). However, IL-6 concentrations after hypnosis did not significantly differ from those found in inactive UC. IL-6 is a major inflammatory cytokine and the principal stimulus for C-reactive protein production by the liver. It is involved in many disease processes and may be important in the pathogenesis of IBD (441). A fall in serum IL-6 could therefore contribute to the reported benefits of hypnotherapy in IBD and other inflammatory diseases. In healthy individuals, IL-6 stimulates a stress-response in the hypothalamus, culminating in the release of glucorticoids, which have mainly anti-inflammatory actions, from the adrenal cortex. This creates an anti-inflammatory

feedback loop serving to reduce peripheral inflammation (442). However, in individuals with chronic inflammatory conditions, such as IBD, the hypothalamic response to IL-6 is blunted: the associated loss of the anti-inflammatory feedback loop may predispose to stress-induced worsening of inflammation (443). A hypnotherapy-induced reduction in serum IL-6 concentration could thus prevent stress-induced worsening of IBD.

NATURAL KILLER CELLS

As reported previously, hypnotherapy transiently decreased NK cell numbers, a change opposite to that we observed in response to acute stress. While it is conceivable that brief decreases in NK cell number could down-regulate mucosal inflammation via the interactions between NK and dendritic cells (444), the short lived nature of the changes observed make them unlikely to explain any possible long-term therapeutic effects of hypnosis in IBD.

8.6 CONCLUSIONS

Hypnosis reduced serum IL-6 concentration and led to a transient fall in NK cell count. These changes may be mediated by the change in autonomic balance observed in response to hypnosis and may be relevant to any therapeutic effects of hypnotherapy in UC.

CHAPTER 9

THE EFFECTS OF HYPNOTHERAPY ON MUCOSAL MEASURES IN PATIENTS WITH ACTIVE ULCERATIVE COLITIS

9.1 SUMMARY

Aim: To assess the effects of hypnotherapy on several inflammatory measures at the mucosal level.

Methods: 25 individuals with active UC underwent a session of hypnotherapy or the control protocol and the rectal release of inflammatory cytokines, rectal mucosal blood flow and reactive oxygen metabolite production were assessed before and after the procedures.

Results: The principal findings were:

1. Hypnotherapy reduced rectal peri-mucosal fluid concentrations of Substance P (SP) by 81% (p=0.001), histamine by 35% (p=0.002) and IL-13 by 53% (p=0.003).
2. Rectal mucosal blood flow was reduced by 18% (p=0.0004) in response to hypnosis.
3. Chronic stress, as assessed by psychometric questionnaires, hypnotisability, as assessed by Spiegel's score (383), and depth perception had no effect on the autonomic or inflammatory response to hypnosis.
4. The control protocol had no effect on any of the variables assessed.

Conclusions: Hypnosis reduced the rectal mucosal release of several potentially pathogenic inflammatory mediators.

9.2 INTRODUCTION

Hypnotherapy is of proven to be of benefit in functional GI disorders such as IBS and dyspepsia (359;363;445). However, the mechanisms by which it leads to therapeutic benefit in these conditions are unknown. Hypnotherapy has been shown to have various effects on GI physiology. It reduces orocaecal transit time and decreases colonic motility (361). Hypnotherapy also reduces sensitivity to rectal distension in patients with IBS, and this may be of relevance to the reduced symptoms described by some patients with UC in response to hypnosis (362). There have, however, been no studies of the effects of hypnotherapy on mucosal inflammatory measures in patients with UC.

In this chapter we will describe the effects of one session of hypnosis on the mucosal measures described in the experimental protocol. The hypothesis underlying this work is that relaxation achieved through hypnosis is capable of reducing inflammation at the mucosal level.

9.3 PATIENT DEMOGRAPHICS (Table 8.1)

17 patients with active UC, as defined by a Baron's sigmoidoscopic score of >1 underwent the hypnotherapy protocol and 8 patients with active UC underwent the control protocol. Their demographics are described in chapter 8.

9.4 RESULTS

The data for ROM production and rectal mucosal blood flow was available in only fifteen of the seventeen patients who underwent hypnotherapy. In the other two patients the data could not be obtained due to technical problems with the equipment.

9.4.1 Cytokine and mediator concentration in rectal peri-mucosal fluid (Figure 9.1, 9.2 and 9.3 and Table 9.1)

In patients with active UC, the median concentrations of SP, histamine and IL-13 in rectal peri-mucosal fluid were decreased by 81% ($p=0.001$), 35% ($p=0.002$) and 53% ($p=0.003$) respectively after hypnotherapy. Mucosal release of TNF-α did not change.

Figure 9.1 The effects of hypnotherapy on substance P concentration (pg/ml) in rectal peri-mucosal fluid in patients with active UC (n=17). Median shown in red.

