

**BLOOD AND PROTEIN LOSS IN WHOLE GUT LAVAGE  
FLUID IN GASTROINTESTINAL DISEASE**

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## **DECLARATION**

**I declare that this thesis has been composed by myself and that the work contained within it, except on occasions that are clearly stated , was performed by myself.**

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08/02/96

## ABSTRACT

During the 20th century many clinical tests have been developed for the assessment of digestive function or diagnosis of gastrointestinal disease. Many of these tests are based on the analysis of faeces, which is readily available and for which the only obvious disadvantage is aesthetic.

Reliable, reproducible tests depend on the biological stability of the substance during passage through the gut, and unfortunately pancreatic and intestinal proteases in gut contents destroy many proteins in faeces which otherwise could yield valuable diagnostic and mechanistic information.

Recently the potential of the analysis of whole gut lavage fluid (WGLF), a clear effluent obtained following routine bowel preparation with a polyethylene glycol based isotonic solution, has been recognised, and has already been shown to be of value in assessing protein loss in inflammatory bowel disease. The fluid can be processed rapidly, to prevent proteolytic degradation enabling its use for accurate protein measurements for protein loss determination and research in gut immune function. Furthermore such analyses can be readily standardised since they are not confounded by biological variables such as diet or transit time which would limit their value.

The object of this thesis is to investigate the value of analysis of WGLF in the assessment of gastrointestinal pathology, specifically in the measurement of blood and protein loss from the gut.

The method for haemoglobin measurement was selected on the basis of a need for sensitivity and the need to detect metabolites of haemoglobin as well as the parent molecule. The measurement of haemoglobin in WGLF has been assessed in a series of patients with clinical conditions known to result in anaemia and which may be related to blood loss from the gut.

The relationship between the subjective assessment of inflammatory bowel disease activity and the measurement of WGLF proteins arising by leakage from plasma has been examined with a view to establishing more objective activity indices.

The specificity of WGLF protein measurements for the diagnosis of active inflammatory bowel disease has been assessed in a large group of patients with a variety of gastrointestinal pathologies.

Gut clearance measurements of plasma proteins and haemoglobin have been undertaken to evaluate quantitative protein and blood loss in normal and pathological groups.

Many workers have used alpha-1-antitrypsin in faeces as an indicator of inflammatory bowel disease, and different molecular weight forms have been described. The molecular weight of alpha-1-antitrypsin in WGLF has been investigated in controls and patients with inflammatory bowel disease.

Hyaluronic acid, a structural glycoprotein found in the submucosa of the intestine has been determined in WGLF in control and patient groups in an attempt to find a direct index of tissue disruption during active disease process in the gut.

This work has been supported by a grant from the Scottish Hospital Endowments Research Trust and has been carried out while I have been employed by the Lothian Health Board as a Clinical Scientist at the Gastrointestinal Laboratory. The research has been compatible with my normal duties in the area of the development of novel tests for the investigation of gastrointestinal diseases.

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## ABBREVIATIONS

AGA	anti gliadin antibodies
$\alpha$ 1-AT	alpha-1-antitrypsin
$\beta$ G	$\beta$ -galactosidase
CA	carcinoma
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
cm	centimetre
CRC	colorectal cancer
CT	chymotrypsin
Cr	chromium
CrCl <sub>3</sub>	chromium chloride
Cu	copper
CV	coefficient of variation
DEA	diethanolamine
EIA	enzyme immuno assay
ELISA	enzyme linked immuno sorbent assay
EDTA	ethylenediaminetetraacetic acid
eg	for example
ESR	erythrocyte sedimentation rate
Fe	iron
fl	femtolitre
FOB	faecal occult blood
g	gramme
g	gravity force
GAG	glycosaminoglycan
GI	gastrointestinal
HA	hyaluronic acid
Hb	haemoglobin
HCl	hydrochloric acid
I	iodine
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ie	that is
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IV	intravenous
kD	kiloDaltons
l	litre
M	multiples
mm	millimolar
MW	molecular weight
MCV	mean corpuscular volume

mg	milligramme
ml	millilitre
N	normal
n	number
Nb	niobium
nm	nanometre
NSAID	non steroidal anti inflammatory drugs
OD	optical density
p	probability
PBS	phosphate buffered saline
PEG	polyethylene glycol
PLE	protein losing enteropathy
PMSF	phenyl methyl sulphonyl fluoride
PTI	Powell-Tuck Index
PVP	polyvinyl pyrrolidone
RCE	relative coefficient of excretion
RID	radial immune diffusion
SAPU	Scottish Antibody Production Unit
SBTI	soy bean trypsin inhibitor
SI	small intestinal
SD	standard deviation
SDS	sodium dilauryl sulphate
SEM	standard error of the mean
UC	ulcerative colitis
TRIS	tris(hydroxymethyl)methylamine
ug	microgramme
UK	United Kingdom
ul	microlitre
VAS	Visual Analogue Scale
vol	volume
WGL	whole gut lavage
WGLF	whole gut lavage fluid
w/w	weight for weight
%	percent
>	greater than
<	less than

## **PRESENTATIONS AND PUBLICATIONS ASSOCIATED WITH THE WORK OF THIS THESIS**

Gut lavage proteins (particularly IgG) correlate with Crohn's Disease Activity Index (CDAI). WG Brydon, CP Choudari, S O'mahony, Anne Ferguson. Proceedings of the ACB National meeting 1991 (poster) A81, p57.

Haemoglobin in gut lavage fluid as a measure of gastrointestinal blood loss. WG Brydon and Anne Ferguson. *Lancet* 1992;340:1381-1382.

Relative specificity for active inflammatory bowel disease of plasma-derived proteins in gut lavage fluid. WG Brydon, CP Choudari, Anne Ferguson. *Eur J Gastro Hepatol* 1993;5:269-273.

Gut lavage fluid protein concentrations: Objective measures of disease activity in inflammatory bowel disease. CP Choudari, S O'Mahony, WG Brydon, O Mwantembe, Anne Ferguson. *Gastroenterology* 1993;104: 1064-1071.

Faecal occult blood testing will fail to detect colonic polyps. WG Brydon, Anne Ferguson. *Gastroenterology (Letter)* 1994;106:277.

Use of whole gut perfusion to investigate gastrointestinal blood loss in patients with iron deficiency anaemia. A Ferguson, WG Brydon, H Brian, A Williams, MJ Mackie. *Gut* 1996;38:120-124.

## FOREWORD

A wide range of functional tests are available for use in clinical gastroenterology which have been well validated and yield valuable information. Many of these tests are based on the analysis of faeces which can reveal:

- a. residues of dietary substances or other molecules used to test absorption
- b. endogenous substances normally found in the gut lumen eg enzymes
- c. various materials which may exude or leak into the gut from blood or tissues, in inflammatory or malignant disease.
- d. labelled substances given systemically and which have leaked into the gut lumen as a result of disease.

Practical issues of sampling are important. Although quantitative, timed collections are ideal, in practice one or more random aliquots are used for routine diagnostic use and screening. Reliable, reproducible tests depend on the biological stability of the substance during passage through the gut, and unfortunately, pancreatic, intestinal and bacterial proteases in gut contents destroy many proteins in faeces which otherwise could yield valuable diagnostic and mechanistic information.

During the last seven years, our research group has developed and exploited a whole gut lavage method for the study of gut immunity. Whole gut lavage fluid, essentially a gut perfusate, is the clear effluent passed per rectum after completion of bowel cleansing, when patients or volunteers take a polyethylene glycol-based isotonic solution. The fluid can be processed rapidly to prevent proteolytic degradation and thus is suitable for assays of immunoglobulins and other proteins. A recent publication from this department has recognised increased levels of both IgG and albumin in patients with active inflammatory bowel disease and so it seemed likely that measurement of protein loss from the gut using whole gut lavage fluid would be feasible. In theory, blood loss into the gut lumen could also be studied with the same

material. Furthermore, with a standardised clinical protocol, the confounding effects of variables such as diet, faecal water content or gut transit time, could be eliminated.

The overall aim of my research has been to investigate the value of analysis of WGLF in the laboratory assessment of patients with GI diseases and symptoms, concentrating particularly on measurements of blood and protein loss from the gut.

Bowel preparation with isotonic polyethylene glycol leads to the rapid evacuation of solid faecal/intestinal contents and prolonged ingestion of this fluid leads to a potential gut perfusion situation with possible steady state characteristics. This has been confirmed by collecting and analysing several sequential aliquots of WGLF after the bowel effluent has gone clear.

The method for haemoglobin measurement was selected on the basis of a need for sensitivity and the need to detect metabolites of haemoglobin as well as the parent molecule. Haemoglobin in WGLF has been assessed in a series of patients with clinical conditions known to result in anaemia and which may be related to blood loss from the gut.

The relationship between subjective assessment of inflammatory bowel disease activity and concentrations of WGLF proteins has been examined, with a view to the development of objective indices of disease activity. The specificity of high WGLF protein concentrations for active inflammatory bowel disease has been assessed.

Gut clearance of plasma proteins and haemoglobin have been calculated to document the GI protein and blood loss in normal and pathological groups.

In an attempt to examine in more detail the nature and sources of proteins in WGLF, two further studies have been carried out. Since many workers have used alpha-1-antitrypsin in faeces as an indicator of inflammatory bowel disease, and different molecular weight forms have been described, I have studied the molecular weight of alpha-1-antitrypsin in faeces and in WGLF in controls and patients with inflammatory bowel disease.

Hyaluronic acid, a structural glycoprotein found in the submucosa of the intestine has been determined in WGLF in control and patient groups in an attempt to find a direct index of tissue disruption during active disease process in the gut.

# CHAPTER 1

## INTRODUCTION AND REVIEW OF THE LITERATURE

### BLOOD LOSS FROM THE GUT

Gastrointestinal (GI) bleeding occurs in various forms. An acute GI bleed is a medical emergency, normally from the oesophagus, stomach or duodenum, but occasionally colonic. Intermittent rectal bleeding, manifested by the passage of small amounts of fresh blood per rectum, can be due to anal disease, such as haemorrhoids, proctitis, diverticulosis, bleeding from a polyp or carcinoma of colon or rectum, and a number of other rare causes. In a study of 319 apparently healthy Australian war veterans aged over 50 (Chapuis et al,1985) 15% had reported overt rectal bleeding during the previous six months. Of this group sigmoidoscopy revealed no colorectal cancers, and only four polyps greater than one cm. About 50% of these subjects had haemorrhoids with/without other associated colonic disease. This study highlighted the poor positive predictive value of overt bleeding (<10%) in otherwise healthy subjects for colorectal polyps and cancer.

Occult GI bleeding is loss of blood into the GI tract which cannot be observed by the patient or detected by the physician on physical examination.

Occult blood loss may be suspected if a patient has an otherwise unexplained iron deficiency, and is detected by analysis of the stool for blood by any one of a range of techniques. The presence of occult blood is important because it may indicate otherwise asymptomatic GI neoplasia, or another GI source of blood loss in the patient with iron-deficiency anaemia. (Greegor,1967, Hardcastle et al,1989).

Both serious and apparently trivial diseases can lead to occult GI blood loss (Winawer et al,1976, Herzog et al,1982, Simon 1988) and studies of the subject are confounded by methodological limitations of the commonly available laboratory techniques.

## **Normal Physiological Blood Loss**

About 0.3 - 1.3 ml of blood is normally lost into the GI tract daily (Cameron 1960, Herzog et al,1982). The precise source of the bleeding is not known.

Increased physiological blood loss has been reported in several situations. Pasteurised cows milk fed to infants significantly increased faecal haemoglobin whilst formulae feeds had no effect (Zeigler et al,1990). It is not known whether this is a toxic effect or due to local damage associated with an immune response, although the fact that milk feeds modified for infants do not seem to have this effect, suggests that it is probably not immunological.

Blood loss has been reported in long distance runners and may be severe enough to produce anaemia. 10-20% of marathon runners may have positive faecal occult tests after a marathon (Fischer et al,1986, Stewart et al,1984). The mechanism of increased physiologic blood loss is unknown, but it has been speculated to involve mesenteric ischaemia. In some high performance runners, gastric and colonic erosions have been identified and these improve with abstinence from running.

Physiologic blood loss does not appear to increase with anticoagulant therapy when prothrombin time is controlled at normal therapeutic levels (Jaffin et al, 1987). When faecal occult blood is detected in asymptomatic outpatients receiving anticoagulant therapy with sodium warfarin in a normal therapeutic range, a pathologic explanation for the occult blood is usually identified. This is not the case for patients with a markedly elevated prothrombin time, in whom frank or occult bleeding may occur without detectable pathology.

## **Iron Deficiency and Hypochromic Anaemias**

Iron deficiency anaemia due to occult GI bleeding must be distinguished from hypochromic microcytic anaemias due to bleeding from sites other than the GI tract, iron malabsorption, or inadequate iron ingestion.

Anaemias with similar morphologic abnormalities of red blood cells may be found secondary to chronic disease.

Thalassemia and sideroblastic anaemia should also be excluded. These can usually be distinguished from iron deficiency anaemia by the presence of Prussian-blue stainable iron in the bone marrow or by measurement of total iron, transferrin and ferritin in the serum.

## **Measurement of faecal occult blood (FOB)**

### ***1. Qualitative chromogenic tests for FOB***

Occult GI bleeding may be detected by a positive FOB test. A variety of simple qualitative FOB tests have been used. Gum guaiac, benzidine, and o-tolidine were used in the 19th and early 20th century by Dutch and German scientists as indicators of GI bleeding in simple colorimetric tests. Other chromogenic tests for haemoglobin were based on the use of ferrocyanide, 3-aminophthalic acid hydrazide, pyramidon, aloin, phenolphthalein and other chromogens. Each of these chemical tests depends on the ability of haemoglobin to oxidise a chromogen, thus producing a coloured product. Such oxidation can be enhanced by hydrogen peroxide, catalysed by the haematin component of haemoglobin which exhibits a pseudoperoxidase activity (similar to other naturally occurring peroxidases and catalases). The phenolic group of guaiac is converted to a quinone structure, which in turn changes colour to blue by an intermolecular reaction.

The major advantages of these qualitative tests for occult blood are simplicity, ease of performance, low cost, and widespread applicability.

There have been several commercial developments of these tests such as 'Hematest' (Ames Co, UK) which uses o-tolidine as chromogen, 'Haemoccult' (Smith Kline Diagnostics, US) which uses guaiac as chromogen; 'Haemoccult 2', 'Fecatst' (Finnpipette Ky), 'Fecatwin' and 'Fecatwin S' ( Labsystems Corp, Finland.) are also guaiac based tests which have been developed all with varying sensitivities.

The 'gold standard' for quantitative blood loss measurement is the  $^{51}\text{Cr}$ -labelled red cell test and there have been relatively few comparisons of qualitative tests with this. (Morris et al ,1976, Hertzog et al ,1982, Macrae et al,1982a, )

The occult blood reaction is not specific for haemoglobin but is affected by peroxidases and catalases in various foodstuffs including meat, fresh fruits and uncooked vegetables (Macrae et al,1982b) and published studies vary in making allowance for this factor. When GI blood loss does not exceed two ml per day, approximately 10% of stool samples collected from patients not on dietary restriction will be Haemoccult positive (Stroehlein et al,1976) which is reduced to 2% on dietary restriction (Bassett and Goulston,1980). The amount of liquid in a faecal specimen also markedly affects the rate of test positivity for any level of haemoglobin concentration. In liquid stool there is enhanced lysis of red cells and dissolution of haemoglobin (Macrae et al,1982b).

A number of drugs can affect the FOB test peroxidase reaction. Ascorbic acid acts as an anti-oxidant and can produce false negative tests. Aspirin and non steroidal anti-inflammatory agents cause GI mucosal inflammation and increased occult GI blood loss (Scott et al,1961, Bjarnsson et al,1989) and should be stopped prior to screening for colonic neoplasia using any FOB test.

Hill and Fyffe (1990) have highlighted the need for quality control material which would assist competent use of such tests.

## ***2. Immunological tests of FOB***

In order to improve the specificity of the FOB test, techniques using immunological recognition of faecal human haemoglobin have been developed, some of which are available commercially (Heme Select, Smith Kline Diagnostics, and FECA-EIA, Lab Systems, Finland). These immunochemical tests are exquisitely sensitive, require no dietary restriction and can detect small amounts of blood passed into the lower digestive tract. However they may fail to detect much larger amounts of bleeding in the upper digestive tract because of haemoglobin degradation by proteolytic and bacterial enzymes. In the last 10-15 years many immunological tests have been described which measure faecal hemoglobin (Songster et al, 1980, McDonald et al, 1984; Saito et al, 1985; Uchida et al, 1990; Nagata et al, 1992), but since metabolites

are not measured these can at best only be considered qualitative indices of blood loss from the lower bowel. Most studies have shown that immunological tests are superior to guaiac based slide tests in the identification of patients with colonic polyps or carcinomas despite yielding more false positive tests (Robinson et al, 1994).

Turunen et al (1984) have combined Fecatwin S (guaiac test) and FECA-EIA (immunological test), when testing for colorectal cancer only using the more expensive immunological test on those samples which are positive for Fecatwin S.

### **3. *Quantitative measurement of faecal occult blood***

#### **a. $^{51}\text{Cr}$ -labelled red cells in faeces**

$^{51}\text{Chromium}$  labelled gamma-emitting red cells were initially used by Owen et al (1954) to measure blood loss in faeces of dogs. Subsequently these were used by Bannerman (1957) in humans and the radioactivity was calculated in a weighed aliquot from a 24 hour collection. However the method is malodourous and cumbersome, and Cameron (1960) improved sensitivity and the practicality of the technique by using freeze dried faeces. Because of the relatively long half life of 28 days for  $^{51}\text{Cr}$ , each patient can be studied for four - six weeks after red cells have been labelled.

Experiments were carried out which showed no absorption of orally-administered Cr-labelled red cells, and insignificant elution of Cr from red cells to plasma. In 10 healthy control subjects the average daily blood loss was 0.6 ml, with a range from 0.3 - 1.3 ml. There are obvious practical limitations in this method which requires complete faecal collections free from contamination with urine.

#### **b. Whole body counting of $^{59}\text{Fe}$ labelled red cells**

A method using whole body counting of  $^{59}\text{Fe}$  labelled red cells was used by Holt et al (1967). When plasma is labelled with  $^{59}\text{Fe}$ , the label is taken up into red cell haemoglobin within 7-10 days. In normal males and post menopausal women the

whole body radioactivity will remain stable and any loss in total body radioactivity must represent loss of blood. A knowledge of blood volume is required (taken as 65.6 ml/kg body weight).

The method is insensitive for blood loss of one to three ml per day but as blood loss increases so accuracy of detection increases. GI or menstrual bleeding may occur erratically and lead to fluctuations in the red cell content of  $^{59}\text{Fe}$  and blood volume. It is not possible to make corrections for these. The principal source of error in measurement is caused by minor variations in the patient's counting position on different occasions. Another disadvantage is the delay of 7 - 10 days while iron is being incorporated into haemoglobin, before counting can occur. The main advantage of this method is the ability to measure intermittent blood loss, since the long half life of the isotope allows measurements over two to three month periods.

### **c. Fluorometric determination of faecal haem and metabolites**

Schwartz et al (1983) described 'Hemoquant', a fluorometric assay which measures haem and haem-derived porphyrin. This is affected by red meat in the diet but not by vegetable peroxidases. By detecting haem metabolites, 'Hemoquant' is more sensitive than other tests for detecting blood loss from upper GI tract sources and since haemoglobin metabolites are also measured a quantitative assessment of blood loss is made. Young et al (1990) have shown that most of the haem lost into the gut lumen enters the colon and is converted by bacteria into a range of haem-derived porphyrins, lacking iron. This conversion is a slow and incomplete process and the amount converted in this way depends on colonic transit rate, site of bleeding and amount of luminal haem. As a result faeces contain variable proportions of haem and haem-derived porphyrins. Ahlquist et al (1985) have shown that faecal haemoglobin levels are less than 2 mg / g of stool in 98% of healthy volunteers.

#### **4. Plasma markers of blood loss**

Moran et al (1993,1995) have used faecal  $\alpha$ 1-AT as an indirect index of faecal occult blood loss and have shown that this gave a more accurate diagnosis of GI blood loss than both guaiac based FOB and immunochemical faecal haemoglobin measurements. Uchida et al (1990), combined immunological measurement of haemoglobin with transferrin to improve detection of blood loss.

#### **Comparison between quantitative techniques of FOB**

Using three different quantitative methods, Leahy et al (1991) assessed blood loss in faeces in five patients with recurrent Fe deficiency. Whole body counting of  $^{59}\text{Fe}$  was relatively insensitive to small blood losses but allowed losses to be followed up over longer periods. In short term studies (12 day) chemical analysis of complete stool collections for haem-derived porphyrins (Hemoquant) gave results which closely correlated with those obtained by measuring stool loss of  $^{51}\text{Cr}$  labelled red blood cells ( $r=0.98$ ), although absolute values were about 10% lower for Hemoquant than for  $^{51}\text{Cr}$  studies.

#### **Intestinal absorption of haem**

None of the chemical or immunological tests for blood loss make allowance for intestinal absorption of haem, and quantitation of GI bleeding derived from measurement of haem and its metabolites in faeces is therefore likely to underestimate the amount of haem delivered into the gut lumen.

Young et al (1993) have shown that recovery of the haem of ingested red cells by Hemoquant is only 63%, compared with 100% using the  $^{51}\text{Cr}$  labelled red cell method. Schwartz et al (1985) examined faecal recovery of ingested haemoglobin haem in blood and compared this with ingested meat and fish. The average faecal recovery of haemoglobin haem from 10-36 ml blood was 88% ( $n=13$ ). All

Haemocult tests remained negative despite greater than 20-fold increases in faecal haem. Up to 83% of the blood haem was converted in the intestinal tract to protoporphyrins. Negligible amounts of haem were found in fish and fowl and their ingestion led to no significant increase in faecal haem. An average of 25% of the haem from ingested meat was subsequently recovered in faeces.

### **Causes of occult GI bleeding**

Disease of the oesophagus, stomach and duodenum is a common clinical problem and frequent cause of occult bleeding. The high prevalence of these conditions makes secondary iron deficiency relatively frequent. Cook et al (1986) in a study where GI investigations were carried out in patients with iron deficiency anaemia, demonstrated a large number with upper GI pathology (44% of diagnoses). The majority of these patients had ulcerative oesophagitis, gastric or duodenal erosions, or were post gastrectomy; large hiatus hernia, gastric cancer and angiodysplasia accounted for most of the other diagnoses.

Non steroidal anti-inflammatory agents are perhaps the most common group of drugs used, both by prescription and over the counter, and these frequently produce gastroduodenal mucosal erosions and ulceration, and small intestinal inflammation. Many patients using these drugs have an increased rate of GI blood loss, which may be clinically apparent or occult, and may result in iron deficiency anaemia. For example approximately 70% of people taking 2.5 g aspirin per day lose 2-5 ml blood daily into the GI tract (Ahlquist et al, 1985).

On a global scale infections that cause occult GI bleeding are very common. In the tropics and subtropics hookworm infection is endemic. Daily faecal blood loss averaged about 12 ml per day and correlated with worm density in the gut (Roche et al, 1966). Other chronic GI infections such as tuberculosis, amoebiasis, ascariasis, and acute infectious gastroenteritis, may also cause occult GI bleeding.

Vascular malformations of the GI tract can lead to occult GI blood loss (Anon,1981, Howard et al, 1982). Angiodysplasia occurs in 0.5-2% of adults over age 50 and occult blood loss may be the only symptom. Blood loss may also occur in the hereditary haemorrhagic telangiectasia syndrome, 'watermelon-stomach', radiation-induced enteritis, and uraemia associated telangiectasias (Richardson et al, 1975; Stafford et al,1970).

All inflammatory bowel diseases including Crohn's disease, chronic ulcerative colitis, eosinophilic gastroenteritis, Whipple's disease, and solitary colonic ulcer, can result in occult bleeding (Guiliani et al,1961). Usually these conditions present with other symptoms referable to the digestive tract such as diarrhoea, weight loss, abdominal pain or fever, but bleeding, although occult, may provide valuable discriminating clinical information.

One of the major uses of FOB measurements is in the diagnosis of colorectal cancer.

## **FOB and colorectal cancer**

### ***1. Sensitivity and predictive value using chromogenic slide tests***

Since it is well established that occult bleeding may be due to a colonic carcinoma which is otherwise asymptomatic, tests for FOB have been used in several important screening programmes in attempts to detect colorectal cancer (Winawer et al,1976, Gilbertson et al,1980, Knight et al,1989, Ahlquist et al,1990).

Initially, Gregor (1967,1971) used guaiac-impregnated slides for his patients and reported the detection of several asymptomatic neoplasms. Subsequently this slide test has been refined and marketed as the 'Haemoccult' test, the most commonly used FOB test. Studies have suggested that neoplasias identified by screening for faecal occult blood are found at an early stage, which may result in a more favorable patient prognosis (Cummings et al 1984, Gilbertson et al 1980, Clayman, 1989, Ransohoff et al, 1991). Nevertheless there are problems in relation to this topic also. Firstly not all colorectal tumours bleed in quantities suitable for detection by presently available FOB tests; many other GI conditions can result in positive FOB tests, and if the

screening test is too sensitive large numbers of expensive, unpleasant and potentially dangerous tests may be required to exclude a diagnosis of carcinoma; finally it is not clear to what extent premalignant tumours (polyps) actually bleed, since because these are extremely common, their presence, even in a patient with a positive FOB may be coincidental.

The predictive value of a positive Haemoccult test for colorectal cancer is less than 10%, usually about 5% (Ahlquist et al,1989, Barry et al ,1987). Polyps are detected in about 20% of people with positive Haemoccult tests. The majority of positive Haemoccult results appear to be due to technical false positives, with no evidence of GI disease or evidence of only benign disease such as haemorrhoids (Johnston, 1989). Fecatwin S and Fecatest tests are reported to be more sensitive than Haemoccult (Adlercreutz et al 1978, Turunen et al 1984).

The commonly used guaiac tests have low sensitivity for detecting bleeding from the proximal colon as they fail to detect haem that has been degraded by bacteria to porphyrin during intestinal transit. Thus both guaiac FOB tests and sigmoidoscopy are biased towards the detection of distal colorectal disease.

<sup>51</sup>Cr-labelled erythrocytes have been used by Hertzog et al (1982) and Macrae et al (1982) to quantitate blood loss from colonic polyps and colorectal cancer respectively. The mean faecal blood loss per day was 1.3 ml per day for polyps compared with 0.6 ml per day in controls; the mean blood loss per day for subjects with caecal and ascending colon cancer was nine ml per day and between 1.5 - 2 ml for all other areas. Ahlquist et al (1985) using Hemoquant and pooling six aliquots taken from separate sites of the same stool showed that 43/44 patients with diagnosed colorectal cancer had faecal haemoglobin concentrations of >2 mg/g stool, median 6.2 mg/g with ulcerated tumours having higher values (median 9.4), than non-ulcerated tumours (median 3.2). Patients with polyps <2 cm had median faecal haemoglobin of 1.6 mg/g , and with polyps >2 cm a median faecal haemoglobin of 3 mg/g.

In a further study records were examined from 160 patients with both a new tissue diagnosis of colorectal adenocarcinoma and a preceding stool blood test by Hemoquant (Ahlquist et al 1990). In this group 71% had suggestive colorectal symptoms ( recent change in bowel habit, frank rectal bleeding, abdominal pain) or anaemia at presentation, and 29% were asymptomatic; faecal blood levels were normal in more than 40% of both symptomatic and asymptomatic patients. Faecal blood levels were higher in more advanced, larger and proximal tumours and before purgation.

The authors concluded that increased faecal blood loss alone is an uncommon manner of colorectal cancer presentation in the clinical practice setting (16%). However cancers so detected are likely to be at an earlier pathological stage.

Both asymptomatic and symptomatic cancers were not uncommon in patients with normal faecal blood levels.

Overall the conclusion was that despite the widespread use of faecal blood tests in case detection, most colorectal cancers present with clinical symptoms.

## *2. Comparison of chromogenic slide tests with other methods in the detection of colorectal cancer*

Ahlquist et al (1993) assessed the ability of both Hemoccult and Hemoquant as screening tests in two patient groups likely to have higher than average prevalence of colorectal neoplasia; patients who had undergone resection of colorectal cancer and first degree relatives of patients with colorectal cancer. Overall sensitivity of either test for cancer detection was only 26%. Haemoccult was positive with 21% of intraluminal recurrences, 33% of all with new primary tumours and 29% of those with Dukes A or B cancers: Hemoquant was elevated in 24%, 28%, and 29% of these groups respectively. Sensitivity for polyps one cm or larger was 13% for Haemoccult and 11% by Hemoquant. In relatives, estimated sensitivity for cancer at one and three years of follow up was 25% and 33% by Haemoccult, 29% to 43% by Hemoquant.

More recent work using immunoassays to measure FOB have produced much better sensitivity for detection of colonic polyps and cancer than Haemoccult (Songster et al,1980; Turunen et al,1984; Saito et al,1985; Uchida et al,1990; Robinson et al,1994), but some studies show that a significant number of colonic cancers do not bleed measureably.

The principal factors limiting FOB testing for the detection of colonic neoplasia/carcinoma are poor patient compliance in providing faecal specimens, the observation that as many as 30% of colorectal neoplasms do not shed sufficient amounts of blood to be detected, and the large number of evaluations that must be performed to identify test-positive patients in order to achieve the benefits of screening (Griffith et al,1981).

## **Rationale for the use of whole gut lavage fluid to investigate occult blood loss**

### ***1. Factors influencing faecal composition***

The biological nature of faeces can be influenced by several physiological/pathological factors. There is great variation in the normal daily stool output varying between 50 - 280 g per day and whole gut transit time can vary between one to seven days. (Eastwood et al,1984). Both these factors are strongly influenced by diet. The consistency of faeces varies to an even greater extent in pathological conditions where the proportion of water can vary between 60% in constipated patients to over 90% in patients with diarrhoea, with an even greater range of stool weights and transit times. With such natural and pathological variation excretion of blood would be expected to vary considerably if random samples of faeces were being analysed and such variation might blur differences between normal and pathological blood loss. In addition Rosenfield et al (1979) have reported non uniform distribution of blood in faeces.

## *2. WGL as a gut perfusion system*

Whole gut lavage has been used for several years to prepare the colon for examination (Thomas et al,1982, Di Palma et al,1984), and recent work from this department (Sallam, Brydon and Ferguson) has confirmed earlier work by O'Mahony et al (1990) that the clear fluid which is passed per rectum, after bowel cleansing is complete, is gut perfusion fluid, and can be used to investigate GI pathology. Because this procedure can be standardised with respect to perfusion rate, problems associated with faecal sampling do not occur.

### **Selection of method likely to be suitable for measurement of Hb in WGLF**

In much of the early literature on occult blood loss relating to iron deficiency anaemia, the presumed site of bleeding has been established radiologically or endoscopically, but without quantitative or qualitative assessment by detection of FOB. In a few cases, radiolabelled red cells have been used (either  $^{51}\text{Cr}$  or  $^{59}\text{Fe}$ ) to measure gut blood loss. These either required several faecal collections or patient monitoring over several weeks; ie these are complex tests and a rapid measurement which might contribute to early diagnosis cannot be made.

Anaemia and GI diseases which may but do not necessarily lead to blood loss may coexist and the link between the two is often assumed without good FOB data being available. The development of a rapid quantitative test of GI blood loss would facilitate investigation of patients with different clinical conditions associated with occult GI bleeding.

The guaiac and tetramethylbenzidine tests give a qualitative index of faecal blood, depend on the pseudoperoxidase activity of intact haematin and cannot detect haem metabolites.

Immunological tests are even more fastidious in that these require an intact haemoglobin molecule for detection, and their extreme sensitivity increases the chance of false positives due to minor bleeds from trivial causes. These tests perform better in the detection of large bowel bleeding rather than more proximal bleeding.

The fluorometric Hemoquant assay quantitates both haem and metabolites, and performs well for both proximal and distal bleeding.

I have investigated the measurement of haemoglobin in WGLF in order to quantify blood loss in GI disease.

## **PROTEIN LOSS INTO THE GUT.**

### **Historical background**

The GI tract plays a major role in the homeostasis of plasma proteins in health and disease. The small intestine is a major site of synthesis of serum proteins, including certain lipoproteins, complement components and immunoglobulins. In addition the mucosal epithelium absorbs the products of protein digestion and under certain circumstances intact plasma proteins are absorbed. For example, IgG antibodies are transported across the GI epithelium of neonatal rats by a saturable transport process that involves binding by enterocyte surface receptors specific for IgG (Jones et al,1972).

For many years it was assumed that the hypoproteinaemia in patients with GI diseases was due to impairment of protein synthesis.

Welsh et al (1937), in a study of three patients with chronic ulcerative colitis made extensive measurements of the nitrogen composition of the faeces, urine and food. They concluded that there was normal absorption from the small bowel (faecal fat normal), increased loss of nitrogen in the faeces, unrelated to food intake, and decreased urinary nitrogen excretion; the metabolic disturbance had a direct relation to disease severity. This study introduced the concept that losses of proteins are important contributing factors in ulcerative gut diseases.

Albright et al (1949) using infused albumin showed that excess catabolism rather than decreased synthesis resulted in reduced plasma albumin in patients with idiopathic hypoproteinaemia.

Evidence for the site of protein catabolism was initially provided by Citrin et al (1957), who showed that a patient with giant hypertrophy of the gastric mucosa and associated hypoalbuminaemia lost excessive amounts of albumin into the lumen of the GI tract. In their experiments an oro-gastric tube was used to collect the gastric secretions from the patient after being given intravenous (IV) iodinated albumin. Protein-bound radioactivity was detected in the gastric juice.

However there are many practical and theoretical difficulties with this approach. Complete retrieval of GI secretions is difficult and furthermore there is rapid catabolism of labelled proteins in the GI lumen with reabsorption of amino acids and the iodine label. Also both salivary glands and the gastric mucosa secrete the free label.

In 1959, Gordon, in an attempt to avoid these problems, used IV-administered radioiodinated polyvinylpyrrolidone (PVP). This is a synthetic polymer with a molecular weight (MW) in the same range as that of the smaller serum proteins, is unaffected by mammalian or bacterial enzymes and poorly absorbed from the GI tract. Since then many other labelled molecules have been used to assess leakage of proteins from blood into the gut, including  $^{51}\text{Cr}$ -labelled serum proteins (Rubini and Sheehy, 1961, Waldman, 1966);  $^{95}\text{Nb}$ -labelled albumin (Jeejeeboy et al, 1968);  $^{59}\text{Fe}$ -labelled dextran (Anderson and Jarnum, 1966); and  $^{67}\text{Cu}$ -labelled ceruloplasmin (Waldman et al, 1967). These have proved to be superior to  $^{131}\text{I}$ -PVP for the study of protein losing enteropathy (PLE).

Tests of GI protein loss have also been of value in determining the relapse and remission of activity of disease and in monitoring effects of therapy.

## **Physiology of protein turnover**

Investigation of the normal metabolism of serum proteins, especially albumin and immunoglobulins, has led to greater understanding of the disturbances of protein metabolism in patients with GI disorders (Berson et al,1953; Rothschild et al,1972). The protein that has been most studied in this context is albumin. Albumin is synthesised by the liver (normal rate 10-14 g/day for a 70kg man) and then delivered into the systemic circulation. Substantial amounts of albumin are distributed in extracellular and extravascular spaces with only 36% to 53% of the body albumin pool in the plasma compartment.

The site of catabolism of the plasma proteins has not been determined, and the role of the GI tract in this process is thought to be minor. Nevertheless albumin, gamma globulins and other serum proteins have been demonstrated in the GI secretions of normal individuals by electrophoretic and immunological techniques (Gullberg and Olhagen,1959, Holman et al,1959, and Steinfield et al,1960).

The removal of the stomach, and small and large intestine of rats resulted in only a 0 - 10% increase in the survival time of iodinated albumin (Gitlin et al,1958, Katz et al, 1961). In addition, clearance studies performed using  $^{51}\text{Cr}$ -albumin (van Tongeren, 1966, Waldman et al, 1969) and a variety of other labelled proteins showed that only 5 - 15% of the normal turnover of albumin and gamma globulin could be accounted for by enteric protein loss.

## **Pathophysiology**

Many GI diseases result in protein loss into the gut and this can occur by a variety of mechanisms. Disorders of the GI lymphatics can lead to a loss of lymph fluid into the gut lumen and inflammation and ulceration of the gut mucosa can result in exudation of plasma proteins. In other conditions the mechanism of loss has not been determined. The half-life of plasma albumin, IgG, IgA, IgM and ceruloplasmin are markedly reduced (Strober et al ,1967, Waldman et al,1967). The fraction of IV pool of these proteins catabolised per day is the same for each protein, which indicates

bulk loss of the serum proteins irrespective of their size, which is in contrast with the selective loss of proteins in the nephrotic syndrome where the sieving properties of the glomerulus are partly retained and lower MW proteins are preferentially lost.

A rate of loss of protein across the gut exceeding the body's capacity to synthesize that protein results in PLE and hypoproteinaemia. In these circumstances protein synthesis rates are normal or can only be increased to a maximum of twice normal as determined indirectly using radioiodinated albumin, or directly using  $^{14}\text{C}$  carbonate (Wochner et al,1968). The synthesis of gamma globulins is usually normal and the serum concentration is reduced in enteropathies that are not associated with an inflammatory disease process (Strober et al,1967).

Immediately after the onset of PLE, the amount of albumin and gamma globulins lost exceeds that synthesised and the serum levels fall until new steady states are reached at lower levels. At these new levels the absolute quantity of the protein degraded daily (determined from the product of the lower than normal protein pool size and the higher than normal fraction of that pool catabolised or lost per day) again equals the daily synthetic rate of the protein.

The reduction in serum protein concentrations varies for individual proteins. GI protein loss has a greater effect on the serum concentration of a protein with a low fractional catabolic rate such as IgG (6%) compared with one with a high catabolic rate such as IgE (92%) (Waldman et al,1972).

The loss of serum components in PLE is not limited to proteins. It has been shown that iron, calcium, copper and lipids may be lost into the GI tract. Also loss of lymphocytes into the GI tract, causing lymphocytopenia and abnormalities of cellular immunity, is a major feature of patients with disorders of lymphatic channels of the bowel (Strober et al,1967, Weiden et al,1972).

## **Disorders associated with protein loss in the gut**

Protein loss from the gut has been demonstrated in association with a large number of disorders affecting the GI tract.

It is not only seen in diseases restricted to the GI tract but may be observed as a significant feature in a number of generalised disorders. Among these are congestive heart failure, immunodeficiency diseases, connective tissue disorders, the nephrotic syndrome and metastatic malignancy.

Diseases associated with PLE can be broadly classified into four groups:

1. Diseases with disorders of the intestinal lymphatics eg intestinal lymphangiectasia, Whipples disease, neoplasms involving mesenteric lymphatics, congestive heart failure.
2. Diseases with demonstrable pathology specific to GI tract, but in which the mechanism of loss is not known eg coeliac disease, jejunal diverticulosis, giardiasis, megacolon, chronic pancreatitis.
3. Diseases with demonstrable pathology (generalized) but in which the mechanism of loss is not known eg cystic fibrosis, dermatitis herpetiformis, scleroderma, toxemia of pregnancy.
4. Diseases with ulceration of a region of the mucosa of the GI tract eg Crohn's disease (CD), ulcerative colitis (UC), carcinoma of the large bowel.

It is this last group which has been investigated predominantly in this thesis in relation to protein loss.

## **Methods of measuring protein loss**

Because most proteins are degraded in the GI tract with subsequent reabsorption of constituent amino acids, conventional nitrogen balance techniques cannot be used to detect protein loss. Different approaches have been used.

1. The magnitude of loss of serum into the GI tract is estimated by measuring the faecal excretion of the radioactive label following IV administration of radiolabelled macromolecules.
2. GI protein loss is quantitated by determining the faecal clearance of alpha-1-antitrypsin ( $\alpha$ 1-AT), a plasma protein that is relatively resistant to catabolism by intestinal proteases.
3. specific protein loss into the gut has been measured using jejunal and colonic closed loop perfusion studies.

### **Faecal excretion of radiolabelled molecules**

The ideal label for GI protein loss measurement should fulfil the following requirements:

- a. the attached label should not alter the metabolic behaviour of the protein to which it is bound.
- b. no reabsorption of the label from the GI tract should occur after catabolism of the protein.
- c. there should be no excretion of the label into the GI lumen except when protein bound.

If these requirements are all met, the labelled macromolecule can be used to determine the fraction of the protein pool lost into the GI tract per day.

None of the radiolabelled molecules available fulfil all of these requirements but they do provide much useful information.

The radioiodinated serum proteins were the first widely used radioactive molecules for the detection of GI protein loss. Following catabolism of the labelled protein, almost all the radioactivity is excreted into the urine and none is reincorporated into serum proteins. The radioactivity left in the serum is determined over the next 2 - 3 week period. In some studies the time course of decline of protein-bound radioactivity in the whole body was measured. This was determined by whole body counting or cumulative subtraction of daily urinary excretion of radioactivity from the initially injected dose.

Iodinated proteins had their limitations, partly because there was rapid reabsorption of the radioiodide label after metabolism of the labelled protein in the GI tract and also because active secretion of radioiodide into the intestinal lumen occurred in the salivary glands, gastric mucosa, and certain other GI sites.

In order to overcome the problem of reabsorption of the free label Citrin et al (1957) eliminated this problem by using direct gastric intubation in a patient with giant rugae who had previously received IV iodinated albumin and identified significant amounts of protein bound radioiodine in gastric secretions.

To avoid the problems inherent in the use of iodinated proteins other labelled macromolecules have been proposed, and the most widely used of these are the  $^{51}\text{Cr}$  labelled serum proteins, including  $^{51}\text{Cr}$ -albumin (Waldman,1961),  $^{51}\text{Cr}$ -transferrin, and serum proteins labelled in vivo by the IV administration of  $^{51}\text{CrCl}_3$  (Rubini and Sheehy,1961, van Tongeren and Reichert,1966). The  $^{51}\text{Cr}$  label is neither significantly absorbed from nor secreted into the GI tract and from 93-100% of an orally administered dose of  $^{51}\text{Cr}$ -albumin appears in subsequent faecal collections.

To use this in a simple screening test, approximately 25 microcuries of  $^{51}\text{Cr}$ -albumin is administered intravenously, and the stools collected over the next four days, in 24 hour lots, free of urine. Following homogenisation an aliquot is counted in a gamma counter. Results are expressed as percentage of injected dose of radioactivity excreted in the stool in the four days. Normal subjects excrete between 0.1 and 0.7% of the administered dose in the stool during this period, whereas patients with excessive protein loss excrete 2 - 40% of the dose (Waldman,1961).

A more meaningful measure of enteric protein loss can be obtained if the clearance of  $^{51}\text{Cr}$ -labelled serum protein is expressed as the fraction of the plasma pool or the volume (ml) of plasma lost into the GI tract per day. This is accomplished by relating the faecal excretion of  $^{51}\text{Cr}$  to the serum radioactivity curve. These studies require faeces to be collected on several consecutive days and processed and counted individually. Serum samples are obtained 10 minutes after injection and daily thereafter, and likewise counted. The volume of plasma cleared into the GI tract per day is then determined as follows:

ml of plasma cleared of protein per day = 24 hour stool counts / counts in one ml of the serum collected one day prior to stool collection.

This value assumes a one day transit time between secretion of protein into the GI tract and its appearance in the stool.

The mean value between three and 12 days is calculated.

Normal individuals clear the protein from 5 - 40 ml of plasma per day (Beeken, 1967; van Tongeren and Reichert, 1966). This is equivalent to GI clearance of 0.2 - 1.6% of the plasma pool of protein daily. Patients with excess protein loss clear from 50 - 1800 ml plasma, which is 2 - 60% of the intravascular pool of proteins into the GI tract daily.

One disadvantage of  $^{51}\text{Cr}$ -albumin is the short half life of survival compared with iodinated albumin. This is due to elution of chromium from albumin, with subsequent association of the label with other serum proteins, especially transferrin. This does not preclude its use for protein loss since it is assumed that the specific activity of protein in plasma is the same as that lost into the GI tract.

Indeed, intravenous administration of sterile  $^{51}\text{CrCl}_3$  with 'in vivo' labelling of a number of serum proteins, has been suggested as an alternative technique for the detection of GI protein loss (Rubini and Sheehy, 1961; van Tongeren and Majoor, 1966). Results of clearance studies using this approach are comparable to those using  $^{51}\text{Cr}$ -albumin, although a higher fraction of the administered radioactivity may be cleared by the kidney and reticuloendothelial system.

Other radiolabelled macromolecules which are not normally secreted or absorbed from the GI tract eg  $^{131}\text{I}$ -PVP (Gordon, 1959),  $^{59}\text{Fe}$ -dextran (Anderson and Jarnum, 1966), have been used in a manner similar to Cr-labelled proteins.

## Faecal alpha-1-antitrypsin

Because of the requirement to administer radioactive macromolecules and furthermore to collect serial faecal samples, such techniques have not been widely used to assess GI protein loss. Also, with the exception of  $^{51}\text{CrCl}_3$ , these materials are not commercially available.

Because of this Crossley and Elliot (1977) assessed whether an endogenous marker, faecal alpha-1-antitrypsin ( $\alpha$ 1-AT) could be used as an alternative approach, since this protease inhibitor is a glycoprotein which is itself resistant to degradation, and is present in significant amounts in serum (2 - 5 g/l). In their initial studies they measured the  $\alpha$ 1-AT concentration in saline extracts of random freeze-dried faecal samples following centrifugation, using commercially available radial immune diffusion plates; samples were first freeze-dried to eliminate the possibility that excessive water content in some faecal samples might give falsely low results. Two children with protein-losing enteropathies had much higher faecal  $\alpha$ 1-AT concentrations and faecal/serum  $\alpha$ 1-AT ratios than did healthy adults or children. Thomas et al (1981) confirmed these findings in a large group of children.

Haeney et al (1979) using an extraction procedure with distilled water did not get a significant correlation between faecal  $\alpha$ 1-AT concentrations and faecal  $^{51}\text{Cr}$ -albumin. Quigley et al (1987) showed a significant correlation between faecal  $\alpha$ 1-AT and faecal  $^{51}\text{Cr}$ -albumin, but because of considerable overlap between controls and patients, sensitivity and specificity of the test were only 58% and 80% respectively, which made it an unreliable measure of enteric protein loss.

About the same time Best et al (1976) in a large national cooperative disease study developed an index of degree of illness for Crohn's Disease (CD), the Crohn's Disease Activity Index (CDAI) which combined eight subjective and objective clinical predictors in a simplified, multiple regression equation, to give a figure which is an index of CD activity. This has subsequently been used as a 'gold standard' when evaluating other predictors of inflammatory activity. Faecal  $\alpha$ 1-AT has been

compared in several studies with the CDAI to assess its value as a measure of disease activity.

Meyers et al (1985) have evaluated faecal  $\alpha$ 1-AT as an index of CD activity in 34 patients, 24 with CD and ten controls. There was a significant correlation between disease activity and faecal  $\alpha$ 1-AT concentration ( $r=0.65$ ,  $p<0.001$ ), although the latter could not be used independently to measure disease activity.

Other workers have determined the intestinal clearance of  $\alpha$ 1-AT. (Bernier et al, 1978; Florent et al, 1981, Quigley et al, 1987)

This is expressed as ml of plasma cleared of  $\alpha$ 1-AT into the intestinal tract per day.

Florent et al (1981) had an upper limit of normal for  $\alpha$ 1-AT clearance of 13 ml/day, and there was a highly significant relationship between this and  $^{51}\text{Cr}$ -protein clearance ( $r=0.96$ ,  $p<0.001$ ) although a regression slope of 0.55 indicated some degradation of  $\alpha$ 1-AT in the intestine. Gastric secretions virtually destroy  $\alpha$ 1-AT, and this test cannot be used to measure gastric loss of serum proteins. Quigley's study showed considerable overlap in clearance results between controls and patients with GI and hepatic diseases.

Karbach et al (1983) showed a weak correlation between faecal  $\alpha$ 1-AT clearance and  $^{51}\text{Cr}$ -albumin clearance in ten cases. In all cases of active CD, faecal  $\alpha$ 1-AT was raised above control values but did not correlate with activity indices.

Kapel et al (1992) have shown that faecal clearance of  $\alpha$ 1-AT is increased in subjects with both inactive and active CD compared with controls but the increase was not statistically related to the grade of the disease.

Strygler et al (1990) investigated plasma clearance of  $\alpha$ 1-AT in normal subjects and in patients with chronic diarrhoea, malabsorption, or unexplained hypoalbuminaemia. Normal subjects were also investigated following induction of diarrhoea secondary to intake of different laxatives. They concluded that induced diarrhoea can cause an increase in  $\alpha$ 1-AT clearance (mean 28 ml per day compared with 14 ml per day without diarrhoea); that there is a highly significant correlation between  $\alpha$ 1-AT clearance and serum albumin; that faecal  $\alpha$ 1-AT concentration is not a reliable index of abnormal  $\alpha$ 1-AT clearance.

Fischbach et al (1987) compared faecal  $\alpha$ 1-AT (concentration and clearance) and excretion of  $^{111}\text{In}$  granulocytes, another putative index of disease activity, in assessing disease activity in chronic inflammatory bowel disease (IBD). These authors got a wide range of overlap for faecal  $\alpha$ 1-AT clearance results between active and inactive IBD, and a poor relationship between clearance results and CDAI. There was however good correlation between  $^{111}\text{In}$  labelled granulocyte excretion and CDAI. The authors concluded that assessment of inflammatory activity in an individual patient cannot be based on faecal  $\alpha$ 1-AT concentration and clearance alone, since enteric protein loss depends not only on mucosal inflammation but may also be influenced by prior resections, complications such as stenosis, extent and location of disease and other factors such as bacterial overgrowth, and secondary intestinal lymphangiectasia.

Faecal  $\alpha$ 1-AT has also been shown to be raised in coeliac disease (Bai et al 1991). Mean clearance of  $\alpha$ 1-AT was 45 ml per day (n=83). Matoth et al (1991) have shown increased concentrations of faecal  $\alpha$ 1-AT in children recovering from burns.

To summarise, faecal  $\alpha$ 1-AT clearance is increased in most studies in patients with IBD, although there is some overlap with control subjects, but does not distinguish between active and inactive disease; faecal  $\alpha$ 1-AT concentration alone is less discriminatory.

I have followed up the principles of GI protein loss studies used by others by measuring IgG, albumin and  $\alpha$ 1AT concentrations in WGLF from patients with IBD and the association with activity indices. The specificity of these tests for patients with active IBD has also been measured.

## GI perfusion studies

Segmental perfusion of the jejunum has been described by Rambaud et al (1981), using a four lumen tube with a proximal occluding balloon, which allows duodenal fluid to be aspirated and discarded and pure jejunal fluid sampled without contamination from other proteins or proteolytic enzymes. Albumin and IgA secretion rates were studied and extrapolating results to the total length of the jejunum the mean jejunal clearance of albumin for the ten subjects studied was 25 ml per day.

Jonard et al (1984) have measured secretion rates of albumin, IgG, IgA, and other proteins in a segmental perfusion study in the human jejunum and a relative coefficient of excretion (RCE), which is jejunum to serum concentration expressed relative to that of albumin was determined for each protein. From radioisotopic studies in two patients the specific activity of albumin in plasma compared with jejunal fluid was 0.9 and 1.1, which indicated that 100% of albumin in jejunal secretion was derived from plasma. RCE values for other proteins were as follows: 1.4 for orosomucoid, 0.83 for IgG, and 0.74 for IgE. This was inversely related to the MW of these proteins and indicated mainly passive diffusion into the jejunum from plasma.

However transferrin (1.1),  $\alpha$ 2-macroglobulin (0.77) and polymeric-IgA (218) had RCEs exceeding the value expected from simple seepage indicating local gut synthesis and possible active transepithelial transport. Colombel et al (1992) have done similar studies and showed that the RCE for IgG compared with albumin was lower (about 0.5); when patients were on elemental diets, albumin secretion increased approximately threefold and IgG secretion about fivefold. Based on these studies clearance of IgG from the small bowel can be predicted to be between ten and 20 ml per day in normal subjects. The influence of MW on secretion into the gut should be carefully considered when using different serum proteins for clearance studies across the healthy and inflamed gut; the RCE differential may be different or abolished in GI disease affecting the mucosa.

Prigent-Dedlecourt et al (1995) using a segmental perfusion technique have perfused the colon in ten normal subjects and have studied secretion rates of immunoglobulins and plasma proteins from the colonic mucosa and measured RCEs for various plasma

and secretory proteins relative to albumin. The RCE for IgG was 1.17, with intracolonic clearance of albumin extrapolated to 24 hours of 3.4 ml per day, and IgG of four ml per day.

