THE EFFECT OF CYTOTOXIC CHEMOTHERAPY ON THE MUCOSA OF THE SMALL INTESTINE

by

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Abstract

This thesis investigated the effect of chemotherapy on the mucosa of the small intestine both in humans and in rats.

Introduction: Mucositis after chemotherapy for cancer is becoming increasingly important, both as a cause of patient morbidity and occasional mortality, and because the resulting toxicity potentially limits the dose, and therefore the chance of cancer cure. The reasons for mucositis becoming more predominant are two-fold; protection from bone marrow toxicity by colony stimulating factors has led to increased doses of drugs being given, and there is a drive to increase doses in order to increase cure rate. The aims of the project were to investigate the prevalence, duration and severity of mucositis in the gastrointestinal tract following chemotherapy.

Literature review: this covers the areas of mucositis, small intestinal morphology, apoptosis, nutrition and malignancy, intestinal sugar permeability, and the effects of chemotherapy on the small intestine. Most chemotherapeutic agents kill rapidly dividing cells, making the gastrointestinal tract particularly vulnerable. Cytotoxic agents kill cells at different levels of the crypt hierarchy, leading to crypt hypoplasia followed by regeneration. The exact mechanism of mucositis is not known, nor is it apparent if there are functional abnormalities of absorption, and how these correlate with symptoms such as bloating, abdominal pain and diarrhoea.

Research plan: the project is split into four areas:

1. Mucositis was studied after high dose chemotherapy and autologous blood stem cell transplantation in forty patients. Symptoms were recorded and mucositis assessed indirectly by an intestinal sugar permeability test. Oral mucositis occurred in 100% of patients, with 50% having grade 3 or 4 oral mucositis. Small intestinal symptoms (diarrhoea, vomiting) of grade 3 or 4 occurred in 41%, permeability peaking at an increase over baseline value of 6.8-fold at day 14. The conclusion from this study was that high dose chemotherapy causes a transient increase in intestinal permeability associated with small intestinal symptoms.

2. A second study was undertaken of small intestinal mucositis after both standard and high-dose chemotherapy, to further define the prevalence, duration and symptom severity at intervals of 3 days up to 14 days, and then at 28 days after chemotherapy. Symptoms were scored by questionnaire, and mucositis was assessed by an iso-osmolar sugar permeability test. Nutritional changes were small. Serum endotoxin and combined breath tests for bacterial overgrowth were unhelpful.

3. A third study assessed small intestinal mucosal histology following chemotherapy. Morphological changes began with a transient increase in crypt apoptosis at day 1 after chemotherapy, followed by a reduction in villus area, crypt length and mitotic index by day 3, the latter two rebounding to greater than baseline levels at day 16. Thus the new finding of this study was that mucositis is due to induction of early crypt apoptosis that precedes hypoplastic villous atrophy.

4. The effect of oral glutamine on ameliorating intestinal mucositis was assessed in the dark agouti (DA) rat given subcutaneous implants of isogeneic mammary adenocarcinoma and treated with methotrexate (MTX). Glutamine had no significant effect on tumour growth, nor did it ameliorate mucositis as assessed by apoptosis and villus area, crypt length and mitotic count. The conclusions were that this is a good model for further study of mucositis, and that glutamine does not protect against small intestinal mucositis.

Conclusions: The conclusion of this thesis is that small intestinal mucositis occurs symptomatically in a significant number of patients, and peaks 3-7 days after treatment. The principal mechanism is apoptosis of intestinal crypts that results in intestinal hypoplasia.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Dorothy Keefe

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ABBREVIATIONS

G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte/macrophage-colony stimulating factor
PDGF	Platelet-derived growth factor
CRTZ	Chemo receptor trigger zone
TGF	Transforming growth factor
IGF	Insulin-like growth factor
TNF	Tumour necrosis factor
EGF	Epidermal growth factor
NCI	National Cancer Institutes
HPTLC	High pressure thin layer chromatography
HPLC	High performance liquid chromatography
ECOG	Eastern Co-operative Oncology Group
CMF	cyclophosphamide, methotrexate, 5-fluorouracil
GVHD	Graft versus host disease
WHO	World health organisation

CHAPTER 1

CYTOTOXIC CHEMOTHERAPY AND THE SMALL INTESTINE

1.1 Introduction

Cytotoxic drugs are used to treat malignancy to eradicate neoplastic cells. Treatment with these drugs may, however, result in side effects as cytotoxic drugs do not always distinguish between normal and malignant cells. The efficacy of chemotherapy relies firstly on the sensitivity of the neoplasm to treatment, and secondly on the ability of normal cells to recover more quickly than cancer cells. Certain tissues, such as the bone marrow and the gastrointestinal tract, are more affected by chemotherapy, but also recover rapidly. The maximum dose of chemotherapy is limited by toxicity to normal organs. The 'dose-limiting toxicity' is that toxicity which prevents a higher dose being used. Until recently this was most commonly bone marrow suppression, with doses not reaching a level where life-threatening gastrointestinal toxicity was a major issue. Since the advent of colony-stimulating factors, which stimulate bone marrow recovery, higher doses of chemotherapy are able to be used, in an attempt to increase cancer cure rates. This has lead to an increase in prevalence of gastrointestinal toxicity, which has become dose-limiting.