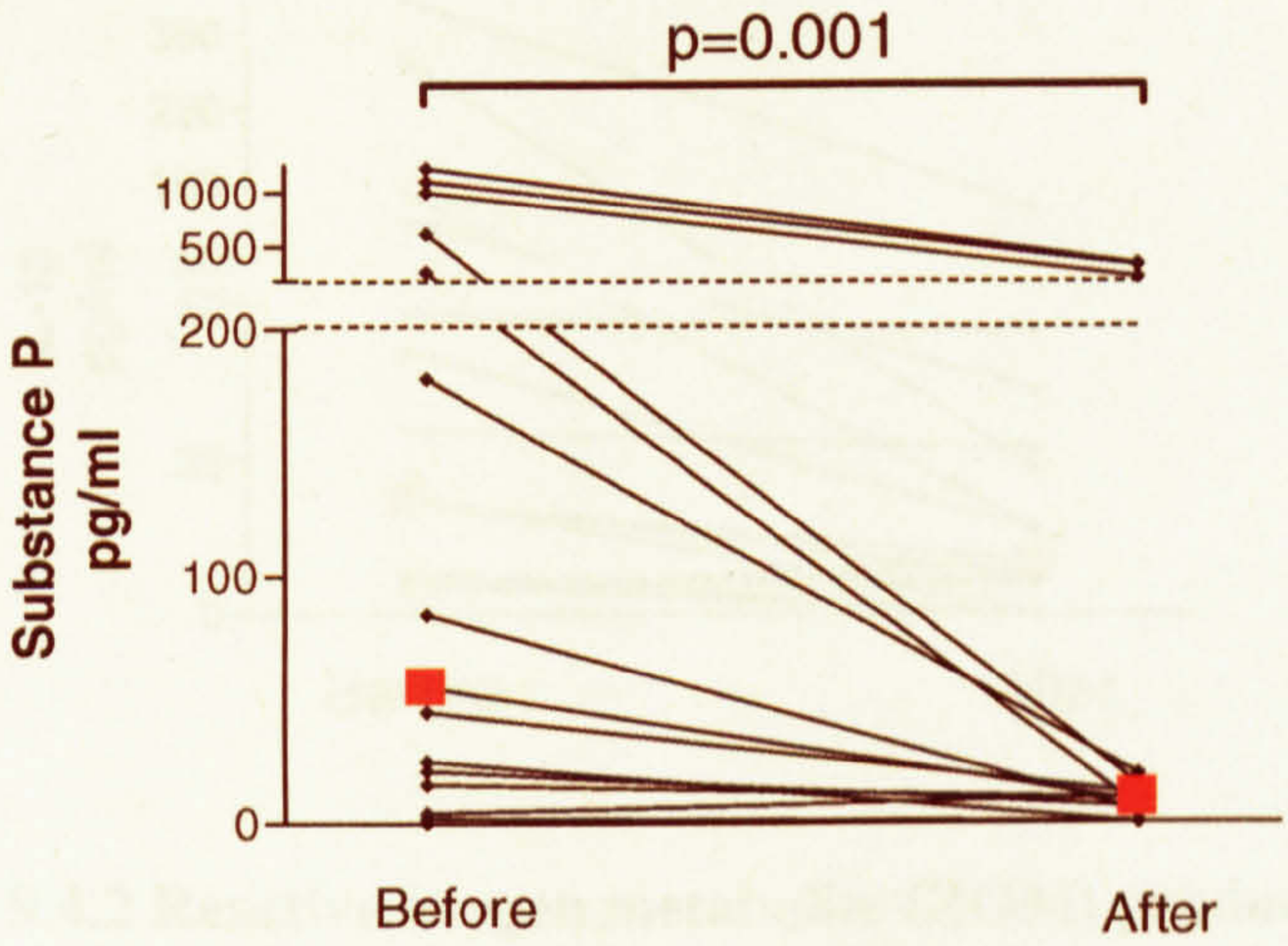


Figure 9.2 The effects of hypnotherapy on histamine concentration (pg/ml) in rectal peri-mucosal fluid in patients with active UC (n=17). Median shown in red.

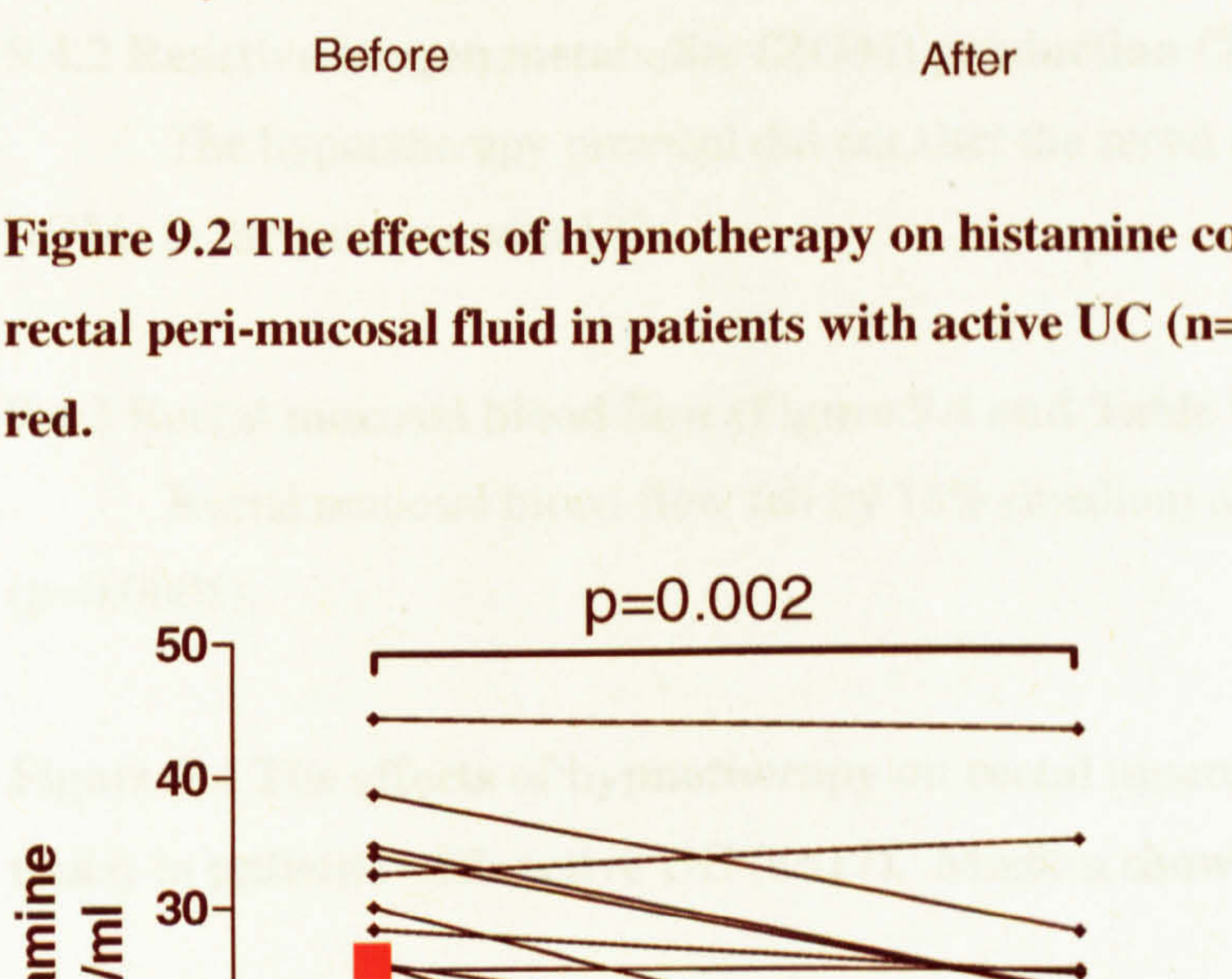
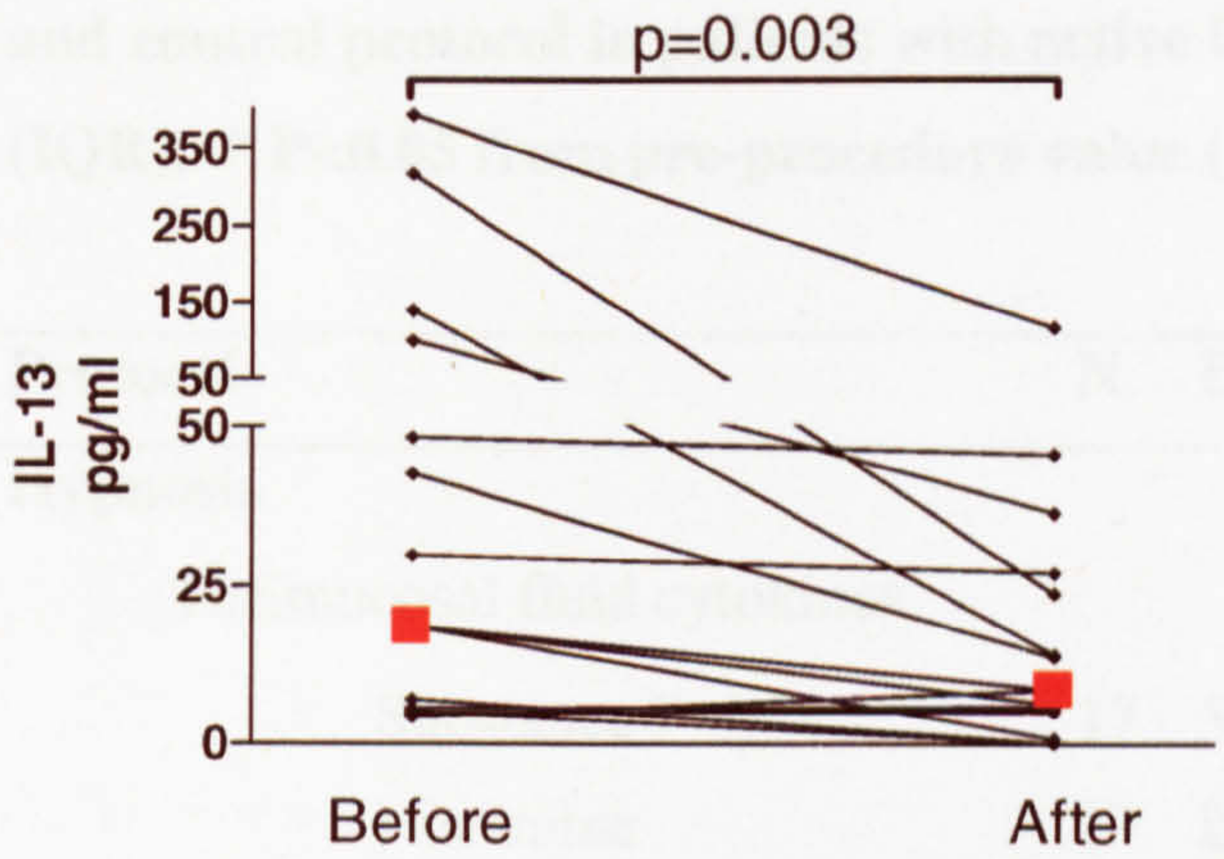