I have discussed protein loss and clearance in WGLF, in relation to previous clearance and perfusion studies.

### **Faecal $\alpha$ 1-AT molecular weight heterogeneity**

Mizon et al (1988) applied sodium dodecyl sulphate - polyacrylamide gel electrophoresis and immunoblotting to analyse  $\alpha$ 1-AT from healthy subjects and patients with CD. A component with MW 38 kiloDaltons (kD) was identified in normal faecal extracts as well as in six pathological samples. In these cases the CDAI was 170 (SEM 47). In contrast  $\alpha$ 1-AT of MW 51 kD was detected in faecal extracts from eight patients with active CD (CDAI 287, SEM 39)

Mizon et al (1991) have sequenced the N-terminal end of the 38 kD molecular form of  $\alpha$ 1-AT, and have shown that it differs from the native plasma form by the loss of 17 N-terminal amino acids; carbohydrate analysis of this form demonstrated a total lack of neutral sugars; in contrast the 51 kD form of  $\alpha$ 1-AT is glycosylated and can be differentiated by virtue of its reactivity with concanavilin A; furthermore analysis of 25 faecal extracts from patients with CD demonstrated that the presence of the glycosylated form of  $\alpha$ 1-AT was closely related to the degree of inflammation; the authors interpretation of these findings was that in active CD there is inhibition of the hydrolytic activity of some glycosidases.

I have extended this work by investigating  $\alpha$ 1-AT MW and  $\beta$ -galactosidase in faeces and WGLF of patients with CD and UC.

## **Hyaluronic acid**

The lamina propria is characterised by a high concentration of hyaluronic acid (HA) which is an essential component of extracellular matrix (Laurent,1987, Gerdin et al,1991). The concentration of HA in intestinal lymph is much higher than in serum and this suggests that there is local synthesis of HA in the intestinal mucosa and considerable turnover of the intestinal HA pool (Tengblad et al,1986). HA can be observed in the perfusate from isolated jejunal segments of healthy individuals (Knutson et al,1989). The recovery is higher in the perfusate from isolated perfused jejunal segments in patients with CD than in controls (Ahrsted et al,1992, Colombel et al,1989).

Colombel et al (1992) using a segmental perfusion technique have studied the effect of intrajejunal elemental diet perfusion on the jejunal secretion of immunoglobulins, albumin, and HA in man, with albumin and immunoglobulin secretion increasing and HA being unaffected. The increased secretion rate was thought to be caused by hyperemia associated with the presence of nutrients in the gut lumen.

I have measured HA in WGLF from normal subjects and patients with GI disease as a potential direct index of intestinal leakage from the sub-mucosa in inflammatory bowel disease.

## **CHAPTER 2**

### **BLOOD LOSS IN GASTROINTESTINAL DISEASE**

#### **SELECTION OF METHOD TO MEASURE GI BLOOD LOSS IN WGLF**

For the current investigations the faecal 'Hemoquant' method of Schwartz et al (1983) has been adapted to measure blood loss in WGLF; this measures GI blood loss from any level of the gut ; using WGLF removes the main disadvantage of red meat haem and the variable amount of faeces per day which may contribute to the unreliability of faecal haemoglobin (Hb) concentrations for FOB detection.

Other methods for measuring FOB have been discussed in the thesis introduction.

#### **WGLF haemoglobin by 'Hemoquant'**

See chapter 2 appendix for full description of method.

The changes from the method as described by Schwartz et al (1983) are as follows:

1. the use of 0.1 ml WGLF as sample for assay instead of 8 mg faeces.
2. adjustment of concentration of oxalic acid reagent constituents to give same final concentration after addition of 0.4 ml to 0.1 ml WGLF as in method described by Schwartz et al (1983).
3. the use of 600 nM instead of 650 nM as the emission wavelength.

#### **Validation of technique for WGLF Haemoglobin measurement**

##### **1. Sensitivity of the method**

Schwartz et al (1983) quote sensitivity for faecal method to <0.01 mg/g using an 8 mg sample of faeces, ie a detection limit of <0.1 ug per sample.

Assuming that the instrument used here is of equivalent detection ability/stability the sensitivity level should be similar ie  $<0.1$  ug per sample or  $<1.0$  ug/ml for a 0.1 ml sample.

## **2. Analytical Precision**

Coefficients of variation for results within and outwith the reference range are less than 10% (between batch variation).

For WGLF Hb of 3 ug/ml, coefficient of variation is 6.3 (n=29)

For WGLF Hb of 27 ug/ml coefficient of variation is 7.2 (n=29)

## **3. Use of 600 nm rather than 650 nm as emission wavelength**

650 nm was used by Schwartz et al (1983) as the emission wavelength, but from their published work it was apparent that there was also a strong emission peak at about 600 nm.

Van den Berg (1988, 1989) using two simpler variations of this assay, where the faeces are extracted in isopropanol/ HCl before the reaction to convert to protoporphyrins, used the emission wavelength of 597 nm and 599 nm.

(Measurement at 650 nm would have required the installation of a red filter in the fluorimeter for my work).

600 nm appeared to have further advantages in that chlorophyll which is a possible interferent in the assay in faeces while being strongly measured at 650 nm is not measured at 600 nm.

625 nm is the peak wavelength at pH 4, and was used by Schwartz et al (1983) in an HPLC comparison with their assay at 650 nm. Their comparison using 150 different faecal samples gave a correlation coefficient of 0.97.

To further validate the use of 600 nm rather than 650 nm, 40 samples were analysed after the second extraction (ethyl acetate/butanol) using an emission wavelength of

625 nm and also measured using the 'Hemoquant' procedure using 600 nm as the emission wavelength.

The results gave a correlation coefficient of 0.99, with the mean at 600 nm (7.9) being slightly higher than the mean at 625 nm (7.6).

The method of van den Berg et al is simpler than the original description of Schwartz in that it involves fewer liquid/liquid extractions, and heating at only 60°C . This method was attempted for WGLF haemoglobin analysis omitting the initial isopropanol/HCl extraction step and directly treating 50 ul WGLF with acetic acid (1 ml) and ferrous sulphate/HCl reagent (50 ul). However results were unsatisfactory and the method was not pursued.

#### **4. Recovery of haemoglobin when added as red cells to WGLF (Table 2.1)**

50 ul of whole blood of known haemoglobin value (Haematology quality control) was added to 10 ml of three patient WGLF samples which were assayed after freezing overnight at -70°C, and centrifuging.

Blood haemoglobin concentration was 123 g/l.

#### **5. Recovery of disodium protoporphyrin when added to WGLF (Table 2.2)**

Haemoglobin has a MW of 67000, with four haem groups each equivalent to a porphyrin.

Disodium protoporphyrin has a MW of 606.6, and each porphyrin has an MW equivalent of 562.6 mg

Therefore an Hb standard of 67 ug/ml is equivalent to 2.25 ug/ml protoporphyrin.

About 40 mg disodium protoporphyrin (Sigma Chem Co Ltd, UK) were weighed out dissolved in 10 ml 6M HCl and made up to 100 ml with distilled water to give a stock solution of about 400 ug/ml.

The molar extinction coefficient of protoporphyrin at 407 nm is  $275 \times 10^3$ .

Dilute stock solution x 200 and read OD

OD at 407 nm = 0.883, ie stock solution is 0.642 mm/l or 361 ug/ml protoporphyrin

Take 1.0 ml stock solution and dilute to 10 ml with distilled water to give a working standard of 36 ug/ml

0.1ml WGLF samples from 2 patients were assayed with and without addition of 0.01 ml 36 ug/ml protoporphyrin in duplicate and recoveries determined.

## 6. Linearity of Assay

50 µl of red cells were added to 5 ml WGLF solution and haemolysed by freezing overnight at  $-70^{\circ}\text{C}$ . After centrifugation at 1500g in a Mistral 3000i centrifuge the supernatant was double diluted x8 with lavage fluid and the samples assayed as for WGLF.

readings were as follows:

>9999, 9500, 4620, 2450, 1150, 620, 300, 150, 70

The first standard (>9999) was diluted x5, and gave a reading of 4100 (20500 when correcting for dilution). This standard has not been included on the dilution curve (Figure 2.1)

The correlation coefficient was 0.999.

The assay is linear up to full range ie 990 µg/ml, with dilutions required beyond this.

## Calculation of blood loss

The blood loss was calculated as clearance of blood from whole blood into the gut in ml per day.

$\text{WGLF Hb (ug/ml)} \times 28.8 / \text{Blood Hb concentration (g/l)}$

This assumes a perfusion rate of 20 ml/min = 28.8 l per day.

### **Reference range for WGLF haemoglobin**

Assays were performed in WGLF from 22 healthy volunteers and 58 patients with simple constipation or trivial, functional GI symptoms.

Values for WGLF Hb ranged from 0-9 ug/ml The 95% percentile range was 1-5 ug/ml. This has been set as the reference range.

This is equivalent to blood loss of 0.2 - 1 ml blood per day.

These results are shown in Figure 2.2

## **ANAEMIA AND GI BLOOD LOSS IN PATIENTS WITH IBD**

### **Introduction**

IBD of small intestine and colon is associated with anaemia.

The anaemia of CD was recognised when the condition was first described (Crohn et al, 1932) and has been described and investigated by many groups since then. For example anaemia was shown to occur in 79% males and 54% females with exacerbation of CD (Dyer et al, 1972).

Since it was established that erythrocytes were often hypochromic, iron deficiency was considered an important cause. This could be due to low iron in the diet, blood loss from the gut, or malabsorption of iron; in addition iron uptake by activated macrophages and inhibition of bone marrow haematopoiesis in response to a chronic inflammatory condition may contribute.

Also macrocytic anaemia occurs due to malabsorption of folate and vitamin B12.

Anaemia has been described in UC for many years. Garvin and Bargen (1937) in a series of 100 cases found a mean blood haemoglobin of 111g /l. Subsequently many authors have investigated anaemia in UC. Ormerod et al (1967) have shown, in common with other series, that the anaemia is mainly due to iron deficiency.

The technique of whole gut lavage now makes it possible to measure the role of any occult GI blood loss as a factor in the anaemia of IBD patients.

I have assayed Hb and IgG in WGLF in patients with both inactive and active CD and UC to assess blood loss on the one hand and disease activity (objectively) on the other. The use of IgG to assess disease activity is described in chapter 3. Results have been compared with blood Hb to assess to what extent the presence of anaemia is related to blood loss on the one hand and inflammatory disease activity on the other.

Patients receiving iron supplements or who had a macrocytic anaemia as assessed by  $MCV >98(fl)$  were excluded from this study.

## **Patients**

93 patients with CD (49 males, 44 females) and 43 patients with UC (19 males, 24 females) were investigated prospectively over two years. 58 had active CD, and 26 had active UC as assessed by WGLF IgG > 10ug/ml.

Blood Hb, and WGLF IgG and Hb were measured in all patients and GI blood loss calculated from blood and WGLF Hb measurements.

Reproduceability of the assay was assessed indirectly by examining results of WGLF Hb measurements in pairs of samples which had been collected from 40 patients or healthy volunteers in the course of other research projects, or as part of their routine clinical care, including ten pairs of specimens from patients with active IBD before and after initiation of treatment.

## **Results**

28/58 patients with active CD and 11/26 patients with active UC had blood Hb below the accepted reference ranges for males and females (<130 and <115 g/l respectively). See Table 2.3 .

GI blood loss results are shown in Figure 2.3.

21/58 patients with active CD and 6/26 with active UC had GI blood loss within reference range (<1.0 ml/day).

The median GI blood loss per day in patients with active CD was 1.5(0 - 12.8) ml per day and was significantly less than patients with active UC, median 4.4(0.3 - 36) ml per day ( $p < 0.005$ ).

There was no significant difference in blood Hb or WGLF IgG for patients with active CD and UC.

## **CD patients**

There were significant differences between active and inactive disease groups for both blood Hb and GI blood loss ( $p < 0.05$ , and  $p < 0.0005$  respectively).

However 6/35 patients with inactive CD were anaemic (low Hb) and 7/35 had GI blood loss, although in all cases this was less than 2 ml per day.

For patients with active disease:

There was a significant correlation between blood loss and WGLF IgG ( $r = 0.34$ ,  $p < 0.01$ ); see Figure 2.4a.

There was no significant correlation between blood Hb and WGLF IgG ( $r = -0.19$ ,  $p = 0.15$ ), or between blood Hb and blood loss ( $r = -0.19$ ,  $p = 0.16$ ).

There was a weak significant relationship between blood Hb and blood loss in females ( $n = 22$ )  $p < 0.05$ , but not in males; see Figure 2.5b.

## **UC patients**

There was a significant difference between GI blood loss in patients with active and inactive disease ( $p < 0.0001$ ), but no significant difference between blood Hb levels ( $p = 0.053$ ).

3/17 patients with inactive UC were anaemic and 2/17 had GI blood loss (2 and 4 ml/day).

For patients with active disease:

There was a significant relationship between blood loss and WGLF IgG ( $r = 0.54$ ,  $p < 0.005$ ); see Figure 2.4b.

There was a weak significant correlation between WGLF IgG and blood Hb ( $r = -0.40$ ,  $p < 0.05$ ); See Figure 2.5a.

There was no significant relationship between blood Hb and blood loss ( $r = -0.05$ ,  $p = 0.6$ ).

### **WGLF haemoglobin reproducibility (biological)**

Thirty sets of duplicate samples were obtained from individuals with normal values for WGLF Hb at the first test. In all cases the value for the second sample was also normal; mean difference in duplicate values was 1.0 ug/ml (range 0 - 3). There were ten pairs of specimens from patients with IBD, one sample collected at relapse and one after a variable clinical improvement on treatment. As shown in Table 2.4, concentrations of WGLF Hb and IgG in WGLF paralleled disease activity; Hb concentrations were lower in the specimen collected after treatment, but in most cases the values were still above the reference range.

### **Discussion**

More than 50% of patients with active CD and UC had no evidence of anaemia based on blood Hb, and about 40% patients with active CD, and 20% with UC had no evidence of GI blood loss.

In neither group was there a significant relationship between blood Hb and blood loss, and this was also the case when subjects with low Hb were considered separately.

This indicates that factors other than gut blood loss contribute to anaemia in patients with active IBD.

In patients with active UC WGLF IgG correlated with both fall in blood Hb and GI blood loss. This may indicate that inflammation causes mucosal and systemic effects with both blood loss and decreased Hb synthesis contributing to anaemia.

Teahon and Bjarnason (1993) used faecal <sup>111</sup>indium labelled neutrophils and faecal <sup>51</sup>Cr labelled red cells as indices of intestinal inflammation and blood loss, and showed a significant relationship between intestinal inflammation and blood loss in patients with UC (n=12) but not in patients with CD (n=15).

Their patients with UC had significantly greater blood loss (median 6.5, range 1.8 - 29.2 ml per day) than patients with CD (median 2.1, range 0.7 - 5.3 ml per day) which is in agreement with my results.

However whilst WGLF IgG reflects altered permeability of the gut to serum proteins, due to tissue damage, neutrophils may be involved in the inflammatory process per se with the parameters used by Teahon and Bjarnason measuring different stages of active inflammation than WGLF IgG.

Others in our group have developed methods to measure luminal neutrophilia by assay of neutrophil elastase in WGLF and further studies of this issue are planned.

Blood loss values were greater in Teahon's study which may reflect the greater recovery of intestinal blood using the <sup>51</sup>Cr-labelled red cells procedure and possibly the detection of intermittent blood loss which would be facilitated by timed faecal collections. Also they used in-patients whereas many of our patients studied were out-patients and the severity of illness may have been less in our group.

In conclusion in this group of patients with active UC and CD anaemia was not related to GI blood loss. Only in patients with active UC was there a relationship between a fall in blood Hb and the inflammatory parameter WGLF IgG. The degree of GI blood loss correlated weakly with WGLF IgG in patients with both active CD and UC, but blood loss was not a consistent feature of active inflammation. Factors in addition to inflammation, such as duration of illness with resulting macrophage uptake of plasma iron and reduced bone marrow haematopoiesis (Fuchs et al, 1991), and poor diet may be important in causing the degree of anaemia in these patients.

## **ASSESSMENT OF BLOOD LOSS BY WGLF HAEMOGLOBIN IN PATIENTS WITH UNEXPLAINED FE-DEFICIENCY ANEMIA**

### **Introduction**

Iron deficiency anaemia may be the only clinical manifestation of ulcerative oesophagitis, benign or malignant gastric ulcer, duodenal ulcer, vascular malformations, large benign colonic polyps or colonic cancer. Thus it is normal clinical practice to examine the upper and lower GI tract by radiological or endoscopic procedures in patients with unexplained iron deficiency; high detection rates for lesions capable of causing blood loss are reported - eg 57%, 60%, 70% in recent series from the USA (Rockey et al,1993), Australia (Cook et al,1986) and England (McIntyre and Long,1993). However accurate measurements of the amounts of blood lost into the gut in such patients have not been performed, and so the cause/effect relationships between the lesions detected and the anaemia remain uncertain.

WGLF Hb has been measured in a series of patients with Fe deficiency anaemia to assess which of the GI lesions detected are chronically bleeding.

### **Patients and Methods**

42 patients referred to GI physicians with unexplained iron deficiency were investigated over an 18 month period. 26 were women aged between 40 and 85 (median 66) and 16 were men aged between 43 and 86 (median 72). Entry criteria were a low blood Hb concentration (<130 g/l in men, <115 g/l in women) together with two of the following: low MCV (<76 fl), low serum ferritin (<10 ug/l), low serum iron (<14 umol/l), reticulocyte response with rise in Hb after oral iron therapy.

## **Clinical Assessment**

As part of the initial clinical interview and examination, as well as GI symptoms and clinical signs, quality of diet, current or recent use of NSAIDs, aspirin or anticoagulants were also recorded.

Upper GI endoscopy, rigid sigmoidoscopy, barium enema and/or colonoscopy were performed on 40 patients. In two patients, when the first endoscopy examination showed a carcinoma (one gastric, one colonic) other investigations were cancelled. These patients have not been excluded from the series since examination of the GI tract was completed at laparotomy. Although the planned protocol included endoscopic duodenal biopsies for the diagnosis of coeliac disease, these were in fact obtained from only 25 patients. Thus, as a supplementary method to rule out coeliac disease, serum IgA class and WGLF IgM class antibodies to gliadin (AGA) were measured by ELISA in patients who had not had a small bowel biopsy (O'mahony et al 1991b). The four with positive results (serum AGA IgA >20, WGLF AGA IgM>15) were recalled and jejunal biopsy performed in three. One patient declined further tests.

## **Results**

These are shown in Table 2.5 and Figures 2.6a and 2.6b.

Initial Hb values in the 16 men ranged from 61 - 119 g/l (mean 90 g/l), and in the 26 women ranged from 45 - 114 g/l (mean 85 g/l). Many patients had symptoms of anaemia, such as tiredness, lethargy and breathlessness, but few had significant GI symptoms.

One woman developed dysphagia while being investigated; five patients reported dyspepsia, three heartburn, one constipation and two diarrhoea.

There were ten patients whose diet was clearly deficient in iron containing foods ( as judged at clinical interview; formal dietary assessment was not performed.) Nine

patients had been taking NSAIDs when the anaemia was diagnosed, but at the time of their GI investigations the drugs had been withdrawn from eight of the nine. Four patients had been on long term anticoagulant therapy, which had been discontinued in one case; there was a further patient with a bleeding diathesis due to idiopathic thrombocytopenic purpura, and one woman with alcoholic liver disease also had abnormal coagulation.

### **Malabsorption**

Twelve patients had gastric pathology likely to lead to iron malabsorption. Of these, seven had had a partial gastrectomy for peptic ulcer disease many years previously, and the other five had atrophic gastritis.

There were two patients in whom it is likely that small bowel disease with malabsorption was present. One woman, with no GI symptoms and a diet deficient in iron, had small bowel biopsy pathology typical of coeliac disease. Coeliac disease was probable in one other patient. She gave a history of pernicious anemia 25 years ago and was receiving regular injections of neo-cytamen. Endoscopy had shown an oesophageal carcinoma, and a duodenal biopsy was not taken. When ELISA results were found to be positive for serum and WGLF antibodies to gliadin, her old case notes were retrieved and examined and it emerged that she had had a macrocytic anaemia; at clinical reassessment she was noted to be of short stature with signs of old rickets, strongly suggestive of coeliac disease. Because of her failing health further tests were declined.

### **Other GI diseases detected**

Standard X-ray and endoscopic investigations produced many positive results, including six carcinomas (Table 2.5). Indeed there were only seven patients in whom no lesion was found; two of these had iron deficient diets, one had alcoholic liver disease, one had previously taken NSAIDs, leaving only 3/42 patients for whom there were completely negative findings (Fig 2.6).

## Measurement of GI blood Loss

25/42 patients with iron deficiency had values for WGLF Hb within the reference range, and there were a further seven with marginally elevated levels (6 - 8 ug/ml, calculated daily GI blood losses 1.2 - 1.7 ml per day), including two patients with malignant disease (gastric cancer, 6 ug /ml, 1.2 ml/day, oesophageal cancer 6 ug/ml, 1.3 ml per day). Two patients had WGLF Hb concentrations of 10 and 12 ug/ml, with calculated blood losses of borderline clinical significance at 2 and 2.1 ml per day, and there were only eight patients with unequivocally high values ( taken as WGLF >10 ug/ml, and blood loss > 2 ml per day) (Figure 2.7). This group included all four patients with colon cancer (blood losses 5 - 132 ml per day).

## Discussion

In view of the very high rates of detection of GI diseases in anaemic patients, it has been entirely reasonable to assume that chronic blood loss from the gut is the commonest cause of iron deficiency anaemia in men and post menopausal women (Beveridge, 1965). Lack of correlation with FOB tests has generally been attributed to deficiencies in the guaiac based FOB methods, which are relatively insensitive and preferentially detect blood loss from the distal GI tract. A further factor is that bleeding may be in the form of multiple intermittent episodes, rather than a continuous ooze.

Review of the results of WGLF Hb measurements highlights the complexity of possible aetiologies in the 42 iron deficient patients studied. Altogether there were 73 clinical, dietary or iatrogenic factors recorded - poor diet (10), gross GI pathology (34 in 28 patients), malabsorption (14), coagulation problems (6), and NSAID use (9). However WGLF haemoglobin test detected only eight patients who were losing >2 ml blood daily into the gut, including all six patients with lesions generally

recognised to be important causes of occult bleeding four with colonic cancer, one with diffuse gastric vascular ectasia, and one with severe, ulcerative oesophagitis.

There are several possible explanations for the findings. In some patients with low or normal values for WGLF Hb, GI bleeding may be intermittent rather than a continuous ooze, as discussed above. The lesion responsible for blood loss might have healed in the interval between diagnosis of anaemia and investigation; this may well have been the case in NSAID treated patients, eight of whom had the drugs discontinued as soon as anaemia was recognised. However it seems likely that in many instances, GI lesions detected by standard investigations are coincidental and are not bleeding. In these patients, iron deficiency is due to poor diet, malabsorption, chronic blood loss from a site other than the gut, or a combination of these (Sayer and Long, 1993). The emphasis which has been placed on the development of better tests for GI bleeding has led to relative neglect of these other clinically important features.

Absorption and bioavailability of dietary iron, and iron malabsorption, are partially interrelated. The amount of inorganic iron which is absorbed is greatly influenced by the nature of other foods taken at the same time (Charlton et al, 1983). A much higher proportion of haem iron than inorganic iron is absorbed (Bjorn-Rasmussen et al, 1974) and malabsorption syndromes may affect one and not the other; for example, in coeliac disease there is malabsorption of ferrous but not haem iron (Anand et al, 1977). Optimal inorganic iron absorption requires healthy gastric and small bowel mucosae, and so will be compromised after gastric surgery, and probably also in patients with atrophic gastritis and hypochlorhydria. There were seven (17%) of the patients in our series who had had a partial gastrectomy many years before. Coeliac disease may be expressed as a single nutrient deficiency, and some form of small bowel biopsy should be included in the work up of all iron deficient patients. Free iron loss into the gut in association with high rates of epithelial cell proliferation and loss, is another potential factor in patients with coeliac disease or gastritis (Sutton et al, 1970).

In iron deficient patients reported in recent literature, serious diseases detected vary somewhat because of different aims of the studies, entry criteria, age and sex. However in all reports, a significant minority of patients with unexplained anaemia are found to have completely asymptomatic colonic cancer - in our study 9.5% of 42 patients, a good yield of potentially curable serious disease. There is no reason to change current clinical practice, that such patients merit colorectal endoscopy or imaging, and probably upper GI endoscopy at the same time. But in the majority who do not have colorectal cancers, more attention needs to be given to the completion of the diagnostic process: separate recognition of bleeding and non bleeding lesions, of dietary iron deficiency, and of iron malabsorption due to hypochlorhydria or coeliac disease.

## **A COMPARISON OF FAECAL AND WGLF HAEMOGLOBIN MEASUREMENTS**

### **Introduction**

WGLF has several theoretical advantages over faeces for quantitating analytes.

With regard to haemoglobin in the gut the 'Hemoquant' method quantitates both Hb and metabolites and so bacterial degradation does not result in loss of quantitative measurement in either faeces or WGLF.

However there is a possible dietary interference from haemoglobin and myoglobin present in meat, which can significantly increase faecal Hb.

In addition unless daily collections of faeces are made blood loss cannot be quantified.

Since the normal stool weight can vary from 50 - 300 g/day, and since this range widens with pathological conditions leading to diarrhoea/constipation, random stools from such patients can only give a semi quantitative measure of blood loss and concentrations determined may be misleading.

Furthermore it has been reported that faeces are non homogeneous and that there is variation in constituents in the same stool sample at different sites. This can happen when the bleeding site is the distal colon or rectum.

### **Subjects**

Faecal samples were obtained from 70 subjects who were having the lavage procedure. Both faecal samples and WGLF were assayed for Hb.

Diagnoses in this group were as follows

CD (13)

UC (13)

IBS (15)

Colorectal Cancer (6)

Anaemia (10)  
Diverticulitis (4)  
Polyps (3)  
Miscellaneous (9).

In a separate study faecal samples were obtained from 15 healthy laboratory volunteers. Faecal samples were taken from three sites on the same stool from each volunteer to determine the sample site variation in faecal haemoglobin results.

Figure 2.8 shows distribution of faecal Hb for control subjects (including patients with IBS) and patients with IBD and colorectal cancer.

## Results

Reference range for Faecal Hemoquant determined from control subjects and patients with IBS was 0.1 - 1.8 mg/g (95 percentile range, n = 30).

The coefficient of variation (CV) of results for triplicate samples taken from a single stool in 15 normal subjects was 18% .

There was a significant correlation between WGLF and faecal haemoglobin. ( $r=0.82$ ,  $p<0.0001$ , Figure 2.9).

39 patients had both WGLF Hb and Faecal Hb within the reference ranges.

20 patients had both WGLF Hb and Faecal Hb outwith reference ranges.

This included patients with Ca colon (5) CD (2), UC (6), diverticulitis (2), polyp (1), and anaemia (3).

11 patients had raised WGLF Hb (median 9, range 6 - 28) and faecal Hb within reference range . Of this group 5 patients had CD, 2 had UC and 1 had colon cancer.

1 patient had WGLF Hb within the reference range with slightly raised faecal Hb.(2.5 mg/g)

Median(range) WGLF Hb = 19 (3 - 2640) ug/ml

Median(range) faecal Hb = 2.7 (0.2 - 129) mg/g

Results for patients with either a raised WGLF or faecal Hb were further expressed as multiples (M) of the respective upper limits of normals in order to compare the relative sensitivities of these tests. See Figure 2.10.

The median and range of M values for WGLF and faecal Hb were 3.7 (0.6 - 528) and 1.5 (0.1 - 72).

The M values for WGLF Hb were significantly higher than faecal Hb M values. ( $p < 0.01$ , Wilcoxon Signed Rank Test for paired data).

This indicates that the WGLF Hb test is more sensitive than random faecal Hb in the detection of FOB.

## **Discussion**

Use of WGLF samples for Hb measurement eliminates two major problems caused by faecal samples.

1. sampling error is minimised by using a steady state perfusate (WGLF) aliquot for analysis which can be quantified. To achieve this the rate of ingestion of gut lavage solution must be controlled and monitored.
2. interference from dietary constituents is eliminated.

Additional knowledge of blood haemoglobin can enable quantitative measurement of blood loss. This is important since in many cases blood haemoglobin concentration may be low which will 'increase' calculated blood loss.

Such problems with faeces can be eliminated by making timed faecal collections over several days, mixing and taking an aliquot; however in practise this is rarely done.

Rose et al (1989) have shown that the mean Hb concentration in faecal samples increased from 0.65 (SD 0.42) on a meat free diet to 2.44 (SD 2.15) when consuming 250 g or more of rare red meat per day.

This variable will increase the normal scatter of results within the reference range for faecal Hb concentration, and hence reduce the sensitivity of the test. This influence can be reduced by avoidance of meat in the diet for 3 days prior to the test.

The stool site sampling study gave a CV of 18%. Variation within samples from normal subjects was minimal and analytical variation will contribute to this.

Several patients with active IBD had normal faecal and raised WGLF Hb; this may have been due to dilution of Hb by the greater faecal mass which is typical of this patient group.

## **WGLF HAEMOGLOBIN IN PATIENTS WITH COLONIC POLYPS AND COLON CANCER**

### **Introduction**

The most widespread use of faecal testing for blood is to assist in the diagnosis of colonic cancer.

Ahlquist et al (1990) in a study of 160 patients with a new tissue diagnosis of colorectal carcinoma (CRC) and a preceding stool blood test (Hemoquant) showed that bleeding was a feature in otherwise symptomless CRC in 16% of cases. However these were detected at a more favorable stage.

Faecal blood levels remained normal in about 40% of both symptomatic and asymptomatic patients.

Symptoms were present in most cases (about 70%) of colorectal cancer prior to diagnosis: these were changes in stool pattern, overt bleeding, melena, abdominal pain, and weight loss.

For the entire group the median faecal blood level was only 2.7 mg/g range 0.1 - 67. Overall sensitivity was 59% for symptomatic patients and 56% for asymptomatic patients.

Since right sided cancers tend to bleed more than left sided ones, the combination of faecal blood testing and flexible sigmoidoscopy appears to be complimentary and in this series would have detected 83% of cancers. In this respect the Hemoquant test is superior to guaiac and immunological tests which are better suited for detection of left sided and rectal tumours.

Because of the relatively high incidence of this disease in the general population over 50, and the high parallel mortality rate (20,000 deaths in the UK annually) methods to improve early detection are constantly being sought and evaluated.

Screening of high risk populations for FOB with 'Haemoccult' cards has been reported but the poor sensitivity and specificity of this test has limited its value.

Nevertheless Mandel et al (1993) randomly assigned almost 50,000 asymptomatic subjects aged 50 - 80 to screening annually, biennially or to an unscreened control

group. Participants who were screened submitted six guaiac impregnated paper slides with two smears from each of two consecutive stools. The 13 year accumulative mortality per 1000 from colorectal cancer was 5.88 in the annually screened group 8.33 in the biennially screened group and 8.83 in the control group. The annually screened group mortality rate was significantly lower (33%) than the control group. Reduced mortality in the annually screened group was accompanied by increased survival in those with CRC and a shift to detection at an earlier stage of cancer.

The guaiac based Haemoccult (Rohm Pharma, Weiterstadt, Germany) which is the most commonly used slide test, requires approximately 5 times the average daily loss to give a positive result - sensitivity 51 - 68% for detection of CRC; animal haemoglobin and vegetable peroxidases interfere with this assay and can cause false positive results; rehydration of slides although increasing sensitivity decreases specificity for CRC detection.

The globin moiety of haemoglobin molecule is specifically detected by immunological techniques which as a result are very specific for human blood and more sensitive than guaiac based methods for CRC detection. Some of these are now commercially available.

Robinson et al (1994) compared guaiac and immunological FOBs for population screening for CRC and found the immunological FOB (Hemeselect) to be much more sensitive in the detection of CRC (9/9 with positive FOBs detected) than Haemoccult (1/9 detected).

Many groups have reported that colonic polyps and neoplasms also bleed although the frequency of such is less than for tumours.

Ransonoff and Lang (1991) have argued that the detection of small adenomas (<1.0 cm in diameter) through FOB screening is purely by chance.

Macrae and St John (1982) showed previously that only adenomas with a diameter of >2 cm cause faecal blood loss of >2 ml per day.

Robinson et al (1994) have shown that Hemeselect is much more sensitive in the detection of adenomatous polyps than Haemoccult. Hemeselect detected adenomas of <1 cm in size in 19 patients, between 1 and 2 cm in size in 22 patients and > 2 cm in 7

patients. Corresponding figures for Haemoccult were one, six, and one. Although this paper gives no information on carcinomas/adenomas missed on the 90% of patients who were FOB negative by either test, it clearly indicates the insensitivity of the Haemoccult test.

An epidemiological comparison of the prevalence of adenomas and carcinoma in Norway (Eide,1986) calculated the annual risk of an adenoma converting to a carcinoma to be 0.25% for all adenomas, 3% for adenomas > 1 cm, 17% for villous adenomas, and 37% for those villous adenomas showing severe dysplasia.

## **Subjects**

In a period of two years 530 patients have undergone whole gut lavage for colonic washout prior to colonoscopy, barium enema, IBD assessment or other investigations. All these patients had WGLF Hb and many had blood haemoglobin measured to assess blood loss.

Of these, 20 were diagnosed as having colonic cancer, one had a large benign rectal adenoma, and 20 had colonic polyps;

## **Results**

Results are shown in Figure 2.11

### **1. Colorectal cancer**

WGLF Hb ranged from 5 - 990 (median 31) ug/ml for patients with CRC. Only 1/20 patients had a result within the reference range (5 ug/ml).

GI blood loss in this group ranged from 1 - 133 (median 6) ml per day.

Of this group ten subjects had raised WGLF IgG, eleven had raised albumin and four had raised  $\alpha$ 1-AT.

## 2. Colonic Polyps

WGLF Hb ranged from 1 - 69 ug/ml (median 3) for patients with colonic polyps, with only three patients having results outwith the reference range (6, 20, and 69 ug/ml, blood loss 1, 4, and 12 ml/day).

One patient with a large benign rectal adenoma had a WGLF Hb of 270 ug/ml.

### Discussion

The WGLF haemoglobin test for the CRC indicates a high detection rate (95%) in a hospital population undergoing GI investigation. This is higher than has been previously reported by some groups who claim that up to 30% of tumours do not bleed. The median blood loss in this group of patients was 6 ml per day, and it is possible that about 50% of these patients might be negative for guaiac based tests, since these require a blood loss of 5 - 10 ml per day to be positive.

WGLF proteins were also raised in some patients which may have been directly related to blood loss in some cases where this was considerable or to inflammation at the site of the tumour.

Including the subject with the rectal adenoma only 4/21 subjects with colonic polyps detected by colonoscopy were losing pathological concentrations of blood from the gut. This was disappointing for a test which otherwise was very sensitive. However most of the polyps were <1 cm in diameter and this further confirms that the majority of colonic polyps detected are small and bleed negligibly.

The greater sensitivity of the WGLF haemoglobin test may contribute to the increase in detection rate of colonic tumours. Further experience is required to substantiate this.



## **SPECIFICITY OF WGLF HAEMOGLOBIN FOR GI BLOOD LOSS**

### **Introduction**

A series of 530 patients was investigated for WGLF Hb over a period of two years.

### **Results**

Raised WGLF haemoglobin was found in 15 of the series of 530 patients in whom the diagnosis was not one generally associated with blood loss. See Table 2.6

### **Discussion**

Only 15 of 530 patients had a raised WGLF haemoglobin which was associated with a condition normally unrelated to GI blood loss and only four of these had blood loss greater than 2 ml per day with radiation enteritis, coeliac disease and idiopathic diarrhoea who are being followed up in case significant pathology emerges.

The specificity of this test for GI blood loss is thus very high (>95%) when used in this population. Exclusion of dietary interference and the variability of stool weights may well contribute to this.

**Table 2.1**

**Recovery of blood haemoglobin added to WGLF**

WGLF haemoglobin ug/ml	WGLF + added blood haemoglobin ug/ml	Recovery %
3	611,588	97
1	610,604	99
4	603,597	97

**Table 2.2**

**Recovery of protoporphyrin added to WGLF**

WGLF haemoglobin ug/ml	WGLF + added protoporphyrin haemoglobin ug/ml	Recovery %
3	102,105	93
15	118,112	104

protoporphyrin standard (3.6 ug/ml ) read 1105,1150 (mean 1128)  
haemoglobin standard (67 ug/ml) set at 670.

**Table 2.3****Blood haemoglobin, WGLF haemoglobin , blood loss, and WGLF IgG in patients with IBD**

	CD		UC	
	ACTIVE (58)	INACTIVE (35)	ACTIVE (26)	INACTIVE (16)
BLOOD HB g/l	123 (92-165)	138 (97-156)	126 (88-156)	134 (111-169)
WGLF HB ug/ml	6 (0-58)	3 (1-9)	16 (1-151)	3 (0-16)
BLOOD LOSS ml/day	1.5 (0-12.8)	0.7 (0.2-1.7)	4.4 (0.3-36)	0.7 (0-4.2)
WGLF IgG ug/ml	37 (10-280)	3 (1-9)	48 (10-318)	4 (0-9)
median(range)				

**Table 2.4**

**Concentrations of IgG (reflecting disease activity) and of haemoglobin in WGLF from a series of patients with IBD who had gut lavage performed twice within a four week period.**

	IgG (ug/ml) in WGLF		Hb (ug/ml) in WGLF	
	Relapsed	Treated	Relapsed	Treated
CD	55	24	10	5
	93	60	22	9
	144	82	162	145
	137	54	58	14
	27	9	12	9
	216	41	15	9
	44	34	9	8
	101	11	14	4
	UC	254	102	151
188		2	23	4

**Table 2.5**

**GI diseases and other factors of possible relevance to iron deficiency anaemia in 42 patients**

**Malabsorption**

Atrophic gastritis	5
Partial gastrectomy without gastritis	4
Partial gastrectomy with gastritis	3
Coeliac definite	1
Coeliac probable	1

**Other GI disease**

Oesophagitis mild	4
Oesophagitis severe ulcerative	1
Oesophageal carcinoma	1
Hiatus hernia	10
Acute gastritis	2
Gastric vascular ectasia	1
Gastric carcinoma	1
Duodenal ulcer	1
CD (ileum)	1
Colon cancer	4
Benign colonic polyps	2
Diverticular disease	6

**Abnormal Coagulation**

On anticoagulants	3
Previously anticoagulated ,	1
Idiopathic thrombocytopenic purpura	1
Alcoholic liver disease	1

**Drugs**

Previously taking NSAIDs	8
Currently on NSAIDs	1

**Poor diet**

Iron deficient diet	10
---------------------	----

**Table 2.6****Patients with WGLF haemoglobin >5 ug/ml having a condition not associated with GI blood loss.**

Diagnosis	No	WGLF haemoglobin (ug/ml)
Constipation	2	7,9
Idiopathic Bile acid diarrhoea	3	6,6,6
Radiation enteritis	1	143
Radiation strictures	1	6
Coeliac	1	15
Idiopathic diarrhoea	3	8,10,32
Travellers diarrhoea	1	8
Diabetic, renal transplant	1	6
Wt loss of unknown cause	1	6
Depression, alcohol abuse	1	9

FLUORESCENCE DILUTION CURVE  
WGLF HAEMOGLOBIN

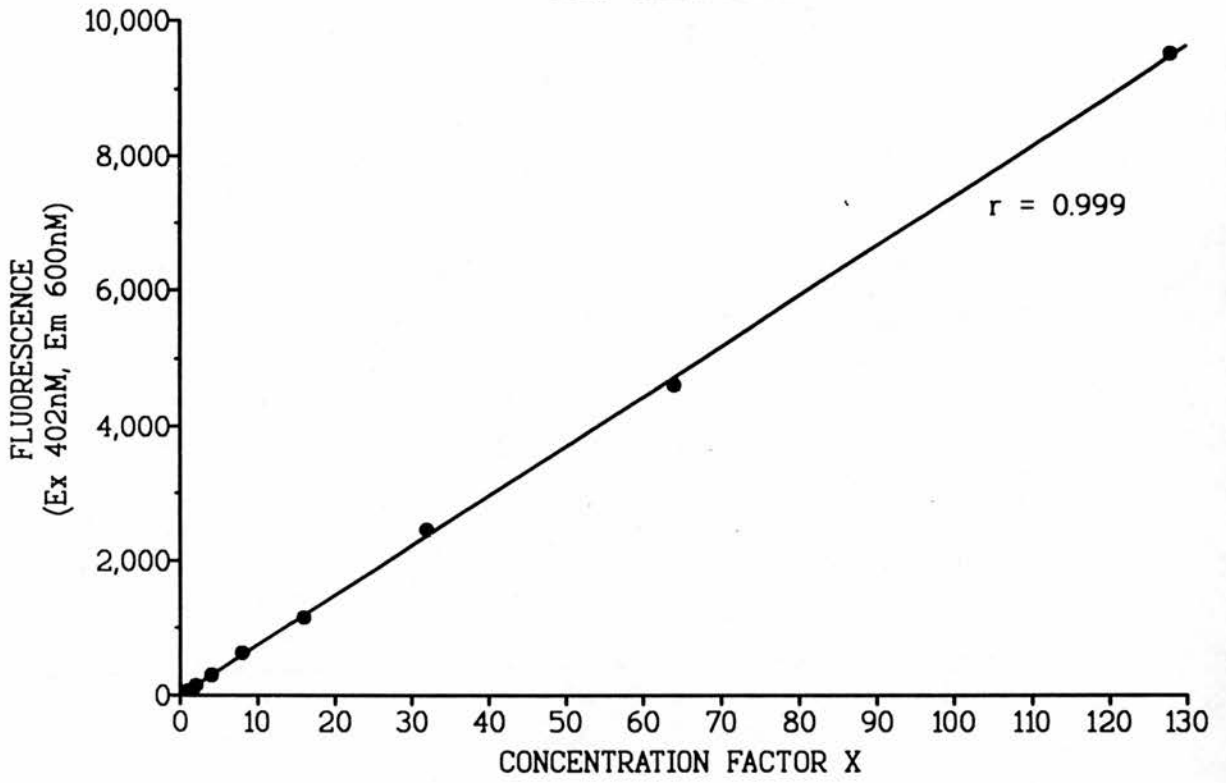


Figure 2.1

WGLF HAEMOGLOBIN  
CONTROLS

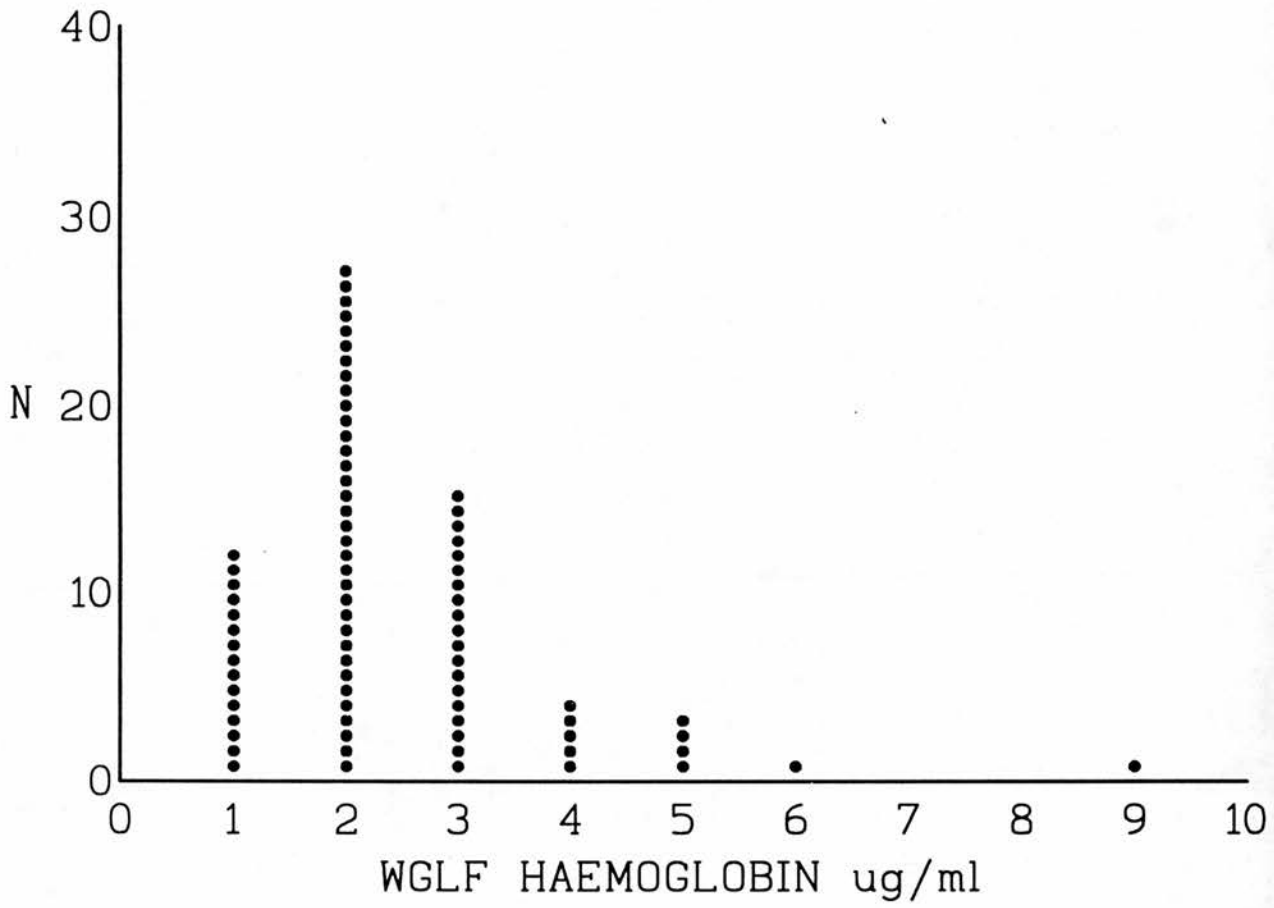


Figure 2.2

GI BLOOD LOSS in IBD

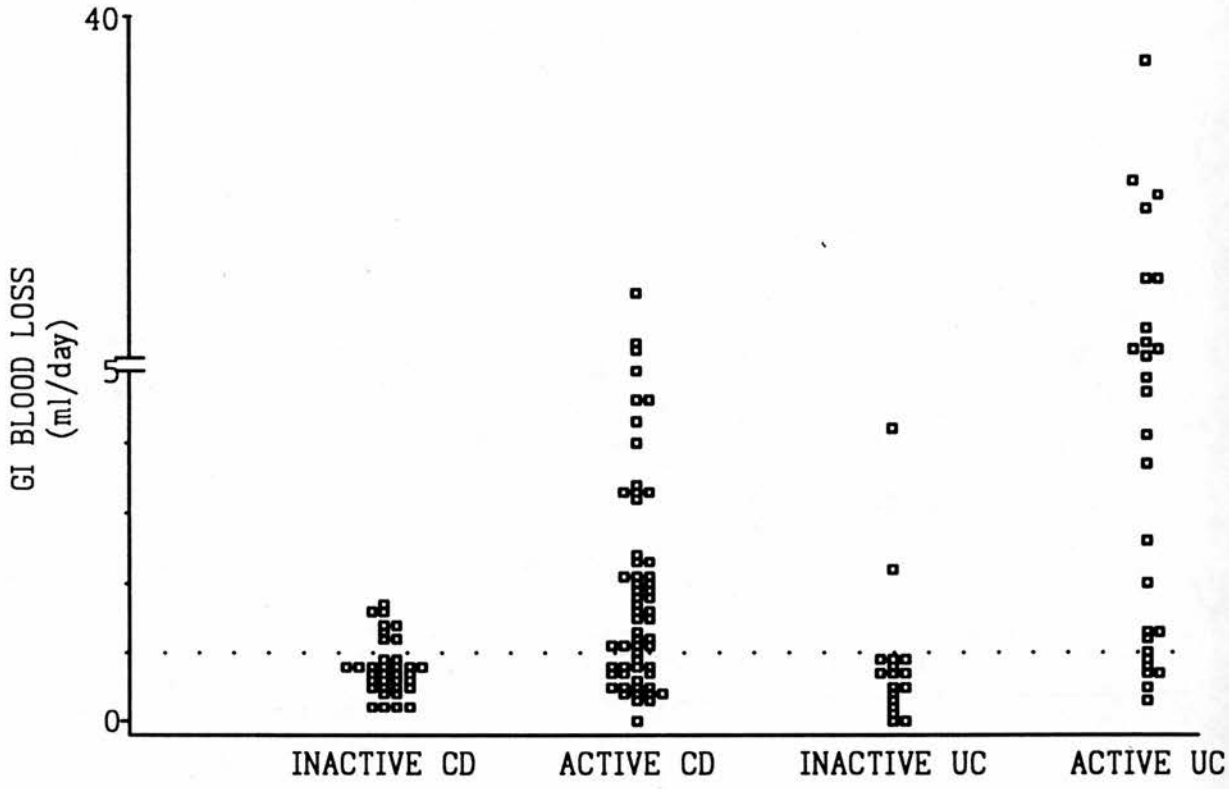


Figure 2.3

WGLF IgG and GI BLOOD LOSS  
in ACTIVE CD

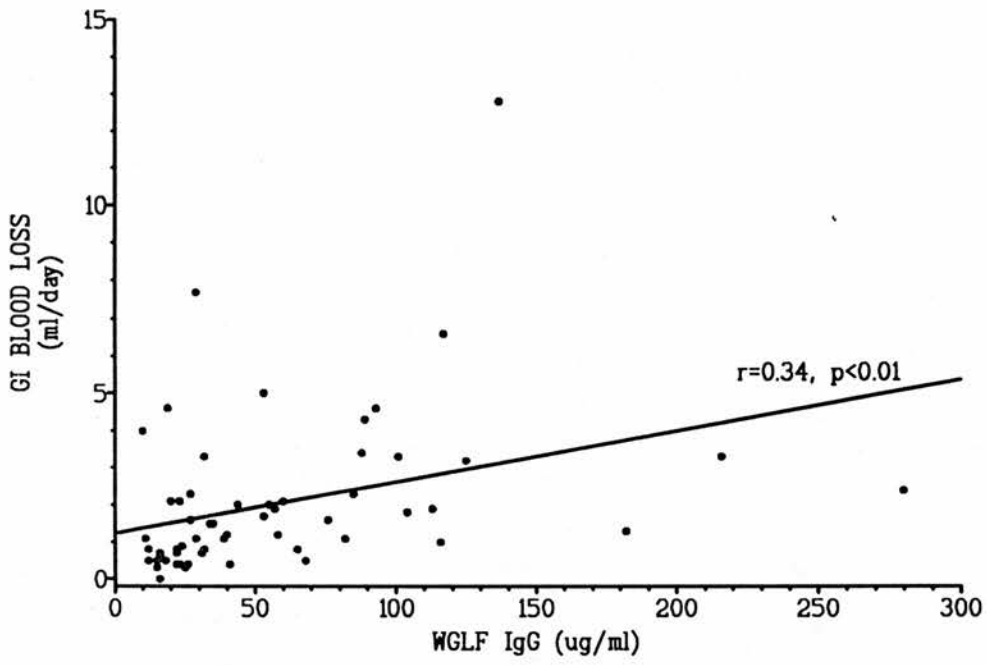


Figure 2.4a

WGLF IgG and GI BLOOD LOSS  
in ACTIVE UC

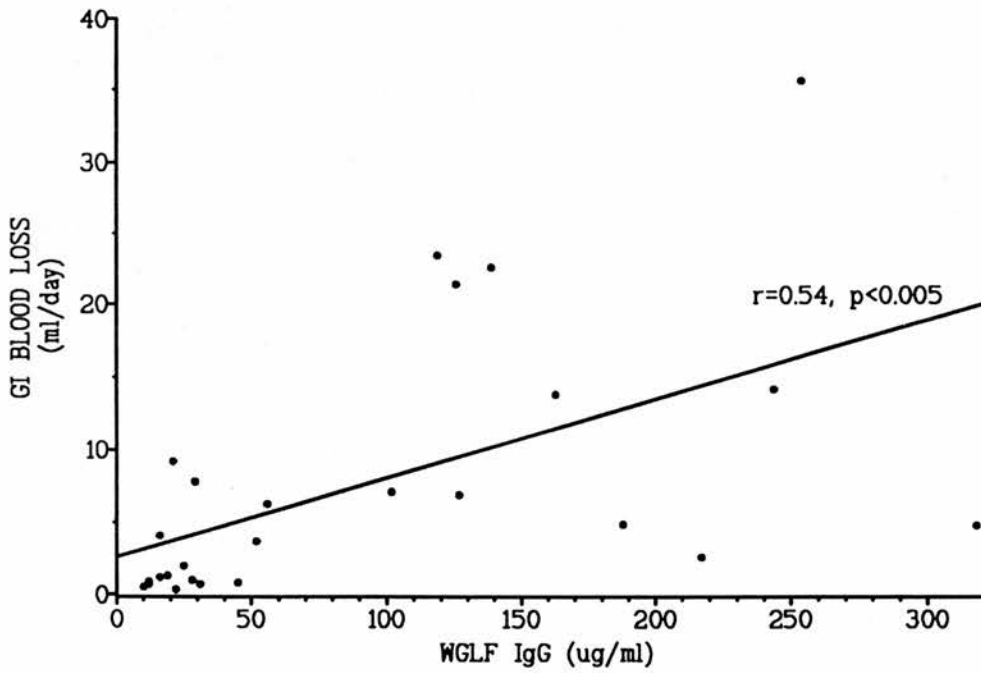


Figure 2.4b

WGLF IgG and BLOOD HAEMOGLOBIN  
in ACTIVE UC

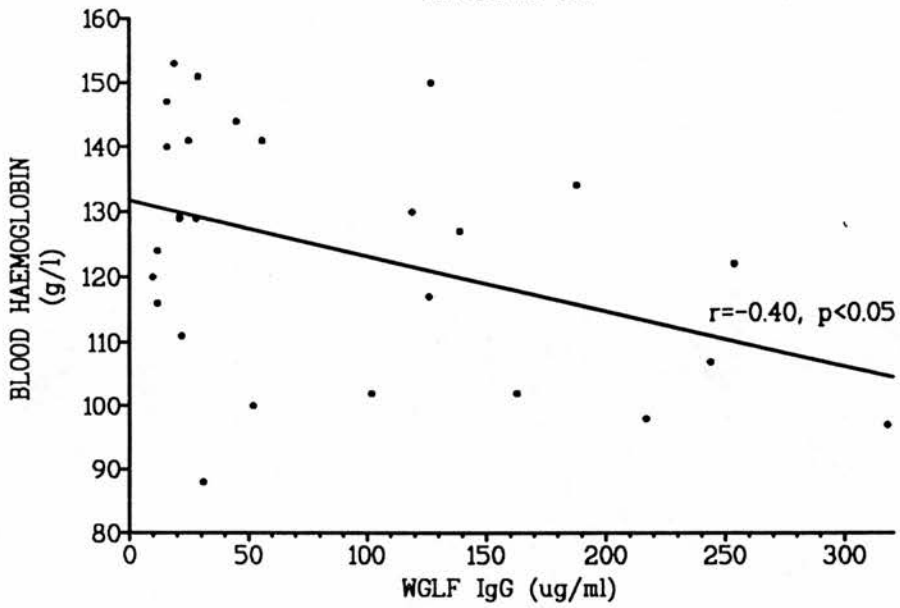


Figure 2.5a

BLOOD LOSS and BLOOD HAEMOGLOBIN  
in FEMALES with ACTIVE CD

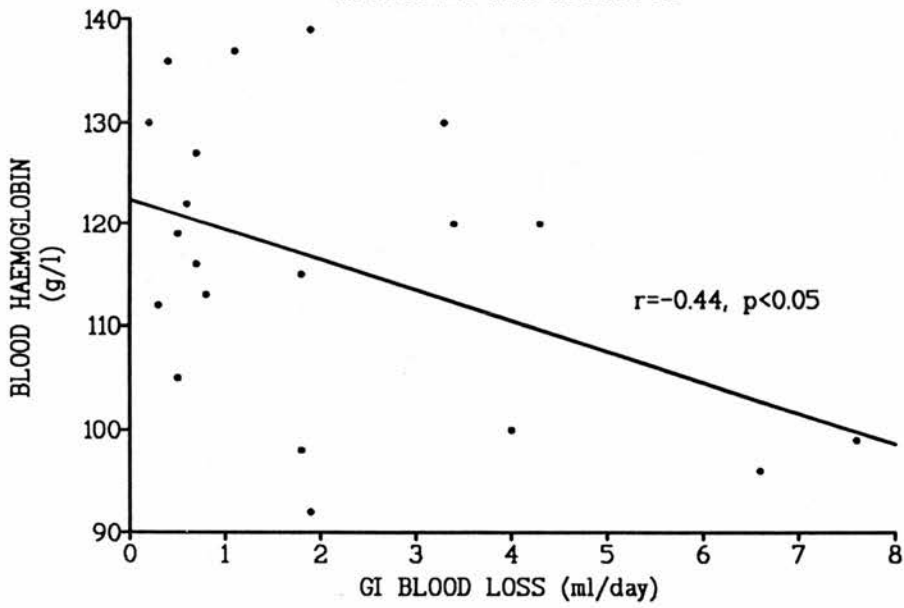
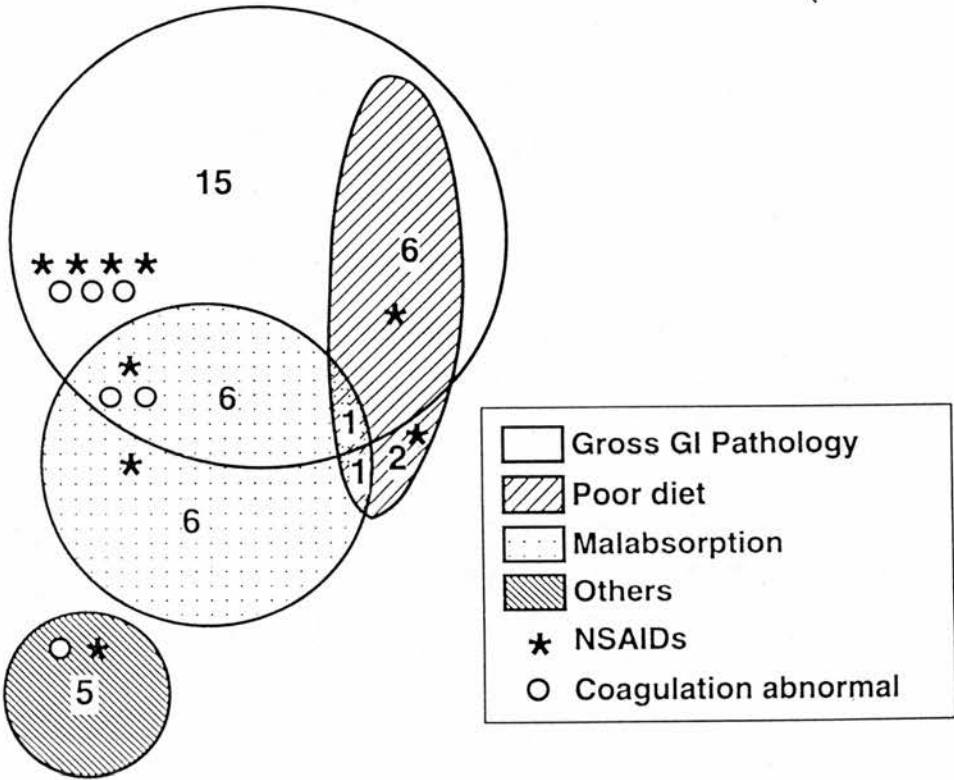