Figure 9.3 The effects of hypnotherapy on IL-13 concentration (pg/ml) in rectal peri-mucosal fluid in patients with active UC (n=17). Median shown in red.



9.4.2 Reactive oxygen metabolite (ROM) production (Table 9.1)

The hypnotherapy protocol did not alter the rectal mucosal production of ROMs in the patients with UC.

9.4.3 Rectal mucosal blood flow (Figure 9.4 and Table 9.1)

Rectal mucosal blood flow fell by 18% (median) after hypnotherapy (p=0.0004).

Figure 9.4 The effects of hypnotherapy on rectal mucosal blood flow (arbitrary units) in patients with active UC (n=17). Median shown in red.

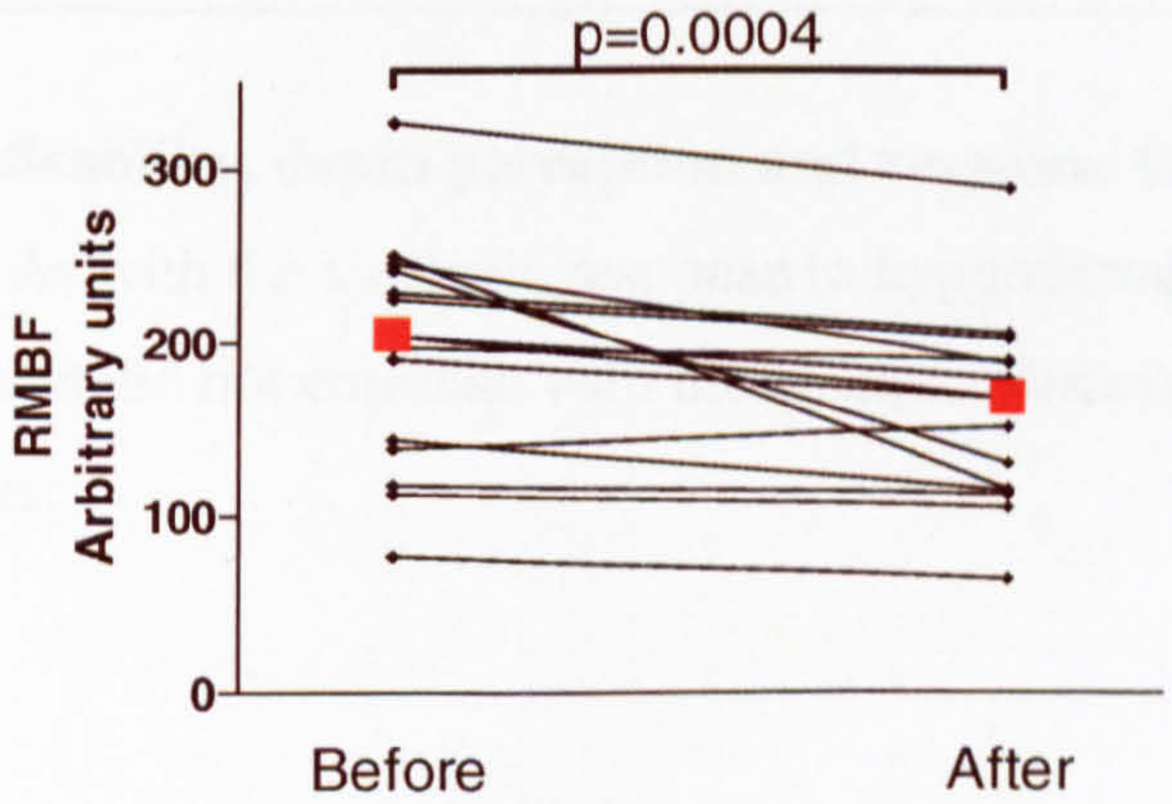


Table 9.1 Rectal peri-mucosal fluid cytokine concentrations (pg/ml), reactive oxygen metabolite (ROM) production by rectal mucosal biopsies (photons/mcg/min), rectal blood flow (arbitrary units) in response to hypnosis and control protocol in patients with active UC. Results are shown as median (IQR). * P<0.05 from pre-procedure value (paired data).

Protocol	N	Before	After
Hypnosis			
Perimucosal fluid cytokines			
Substance P	17	55 (18-610)	10 (5-129)*
Histamine	17	26 (15-34)	17 (6-25)*
IL-13	17	19 (5-99)	9 (5-27)*
TNF-α	17	111 (42-251)	140 (62-253)
ROM production	15	275 (38-1175)	463 (175-666)
Blood flow	15	205 (139-245)	169 (115-198)*
Control			
Peri-mucosal fluid cytokines			
Substance P	8	57 (38-451)	99 (24-363)
Histamine	8	21 (9-31)	19 (5-32)
IL-13	8	17 (11-21)	20 (13-23)
TNF-α	8	99 (65-224)	135 (54-319)
ROM production	8	199 (31-502)	293 (65-600)
Blood flow	8	152 (133-223)	183 (145-232)

Hypnotisability, depth perception and response to hypnosis

As with the systemic response to hypnotherapy, hypnotisability and depth perception did not correlate with the changes observed in the rectum in response to hypnosis.

Psychometric questionnaires

None of the measures of long term stress (STAI-T, HADS, PSQ or BSI) correlated with the changes observed at the mucosal level in response to the hypnotherapy protocol. Neither did scores on the STAI-S, a measure of anxiety at that time, correlate with the changes observed.

Relationships of age to response to hypnotherapy protocol

Age did not correlate with any of the rectal changes occurring in response to the hypnosis.

Effects of control protocol

The control protocol caused no changes in any of the rectal mucosal variables assessed.

9.5 DISCUSSION

Hypnotherapy reduced the levels of several of the rectal mucosal inflammatory measures assessed. Each of the measures found to be affected will be discussed in turn.

MUCOSAL SUBSTANCE P AND HISTAMINE RELEASE.

Baseline concentrations of SP and histamine in peri-mucosal fluid in patients with active UC were more than twice those found in quiescent UC (see Chapter 5). Hypnosis reduced the peri-mucosal concentrations of both, in the case of SP to values similar to those found in inactive disease.

SP is found in neurones throughout the enteric nervous system, many of which are found in close association with mast cells (189). As described previously, SP has a range of inflammatory actions (see Chapter 1), some of which may contribute to the aetiology of IBD. Increases in numbers of neurones containing SP have been reported in the colon of patients with IBD (189). The possibility of SP acting as a stimulus for mucosal mast cell degranulation is supported by the

observation that incubation with SP of colonic biopsies from patients with IBD increases mast cell-mediated histamine release (190). This may contribute to the increased activation of mast cells (122) and enhanced rectal mucosal release of histamine observed in active UC (123). Acute physical stress stimulates mast cell degranulation in patients with IBD (279), while, in animal models, the presence of mast cells is required to mediate the increases in intestinal permeability and mucosal bacterial uptake caused by stress (311;314).

Our results suggest that hypnotherapy, by reducing neuronal release of SP, decreases release of histamine, and by implication possibly that of other pathogenic mast cell mediators such as tryptase. However, although it is also released by mast cells, we did not find that a single session of hypnosis alters the mucosal release of TNF-α (121).

MUCOSAL IL-13 RELEASE.

Recent work has suggested that a mucosal population of IL-13-secreting NKT cells may be important in the aetiology of UC (446). These NKT cells are cytotoxic to colonic epithelial cells *in vitro* whilst (447), also *in vitro*, IL-13 has been shown to impair colonic epithelial barrier function (180). Using the filter paper method, we have found baseline mucosal production of IL-13 in active UC to be nearly three times that found in inactive disease. After hypnosis the median peri-mucosal IL-13 concentration was less than twice that found in inactive UC. If IL-13 is important in the pathogenesis of IBD, then the reductions we observed in the mucosal release of IL-13 in response to hypnosis could contribute to any therapeutic effect.

RECTAL MUCOSAL BLOOD FLOW

RMBF was reduced by hypnotherapy. In the normal rectum, RMBF reflects autonomic tone, being decreased by higher sympathetic and recuced parasympathetic tone (407). While in the normal rectum, therefore, hypnosis might be predicted to increase RMBF, mucosal blood flow in active UC is likely to reflect not only autonomic tone but also vasodilation caused by local production of inflammatory mediators (448). Baseline RMBF was 75% higher in patients with active UC

compared with inactive disease, but only 50% greater after hypnosis. This may reflect reduced mucosal production of inflammatory mediators as a result of hypnosis.

9.6 CONCLUSION

Hypnosis markedly reduced the rectal mucosal release of SP and to a lesser extent histamine. If SP does act as a stimulus for mast cell degranulation, and the latter is important in the aetiology of UC, then these changes may be important in mediating hypnotherapy-induced reductions in mucosal inflammation. Hypnosis also reduced IL-13 release, an inflammatory cytokine of possible importance in the aetiology of UC, and RMBF.

CHAPTER 10

SUMMARY AND CONCLUSIONS

10.1 INTRODUCTION

The rationale for studying the effects of acute experimental stress in UC was based on the observation that for many years by patients with UC have identified psychological stress as being an important factor in provoking relapse of their disease (449). Studies assessing the effects of adverse life events on disease activity in UC have produced conflicting results (270;450), although studies assessing the effects of chronic perceived stress seem more conclusive (273). There is strong evidence from animal models of colitis to suggest that stress can act as a contributing factor to relapses of mucosal inflammation (299;300).

The aim of this work was therefore to study the effects of psychological stress and relaxation achieved though hypnosis, on a range of inflammatory variables in UC. The hypotheses were that stress might increase and that hypnosis might reduce these measures. We also tested whether patients with UC had a different response to stress at the systemic level compared to healthy volunteers, and whether chronic stress affected the response to acute stress.

10.2 SUMMARY OF MAIN FINDINGS

COMPARISON OF BASELINE VALUES IN PATIENTS WITH QUIESCENT AND ACTIVE UC

Several of the inflammatory measures assessed in this study have not been measured previously in patients with active and inactive UC. The concentration of SP and IL-13 in rectal peri-mucosal fluid was found to be greater in active than inactive UC.

Substance P is a neurotransmitter which has several pro-inflammatory actions (189). SP containing neurones have been reported as being increased in UC, although studies are conflicting (see Chapter 1) (189). This is the first time that the *in vivo* rectal release of SP has been measured and found to be increased in active UC

compared to that in inactive disease. Our results are compatible with the proposal that SP may play a role in the pathogenesis of UC.

Recent evidence suggests that an IL-13 producing population of NKT cells may be important in the pathophysiology of UC (180;451). LPMCs from patients with UC produce more IL-13 in vitro in response to stimulation with antibodies to CD2 and CD28 than LPMCs from individuals with Crohn's disease (452). As with SP, this is the first occasion that the in vivo rectal release of IL-13 has been measured and shown to be increased in active UC compared to inactive disease.

In addition to the rectal release of IL-13, we also found serum levels of IL-13 to be increased in active UC compared to inactive disease. To the best of our knowledge this has not been reported previously.

THE EFFECTS OF STRESS

In this study, psychological stress was found to increase a range of inflammatory variables at both the systemic and mucosal levels. It is possible that increases in some of these variables may be involved in the stress-related relapses in colonic inflammation reported by both patients with UC and seen in animal models of colitis. Of particular note, this is the first occasion that acute stress has been shown to increase rectal mucosal release of TNF-α and reactive oxygen metabolite production by mucosal biopsies.

Stress increased a range of inflammatory variables systemically. It is possible that some of the increases are relevant to other inflammatory conditions where stress is thought to play a role. For example stress-related increases in platelet activation and PLA formation may be important in myocardial infarction.

We did not find that participants with high chronic stress, as assessed by psychometric questionnaires, showed a greater autonomic and inflammatory response to acute stress. This is in contradiction to other reported studies involving both humans and animals (137;223;231;297;309;453). This may in part be due to the relatively low levels of chronic stress present in the individuals in our study. Most of the reported data from human studies showing an increased response to acute stress in the presence of chronic stress has focused on individuals in chronically highly

stressful situations. For instance, chronic caregivers showed a greater autonomic response to acute stress than healthy volunteers (137). In animal studies, the model used to simulate chronic stress is usually maternal separation which seems to induce prolonged depression and augements the pro-inflammatory effects of acute stress. In both cases it is likely that the level of chronic stress affecting the response to acute stress is greater than that present in the patients in our study.

We did not find that patients with UC had more abnormal autonomic or inflammatory responses to acute stress than healthy volunteers. As stated in chapter 6, it is likely that whilst stress may contribute to exacerbations in UC, an abnormal stress response is not a necessary aetiological factor.

One weakness of this study is that it is difficult to know if the stress induced by this protocol and the magnitude of the immune response are physiologically relevant. However stress did relevant

THE EFFECTS OF HYPNOTHERAPY

The effects of hypnotherapy were not as wide ranging as those seen in association with stress (see below). Hypnotherapy reduced some, but not all of the inflammatory measures assessed in this protocol. Of particular note, hypnotherapy reduced the rectal mucosal release of SP, histamine and IL-13.