Figure 2.5b

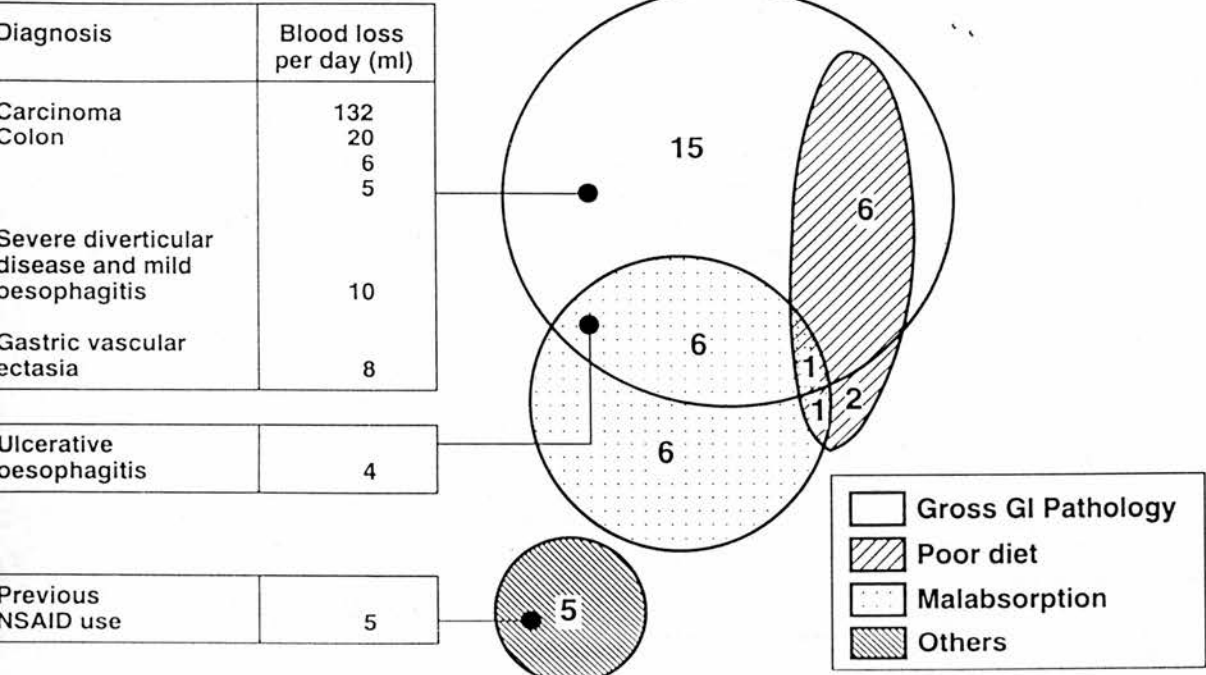
**Presence of gastrointestinal diseases and other aetiological factors in 42 patients with iron deficiency anaemia.**



**Figure 2.6**

In 28 patients, one or more GI disease was detected by imaging or endoscopy, or both; in 10 the diet was judged by an experienced clinician to be deficient in iron; 14 patients had diseases associated with iron malabsorption. As shown there was significant overlap between these groups and with the presence of other relevant factors such as use of NSAIDs or coagulation abnormality

**Distribution (within the classification of Figure 2.6) and diagnosis for the eight patients in whom GI blood loss was estimated to be > 2ml per day**



**Figure 2.7**

FAECAL HAEMOGLOBIN in CONTROL SUBJECTS  
and in PATIENTS with GI DISEASE

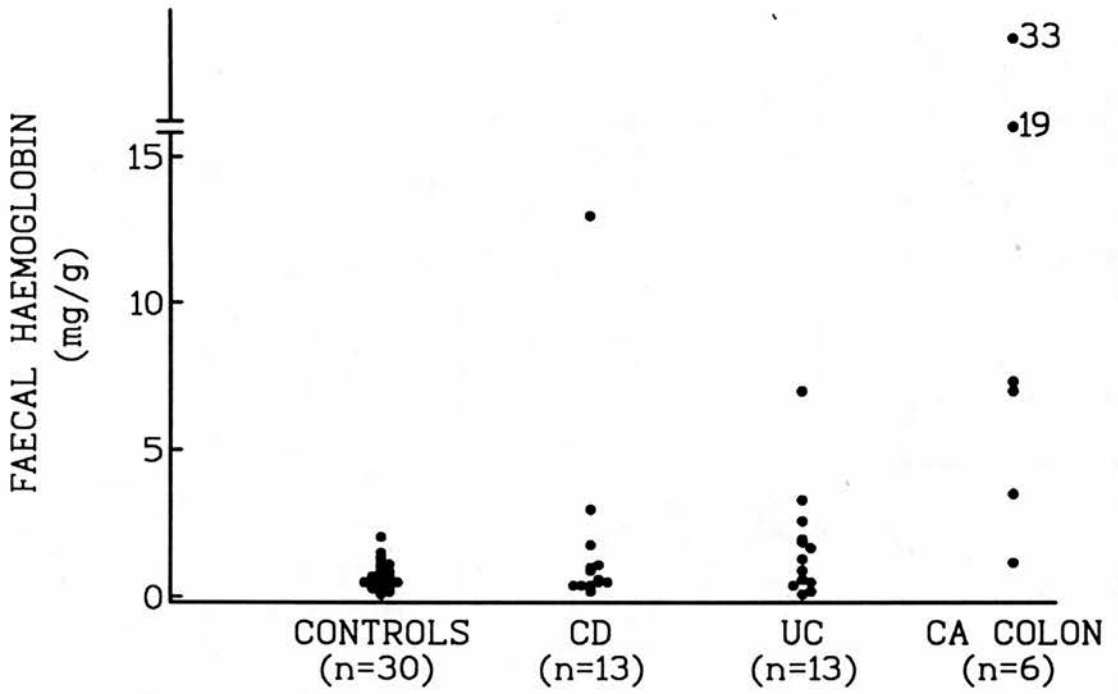


Figure 2.8

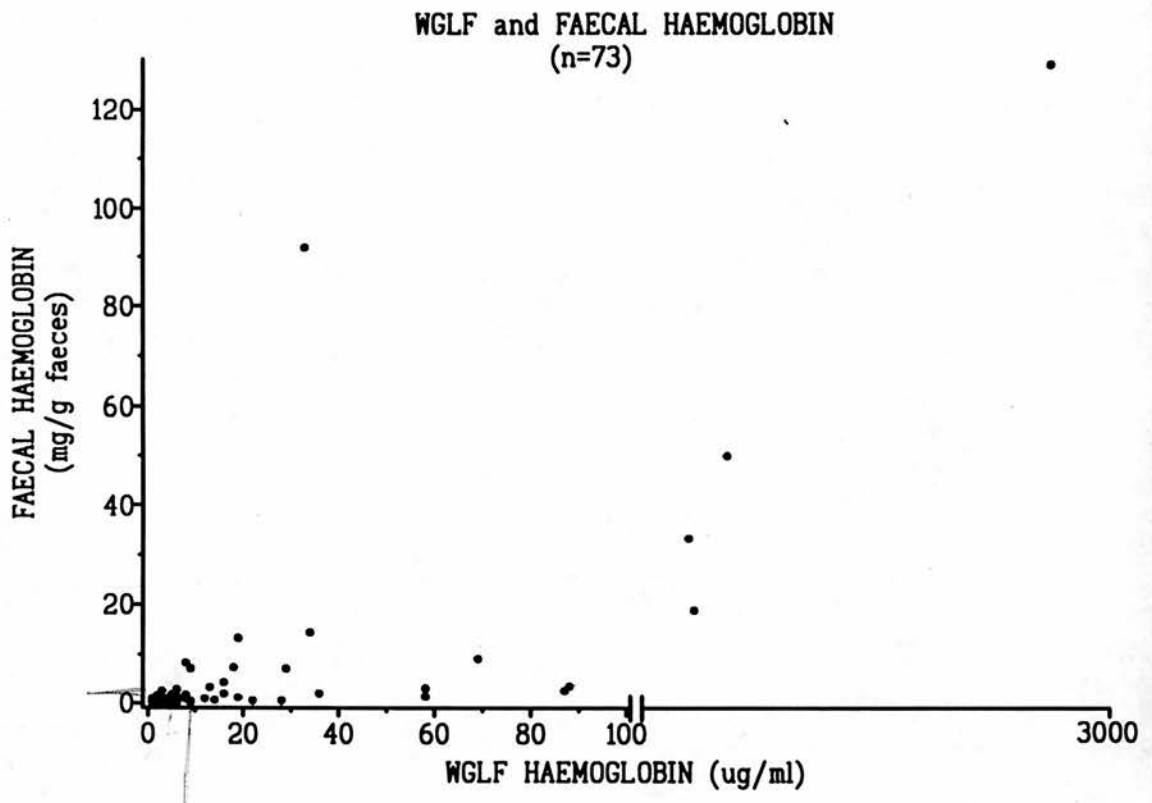


Figure 2.9

### WGLF and Faecal Haemoglobin: Multiples outwith reference range

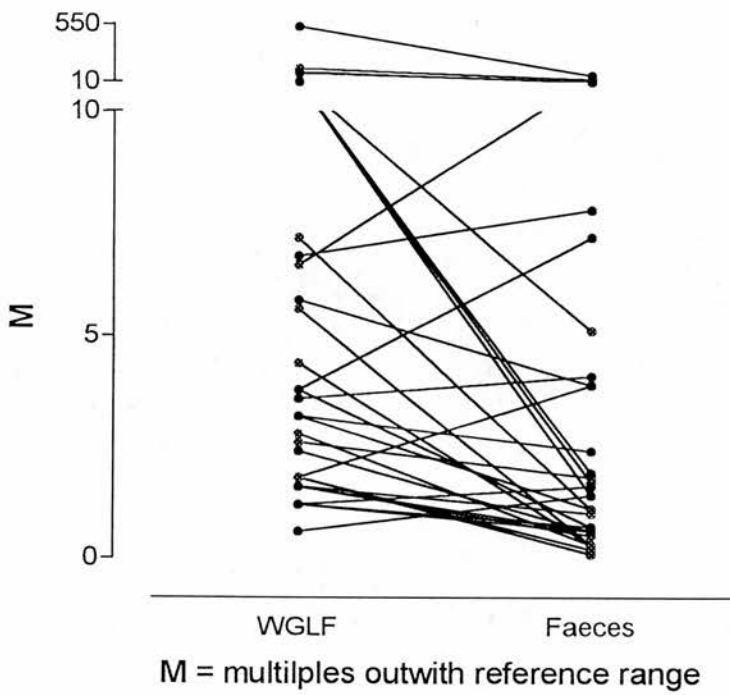


Figure 2.10



## APPENDIX : CHAPTER 2

### 1. Protocol for whole gut lavage solution administration to patients

#### Introduction:

The whole gut lavage procedure is carried out using 'Kleen-Prep' or 'Golytely' which are highly effective, commercially prepared orally administered bowel cleansing agents which produce a consistently high quality bowel preparation when used prior to barium enema or colonoscopy tests. Gut lavage with these preparations involves no dietary restrictions, no net loss of fluids or electrolytes and enables a patient to be prepared in as little as four hours in complete safety even in those patients who are elderly, poorly hydrated or have cardiac or renal impairment.

It is well tolerated by over 90% of patients and over three million doses have now been given worldwide.

The composition of the whole gut lavage solution is designed to maintain fluid and electrolyte balance while rapidly cleansing the GI tract.

Since the patient has to swallow a large amount of liquid it is very important that the composition of the solution does not deviate from the tightly specified values which otherwise may compromise patient safety.

#### Pharmacology and preparation of WGL solution:

Whole gut lavage solution contains sodium chloride, sodium bicarbonate, sodium sulphate, potassium chloride, and polyethylene glycol 3350 (see below). The sodium, potassium, chloride and bicarbonate counteract the absorption or secretion of these major serum electrolytes. Sulphate ions inhibit the active absorption of sodium and chloride when substituted for the latter. This allows the electrolyte balance of the patient to be undisturbed. The inert, osmotically active PEG balances the osmolarity of the solution to that of plasma which results in no water absorption or secretion so that patients become neither dehydrated nor overloaded with fluid. The product comes as sachets of dry powder which are reconstituted to 1 litre with tap water.

Each sachet contains the following:

sodium sulphate (anhydrous)	5.685g
sodium bicarbonate	1.685g
sodium chloride	1.465g
potassium chloride	0.743g
PEG 3350	59g
aspartame	0.05g

The solution has a slightly salty taste but is still acceptable to the majority of patients. The palatability is improved if the solution is chilled overnight before use.

Sugar or fruit juice must not be added to the solution as this would upset the osmotic balance.

## Administration of WGL solution to the patient:

It is recommended that the patient may have a normal diet up to midnight before starting the procedure at 0900 hrs.

No food or fluids should be given during the procedure.

The patient should drink one glass of solution (200 ml) every ten minutes. It is easier for the patient to drink this quickly rather than by sipping ( half a glass every five minutes is typical).

Initially the patient may feel full and bloated but this will pass after the first bowel movement which usually occurs after about 60 minutes.

If the patient feels very uncomfortable, it may be necessary to reduce the rate of drinking; in extreme cases the patient may feel very nauseated and will vomit if pressed to continue; in such cases an injection of a suitable anti-emetic such as metoclopramide should be considered.

The patient should continue drinking the solution until they are passing clear faecal fluid containing no solid faecal matter. This usually occurs between three to four hours after commencing drinking the solution. After the patient has ceased to drink the lavage solution, they will pass faecal fluid several more times. Most patients will not experience the urgency and discomfort associated with purgatives.

## **APPENDIX : CHAPTER 2**

### **Measurement of haemoglobin and metabolites in WGLF**

#### **Principle of Method**

Haemoglobin is converted to fluorescing porphyrins by the removal of Fe. Total haemoglobin is determined by reaction with heated oxalic acid:FeSO<sub>4</sub> reagent which converts haem to porphyrin without loss of preformed porphyrins. A 3 step purification procedure eliminates other interfering fluorescent materials which may be present.

#### **Specimen Requirements**

1.WGLF: 2 x 1.5 ml samples of WGLF (unfiltered) are aliquoted into 2 ml microtubes with screw lids (Sarstedt, UK). 30 µl sodium azide (2 g/100 ml distilled water) is added as preservative, and samples stored at -70°C.

#### **Sample storage and stability**

Samples of WGLF should be kept stored at -70°C, and analysed within 2 months. There is no data on stability of samples beyond this time.

#### **Instrumentation**

Fluorescence is measured using the LS-5B Luminescence Spectrometer (Department of Medicine). Switch on main power (rear of instrument, rhs). Set excitation slit width to 15 nm (lhs), and emission slit width to 5 nm (rhs). Set excitation wavelength to 402 nm and emission wavelength to 600 nm in the following way

- a. Press low high reset simultaneously.
- b. Press 402 goto EX
- c. Press 600 goto EM and leave instrument for 5 minutes to stabilise.
- d. Place blank sample in cell carrier (nearest the instrument) and press AUTOZERO (light on)
- e. Place standard sample in the cell carrier set value eg 1000 for 100 µg/ml standard and press AUTOCONC (light on).
- f. Repeat steps 4 and 5 to finalise calibration.
- g. Test samples can now be read.

#### **Reagents**

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. These are stored in room 7.

a. Oxalic Acid Reagent: 4.0 g oxalic acid (analar) is made up to a 10 ml volume with distilled water, and dissolved in a water bath at 100°C. 0.31 g FeSO<sub>4</sub> is added to the oxalic acid reagent which is returned to 100 C. 0.10 g uric acid and 0.11 g mannitol are then added with mixing, final volume adjusted to 10 ml and the reagent left at 100°C for 5 minutes. The suspension is pipetted while hot in the fume cupboard. The reagent is prepared fresh before use. Dispose of excess reagent by washing down sink immediately after use.

b. Ethyl acetate/acetic acid 10/1 v/v. Add 50 ml glacial acetic acid to 500 ml ethyl acetate in a fume cupboard. Mix and store in solvent cupboard. Label corrosive/inflammable.

c. 3M Potassium Acetate (294 g/l). Dissolve 29.4 g potassium acetate (BDH GPR) in 100 ml distilled water. Store at room temperature.

d. 3M Potassium Acetate in 1M Potassium Hydroxide (56 g/l). Dissolve 147 g potassium acetate in about 300 ml distilled water, add 28 g potassium hydroxide (fume cupboard), dissolve with stirring and make up to 500 ml with distilled water. Store at room temperature. Label corrosive.

e. N-butanol (Rathburn Chemicals Ltd, Walkerburn). Store in solvent cupboard.

f. 2M H<sub>3</sub>PO<sub>4</sub>/Acetic acid 9/1 v/v. Dissolve 68 ml orthophosphoric acid in 300 ml distilled water (fume cupboard). When cool, make up to 500 ml with distilled water and add 58 ml glacial acetic acid. Store in corrosives cupboard. Label corrosive.

g. PEG 4000 reagent: Dissolve 60 g PEG 3350, 9 g NaCl, and 0.2 g sodium azide in 1 litre of distilled water. Use to make Drabkins reagent. Do not keep any excess reagent. Flush down sink with copious water.

h. Drabkins Reagent: (Sigma cat. no. 525-2) 6 vials.

Each vial contains 1 g sodium bicarbonate, 0.2 g potassium ferricyanide, and 0.05 g sodium cyanide.

Take 1 vial and reconstitute to 1 litre with PEG 4000 reagent.

Store at room temperature. Label poisonous.

### Standard Material

a. Cyanomethaemoglobin Standard: Dissolve 50 mg haemoglobin (Sigma - H 7379) in 100 ml Drabkins reagent. Leave at room temperature for 15 minutes. To calculate actual haemoglobin concentration read OD at 540 nm against Drabkins reagent (blank).

Molar extinction coefficient (540 nm) = 44.0

Dilute x5 in Drabkins reagent to give a standard value of about 100 ug/ml. Aliquot (0.5 ml) and store at -70°C. Label poisonous. Stable for up to 6 months.

### Procedure

a. WGLF samples should be frozen at -70°C before assay to allow haemolysis of any intact red cells. After thawing these should be centrifuged at 2000 rpm in the Mistral 3000i (Room 1), and supernatant used for assay.

b. Add 0.4 ml of the oxalic acid reagent to 0.1 ml WGLF supernatant, quality control, haemoglobin standard, and blank (golytely) in a 10 ml stoppered quickfit tube. Mix thoroughly and heat at 100°C for 30 minutes in a water bath. Remove stoppers and cool for 2 minutes.

c. Add 1 ml of the 3M potassium acetate reagent, followed by 3 ml ethyl acetate/acetic acid reagent. Vortex mix for 30 seconds. This extracts the porphyrin analytes into the upper organic phase.

d. Transfer 2 ml of the upper organic phase to a 30 ml stoppered quickfit tube and add 0.8 ml butanol and 6.0 ml of 3M potassium acetate in 1M KOH. Mix for 30 seconds. This removes coproporphyrin and other porphyrins not derived from haemoglobin haem (contain more than 2 carboxyl groups) - extracted into the lower alkaline aqueous phase.

e. Transfer 1 ml of the upper organic phase to a 10 ml stoppered quickfit centrifuge tube, and add 3 ml phosphoric acid/acetic acid reagent. Mix for 30 seconds. Remove the top layer which contains chlorolorphyll. Read the fluorescence of the lower acid extract.

f. For instructions in use of the spectrofluorometer see **Instrumentation** section.

Tests readings / 10 give results in µg/ml.

g. For faecal haemoglobin, weigh out about 0.1 g faeces, and add whole gut lavage solution to this to make the final faecal concentration 0.1 g faeces per ml lavage fluid. Break up the faeces into a fine suspension using wooden applicators and rotaxixer. Use 0.1 ml faecal suspension as sample and then analyse as for lavage.

spectrofluorometer readings / 1000 give results in mg haemoglobin per g faeces.

### Interferences and Limitations

Other haem containing proteins have the potential to interfere in this assay; eg haemoglobin, myoglobin in meat, peroxidases in vegetables, although neither of these should cause significant interference in lavage samples; WGLF still contaminated with faecal material, where any blood cells may be concentrated should be avoided. Faecal

haemoglobin values can be raised by up to 1 mg/g faeces for a daily red meat intake of 100 g, given an average stool output of 150 g. Vegetable peroxidases will cause no significant interference taken even in large amounts. WGLF haemoglobin will be unaffected by dietary constituents as any intestinal dietary residues will be flushed through by the lavage solution before samples are collected.

## **CHAPTER 3**

### **PROTEIN LOSS IN GASTROINTESTINAL DISEASE**

#### **PROCEDURES DEVELOPED TO MEASURE WGLF PROTEINS**

##### **Introduction**

Most previous studies of protein loss from the gut involve the measurement of radiolabelled proteins in faeces after intravenous administration or the measurement of faecal components which are stable during gut transit.

However the vast majority of proteins excreted in faeces have been partially or completely degraded.

The WGL procedure allows proteins secreted into or diffusing across the gut lumen to be obtained quickly, before significant degradation occurs and procedures which inhibit protein degradation allow more accurate measurement of potentially labile proteins.

Gaspari et al (1988) inhibited proteases in WGLF using gauze filtration and a two stage centrifugation procedure in combination with addition of a series of reagents, and the success of this procedure was assessed using a non specific protease reagent. This procedure was considered protracted and labour intensive and an alternative using a one step filtration procedure has been developed.

O'Mahony et al (1990,1991a) have previously shown in preliminary studies in patients with active inflammatory bowel disease that both IgG and albumin are raised in WGLF, and that IgA levels decrease with time unless proteases are inhibited.

Since plasma may be a source of IgG in the gut and is almost certainly the major source of albumin in patients with intestinal disease, both these proteins as well as  $\alpha$ 1-AT, which is known to resist proteolysis in gut contents, have been measured. Methods have been developed to measure  $\alpha$ 1-AT and albumin in WGLF which have potential as rapid first line indices of inflammatory bowel disease.

Depending on the site of lesion in the bowel, proteins once leaked or secreted into WGLF may be in the gut lumen for between 5 and 120 minutes approximately before being passed per rectum. Hence it is important to assess the biological stability of such proteins, as this will affect the reliability of their measurement.

### **Development of a simple, rapid method for processing WGLF samples.**

In order to protect immunoglobulins and other proteins in WGLF from degradation by pancreatic, intestinal and bacterial proteases, Gaspari et al (1988) devised a relatively complex and time consuming procedure involving both filtration, centrifugation, and reagent addition and although suitable for research into a small number of samples it was felt that a simplified version would facilitate the procedure and make it more suitable for wider use with the potential for increased protein protection if more rapid.

As a result a single filtration stage was undertaken using Whatman GFA/A glass fibre filter papers with a nominal cut off size of 1.6  $\mu$ M which retains cells and cell debris. Protease inhibitors and preservatives were added in the same order and concentrations as used by Gaspari et al, and the procedure was completed within 15 minutes. Both WGLF processing procedures are described in chapter 3 appendix.

### **Comparison of processing procedures**

In order to validate the new processing procedure WGLF samples which had been simultaneously processed by the original and new procedures were assayed for protease activity using a non specific protease assay (chapter 3, appendix) and WGLF proteins. See Table 3.1 for results.

Two groups of patients were used for 1. IgG and protease inhibition and 2. albumin and  $\alpha$ 1-AT measurements since these were carried out at different stages of method development.

There was no significant difference between IgG, albumin,  $\alpha$ 1-AT or percent protease inhibition when processed by either procedure - Spearman's Rank Sum Test for paired data.

## **Comparison of filtration processing procedure with protease inhibition by addition of PMSF reagent only**

The serine protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) has been used to treat aspirates of gut contents (Hohmann et al, 1983). For this reason it was decided to compare the processing procedure as devised with the simple addition of PMSF reagent alone to WGLF as this would further facilitate sample collection and also collection tubes could be prepared in advance.

Ten WGLF samples were collected and processed as per normal using the filtration procedure, and also by addition of PMSF alone to a final concentration of 2 mmolar. WGLF samples were also retained to which sodium azide alone was added which provided baseline protease values.

All samples were then assayed for non-specific protease (Asacoll) activity and chymotrypsin (chapter 3, appendix).

Results for non specific protease percent inhibition are shown in Figure 3.1. In all cases the full processing procedure resulted in a >95% inhibition of protease activity. Although PMSF gave >70% inhibition in all cases the inhibition was less complete for each sample (Spearman's Rank test  $p < 0.001$ ).

CT results ranged from 0.8 - 3.4 u/ml before and between 0 - 0.02 u/ml after the different processing procedures. Inhibition of CT was similar by both procedures (>99%)

## **Development of techniques for the measurement of WGLF albumin and $\alpha$ I-AT**

Simple manual procedures based on immunoturbidimetric measurement have been developed according to Whicher et al (1983) to measure both albumin and  $\alpha$ 1-AT in WGLF. The main advantage of these assays is that they can be performed in less than 1 hour.

Turbidimetry involves the measurement of light scattering species in solution by means of the decrease in intensity of the incident beam as it passes through the sample. The light has been lost due to absorption, reflection and scatter. Turbidimetric

measurements can be made with a spectrophotometer and the signal will be a function of several factors including monochromator wavelength, spectral band width, stray light, cuvette path length and geometry, light source and detector stability.

Optimal concentrations of polyethylene glycol (PEG) 6000 and antibody, and time course of turbidity development were determined.

PEG concentrations of 2,3 and 4% were chosen for turbidity enhancement and turbidity development of 100 ug/ml standard of both albumin and  $\alpha$ 1-AT followed for 60 minutes at 340 nM.

Calibration curves for albumin and  $\alpha$ 1-AT were followed using 1 - 3% antibody concentration.

For standardisation human serum was used (SPS-01, PRU, Sheffield). This was diluted in isotonic saline containing 48 g/l PEG 3350 and 1 g/l sodium azide prior to preparing standard curves for both tests.

## **Results**

Results are presented in Figures 3.2 and 3.3. Turbidity development was complete within two minutes using 4% PEG 6000 concentration, for both albumin and  $\alpha$ 1-AT assays, and remained constant for at least 60 minutes.

2.5% concentration of antibody gave a standard curve for both assays with antigen excess at least twofold beyond the expected possible pathological values for the assays.

Calibration curves for albumin and  $\alpha$ 1-AT are shown in Figures 3.4a and 3.4b.

## **Recovery Experiments**

Standard serum was added to four different WGLF samples one processed and one unprocessed. For  $\alpha$ 1-AT 0.02 ml standard serum (1.38 g/l  $\alpha$ 1-AT) was added to 0.53 ml WGLF. For albumin 0.02 ml of a 1 in 20 dilution of standard serum (42 g/l albumin) was added to 0.53 ml WGLF. Results are shown in Table 3.2.

Recoveries ranged from 94 - 110% for unprocessed samples and 92 - 110% for processed samples for  $\alpha$ 1-AT and 96 - 104% for unprocessed samples and 97 - 110% for processed samples for WGLF albumin.

### **Comparison of $\alpha$ 1-AT by immunoturbidimetric and radial immune diffusion methods**

WGLF  $\alpha$ 1-AT was measured initially using a radial immune diffusion (RID) procedure (LC-Partigen, Behring, France), which is commonly used to measure faecal  $\alpha$ 1-AT. This has a working range of 8 - 125 ug/ml. Many of the subjects with active IBD were found to have values within this range. Subsequently parallel immunoturbidimetric methods were set up for both  $\alpha$ 1-AT and albumin.

WGLF samples (n = 62) have been analysed by both immunoturbidimetric and RID procedures. The standard curve for the RID procedure is shown in Figure 3.5, and the comparison in Figure 3.6b. Methods are described in full in Chapter 3, appendix. Correlation coefficient was 0.97 and slope 1.17.

### **Comparison of albumin by immunoturbidimetric and centrifugal analyser immunoturbidity**

WGLF samples (n = 54) have been analysed by both procedures and results presented in Figure 3.6a. Methods are described in full in Chapter 3, appendix. Correlation coefficient was 0.99 and slope 1.19.

### **Investigation of the stability of IgG, albumin, and $\alpha$ 1-AT in WGLF**

In order to determine the stability of individual proteins in WGLF patient sera (0.5 ml), was added to WGLF (50 ml) on eight occasions and samples processed immediately and after two hours at 37°C, prior to analysis for WGLF proteins.

Concentrations of both IgG and  $\alpha$ 1-AT were not significantly different before and after incubation. Concentrations of albumin decreased in all WGLF samples after incubation at 37°C. See Figure 3.7. Mean decrease in WGLF albumin was 49%.

From these results it can be concluded that both IgG and  $\alpha$ 1-AT leaking into the gut lumen will be recovered from WGLF, whilst albumin will be lost to a variable degree.

### **Total Protein in WGLF**

The total protein found in WGLF will reflect protein secreted in biliary and pancreatic fluid, intestinal secretions and mucus, and in disease, any protein loss across the mucosa. Much of this protein is normally metabolised and reabsorbed as amino acids and peptides, and so total protein in WGLF is a measure of protein movement into the gut rather than protein loss. However this information is not available from faeces, and may be of significance in intestinal disease.

Total protein has been measured in WGLF in patients without or with minimal GI disease (normal or constipated) and subjects with IBD, using the Bio-Rad protein assay (chapter 3, appendix). Results are shown in Figures 3.8a,b.

Control subjects have protein concentrations median 205, range 110 - 380 ug/ml. Much of this will be IgA which is present in WGLF at a concentration of 50 - 200 ug/ml. Subjects with IBD have significantly higher values: CD: median 410, range 210-1300 ug/ml ( $p < 0.001$ ); UC: median 925, range 430 - 1800 ug/ml ( $p < 0.02$ ). This may in part reflect leakage of plasma protein across the gut lumen.

These results should be interpreted with caution as individual proteins react differently with the protein reagent used for this measurement. A composite human serum standard (SPS01, Sheffield, UK) was used to calibrate this assay.

### **Discussion**

The WGL procedure has facilitated the measurement of proteins which are released into the gut lumen. Adequate treatment of this fluid to prevent protein metabolism is essential to quantify proteins present. The use of a simple filtration procedure, with subsequent addition within minutes of stabilising reagents, has facilitated this

procedure. The filter selected GFA/A (Whatman,UK) allows a rapid flow of WGLF filtrate which can then be treated with the series of inhibitors. There was no significant difference in a series of proteins measured when processed by the Gaspari or filtration procedures which confirmed the efficacy of protein protection, with non specific protease inhibition being >95% for both procedures.

The action of the various reagents is as follows: Soybean trypsin inhibitor inactivates pancreatic trypsin; PMSF inhibits serine proteases, which includes chymotrypsin and trypsin; ethylenediamine tetraacetic acid complexes calcium which is a co-factor for some proteases; sodium azide inhibits bacterial growth; newborn calf serum may slow down proteolysis by acting as an alternative substrate for any remaining proteases.

The use of PMSF alone to inhibit proteolysis was shown to be less effective than addition of all reagents. Although PMSF inhibits the major proteases of the GI tract ie trypsin and chymotrypsin, others such as aminopeptidases, and carboxypeptidases will remain active and the full processing procedure was considered superior and necessary for biological recovery of gut proteins.

The method for measuring WGLF IgG is by enzyme linked immunosorbent assay (ELISA-Gaspari et al,1988) and has been established in the laboratory for some time. Albumin measurements have been measured using a micro albumin centrifugal analyser technique in the Clinical Chemistry Department, Western General Hospital, Edinburgh. Manual immunoturbidimetric methods for the measurement of albumin and  $\alpha$ 1-AT have been developed. Their main advantage over other analytical procedures eg ELISA or radial immune diffusion are simplicity and speed of analysis. Sensitivity for these tests was reasonable in the pathological range and added standard recovered well for both assays in a series of processed and unprocessed WGLFs. Buffone and Shulman (1985) have compared radial immune diffusion and nephelometry for measurement of  $\alpha$ 1-AT in faecal extracts with both tests giving comparable results although the RID test was more influenced by complexing with elastase.

The stability of both IgG and  $\alpha$ 1-AT in WGLF over a two hour period without protease inhibition was satisfactory with no significant inhibition. Albumin was less stable (mean destruction - 49% over two hours) and this may influence its diagnostic

value.

**Table 3.1**

**Comparison of results for WGLF proteins and protease when samples are processed using filtration or centrifugation procedures**

No	IgG ug/ml		Protease %inhibition		Albumin		$\alpha$ 1-AT	
	F	C	F	C	F	C	F	C
1	0	0	100	98	40	36	16	15
2	2	2	98	99	3	2	0	1
3	2	1	98	99	3	3	2	2
4	222	160	96	98	15	17	5	6
5	0	0	100	95	220	205	36	33
6	1	1	99	99	25	25	4	5
7	4	4	99	90	56	58	9	7
8	37	38	97	98	3	3	0	1
9	37	30	98	97				
10	7	6	99	90				

F = filtration, C=centrifugation.

**Table 3.2****Recovery of added  $\alpha$ 1-AT**

Sample no	WGLF $\alpha$ 1-AT ug/ml	WGLF $\alpha$ 1-AT + added standard ug/ml	%recovery
1u	4	59	110
1	2	48	92
2u	0	48	96
2	1	55	108
3u	35	85	102
3	31	85	110
4u	7	54	93
4	6	55	98

u = unprocessed

**Recovery of added albumin**

Sample no	WGLF albumin ug/ml	WGLF albumin + added standard ug/ml	%recovery
1u	120	194	103
1	95	166	97
2u	59	134	101
2	58	138	108
3u	8	87	104
3	7	91	110
4u	7	79	96
4	6	87	107

u=unprocessed

PERCENT INHIBITION  
NON SPECIFIC PROTEASE

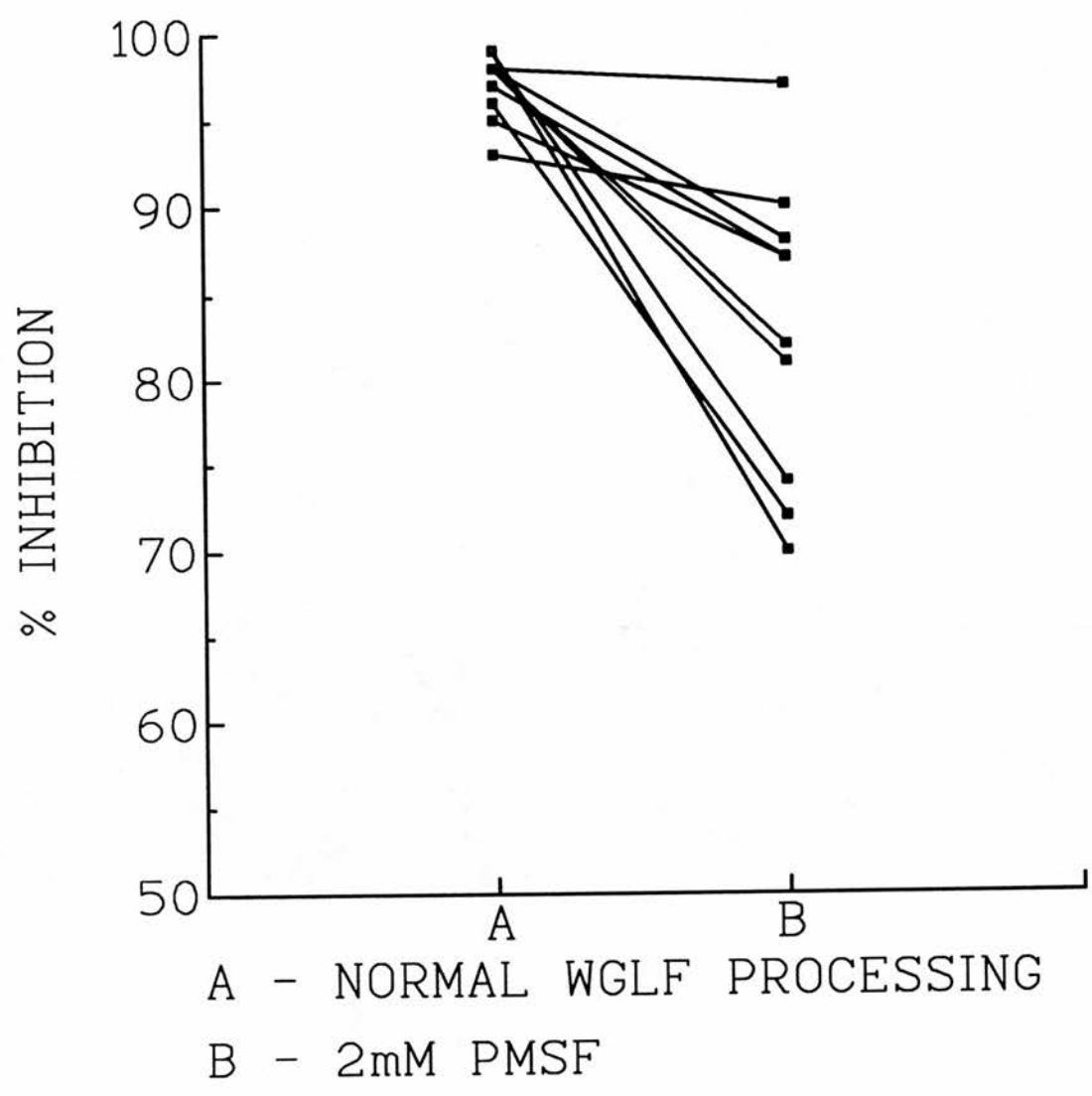


Figure 3.1

WGLF ALBUMIN (100ug/ml)  
TIME COURSE - IMMUNOTURBIDITY

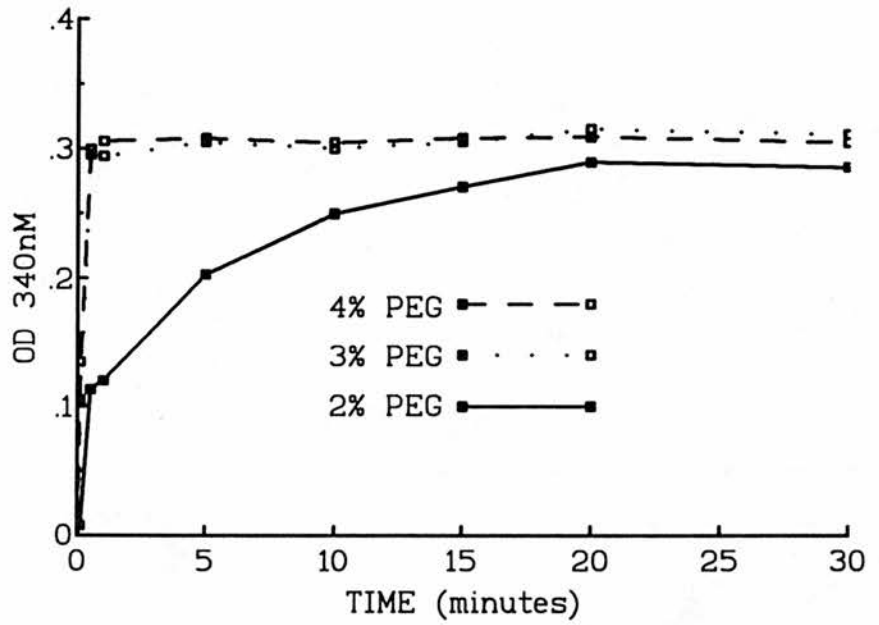


Figure 3.2a

WGLF  $\alpha$ 1-AT (100ug/ml)  
TIME COURSE - IMMUNOTURBIDITY

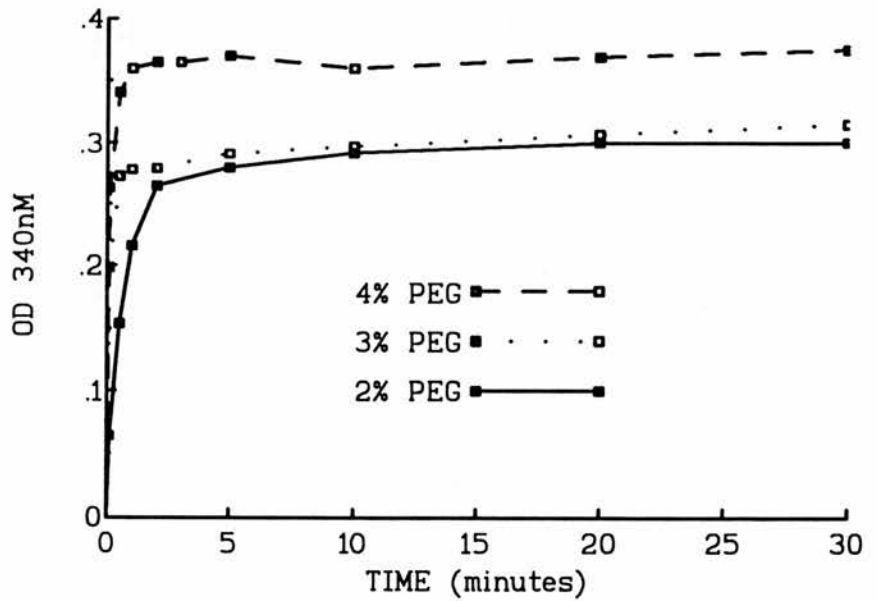


Figure 3.2b

ALBUMIN CALIBRATION CURVE  
INFLUENCE OF ANTIBODY CONCENTRATION

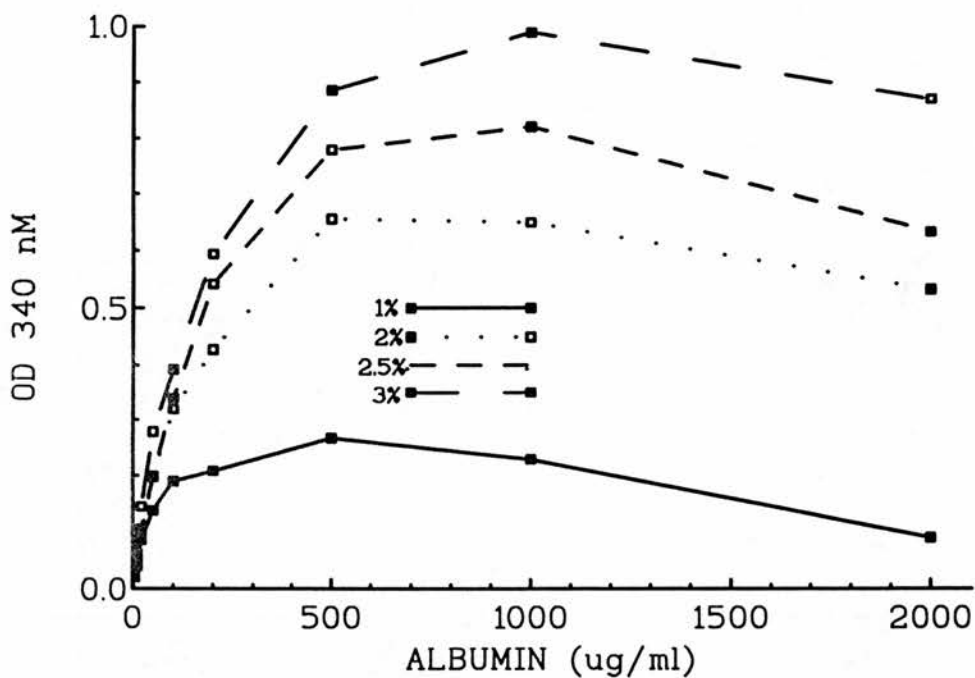


Figure 3.3a

$\alpha$ 1-ANTITRYPSIN CALIBRATION CURVE  
INFLUENCE OF ANTIBODY CONCENTRATION

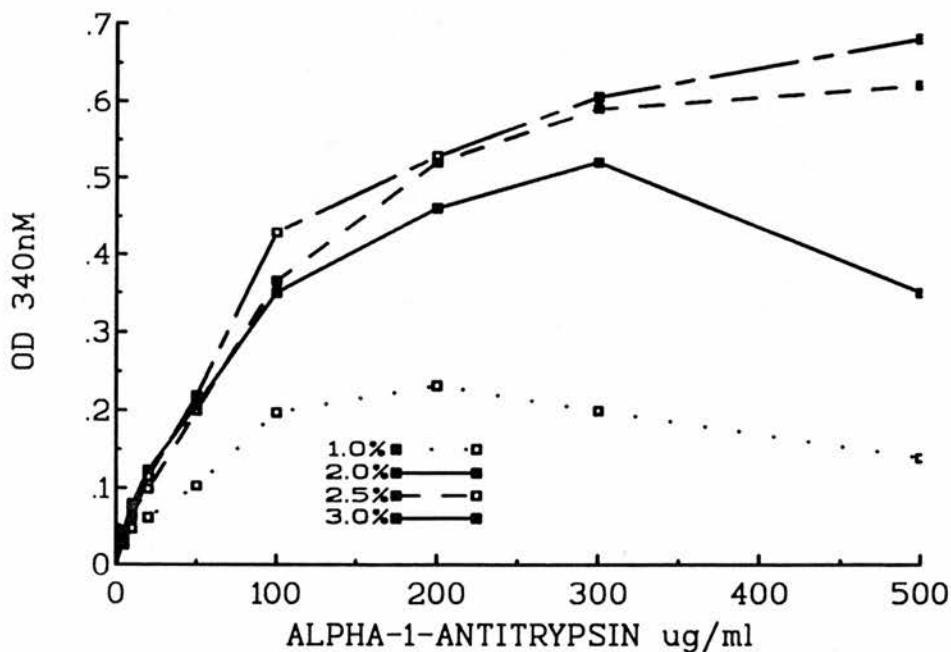


Figure 3.3b

CALIBRATION CURVE  
ALBUMIN

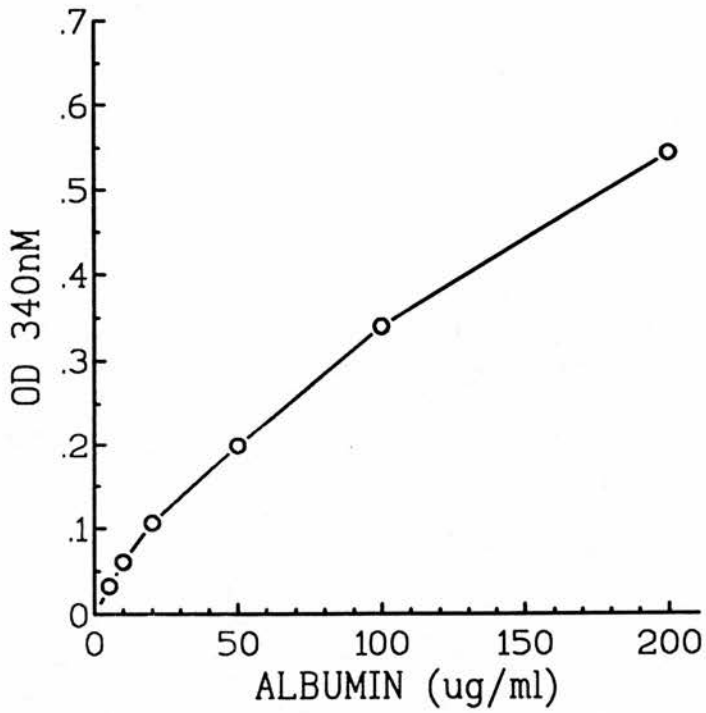


Figure 3.4a

CALIBRATION CURVE  
 $\alpha$ 1-ANTITRYPSIN

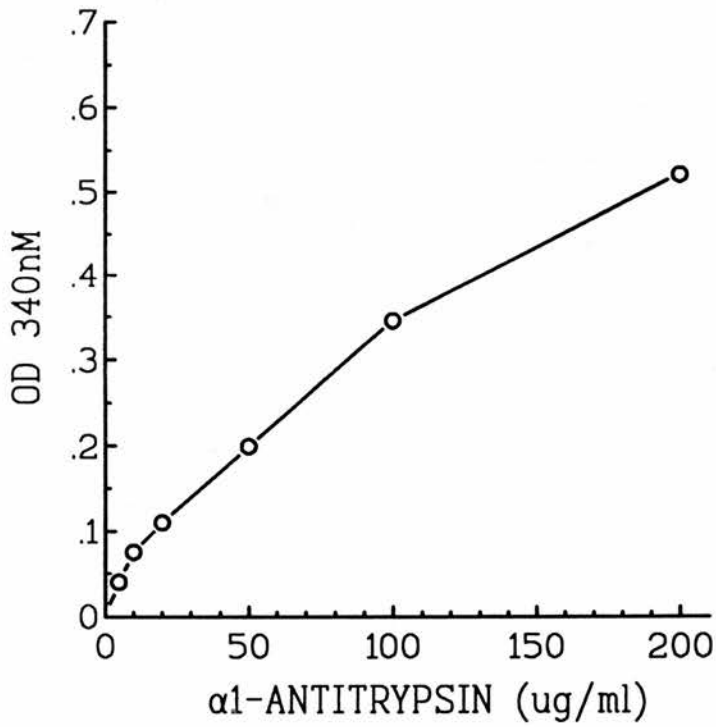


Figure 3.4b

RID -  $\alpha$ 1-ANTITRYPSIN  
(LC-PARTIGEN)

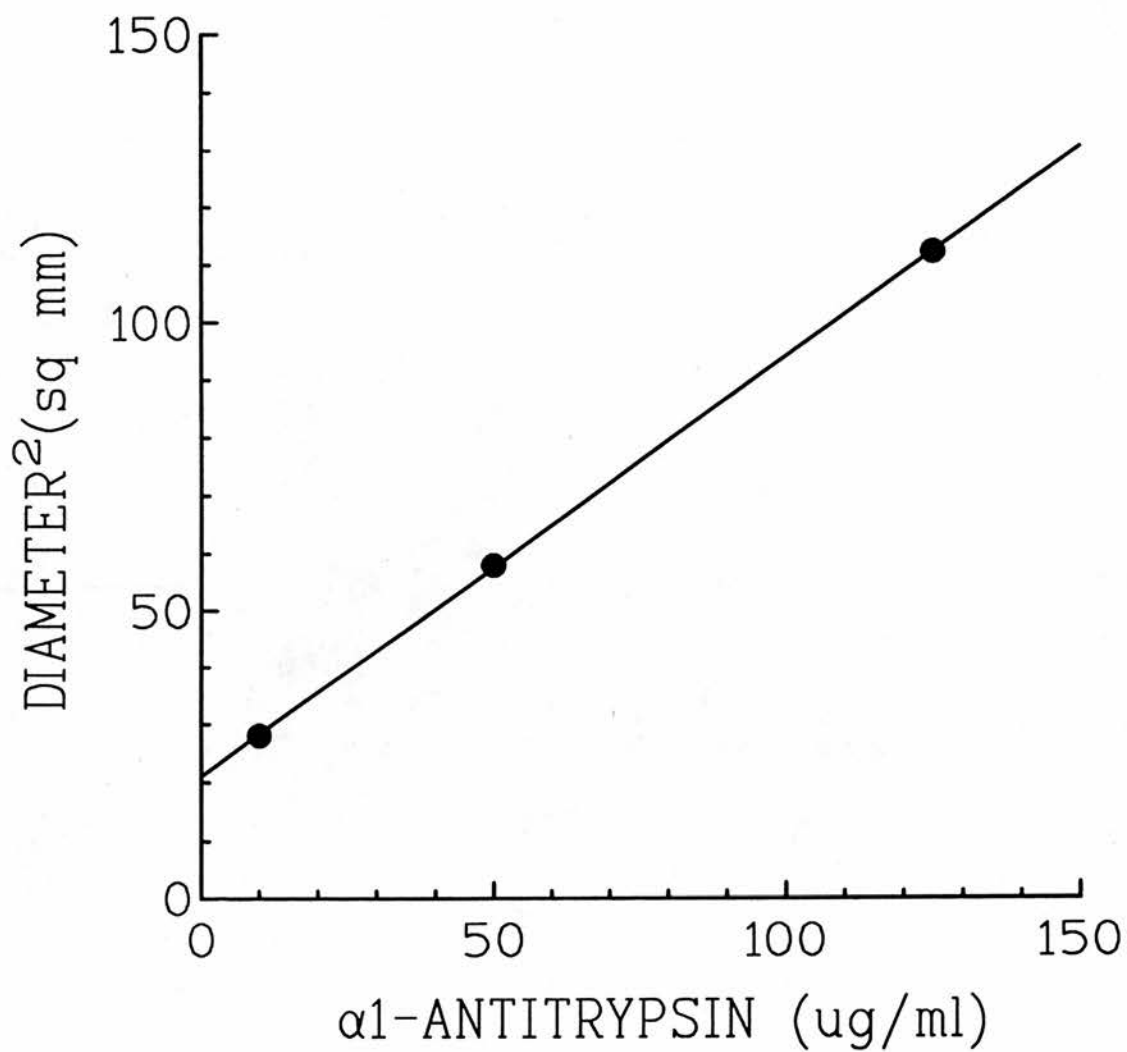


Figure 3.5

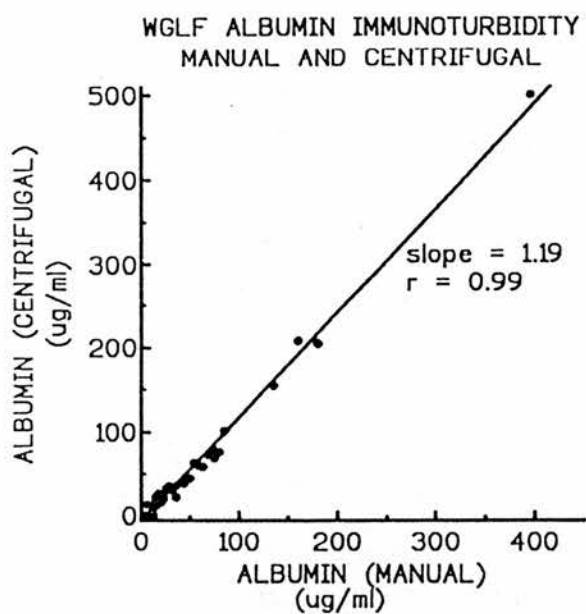


Figure 3.6a

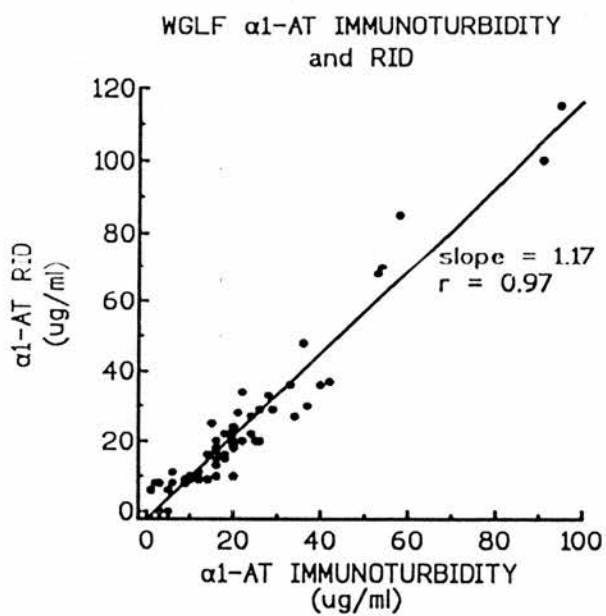


Figure 3.6b

STABILITY OF ALBUMIN IN  
WHOLE GUT LAVAGE FLUID

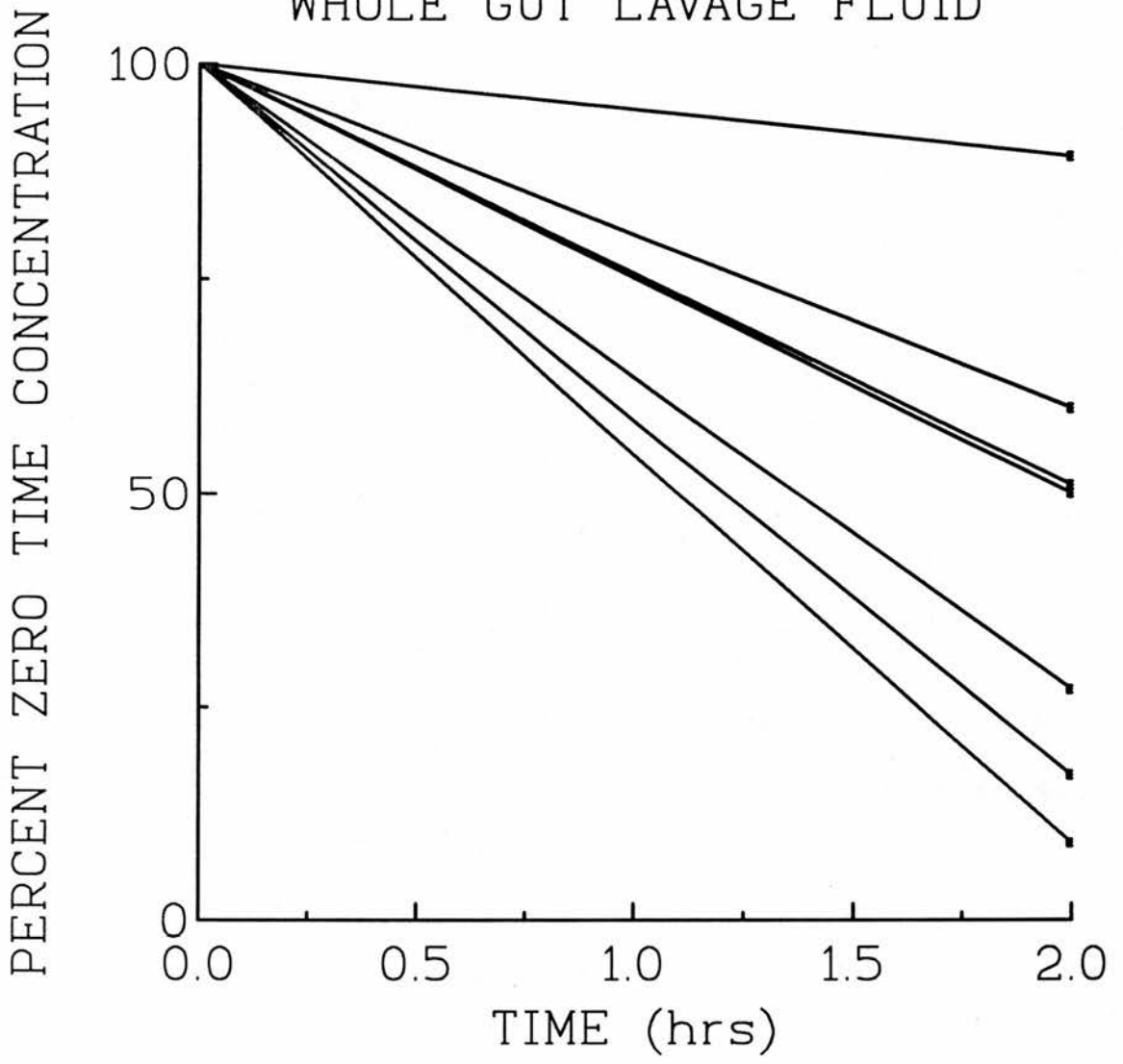


Figure 3.7

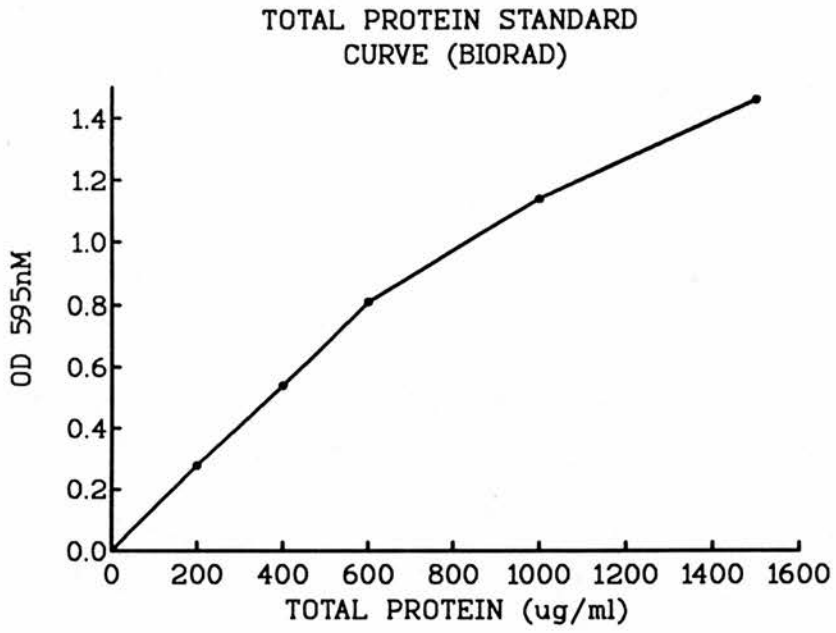


Figure 3.8a

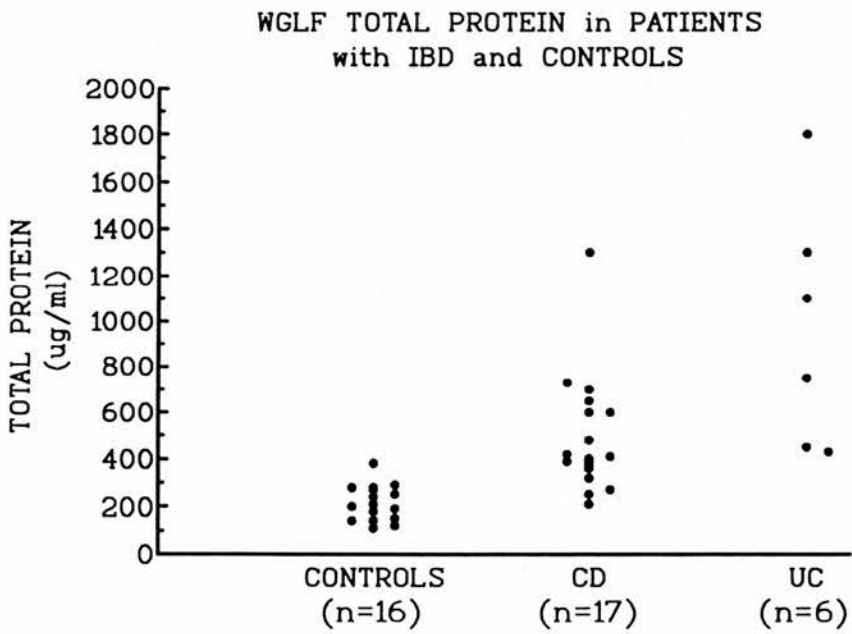


Figure 3.8b

# **REFERENCE VALUES AND RELATIVE SPECIFICITY FOR ACTIVE IBD OF WGLF PROTEINS**

## **Introduction**

Normally WGLF contains only trace amounts of IgG, but in two separate groups of ten and 44 patients with active IBD O'mahony et al (1990,1991a), found concentrations of IgG that were significantly higher than those of controls. High concentrations of WGLF albumin were also found with a positive correlation between IgG and albumin content, suggesting that these tests are measuring plasma leakage across inflamed mucosa.

WGLF from a further series of patients have been assayed, in order to determine reference values for WGLF protein concentrations, and to establish to what extent abnormally high values are found in diseases other than IBD.

## **Subjects and Methods**

### **Patients and volunteers**

WGL was performed on 263 occasions. The indications were as bowel preparation for barium enema, colonoscopy or colorectal surgery; as a treatment for intractable constipation; and, in volunteer members of staff and patients, as part of a research project on secretory antibodies to food proteins.

### **Analytical methods**

WGLF IgG, albumin, and  $\alpha$ 1-AT were measured by methods described in chapter 3 appendix.

The data presented below relate to 70 specimens processed by the Gaspari method and 193 by filtration. Protein results have not been corrected for dilution with processing reagents.

## **Results**

### **Reference values for WGLF proteins**

The WGL procedure has been carried out in 63 individuals who were classified as "normal". There were 20 men, age range 15 - 80, mean 44 years, SD 22.3; and 43 women, age range 24 - 88, mean 51 years, SD 20.0. There were seven healthy volunteers, 19 patients with simple constipation, eleven with small benign colonic polyps, 18 who had had minor GI symptoms, and in whom no abnormality had been found, and eight patients with minor, non-inflammatory GI conditions. All were taking a normal diet, and none was being treated with immunosuppressive drugs or NSAIDs.

Values for WGLF IgG, albumin and  $\alpha$ 1-AT had a skewed distribution (Figure 3.9), which was not normalised by using transformed data. The upper and lower values which enclose 95% of the results (Altman 1991), have been taken as the working normal ranges and are as follows: WGLF IgG 0 - 10 ug/ml, albumin 0 - 26 ug/ml, and  $\alpha$ 1-AT 0 - 19 ug/ml.

### **WGLF proteins in patients with IBD**

102 WGLs were performed in patients with IBD, 71 with CD, 31 with UC, heterogeneous with respect to disease distribution, activity and treatment. Abnormally high concentrations of WGLF IgG were present in 64% of these (44 CD, 21 UC); of albumin in 52% (35 CD, 19 UC); and of  $\alpha$ 1-AT in 36% (28 CD, 9 UC). See Figure 3.10.

## **WGLF proteins in patients with colorectal cancer**

Apart from IBD, colorectal cancer (CRC) was the only diagnostic category for which a substantial number of patients had abnormal results. Data was available for ten patients, and in seven of these, one or more abnormal results was obtained. Concentration of WGLF IgG was high in four cases, albumin high in six and  $\alpha$ 1-AT high in two. See Figure 3.10.

## **WGLF proteins in other diseases**

Details of the diagnoses and WGLF results in 95 patients with other conditions, are given in Table 3.3

### **Small bowel disease**

Only four of 29 patients with small bowel disease had a high value for one or more of the WGLF proteins studied; unequivocally abnormal findings in two patients - a man with lymphangiectasia and protein-losing enteropathy, and a male coeliac with enteropathy-associated T cell lymphoma, which was extensively ulcerated, with local perforation and an abscess cavity communicating with the gut. Two of eight patients with idiopathic diarrhoea (ie. severe watery diarrhoea with no diagnosis reached after investigation by a consultant gastroenterologist) had high values for  $\alpha$ 1-AT (21 and 31 ug/ml).

### **Colorectal disease**

Three of 27 patients with various non-malignant colorectal disease had high values for WGLF protein concentrations - a young man with immunodeficiency, several autoimmune diseases and lymphocytic colitis, whose diarrhoea was steroid responsive, a patient with pouchitis, and an 81-year-old woman with severe diverticulitis, local perforation, peri-diverticular abscess and a colo-vesical fistula.

## **Other diseases**

WGLF protein concentrations were normal in 41 of 42 patients with various other GI and systemic disorders, the exception being a woman with lung cancer and widespread abdominal metastases.

## **WGLF proteins: analytical precision (reproduceability)**

The coefficient of variation for measurement of patient samples for the proteins were as follows: (n=30)

for IgG of 12 ug/ml = 13%

for albumin of 87 ug/ml = 6%

for  $\alpha$ 1-AT of 12 ug/ml = 13%

## **Discussion**

This work has confirmed a high frequency of positive results for plasma derived proteins in IBD patients.

When cases judged to be normal are excluded, there were 98 examinations in patients without IBD. In 15 of these, a positive result was obtained for one or more of the assays performed. In the majority of cases, and in all those with multiple abnormal results, the existence of GI protein loss was in keeping with the clinical picture - one patient with lymphangiectasia, seven with malignant colorectal tumours, two with intra-abdominal abscesses communicating with the gut, one pouchitis and one unusual lymphocytic colitis. The phenomenon detected by WGLF protein assays clearly can occur in a range of clinical situations, although the majority of positive results are in IBD patients.

Further experience continues to give the same type of results, although healthy volunteers have reference ranges which are lower than those given by hospital controls. One further patient with the rare condition of lymphangiectasia has since been investigated who also shows a disproportionate increase in WGLF  $\alpha$ 1-AT with

protein values of 78 for IgG, 15 for albumin and 120 for  $\alpha$ 1-AT. The mechanism for protein loss in this condition may be different from that seen in active IBD.

**TABLE 3.3****Proteins in WGLF**

Diagnosis	Number of lavages	WGLF proteins normal	Values in abnormal cases (ug/ml)		
			IgG	albumin	$\alpha$ 1-AT
<b>Small bowel disease</b>					
coeliac disease	4	4			
bacterial overgrowth	4	4			
ileal resection	3	3			
Lymphangiectasia	1	-	14	36	195
Giardiasis	1	1			
Idiopathic diarrhoea	15	13	1	3	21
			2	5	31
Enteropathy-associated T cell lymphoma	1	-	41	70	19
<b>Other benign colo-rectal disease</b>					
Colonic diverticulosis (uncomplicated)	10	10			
Perforated diverticulitis	1	-	85	18	10
Eosinophilic colitis	1	1			
Microscopic colitis	1	1			
Ischaemic colitis	1	1			
Collagenous colitis	1	1			
lymphocytic colitis and common variable immuno-deficiency	1	-	18	68	29
radiation proctitis (treated)	3	3			
solitary rectal ulcer	2	2			
rectal prolapse	1	1			
Pelvic ileal reservoir	4	4			
Pelvic ileal reservoir with pouchitis	1	-	120	34	27
<b>Other significant disease</b>					
Gastrointestinal	27	27			
Systemic	15	14	18	48	1*

\* lung cancer with large intra-abdominal metastases

# WGLF PROTEINS in NORMAL PATIENTS and VOLUNTEERS (n=63)

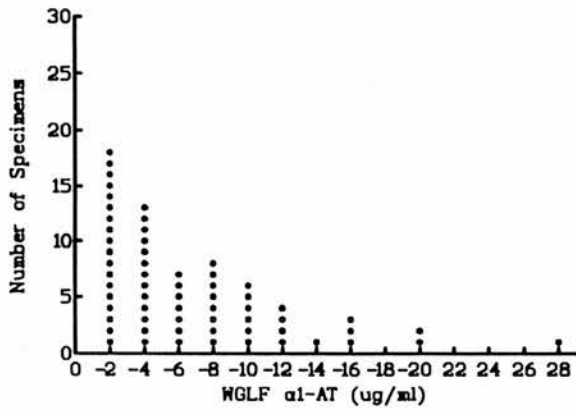
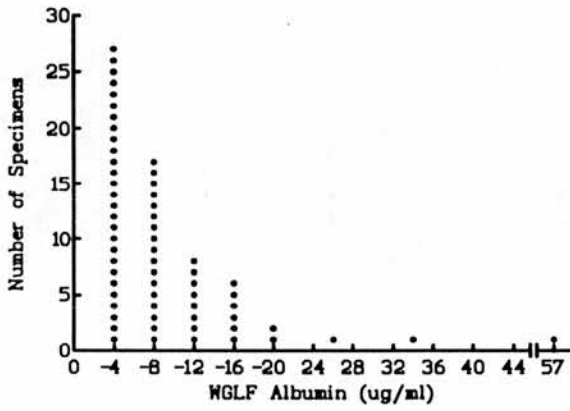
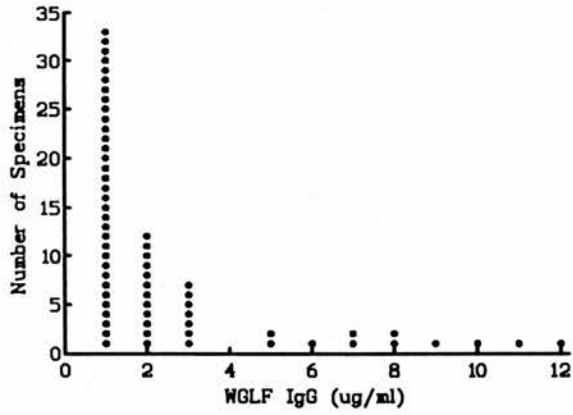


Figure 3.9

WGLF PROTEINS  
in IBD and CA COLON

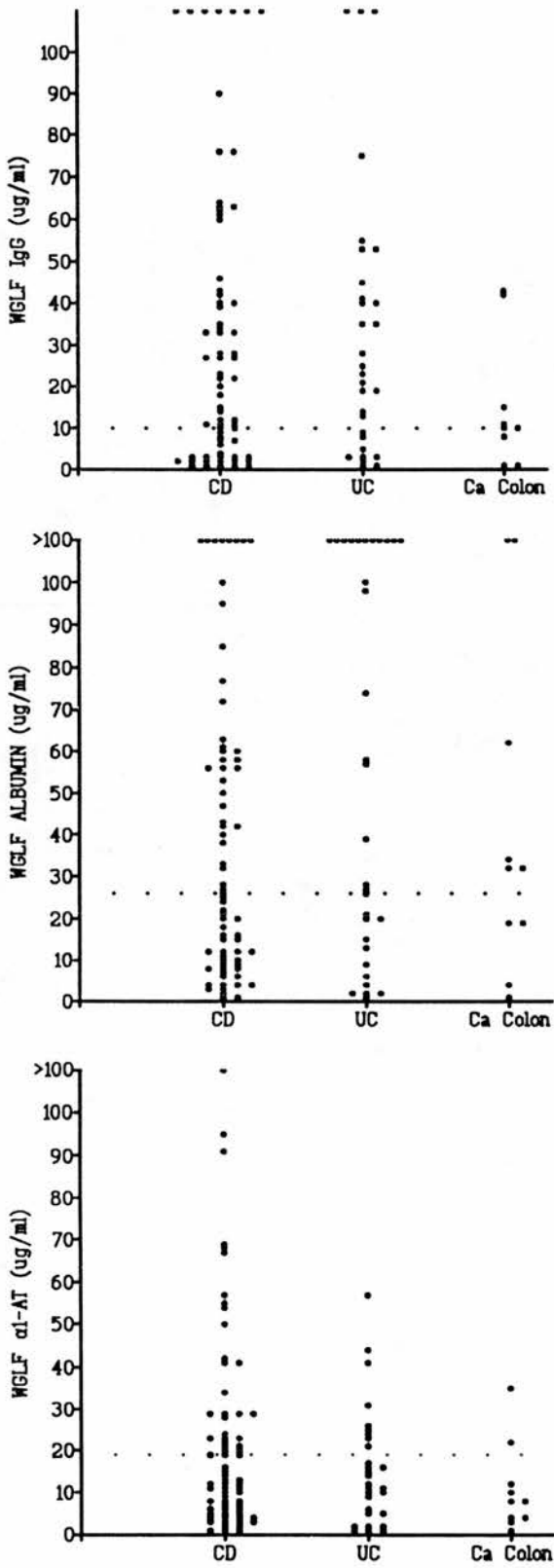


Figure 3.10

# **WGLF PROTEIN CONCENTRATIONS: OBJECTIVE MEASURES OF DISEASE ACTIVITY IN IBD**

## **Introduction**

### **Subjective indices of IBD activity**

The Crohn's Disease Activity Index (CDAI), developed by Best et al (1976) has proved a reliable and reproducible measure of health status in patients with CD. In theory, the CDAI is vulnerable to criticism because it is heavily weighted by subjective factors of general well-being and abdominal pain, and investigators have argued that the CDAI is not so much a measure of disease activity as of general disability. Many factors apart from intestinal inflammation will contribute to the index, including infection, obstruction, malnutrition, psychological dysfunction and the side effects of drugs. Nevertheless, the CDAI, and similar indices for UC activity such as the Powell-Tuck Index (PTI), developed by Powell Tuck et al (1978) are used almost exclusively as measures of remission in clinical trials and are recognised as standards for the assessment of any new approach to the measurement of disease activity in IBD.

The degree of illness in a patient with IBD is not directly related to the length of bowel grossly affected. Factors such as the severity of local inflammation and non-specific "disease activity" are also relevant. There is an urgent need for simple and objective indices of these separate components of overall disability, for use in clinical practice and research.

### **WGLF proteins as potential objective indices of IBD**

The aims were

1. to establish whether assay of WGLF proteins could be used as a diagnostic test for IBD

2. to assess to what extent concentrations of proteins in WGLF parallel the global indices, CDAI and PTI.
3. to determine whether it is possible to grade the severity within "active" disease, or to detect minimal abnormalities in patients with CDAI at 150 or below, or PTI at 4 or below (i.e. classified as inactive).
4. to determine whether WGLF proteins offer any advantage over conventional clinical tests (ESR, C-reactive protein, platelet count), or a physician's global assessment, for the purpose of this investigation, recorded on a visual analogue scale (VAS).

In the present prospective study, the WGL procedure has been performed on 53 occasions in 45 well characterised patients with CD or UC, and WGLF concentrations of IgG, albumin and  $\alpha$ 1-AT measured. At the same visit the CDAI or PTI as appropriate was calculated, blood taken for measurements of ESR, platelet count and C-reactive protein (frequently used, single indices of activity), and the physician's global assessment was quantified by using a VAS of 0-120.

## **Material and methods**

### **Subjects**

This study was undertaken in collaboration with Dr C.P.Choudari who took responsibility for the clinical descriptions, and patient evaluation and collaborated in result interpretation.

A total of 45 patients with IBD were studied, 27 with CD (11 male, 16 female), 18 with UC (8 male, 10 female). The diagnosis and disease extent in all patients were confirmed by histology, endoscopy and radiology. Full details are given in Table 3.4. The patients were classified into various sub-groups by the nature of previous resections and current macroscopic disease. Five CD patients and one UC patient were studied twice, and one man with UC was studied three times. In 44 of the 53 studies, WGL procedure was being performed as bowel preparation for colonoscopy

or barium enema examination; in four cases as preparation for surgery; and in five cases, to empty the GI tract prior to treatment with an elemental diet.

### **Clinical assessments**

Patients completed a diary card of symptoms for the week preceding the WGL procedure. Haematological and biochemical indices measured included haemoglobin, platelet count, ESR, serum total protein, albumin,  $\alpha$ 1-AT, C-reactive protein, and immunoglobulins, and were carried out in the Departments of Haematology and Clinical Chemistry, Western General Hospital, Edinburgh.

All patients were clinically assessed, and the CDAI or PTI was calculated in the standard fashion ( chapter 3, appendix). In addition, the physician's assessment of overall disease activity for the day of lavage was recorded on a VAS, this being based on symptoms, signs and results of the blood tests.

### **WGL protocol**

This was carried out as previously described. The procedure was supervised by an experienced nurse and performed either in a general in-patient ward or in the Gastrointestinal out-patient investigation area of the Western General Hospital.

### **Results**

The patients studied were heterogeneous in disease distribution, and in their previous and current treatment. In CD, the CDAI range was from 24 to 321; 24 lavages were in patients with CDAI of  $>150$  (described below as having active disease) and eight in patients with a CDAI  $<150$  (in remission). In patients with UC, the PTI ranged from zero to 13; 13 lavages were in patients with active disease (PTI of  $>4$ ) and eight were in patients in remission with a PTI of  $< \text{or} = 4$ .

## **WGLF proteins**

Values for WGLF protein concentrations in CD and UC patients were plotted against CDAI or PTI respectively and are shown in Figures 3.11 and 3.12. These results have not been corrected for dilution with processing reagents.

### **Diagnostic potential of WGLF proteins**

If WGLF protein concentrations are always high when there is overt intestinal mucosal inflammation (on radiological or endoscopic visualisation), this would provide a means of screening patients for the presence or absence of IBD. This issue has been considered by analysis of the results for WGLF IgG. High values for IgG concentration were present in only 24 of the 27 lavages performed in patients who had unequivocal macroscopic small bowel or colonic CD, and in 13 of the 15 lavages in patients with UC who had positive X-ray or endoscopic findings. There was also one patient with microscopic evidence of colonic CD, no macroscopic disease but high WGLF IgG concentration.

Further experience is clearly showing that quite extensive disease, if inactive, gives normal values for IgG and albumin, whilst active diffuse disease which cannot readily be seen radiologically gives raised values for these proteins especially IgG.

### **WGLF proteins and disease activity**

#### **a. IgG**

Values for WGLF IgG concentration were normal in nine lavages in CD patients in this study. Seven of these were in patients in remission and one in an 18-year old man with a borderline CDAI of 168. He had ankylosing spondylitis, and his anorexia and high ESR shortly after right hemicolectomy for CD were thought to reflect activity of arthritis rather than of CD. There was one patient with WGLF IgG concentration of 10 ug/ml and CDAI of 185.

Abnormally high concentrations of WGLF IgG were present in 23 cases, of whom 22 had active disease. A 13 year-old boy with perianal CD and unexplained abdominal pain had a CDAI of 119 and WGLF IgG of 20 ug/ml. During the next six months his growth stopped and pain became worse; both problems have resolved with systemic steroid therapy.

In 21 lavages from patients with UC, values for WGLF IgG concentration were normal in eight cases (of whom seven were in remission) and high in 13 cases (12 with active disease). Both discrepant cases were patients who had had unequivocally active disease two weeks before and were in the early stages of sulphasalazine or oral corticosteroid treatment respectively.

#### **b. Albumin**

In 32 lavages from patients with CD, values for WGLF albumin concentration were normal in 15 cases (of whom eight were in remission, CDAI in the others ranging from 168 - 283).

Albumin concentrations were high in 17 cases, all with active disease.

In 21 lavages from patients with UC, values for WGLF albumin concentration were normal in 12 cases, (of whom eight were in remission), and abnormally high in nine cases, all with active disease.

#### **c. $\alpha$ 1-AT**

In 32 lavages from patients with CD, values for WGLF  $\alpha$ 1-AT concentration were normal in 17 cases (of whom seven were in remission), and were high in 15 cases (14 with active disease).

In 21 lavages from patients with UC, values for WGLF  $\alpha$ 1-AT concentration were normal in 14 cases (of whom seven were in remission), and high in seven cases, (six with active disease).

### **Correlation of WGLF protein concentrations with activity indices in active and inactive disease**

Correlation co-efficients for WGLF protein concentrations against activity indices are detailed in Table 3.5. For both CD and UC, and for all three proteins studied, there was a highly significant positive correlation between activity index and WGLF protein concentration, with  $r$  values ranging from 0.44 to 0.72.

Since, as shown clearly in Figure 3.11, the relationship between activity index and WGLF proteins is not linear but biphasic, correlations were separately calculated for cases with active and inactive disease (as defined by CDAI and PTI). Calculations were also performed for the subsets of CD patients with active colonic and active small bowel disease.

The results show that only in active disease were there significant correlations; that the highest  $r$  values were found for IgG versus CDAI in active CD ( $r=0.82$ ), and particularly in the subset with active small bowel CD ( $r=0.97$ ); and that in active UC, there was good correlation between WGLF IgG concentration and the PTI ( $p<0.01$ ), with the other two proteins correlating less well with the PTI ( $p<0.05$ ). In inactive CD and UC, WGLF protein concentrations were independent of activity indices.

### **Conventional laboratory indices of disease activity**

As described above for the purposes of this analysis, patients with CD who had a CDAI of  $>150$ , and those with UC who had a PTI of  $>4$ , have been designated as having active disease. The numbers of cases with abnormal and normal values for ESR, C-reactive protein and platelet count in the active and remission groups are given in Table 3.6. This table also includes, for comparison, numbers with normal

and abnormal WGLF IgG, albumin and  $\alpha$ 1-AT values, and the physician's overall assessment based on a VAS.

The three blood indices were normal in most patients with inactive disease. However these were also normal in many patients with unequivocally active disease, and this was particularly so in the group of 13 assessments in active UC - ESR was normal in eight cases, C-reactive protein was normal in ten and platelet count was normal in eight.

## **Discussion**

There have been many attempts to develop a single index of illness in IBD (Best et al, 1976, Powell-Tuck et al, 1978, Harvey et al, 1980, Van Hees et al, 1980, Myren et al 1984, Sandler et al, 1988) by combining a few clinical and/or laboratory criteria, and claims have been made for the significance of single laboratory determinations in some situations (Sachar et al, 1986, Fegan et al, 1982, Jansen et al, 1976, Meyers et al, 1985).

This prospective study is based on 53 lavages in patients with CD or UC, fully characterised with regard to anatomical distribution of disease and investigated for various single and composite activity indices at the time of the lavage.

The study demonstrates that WGLF protein concentrations, particularly IgG, discriminate well between active and inactive IBD; and also, within the subsets of patients with active disease, closely parallel disease activity as defined by CDAI and PTI. Furthermore, these sensitive tests were completely normal in most patients with CDAI <150 and PTI <5, strongly supporting the present clinical trials practice whereby such values are taken as successful end-points, indicating that remission has been achieved.

However WGLF protein assays cannot be used as screening or diagnostic tests for IBD. Although high values are found in many patients, the data in this paper clearly show that values are normal in clinically inactive disease, even when there are

unequivocal radiological or endoscopic features of IBD such as cobblestone ulcers and long, string-like strictures. Nevertheless in clinical practice the assay of WGLF IgG is proving to be a valuable aid to diagnosis in some clinical situations eg in patients with microscopic CD, when IBD co-exists with severe diverticular disease, and in separating the effects of intestinal and joint disease in IBD patients with ankylosing spondylitis.

There is abundant evidence to implicate IgG in the pathological lesions of IBD, particularly CD - immunohistochemistry reveals an excess of IgG-containing cells, deep in the mucosa (Harries et al,1983, Baklien et al,1976); isolated intestinal mononuclear cells from IBD patients spontaneously secrete high amounts of IgG (Rosekrans et al,1980, MacDermott et al,1981); and there are differences in intestinal plasma cell IgG subclass distribution between controls, UC and CD patients (Verspaget et al,1988). Thus, when the presence of IgG in WGLF was first noted, it was thought that this was probably derived from the abundant IgG cells in diseased mucosa. However the findings of high concentrations of two plasma proteins, albumin and  $\alpha$ 1-AT, in WGLF from patients with active IBD suggests that at least some of the IgG is plasma-derived. Furthermore, all three protein concentrations were low in WGLF from some inactive CD patients who have extensive chronic ulceration of the intestine (diseased tissues known to be replete in IgG cells).

It could be argued that these sensitive tests are merely measuring plasma proteins entering the gut lumen as a result of bleeding. However, in active IBD, when abundant amounts of the three proteins are present in WGLF, their relative concentrations sometimes differ from those in plasma. The three proteins differ in molecular weight, IgG 150 kD, albumin 69 kD,  $\alpha$ 1-AT 54 kD, and it could be that there is a selective increase in mucosa-to-lumen permeability in active IBD, operating either at the level of the capillary or the epithelial basal lamina. Although there is blood loss in some patients with active inflammatory bowel disease in the majority increase in WGLF protein concentration is not accompanied by a corresponding increase in WGLF haemoglobin concentration (Chapter 2).

Jonard et al (1984) and Colombel et al (1992) have shown greater plasma to jejunum excretion of albumin compared with IgG in normal subjects. The lower clearance of IgG in control subjects may contribute to the greater sensitivity of this test if the excretion differences are abolished in disease state.

It has also been considered whether analytical factors could explain the different results for the three proteins studied, in relation to discrimination between active and inactive disease. Although fluids are processed within minutes of collection, proteins leaking into the gut lumen proximally will have been exposed to pancreatic and intestinal proteases during the transit of fluid along the gut (0.5-2 hours). Experiments have shown that at 37°C, degradation of albumin by unprocessed WGLF is significant (loss of 50% of measured albumin in two hours), whereas IgG and  $\alpha$ 1-AT are resistant to such proteolysis. This could explain the differences in results for IgG and albumin in active disease. The relative insensitivity of WGLF  $\alpha$ 1-AT concentrations may be due to the low plasma levels of this protein in relation to IgG (x4-5) and albumin (x15 - 20).

Various scientific approaches to the measurement of disease activity in IBD have recently been reviewed (Kett et al 1987, Singleton,1987). Laboratory tests such as ESR, platelet count, and acute phase proteins are useful. However these may be normal in active IBD, particularly in UC and in small bowel CD (Table 3.6) and will be positive in situations of active inflammation or infection without gut involvement. Other techniques such as labelled leucocyte studies (Camilleri et al,1989, Saverymuttu et al,1986, and Crama-Bohbouth et al,1988) and measures of GI protein loss by  $\alpha$ 1-AT clearance (Florent et al,1981, Boirivant et al,1991, Strygler et al,1990), have advantages and disadvantages which are discussed later in this chapter. In general, they have been developed and applied without being subjected to prospective evaluation against the carefully developed standard activity indices such as CDAI. Furthermore, labelled leucocyte studies are expensive, involve exposure to radioactivity, and depend on complete faecal collection. The findings with these tests relate to events in macroscopically affected gut and thus differ from GI protein loss

which appears to measure a more diffuse phenomenon. Work recently undertaken in our laboratory ( L. Handy and S.Ghosh) suggest that leucocyte tests measure different aspects of the inflammatory process compared with tests of protein loss.

There may be problems in interpretation of isotope-based or  $\alpha$ 1-AT clearance studies if the treatment used, such as elemental diet therapy, changes intestinal transit rate or otherwise influences defaecatory function; this could either mask or mimic changes due to specific anti-inflammatory effects of the test treatment. Since the standard protocol for collection of WGLF is not influenced by concurrent dietary or pharmacological treatment, this method should lend itself to clinical trials.

**TABLE 3.4**

**Clinical details of 45 patients with IBD, undergoing WGL on 53 occasions**

Parts of GI tract previously resected	Number of patients	Macroscopic disease at time of lavage	Number of lavages
<b>1. CD</b>			
None	15	small bowel	6
		ileo-colonic	2
		colonic	4
		recto-sigmoid	3
		peri-anal	2
		* microscopic	1
Small bowel	2	small bowel	2
Ileo-caecal + terminal ileum	8	no macroscopic disease	5
		small bowel	3
		ileo-colonic	1
Segmental colonic resection	2	ileo-colonic	1
		colonic	2
Total	27		32
<b>2. UC</b>			
Previously documented macroscopic disease			
recto-sigmoid	11	no macroscopic disease	3
		recto-sigmoid	9
left sided	3	no macroscopic disease	1
		left sided	2
pancolitis	4	no macroscopic disease	1
		pancolitis	5
Total	18		21

**TABLE 3.5****WGLF proteins in patients with active and inactive IBD:  
correlations with CDAI and PTI**

	Number of lavages	IgG		Albumin		$\alpha$ 1-AT	
		r	p	r	p	r	p
<b>CD</b>							
(CDAI)							
All	32	0.72	<0.0001	0.59	<0.0001	0.44	<0.01
Active	24	0.82	<0.0001	0.55	<0.01	0.29	NS
Inactive	8	0.22	NS	-0.35	NS	0.16	NS
Active colonic	8	0.78	<0.05	0.51	NS	0.47	NS
Active SI	10	0.97	<0.0001	0.55	NS	0.36	NS
<b>UC</b>							
(PTI)							
All	21	0.71	<0.0001	0.70	<0.0001	0.53	<0.02
Active	13	0.74	<0.01	0.61	<0.05	0.60	<0.05
Inactive	8	-0.32	NS	-0.38	NS	-0.04	NS

NS - not significant,  $P > 0.05$

**TABLE 3.6**

**Presence of normal or abnormal values for conventional laboratory indices of disease activity, global assessment, WGLF IgG, albumin, and  $\alpha$ 1-AT in patients with CD and UC arbitrarily subdivided into active or inactive IBD on the basis of CDAI or PTI respectively**

	CD		UC	
	Active CDAI>150	Inactive CDAI<150	Active PTI>4	Inactive PTI<4
No of lavages	24	8	13	8
<b>ESR</b>				
Normal (<20)	5	5	8	8
Abnormal	19	3	5	0
<b>CRP</b>				
Normal (<1.5)	8	8	10	8
Abnormal	16	0	3	0
<b>Platelets</b>				
Normal	6	7	8	7
Abnormal	18	1	5	1
<b>VAS</b>				
Normal (<60)	1	7	2	6
Abnormal	23	1	11	2
<b>WGLF IgG</b>				
Normal	2	7	1	7
Abnormal	22	1	12	1
<b>WGLF Albumin</b>				
Normal	7	8	4	8
Abnormal	17	0	9	0
<b>WGLF <math>\alpha</math>1-AT</b>				
Normal	10	7	7	7
Abnormal	14	1	6	1

# WGLF PROTEINS and CDAI

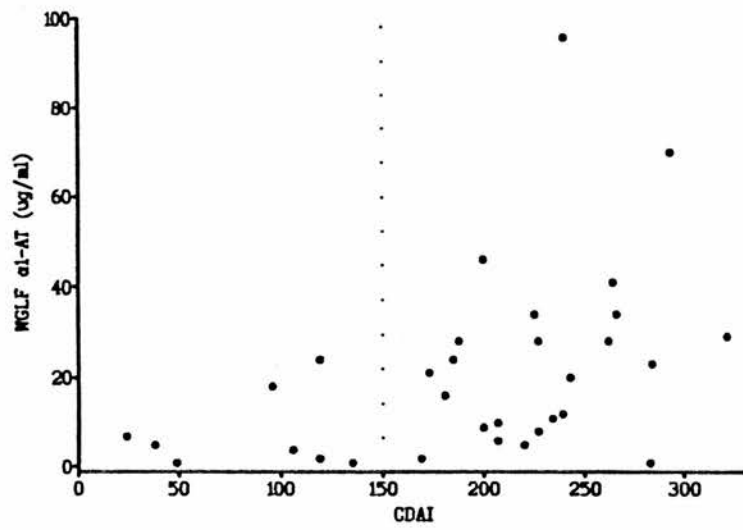
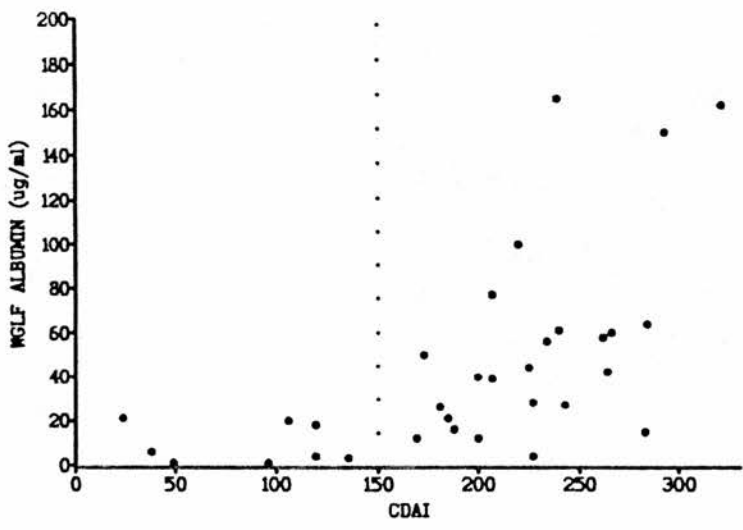
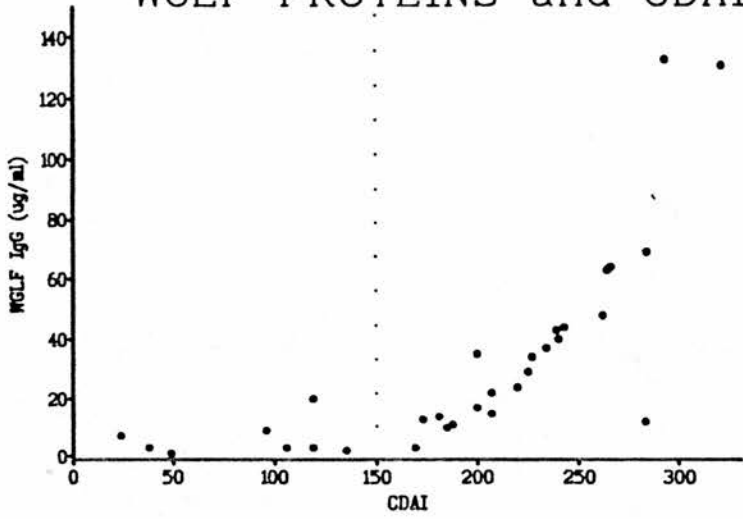


Figure 3.11

# WGLF PROTEINS and PTI

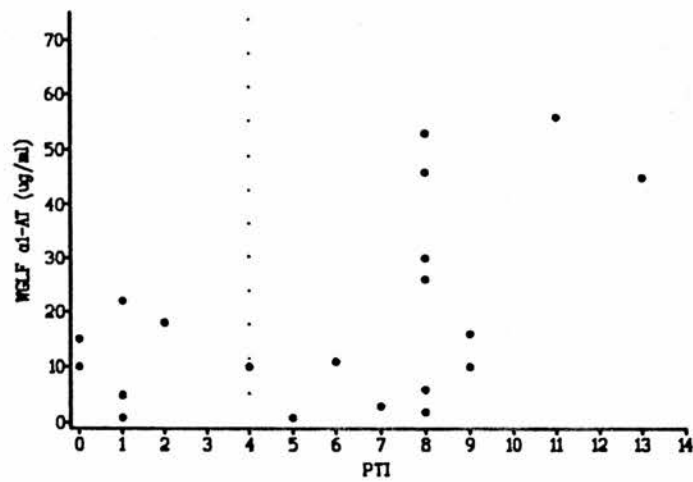
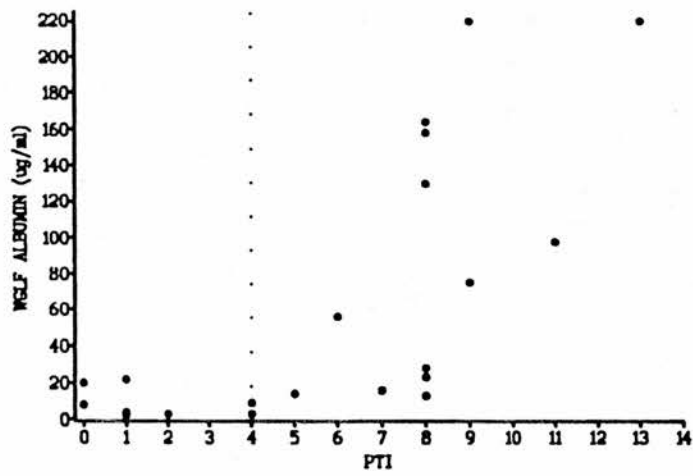
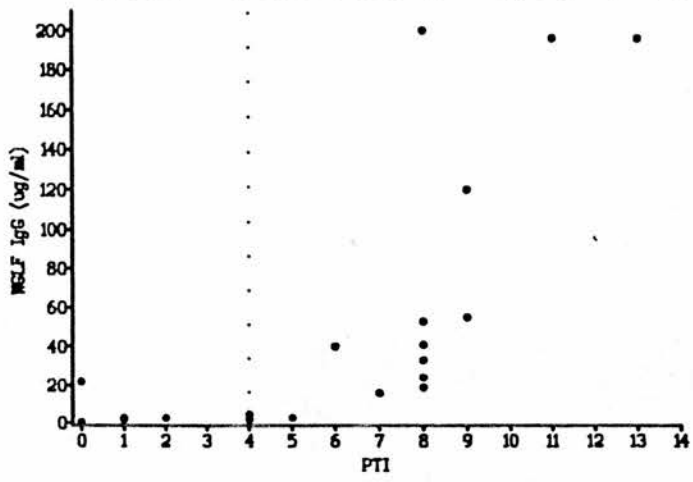


Figure 3.12

# INTESTINAL CLEARANCE OF PROTEINS IN INFLAMMATORY BOWEL DISEASE

## Introduction

The amount of plasma-derived protein lost into the gut (protein clearance per ml of plasma per unit time) has usually been measured using faecal loss of chromium labelled albumin or faecal  $\alpha$ 1-AT.