Hypnotherapy has been shown to be beneficial in IBS although the mechanism by which this occurs is unknown (360). Hypnosis has also been shown previously to affect GI physiology with reduced colonic transit and rectal sensitivity (454). This is the first occasion that hypnosis has been shown to reduce inflammatory variables in UC. It is possible that some of the effects described in this present study are relevant to the anecdotally reported beneficial effects of hypnotherapy by patients with UC. The fall in serum IL-6 in response to hypnosis may also be relevant to the reported beneficial effects of hypnotherapy in other inflammatory diseases (355;357).

RELATIONSHIP BETWEEN THE EFFECTS OF HYPNOTHERAPY AND OF STRESS.

Although we have found that hypnotherapy reduced several of the inflammatory measures assessed in patients with active UC, we did not find that it reduced most of the measures that were increased by experimental stress in inactive disease. In some instances, this may reflect the different suitability of the assays used for assessing inflammation in patients with active and inactive UC. In particular, our failure to find a reduction in ROM production in response to hypnosis may reflect the wide variation in ROM production by biopsies from patients with active UC, and could represent a type II statistical error. Although chemiluminescence is very sensitive for detecting increases from baseline of ROM production in inactive UC, it is less sensitive for detecting reductions in ROM release in active disease.

There is also no *a priori* reason why the effects on the systemic and mucosal inflammatory responses of hypnosis in active UC and of acute stress in quiescent disease should be mirror images of each other. Hypnotherapy may act through different pathways from those involved in the pathophysiology of stress. Furthermore, systemic and mucosal inflammatory responses in quiescent UC are likely to be different from those in established active disease.

10.3 LIMITATTIONS OF THE STUDY

HETEROGENICITY OF PATIENTS

In order to include sufficient patients in both the stress and hypnosis sections of this study, patients were not differentiated on the basis of disease characteristics or medical treatment. Clearly patients with UC represent a heterogeneous group depending on disease distribution and duration. It is quite possible that these differences may have affected the response to stress or hypnosis. Similalry it must be acknowledged that drugs such as immunosuppressants (thiopurines and methotrexate) and to a lesser extent 5-ASA may have also affected the immune responses to either stress or hypnosis.

HETEROGENICITY OF RESPONSE TO HYPNOSIS

The ability of an individual to be hypnotised is known to vary depending on the individual and the hypnotic technique used. We assessed hypnotisability using Spielberger's score but did not find it to relate to the physiological or immune responses observed in response to hypnosis. It could be suggested that only individuals with high hypnotisabilty scores should have been included in the study. This may have increased the likelihood of detecting an immune response to hypnosis. However, this would have limited the number of patients eligible for the study and in fact the vast majority of patients were hypnotisable to some degree. Furthermore it could also be argued that by limiting the study in this way it would reduce the applicability of the findings to the general population with ulcerative colitis.

The technique used to induce trance in this study relied on visualisation. Whilst Spielberger's score assesses suggestibility it does not directly assess visualistion. The effects of hypnosis in this study may therefore have been limited in patients who are unable to visualise. This may have added further heterogenicity to the patient group

PHYSIOLOGICAL RELEVANCE OF THE CHANGES OBSERVED IN RESPONSE TO STRESS AND HYPNOSIS

Since the inflammatory variables assessed in this study were measured only up to 30 minutes after stress and hypnosis, we do not know if these changes are sustained over a longer period. This may limit the physiological relevance of our findings. Further studies of different design would be needed to explore the long-term anti-inflammatory effects of a single (or serial) sessions of hypnosis.

It is difficult to know if the magnitude of the changes observed in response to stress and hypnosis have physiological relevance. However ROM production by mucosal biopsies from patients with inactive disease after the stress protocol was similar in magnitude to the baseline ROM production by mucosal biopsies from patients with inactive disease undergoing hypnosis. Similarly serum IL-6 concentration and peri-mucosal fluid levels of Substance P and IL-13 were similar

after hypnosis in patients with active UC to the baseline values observed in patients with inactive disease.

10.4 POSSIBILITIES FOR FURTHER STUDY

STUDIES RELATING TO THE EFFECTS OF STRESS

We did not find that our model of stress increased the mucosal release of histamine. This is in contrast to the majority of studies in humans and animals, where stress has been found to lead to mast cell degranulation (278;279;311;314). Indeed, mast cell degranulation is thought to be a key step in mediating the effects of stress on the GI tract. In our pilot studies using immunofluorescnce we did find a trend to an increased percentage of degranulating mast cells in biopsies taken after stress compared to those taken before (see Chapter 7). Further samples and a control group are required to confirm this finding.

Stress has been shown to increase GI bacterial adherence and internalisation in animal models (316;318). In this study we have presented limited data which hints this may also be the case in patients with UC (see chapter 7). However, currently the number of patients studied is too few to make definite conclusions and there is no control group with which to compare.

The changes observed in response to stress and hypnosis occurred over the relatively short time span of approximately 90 minutes. Further studies should be undertaken to establish whether these changes continue to be present over longer time periods. This would clearly add weight to the theory that the alterations we observed are important in mediating the effects of stress or hypnosis in UC.

The stress protocol used was effective in inducing stress subjectively and provoking a cardiovascular and immune response in the majority of patients studied. It would therefore seem sensible to continue using this model for future studies. However, the studies could also be repeated using the different models of stress described in Chapter 3. Immersion of the hand in iced water has the advantage of being a technique which is easy to administer and less subject to psychological overlay by the subject and could therefore be very reliable and useful in future

studies. However, as described previously it is strictly a physical stress and not sustainable for fifty minutes as was required for this protocol. Various emotions can be induced during hypnosis and this technique has been used to study the effects of various emotions, including anger and happiness on the GI tract (455). It might therefore be possible to induce stress via hypnosis and use this technique in future studies. However there is no data relating to the immune effects of stress induced via hypnosis and clearly the technique depends on a patient being hypnotisable. For these reasons this technique was not used for this protocol.

STUDIES RELATING TO THE EFFECTS OF HYPNOTHERAPY IN UC

The data presented in this study suggests that hypnotherapy can reduce certain inflammatory variables in UC. As already discussed, only a single session of hypnosis was given. In trials of hypnosis in IBS, the maximum benefits of hypnotherapy occur after several sessions and this may also be the case in IBD (360;456;457). It might have been more effective to study the effects of several sessions of hypnosis on the immune response in patients with IBD. This may not have required multiple sessions of hypnosis with the hypnotherapist as hypnosis and self-hypnosis can be practiced with the aid of recorded script. However as hypnosis was given to patients with active disease, to avoid the effects of confounding medication changes, patients with active UC would have had to remain untreated for several weeks whilst hypnosis was performed. Alternatively a group of patients with chronic refractory active UC would have had to be identified for whom medication changes were unnecessary.

As yet the only reports of the benefits of hypnotherapy in UC are anecdotal accounts and one published abstract which showed a trend to clinical improvement (365). It would now seem appropriate to undertake a formalised trial of the therapeutic benefit of hypnotherapy in UC.