When WGL solution is taken by the patient at the rate of 200 ml every 10 minutes, which is equivalent to 20 ml per minute or 28.8 l per day, knowledge of both the serum protein concentration and the corresponding WGLF concentration should allow an estimate of clearance values (ml/day), to be determined for these proteins, and thus provide a measure of protein loss.

The data presented in the previous chapter from patients with IBD in whom the disease activity indices, CDAI and PTI, have been determined have been reexamined to determine clearance values for the three proteins.

Additional patients with both active and inactive CD and UC have been included in this group and 17 healthy volunteers have been included as a control group.

The aim was to establish whether intestinal clearance of proteins correlated better with activity indices than the simple measure of concentration in WGLF. Clearance values using the three WGLF proteins have been compared with published results based on existing methods described for faecal clearance of proteins.

## Subjects and methods

42 patients with CD, 27 active and 15 inactive as assessed by CDAI; 23 patients with UC, 13 active and ten inactive assessed by PTI; 17 control subjects (normal volunteers). Clearance of protein per ml plasma per day was calculated from the

concentration of protein in WGLF (ug/ml) x 28.8 / concentration of protein in plasma (g/l).

For this study protein results have been corrected for dilution with processing reagents to give absolute clearance values.

Serum proteins were measured by immunoturbidity at the Department of Clinical Chemistry, Western General Hospital, Edinburgh.

## Results

These are presented in Tables 3.7 - 3.10.

CDAI and intestinal clearance of proteins is shown in Figure 3.13

PTI and intestinal clearance of proteins is shown in Figure 3.14

Clearance values for  $\alpha$ 1-AT are consistently higher for all groups including controls compared with IgG which in turn gives clearance values about twofold higher than albumin.

For IgG and albumin there is good separation between active and inactive CD ( $p < 0.0001$ ) but there is greater overlap for  $\alpha$ 1-AT clearance values ( $p < 0.05$ ). Clearance of albumin gave a consistently better correlation with activity indices than WGLF albumin concentration.

There was no significant relationship between activity indices and clearance of  $\alpha$ 1-AT.

There was a significant correlation between serum albumin and WGLF albumin clearance in CD but not in UC patients (Figure 3.15, Table 3.9).

There were significant correlations between serum albumin and WGLF IgG clearance in both CD and UC patients. (Figure 3.16, Table 3.9).

There was a significant correlation between serum albumin and both CDAI and PTI, which may contribute to the stronger relationship between clearance of albumin and activity indices (Figure 3.17, Table 3.10).

There was a significant correlation between serum  $\alpha$ 1-AT and CDAI in CD patients, but not PTI in UC patients (Table 3.10).

## Discussion

In order to measure protein loss into the gut from plasma the following criteria should be fulfilled.

1. The protein in the gut only arises from leakage from blood plasma.
2. The protein is not metabolised in the gut lumen.
3. The protein is not reabsorbed from the gut lumen.

Criteria 2 and 3 are minimised by using the WGL procedure.

### IgG clearance

Jarnum and Jensen (1975) compared the faecal excretion of  $^{131}\text{I}$  labelled albumin and  $^{125}\text{I}$  labelled IgG following intravenous injection in IBD patients. The ratio of excretion of IgG to albumin was near to unity for patients with colonic disease whilst in patients with small bowel disease the ratio was higher and significantly more labelled IgG was excreted, and was highest in cases with jejunal involvement. These results may have been caused by greater metabolism of albumin with release and reabsorption of the labelled iodine in the more proximal cases.

In my study median (range) clearance values were as follows : for non hospital controls 4 (3 - 9); active CD 98(6 - 501); inactive CD 13(4 - 49); active UC 140(9 - 1054); inactive UC 9(4 - 88). These results are lower for controls as judged from clearances reported for segmental perfusion studies (Jonard et al,1984, Colombel et al,1992, Prigent-Delecourt et al,1995) but similar for IBD patients when compared with  $^{51}\text{Cr}$ -albumin measurements (Quigley et al,1987, Fishbach et al,1987).

### Albumin clearance

The clearance of albumin from plasma into the GI tract has been studied by several groups using  $^{51}\text{Cr}$ -albumin. van Tongeren et al,(1966) showed that there is a redistribution of chromium between different plasma protein fractions especially transferrin; ie this measurement gives a value for the clearance of plasma proteins

rather than specifically albumin ; however in practice there is little difference, while the specific activity of the labelled proteins does not change between plasma and gut.

Various control values have been reported:

van Tongeren et al (1966): 2 - 23 (mean 13); Waldman et al (1969) 6 - 35 (mean 16); Beeken et al (1972) up to 35 ml. These are similar to values obtained in segmental perfusion studies where Rambaud et al (1981) found a mean albumin clearance (jejunum) of 25 ml per day, and Prigent-Delecourt (1995) found a mean albumin clearance (colon) of 3.7 ml per day; ie a total of about 30 ml per day.

Several groups have used this test to assess protein loss in IBD. For Beeken et al (1972), the median (range) clearance of  $^{51}\text{Cr}$ -albumin in active IBD was 78(18 - 750)

From studies of Quigley et al (1987), and Fishbach et al (1987) median clearance was estimated to be between 100 and 200 from published graphs.

Results from this study are lower possibly due to the lability of albumin in the GI tract; however there is good diagnostic separation between active and inactive disease.

Median and range of clearance of albumin for non hospital controls : 5(0 - 13); active CD : 39 (4 - 218); inactive CD : 6 (1 - 20); active UC : 69(13 - 273); inactive UC : 5 (1 - 99).

### **$\alpha$ 1-AT clearance**

Many groups have investigated the clearance of  $\alpha$ 1-AT in faeces . Median and range of clearances of 6(0 - 46), 3(0 - 8), and 30(0 - 150), have been reported in control groups (Quigley et al,1987, Bernier et al,1978, Fishbach et al,1987), with corresponding figures for active IBD groups being 35(4 - 890), 92(17 - 218), 50(3 - 705).

Strygler et al (1990) had an additional control group with induced osmotic diarrhoea with a mean clearance of 28 ml per day, which was significantly higher than the control group without diarrhoea (mean 14 ml per day).

In the study by Fishbach et al (1987) subjects with inactive CD had a higher median clearance 80(30 - 320) than the corresponding active group. In most other studies inactive IBD cases are not separated from active cases or are not studied.

Quigley et al (1987) showed  $\alpha$ 1-AT clearance in faeces had poor sensitivity, with many false negatives (10/18 with raised  $^{51}\text{Cr}$ -albumin). Furthermore they showed that the in-patient control population had a skewed distribution which raised the upper limit of normal, reducing sensitivity.

The clearance of  $\alpha$ 1-AT compared with  $^{51}\text{Cr}$ -labelled albumin had a regression slope 0.55 (Florent et al,1981) and 0.64 (Quigley et al,1987) indicating possible different mechanisms of clearance of these two proteins. Florent et al (1981) showed good separation between control and IBD patients with a strong correlation between  $^{51}\text{Cr}$ -albumin and faecal  $\alpha$ 1-AT but did not separate active and inactive IBD patients.

In my study the results were higher compared with most other studies. Median clearance for non hospital controls: 38 (0 - 120), active CD: 198(0 - 1730); inactive CD: 79(11 - 324); active UC: 260(24 - 764); inactive UC: 110(18 - 292).

The influence of WGL procedure may be similar to that of induced osmotic diarrhoea, where the control values approximate to those for Strygler et al (mean 28, range 10 - 100 ml) with reduced likelihood of digestion and/or absorption of  $\alpha$ 1-AT, or increased leakage or exudation of  $\alpha$ 1-AT because of the increase in flow of fluid through the intestine. This effect was not evident for albumin or IgG. Geboes et al (1982) have identified  $\alpha$ 1-AT in human small intestinal epithelia using immunofluorescence techniques in both healthy subjects and patients with CD. Molmenti et al (1993) using immunohistochemical procedures have shown that  $\alpha$ 1-AT is expressed in human and jejunal enterocytes and is also a product of Paneth cells, and suggest that it is possible that  $\alpha$ 1-AT detected in faecal  $\alpha$ 1-AT may be derived from sloughed enterocytes. It may be that the WGL procedure, which involves the administration of a PEG 3350, a non absorbable osmotic agent, leads to release of this protein in some normal subjects and to a greater extent in subjects with IBD. This would explain the higher results for the clearance in WGLF compared with that reported for faeces. No RCE values for  $\alpha$ 1-AT have been reported but the smaller MW compared with albumin and IgG ( 54 kD compared with 65 kD and 145

kD) may lead to higher seepage from plasma to gut lumen in normal subjects compared with albumin and IgG.

The separation between active and inactive IBD cases was not clear. This is similar to other reported studies, and may indicate a different mechanism for loss/exudation of  $\alpha$ 1-AT in these patients. Alternatively interaction between  $\alpha$ 1-AT and elastase may confound interpretation of these results (James et al 1978).

Preliminary studies using a more sensitive test for measurement of WGLF  $\alpha$ 1-AT, a modification of the ELISA technique of Michalski et al (1985), gives a similar reference range for normal subjects which confirms the increased clearance values.

### **Protein clearance and activity indices**

The correlations between protein clearances and activity indices in patients with active IBD in general are similar to corresponding correlations using protein concentrations, although the correlation for clearance of albumin is consistently better; this probably reflects the fact that serum albumin concentrations were decreased in some of these cases, thus increasing the clearance values. (Table 3.10, Figure 3.15)

To enable more accurate values of clearance to be determined timed collections of WGL fluid would be required ; a study by Sallam et al (1995) from this unit demonstrates that WGLF output rates are very similar to intake rates; further work measuring sequential WGL collections is in progress.

**TABLE 3.7****WGLF proteins - concentrations and clearances.**

PATIENT GROUP	IgG		Albumin		$\alpha$ 1-AT	
	ug/ml	ml/day	ug/ml	ml/day	ug/ml	ml/day
CD (42)	17 (1-248)	56 (4-600)	26 (1-248)	19 (1-219)	14 (0-125)	145 (0-1730)
CD (27) ACTIVE	42 (4-248)	98 (6-600)	52 (5-248)	39 (4-218)	25 (0-124)	198 (0-1730)
CD SI (14) ACTIVE	32 (12-86)	79 (30-442)	41 (5-79)	31 (4-73)	30 (11-57)	233 (116-568)
CD COLON ACTIVE (13)	42 (4-248)	108 (6-600)	76 (15-248)	59 (13-219)	12 (0-124)	96 (0-1730)
CD (15) INACTIVE	4 (1-25)	13 (4-49)	10 (1-26)	6 (1-20)	6 (1-30)	79 (11-324)
UC (23)	27 (1-248)	28 (1-275)	12 (1-69)	59 (4-1055)	19 (1-69)	129 (18-765)
UC (13) ACTIVE	51 (4-248)	139 (9-1054)	93 (16-273)	69 (13-273)	20 (1-69)	260 (24-764)
UC (10) INACTIVE	4 (1-32)	9 (4-88)	7 (1-161)	5 (1-99)	12 (1-27)	110 (18-417)
NORMAL (17) SUBJECTS	1 (1-2)	4 (3-9)	6 (0-20)	5 (0-13)	4 (0-10)	38 (0-120)

number of patients in brackets

median (range)

concentrations and clearances corrected for processing dilution

**TABLE 3.8**

**Correlations between activity indices and WGLF protein clearance and concentration in patients with active IBD.**

Patient group	correlation protein concentration ug/ml and activity index CDAI or PTI			correlation protein clearance ml/day and activity index CDAI or PTI		
	IgG	Albumin	$\alpha$ 1-AT	IgG	Albumin	$\alpha$ 1-AT
CD ACTIVE	0.84 (0.001)	0.63 (0.001)	0.36 (ns)	0.69 (0.001)	0.66 (0.001)	0.24 (ns)
CD SI ACT	0.96 (0.001)	0.65 (0.01)	0.38 (ns)	0.73 (0.003)	0.70 (0.005)	0.44 (ns)
CD COL ACT	0.85 (0.001)	0.59 (0.03)	0.42 (ns)	0.68 (0.01)	0.63 (0.02)	0.22 (ns)
UC ACTIVE	0.74 (0.004)	0.61 (0.03)	0.60 (0.03)	0.82 (0.001)	0.72 (0.005)	0.44 (ns)

figures in brackets = significance (p<) levels  
 SI ACT = small intestinal active; COL ACT = colonic active

**TABLE 3.9**

**Correlation between serum albumin and WGLF protein concentration and clearance.**

PATIENT GROUP	Correlation-S.Albumin and WGLF proteins			Correlation-S.Albumin and WGLF protein clearance		
	IgG	Albumin	$\alpha$ 1- AT	IgG	Albumin	$\alpha$ 1-AT
CD	-0.45 (0.01)	-0.39 (0.02)	-0.28 (ns)	-0.46 (0.002)	-0.45 (0.003)	-0.15 (ns)
UC	-0.48 (0.02)	-0.16 (ns)	-0.30 (ns)	-0.57 (0.005)	-0.31 (ns)	-0.15 (ns)

figures in brackets = significance (p<) levels

42 patients in CD group, 23 patients in UC group

**TABLE 3.10**

**Serum Albumin, IgG, and  $\alpha$ 1-AT in patients with CD and UC and controls (median and range), and correlations with activity indices**

	IgG g/l	Albumin g/l	$\alpha$ 1-AT g/l
CD (42)	10.4 (5.2-36)	38 (27-45)	3.0 (1.8-6)
CDAI CORRELATION	0.12 (ns)	-0.61 (P<0.001)	0.53 (P<0.001)
UC (23)	10.4 (5.5-15.6)	41 (29-47)	3.0 (1.5-5.1)
PTI CORRELATION	-0.16 (ns)	-0.54 (P<0.001)	0.20 (ns)
CONTROLS (17)	9.8 (6.5-14.9)	45 (39-49)	3.0 (2.2-4.0)

number of patients in brackets  
median (range)

# CDAI and INTESTINAL CLEARANCE of PROTEINS

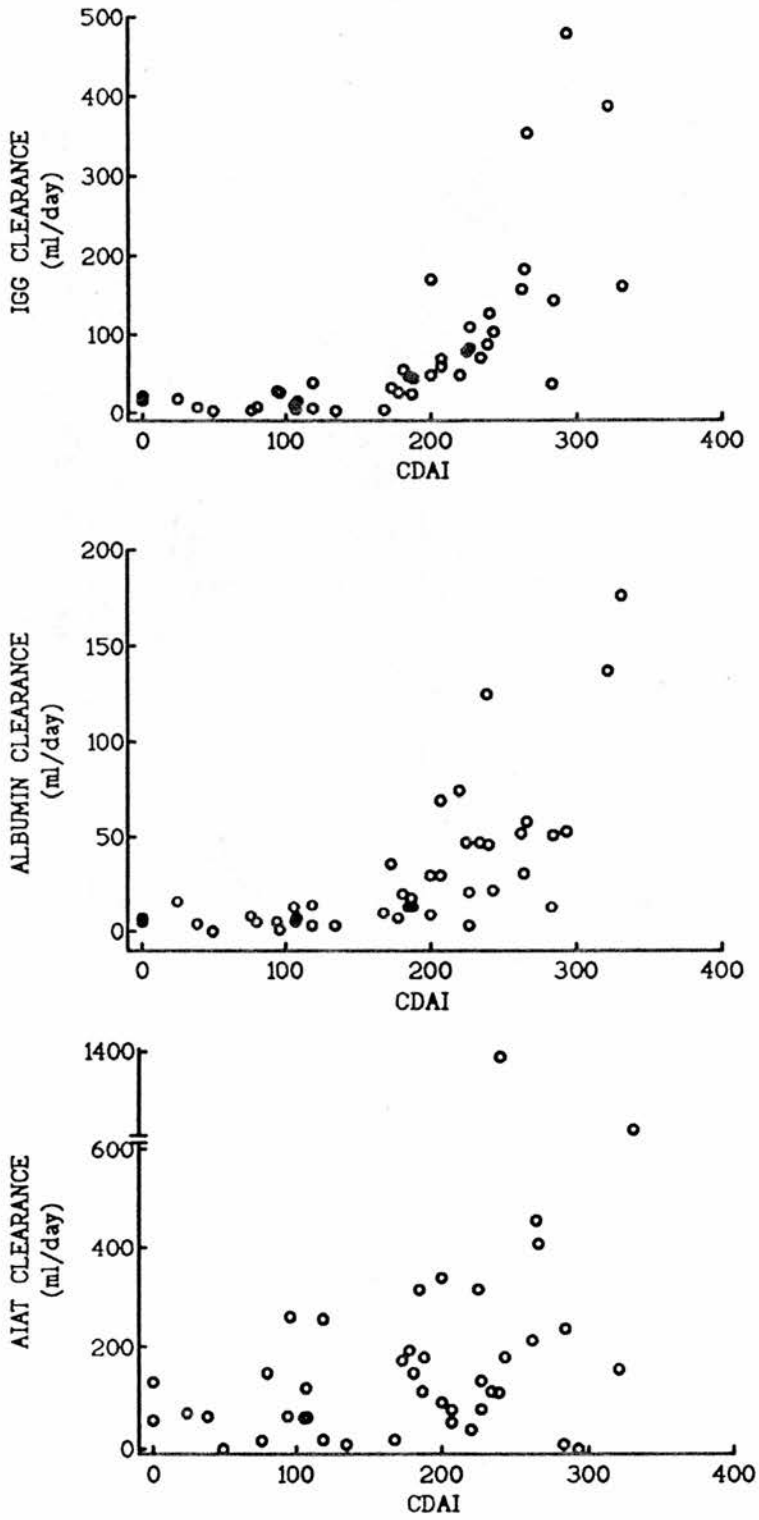


Figure 3.13

# PTI and INTESTINAL CLEARANCE of PROTEINS

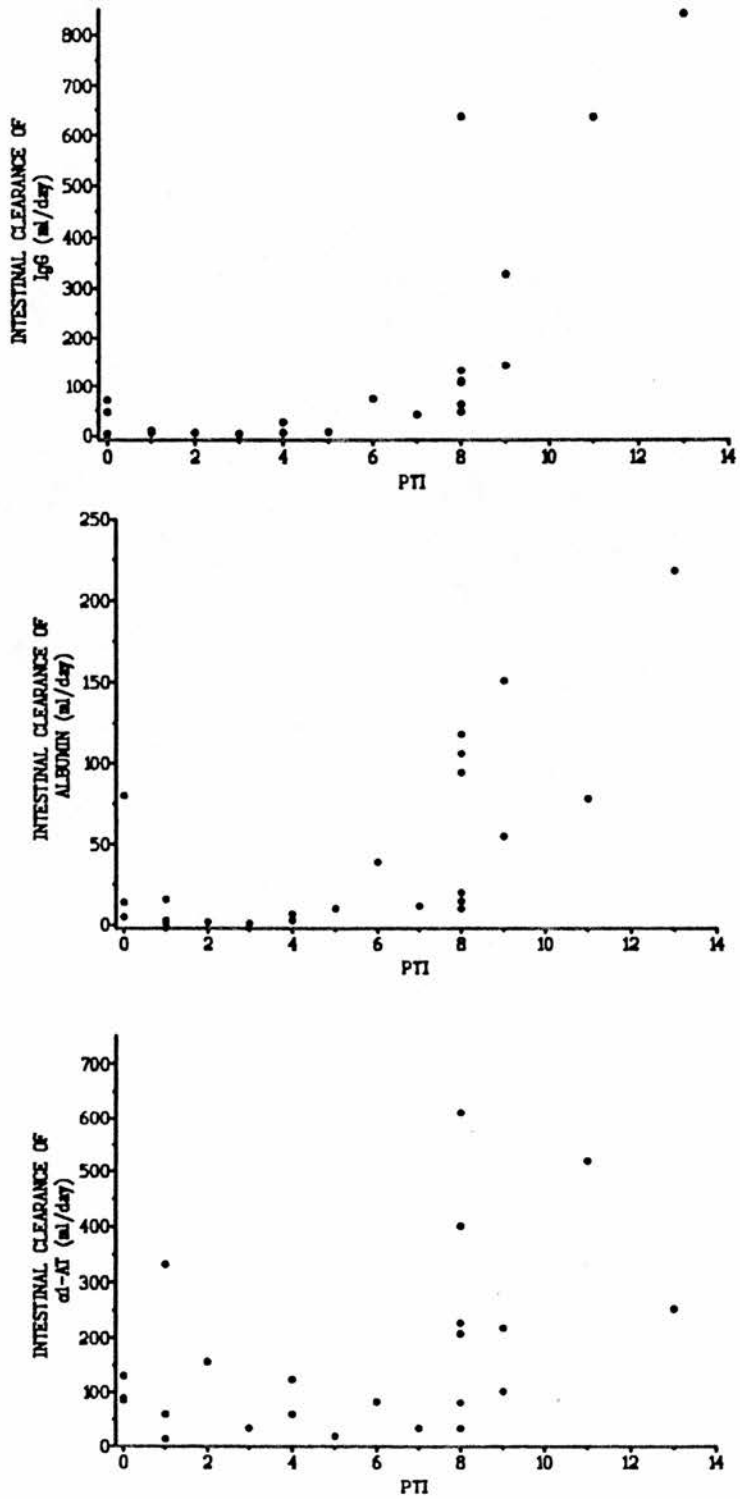


Figure 3.14



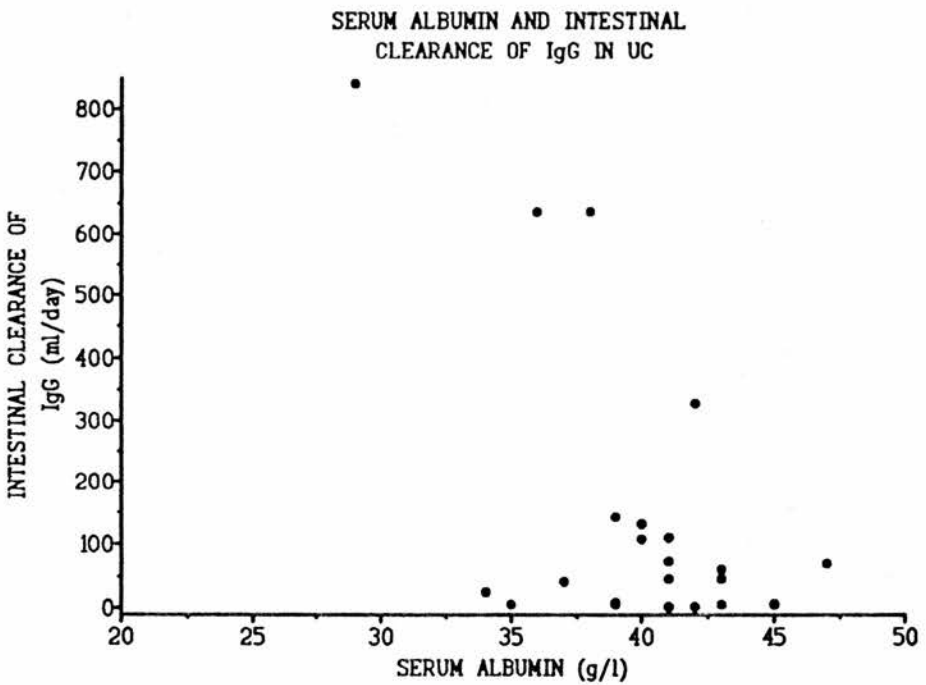
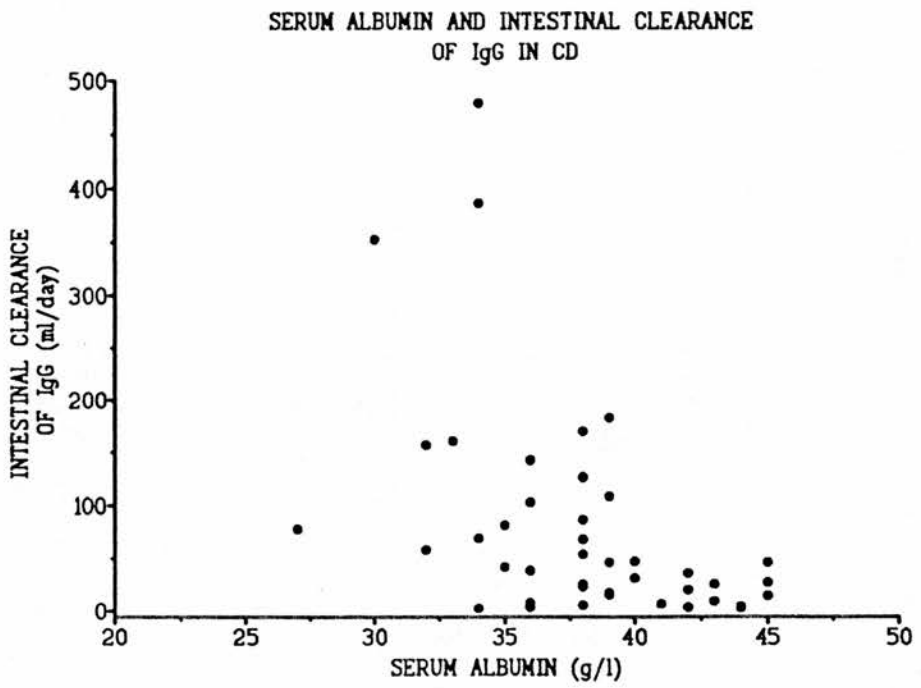


Figure 3.16

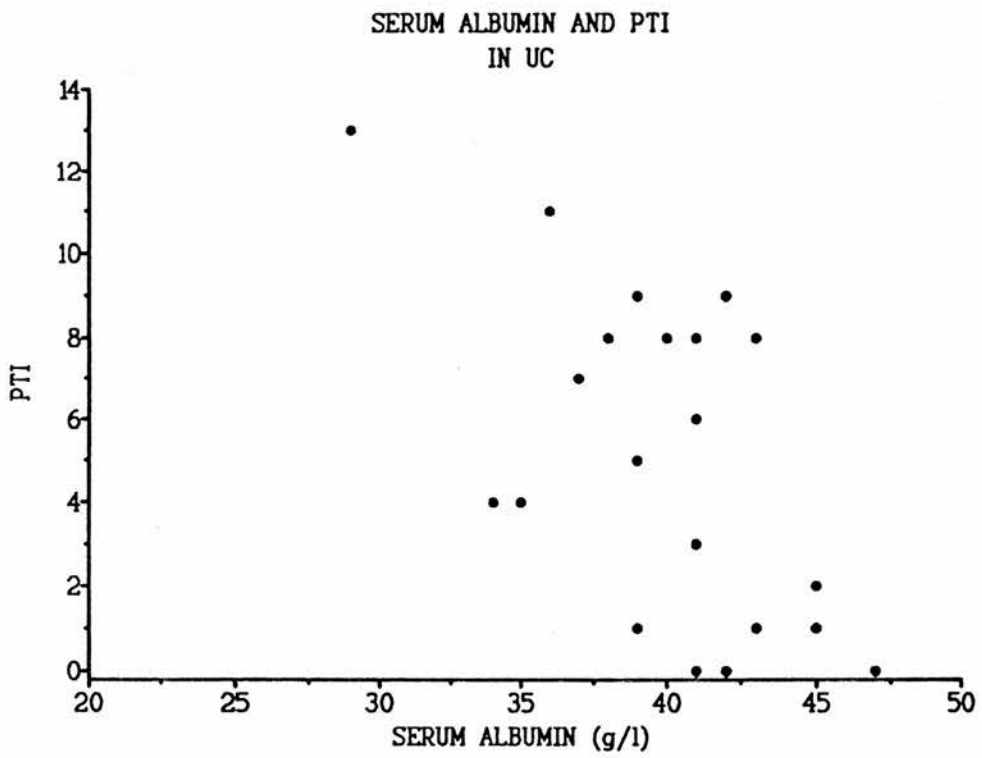
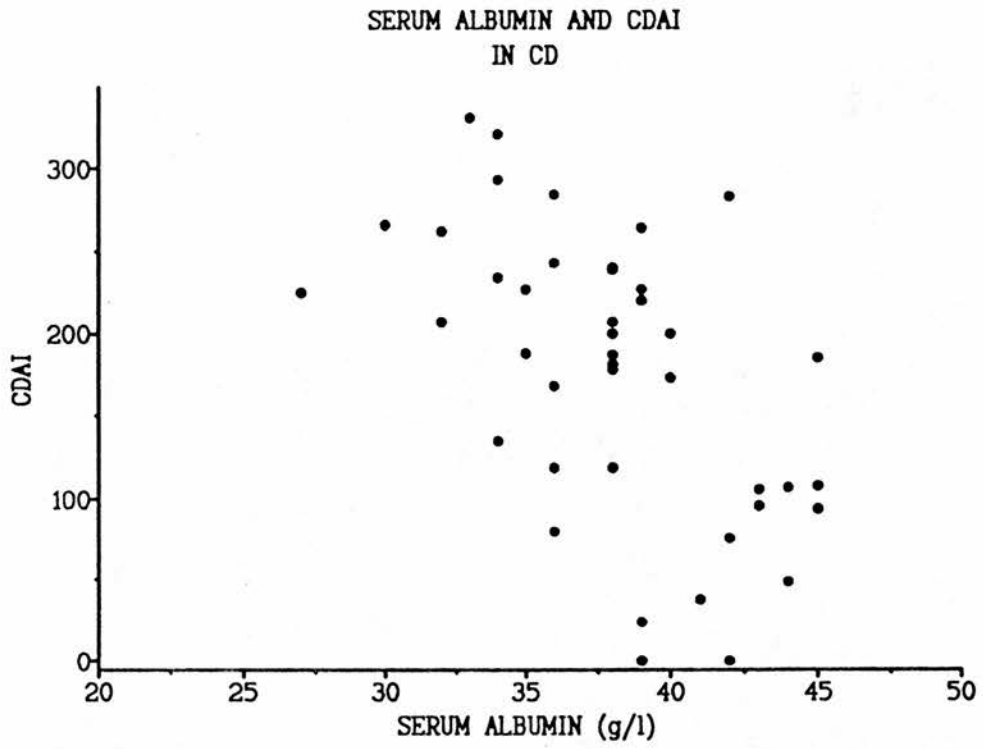


Figure 3.17

## APPENDIX : CHAPTER 3

### 1. WGLF processing (Gaspari et al, 1988)

#### Reagents

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. All working reagents are kept at 4°C unless otherwise stated.

- a. Phosphate buffered saline: (PBS - Sigma) 1 tablet dissolved in 200 ml distilled water to give pH 7.2.
- b. Soy bean trypsin inhibitor: (SBTI - Sigma): Dissolve 0.1g per 100 ml PBS. Solid kept at -20°C.
- c. Disodium EDTA: Add 11 g to 100 ml distilled water. Dissolve to form a saturated solution. Store at room temperature.
- d. Phenylmethylsulphonylfluoride: (PMSF - Sigma) 0.1M in 95% ethyl alcohol.
- e. Sodium Azide: 2g/100 ml distilled water.
- f. New Born Calf Serum: (Heat inactivated - Sigma Chem Co,UK.)

Approximately 200-400ml WGLF is collected and filtered through cheesecloth into 50 ml polypropylene centrifuge tubes. SBTI was added to each tube to a final concentration of 10% (v/v) followed by addition of 1.0M EDTA in PBS to 5% (v/v). Tubes were centrifuged at 550g for 10 minutes.

The supernatant was transferred into another centrifuge tube and PMSF reagent added to a final concentration of 1%(v/v). Tubes were then centrifuged at 2200g for 30 minutes. The supernatant was transferred to another tube and PMSF again added at 1% (v/v) followed by the addition of sodium azide reagent to a final concentration of 1%. After 15 minutes, 4% v/v heat inactivated calf serum was added. Samples were divided into aliquots and stored at -70°C until assayed.

## **2. WGLF processing by filtration**

1. 2 x 25 ml samples of clear WGLF are collected from the patient.
2. 10 - 15 ml is filtered into a universal container, using Whatman GF/A 12.5 cm filter paper. 10 ml is transferred to another universal container.
3. The following reagents are added with mixing between each addition:

1 ml SBTI, 0.56 ml Na EDTA, 0.24 ml PMSF, 0.12 ml sodium azide. (Leave for 2 minutes.) 0.6 ml new born calf serum. Mix, and dispense 6 x 1.5ml aliquots into 2 ml microtubes with screw lids (Sarstedt, UK). Samples are stored at  $-70^{\circ}\text{C}$ .

### 3. WGLF albumin and $\alpha$ 1-AT

#### Chemicals and Reagents

- a. Anti human albumin (sheep): SAPU - code S034-205.
- b. Anti human  $\alpha$ 1-AT (goat): PRU Central Antiserum Procurement Unit, Sheffield, UK.
- c. Polyethylene glycol 3350 (PEG 3350-Sigma Chem Co, UK)
- d. Polyethylene glycol 6000 (PEG 6000-Biochemical Grade) Merck, UK.
- e. Sodium Chloride (Analar) Merck, UK.
- f. Standard - Human serum SPS-01 (Protein Reference Unit, Sheffield, UK)
- g. Tris(hydroxymethyl)methylamine - TRIS (Analar) : Merck, UK.
- h. Tween 20 ( Polyoxyethylene sorbitan monolaurate)(Sigma, UK)

#### Reagents

- a. PEG 6000 reagent: 40g PEG 6000, 6g TRIS, 2g TWEEN 20 , and 1g sodium azide are dissolved in 800ml distilled water, adjusted to pH 7.0 with dilute hydrochloric acid and made up to 1 litre volume with distilled water. Store at +4<sup>0</sup>C.
- b. Antibody Reagent -  $\alpha$ 1-AT. Dilute anti-human  $\alpha$ 1-AT 1 in 40 with filtered PEG 6000 reagent just prior to analysis. Allow reagent to come to room temperature before using.
- c. Antibody Reagent - Albumin - Dilute anti-human albumin 1 in 40 with filtered PEG 6000 reagent just prior to analysis. Allow reagent to come to room temperature before using.
- d. Standard Diluent - Dissolve 9g sodium chloride, 48g PEG 3350, and 1g sodium azide in 1 litre solution distilled water.
- e. Working Standards - Dilute standard serum with standard diluent to give standards of 0,5,10,20,50,100,and 200ug/ml.
- f. Sample blank - lavage solution processed as per WGLF.

#### Procedure

The procedure for both assays is essentially the same using the appropriate antibody reagent.

- a. Centrifuge processed lavage samples at 1500g for 10 minutes.

- b. Bring PEG 6000 reagent and standard diluent reagents to room temperature. Filter sufficient PEG 6000 reagent through a 0.22 $\mu$  filter.
- c. Using a Dilugil dispenser, dilute 0.05ml standards, blank and test samples with 0.95ml PEG reagent in 2ml polystyrene tubes (LIP,UK), in duplicate.
- d. Repeat as for c with antibody reagent.
- e. Leave tubes for 15 minutes at room temperature and read turbidity at 340nm using a flow through spectrophotometer (PU8610 - ATI Unicam, Cambridge, UK).
- f. After blank subtraction plot standard curve and read test concentration from calibration curves.

Note: in practice the zero standard and sample blank give same values, with no cross reaction between antibodies used and calf serum for either assay.

#### **4. WGLF $\alpha$ 1-AT by radial immune diffusion**

Commercial plates supplied by Behring suitable for measuring low protein concentrations (LC-Partigen) are used. Each plate has 12 wells, 3 for standardisation and 9 for test samples. To use the plate remove the plastic container from the aluminium sealed container, open the plate and leave at room temperature for 5 minutes to allow any condensation in the wells to evaporate. Using a Hamilton micro syringe add 20ul standards and samples to the wells taking care to avoid damaging the agar-gel layer. After loading, allow the plates to stand open for about 10 - 20 minutes, then close them with the plastic lid and leave them standing at room temperature. Diameters of precipitates should be read off after 2 - 3 days. For standardisation the diameters (in mm to the nearest 0.2 mm) are squared and plotted against  $\alpha$ 1-AT standard concentrations.

Standard used was SPS-01 diluted in standard diluent (method 3) to give standards of 10, 50, and 125 ug/ml.

## 5. WGLF Albumin immunoturbidity - centrifugal analyser

Standard curve prepared: 12.8, 25.6, 51.3, 102.5, 164, 205 mg/l

Antibody - SAPU - anti-human albumin (Sheep) - SAPU.

Dilute 1 in 37.5 with 4% PEG

Assay run on Monarch Centrifugal analyser

Sample volume 40ul

Diluent volume = 26 ul

Antibody volume = 120ul

Temperature 37<sup>0</sup>C

Delay time 5 seconds

interval time 30 seconds

no of data points 10

filter 1 = 340 nm

filter 2 = 340 nm

run time 275 seconds

## **6. Protease - non specific - Azacoll**

Azacoll is a dye-derivatised collagen. Proteolytic enzymes release the blue dye from this product which can be measured at 530nm.

### **Reagents**

- a. Phosphate buffered saline (PBS-0.01M pH7.0)
- b. Azacoll reagent (Sigma Chem Co, Ltd) 10mg per ml PBS

### **Procedure**

- a. Add 0.05ml WGLF to 0.05ml Azacoll suspension reagent in 2 ml polystyrene tubes. Mix and incubate at 37°C for 15 minutes .
- b. Stop reaction by placing reaction tubes in iced water for 2 minutes.
- c. Add 1.5 ml PBS and centrifuge at 1000g for 5 minutes to sediment residual substrate.
- d. Transfer 1.0 ml supernatant to similar tubes and read OD at 530nm.
- e. Both processed and unprocessed WGLF are assayed by this procedure and percent inhibition calculated.

## 7. Chymotrypsin

The inactive precursor chymotrypsinogen is secreted by the acinar cells of the pancreas and hydrolysed by trypsin in the small intestine to the active peptidase chymotrypsin. (CT)

CT hydrolyses peptide links in which the carboxyl group is provided by an aromatic amino acid and the amino group of any amino acid other than aspartate or glutamine. Measurement is by the hydrolysis of a synthetic polypeptide labelled with p-nitroaniline. The production of the p-nitroaniline is measured kinetically in a spectrophotometer at 405nm.

### Reagents

a. Solvent - Boehringer Mannheim, UK contains 0.7% lauryl trimethylammonium chloride, 0.5M sodium chloride, and 0.1M calcium chloride

b. Substrate and control are supplied with the kit in freeze dried ampoules, reconstituted with 2 and 1 ml water respectively.

The chromogenic substrate is succ-ala-ala-pro-phe-p-nitroanilide.

### Procedure

a. Switch on the spectrophotometer, check programme parameters and allow temperature stabilisation.

b. The spectrophotometer settings for this test are as follows:

WAVELENGTH	405nm
MODE	KINETIC
FACTOR	5.3
DELAY	0.50
TIME	1.00
CYCLES	2
TEMPERATURE	37°C
VOLUME	0.8ml

c. Assay water to check cycle function.

d. Add 20 ul WGLF to 1 ml substrate.

e. Aspirate into spectrophotometer.

f. Repeat for control and further test samples.

The spectrophotometer printout gives the absorbance of the sample, the calculated enzyme activity for each of the two cycles and the mean activity of the two cycles in u/ml.

## 8. IgG in WGLF

### Principle of Method

Immunoglobulins are absorbed by class-specific antibodies bound to the solid phase (ELISA plate). Then an enzyme-labelled anti-globulin conjugate (alkaline phosphatase) is added and binds to the specific immunoglobulin required.

### Instrumentation

Optical densities are read on a Dynatech microplate reader MR5000.

- a. Place plate in the plate carrier.
- b. Press - MANUAL
- c. Wavelength - DUAL
- d. Test filter 405nm
- e. Reference filter 630nm
- f. This will read the plate and hold the results, enabling you to see when the standard has reached the desired level.
- g. When Standard has reached the desired O.D. press - START
- h. TEST NUMBER = Type number desired e.g. 11 for Total IgG,
- i. Lastly PLATE = you can type letters or numbers or a combination of the two.
- j. The plate will now be read and the results passed on to the printer.

This assay is carried out on IMMULON 1, flat-bottomed plates (DYNATECH, UK).

### Reagents

- a. Carbonate/bicarbonate coating buffer: dissolve the contents of 10 capsules (Sigma C-3041) in 1 litre of distilled water.
- b. Coating antibodies used are:  
Goat anti Human IgG Affinity purified. (Sigma I-2136) Store at +4°C.
- c. Elisa wash: 0.9% saline + 0.05% Tween 20 (0.5ml Tween 20/litre of saline. Store at +4°C.
- d. Elisa diluent: 0.9% saline + 0.05% Tween 20 + 1% adult bovine serum (filter sterilised before adding) ie 0.5ml Tween 20/litre of saline and 10ml of filtered adult bovine serum.
- e. Alkaline phosphatase conjugate: goat anti Human IgG (Sigma A-3187. Store at +4°C.

f. Diethanolamine liquid (Sigma, UK). Store at room temperature.

Working diethanolamine reagent:

Diethanolamine                    100 ml  
Magnesium chloride( $MgCl_2 \cdot 6H_2O$ ) 0.102 g  
Sodium azide ( $NaN_3$ )            0.2 g  
Distilled water                    800 ml  
Adjust pH to 9.8 with 6N HCl, and make up to 1 litre.  
Store at 4°C

g. p-nitrophenyl phosphate (Sigma 104-105 Phosphatase substrate tablets) 5 mg/tablet. One tablet used per 5 ml of diethanolamine working reagent.

### Standard

1. Standard SPS 01 (Protein Reference Unit, Sheffield, UK). Store at 4°C.

The starting concentration is adjusted to 1.0 µg/ml and a further 6 doubling dilutions are made, giving a standard range of 1000 - 15.6 ng/ml.

### Procedure

a. Coat plates with 1/5000 dilution of IgG coating antibody in coating buffer, and store at 4°C overnight in a moist box.

b. After overnight incubation plates are washed x3 with Elisa wash, then inverted and tapped upside down on tissue to drain.

All wells are then filled with Elisa diluent and incubated at 20°C to block non-specific binding for 1 hour.

c. While plates are blocking make all standard, sample, and QC dilutions in Elisa diluent. Lavage samples are diluted 1/25 for the measurement of IgG, and then double diluted.

d. After at least 1 hour of blocking, plates are decanted and drained upside down on tissue.

e. Plates are loaded with the desired samples, standards, etc. (volume used in all wells is 125 µl.)

f. Incubate plates overnight at 4°C. in covered, moist box.

g. Next day plates are washed with Elisa wash x3, decanted and drained on tissue.

h. Conjugate is added to the plates at a dilution of 1/5000 for IgG to all wells, and the plates covered, put in a moist box and incubated for 3hrs at room temperature.

i. Plates are washed x3, drained and substrate added to all wells.

j. Allow plates to stand on the bench for about 3-4 mins, by which time colour development of top standards, high samples and QCs will occur.

k. Place plates on the plate shaker - checking the OD of the top standard, via the manual mode on the Elisa reader.

l. When OD has reached 1.000 , the plate is ready to read. (Test 11 for total IgG)  
Programs will subtract background, read all wells, draw standard curve, and perform regression on the results.

## **9. Total Protein - Bio-Rad Protein Assay**

### **Introduction**

This is based on the Bradford dye binding procedure (Bradford, 1976) and is a simple colorimetric assay for measuring total protein concentration. The standard assay is used with samples having a protein concentration between 200 and 1400 µg/ml. The assay is based on the colour change of Coomassie brilliant blue G-250 in response to various concentrations of protein. The dye binds primarily to basic (especially arginine) and aromatic amino acid residues.

### **Reagents**

The dye reagent is provided as a five fold concentrate, which should be diluted 1 in 4 with distilled water and filtered prior to use in the assay. This can be stored in a glass container for up to 2 weeks at room temperature.

### **Standard**

SPS-01 (Protein Reference Unit, Sheffield, UK) diluted with lavage solution to give concentrations in the range 200-1400 µg/ml.

### **Test samples**

Whole gut lavage fluid treated with PMSF (final concentration 2mM) and sodium azide (final concentration 200mg/l)

### **Assay procedure**

1. Place 0.05 ml of standards and samples in polypropylene tubes, and 0.05 ml lavage solution in blank tube.
2. Add 2.5 ml diluted dye reagent.
3. Vortex (avoid excess foaming).
4. After a period of 5 minutes to 1 hour, measure OD at 595 nm versus reagent blank.
5. Plot OD at 595nm versus concentration of standards (see Figure 3.8a).

## 10. Development of Crohn's Disease Activity Index

This was undertaken by the National Cooperative Crohn's Disease Study group (USA) in 1970. This group contained gastroenterologists from 13 universities or university affiliated medical centres.

Their objective was to derive an index which satisfied the following requirements:

a. it should incorporate factors considered by most knowledgeable gastroenterologists to be important indicators of disease activity.

b. it should utilise observations readily available at the time of a patient visit

c. it must weigh the component factors in an intuitively appropriate direction.

d. it should require only relatively simple computations.

e. it should demonstrate visit to visit changes consistent with the physician's overall appraisal of changes in the patients condition.

f. it must correlate well with the physicians overall appraisal of disease activity.

g. it should weigh the component factors so as to optimise the accuracy with which it predicts physicians overall appraisals.

Eight independent variables were coded in such a way that the expected value in a normal individual was zero, and progressively larger positive values were expected to reflect progressively greater activity of Crohn's disease.

Examples of such variables were : number of liquid or soft stools in a week; abdominal pain; general well being; symptoms presumed related to Crohn's disease; taking anti diarrhoeal drugs; body weight changes; hematocrit; abdominal mass.

Appropriate coefficients were determined which best predicted dependent variable values using the eight independent variables.

Variables with a major contribution to the final index are stools, pain, well being, and haematocrit; each of the others contributes less than 10%. Two of the variables are purely subjective - pain and well being, and are recorded by the patient on a daily basis; the objective variables are recorded by the physician.

In defining a limit between active and quiescent disease, one might select various cut off values in the range 100-200. 150 has been chosen as a reasonable compromise.

## 11. Powell-Tuck Index

This is used to assess disease activity in patients with ulcerative colitis.

This is a more simple index than the CDAI and is based on the following variables: general health, abdominal pain, bowel frequency, stool consistency, blood in stool, anorexia, nausea/vomiting, abdominal tenderness, other related lesions - eye inflammation, arthralgia, oral ulceration, related skin lesions, body temperature, sigmoidoscopy.

Scores varying between 0-10 are allocated based on the findings.

A total score of greater than 4 indicates active disease.,

## CHAPTER 4

### INVESTIGATION OF MOLECULAR HETEROGENIETY OF $\alpha$ 1-ANTITRYPSIN AND $\beta$ -GALACTOSIDASE ACTIVITY IN FAECES AND WGLF OF SUBJECTS WITH ACTIVE IBD.

#### Introduction

Many proteins are secreted into the GI tract as a result of physiological and pathological processes. Most of these undergo extensive degradation such that measurement in faeces grossly underestimates their secretion rate. However  $\alpha$ 1-AT is resistant to extensive proteolysis and has been used to measure protein loss from the gut. Mizon et al (1988) and Boege and Fischbach (1991) have recently investigated the molecular heterogeneity of  $\alpha$ 1-AT in the GI tract, and have presented evidence for partial degradation of this protein by enzymes which are secreted by gut bacteria in healthy individuals.

Mizon et al (1988) applied sodium dodecyl sulphate - polyacrylamide gel electrophoresis and immunoblotting to analyse  $\alpha$ 1-AT from healthy subjects and patients with CD. A component with molecular weight 38 kD was identified in normal faecal extracts as well as in six pathological samples. In these cases the CDAI was 170 (SEM 47). In contrast  $\alpha$ 1-AT of 51 kD was detected in faecal extracts from eight patients with very active CD (CDAI 287, SEM 39)

Mizon et al (1991) have sequenced the N-terminal end of the 38 kD  $\alpha$ 1-AT, and have shown that it differs from the native plasma form by the loss of 17 N-terminal amino acids; carbohydrate analysis of this form demonstrated a total lack of neutral sugars; in contrast the 51 kD  $\alpha$ 1-AT is glycosylated, and an intermediate 44 kD molecule is partially deglycosylated; furthermore analysis of 25 faecal extracts from patients with CD demonstrated that the presence of the glycosylated form of  $\alpha$ 1-AT was related to the degree of inflammation, although some patients with raised CDAI

have only the 38 kD  $\alpha$ 1-AT; the author's interpretation of these findings was that in active CD there is inhibition of the hydrolytic activity of some glycosidases.

This was confirmed by the same group (El Yamani et al, 1992) who showed decreased exoglycosidase activity, especially  $\beta$ -galactosidase ( $\beta$ G) in patients with active CD with the 51 kD  $\alpha$ 1AT.

Hoskins et al (1981) have shown that bacterial subpopulations, producing glycosidases as extracellular enzymes, average about 1% of the viable faecal flora, and once diluted in culture cease to produce the enzymes extracellularly; the intracellular enzymes which are present in the majority of the bacteria have no influence on the metabolism of luminal colonic glycoproteins.

Using concanavalin A detection El Yamani et al (1992) have further shown that alterations in the deglycosylation process in some patients with CD could be extended to all faecal glycoproteins. Subjects with faecal 51 kD  $\alpha$ 1-AT in addition had several other concanavilin A-reactive bands, which were not present in subjects with faecal 38 kD  $\alpha$ 1-AT. Because of its particular resistance to proteolysis, it may be possible to use faecal  $\alpha$ 1-AT MW as a marker for assessing the deglycosylation process in the gut.

In order to confirm and extend these studies I have a. measured MW distribution of  $\alpha$ 1-AT and  $\beta$ G activity in WGLF and faeces from control subjects and patients with active UC and CD; b. compared results in WGLF and faeces.

## **Principles of procedures used**

### **1. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE):**

A gel is formed by polymerising acrylamide and a suitable cross linking agent, N,N'-methylene bisacrylamide. The polymerisation occurs rapidly at room temperature in the presence of an initiator and catalyst and porosity of the gel can be adjusted by varying the acrylamide concentration and degree of cross linking.

Denaturing conditions are used for the PAGE. When a strongly ionic detergent is used such as sodium dilauryl sulphate (SDS), proteins are denatured and combine with a constant ratio (w/w) of SDS. The overwhelming negative charge provided by the SDS coating makes any charge contributed by the protein negligible, and so separation of such protein-SDS complexes by PAGE is almost entirely due to sieving, and is therefore dependent on the molecular size of the protein.

The molecular sieving of the gel depends on the concentration of polyacrylamide. A reasonably accurate value for the molecular weight of most proteins (except those which are highly glycosylated) can be obtained by comparison of their electrophoretic mobility with those of standard proteins of known MW.

The protein mixture is denatured and reduced (disulphide bonds split) by heating in SDS with a reducing agent, prior to electrophoresis.

## **2. Transfer of proteins from gels to porous membranes (immunoblotting).**

Protein blotting transfers the protein pattern generated in a gel to a porous membrane (nitrocellulose) to produce a precise replica.

The purpose of this transfer is usually to facilitate binding of macromolecular ligands (usually antibodies) to the proteins on the membrane. By this simple manipulation, a ligand too large to penetrate the pores of a gel may now easily reach its target proteins. When an antibody is used in this way the procedure is called immunoblotting or Western blotting.

A typical immunoblotting experiment may be divided into five steps.

1. sample preparation and electrophoresis.
2. transfer to a membrane.
3. blocking (quenching) of the residual binding capacity of the membrane
4. incubation with probe (antibody)
5. detection of bound ligand with secondary labelled reagents.

The reagents, equipment and procedure used are described in the appendix to this chapter. The PAGE procedure is essentially that described by Laemmli (1970).

### **3. $\beta$ -Galactosidase**

P-nitrophenyl-galactoside was incubated with faecal extract or WGLF with release of p-nitrophenol by  $\beta$ G. The reaction was stopped by dilution with sodium carbonate and p-nitrophenol measured at 405 nM.

#### **Molecular weight marker standards**

(Gibco BRL, Life Technologies Ltd, Paisley UK)

This contained proteins in the range 94 - 14 kD:

phosphorylase b (94), albumin (67), ovalbumin (44), carbonic anhydrase (29), trypsin inhibitor (20),  $\alpha$ -lactalbumin (14). The proteins were at a concentration of 1 mg/ml.

Also used was wide range MW marker (Sigma Chem Co, UK, M-4038). This contained proteins in the range 205 - 6.5 kD:

myosin (205),  $\beta$ -galactosidase (116), phosphorylase-B (97), fructose-6-phosphate kinase, rabbit (84), albumin, bovine (66), glutamate dehydrogenase, bovine (55), ovalbumin, chicken (45), glyceraldehyde-3-phosphate dehydrogenase, rabbit,(36), carbonic anhydrase, bovine (29), trypsinogen, bovine (24), trypsin inhibitor, soybean (20),  $\alpha$ -lactalbumin, bovine (14), aprotin, bovine (6.5)

#### **Patient samples investigated**

Faecal homogenates were prepared from 17 patients ; nine with active CD, eight with active UC, as assessed by raised WGLF IgG (>10ug/ml).

0.5 g faeces were suspended in 5 ml 0.9% sodium chloride and mixed on a rotomixer periodically for 15 minutes. Samples were then centrifuged at 25,000g for 30 minutes at 4°C in an MSE Superspeed 50 centrifuge. PMSF was added to a final concentration of 2 mM to supernatants before freezing at -70°C for subsequent  $\alpha$ 1AT MW distribution and  $\beta$ G activity measurements.

WGLF samples (filtered, processed), were also investigated in 24 patients with active IBD (15 CD, nine UC) for  $\alpha$ 1-AT MW distribution and  $\beta$ G activity.

These were filtered through 0.22  $\mu$ M filters prior to assay for  $\beta$ G.

WGLF from nine control subjects were also assayed for  $\beta$ G.

In addition 0.1 ml human  $\alpha$ 1-AT (1 mg/ml PBS, Sigma Chem Co Ltd) was incubated for 24 hours at 37°C with

- a. 0.2 ml phosphate buffered saline (PBS).
- b. 0.1 ml faecal suspension (one part faeces and four parts isotonic saline), and 0.1 ml PBS.
- c. 0.05 ml faecal suspension, 0.05 ml WGLF, and 0.1 ml PBS
- d. 0.1 ml WGLF, and 0.1 ml PBS.

The faecal suspension and WGLF (filtered, processed) were prepared from a patient with inactive CD.

Samples were then centrifuged as for faecal homogenates before electrophoresis and blotting.

## Results

The relative mobility of the MW markers were determined from the blots initially stained with Ponceau S dye and plotted on semi-log graph paper against MW.

Relative mobilities were determined as a ratio of distance moved by band divided by total length of electrophoretic run.

MWs of  $\alpha$ 1-AT bands revealed by blot probe were read from a calibration graph after relative mobility calculations.

Plates 1,2 demonstrate blots of faecal and WGLF  $\alpha$ 1-AT.

Plates 3,4 demonstrate a blot developed for WGLF  $\alpha$ 1-AT (3) previously stained with Ponceau-S dye (4)(photograph enlarged).

Plate 5 shows PAGE gel corresponding to plate 3 and 4 stained with coomassie blue.

Plate 6 shows the influence of WGLF and faeces on native human serum  $\alpha$ 1-AT.

WGLF reduced the MW to 51 kD and faeces to 38 kD.

The calculation for MW is given in detail for plates 3 and 4 in table 4.1 and figure 4.1 (standard calibration).

Bands in the region 49 - 52 kD are reported as 51 kD, and in the region 36 - 38 kD are reported as 38 kD.

Figures 4.2a and 4.2b give results for MW distribution of  $\alpha$ 1-AT in faeces and WGLF of patients with IBD and corresponding  $\beta$ G activities.

Figure 4.3 shows the correlation between WGLF and faecal  $\beta$ G activities in patients with active IBD. ( $r=0.79$ ,  $p<0.0002$ ).

Tables 4.2 and 4.3 show results for individual patients with active CD or UC.

Four patients with active CD had  $\alpha$ 1-AT with MW of 51 kD in both faeces and WGLF with low  $\beta$ G activity in both.

All other patients with either active CD or UC had  $\alpha$ 1-AT MW of 38 kD in faeces.

One patient had  $\alpha$ 1-AT MW of 44 kD in addition to 38 kD in faeces.

More patients had  $\alpha$ 1-AT of 51 kD in WGLF, which included 6 patients with active UC who had 38 kD  $\alpha$ 1-AT in faeces.

Four patients with CD and one with UC had 2 bands corresponding to 38 kD and 51 kD with a further intermediate band at 44 kD in 2 cases.

$\beta$ G activity was significantly lower in patients with 51 kD compared with 38 kD  $\alpha$ 1-AT for both faecal extracts ( $p<0.01$ ) and WGLF ( $p<0.001$ ).

WGLF  $\beta$ G activity in nine control subjects who had no GI disease ranged from 0.1-1.7 u/l.

### **WGLF IgG and $\alpha$ 1-AT in patients studied with active IBD**

Median (range) WGLF IgG for patients with active CD was 50(22 - 455) ug/ml.

Median(range) WGLF IgG for patients with active UC was 75(11 - 139) ug/ml.

Of the 4 patients with faecal 51 kD  $\alpha$ 1-AT, WGLF IgG were 32, 50, 113, and 455 ug/ml.

WGLF IgG were not significantly different between active UC and active CD patients.

WGLF IgG were not significantly different between patients with active CD with 38 or 51 kD  $\alpha$ 1-AT.

WGLF  $\alpha$ 1-AT ranged from 17 - 92 (median 31) ug/ml in subjects with active IBD.

## **Discussion**

Previous studies (Mizon et al,1988,1991) have shown that the faecal form of  $\alpha$ 1-AT is related to the exoglycosidase (particularly  $\beta$ G) activity in patients with active CD; patients with active disease and 51 kD molecular form  $\alpha$ 1AT had a significantly lower glycosidase activity than patients with inactive or active CD and the 38 kD form.

In addition El Yamani et al (1992) have shown that the loss of  $\beta$ G activity could not be explained by destruction of the enzyme by protease activity since  $\beta$ G activity was resistant to faecal proteases for several hours at 37°C; neither could the low exoglycosidic activity of the samples containing molecular form 51 kD be explained by the presence of an inhibitor since mixing such an extract with another containing molecular form 38 kD resulted in deglycosylation, and a  $\beta$ G activity equal to the theoretical enzyme activity calculated from that of the two mixed samples.

The results in my studies in patients with active CD agree with those of El Yamani et al. 4/9 patients with active CD have both the 51 kD molecular form of  $\alpha$ 1-AT and a low  $\beta$ G activity in faeces.

All patients with active UC studied had the 38 kD  $\alpha$ 1-AT in faeces.

Faecal exoglycosidase activities have previously been studied in patients with CD and UC and compared with healthy control subjects and amounts found to be widely distributed;  $\beta$ G activity was not different in patients and control subjects and was not related to disease activity (Rhodes et al,1985). Also if one of the exoglycosidase enzyme activities was increased, the other activities were also elevated.

In my study control subjects had significantly greater faecal  $\beta$ G activity than subjects with active UC, and active CD with  $\alpha$ 1-AT 51 kD but were not different from patients with active CD and  $\alpha$ 1-AT 38 kD.

In WGLF the 51 kD molecular form of  $\alpha$ 1-AT was present in patients with active IBD to a greater extent than in faeces. This included six patients with UC all of whom had low  $\beta$ G (0.2 - 1.7 u/l). All of these patients had 38 kD  $\alpha$ 1-AT in faeces and  $\beta$ G 2 - 13 u/g.

Of the patients with CD the four subjects with low levels of  $\beta$ G in the faeces and 51 kD  $\alpha$ 1-AT also had low  $\beta$ G in WGLF (0.2 - 0.8 u/l) and 51 kD  $\alpha$ 1-AT.

The low exoglycosidic activity may be explained by changes in the intestinal bacterial flora or its metabolic activity, resulting in a decreased secretion of these enzymes. The combination of 51 kD  $\alpha$ 1-AT in faeces and low  $\beta$ G activity was not found in patients with active UC and appears to be specific for CD.

The greater degree of 51 kD  $\alpha$ 1AT in WGLF compared with faecal extracts may have been due to the rapid transit of proteins (less than two hours maximum) in WGLF compared with faeces with less time being available for deglycosylation. Alternatively the greater dilution of bacteria by liquid may have inhibited secretion of exoglycosidase enzymes. However the relatively high activity of  $\beta$ G and the presence of 38 kD  $\alpha$ 1-AT in WGLF in some subjects with active IBD indicates the potential for rapid deglycosylation and may indicate a mucosal site for these bacteria since the majority of bacteria in the lumen will be washed out by the WGL procedure.

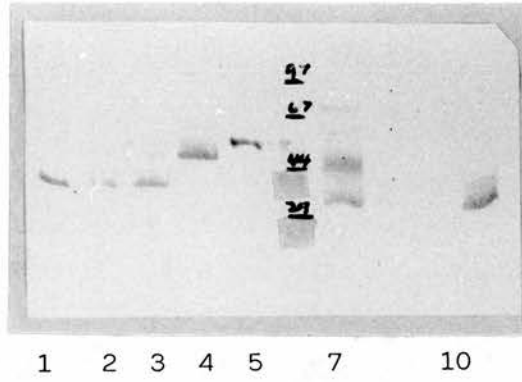
Control values for  $\beta$ G activity in WGLF were similar to those for patients with 51 kD  $\alpha$ 1-AT and lower than those with 38 kD  $\alpha$ 1-AT.

Control values for  $\beta$ G were much lower in WGLF than in faeces for control subjects ie these did not parallel each other as seen in active IBD patients. The higher level in faeces may relate to the longer transit time of these subjects compared with patients with active IBD with  $\beta$ G possibly being more stable in faeces than other proteins released into the gut - the longer the transit time the lower the total protein may become thus artificially raising  $\beta$ G activity when expressed as u/g protein. In patients with active IBD transit time is usually shorter and protein excretion greater.

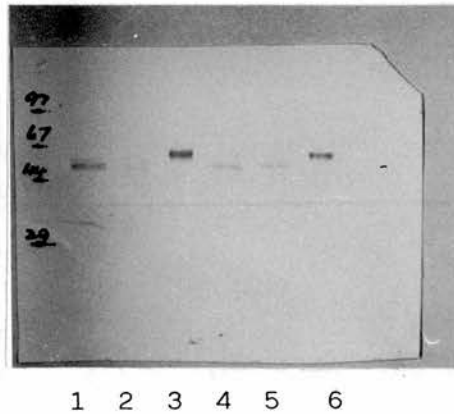
This highlights the advantage of using a standardised WGL procedure where controls and test subjects are treated in a similar way.

Although previous studies have shown no difference between faecal  $\beta$ G activities in control and active IBD groups, there may be greater secretion of  $\beta$ G by gut bacteria in some patients with active IBD in response to loss of glycoproteins into the gut.

**Plate1**



**Plate 2**



**Plate 1: Immunoblot - Faecal  $\alpha$ 1-AT in active CD and UC**

Lane 1, 2 - active UC (38 kD); Lane 3 - active CD (38 kD); Lane 4 - active CD (51 kD);  
Lane 5 - human  $\alpha$ 1-AT standard (54 kD); Lane 7 - active CD (38 and 44 kD);  
Lane 10 - active UC (38 kD).

**Plate 2: Immunoblot - WGLF and faecal  $\alpha$ 1-AT (51 kD) in patients with active CD**

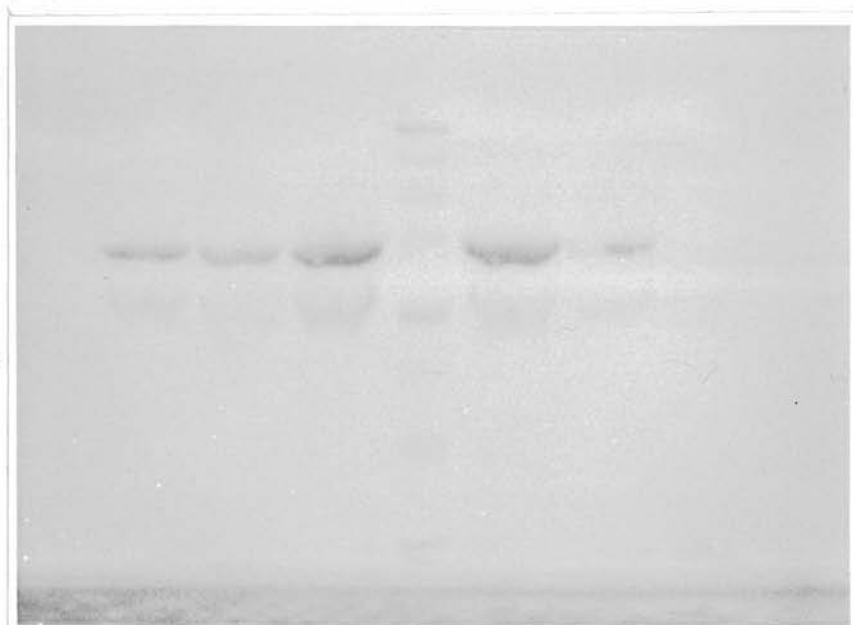
Lane 1,2 - faecal and WGLF  $\alpha$ 1-AT in CD (51 kD); Lane 3,6 - human  $\alpha$ 1-AT standard (54 kD)  
Lane 4,5 - faecal and WGLF  $\alpha$ 1-AT in CD (51 kD).

**Plate 3**



1 2 3 4 6 7 9

**Plate 4**



— 66 kD  
— 55 kD  
— 45 kD  
— 36 kD

5

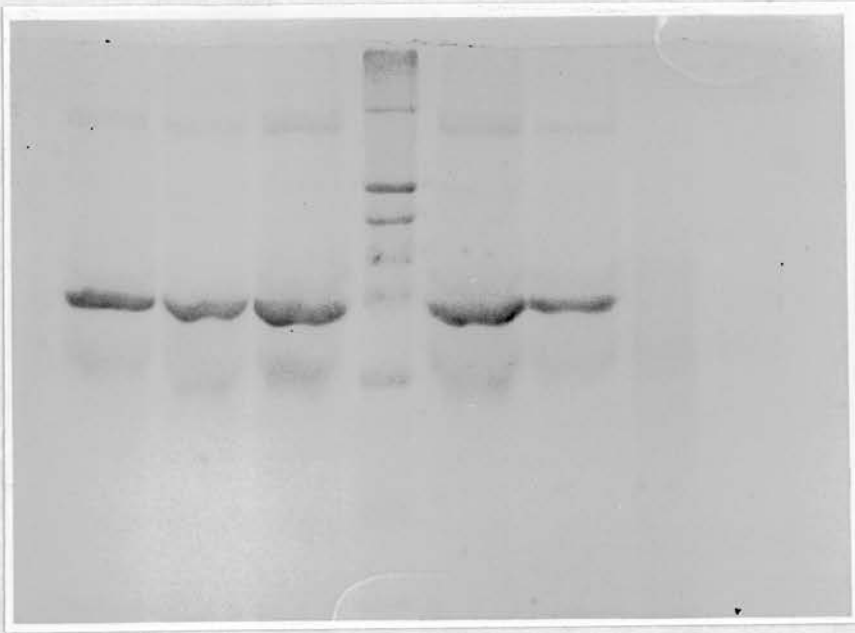
**Plate 3 : Immunoblot - WGLF in active CD**

Lanes 1, 9 - human  $\alpha$ 1-AT standard (54 kD); Lane 2 - 51 kD; Lane 4 - mixture 37-51 kD;  
Lanes 3, 6,7 - 38 kD.

**Plate 4: Ponceau S protein stain of immunoblot**

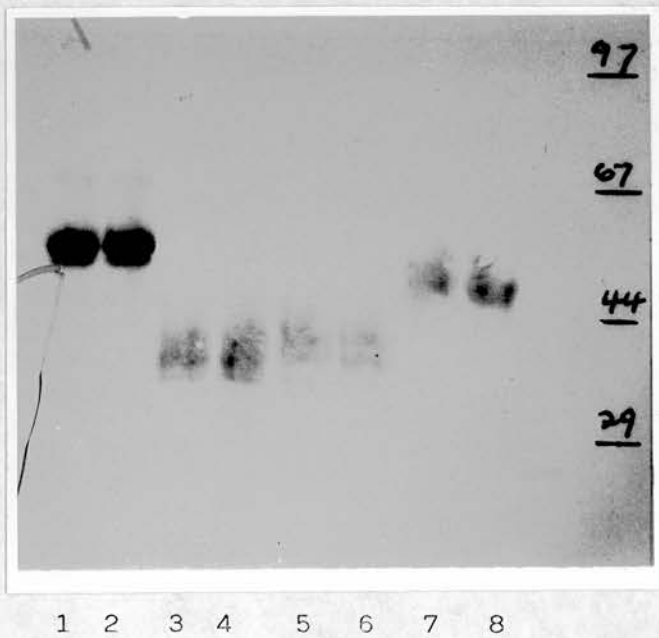
Lane 5 - protein marker (MW)

**Plate 5**



5

**Plate 6**



**Plate 5: PAGE separation corresponding to plates 3,4 showing proteins stained with coomassie blue**

Lane 5 : protein marker (MW)

**Plate 6: Influence of incubation of human  $\alpha$ 1-AT with WGLF and faeces (24 hour at 37°C)**  
Lanes 1,2 - human  $\alpha$ 1-AT (54 kD); Lanes 3,4 - human  $\alpha$ 1-AT incubated with faecal suspension (38 kD); Lanes 5,6 - human  $\alpha$ 1-AT incubated with faecal suspension and WGLF (38 kD); Lanes 7,8 - human  $\alpha$ 1-AT incubated with WGLF only.

**Table 4.1**

Example of calculation of molecular size of WGLF  $\alpha$ 1-AT in active CD (plates 3-5).  
Calculation of MW of principal bands.

	distance from origin (mm)	relative mobility	MW (kD)
Lane 1	36	0.46	55 (human standard - 54 kD)
Lane 2	43	0.55	49
Lane 3	57	0.73	36
Lane 4	57	0.73	36
Lane 5		Composite Standard	
Lane 6	58	0.74	35
Lane 7	55	0.71	37
Lane 9	36	0.46	55 (human standard 54 kD)

full distance = 78 mm

**TABLE 4.2** **$\beta$ -Galactosidase activity and  $\alpha$ 1-AT MW distribution in patients with active CD**

Patient No	$\alpha$ 1-AT MW (kD)		$\beta$ -galactosidase	
	WGLF	FAECES	WGLF(u/l)	FAECES(u/g)
1	38	38	4.5	50
2	38	38	8.1	39
3	38	-	3.5	-
4	38	-	1.7	-
5	38-51	-	4.1	-
6	38-51	-	1.2	-
7	38-51	-	0.5	-
8	38-51	38, 44	2.3	2.0
9	51	51	0.8	0.2
10	51	38	1.6	17
11	51	38	1.1	9
12	51	51	0.2	0.1
13	51	51	0.6	2.2
14	51	51	0.6	2.0
15	51	-	0.1	-

**Table 4.3** **$\beta$ -Galactosidase activity and  $\alpha$ 1-AT MW distribution in patients with active UC**

Patient No	$\alpha$ 1-AT MW (kD)		$\beta$ -Galactosidase	
	WGLF	FAECES	WGLF (u/l)	FAECES (u/g)
1	38	38	3.0	8.0
2	38	-	6.2	-
3	38-51	38	2.0	12
4	51	38	0.4	3.2
5	51	38	0.4	13
6	51	38	1.3	7.1
7	51	38	1.1	12
8	51	38	1.7	2.1
9	51	38	0.2	2.5

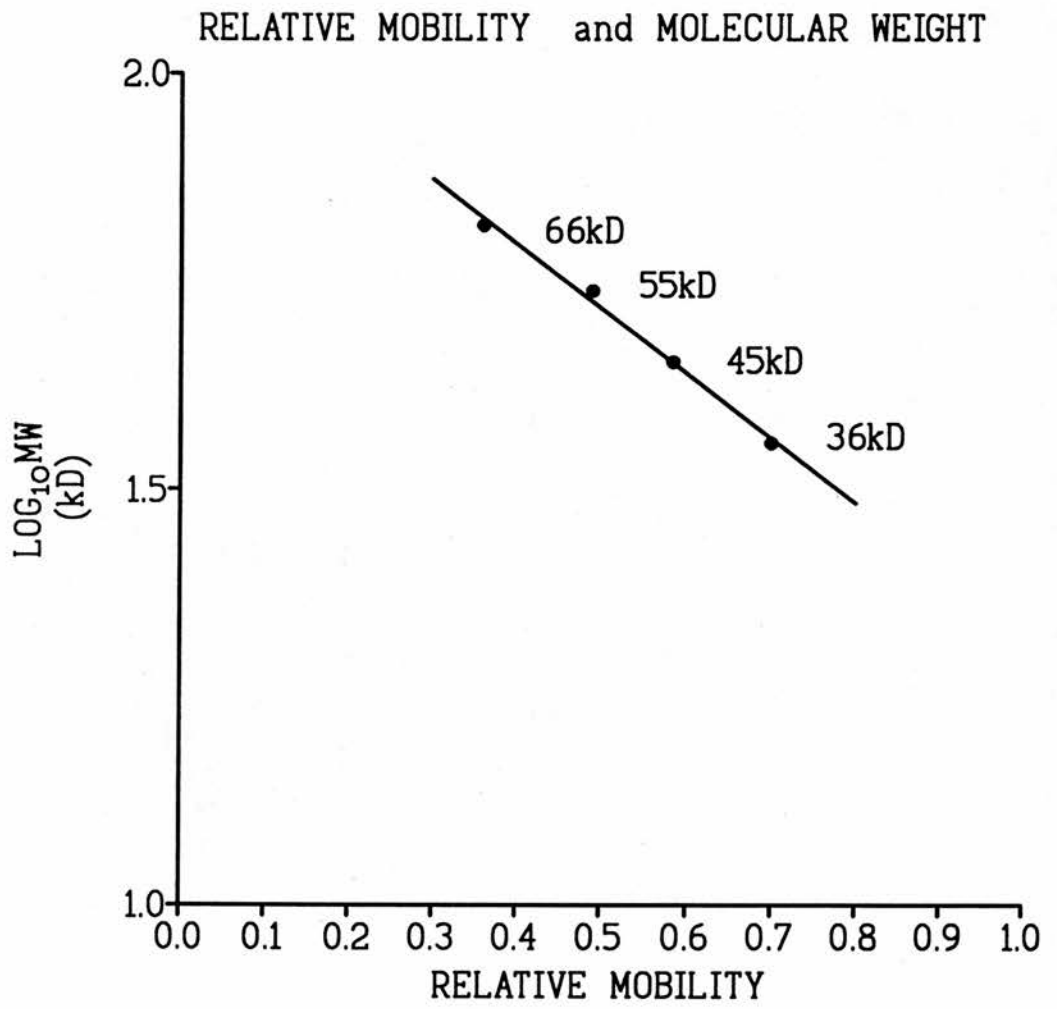


Figure 4.1

FAECAL  $\alpha$ 1-AT MW DISTRIBUTION  
and  $\beta$ -GALACTOSIDASE ACTIVITY

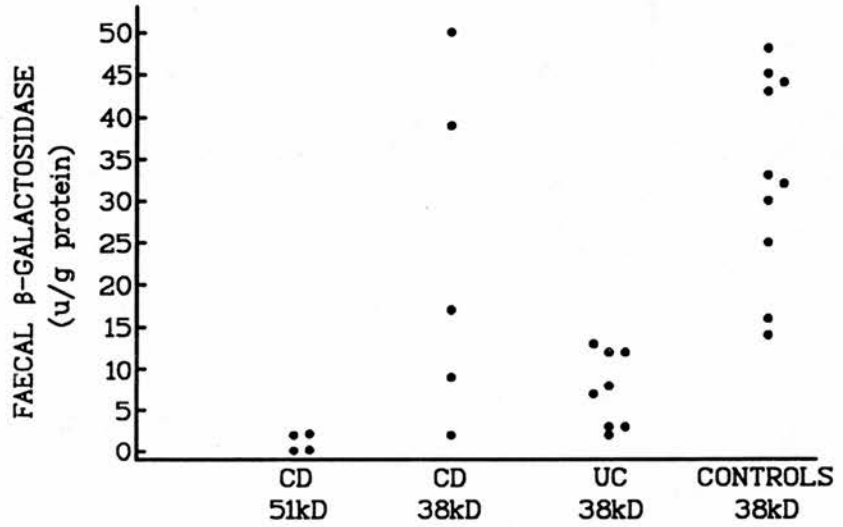


Figure 4.2a

WGLF  $\alpha$ 1-AT MW DISTRIBUTION  
and  $\beta$ -GALACTOSIDASE ACTIVITY  
in PATIENTS with ACTIVE IBD

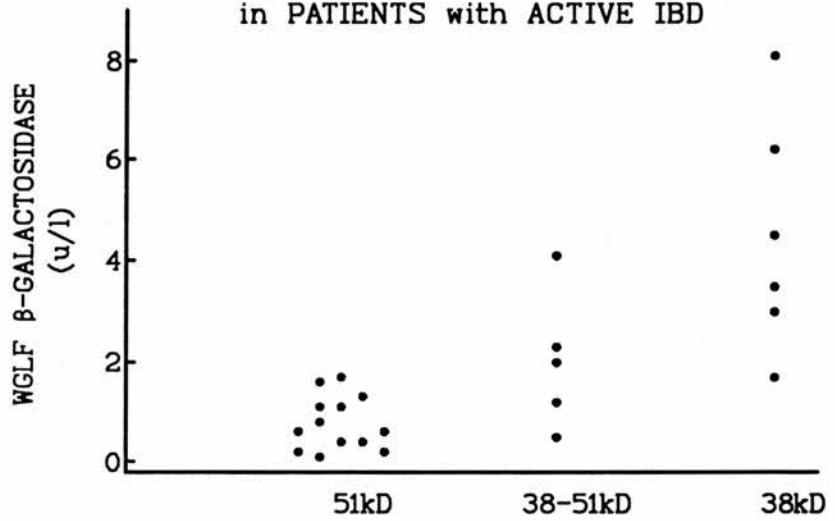


Figure 4.2b

WGLF AND FAECAL  $\beta$ -GALACTOSIDASE  
IN PATIENTS WITH ACTIVE IBD

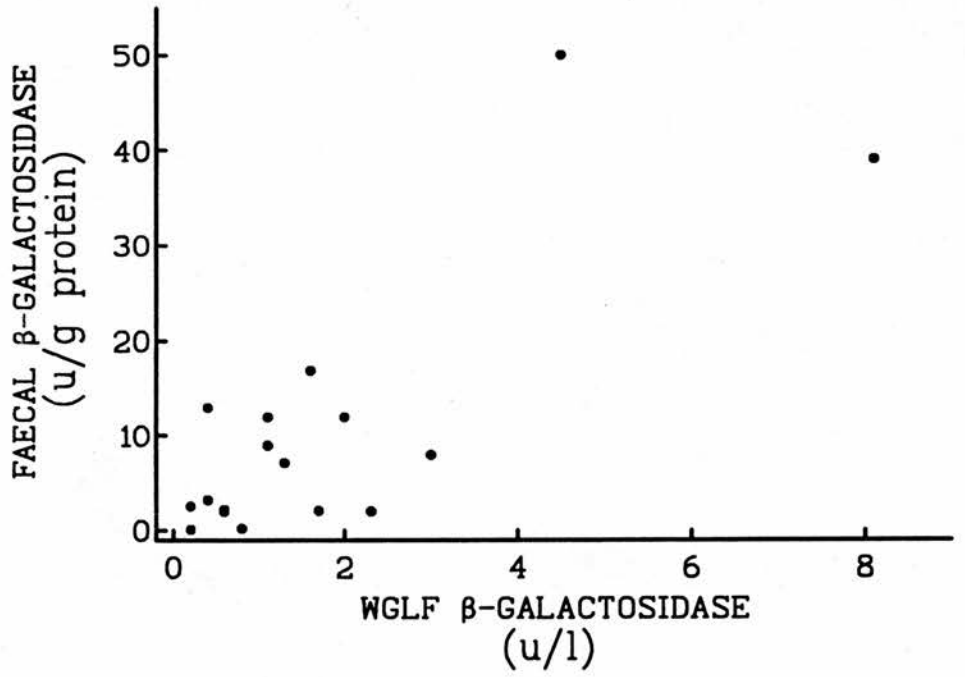


Figure 4.3

## APPENDIX : CHAPTER 4

### 1. Poly Acrylamide Gel Electrophoresis and Immunoblotting - $\alpha$ 1AT.

#### Equipment

SE 250 - MIGHTY SMALL 11 SLAB GEL ELECTROPHORESIS UNIT (Hoeffer Scientific Instruments, San Francisco, USA).

The SE 250 basic unit has upper and lower buffer chambers, lid assembly with cables, alumina plates (notched), glass plates, red clamps, and celloseal to seal plates.

In addition SE 250-10A -.75 includes the above as well as a SE245 dual caster, 2x10 well combs and 4xT shaped spacers, 0.75 mm thick

This permits the preparation of 2 minigels approx 80 x 10 x 0.75 mm.

#### Reagents

Chemicals are from Sigma Chem Co Ltd, UK unless otherwise stated.

- a. Acrylamide-bisacrylamide (30:0.8) dissolve 30 g acrylamide and 0.8g bisacrylamide in 100 ml water (total volume). Filter through Whatman no 1 filter paper (BDH Ltd, Merck, UK) and store at 4°C in a dark bottle. Prepare fresh every 1 - 2 months.
- b. TEMED : used as supplied: store at room temperature.
- c. Ammonium persulphate (1.5% w/v) dissolve 150 mg in 10 ml water on day of use.
- d. Sodium dilauryl sulphate (SDS) (10% w/v) dissolve 10g SDS in water to 100 ml. Heat in warm water to ensure dissolved before use.
- e. Resolving gel buffer : 3.0M Tris/HCl (pH 8.8) 36.3 g tris and 48 ml 1.0M HCl to 100 ml volume. Filter and store at 4°C.
- f. Stacking gel buffer : 0.5M Tris/HCl (pH 6.8): Dissolve 6g Tris in 40 ml water, adjust to pH 6.8 with 1M HCl ( about 48 ml) and bring to 100 ml volume. Filter and store at 4°C.
- g. Reservoir buffer : 0.25M Tris, 1.92M glycine, 1% SDS (pH 8.3). Dissolve 30.3 g Tris, 144 g glycine, and 10 g SDS to 1 litre. Store at 4°C. Dilute 1 : 10 before use with water.
- h. Sample buffer: 6% SDS, and 6% 2-mercaptoethanol, 30% sucrose and 0.02% bromophenol blue marker, in 0.15 M Tris HCl pH 6.8.

For 2 x 10% resolving gels mix:

10 ml acrylamide/bis  
3.75 ml buffer  
0.3 ml 10% SDS  
14.45 ml water

Degas using water suction pump

add 1.5 ml ammonium persulphate and 0.015 ml TEMED and gently mix.

Cast gels, loading with a pastette.

Apply 1 ml 0.3% SDS solution to surface of the gels. Allow to set (20 - 30 minutes);  
pour off 0.3% SDS.

For 2 stacking gels use:

2.5 ml acrylamide/bis  
5 ml stacking gel buffer  
0.2 ml 10% SDS  
11.3 ml water

Degas using water suction pump

1.0 ml ammonium persulphate  
0.015 ml TEMED.

Pour to the top and insert perspex comb before gel polymerises (from left to right).  
Gel polymerises within 30 minutes.

Remove from casting block and clamp to electrophoresis tank after greasing rubber seal. Pour reservoir buffer into top (cathode) above the level of the comb and check for any leaks. Add reservoir buffer to lower compartment (anode). Remove comb and add samples.

### **Sample preparation**

Add sample buffer to sample (1 part buffer to 2 parts sample). Boil in eppendorfs for 3 - 5 minutes maximum at 100 °C.

Cool and apply samples (3 ul for MW marker and 1 uL for 1mg/ml  $\alpha$ 1-AT standard, 2 ul faecal supernatants and 10ul WGLF samples).

Attach lid assembly with cables to anode and cathode, and circulate cold water.

Run gels for 1 -2 hours at constant current (20 mA per gel)

### **Protein Transfer Blot**

#### **Equipment:**

1. Milliblot - SDE Transfer system.
2. Consort E741 Power Supply.

Use a constant voltage supply; and do not allow initial voltage to exceed 100 volts DC with a single layer of gels; with an 8 cm x 5.5 cm resolving gel the power supply

adjusted to provide an initial current of 110 mA ( 2.5 x the area of gel). The normal operating voltage should be between 10 and 100 volts at this current setting when running one gel. (Voltages above 100 indicate the end of the electrodes usable life, and must be replaced.)

The Mylar insulating matrix must be placed directly on top of the lower electrode to prevent top electrode from touching lower electrode because of overtightening or misalignment.

Transfers should be run for 30 minutes.

## **Reagents**

### **a. Methanol**

b. Anode buffer 1: (0.3M Tris, 10% methanol, pH 10.4). 36.3 g Tris, 100 ml methanol in 800 ml with water and adjusted to pH 10.4 as required with 1M HCl before adjusting to 1L volume.

c. Anode buffer 2: (0.025M Tris, 10% methanol, pH 10.4.) 3.03 g Tris and 100 ml methanol in 800 ml water adjusted to pH 10.4 as required with 1M HCl before adjusting to 1L volume.

d. Cathode buffer: (.025M Tris, .04M glycine, 20% methanol, pH 9.4). 3.03 g Tris , 2.52 g glycine and 200 ml methanol in 800 ml water and adjusted to pH 9.4 as required with 1M HCl before adjusting to 1 L volume.

e. Chromatography paper : grade 3MM CHR (Whatman, UK)

f. Nitrocellulose membrane: grade NCHAHY, 0.45um (Millipore, UK)

## **Procedure**

a. Align the Mylar insulating matrix over the lower (anode) electrode.

b. Cut chromatography papers (x6) and nitrocellulose membrane to size of PAGE gel (approx 8x6 cm).

c. Soak 1 layer of chromatography paper in anode buffer 1 and then allow excess buffer to drain.

d. Place a drop of anode buffer 1 in the middle of the lower electrode surface, then place the soaked filter paper on the lower electrode surface. Centre the paper and smooth out to make a good contact.

e. Soak 2 layers of filter paper in anode buffer 2. Drain and place on top of the filter paper already installed on the lower electrode surface. Roll a glass rod over the filter paper to smooth the paper and remove trapped air bubbles.

f. Cut off a small corner of nitrocellulose membrane to mark. Wet with deionised water for 5 minutes and rinse the membrane in anode buffer 2, before carefully placing on top of the previous three layers of chromatography paper. Wear gloves when handling membranes to avoid protein contamination.

g. Cut off a small corner of gel to mark. Centre the gel onto the membrane, and make sure air bubbles are not trapped between the transfer membrane and the acrylamide gel ; note : air will show up as white spots on the membrane and can be removed by adding a few drops of cathode buffer to the gel. Ease out the bubbles with a gloved finger or use a stirring rod dipped in cathode buffer to gently roll the trapped air out.

h. Place 3 layers of filter paper soaked in the cathode buffer on top of the acrylamide gel.

i. Carefully place the lid over the 4 studs so that the top electrode (cathode) rests on the trans unit sandwich, slip a knob over each of the 4 studs and then alternately tighten each until a slight resistance is felt. Do not overtighten.

j. Attach the power cables to a Consort E741 power supply and set as follows: Switch on at mains and instrument; comes up TEST, OFF. Press SET to set parameters as follows : Time Down; End; constant voltage 20 volts; current 0.14 Amps ;0.5 (KVH); power 300W.

k. Connect leads to power supply and transfer system and press RUN. Electrophoresis will take place for 30 minutes at a current of 140 mA, with a constant voltage of 20V.

At the end of the run remove knobs and transfer gel to Coomassie blue stain for 1 hour to stain protein bands, and treat blot as below (wear gloves).

### **Immunoperoxidase staining of nitrocellulose blots.**

#### **Reagents**

a. Ponceau S concentrate (protein stain - Sigma Chem Co, UK). Dilute 1 in 10 with distilled water before use.

b. Tris Buffered Saline (TBS) : 10 mM Tris (1.21 g/l) and 140 mM NaCl (8.18 g/l) adjusted to pH 7.4 with 1M HCl.

c. TBS - Tween : TBS containing 0.1% (v/v) Tween 20.  
Reagents 1 and 2 can be prepared and stored as x10 concentrates.

d. 5% lo fat milk (Asda) in TBS - Tween. Prepare fresh on day and store at 4°C.

e. Goat anti human alpha-1-antitrypsin (PRU-Sheffield). Prepare just before use as a 1 in 100 dilution in 5% lo fat milk in TBS-Tween.

f. HRP labelled Anti-Sheep/Goat IgG (donkey) (SAPU code S084-201). Dilute 1 in 500 with TBS-Tween just prior to use.

g. Substrate solution : Dissolve 18 mg 4-chloro-1-naphthol in 6 ml methanol. Add 94 ml TBS and 0.025 ml 30% H<sub>2</sub>O<sub>2</sub>. Mix well and use immediately.

### **Procedure**

a. Wash blot in TBS-tween for 2 minutes and transfer to Ponceau-S working reagent for 5 minutes to highlight proteins. Decant reagent and measure mobility of marker proteins.

b. Wash the blot in TBS Tween for 10 minutes, before blocking in 5% lo fat milk in TBS Tween for 1 hour on rocker.

c. Decant solution and wash x6 with TBS-Tween (5 minutes per wash) on rocker.

d. Add 20 ml antibody solution and place on mixer for 1 hour.

e. Decant and wash x6 in TBS-Tween as before.

f. Add 20 ml HRP-labelled antibody solution and place on rocker for 1 hour.

g. Decant and wash in TBS-Tween x3, followed by TBS x3 as before.

h. Prepare substrate solution and use immediately. Place nitrocellulose membrane in substrate and agitate gently on rocker. Purple spots develop and darken over 30 minutes.

i. Stop the reaction by washing the blot in several changes of distilled water for a minimum of 10 minutes. Surface dry the nitrocellulose using filter paper and measure mobility of  $\alpha$ 1-AT bands. Photograph using reflected light. Store blots protected from light.

## 2. Faecal and WGLF $\beta$ -Galactosidase

### Reagents

1. p-nitrophenyl  $\beta$ -D-galacto-pyranoside (Sigma Chem Co, UK)
2. Phosphate Buffered Saline(PBS) pH 7.4 0.01M (Sigma Chem Co, UK)
3. 0.25M sodium carbonate (Merck,UK)

### Procedure

The method used was that of Sharfman et al (1975).

P-nitrophenyl-galactoside (0.1 ml of 1 mg/ml in PBS) is incubated with faecal supernatant or filtered/processed WGLF (0.05ml) at 37°C for 1 hour. The reaction is stopped by addition of 0.25M sodium carbonate (1 ml), and OD of reaction product, p-nitrophenol, read at 405 nM. Tests are read against faecal supernatant blanks where sodium carbonate is added at time zero.

Calculation of  $\beta$ -Galactosidase activity

Extinction coefficient of p-nitrophenol at 405 nM is  $18.8 \times 10^3$

Therefore a 1 umole/litre solution has an OD at 405 nM of 0.018 OD units, and 1 umole in final reaction volume, 1.15ml, has an OD of 15.7 OD units

To express results in umols/min/g protein or units/g protein

- a. divide by 60 to give OD/minute
- b. divide by protein concentration in mg/ml (g/l)
- c. multiply by 20 and 1000 since 0.05 ml sample is used and activity is per g protein
- d. Divide by 15.7 since 1 umol in the final reaction volume has an OD of 15.7 OD units.

This simplifies to change in OD/hour  $\times$  21.2/protein concentration in mg/ml.

For WGLF calculation simplifies to OD/hour  $\times$  21.2 u/litre.

### 3. Total protein

The assay used is the 'Pierce BCA Protein Assay' and is a highly sensitive method for the spectrophotometric determination of protein concentration. Bicinchoninic acid (BCA) in the form of its sodium salt is a sensitive, stable and highly specific reagent for the cuprous ion. Macromolecular structure and the four amino acids (cystine, cysteine, tryptophan and tyrosine) are responsible for the colour formation when assayed with BCA. The purple reaction product formed by the reaction of two molecules of BCA with one cuprous ion, in an alkaline solution is water soluble and exhibits a strong absorbance at 562 nM.

#### Reagents

- a. Reagent A: 1000 ml of base reagent which contains sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2N sodium hydroxide.
- b. Reagent B: 25ml of 4% copper sulphate solution
- c. Working Reagent: Mix sufficient reagents A and B in the ratio 50:1 just prior to use.

#### Procedure

- a. Prepare a set of protein standards of known concentration by diluting the albumin standard solution (2 mg/ml) provided with the kit in the same diluent as the unknown samples.
- b. Pipette 0.05 ml of each standard or unknown protein sample into the appropriately labelled test tube (3 ml polystyrene). For blanks use 0.05 ml isotonic saline.
- c. Add 2 ml working reagent to each tube and mix well.
- d. Incubate all tubes for 30 minutes at 37°C
- e. Cool all tubes to room temperature and measure absorbance at 562 nM against water reference.
- f. Prepare a standard curve by plotting net absorbance at 562 nM vs protein concentration. Using the standard curve determine the protein concentration for each unknown.

## CHAPTER 5

### MEASUREMENT OF HYALURONIC ACID IN WGLF IN GI DISEASE

#### Introduction

Connective tissues are rich in the glycosaminoglycans (GAGs): chondroitin sulphate, dermatan sulphate, heparin sulphate, keratan sulphate, and hyaluronic acid. These are heteropolymers of an aminosugar and a uronic acid or a sugar plus a small proportion of protein. These very large polyanions bind water and cations and form the ground substance of connective tissue along with collagen and elastin.

Hyaluronic acid (HA) is an unbranched polysaccharide built from repeating disaccharide units of D-glucuronic acid and N-acetyl-glucosamine linked by  $\beta(1-3)$  and  $\beta(1-4)$  glycosidic linkages respectively, and the major MW component is in the range of 400 kD - 1000 kD. Unlike the other GAGs, HA contains no sulphate and less protein.

The main sites of production of HA are the connective tissue and synovial membranes. HA forms an important component of the tissue ground substance where there is interaction with fibronectin, other GAGs and collagen, with the cytoskeleton and with specific cell surface binding proteins.

Most HA is degraded in lymph nodes but a small amount diffuses from the tissues and joint fluid to enter the circulation via the lymph and its concentration in blood is 10 - 100ug/ litre. Clearance from blood is rapid mainly as a result of uptake by receptors on hepatic endothelial cells where it is degraded; studies in man using labelled HA have shown a serum half life of 2.5 - 5.5 minutes.

Colombel et al (1989) and Lavo et al (1990) have reported increased jejunal secretion of HA in patients with IBD and in coeliac patients when challenged with gliadin, respectively. Concentrations of HA in health and disease were much higher than could

be explained by passive leakage from plasma. Farzad et al (1993) induced intestinal epithelial denudation in rats by reversible ischaemia, and showed an increased HA concentration in the jejunal perfusate with loss from the villous tips, and have suggested that increased denudation of epithelial cells due to inflammatory or toxic responses will result in increased release of HA from the exposed lamina propria to the intestinal lumen.

WGLF proteins such as IgG and albumin have been measured as indices of protein leakage from plasma as a result of inflammatory changes in the intestinal mucosa. HA has now been measured in WGLF in control subjects and in subjects with IBD and colon cancer as a possible direct measure of leakage from the lamina propria in small and large bowel disease.

### **1. Experiments investigating measurement of HA in WGLF**

HA was measured using a radioimmunoassay kit supplied by Pharmacia, Uppsala, Sweden (Chapter 5, appendix).

The calibration curve for HA is shown in Figure 5.1

#### **a. Comparison of processed and unprocessed WGLF samples for HA measurement**

Three samples from control subjects, and three samples from patients with IBD were measured for HA using a. unfiltered unprocessed samples b. filtered processed samples. See table 5.1

Results were significantly higher for all unprocessed, unfiltered samples compared with their respective samples processed with protease inhibitors. This may indicate a positive interference in the unfiltered, unprocessed samples or a quenching of HA measurement in processed samples.

### **b. Recovery of standard HA from WGLF**

0.04 ml 500ug/l HA standard was added to two patient samples of processed WGLF (0.1ml). Samples were then assayed for HA.

See Table 5.2

Quantitative recovery of HA from processed WGLF indicated no interference causing reduction in HA measurement in these samples.

### **c. Investigation of interfering compound using unfiltered unprocessed samples.**

A sample of WGLF was treated in four different ways in order to attempt to identify the source of the interference.

The sample was assayed a. unfiltered and unprocessed, b. unfiltered and processed c. filtered and processed and d. filtered through a 0.22u filter and unprocessed.

See Table 5.3

These results on this single sample indicate that removal of bacteria has no influence on the results, whilst processing with protease inhibitors significantly lowered the results.

### **d. Influence of time delay on filtering and processing samples**

A sample of WGLF was divided into three and filtered and processed at time zero and subsequently after +60 and +120 minutes at 37<sup>0</sup>C.

The HA concentrations for time 0, +60, and +120 minutes before processing were 58, 60 and 56 ug/l; it can be concluded that there is no increase or decrease in HA concentration caused by delay in protease inhibition.

### **e. Influence of pancreatic enzyme preparations on HA assay**

Two different pancreatic enzyme preparations were treated as WGLF and assayed before and after filtering and processing.

The enzymes used were a. chymotrypsin and b. collagenase and were obtained from Sigma Chem Co Ltd, UK. Both were used at a concentration of 0.5 mg/ml

Chymotrypsin gave a value for measured HA in excess of 500 ug/l before processing, whilst collagenase gave a value of 12 ug/l; processing of both samples led to a significant reduction in the HA in the chymotrypsin sample to 15 ug/l whilst the collagenase value remained the same.

It was concluded that chymotrypsin was interfering with the HA assay in the unprocessed samples. It may do this by partial proteolysis of the labelled HA binding protein making this less available for reaction with HA or with the HA-Sepharose binding reagent thus reducing the 'bound counts' and increasing the apparent concentration of HA in the sample.

## **2. WGLF HA in patients with active IBD and colon cancer**

HA was measured in 20 control subjects, 19 patients with active CD, seven patients with active UC, five patients with colon cancer, and in seven other patients with miscellaneous GI disease. Significant differences were determined using a Mann Whitney test for non parametric data.

WGLF HA was correlated with WGLF IgG for all subjects (n=58)

### **Results**

Results are shown in Figure 5.2a and Figure 5.2b.

Median (range) for the different groups was as follows: controls 24(4 - 608), active CD 159 (6 - 620), active UC 301(14 - 740), CRC 43 (19 - 123).

There was a significant difference between control subjects and patients with active CD ( $p < 0.001$ ) and active UC ( $p < 0.02$ ) but no significant difference between controls and patients with colon cancer. There was a weak but significant correlation between WGLF HA and WGLF IgG ( $r = 0.46$ ,  $p < 0.002$ ). However there was considerable scatter of points and some patients with high HA had a normal IgG and vice versa.

A single patient in the control group had a very high HA concentration (608 ug/l). The protease and chymotrypsin activity of this sample was found to be low as expected in processed samples, and so this could not explain the raised value. A subsequent sample taken on this patient gave an HA concentration of 36 ug/l.

## **Discussion**

In the small bowel HA can be observed in large quantity in the loose connective tissue of the villi and lamina propria of human jejunum using a histochemical procedure with biotin labelled protein binding to the HA and identification with avidin enzyme and substrate (Gerdin and Hallgren, 1991). It has been concluded that the accumulation of HA in the subepithelial layer of the jejunal mucosa indicated that the high jejunal secretion of HA in normal subjects is due to passive diffusion from the subepithelial interstitium.

HA has physicochemical properties promoting a high ability to bind water and create microenvironments where macromolecules are excluded (Laurent, 1987). A considerable proportion of HA (about 60%) can be readily mobilised from the interstitium by enhanced interstitial fluid flux (Reed et al, 1992). This is substantiated by the HA concentration in intestinal lymph, which is considerably higher than that found in lymph from other regions of the body (Tengblad et al, 1986). During intestinal absorption intestinal lymph flow increases as does lymphatic HA concentration probably as a result of release of HA from jejunal lamina propria. The decreased interstitial concentration of HA could be expected to have important

implications for interstitial exclusion of macromolecules as well as for transport of water and protein.

Colombel et al (1989) and Lavo et al (1990) have reported jejunal secretion of HA in both normal subjects and patients with IBD and coeliac disease. Increased jejunal secretion of HA was observed in the disease groups and this may be indicative of enhanced connective tissue response due to inflammation, reduced lymphatic drainage, or direct leakage through denuded epithelial cells.

In the present study the difficulties of measuring HA in the presence of proteolytic enzymes has once more been identified and a direct effect of chymotrypsin on the assay observed. This difficulty has previously been identified by Engstrom-Laurent et al (1985) who observed that other authors using proteolytic digests of serum had obtained unusually high values when measuring serum HA.

The increased WGLF HA of patients with active IBD is in agreement with previous jejunal perfusion studies.

Ahrenstedt et al (1992) in a study of patients with CD of the terminal ileum perfused the proximal jejunum and observed increased secretion of HA from 'uninvolved' jejunum compared with controls and patients with inactive disease. This is compatible with a subclinical alteration in the connective tissue of macroscopically unaffected mucosa in active CD. They suggest that a reasonable mechanism for the access of HA to the intestinal lumen is through the many denuded villous tips which are formed during the continuous turnover of the villous epithelium. This has also been suggested by Farzad et al (1993). Colombel et al (1992) have shown that elemental diets cause an increase in leakage of plasma proteins albumin, and IgG and also stimulate secretory immunity (IgA) but have no effect on the secretion of HA from the jejunum, which highlights the different mechanisms resulting in the appearance of these proteins.

This has further been observed with this study in WGLF where the relationship between plasma leakage (IgG) and interstitial leakage (HA) is only weakly significant. The reference values for HA in WGLF (median of 24 ug/l with an average perfusion rate of 20 ml per minute) would appear to be higher than those from the jejunal perfusion studies. Ahrenstedt et al (1992) obtained control values of mean (SD) of

42 (23) at a flow rate of 3 ml pr minute, and Colombel et al (1989) obtained control values of mean (SEM) of 12 (2) at a flow rate of 10 ml per minute

Preliminary values for patients with active UC show some high values and the normal colonic mucosa may contribute significantly to baseline WGLF HA values.

A single sample of human gastric aspirate gave a value of 150 ug/l. HA from sources within the GI tract other than the small and large bowel may influence the WGLF reference range. Alternatively the rate of flow of perfusate itself may influence HA loss.