The identification of the correct group of patients for this study will be essential. A course of hypnotherapy requires several weeks which may make its use in patients with active UC unethical when compared to the response to steroids which often requires only a few days. Hypnotherapy could be given to patients with

chronic active disease in whom other treatments for acute UC have failed. However, given that these patients are already refractory to most conventional medical treatments, in cluding immunomodulators and immunosuppressives, the likelihood of detecting a therapeutic effect for hypnotherapy is low. Alternatively one could study hypnosis as a treatment to prevent relapse in patients with quiescent disease. However, the low relapse rate observed in most patients with quiescent UC may lead to the number of patients required to power a study of the benefits of hypnotherapy in quiescent UC being prohibitively large. An alternative design might to identify a group of patients with quiescent disease, but in whom the predicted relapse rate is higher, for example patients stopping thiopurine treatment (458).

We are in fact now undertaking a trial of hypnotherapy to prevent relapse in patients who are stopping therapy with azathioprine or 6-mercaptopurine (6-MP). Patients who have been stable on azathioprine/6-MP and who now wish to stop are randomised to either the hypnotherapy or control arm (simple discussion regarding their condition). Patients are then followed for six months and the occurrence of relapse recorded. They are allowed to continue with their other medications. The principal investigator is blinded to which therapy the patients have received. As the number of patients who fulfil these criteria is small, patients have not been excluded on the basis of their disease characteristics or hypnotisability.

If hypnosis does in fact transpire to have therapeutic benefit in UC it is tempting to speculate how it might be administered and where it might fit in the UC treatment algorithm. In the first instance hypnosis is labour intensive and time consuming. Although a relatively simple technique its administration requires practice and experience. It is therefore unlikely to be administered by gastroenterologists but rather by trained hypnotherapists. The best model might in fact be a trained hypnotherapist operating within specialist gastroenterology unit and under the supervision of a gastroenterologist. Alternatively it is possible that a gastroenterology nurse specialist could be trained in hypnotherapy and provide this service within a gastroenterology department. After the initial period, self hypnosis can be practiced at home and improvement in patients with IBS with this technique have been demonstrated up to five years (360).

One of the key factors in the success of hypnosis is the willingness of the individual to participate in the technique. I suspect this will be the key factor in determining to whom it is applicable. It may also be especially useful for individuals who find psychological stress an important factor in their disease. For these reasons I think it is unlikely to have a specific place in any treatment algorithm but rather could serve as an adjunctive treatment at any point for individuals who find it helpful. Clearly it has advantages over pharmacotherapy of having few or no side-effects.

STUDIES RELATING TO SERUM IL-13 CONCENTRATION

Assessment of disease activity in inflammatory bowel disease (IBD) is important in selecting and monitoring the effects of medical therapy. However, objective measurement of disease activity in both Crohn's and UC remains problematic. Self-reported symptoms are often unreliable and can be confounded by co-existent conditions such as irritable bowel syndrome (IBS). Clinical indices, combining both objective and subjective measurements, are useful in assessing clinical response in therapeutic trials, but are usually too cumbersome for day-to-day use (262). Endoscopic assessment is invasive, unpleasant for the patient, and may be unsafe in active disease (376).

A variety of laboratory measures including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelet count, haemoglobin, haematocrit and albumin have been used to assess disease activity in IBD, but none is sufficiently sensitive or specific (459;460). Faecal calprotectin shows promise as a marker of disease activity but requires stool collection and is not, as yet, widely available (461).

As yet there appears to have been no data published regarding the systemic levels of IL-13 in patients with UC. In this study we found serum IL-13 concentrations to be raised in active disease. Serum IL-13 concentration might prove to be a useful marker of disease activity in UC. In order to answer this question, serum IL-13 concentrations would need to measured in more patients with UC and should be compared with other commonly used laboratory markers to find the best discriminator of disease activity. The concentration of serum IL-13 should also be

measured in patients with Crohn's and other inflammatory conditions, such as infective diarrhoea, to act as control groups.

10.5 CONCLUSIONS

Ulcerative colitis and Crohn's disease were initially considered examples of psychosomatic diseases in which psychological factors played a major role. However, as knowledge of the genetic, environmental and molecular pathogenesis of IBD increased, the possible contribution to its aetiology of psychological stress was progressively neglected. Indeed, stress was often dismissed as a vague subjective concept, a view which some of the early and methodologically flawed studies of stress in relation to IBD did nothing to diminish.

In recent years, however, considerable evidence has accumulated that psychological stress does indeed contribute to the risk of relapse in IBD. Furthermore, laboratory research has indicated a variety of mechanisms by which stress can affect both the systemic and gastro-intestinal immune and inflammatory responses. In this study we have to some degree linked these separate lines of enquiry by showing that acute experimental stress can increase a range of inflammatory variables in patients with UC.

Translating these findings into therapeutic interventions based on stress reduction has proved a challenge. This is in part due to the wide range of stress-reducing psychotherapeutic interventions available. In this study, we have shown that hypnotherapy can reduce a range of both systemic and mucosal inflammatory measures in patients with active UC. We believe that this data justifies a clinical trial to assess whether hypnotherapy has clinical benefit in UC. The solution to this question may not only benefit patients, but also shed further light on the pathogenesis of IBD.

APPENDIX I

PSYCHOMETRIC QUESTIONNAIRES AND HYPNOTISABILITY TESTS

Below are reproductions of the Perceived Stress Questionnaire (PSQ) (373) and the Bradford Somatic Inventory (BSI) (374). The State Trait Anxiety Inventory (STAI) (462) and Hospital Anxiety Depression Scale (HADS) (387) could not be reproduced as they are under copyright. Similarly Spiegel's test of hypnotisability could not be reproduced (383).

Perceived Stress Questionnaire (373)

Instructions

For each sentence circle the number that describes how often it applies to you in general during the last year or two. Work quickly without bothering to check your answers and be careful to describe your life in the long run.