**Table 5.1****WGLF HA before and after filtering and processing**

	HA pre ug/l	HA post ug/l
1 control 1	>500	8
2 control 2	350	9
3 control 3	350	14
4 IBD 1	>500	114
5 IBD 2	>500	180
6 IBD 3	>500	35

**Table 5.2****Recovery of HA from WGLF samples**

	WGLF HA ug/l	WGLF + added HA ug/l	Recovery
1.	116	240	112%
2.	42	163	92%

**Table 5.3****Processing procedure for WGLF prior to analysis**

Treatment	HA ug/l
a. unfiltered unprocessed	650
b. unfiltered processed	30
c. filtered processed	28
d. filtered 0.2u, unprocessed	636

HYALURONIC ACID  
CALIBRATION CURVE

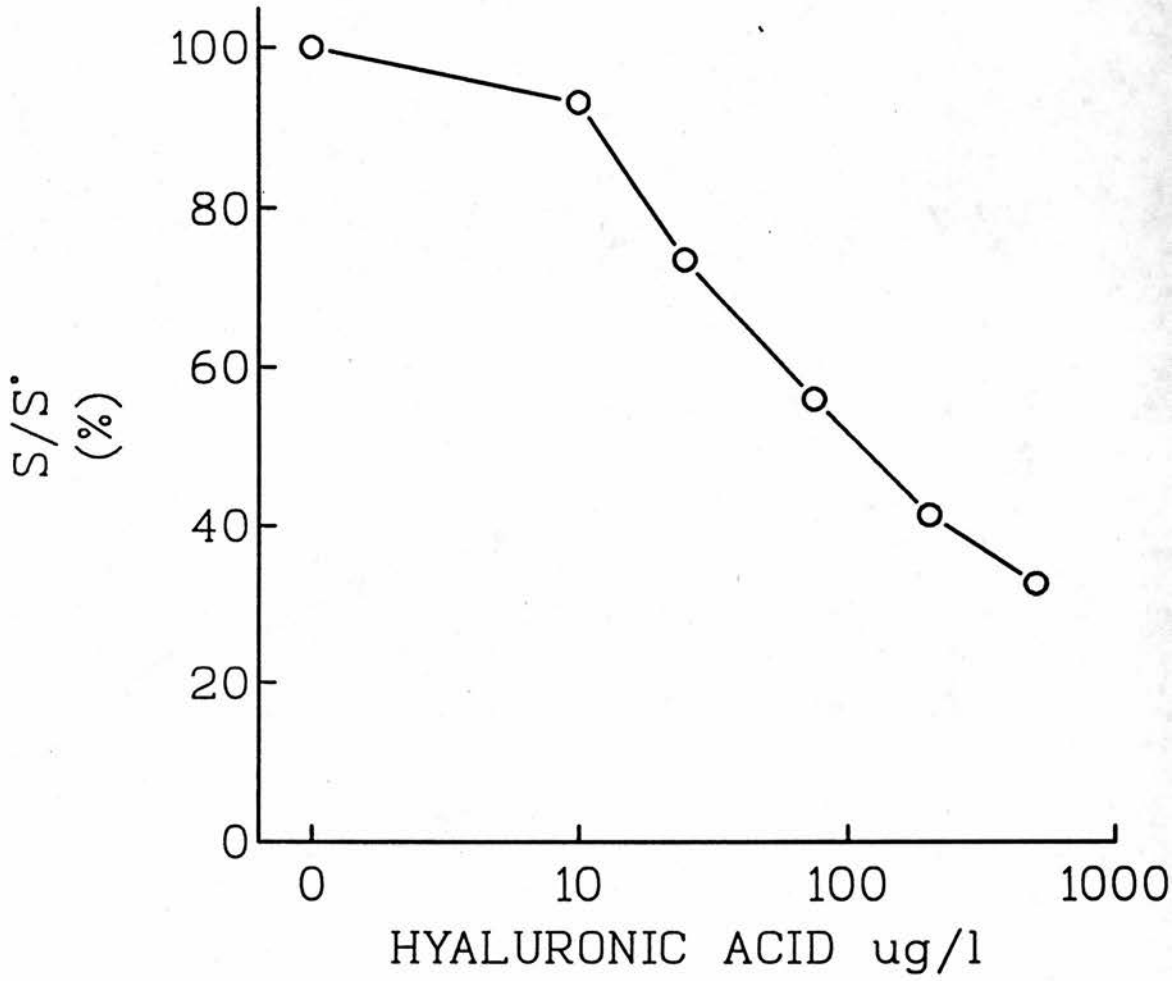


Figure 5.1

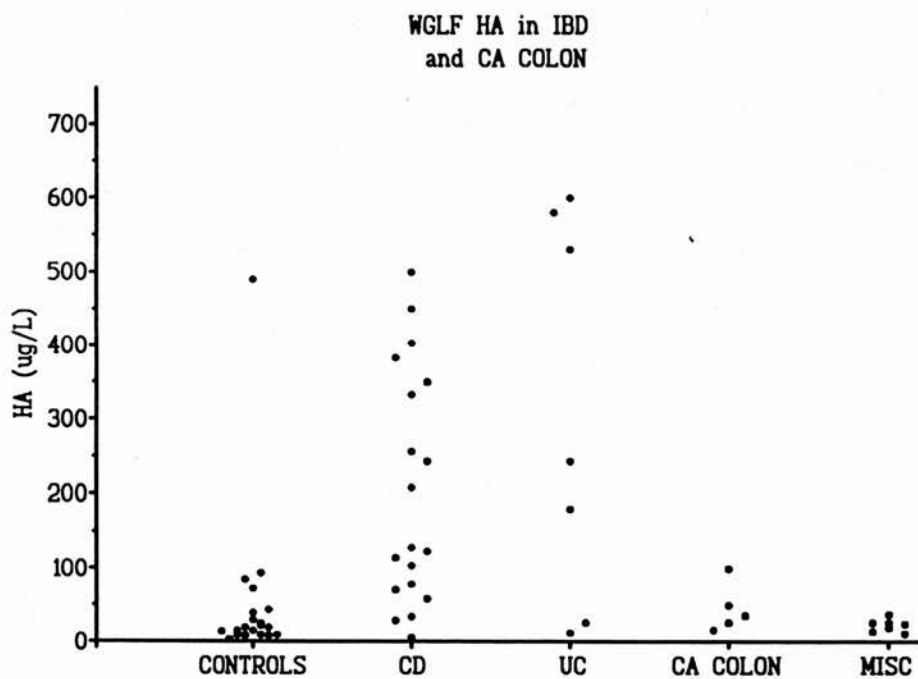


Figure 5.2a

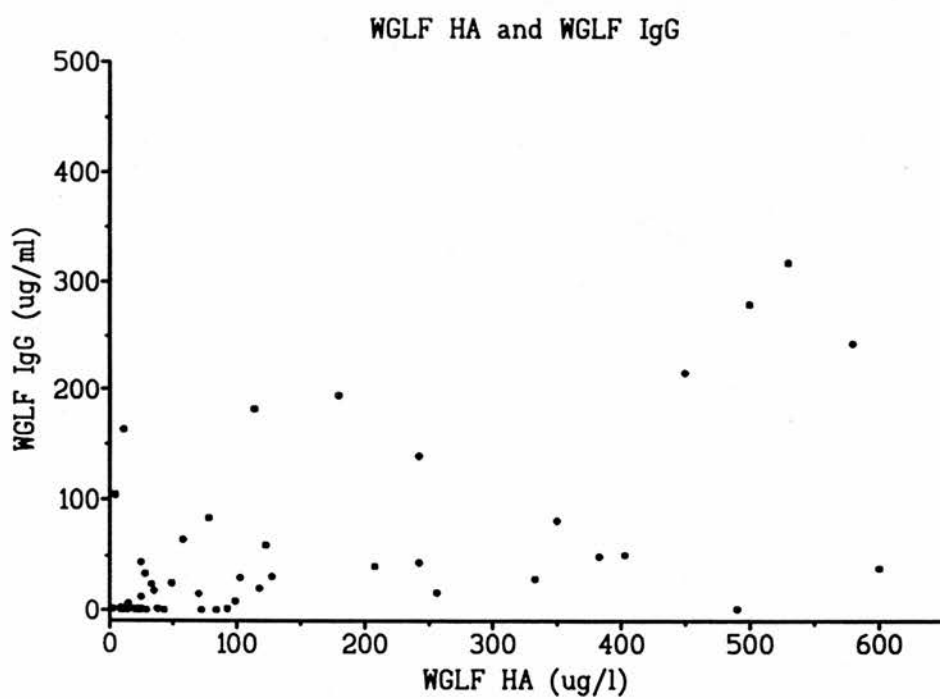


Figure 5.2b

## **APPENDIX: CHAPTER 5**

### **Measurement of WGLF Hyaluronic Acid**

#### **Principle of Test Procedure:**

This is a radiometric assay which is based on the use of specific hyaluronic acid binding proteins, isolated from bovine cartilage ( Pharmacia Diagnostics AB, Uppsala, Sweden). The hyaluronic acid in the patient sample reacts with HABP-<sup>125</sup>I in solution. The unbound HABP-<sup>125</sup>I is then quantified by incubating with HA covalently coupled to sepharose particles of small size and low density. Separation is performed by centrifugation followed by decanting. The radioactivity bound to the particles is measured in a gamma counter and the response is inversely proportional to the concentration of HA in the sample.

#### **Reagents:**

1. HA standards: 10, 25, 75, 200, and 500 ug/l. Store at 2 - 8°C.
2. Sample diluent and zero standard.
3. HABP-<sup>125</sup>I, 200 K bq: Dilute 1/30 in tracer diluent. 200ul HABP -<sup>125</sup>I in 6 ml tracer diluent. Prepare weekly.
4. Tracer diluent (blue) 15 ml : ready for use.
5. HA-Sepharose Ready for use; store at 2 - 8°C.
6. Decanting suspension 220 ml. Ready for use ;store at 2 - 8°C.

#### **Procedure:**

- a. Pipette 100 ul of standards, processed WGLF samples, and WGLF sample blank consisting of processed lavage solution in duplicate.
- b. Add 200 ul of HABP-<sup>125</sup>I solution.
- c. Incubate for at least 60 minutes at 8 -10°C
- d. Add 100 ul of HA-Sepharose. Resuspend thoroughly before use.
- e. Incubate for 45 minutes but not more than 60 minutes at 8 -10°C.
- f. Mix decanting suspension and add 2 ml to each tube.

g. Centrifuge for 10 minutes at 1500g. Decant the tubes immediately in one movement and let the tubes stand for 30 seconds inverted on absorbent paper. Tap the tubes x2 against the absorbent paper to get rid of residual drops. The precipitate is firmly packed and will not be lost even if the tubes are tapped vigorously.

h. measure the radioactivity in gamma counter.

**Results:**

a. Express the counts (B) for standards and unknowns as a percentage of the mean counts of the 0-standard ( $B_0$ )

% activity bound =  $B/B_0 \times 100$ .

b. Plot the percentage values of the standards against the HA concentration on a lin-log paper and construct a standard curve.

c. Read the concentration of the unknown samples from the standard curve.

d. Subtract the WGLF sample blank result from WGLF sample test results.

e. Results are multiplied by 1.24 to correct for dilution when processing samples.

**Interference:**

There is no measureable interference of chondroitin sulphate at concentrations <100mg/l, keratan sulphate at concentrations <50mg/l or fibronectin at concentrations <500mg/l.

## CHAPTER 6

### CONCLUSIONS

#### **An appraisal of the whole gut lavage procedure for diagnosis of blood and protein loss from the gut**

The WGL procedure which is routinely carried out as a bowel preparation prior to clinical investigations has provided a novel opportunity for investigating blood and protein loss from the gut. My work has shown that this type of test is highly relevant to patient management and to further research in clinical gastroenterology. The procedure has many advantages when compared with the more traditional investigative methods such as those involving faecal collections, but there are also some disadvantages.

The WGL procedure involves the administration of an isotonic solution which is not absorbed from the gut, is safe and well tolerated by patients, clears the gut of faecal debris and leads to the production of a clear fluid per rectum within three to four hours. The procedure is non-invasive and only involves passing tubes in a minority of patients who have a low tolerance for the oral lavage procedure. There are no substrates in the lavage solution which may be metabolised to explosive gases, (when mannitol solution is used as a bowel preparation solution it is fermented and produces hydrogen), so subsequent patient investigations such as diathermy are completely safe. In contrast as the WGL procedure is commonly used prior to colonic examination, collection, processing and analysis of the resultant fluid does not require further patient involvement or inconvenience. The fluid can be processed, for example, as serum and urine are processed; thus, filtration, centrifugation, or direct addition of preservatives into solution are all easily accomplished; these procedures are much less easily done with faecal specimens, which cannot be immediately dealt with in these ways.

After adequate treatment with protease inhibitors WGL fluid can yield valuable information in both healthy and diseased subjects by measurement of endogenous

secretions present in the WGL solution. Since the whole gut is perfused during the procedure, inflammatory lesions in areas which are difficult to investigate by direct access such as the ileum yield products that can be detected.

It has been confirmed by sequential analysis of WGL samples for different proteins that there is a steady state perfusion once the clear fluid is obtained. This however does require monitoring of the patient by trained nursing staff to maintain the correct fluid intake. Measurements of fluid intake rate and concentration of analyte in WGLF can allow quantitative determination of the daily output and additional knowledge of the blood or serum concentration of the substance permits calculation of blood or serum clearance of the analyte. This eliminates the need for 24 hour faecal collections. Quantitative faecal measurements are not easily made, because the discipline required of the patient and staff and the necessary facilities of an investigative metabolic unit are not generally forthcoming or available.

Often in studies where pathological faecal samples are compared with controls, the pathological samples may be of a different form (diarrhoea) from the controls and this itself may introduce a physiological difference as highlighted by Strygler et al (1990). Since a standard WGL procedure is used for controls and patients any such sample differences are eliminated when WGLF samples are studied.

A further advantage of WGLF compared with faeces in investigative work is the elimination of dietary interference by substances that may give false readings. For example a dietary component such as red meat can make a significant contribution to the measurement of faecal haemoglobin, thus reducing the specificity and sensitivity of the assay.

Some physicians perceive the WGL procedure as being very invasive and impractical; however it is well tolerated, and has been successfully used in a wide range of subjects from children to the elderly; it is much less invasive than tests that might give equivalent information but which involve passing tubes; the patient has more control and in this sense the procedure is much less disturbing.

The acknowledged disadvantages and potential disadvantages of the WGL procedure merit consideration:

1. The WGL procedure induces an osmotic diarrhoea, a non physiological state which may lead to altered gut losses compared with normal.
2. Since the whole gut is perfused, pathological areas are not specifically located as in closed loop studies. Accordingly it is not possible to link protein measured in WGLF to intestinal cell or tissue type and so such investigations require to be combined with studies of biopsies or imaging ; however WGL here provides an extra dimension similar to the sugar permeability tests which give diagnostic information complementary to that of jejunal biopsy in patients with malabsorption.
3. Some proteins such as albumin are labile and are still degraded to some extent during the lavage procedure, despite the prompt addition of protease inhibitors when the fluid is collected. The amount of degradation will depend on the site of protein secretion and activity of the patient's proteases, with more proximal protein secretion being more affected.
4. Patients on average pass samples every 10-20 minutes once clear lavage fluid is being produced per rectum and so gut losses are measured only during this time period; ie only continuous loss of protein or blood is measured by this procedure; occasionally patients with active UC have been observed to be passing fresh blood per rectum, have subsequently undergone the WGL procedure, and have been found to have a normal WGL haemoglobin. This highlights the difference between continuous ooze and acute intermittent bleeding which may stop completely between episodes. Little is known about the intermittency of protein loss although reassuringly, sequential samples show little variation.

### **Blood Loss in WGLF in GI disease**

WGLF haemoglobin concentration is not subject to the variations of faecal haemoglobin concentration caused by natural and pathological variation of stool weight and diet differences and has been shown to be a more sensitive index of blood loss. A condition known to be associated with GI bleeding was present in more than 97% of patients in whom WGLF Hb was above the reference range.

Three groups of patients were studied in more detail with regard to GI blood loss.

a. Patients with inflammatory bowel disease:

Anaemia in these patients was unrelated to GI blood loss, and GI blood loss was not a consistent feature of active inflammation. There was a weak relationship between disease activity as measured by WGLF IgG and GI blood loss, but only in patients with active UC was there a relationship between fall in blood haemoglobin and WGLF IgG. Other factors such as duration of illness, malabsorption and poor diet may contribute to anaemia in these patients.

b. Patients with unexplained Fe deficiency anaemia:

Most studies in the past have identified GI lesions which are assumed to bleed and be the cause of Fe deficiency anaemia; this has usually not been confirmed by blood loss measurement. In the present study there were 73 clinical, dietary, or iatrogenic factors recorded in 42 iron-deficient patients all of which could contribute to Fe deficiency anaemia. However the WGLF haemoglobin test detected only eight patients who were losing more than two ml of blood per day, including all six patients with lesions generally recognised to be important causes of occult bleeding, - four with colonic cancer, one with diffuse gastric vascular ectasia, and one with severe ulcerative oesophagitis. Although the WGLF haemoglobin measurement may miss intermittent bleeding, it seems likely that in many instances, GI lesions detected in Fe deficiency patients by standard investigations are coincidental and are not bleeding. In these patients iron deficiency is likely to be due to poor diet, malabsorption, chronic blood loss from a site other than the gut, or a combination of these.

c. Patients with colon cancer:

Occult tests for GI blood loss have mainly been used in the detection of colon cancer. However it is now generally accepted that a substantial percentage of patients with colon cancer are not detected with 'Haemoccult screening' either for technical reasons and lack of sensitivity of the test, or because the tumours are not bleeding. In my series, 19 of 20 patients with colon cancer had elevated WGLF Hb concentrations. I am now extending this work by studying a much larger series.

Practical consequences of this test of occult blood loss have arisen in at least two situations 1. patients may have raised WGLF Hb levels with no apparent disease; in my series there were two cases with raised WGLF haemoglobin had reportedly normal barium enemas but were each subsequently shown to have a cancer at colonoscopy (carried out because of the positive WGLF Hb result).

2. As discussed at (b) above where patients with Fe deficiency have normal WGLF Hb levels, deficient diet and malabsorption should be considered as the cause and treated appropriately.

### **Protein Loss in GI disease**

In healthy control subjects the mean clearances of IgG and  $\alpha$ 1-AT were four and 38 ml per day respectively; albumin is affected by proteolysis but in patients with cystic fibrosis who are deficient in pancreatic enzymes, there was a mean clearance of 14 ml per day (Croft et al,1995). This might indicate that clearance in controls may relate directly to MW size:  $\alpha$ 1-AT (54 kD), albumin (66kD), and IgG (166kD).

Alternatively the higher clearance of the smaller MW  $\alpha$ 1-AT in controls and patients with IBD may be influenced by osmotic diarrhoea ( Strygler et al 1990). The osmotic diarrhoea may lead to greater release of  $\alpha$ 1-AT which has been shown to be present in epithelial cells in both normal and diseased subjects.

IgG determination in WGLF shows a strong relationship with CDAI in active IBD. Raised IgG and albumin levels in WGLF were seen in active but not inactive IBD.  $\alpha$ 1-AT was raised in WGLF from some patients with inactive as well as active IBD. This may be due to the permeability of smaller MW  $\alpha$ 1-AT in damaged but not inflamed tissue as has been shown in other studies where  $\alpha$ 1-AT was raised in inactive as well as active IBD.

Tissue inflammation may be required to allow significant leakage of the higher MW IgG and then in states of inflammation, clearances may become independent of molecular weight. The low rate of leakage for IgG in controls may enhance the sensitivity of WGLF determinations of IgG as an index of active IBD when hyperaemia and inflammation result in leakage from plasma. It is worth noting that

patients with abnormal X-ray findings may have no current inflammation; on the other hand, patients with normal X-ray findings can be very ill despite having no strictures or fistulae but still having active microscopic inflammation which cannot be readily seen on X-Ray. These considerations indicate the importance of tests such as WGLF IgG and other proteins and mediators, that provide objective indices of IBD activity; accordingly our group is continuing to extend this work.

Determinations of WGLF proteins can not be used to make a diagnosis of IBD as these are merely indices of protein exudation into the gut which occurs in other diseases including intestinal neoplasia. Similarly normal values of WGLF proteins do not exclude IBD as these are normal in most patients in the quiescent phase of their illness.

Patients with lymphangiectasia ( only two seen to date) appear to lose more  $\alpha$ 1-AT relative to IgG and albumin, which indicates a different mechanism than that seen with inflammation with  $\alpha$ 1-AT being preferentially lost through dilated lymph vessels. Studies of hyaluronic acid (HA) which is thought to be secreted into the gut transepithelially, have demonstrated only a weak relationship between WGLF IgG and WGLF HA concentrations; this suggests a different mechanism for loss of these proteins, IgG mainly being derived from plasma and HA directly from exposed sub-mucosal tissue.

The selectivity of protein transport into the gut may have different mechanisms or combinations of mechanisms for different types of mucosal inflammation and disease and this whole topic requires further close scrutiny.

It has previously been shown using faeces that  $\alpha$ 1-AT appears in different MW sizes in patients with CD. I have been able to assess this point in much detail, using WGLF. In some patients with active CD the 51 kD form was present and this appeared to relate to deficient levels of  $\beta$ -galactosidase. In contrast no patients with active UC studied had 51 kD  $\alpha$ 1-AT in faeces. Reduced deglycosylation of proteins in patients with CD may influence response to treatment of this group of patients with certain drugs which require to be metabolised into active forms by bacterial enzymes prior to being therapeutic. In addition changes in the metabolism of commensal bacteria as

indicated by reduction in protein deglycosylation may contribute to either exacerbations of, or predispose to, CD.

### **Current perspective on the use of whole gut lavage in diagnosis and research**

Initially the WGL procedure was set up in our department to measure intestinal antibodies, and it was observed incidentally that there was protein loss of albumin and IgG in IBD. Subsequently this has been further explored and the phenomenon is now used by clinicians as a diagnostic test to monitor protein loss in IBD. The procedure was then exploited further to measure blood loss in IBD and to elucidate other conditions notably unexplained Fe deficiency anaemia and colon cancer. More recently, assays for granulocyte elastase ( a measure of neutrophil migration into the gut), growth factors, and cytokines have been set up and used in attempts to gain further insight into disease mechanisms in IBD. Combinations of these tests have been used to investigate colon damage in patients with cystic fibrosis and after radiotherapy.

A clinical trial of a new steroid used in the treatment of CD is underway and this will be supported by WGLF studies along lines developed to monitor IBD. We are also developing protocols for studies of intestinal inflammation in patients with tropical diarrhoeas.

There is a need to separate protein 'loss' into (a) protein delivered physiologically into the gut; (b) protein leaked into the gut; and (c) protein elaborated in the gut. This may require investigation of immunoglobulin subclasses (eg of IgG) which are sometimes present in very low concentrations.

There are many technical problems that still confront us. WGLF contains a large concentration of PE 3350 (59g/l) and so it is difficult to concentrate further when small concentrations of analytes are to be measured. A research colleague within the laboratory has investigated various approaches to reduce or eliminate this problem. Different physical methods have been used to eliminate PEG from the solution prior to further analysis of proteins. Ammonium sulphate precipitation, dialysis, gel filtration, and ion exchange chromatography have been used to separate PEG from

proteins, with varying degrees of success. Both anion exchange chromatography and ammonium sulphate precipitation separated PEG from proteins. Affinity chromatography, using media to selectively extract proteins, has also been used to look at specific groups of proteins eg using thiophilic (T) gels to extract immunoglobulins from WGLF. Now, methods are being explored to examine degrees of degradation of immunoglobulins using SDS/PAGE/ Western blotting techniques and ELISAs for total and specific Ig antibodies.

Exfoliated cells present in the fluid are currently being harvested for DNA extraction in patients with colon cancer and their family members ; recruitment of patients for this molecular study will provide abundant material for my investigation of patients with polyps and colon cancer and the issue as to whether polyps/cancers bleed or not can be further investigated ; also other proteins lost in association with blood loss eg  $\alpha$ 1-AT, or more specific proteins secreted by polyps or carcinomas can also be measured.

The use of WGLF has added a new dimension to research into gut pathology. We feel that it merits continuing exploitation and that it promises to yield a rich harvest of material for the further elucidation of intestinal disease mechanisms and for the clinical diagnosis and management of patients.

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**APPENDIX**

**PUBLISHED PAPERS**

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# Relative specificity for active inflammatory bowel disease of plasma-derived proteins in gut lavage fluid

W. Gordon Brydon, Chintamaneni P. Choudari and Anne Ferguson

**Objective:** Whole gut lavage with isotonic non-absorbable fluid is essentially a whole gut perfusion; whole gut lavage fluid (WGLF) can be used for clinical evaluation of intestinal immunity. High concentrations of WGLF immunoglobulin (Ig)G, albumin and  $\alpha$ -1-antitrypsin (A1AT; probably reflecting plasma leakage) are characteristics of active inflammatory bowel disease. We aimed to establish reference values for concentrations of these proteins in WGLF and to assess the extent of high concentrations in conditions other than active inflammatory bowel disease.

**Methods:** Gut lavage was performed on 63 immunologically normal patients or volunteers and on 254 occasions in patients with gastrointestinal symptoms or disease. After filtration and addition of protease inhibitors, WGLF content of IgG was assayed by enzyme-linked immunosorbent assay; albumin and A1AT by immunoturbidimetry.

**Results:** Normal ranges were established: IgG <1–10  $\mu$ g/ml; albumin <1–26  $\mu$ g/ml and A1AT <1–19  $\mu$ g/ml WGLF. In 102 tests on patients with inflammatory bowel disease of varying activity, high concentrations of WGLF IgG were present in 64%, of albumin in 52% and of A1AT in 36%. High concentrations of one or more proteins were present in 15 out of 152 patients with other conditions. In the majority, the existence of gastrointestinal protein loss was consistent with the clinical picture (one lymphangiectasia, seven colorectal cancers, one gut lymphoma, one perforated diverticulitis, one pouchitis and one lymphocytic colitis).

**Conclusion:** Studies of gut lavage fluid proteins provide a new approach to screening of clinically complex patients with inflammatory bowel disease and other ulcerating and inflammatory lesions. This simple technique offers an alternative to faecal isotope excretion in the diagnosis of protein-losing enteropathy.

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**Keywords:** Ulcerative colitis, Crohn's disease, gastrointestinal protein loss, measure of disease activity.

## Introduction

Whole gut lavage is a relatively new technique for cleansing the bowel prior to barium enema or colonoscopy. Whole gut lavage fluid (WGLF), essentially a gut perfusate, is an excellent source of material for clinical investigation and research concerning intestinal immunity and inflammation [1–6]. By using appropriate immunoassay and biochemical techniques, WGLF can be used to investigate intestinal immunoglobulin and antibody production, locally produced immunoregulatory cytokines and occult gastrointestinal bleeding.

We have also observed that patients with active inflammatory bowel disease have high concentrations of WGLF immunoglobulin (Ig)G, albumin and  $\alpha$ -1-antitrypsin (A1AT) and in a prospective study, we found that WGLF protein concentrations correlated well with various clinical indices of inflammatory bowel disease

activity [2,7,8]. It seems likely that a test based on gut lavage fluid (probably measuring plasma leakage across inflamed mucosa) would have many applications as a safe, inexpensive and objective measure of inflammatory bowel disease activity.

With a view to the introduction of this new test into clinical practice, we have examined WGLF from a series of patients to determine reference values for WGLF protein concentrations and to establish whether abnormally high values are found in diseases other than inflammatory bowel disease.

## Methods

### Patients and volunteers

Gut lavage was performed on 317 occasions. The reasons were bowel preparation for barium enema,

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colonoscopy or colorectal surgery; a treatment for intractable constipation, and in volunteer members of staff and patients as part of a research project on secretory antibodies to food proteins.

Patients and volunteers drank 4 litres of isotonic non-absorbable polyethylene glycol-based lavage fluid (Golytely) at a rate of 250 ml every 10–15 min. Within 2 h, solid then liquid faeces were passed followed by several large volume, virtually clear fluid 'stools'. An aliquot of the first completely clear specimen was collected for analysis.

### Processing of whole gut lavage fluid

In the first phase of our work, specimens were processed as described by Gasparly *et al.* [1]. An aliquot of 200 ml lavage fluid was filtered through gauze into 50 ml polypropylene tubes. Specimens were centrifuged twice and treated with a series of reagents to inhibit proteolysis, and with calf serum which provides an alternative substrate for any remaining proteases. Further experiments showed that the fluids could be clarified by filtration as efficiently as by centrifugation and a simpler method for specimen preparation has been developed.

The fluid (20 ml) was filtered through GF/A (Whatman, Maidstone, UK) glass fibre filters. The following reagents were added to 10 ml of the filtered fluid (final concentrations in brackets): protease inhibitor which comprised soya bean trypsin inhibitor in PBS (80 µg/ml), sodium ethylene diamine tetracetic acid in PBS (15 mmol/l), phenyl methyl sulphonyl fluoride in 95% ethanol (2 mmol/l), preservative: sodium azide (1 mmol/l), and newborn calf serum (5% v/v). Aliquots of processed WGLF were then stored at -70°C for later analyses.

### Analytical methods

Sample analysis for IgG was by enzyme-linked immunosorbent assay (ELISA) [2] using affinity-purified goat anti-human IgG (North East Laboratories) and human reference serum from the Protein Reference Unit (PRU), Royal Hallamshire Hospital, Sheffield, UK. The between run coefficient of variation of the assay was 15.5%.

The technique we used previously for albumin detection had a lower limit of 18 µg/ml. Therefore, we developed a more sensitive, immunoturbidimetric procedure for assaying albumin; a similar method was used for A1AT. Anti-human albumin was supplied by the Scottish Antibody Production Unit and anti-human A1AT was obtained from the PRU. WGLF was diluted by polyethylene glycol (PEG) 6000 reagent in tris buffer (pH 7) with and without antibody. It was then incubated for 15 min and the turbidity was measured at 340 nmol/l in a flow-through spectrophotometer (PU8610, Philips, Cambridge, UK). Standard plots of albumin and A1AT values over 0–200 µg/ml were prepared from Human Reference Serum which

had been diluted in 0.9% NaCl containing 48 g/l PEG 3350. Between run coefficients of variation for these assays were 11% for albumin and 6% for A1AT.

## Results

### Technical aspects

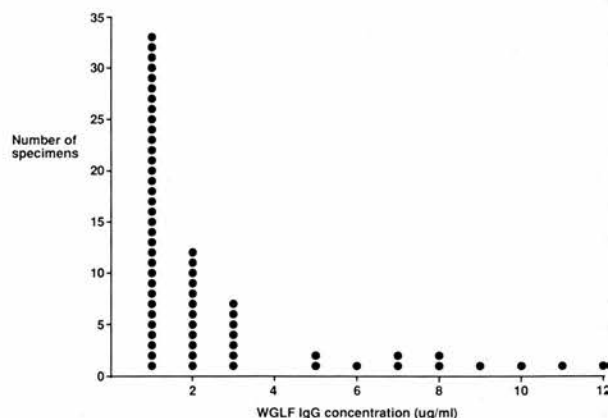
The data presented below relate to 70 specimens processed by the centrifugation method and 246 by filtration. Experiments in a separate group of WGLF specimens, with duplicates processed both by centrifugation and filtration techniques, showed no significant differences in residual protease activity (n = 10), immunoglobulin concentrations (n = 11) or in the concentration of a representative secretory antibody, IgA anti-gliadin antibody (n = 8).

### Normal values for whole gut lavage fluid proteins

Gut lavage was carried out in 63 individuals whom we classified as 'normal'. There were 20 men (age range 15–80 years, mean 44.1 years) and 43 women (age range 24–88 years, mean 51.4 years).

There were seven healthy volunteers, 19 patients with simple constipation, 11 with small benign colonic polyps, 18 who had had minor gastrointestinal symptoms in whom no abnormality had been found and eight patients with minor, non-inflammatory gastrointestinal conditions. All were on a normal diet and none was being treated with immunosuppressive or non-steroidal anti-inflammatory drugs.

Values for WGLF IgG, albumin and A1AT had a skewed distribution (Figs 1–3) which was not normalized by using transformed data. Since only the upper limit of normal is relevant in relation to a diagnosis of active inflammatory bowel disease, we have taken the upper and lower values which include 95% of the results [9] as the working normal ranges: <1–10 µg/ml for WGLF IgG, <1–26 µg/ml for albumin and <1–19 µg/ml for A1AT.



**Fig. 1.** Concentration of immunoglobulin (Ig) G in whole gut lavage fluid (WGLF) from 63 immunologically normal patients and volunteers.

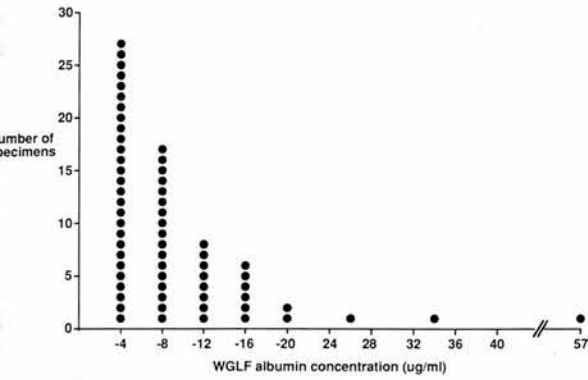


Fig. 2. Concentrations of albumin in whole gut lavage fluid (WGLF) from 63 immunologically normal patients and volunteers.

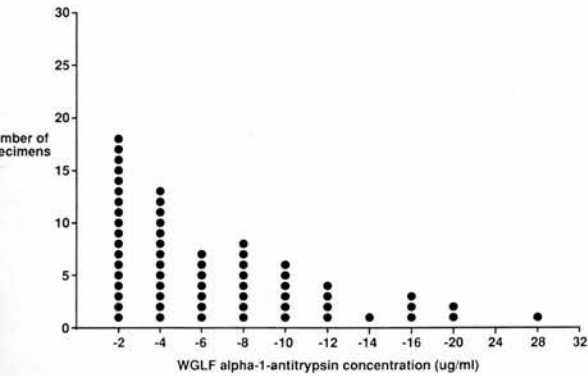


Fig. 3. Concentrations of  $\alpha$ -1-antitrypsin in whole gut lavage fluid (WGLF) from 63 immunologically normal patients and volunteers.

**Whole gut lavage fluid proteins in a further series of immunologically normal subjects**

Once these reference ranges were established, gut lavage has been performed in a further 44 patients considered to be immunologically normal with diagnoses of constipation, benign polyp or with minor gastrointestinal symptoms. In all cases, values for WGLF proteins were within the reference ranges stated above.

**Whole gut lavage fluid proteins in patients with inflammatory bowel disease**

Lavages were performed in patients with inflammatory bowel disease (71 with Crohn's disease and 31 with ulcerative colitis), heterogeneous with respect to disease distribution, activity and treatment. Abnormally high concentrations of WGLF IgG were present in 64% of these (44 with Crohn's disease and 21 ulcerative colitis), of albumin in 52% (35 with Crohn's disease and 19 ulcerative colitis) and of A1AT in 36% (28 with Crohn's disease and nine with ulcerative colitis; Figs 4-6).

**Whole gut lavage fluid proteins in patients with colorectal cancer**

Part from inflammatory bowel disease, colorectal cancer was the only diagnostic category for which a substantial number of patients had abnormal results. Data were available for 10 patients and one or more abnormal result was obtained in seven. Concentration

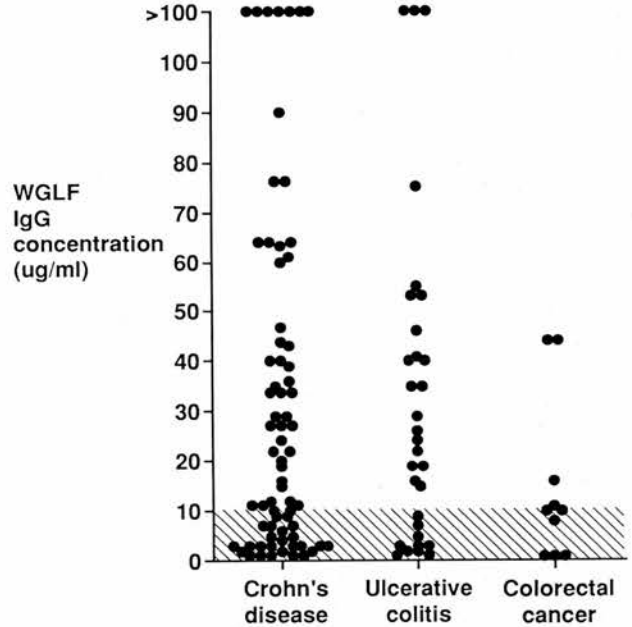


Fig. 4. Concentrations of immunoglobulin (Ig) G in whole gut lavage fluid (WGLF) from patients with Crohn's disease, ulcerative colitis or colorectal cancer.

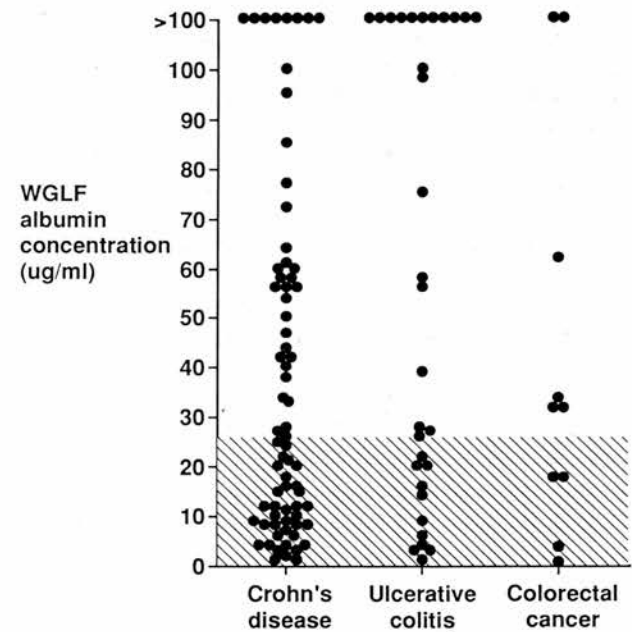


Fig. 5. Concentrations of albumin in whole gut lavage fluid (WGLF) from patients with Crohn's disease, ulcerative colitis or colorectal cancer.

of WGLF IgG was high in four cases, albumin high in six and A1AT high in two (Figs 4-6).

**Whole gut lavage fluid proteins in other diseases**

Details of the diagnoses and WGLF results in 98 patients with other conditions are given in Table 1.

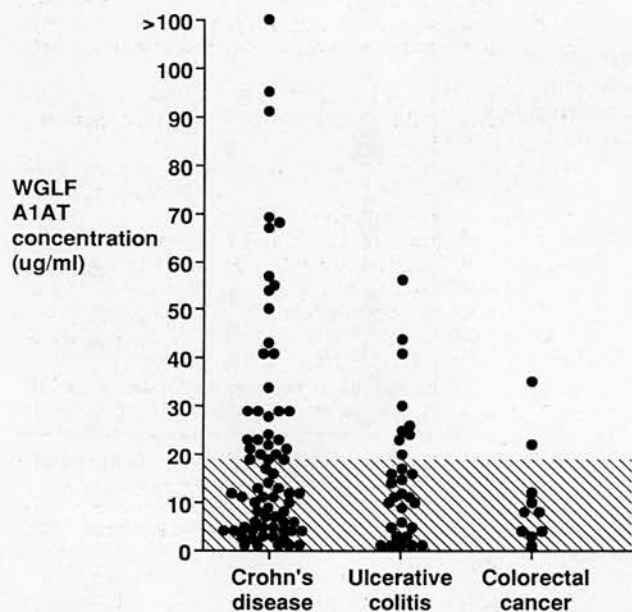
**Small bowel disease**

Only four out of 29 patients with small bowel disease had a high value for one or more of the WGLF proteins studied. Abnormal results were found in two

**Table 1.** Proteins in whole gut lavage fluid (WGLF).

	No. of lavages performed	No. of patients with normal WGLF proteins	Abnormal WGLF proteins ( $\mu\text{g/ml}$ )		
			IgG	Albumin	A1AT
Small bowel disease					
coeliac disease	4	4			
bacterial overgrowth	4	4			
ileal resection	3	3			
lymphangiectasia	1	—	14	36	195
giardiasis	1	1	1	3	21
idiopathic diarrhoea	15	13	2	5	31
enteropathy-associated T cell lymphoma	1	—	41	70	19
Other benign colorectal disease					
colonic diverticulosis (uncomplicated)	10	10			
perforated diverticulitis	1	—	85	18	10
eosinophilic colitis	1	1			
microscopic colitis	1	1			
ischaemic colitis	1	1			
collagenous colitis	1	1			
lymphocytic colitis and common variable	1	—	18	68	29
immuno-deficiency radiation proctitis (treated)	3	3			
solitary rectal ulcer	2	2			
rectal prolapse	1	1			
pelvic ileal reservoir	4	4			
pelvic ileal reservoir with pouchitis	1	—	120	34	27
Other significant disease					
gastrointestinal	27	27			
systemic	15	14	18	48	1*

Ig, immunoglobulin; A1AT,  $\alpha$ -1-antitrypsin. \*Lung cancer with large intra-abdominal metastases.



**Fig. 6.** Concentrations of  $\alpha$ -1-antitrypsin (A1AT) in whole gut lavage fluid (WGLF) from patients with Crohn's disease, ulcerative colitis or colorectal cancer.

patients; a man with lymphangiectasia and protein-losing enteropathy and a coeliac man with enteropathy-associated T cell lymphoma, which was extensively ulcerated with local perforation and an abscess cavity communicating with the gut. Two out of eight patients with idiopathic diarrhoea (i.e. severe watery diarrhoea

with no diagnosis reached after investigation had high values for A1AT (21 and 31  $\mu\text{g/ml}$ , respectively),

### Colorectal disease

Three out of 27 patients with various non-malignant colorectal diseases had high values for WGLF protein concentration. These were a young man with immunodeficiency, several auto-immune diseases and lymphocytic colitis whose diarrhoea is steroid-responsive; a patient with pouchitis, and an 81-year-old woman with severe diverticulitis, local perforation, peridiverticular abscess and a colovesical fistula.

### Other diseases

WGLF protein concentrations were normal in 41 of 42 patients with various other gastrointestinal and systemic disorders; the exception was a woman with lung cancer and widespread abdominal metastases.

### Discussion

Our finding of increased concentrations of IgG and WGLF from patients with Crohn's disease was a chance as we were merely evaluating the use of ELISA techniques for immunoglobulin and antibody measurements which had originally been developed for saliva [2]. Since then, we have conducted a prospective study of WGLF protein concentrations in 53 inflammatory bowel disease patients and found a remarkable

close correlation between the concentration of WGLF proteins, particularly IgG and albumin, and various general measures of activity in inflammatory bowel disease [8]. This study also showed that WGLF protein measurements cannot be used to screen for the presence or absence of inflammatory bowel disease. Normal results were obtained in several patients with active disease but who still had striking radiological pathological changes such as ulceration and strictures.

In this series, when cases judged to be immunologically normal are excluded, there were 98 examinations in patients without inflammatory bowel disease. Of these, a positive result was obtained for one or more of the assays performed. In the majority of cases, and in all those with multiple abnormal results, the existence of gastrointestinal protein loss was keeping with the clinical picture; one patient with lymphangiectasia, seven with malignant colorectal tumours, two with intra-abdominal abscesses communicating with the gut, one with pouchitis and one with usual lymphocytic colitis. Plasma leakage detected by WGLF protein assays (presumably gastrointestinal protein loss) clearly can occur in a range of clinical situations although the majority of positive results are in inflammatory bowel disease patients.

'Disease activity' in inflammatory bowel disease is a rather nebulous entity but well recognized by clinicians in their overall clinical assessments which rely heavily on subjective factors such as general well-being and presence or absence of abdominal pain [9,11]. Objective tests such as erythrocyte sedimentation rate, platelet count and blood concentrations of acute phase proteins provide supportive evidence in some patients, and the present clinical and laboratory techniques of assessment of inflammatory bowel disease activity are satisfactory in 80 to 90% of patients. However, we suggest that a direct approach to the measurement of active intestinal inflammation will be useful from time to time in inflammatory bowel disease patients. For example, there are patients with Crohn's disease in whom blood parameters of inflammation are negative yet active disease is suspected. This is often the case in patients with duodenojejunal disease with recurrent small bowel disease after proctocolectomy. Our tests may also allow an assessment of the relative contribution of primary inflammatory bowel disease activity to overall disability in patients who also have pyogenic complications such as wound or urinary tract infections, or inflammatory diseases of other systems such as ankylosing spondylitis.

Gut lavage is now widely used for bowel preparation prior to endoscopy, radiology and surgery, WGLF or laboratory analysis can often be obtained in the course of a standard clinical workup. Lavage fluid, collected as described above, is aesthetically acceptable to laboratory staff, resembling urine rather than faeces

and specimens can be stored at  $-70^{\circ}\text{C}$  for several months without deterioration. We suggest that this new and direct approach to clinical investigation of gut immunity will prove useful in many clinical and research situations, including the analysis of illness and of response to treatment in some complex inflammatory bowel disease patients. We have had the opportunity to study a single patient with an established disease of chronic protein-losing enteropathy, lymphangiectasia, but our experience suggests that protein assays in WGLF could replace the tedious isotope-based techniques traditionally used to detect and monitor this condition.

## Acknowledgements

We thank the nursing staff of the Clinical Investigation Area for their work in specimen collection, and Mrs Jackie Johnstone and Mr Norman Anderson for technical assistance. This work has been supported by grants from the Scottish Hospitals Endowments Research Trust (SHERT), the National Association for Colitis and Crohn's disease (NACC).

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# Gut Lavage Fluid Protein Concentrations: Objective Measures of Disease Activity in Inflammatory Bowel Disease

CHINTAMANENI P. CHOUDARI, SEAMUS O'MAHONY, GORDON BRYDON, OBEDY MWANTEMBE, and ANNE FERGUSON

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**Background:** Fluid obtained by whole gut lavage normally contains traces of immunoglobulin (Ig) G, albumin, and  $\alpha$ -1-antitrypsin; higher concentrations have been found in patients with inflammatory bowel disease (IBD). **Methods:** In a prospective study, 53 lavages were performed in 45 IBD patients (27 Crohn's disease, 18 ulcerative colitis), in whom disease activity was simultaneously assessed by Crohn's Disease Activity Index or Powell Tuck index. Concentration of IgG in lavage fluid was measured by enzyme-linked immunosorbant assay, and of albumin and  $\alpha$ -1-antitrypsin by immunoturbidimetry. **Results:** For IgG, concentrations in lavage fluid correlated closely with activity indices: in Crohn's disease,  $r = 0.723$  ( $P < 0.0001$ ), in ulcerative colitis,  $r = 0.714$  ( $P < 0.0001$ ). Results for albumin and  $\alpha$ -1-antitrypsin concentrations were similar to those for IgG, but they were less sensitive in detecting active disease. However, this method cannot be used as a diagnostic test for IBD; normal results were obtained for IgG in 6 (all inactive) of 42 lavages in patients who had unequivocal radiological or endoscopic abnormalities. **Conclusions:** Assay of protein concentrations in gut lavage fluid is a simple, objective means of grading disease activity in patients with IBD; its potential uses are likely to be in the evaluation of complex cases and in clinical trials.

The Crohn's Disease Activity Index (CDAI), developed in the 1970s,<sup>1</sup> has proved a reliable and remarkably reproducible measure of health status in patients with Crohn's disease (CD). In theory, the CDAI is vulnerable to criticism because it is heavily weighted by subjective factors of general well-being and abdominal pain, and investigators have argued that the CDAI is not so much a measure of disease activity as of general disability. Many factors apart from intestinal inflammation will contribute to the index, including infection, obstruction, malnutrition, psychological dysfunction, and the side effects of drugs. Nevertheless, the CDAI and similar indices for ulcerative colitis (UC) activity, such as the Powell-Tuck index (PTI),<sup>2</sup>

provide standards for the assessment of any new approach to the measurement of disease activity in IBD.

Whole gut lavage is now widely used as a bowel preparation for barium enema, endoscopy, or colorectal surgery.<sup>3</sup> The clear fluid passed after initial colonic cleansing is essentially a gut perfusate. It contains secreted immunoglobulins (Ig) and antibodies and, after appropriate processing, provides ideal material for the clinical investigation of intestinal humoral immunity.<sup>4,5</sup> Normally, whole gut lavage fluid (WGLF) contains only trace amounts of IgG, but in two separate groups of 10 and 44 patients with active IBD we found concentrations of IgG that were significantly higher than those of controls.<sup>5,6</sup> We also found high concentrations of WGLF albumin with a positive correlation between lavage IgG and albumin content, suggesting that these tests are measuring plasma leakage across inflamed mucosa.<sup>6</sup>

In the present prospective study, gut lavage has been performed on 53 occasions in 45 well-characterized patients with CD or UC, and WGLF concentrations of IgG and of two plasma-derived proteins, albumin and  $\alpha$ -1-antitrypsin (A1AT), were measured. At the same visit the CDAI or PTI as appropriate was calculated, blood was taken for measurements of erythrocyte sedimentation rate (ESR), platelet count, and C-reactive protein (frequently used, single indices of activity), and the physician's global assessment was quantified by using a visual analogue scale (VAS) of 0-120.

We aimed to establish whether assay of WGLF proteins could be used as a diagnostic test for IBD and to assess to what extent concentrations of proteins in WGLF parallel the global indices, CDAI and PTI. Do the results of this investigation make it possible to

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*Abbreviations used in this paper:* A1AT, alpha-1-anti-trypsin; ELISA, enzyme-linked immunosorbant assay; ESR, erythrocyte sedimentation rate; PRU, Protein Reference Unit; PTI, Powell-Tuck index; VAS, visual analogue scale; WGLF, whole gut lavage fluid.

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grade severity within "active" disease or to detect minimal abnormalities in patients with CDAI of  $\leq 150$  or PTI of  $\leq 4$  (i.e., classified as inactive)? Does this approach offer any advantage over conventional clinical tests (ESR, C-reactive protein, platelet count) or a physician's global assessment (recorded on a VAS for the purpose of this investigation)?

## Material and Methods

### Subjects

A total of 45 patients with IBD were studied, 27 with CD (11 male, 16 female) and 18 with UC (8 male, 10 female). The diagnosis and disease extent in all patients were confirmed by histology, endoscopy, and radiology. Full details are given in Table 1. The patients were classified into various subgroups by the nature of previous resections and current macroscopic disease. Five CD patients and 1 UC patient were studied twice, and 1 man with UC was studied three times. In 44 of the 53 studies, gut lavage was being performed as bowel preparation for colonoscopy or barium enema examination; in 4 cases lavage was performed as preparation for surgery; and in 5 cases it was performed to empty

the gastrointestinal tract before treatment with an elemental diet.

### Clinical Assessments

Patients completed a diary card of symptoms for the week preceding the lavage. Haematological and biochemical indices included haemoglobin, platelet count, ESR, C-reactive protein, total protein, and albumin and serum Igs. All patients were clinically assessed by one of us (C.P.C.), and the CDAI or PTI was calculated in the standard fashion. In addition, the physician's assessment of overall disease activity for the day of lavage was recorded on a VAS, this being based on symptoms, signs, and results of the blood tests.

The study was approved by the Medicine Subcommittee of the Lothians Area Ethics of Research Committee.

### Lavage Protocol

Patients drank 4 L of isotonic nonabsorbable polyethylene glycol-based lavage fluid (Golytely; Braintree Laboratories, Inc., Braintree, MA) at a rate of 250 mL every 10–15 minutes. Usually within 2 hours solid feces were passed followed by liquid feces, which were followed by several large-volume, virtually clear fluid "stools." An aliquot of the first completely clear specimen was collected for analysis. The procedure was supervised by an experienced nurse and performed either in a general in-patient ward or in the gastrointestinal out-patient investigation suite.

### Processing of Whole Gut Lavage Fluid

Specimens were processed within 10 minutes of collection. Fluid, 20 mL, was filtered through a GF/A (Whatman Scientific Ltd., Kent, England) glass fibre filter. The following reagents were added to 10 mL of the filtrate, with mixing after each addition (final concentrations in brackets): soya bean trypsin inhibitor in phosphate buffered saline (80  $\mu\text{g}/\text{mL}$ ); sodium ethylenediaminetetraacetic acid in phosphate buffered saline (15 mmol/L); phenyl methyl sulphonyl fluoride in 95% ethanol (2 mmol/L); sodium azide (1 mmol/L); and newborn calf serum (5% volume).

Aliquots of processed WGLF were then stored at  $-70^\circ\text{C}$ .

### Analytical Methods

Sample analysis for IgG was by enzyme-linked immunosorbent assay (ELISA),<sup>5</sup> using affinity-purified goat anti-human IgG (North East Laboratories) and, as standard, human reference serum from the Protein Reference Unit (PRU), Royal Hallamshire Hospital, Sheffield.

Albumin and A1AT were assayed by immunoturbidimetric procedures. Anti-human albumin was supplied by the Scottish Antibody Production Unit, and anti-human A1AT was obtained from the PRU. WGLF was diluted in PEG 6000 reagent in tris buffer pH7 with and without antibody and incubated for 15 minutes. Turbidity was measured at 340 nmol/L in a flow-through spectrophotometer (PU8610, Phillips). Standard plots of albumin and A1AT

**Table 1.** Clinical Details of 45 Patients With IBD Undergoing Whole Gut Lavage on 53 Occasions

Parts of gastrointestinal tract previously resected	No. of patients	Macroscopic disease at time of lavage	No. of lavages
Crohn's disease	15	Small bowel	6
		Ileocolonic	2
		Colonic	4
		Rectosigmoid	3
		Perianal	2
		Microscopic only	1
Small bowel	2	Small bowel	2
Ileocecal $\pm$ terminal ileum	8	No macroscopic disease	5
		Small bowel	3
Segmental colonic resection	2	Ileocolonic	1
		Ileocolonic	1
Total	27	Colonic	2
Ulcerative colitis			32
Previously documented macroscopic disease	11	No macroscopic disease	3
		Rectosigmoid	9
Left sided	3	No macroscopic disease	1
		Left sided	2
Pancolitis	4	No macroscopic disease	1
		Pancolitis	5
Total	18		21

values over the range 0–200  $\mu\text{g}/\text{mL}$  were prepared from Human Reference Serum (PRU), diluted in 0.9% NaCl containing 48 g/L PEG 3350.

### Statistical Analysis

Correlation coefficients were calculated by using the Spearman Rank Correlation test.

### Results

The patients studied were heterogeneous in disease distribution and in their previous and current treatment. In CD, the CDAI range was from 24 to 321; 24 lavages were in patients with CDAI of  $>150$  (described below as having active disease) and 8 in patients with a CDAI  $\leq 150$  (in remission). In patients with UC, the PTI ranged from 0 to 13; 13 lavages were in patients with active disease (PTI of  $>4$ ), and 8 were in patients in remission with a PTI of  $\leq 4$ .

#### Whole Gut Lavage Fluid Protein Concentrations

Reference ranges for WGLF IgG, albumin, and A1AT have previously been established by studies of 63 immunologically normal subjects: 20 men, age range 15–80, and 43 women, age range 24–88, who were all taking a normal diet without being given immunosuppressive or anti-inflammatory drugs. Normal values are for IgG  $\leq 10 \mu\text{g}/\text{mL}$ , for albumin  $\leq 26 \mu\text{g}/\text{mL}$ , and for A1AT  $\leq 19 \mu\text{g}/\text{mL}$ .<sup>7</sup>

Values for WGLF protein concentrations in patients with CD and UC are shown in Figure 1 and are plotted against CDAI or PTI as appropriate.