1. You feel rested

Almost never	Sometimes	Often	Usually
1	2	3	4

2. You feel that too many demands are being made on you

Almost never	Sometimes	Often	Usually
1	2	3	4

3. You are irritable or grouchy

Almost never	Sometimes	Often	Usually
1	2	3	4

4. You have too many things to do

Almost never	Sometimes	Often	Usually
1	2	3	4

5. You feel lonely or isolated

Almost never	Sometimes	Often	Usually
1	2	3	4

6. You find yourself in situations of conflict

Almost never	Sometimes	Often	Usually
1	2	3	4

7. You feel you are doing things you really like

Almost never	Sometimes	Often	Usually

1	2	3	4

8. You feel tired

Almost never	Sometimes	Often	Usually
1	2	3	4

9. You fear that you may not manage to attain your goals

Almost never	Sometimes	Often	Usually
1	2	3	4

10. You feel calm

Almost never	Sometimes	Often	Usually
1	2	3	4

11. You have too many decisions to make

Almost never	Sometimes	Often	Usually
1	2	3	4

12. You feel frustrated

Almost never	Sometimes	Often	Usually
1	2	3	4

13. You are full of energy

Almost never	Sometimes	Often	Usually
1	2	3	4

14. You feel tense

Almost never	Sometimes	Often	Usually
1	2	3	4

15. Your problems seem to be piling up

Almost never	Sometimes	Often	Usually
1	2	3	4

16. You feel you are in a hurry

Almost never	Sometimes	Often	Usually
1	2	3	4

17. You feel safe and protected

Almost never	Sometimes	Often	Usually
1	2	3	4

18. You have too many worries

Almost never	Sometimes	Often	Usually
1	2	3	4

19. You are under pressure from other people

Almost never	Sometimes	Often	Usually
1	2	3	4

20. You feel discouraged

Almost never	Sometimes	Often	Usually
1	2	3	4

21. You enjoy yourself

Almost never	Sometimes	Often	Usually
1	2	3	4

22. You are afraid for the future

Almost never	Sometimes	Often	Usually
1	2	3	4

23. You feel that you are doing things because you have to and not because you want to

Almost never	Sometimes	Often	Usually
1	2	3	4

24. You feel criticised or judged

Almost never	Sometimes	Often	Usually
1	2	3	4

25. You are light hearted

Almost never	Sometimes	Often	Usually
1	2	3	4

26. You feel mentally exhausted

Almost never	Sometimes	Often	Usually
1	2	3	4

27. You have trouble relaxing

Almost never	Sometimes	Often	Usually
1	2	3	4

28. You feel loaded down with responsibility

Almost never	Sometimes	Often	Usually
1	2	3	4

29. You have enough time for yourself

Almost never	Sometimes	Often	Usually
1	2	3	4

30. You feel under pressure from deadlines

Almost never	Sometimes	Often	Usually
1	2	3	4

Scoring the questionnaire

Score 5-circled number for items 1, 7, 10, 13, 17, 21, 25, 29. Score number circles for all other items.

Bradford Somatic Inventory (374)

During the past one month……..

1.	Have you had severe headaches ?	YES/NO
2.	Have you had fluttering or a feeling of something moving in your stomach?	YES/NO
3.	Have you had a pain or tension in your neck and shoulders?	YES/NO
4.	Has your skin been burning or itching all over?	YES/NO
5.	Have you had a feeling of constriction in your head as if being gripped from outside?	YES/NO
6.	Have you felt pain in the chest or heart?	YES/NO
7.	Has your mouth or throat felt dry?	YES/NO
8.	Has there been darkness or mist in front of your eyes?	YES/NO
9.	Have you felt a burning sensation in your stomach?	YES/NO
10.	Have you felt a lack of energy much of the time?	YES/NO
11.	Has your head felt hot or burning?	YES/NO
12.	Have you been sweating a lot?	YES/NO
13.	Have you felt as if there were a pressure or tightness on your chest or heart?	YES/NO
14.	Have you been suffering from a discomfort in the abdomen?	YES/NO

15.	Has there been a choking sensation in your throat?	YES/NO
16.	Have your hands or feet had pins or needles or	YES/NO
17.	Have you felt aches or pains all over the body?	YES/NO
18.	Have you had a feeling of heat inside your body?	YES/NO
19.	Have you been aware of palpitations (heart thumping)?	YES/NO
20.	Have you felt pain or burning in your eyes?	YES/NO
21.	Have you suffered from indigestion?	YES/NO
22.	Have you been trembling or shaking?	YES/NO
23.	Have you been passing urine more frequently?	YES/NO
24.	Have you been having lower back trouble?	YES/NO
25.	Has your stomach felt swollen or bloated?	YES/NO
26.	Has your head felt heavy?	YES/NO
27.	Have you been feeling tired even when you are not working?	YES/NO
28.	Have you been getting pain in your legs?	YES/NO
29.	Have you had difficulty in breathing even when resting?	YES/NO
30.	Have you had a feeling of pressure inside your head?	YES/NO
31.	Have you had difficulty in breathing even when resting?	YES/NO
32.	Have you felt pins and needles all over your body?	YES/NO
33.	Have you been troubled by constipation?	YES/NO
34.	Have you wanted to open your bowels (go to the toilet) more often than usual?	YES/NO
35.	Have your palms been sweating a lot?	YES/NO

36.	Have you had difficulty in swallowing as if there were a lump in your throat?	YES/NO
37.	Have you been feeling dizzy or giddy?	YES/NO
38.	Have you had a bitter taste in your mouth?	YES/NO
39.	Has your whole body felt heavy?	YES/NO
40.	Have you had a burning sensation when passing urine?	YES/NO
41.	Have you been hearing a buzzing noise in your ears or head?	YES/NO
42.	Has your heart felt weak or sinking?	YES/NO
43.	Have you suffered from excess wind or belching?	YES/NO
44.	Have your hands or feet felt cold?	YES/NO

Scoring the questionnaire

Total the number of questions answered yes

APPENDIX II

HYPNOTHERAPY SESSIONS

Below is given a brief overview of each hypnotherapy session

Pre-Hypnotherapy Session Discussion

Each patient was interviewed for 10-15 minutes prior to the hypnotherapy session. Firstly, an explanation of the nature of hypnosis was given incorporating the ideas of suggestion and trance. The subject was told that hypnosis required compliance but that they could not be made to do things against their will, as their unconscious mind would only accept suggestions congruent with their belief system.

The therapist also gave a brief explanation of the role that stress and psychological factors might play in the aetiology of UC. Particular attention was paid to explaining how common stress is and that even a person who considers themself not particularly stressed often experiences considerable worry and anxiety in day to day life. The subject was asked to identify the things they found stressful.

Depth Perception and Hypnotisability

Before commencing the hypnotherapy session, the subject was told that they would be assessed whilst in the process of hypnosis. The subject was informed that they would be requested to give a score between one and ten at three time points during the session to signify how deep they felt themselves to be in the process. A score of ten indicated that they were deeply into hypnosis and a score of one only slightly. They were then tested for hypnotisability by the Spiegel score (383).

Induction (347)

A relaxation induction procedure was used, based on the personal experience of the individual. A relaxing situation was identified, for instance a recent enjoyable holiday or hobby. The person was instructed to imagine themselves in that situation: great care was taken to identifying the sights, sounds, smells, noises and sensory feelings of the surroundings. They were encouraged to imagine themselves relaxing and instructions for progressive muscular relaxation were given, using words such as these:

"The muscles of your ankles will become limp and slack and they will be relaxed. Then the muscles of your calves will go limp and slack, a pleasant feeling is spreading up your body as the muscles of your calves relax."

As each muscle group was mentioned, the idea of relaxation was encouraged until it was suggested that the whole body was sinking into the couch. During the process particular attention was paid to relaxing the muscles of the face.

"All the worry wrinkles and care creases are disappearing as the muscles of your face and scalp relax".

Following this the patient was told that they were feeling "Cool, calm and comfortable. Secure and serene". This phrase was repeated several times.

On average this initial stage of relaxation lasted 15 minutes. Before moving on to the next segment, the patient was told to prepare for their pulse and blood pressure to be measured. They were also asked to make their first estimation of depth of trance.

Further relaxation and stress reduction techniques

First an acknowledgement was made of the patents willingness to participate in hypnotherapy. It was suggested that their UC would benefit from their decision to participate in the study.

A further relaxation technique was then employed using a breathing exercise. Subjects were instructed to take three deep breaths and were told that with each exhalation a phrase would be given to help them relax further. With the first exhalation the phrase "Exit toil and trouble" was given.

With the second breath the phrase "Exit pain and problems" was stated and with the third, the phrase "Exit today's dross".