#### Diagnostic Potential of Whole Gut Lavage Fluid Tests

The possibility that WGLF protein concentrations are always high when there is overt intestinal mucosal inflammation (on radiological or endoscopic visualization) would provide a means of screening patients for the presence or absence of IBD. This issue has been considered by analysis of the results for WGLF IgG. High values for IgG concentration were present in only 24 of the 27 lavages performed in patients who had unequivocal macroscopic small bowel or colonic CD and in 13 of the 15 lavages in patients with UC who had positive radiograph or endoscopic findings. There was also one patient with microscopic evidence of colonic CD and no macroscopic disease; however the WGLF IgG concentration was high.

#### Whole Gut Lavage Fluid Proteins and Disease Activity

**IgG, Crohn's disease.** In 32 lavages from patients with CD, values for WGLF IgG concentration

were normal in 9. Seven of these were in patients in remission, and 1 was in an 18-year-old man with a borderline CDAI of 168. He had ankylosing spondylitis, and his anorexia and high ESR shortly after right hemicolectomy for CD were thought to reflect activity of arthritis rather than of CD. There was one patient with WGLF IgG concentration of 10  $\mu\text{g}/\text{mL}$  and CDAI of 185.

Abnormally high concentrations of WGLF IgG were present in 23 patients of whom 22 had active disease. A 13-year-old boy with perianal CD and unexplained abdominal pain had a CDAI of 119 and a WGLF IgG concentration of 20  $\mu\text{g}/\text{mL}$ . During the next 6 months his growth stopped and pain became worse; both problems have resolved with systemic steroid therapy.

**IgG, ulcerative colitis.** In 21 lavages from patients with UC, values for WGLF IgG concentration were normal in 8 patients (of whom 7 were in remission) and high in 13 patients (12 with active disease). Both discrepant cases were patients who had had unequivocally active disease two weeks before and were in the early stages of Sulphasalazine or oral corticosteroid treatment respectively.

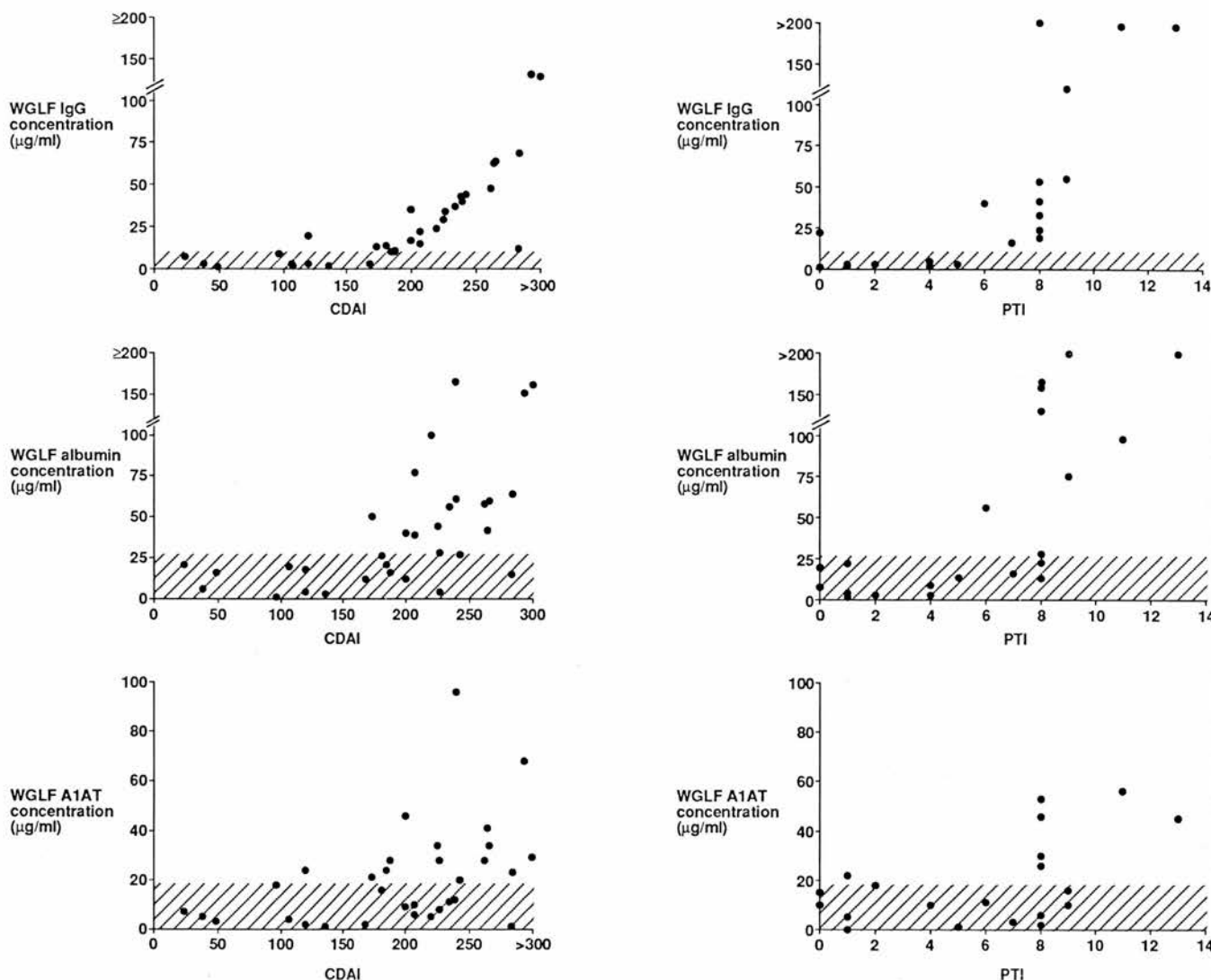
**Albumin.** In 32 lavages from patients with CD, values for WGLF albumin concentration were normal for 15 patients (8 of these patients were in remission and CDAI in the others ranged from 168–283). Albumin concentration levels were high in 17 patients, all of whom had active disease.

In 21 lavages from patients with UC, values for WGLF albumin concentration were normal for 12 patients (of whom 8 were in remission) and were abnormally high in 9 patients, all of whom had active disease.

**A1AT.** In 32 lavages from patients with CD, values for WGLF A1AT concentration were normal for 17 patients (of whom 7 were in remission) and were high in 15 patients (14 with active disease). In 21 lavages from patients with UC, values for WGLF A1AT concentration were normal in 14 patients (of whom 7 were in remission) and high in 7 patients (6 with active disease).

#### Correlation of Lavage Proteins Concentrations With Activity Indices in Active and Inactive Disease

Correlation coefficients for WGLF protein concentrations against activity indices are detailed in Table 2. For both CD and UC and for all three proteins studied, there was a highly significant positive correla-



**Figure 1.** Concentrations of IgG, albumin, and A1AT in WGLF taken from patients with CD and UC and plotted against CDAI and PTI, respectively.

tion between activity index and WGLF protein concentration, with *r* values ranging from 0.439 to 0.723.

Because, as shown clearly in Figure 1, the relationship between activity index and lavage protein concentration is not linear but biphasic, correlations were separately calculated for cases with active and inactive disease (as defined by CDAI and PTI). Calculations were also performed for the subsets of CD patients with active colonic and active small bowel disease.

The results (Table 2) show that there are significant correlations only in active disease; that the highest *r* values were found for IgG vs. CDAI in active CD (*r* = 0.821), particularly in the subset with active small bowel CD (*r* = 0.970); and that in active UC, although there was good correlation between WGLF IgG concentration and the PTI (*r* = 0.740), the other two proteins did not show significant correlation with the

PTI. In inactive CD and UC, WGLF protein concentrations were independent of activity indices.

**Conventional Laboratory Indices of Disease Activity**

For the purposes of this analysis, patients with CD who had a CDAI of >150 and those with UC who had a PTI of >4 have been designated as having active disease. The numbers of cases with abnormal and normal values for ESR, C-reactive protein, and platelet count in the active and remission groups are given in Table 3. This table also includes, for comparison, numbers with normal and abnormal WGLF IgG, albumin and A1AT values, and the physician's overall assessment based on a VAS.

The three blood indices were normal in most patients with inactive disease. However, they were also

**Table 2.** Concentrations of Proteins in WGLF From Patients With Active and Inactive IBD; Correlations With CDAI and PTI

	No. of lavages	IgG concentration vs. activity index		Albumin concentration vs. activity index		A1AT concentration vs. activity index	
		r	P	r	P	r	P
<b>Crohn's disease</b>							
All	32	0.723	<0.0001	0.588	<0.0001	0.439	<0.01
Active	24	0.821	<0.0001	0.547	<0.01	0.293	NS
Inactive	8	0.217	NS	-0.352	NS	0.160	NS
Active colonic	8	0.778	<0.05	0.507	NS	0.468	NS
Active small bowel	10	0.970	<0.0001	0.547	NS	0.359	NS
<b>Ulcerative colitis</b>							
All	21	0.714	<0.0001	0.701	<0.0001	0.527	<0.02
Active	13	0.740	<0.01	0.610	<0.05	0.600	<0.05
Inactive	8	-0.318	NS	-0.378	NS	-0.041	NS

NOTE. P > 0.05. NS, not significant.

normal in many patients with unequivocally active disease, and this was particularly so in the group of 13 assessments in active UC; ESR was normal in 8 cases, C-reactive protein was normal in 10, and platelet count was normal in 8.

**Discussion**

The degree of illness in a patient with IBD is not directly related to the length of bowel grossly affected. Factors such as the severity of local inflammation and nonspecific "disease activity" are also relevant. There is an urgent need for simple and objective indices of these separate components of overall disability for use in clinical practice and research.

There have been many attempts to develop a single index of illness in IBD<sup>1,2,8-13</sup> by combining a few clinical and/or laboratory criteria, and claims have been made for the significance of single laboratory determinations in some situations.<sup>14-18</sup> We have been exploiting the relatively new method of bowel cleansing by whole gut lavage and have reported that whole gut lavage fluid (WGLF), appropriately processed, has

considerable potential as a source of material for clinical evaluation of intestinal immunity and inflammation.<sup>5</sup>

In an initial technical appraisal of ELISA as applied to WGLF, we found high concentrations of IgA but only trace amounts of IgG (<5 µg/mL) in fluid from control subjects, whereas there were significantly higher IgG concentrations in WGLF from 10 patients with active CD.<sup>5</sup> This was confirmed in a separate cohort of patients with active CD who also had substantial amounts of albumin in WGLF, indicating that these tests may be detecting plasma leakage into the gut.<sup>6</sup> In further work, assay of A1AT has been included in the protocol because fecal A1AT concentration and clearance are widely accepted as indices of GI protein loss.

With a view to the introduction of this new test into clinical practice, we also carried out a retrospective review of results for 140 lavages in symptomatic patients<sup>7</sup> to establish whether concentrations of these proteins in WGLF are high in conditions other than active IBD. Assays of WGLF obtained on 102 occa-

**Table 3.** Presence of Normal or Abnormal Values for Conventional Laboratory Indices of Disease Activity, Global Assessment, WGLF IgG, Albumin, and A1AT Concentrations in Patients With CD and UC Arbitrarily Subdivided Into Active or Inactive IBD on the Basis of CDAI or PTI, Respectively

No. of lavages	ESR		C-reactive protein		Platelet count		VAS		WGLF IgG concentration		WGLF albumin concentration		WGLF A1AT concentration		
	Normal (<20)	High (>20)	Normal (<1.5)	High (>1.5)	Normal	High	Normal (0-60)	High (61-120)	Normal (≤10)	High (>10)	Normal (≤26)	High (>26)	Normal (≤19)	High (>19)	
<b>Crohn's disease</b>															
Active (CDAI > 150)	24	5	19	8	16	6	18	1	23	2	22	7	17	10	14
Inactive (CDAI ≤ 150)	8	5	3	8	0	7	1	7	1	7	1	8	0	7	1
<b>Ulcerative colitis</b>															
Active (PTI > 4)	13	8	5	10	3	8	5	2	11	1	12	4	9	7	6
Inactive (PTI ≤ 4)	8	8	0	8	0	7	1	6	2	7	1	8	0	7	1

sions from 95 patients with IBD of varying activity revealed high concentrations of IgG in 65 (64%), of albumin in 53 (52%), and of A1AT in 37 (36%). High WGLF IgG concentrations were present in a patient with lymphangiectasia, in 1 of 5 patients with non-IBD forms of colitis, and in only 1 of 32 patients with other conditions (a value of 11  $\mu\text{g}/\text{mL}$ ). These results and comparable findings for albumin and A1AT supported the view that high WGLF protein concentrations are relatively specific markers of active IBD.

We now report a prospective study based on 53 lavages in patients with UC or CD fully characterized with regard to anatomical distribution of disease and investigated for various single and composite activity indices at the time of the lavage. The aims of this study were to determine whether assay of WGLF proteins can be used as a diagnostic test for IBD and whether this completely objective procedure can substitute for or complement currently used clinical tests and indices of disease activity. We have used the best currently available index, the CDAI, as the reference measure of disease activity in CD and a similar index for UC, the PTI.

The present study shows that WGLF protein concentrations, particularly IgG, discriminate well between active and inactive IBD and, within the subsets of patients with active disease, closely parallel disease activity as defined by CDAI and PTI. Furthermore, these sensitive tests were completely normal in most patients with  $\text{CDAI} \leq 150$  and  $\text{PTI} \leq 4$ , strongly supporting the present clinical trials practice whereby such values are taken as successful end points, indicating that remission has been achieved.

However WGLF protein assays cannot be used as screening or diagnostic tests for IBD. Although high values are found in many patients, the data in this paper clearly show that values are normal in clinically inactive disease, even when there are unequivocal radiological or endoscopic features of IBD such as cobblestone ulcers and long, stringlike strictures. Nevertheless in our clinical practice we are finding that assay of WGLF IgG is a valuable aid to diagnosis in some clinical situations, e.g., in patients with microscopic CD, when IBD coexists with severe diverticular disease, and in separating the effects of intestinal and joint disease in IBD patients with ankylosing spondylitis.

There is abundant evidence to implicate IgG in the pathological lesions of IBD, particularly CD: immunohistochemistry reveals an excess of IgG-containing cells deep in the mucosa<sup>19,20</sup>; isolated intestinal mononuclear cells from IBD patients spontaneously secrete high amounts of IgG<sup>21,22</sup>; and there are differences in

intestinal plasma cell IgG subclass distribution between controls, UC and CD patients.<sup>23</sup> Thus, when we first noted the presence of IgG in WGLF, we considered that it was probably derived from the abundant IgG cells in diseased mucosa. However, the findings of high concentrations of two plasma proteins, albumin and A1AT, in lavage fluid from patients with active IBD suggests that at least some of the IgG is plasma-derived. Furthermore, all three protein concentrations were low in WGLF from some inactive CD patients who have extensive chronic ulceration of the intestine (diseased tissues known to be replete in IgG cells).

It could be argued that these sensitive tests are merely measuring plasma proteins entering the gut lumen as a result of bleeding. However, in active IBD when abundant amounts of the three proteins are present in WGLF, their relative concentrations differ substantially from those in plasma. The three proteins differ in molecular weight (IgG, 150,000 daltons; albumin, 69,000 daltons; A1AT, 45,000 daltons), and we suggest that there is a selective increase in mucosa-to-lumen permeability in active IBD operating either at the level of the capillary or the epithelial basal lamina.

We have also considered whether analytical factors could explain the different results for the three proteins studied in relation to discrimination between active and inactive disease. Although the techniques used differ, co-efficients of variation, between-run and within-run, are similar for the three assays at 10%–15%.<sup>7</sup> Although fluids are processed within minutes of collection, proteins leaking proximally into the gut lumen will have been exposed to pancreatic and bacterial proteases during the transit of fluid along the gut (1–2 hours). Ex vivo experiments show that at 37°C, degradation of albumin by unprocessed WGLF is significant (loss of 10%–40% of measured albumin in 1 hour), whereas IgG and A1AT are relatively resistant to such proteolysis. This could explain the differences in results for IgG and albumin in active disease but not the relative insensitivity of WGLF A1AT concentrations.

Various scientific approaches to the measurement of disease activity in IBD have recently been reviewed.<sup>24,25</sup> Laboratory tests such as ESR, platelet count, acute phase proteins, are useful. However they may be normal in active IBD, particularly in UC and in small bowel CD (e.g., Table 3) and will be positive in situations of active inflammation or infection without gut involvement. Other techniques, such as labeled leucocyte studies<sup>26,27</sup> and measures of GI protein loss by A1AT clearance,<sup>28–30</sup> have advantages and disad-

antages, which have been fully discussed elsewhere.<sup>6,24,25</sup> In general, they have been developed and applied without being subjected to prospective evaluation against the carefully developed standard activity indices such as CDAI. Furthermore, labeled leucocyte studies are expensive, involve exposure to radioactivity, and depend on complete fecal collection. The findings with these tests relate to events in macroscopically affected gut and thus differ with those obtained with the gut lavage technique, which appears to measure a more diffuse phenomenon.

There may be problems in interpretation of isotope-based or A1AT clearance studies if the treatment used, such as elemental diet therapy, changes intestinal transit rate or otherwise influences defecatory function; this could either mask or mimic changes resulting from specific anti-inflammatory effects of the test treatment. Because the standard protocol for collection of whole gut lavage fluid is not influenced by concurrent dietary or pharmacological treatment, this method should lend itself to clinical trials.

Because gut lavage is now widely used for bowel preparation before endoscopy, radiology, and surgery, WGLF for laboratory analysis can often be obtained in the course of a standard clinical work-up. Lavage fluid, collected as described above, is aesthetically acceptable to laboratory staff, resembling urine rather than feces, and specimens can be stored at  $-70^{\circ}\text{C}$  for several months without deterioration. Concentration of IgG in WGLF assayed by ELISA discriminates very well between active and inactive IBD and accurately grades the degree of activity. We suggest that this new and direct approach to clinical investigation of gut immunity will prove useful in the analysis of illness and of response to treatment in some complex IBD patients and has considerable potential in clinical trials of anti-inflammatory and immunomodulatory regimens.

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## Fecal Occult Blood Testing Will Fail to Detect Colonic Polyps

Dear Sir:

In the June 1993 issue of *GASTROENTEROLOGY*, an original report<sup>1</sup> and an editorial<sup>2</sup> debate at length the evaluation of fecal occult blood (FOB) tests for the detection of colorectal neoplasia. Improvements in the screening or case finding of tumors by this approach will require not only refinements in specimen collection, handling, and analytical techniques, but also assume that the lesions to be detected are, in fact, bleeding.

When gut lavage with polyethylene glycol-based solutions is used for bowel cleansing, the clear gut lavage fluid passed at the end of the procedure, whole gut lavage fluid (WGLF), is essentially a gut perfusate and can be used for immunological, biochemical, and cytological studies. For research purposes, the lavage is supervised by an experienced nurse with a defined protocol for fluid ingestion and is administered at a gut perfusion rate of around 20 mL/min.

In relation to the investigation of gastrointestinal bleeding, we have found that hemoglobin (Hb) can readily be assayed in WGLF by using the HemoQuant technique; this allows detection and quantification of chronic bleeding from any level of the gastrointestinal tract without interference in the analysis by dietary constituents and uninfluenced by patient-related variables such as intestinal transit time. We have already reported our preliminary experience of the application of this approach<sup>3</sup>; our further data are relevant to the debate on the potential values of FOB testing.

Reference values for WGLF Hb, based on results in 43 normal individuals, are  $2.50 \pm 1.13 \mu\text{g/mL}$  (mean  $\pm$  SD; range, 1-5.4  $\mu\text{g/mL}$ ).

In the course of immunological research, we have collected WGLF from a series of unselected hospital inpatients (to use as "diseased controls"; these include 25 patients with colorectal tumors). Values for WGLF Hb were abnormally high in all of 12 patients with

colorectal cancer ( $198 \pm 313$ ; range, 13.6-990  $\mu\text{g/mL}$ ). There were 13 patients with benign colorectal polyps. In the 5 cases with small polyps (<2 cm) and in 6 of 8 with larger polyps, WGLF Hb concentration was in the range 1-4  $\mu\text{g/mL}$ , i.e., completely normal. The two patients with high values were a woman with multiple sessile polyps (WGLF Hb, 6.3  $\mu\text{g/mL}$ ) and a man with three polyps, one of which (4  $\times$  4 cm) had areas of moderate dysplasia (WGLF Hb, 20  $\mu\text{g/mL}$ ).

These data show that colorectal cancers diagnosed in general clinical practice have a high frequency (in this small series, 100%) of occult bleeding, but only a minority of benign polyps bleed. A screening strategy based on FOB testing is entirely rational for cancer detection but will miss the majority of benign colorectal polyps.

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**Reply.** My colleagues and I thank Drs. Brydon and Ferguson for their comments on our report.<sup>1</sup> Their data based on HemoQuant assay of whole gut lavage fluid suggests that blood loss is normal in most patients with large adenomas. There are several reasons why we would challenge that conclusion. First, it is not clear whether blood loss measured in lavage fluid equates with blood loss in "real life," and their assays presumably were performed on single samples, not on samples collected over several days as is usual with fecal occult blood testing.

Second, despite the precision of the assay,<sup>2</sup> HemoQuant is not the best method for detection of low grade bleeding from the large bowel; immunochemical tests that detect only hemoglobin and guaiac tests that detect only hemoglobin and heme provide better discrimination between normal subjects and those with low-grade bleeding.<sup>1,3</sup> Although we found considerable overlap in HemoQuant estimates of blood loss between patients with adenomas and normal subjects, mean blood loss was higher in the patients with adenomas.<sup>3</sup>

Perhaps the most compelling evidence comes from the randomized controlled trials of occult blood testing for colorectal cancer, in which the yield of large adenomas exceeds that of small adenomas in subjects with positive Hemoccult tests.<sup>4</sup> If detection of adenomas was caused by serendipity, the reverse would be expected. The high yield of large adenomas supports the view that the positive Hemoccult tests were indeed produced by low-grade bleeding from the adenomas.

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# Use of whole gut perfusion to investigate gastrointestinal blood loss in patients with iron deficiency anaemia

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## Abstract

**Iron deficiency anaemia may be due to occult bleeding into the gut. However, although clinical investigations may show a high frequency of gastrointestinal tract disease in these patients, the cause-effect relationship between the lesions detected and anaemia remain uncertain. This study aimed to establish whether lesions detected by endoscopy or imaging of the gastrointestinal tract in patients with unexplained iron deficiency anaemia are bleeding continuously. Routine clinical tests were performed in 42 patients with unexplained iron deficiency anaemia referred to this unit. Whole gut lavage and assay of haemoglobin in the gut perfusate were also performed. The main outcome measures were clinical diagnoses (by imaging and endoscopy of the upper gastrointestinal tract and colon); the concentration of haemoglobin in whole gut lavage fluid; and the calculated gastrointestinal blood loss per day. There were 73 clinical, dietary, or iatrogenic factors of possible aetiological importance in the 42 patients – poor diet (10), gross gastrointestinal abnormality (34 in 28 patients), malabsorption (14), coagulation problems (6), and NSAID use (9). The gut lavage test showed, however, that at the time the test was performed, only eight patients were losing more than 2 ml blood daily into the gut, including all four with colonic cancer, one with diffuse gastric vascular ectasia, and one with severe ulcerative oesophagitis. It is concluded that occult gastrointestinal bleeding sufficient to cause anaemia was evident in only 19% of 42 patients. There was a high frequency of other potential causes of iron deficiency in the remainder, suggesting that most of the gastrointestinal diseases and lesions detected in them were probably coincidental. Factors other than blood loss should be considered and treated in patients referred for anaemia assessment.** (*Gut* 1996; 38: 120-124)

**Keywords:** occult gastrointestinal bleeding, colon cancer detection, whole gut perfusion, dietary iron deficiency, iron malabsorption.

Iron deficiency anaemia may be the only clinical manifestation of ulcerative oesophagitis, benign or malignant gastric ulcer, duodenal ulcer, large benign colonic polyps, or

colonic cancer. Thus, it is normal clinical practice to examine the upper and lower gastrointestinal tract by *x* ray or endoscopy, or both, in patients with unexplained iron deficiency. High detection rates for lesions capable of causing blood loss are reported – for example, 57%, 60%, and 70% respectively in recent series from the USA,<sup>1</sup> Australia,<sup>2</sup> and England.<sup>3</sup> Accurate measurements of the amounts of blood lost into the gut in these patients have not been performed, however, and so the cause-effect relationships between the lesions detected and the anaemia remain uncertain.

We have recently reported that when peroral gut lavage with a non-absorbable fluid is used for bowel cleansing, the clear fluid passed per rectum at the end of the procedure (whole gut lavage fluid, WGLF) is essentially a gut perfusate.<sup>4</sup> For research purposes the lavage is supervised by an experienced nurse with a defined protocol for fluid ingestion, to give a gut perfusion rate of around 20 ml per minute. Blood loss during the test, from any level of the gastrointestinal tract, can be detected and measured by assay of haemoglobin (Hb) in WGLF.<sup>5</sup> We have used this new test in a series of patients with iron deficiency anaemia in order to assess which of the gastrointestinal lesions detected are chronically and continuously bleeding, and which are probably not doing so.

## Patients and methods

### PATIENTS

Forty two patients referred to gastrointestinal physicians with unexplained iron deficiency anaemia were investigated over an 18 month period. Twenty six were women aged between 40 and 85 years (median 66) and 16 were men aged between 43 and 86 years (median 72). Entry criteria were a low blood Hb concentration (<130 g/l in men, <115 g/l in women) together with two of the following: low mean cell volume (<76 fl), low serum ferritin (<10 µg/l), low serum iron (<14 µmol/l), and reticulocyte response with rise in Hb after oral iron treatment.

In order to establish reference values for the WGLF Hb concentration, assays were performed in WGLF from 22 healthy volunteers and 15 patients with simple constipation or trivial, functional gastrointestinal symptoms; there were 23 men and 14 women, median age 32 years, range 19-86.

Reproducibility of the technique was

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assessed indirectly, by examining results of WGLF Hb measurements in pairs of samples which had been collected from 40 patients or healthy volunteers in the course of other research projects, or as part of their routine clinical care, including 10 pairs of specimens from patients with active inflammatory bowel disease, before and after initiation of treatment.

#### CLINICAL ASSESSMENT

As part of the initial clinical interview and examination, we recorded not only gastrointestinal symptoms and clinical signs but also the quality of the diet and current or recent use of NSAIDs, aspirin, or anticoagulants.

Upper gastrointestinal endoscopy, rigid sigmoidoscopy, and barium enema or colonoscopy, or both, were performed in 40 patients. In two patients, when the first endoscopy examination showed a carcinoma (one gastric, one colonic) other booked investigations were cancelled. These patients have not been excluded from the series since examination of the gastrointestinal tract was completed at laparotomy. Although the planned protocol included endoscopic duodenal biopsies for the diagnosis of coeliac disease, these were, in fact, obtained from only 25 patients, and a further three had a Watson capsule biopsy of the jejunum.

#### GUT LAVAGE PROCEDURE

Gut lavage was performed as preparation for barium enema or colonoscopy examination. Supervised by a research nurse, patients drank isotonic lavage fluid (Klean-Prep, Norgine Ltd, UK), at a rate of one glass (200 ml) every 10–15 minutes, until clear fluid was being passed per rectum. A sample was collected and stored at  $-70^{\circ}\text{C}$ .

#### ASSAY OF HAEMOGLOBIN AND CALCULATION OF BLOOD LOSS

Haemoglobin in WGLF was assayed by the Hemoquant technique, which measures bacterially degraded as well as intact Hb.<sup>6</sup> The blood Hb concentration was measured in a sample taken on the day of lavage and gastrointestinal blood loss per day was calculated as:

$$\frac{\text{WGLF Hb concentration } (\mu\text{g/ml}) \times 28.8}{\text{Blood Hb concentration } (\text{g/l})} = \text{ml blood lost/d}$$

(this assumes a perfusion rate of 20 ml/min = 28.8 l/d).

## Results

#### CLINICAL FEATURES

Initial Hb values in the 16 men ranged from 61–119 g/l (mean 90 g/l), and in the 26 women they ranged from 45–114 g/l (mean 85 g/l). Many patients had symptoms of anaemia such as tiredness, lethargy, and breathlessness but few had significant gastrointestinal symptoms. One woman developed dysphagia while

being investigated; five patients reported dyspepsia, three heartburn, one constipation, and two diarrhoea.

There were 10 patients whose diet was clearly deficient in iron-containing foods (as judged at clinical interview by one of us (AF, AW, or MM)); formal dietary assessment was not performed. Nine patients had been taking NSAIDs when the anaemia was diagnosed but at the time of their gastrointestinal investigations the drugs had been withdrawn from eight of the nine. Four patients had been on long term anticoagulant therapy, which had been stopped in one case. There was a further patient with a bleeding diathesis due to idiopathic thrombocytopenic purpura and one woman with alcoholic liver disease also had abnormal coagulation.

#### MALABSORPTION

Twelve patients had a gastric abnormality likely to lead to iron malabsorption. Seven of the 12 had had a partial gastrectomy for peptic ulcer disease many years previously and the other five had atrophic gastritis.

Small bowel biopsy tissue from one woman with no gastrointestinal symptoms and a diet deficient in iron showed pathology typical of coeliac disease. Coeliac disease was probable in one other patient with an oesophageal carcinoma. She had old rickets and gave a history of macrocytic anaemia with a normal Schilling test 25 years ago; unfortunately the endoscopist had not taken a duodenal biopsy but ELISAs were positive for serum IgA and WGLF IgA antibodies to gliadin.<sup>7</sup>

#### OTHER GASTROINTESTINAL DISEASES DETECTED

Standard x ray and endoscopic investigations produced many positive results, including six carcinomas (Table I). Indeed there were only seven patients in whom no lesion was found:

TABLE I *Gastrointestinal diseases and other factors of possible relevance to iron deficiency anaemia in 42 patients*

	No of patients
Malabsorption:	
Atrophic gastritis	5
Partial gastrectomy without gastritis	4
Partial gastrectomy with gastritis	3
Coeliac-definite	1
Coeliac-probable	1
Other gastrointestinal diseases:	
Oesophagitis mild	4
Oesophagitis severe ulcerative	1
Carcinoma oesophagus	1
Hiatus hernia	10
Acute gastritis	2
Gastric vascular ectasia	1
Gastric carcinoma	1
Duodenal ulcer	1
Crohn's (ileum)	1
Colon cancer	4
Benign colonic polyps	2
Diverticular disease	6
Abnormal coagulation:	
On anticoagulants	3
Previously anticoagulated now stopped	1
Idiopathic thrombocytopenic purpura	1
Alcoholic liver disease	1
Drugs:	
Previously taking NSAID	8
Current on NSAID	1
Poor diet:	
Iron deficient diet	10

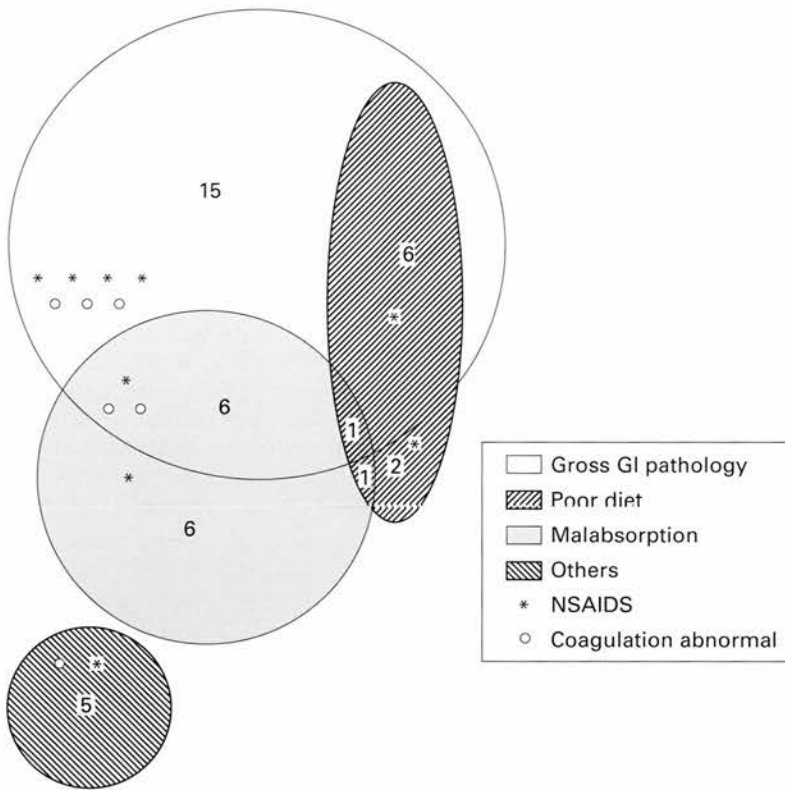


Figure 1: Presence of gastrointestinal (GI) diseases and of other aetiological factors in 42 patients with iron deficiency anaemia. In 28 patients, one or more gastrointestinal disease was detected by imaging or endoscopy, or both; in 10 the diet was judged by an experienced clinician to be deficient in iron; and 14 patients had diseases associated with iron malabsorption. As shown, there was significant overlap between these groups and with the presence of other relevant factors such as use of non-steroidal anti-inflammatory drugs (NSAIDs) or coagulation abnormality.

two of these had iron deficient diets, one had alcoholic liver disease, one had previously taken NSAIDs, leaving only three of the 42 patients for whom we had completely negative findings (Fig 1).

#### MEASUREMENT OF GASTROINTESTINAL BLEEDING

Values for WGLF Hb in the 37 volunteers and patients with a normal gastrointestinal tract, ranged from 1.0–5.4  $\mu\text{g/ml}$  (mean 2.50, SD 1.13). We have therefore set the reference range for this measurement as 1–5  $\mu\text{g/ml}$ .

Thirty sets of duplicate samples were from individuals with normal values for WGLF Hb at the first test. In all cases the value for the second sample was also normal; mean

TABLE II Concentrations of IgG (reflecting disease activity) and of haemoglobin, in whole gut lavage fluid (WGLF) from a series of patients with inflammatory bowel disease who had gut lavage performed twice within a four week period

	IgG ( $\mu\text{g/ml}$ ) in WGLF		Hb ( $\mu\text{g/ml}$ ) in WGLF	
	Relapse	On treatment	Relapse	On treatment
Crohn's disease	55	24	10	5
	93	60	22	9
	144	82	162	145
	137	54	58	14
	27	9	12	9
	216	41	15	9
	44	34	9	8
Ulcerative colitis	101	11	14	4
	254	102	151	25
	188	2	23	4

difference in duplicate values was 0.96  $\mu\text{g/ml}$  (range 0–3). There were 10 pairs of specimens from patients with inflammatory bowel disease, one sample collected in relapse, and one after a variable clinical improvement on treatment. As shown in Table II, concentrations of IgG and of Hb in WGLF paralleled disease activity: Hb concentrations were lower in the specimen collected after treatment, but in most cases the values were still above the reference range.

Twenty five of the 42 patients with iron deficiency had values for WGLF Hb within the normal range. There were a further seven patients with marginally raised concentrations (6–8  $\mu\text{g/ml}$ , calculated daily gastrointestinal blood losses 1.2–1.7 ml/d), including two patients with malignant disease (gastric cancer, 6  $\mu\text{g/ml}$ , 1.2 ml/d, oesophageal cancer 6  $\mu\text{g/ml}$ , 1.3 ml/d). Two patients had WGLF Hb concentrations of 10 and 12  $\mu\text{g/ml}$ , with calculated blood losses of borderline clinical significance at 2.0 and 2.1 ml/d, and there were only eight patients with unequivocally high values (taken as WGLF Hb >10  $\mu\text{g/ml}$ , and calculated blood loss >2 ml/d during the lavage procedure) (Fig 2). This group included all four patients with colon cancer (blood losses 5–132 ml/d).

#### Discussion

In view of the very high rates of detection of gastrointestinal diseases in anaemic patients, it has been entirely reasonable to assume that chronic blood loss from the gut is the commonest cause of iron deficiency anaemia in men and in postmenopausal women.<sup>8</sup> Lack of correlation with positive faecal occult blood test has generally been attributed to deficiencies in the guaiac based faecal occult blood methods, which are relatively insensitive and preferentially detect blood loss from the distal gastrointestinal tract. A further factor is that bleeding may occur in the form of multiple intermittent episodes, rather than as a continuous ooze.

The accurate measurement of bleeding into the gut requires a suitable assay for Hb or some other component of red blood cells, applied to a specimen representative of gut luminal contents – ideally a complete five or seven day faecal collection or several separate faecal samples. Problems associated with tests on faeces include not only the resistance to collection and handling of faecal specimens by patients, nurses, and laboratory staff, but also the potential interference in analyses by residues of meat and other dietary constituents. Patient-related variables such as intestinal transit time and faecal bulk are also relevant. The Hemoquant method is a highly sensitive analytical technique, which measures both intact and bacterially metabolised haemoglobin<sup>7</sup>; when this is used to test a timed specimen of faeces collected after a period on a meat free diet, it can detect and quantify blood loss from the proximal or distal gut. We have previously reported that this method can also readily be used with WGLF.<sup>5</sup> Use of gut

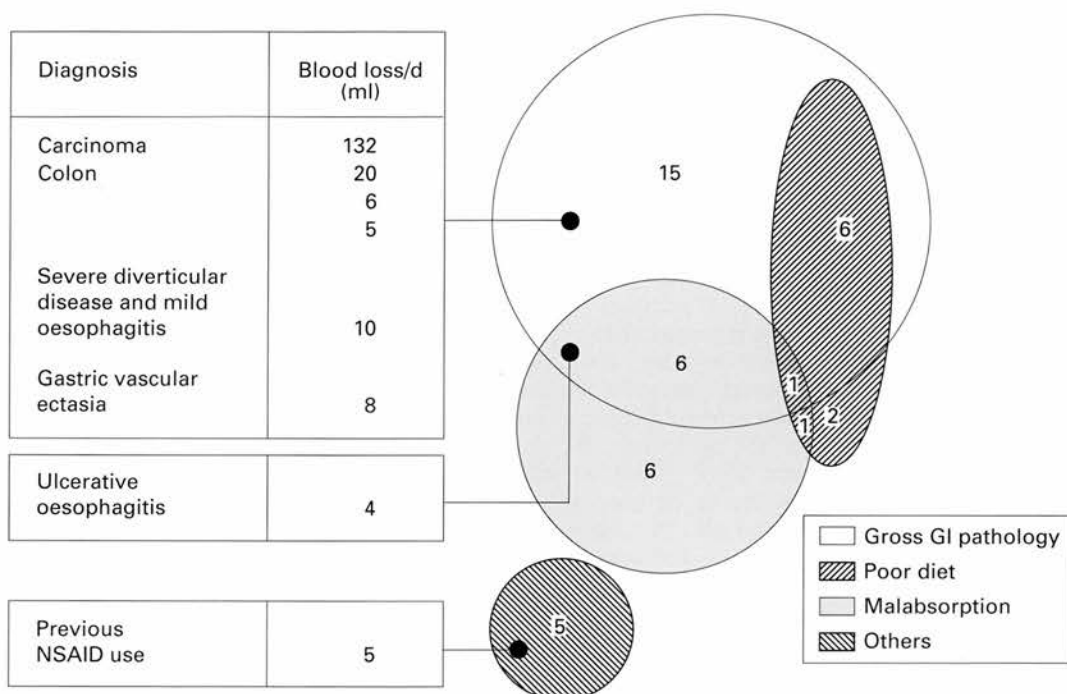


Figure 2: Distribution (within the classification shown in Figure 1) and diagnosis, for the eight patients in whom gastrointestinal blood loss (based on the whole gut lavage fluid test) was estimated to be more than 2 ml daily.

perfusion fluid as an assay material overcomes the need for dietary restrictions and unpleasant faecal collections, and specimens can be obtained when peroral gut lavage is being used to cleanse the bowel before colonic investigations. The reference range for WGLF Hb concentrations, 1–5  $\mu\text{g/ml}$ , equates to daily blood losses of around 0.2–1 ml/d. The main theoretical disadvantage of this new test of gastrointestinal bleeding is that the sampling time is limited to a few hours; further experience, and comparisons between WGLF and five day faecal collections, should show how frequently occult bleeding is discontinuous, and in what time frame.

Review of the results of WGLF Hb measurements highlights the complexity of possible aetiologies in the 42 iron deficient patients studied. Altogether 73 clinical, dietary, or iatrogenic factors were recorded – poor diet (10), gross gastrointestinal abnormality (34 in 28 patients), malabsorption (14), coagulation problems (6), and NSAID use (9). However, our test of gastrointestinal bleeding detected only eight patients who were losing more than 2 ml blood daily into the gut, including all six patients with lesions generally recognised to be important causes of occult bleeding – four with colonic cancer, one with diffuse gastric vascular ectasia, and one with severe, ulcerative oesophagitis.

There are several possible explanations for the findings. In some patients with low or normal values for WGLF Hb, gastrointestinal bleeding may be intermittent rather than a continuous ooze, as discussed above. The lesion responsible for blood loss might have healed in the interval between diagnosis of anaemia and investigation. This may well have been the case in NSAID treated patients, eight of whom had the drugs stopped as soon as anaemia was recognised. It seems likely,

however, that in many instances, the gastrointestinal lesions detected by standard investigations were simply not bleeding, and the anaemia was the result of some other cause. In the patients with calculated daily blood loss of less than 2 ml/d, based on the WGLF test, there was a high frequency of other conditions which might lead to iron deficiency, such as poor diet or malabsorption. Regrettably, in developing the protocol for the study we did not arrange a formal assessment of dietary iron intake, but a general appraisal of the diet was made by an experienced and nutritionally aware clinician. Nevertheless, this is an example of how the emphasis which we and others have placed on the development of better tests for gastrointestinal bleeding leads to relative neglect of these other clinically important features.

Absorption and bioavailability of dietary iron, and iron malabsorption, are partly inter-related. The amount of inorganic iron which is absorbed is greatly influenced by the nature of other foods taken at the same time.<sup>10</sup> A much higher proportion of haem iron than inorganic iron is absorbed,<sup>11 12</sup> and malabsorption syndromes may affect one and not the other – for example, in coeliac disease there is malabsorption of ferrous but not of haem iron.<sup>13</sup> Optimal inorganic iron absorption requires healthy gastric and small bowel mucosae, and so will be compromised after gastric surgery, and probably also in patients with atrophic gastritis and hypochlorhydria. There were seven (17%) patients in our series who had had a partial gastrectomy many years before. Coeliac disease may be expressed as a single nutrient deficiency, and some form of small bowel biopsy should be included in the investigation of all iron deficient patients. Free iron loss into the gut in association with high rates of epithelial cell proliferation and loss, is another

potential factor in patients with coeliac disease or gastritis.<sup>14</sup>

In iron deficient patients reported recently, serious diseases detected vary somewhat because of different aims of the studies, entry criteria, age, and sex. However, in all reports, a significant minority of patients with unexplained anaemia is found to have completely asymptomatic colonic cancer – 9.5% of our 42 patients – a good yield of potential curable, serious disease. There is no reason to change current clinical practice; patients with unexplained iron deficiency should have colorectal endoscopy or imaging, and probably upper gastrointestinal endoscopy at the same time. But in the majority who do not have colorectal cancers, more attention needs to be paid to the completion of the diagnostic process – separate recognition of bleeding and non-bleeding lesions, or dietary iron deficiency, and of iron malabsorption due to hypochlorhydria or coeliac disease.

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providing a source of the deficient complement protein, allowed generation of the membrane-attack complex, thus causing bacterial lysis and endotoxin release. Serum from two C6-deficient patients showed impaired bactericidal and endotoxin-releasing activity in vitro. Thus, whereas C6-deficient serum has no intrinsic bactericidal or endotoxin-releasing activity, addition of normal plasma or purified C6 reconstitutes this activity by allowing assembly of the membrane-attack complex. These data suggest that, compared with immunocompetent patients, patients with a terminal-complement-component deficiency show a reduction in endotoxin release during gram-negative infection coincident with an inability to generate the membrane-attack complex and cause bacterial lysis. Therefore, although unable to deal effectively with the organism, terminal-complement-component-deficient patients may have a decreased morbidity because they are not exposed to the sudden release of endotoxin that can occur after meningococcal infection.

Evidence that deficiency of the terminal complement component may offer a selective advantage comes from a study from the Cape area of South Africa. Among families with a history of neisserial infection, Orren and colleagues<sup>9</sup> found more C6-deficient individuals than expected. They reasoned that C6-deficient individuals show an exclusive susceptibility to neisserial infections. However, tissue damage after all gram-negative infections may be less severe, suggesting a biological advantage in having a terminal-complement-component deficiency. Decreased endotoxin release could account for the limited tissue damage, and our in-vitro studies with *E coli* suggest that the mechanism for limiting endotoxin release may operate for gram-negative organisms other than neisseria.

The administration of FFP to complement-deficient patients may be hazardous because of the possibility of provoking acute endotoxin release.

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## Haemoglobin in gut lavage fluid as a measure of gastrointestinal blood loss

W. GORDON BRYDON ANNE FERGUSON

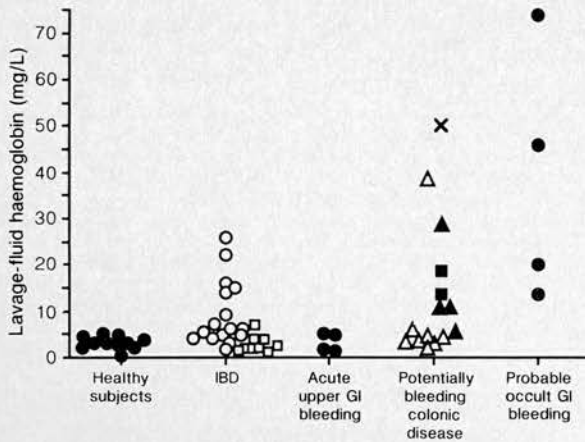
To detect and measure occult gastrointestinal bleeding, we have measured haemoglobin concentrations (by HemoQuant) in the clear fluid obtained after whole-gut lavage. In subjects with healthy gastrointestinal tracts, lavage-fluid haemoglobin concentrations were 0.5-5.1 mg/L, equivalent to daily blood loss of 0.1-1.1 mL. High concentrations were found for patients with colorectal cancer, severe diverticular disease, and rectal varices, in seven of sixteen patients with active inflammatory bowel disease, and in four patients with iron-deficiency anaemia thought to be due to gastrointestinal bleeding. In these four patients, estimated blood loss ranged from 2.6-24.5 mL per day. This method could have various research and clinical applications.

*Lancet* 1992; **340**: 1381-82.

Available methods for the detection and quantification of gastrointestinal bleeding are accompanied by such difficulties as interference by dietary constituents, dilution by faecal bulk, sensitivity too high or too low, inability to detect bleeding from the upper gastrointestinal tract, the need for complete faecal collections over several days, and the use of radioactive tracers.<sup>1</sup> Whole-gut lavage is used to cleanse the bowel for barium enema or colonoscopy, and the lavage fluid is useful for clinical investigation and research in intestinal immunity.<sup>2-6</sup> We tried to find out whether measurement of haemoglobin in whole-gut lavage fluid was a feasible approach to detection and quantification of gastrointestinal blood loss in clinical practice.

Whole-gut lavage was done as bowel preparation for barium enema, colonoscopy, or colonic surgery, in the treatment of severe constipation, and in healthy volunteers for research purposes. A diagnosis was assigned to each patient after review of the case notes several weeks after the lavage procedure, by a physician unaware of the lavage fluid test results. Patients with inflammatory bowel disease were judged to have active disease if the IgG concentration in whole-gut lavage fluid was greater than 10 mg/L.<sup>5</sup>

Subjects drank 4 L isotonic non-absorbable polyethylene-glycol-based lavage fluid (Golytely, Seward Medical, London, UK), 250 mL every 10-15 min. Solid then liquid faeces were passed, within 2 h in most patients, followed by several large volumes of virtually clear fluid. The first volume of completely clear fluid was collected



Concentrations of haemoglobin in whole-gut lavage fluid.

IBD = inflammatory bowel disease (○ = active; □ = inactive). GI = gastrointestinal. △ = benign colonic polyp; ■ = colorectal cancer; X = rectal varices; ▲ = severe diverticular disease.

for analysis; a sample was stored at  $-70^{\circ}\text{C}$ , without filtration or other processing.

Lavage-fluid haemoglobin was measured by the HemoQuant method, in which haemoglobin is converted to fluorescing polyphyrins; the reported sensitivity is less than  $1.0\text{ mg/L}$ .<sup>7</sup> A three-step purification process eliminates any interfering fluorescent material, and fluorescence of the acid extract is measured at  $600\text{ nm}$  in a spectrofluorimeter. The haemoglobin standard used was a simulated lavage fluid, containing  $100\text{ mg/L}$  human haemoglobin in  $60\text{ g/L}$  polyethylene glycol 3350,  $0.9\%$  sodium chloride, and  $0.02\%$  sodium azide.

Haemoglobin concentrations in lavage fluid from eleven subjects diagnosed as having healthy gastrointestinal tracts ranged from  $0.5$  to  $5.1\text{ mg/L}$ . The rate of fluid passage along the gut during lavage is about  $0.02\text{ L/min}$ , so the estimated  $24\text{ h}$  haemoglobin loss into the gut is  $14\text{--}147\text{ mg}$ . The daily blood loss (in mL) is equal to  $28.8$  times the lavage-fluid haemoglobin concentration divided by the blood haemoglobin concentration (taken as  $140\text{ g/L}$ ).

Seven of sixteen patients with active inflammatory bowel disease (six Crohn's disease, one ulcerative colitis) had high concentrations of haemoglobin in gut lavage fluid, whereas concentrations were normal or borderline in patients with inactive disease (figure). Four patients who had lately bled acutely from gastric lesions had normal haemoglobin concentrations in lavage fluid, as did six of seven patients with benign colonic polyps; the exception was a woman with a polyp of  $2\text{ cm}$  diameter, who was on long-term anticoagulant therapy. High concentrations were also found in patients with colorectal cancer, portal hypertension and rectal varices, severe diverticular disease of the colon, and iron-deficiency anaemia thought to be due to occult gastrointestinal bleeding. In the last group, the calculated daily blood losses were  $2.6\text{ mL}$  (von Willebrand's disease),  $5.3\text{ mL}$  (severe oesophagitis, on aspirin treatment),  $11.8\text{ mL}$  (previous gastric surgery and stomal ulcer), and  $24.5\text{ mL}$  (radiation proctitis and a symptomless gastric ulcer). Of twenty-four other patients with no clinical features to suggest gastrointestinal bleeding, twenty-two had normal lavage-fluid haemoglobin concentrations and two borderline concentrations ( $8.8\text{ mg/L}$  in a man with coeliac disease and lymphoma and  $8.9\text{ mg/L}$  in a man with pemphigoid).

Whole-gut lavage is safe and simple. After completion of bowel cleansing, the clear fluid passed per rectum can be used for various clinical investigations and research

techniques. Lavage fluid usually contains  $0.5\text{--}2.0\text{ mg}$  protein per mL (unpublished) of which  $20\text{--}30\%$  is IgA.<sup>2-4</sup> There are trace amounts of plasma-derived proteins in lavage fluid from healthy subjects, and gastrointestinal protein loss is greatly increased in active inflammatory bowel disease.<sup>5</sup> This study shows that gut losses of haemoglobin can be detected and measured by means of whole-gut lavage. The estimated daily blood loss among our normal subjects is similar to values calculated from studies with radiolabelled red cells.<sup>8,9</sup>

The HemoQuant method detects blood loss from the proximal gastrointestinal tract as well as from the colon. Thus it could be used to measure daily blood loss, in accurately timed faecal collections taken with the patient on a meat-free diet, as an alternative to studies with radiochromium-labelled red cells. Whole-gut lavage offers many practical and aesthetic advantages over faecal collections, and preparative and analytical laboratory methods are simpler.

Since gut lavage is now widely used before endoscopy, radiology, and surgery, lavage fluid for laboratory analysis can often be obtained without additional cost or patient discomfort. Measurement of lavage-fluid haemoglobin could have applications in the investigation of patients with iron-deficiency anaemia and suspected occult gastrointestinal bleeding and in assessing the importance of blood loss in patients, such as those with inflammatory bowel disease, in whom poor diet and malabsorption may also contribute to chronic iron-deficiency anaemia. Research studies based on this method should define the frequency and magnitude of gastrointestinal bleeding in patients taking anticoagulants or non-steroidal anti-inflammatory drugs and those with bleeding disorders and will establish whether colorectal polyps and cancers bleed into the gut.

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