The subject was then asked to take a further three deep breaths and on this occasion, a phrase given with inhalation to increase relaxation. With the first breath the statement "Fresh oxygen and energy" was made. The subject was asked to imagine the oxygen reaching all the cells in their body, bringing new energy and taking away all the toxins. With the second inspiration, the phrase "renewed, refreshed restored" was used and with the third the phrase "Strength for the problems of today".

For each individual, specific relevant stressful situations were identified and described. However, the therapist suggested and re-enforced repeatedly that, rather than feeling stressed, as was the usual in these situations, the subject would continue to feel relaxed as they did now. It was suggested that each patient would no longer find these situations stressful in the future. Subjects were asked to imagine themselves feeling relaxed in these previously stressful situations. It was suggested that the feeling of deep relaxation that they were currently experiencing, would be carried forward into their lives once the hypnosis session was over.

This second session took approximately another fifteen minutes at the end of which time a second pulse and blood pressure reading was taken and a second self-assessment of depth.

Disease specific therapy

Several techniques of visualisation were used which were disease-focussed. The first technique involved the subject imagining the lining of their bowel to be a bright red colour in accordance with the associated inflammation. It was suggested that the subject could then imagine this redness slowly turning to orange. An image an orange with its associated smell and texture was used to reinforce this colour. The patient was asked to confirm that they had changed the red to orange by nodding their head. Thereafter, a further shift in colour was made to yellow and an association to an image of a field of corn. Finally the yellow colour was then changed to a cream. The suggestion was then made that this cream was the colour of the normal un-inflamed mucosa of the gut. It was suggested that with the same technique the subject

could imagine the lining of the gut changing from an inflamed red to a normal cream colour.

The second technique involved imaging the gut as a canal or river. The patient was invited to imagine a canal where the water was flowing in a stately, steady and controlled fashion; the banks of the canal were smooth and solid and there was no erosion of the banks. The patient was invited to imagine taking a boat from the bank and setting out on the canal. Attention was paid to the calmness and steadiness of the water. The idea of a lock was introduced, the process of controlled passage through the lock described in great detail. The idea was then introduced of the gut being a canal and that all of these beneficial characteristics might apply. For example, the motion of the gut contents would be stately and smooth with no turbulence. It was also suggested that the process of evacuation might be similar to that of negotiating the lock with the process being calm and under the patient's control.

In a third technique, patients were asked to place a hand on their abdomen. They were asked to imagine a feeling of warmth emanating from their hand and radiating throughout their abdomen. Associated with this feeling of warmth would be a process of healing throughout the gut.

It was also suggested to the patient that science does not, as yet, fully understand either the process of hypnotherapy or the aetiology of UC. It was suggested that hypnotherapy may work via currently unknown mechanisms to improve disease activity in UC. A brief description of the complexity of the brain and its links to inflammatory processes was given. It was hoped that this would improve patients' confidence in the value of hypnotherapy as a therapeutic technique for UC.

Ego-Strengthening (347)

Prior to emergence, a brief period of time was given to ego-strengthening. Individuals were reminded of their past achievements and the characteristics they had

shown which had enabled their success. It was suggested that these same character traits would enable them to cope with their UC successfully in the future.

Prior to emergence a third pulse and blood pressure reading was taken and a final self-assessment of depth.

Emerging Technique

Before emergence, patients were asked to imagine themselves in their place of comfort once more. They were then woken by counting from 1 to 5 and being told that by the count of 5 they would be wide awake feeling refreshed and ready for the day ahead.

APPENDIX III

PUBLICATIONS RESULTING FROM THIS THESIS

Articles

Mawdsley JED, Macey MG, Feakins RM, Langmead L, Rampton DS. The effect of acute psychological stress on systemic and rectal mucosal measures of inflammation in UC. Gastroenterology 2006;131(2):410-419

Mawdsley JED, Rampton DS. Psychological stress in IBD; new insights into pathogenic mechanisms and therapeutic implications. Gut. 2005;54(10):1481-91.

Mawdsley JED, Rampton DS. The role of psychological stress in inflammatory bowel disease. Neuroimmunomod. 2007. In Press

Mawdsley JED, Jenkins DG, Macey MG, Langmead L, Rampton DS. The effect of hypnotherapy on systemic and rectal mucosal measures of inflammation in UC. Am J Gastro 2007. Submitted.

Book Chapters

Mawdsley JED, Rampton DS. Psychological stress in IBD; Something to worry about? P Irving, D.Rampton & F Shanahan, eds. *Clinical Dilemmas in IBD.* Blackwell Scientific Publishing 2006:129-132

PRESENTATIONS AT INTERNATIONAL MEETINGS

Oral presentations

Mawdsley JED, Rampton DS. The effects of acute and chronic stress on sytemic and mucosal measures of inflammation in ulcerative colitis. IBD Symposia at American Psychosomatic Society Meeting 2007. Psychsomatic Medicine 2007; 69 (1); A11.

Mawdsley JED, Rampton DS. 'Acute psychological stress increases rectal mucosal and LPS-stimulated whole blood release of TNF-α in patients with inactive UC. IBD free paper session at BSG. Gut 2006; 55 (Suppl II): A14

Mawdsley JED, Jenkins DG, Macey MG, Rampton DS. Natural Killer cells are increased by psychological stress and decreased by hypnotherapy in ulcerative colitis. IBD plenary session at BSG. Gut 2005; 54 (Suppl II): A23

Poster Presentations

Mawdsley JED, Jenkins DG, Rampton DS. Hypnotherapy decreases rectal mucosal release of substance P, Histamine and IL-13 in patients with active UC. BSG and DDW 2006. Gastroenterology 2006; 130 (Suppl II); A146 and Gut 2006;55 (Suppl II): A75

Mawdsley JED, Rampton DS. Acute psychological stress increases rectal mucosal and LPS-stimulated whole blood release of TNF-α in patients with inactive UC. DDW 2006. Gastroenterology 2006;130 (Suppl II); A146

Mawdsley JED, Rampton DS. Acute psychological stress increases reactive oxygen metabolite production by rectal biopsies in patients with inactive ulcerative colitis. UEGW 2005. Gut 2005; 54 (Suppl VII); A223

Mawdsley JED, Jenkins DG, Rampton DS. Acute psychological stress increases IL-6 production by lipopolysaccharide stimulated blood whilst hypnotherapy decreases serum IL-6 levels in patients with ulcerative colitis. UEGW 2005. Gut 2005; 54 (Suppl VII); A223

Mawdsley JED, Rampton DS. Serum IL-13 concentrations are raised in active ulcerative colitis and correlate with disease activity and mucosal inflammation. UEGW 2005. Gut 2005; 54 (Suppl VII); A224

Mawdsley JED, Macey MG, Rampton DS. Acute psychological stress increases platelet activation and platelet-leukocyte aggregate formation in patients with inactive ulcerative colitis. BSG meeting Gut 2005; 54 (Suppl II): A91

Mawdsley JED, Jenkins DG, Rampton DS. Rectal Blood flow is increased in active ulcerative colitis and decreased by hypnotherapy Poster at UEGW. Gut 2004; 53 (Suppl VI): A224

Mawdsley JED, Jenkins DG, Macey MG, Rampton DS. Natural Killer cells are increased by psychological stress and decreased by hypnotherapy in ulcerative colitis. AGA-BSG meeting on immunology of IBD Oxford Sept 2004

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