Mucositis is a clinical term used to describe damage to mucous membranes after cancer chemotherapy. It is often used synonymously with oral stomatitis, but is not confined to the oral mucosa (1). It rather occurs throughout the gastrointestinal tract, and to a lesser extent in other mucosal surfaces. It is common after cancer chemotherapy, with severity and duration varying with the dose and the chemotherapeutic drugs used. The mechanism and exact anatomical distribution have not been clearly defined, although increasing work is being done

in this area. The importance of mucositis is that it limits the dose of chemotherapy. Once the exact mechanism of gastrointestinal mucositis has been defined, it should be possible to prevent mucositis without reducing the efficacy of chemotherapy, and to increase the dose of chemotherapeutic drugs in order to increase cure rates.

1.2 Definition

Mucositis refers to mucosal damage after cancer chemotherapy. The term is usually used interchangeably with oral stomatitis, but strictly applies to the entire gastrointestinal and genitourinary tracts. It is herein used to describe the damage to the small intestinal mucosa caused by chemotherapy for cancer. This thesis concentrates on small intestinal mucositis.

1.3 Hypothesis

The hypothesis of this thesis is that chemotherapy for cancer causes a dose-dependent, transient abnormality in the small intestinal mucosa, which is clinically manifest by abdominal pain, bloating and diarrhoea, by abnormal intestinal function and by morphological changes.

1.4 Aims

1. To assess the prevalence, severity and duration of gastrointestinal symptoms and nutritional changes in patients receiving cancer chemotherapy

2. To measure functional changes in the small intestine using intestinal permeability and associated changes in serum endotoxin.

3. During these studies, to assess bacterial overgrowth using a combined lactulose breath hydrogen, and ^{14}C-D-xylose breath test.

4. To measure morphological changes of mucositis in the small intestine by histomorphometry (villus area, crypt length, mitotic count), apoptotic cell count per crypt (TUNEL method), by enterocyte cell height, by brush border enterocyte height, and by integrity of enterocyte tight gap junctions.

5. To correlate gastrointestinal symptoms with functional changes, (including nutritional changes), morphological changes, dose and type of chemotherapy used, and diagnosis.

6. To assess the efficacy of oral glutamine in reducing small intestinal mucositis in the dark agouti rat mammary adenocarcinoma model.

1.5 Research Plan

The study is divided into four main stages,

1. The first study (Chapter 3) investigates the effect of high dose chemotherapy and autologous blood stem cell transplant on intestinal sugar permeability.

2. The second study (Chapter 4) investigates the symptoms, nutritional changes, and functional changes in the small intestine after chemotherapy of varying doses.

3. The third study (Chapter 5) investigates the effects of chemotherapy on the histology of the mucosa of the small intestine

4. The fourth study (Chapter 6) investigates the effect of glutamine on tumour growth and proliferation, and on methotrexate-induced small intestinal mucositis in the dark agouti rat mammary adenocarcinoma model.

CHAPTER TWO

A REVIEW OF THE LITERATURE CONCERNING CHEMOTHERAPY, MUCOSITIS AND THE SMALL INTESTINE

"The small intestinal mucosa consists of a single layer of polarised cells. This sheet of cells is moulded during embryogenesis into a complex-shaped series of villi and crypts, forming a folding of the mucosa that causes further polarisation, since the villi are the differentiated functional aspect of the tissue from which cells senesce, die and are shed into the lumen. At the opposite pole, this cell loss is precisely balanced by cell replacement in the crypts. As a consequence, there is a constant movement of cells from the crypt to the villus. This cell migration can be studied..." Potten (2).

2.1 Principles of the action of chemotherapy on tumours

The term 'chemotherapy' was coined by Paul Ehrlich, in the 1890's, in referring to the use of a drug for a selective action, the so-called 'magic bullet'. Chemotherapy for use in the treatment of cancer was introduced in the 1940's and 50's (3). The major problem with chemotherapy is the unwanted toxicity to normal tissues, as chemotherapeutic agents are unable to distinguish between normal and malignant cells. However, tumour cells are more vulnerable to the cytotoxic action of drugs than normal cells, and when drugs are used in combination, and at regular intervals, some cancers can be cured, and even more are palliated. The aim of cancer chemotherapy is to reduce the tumour cell population to zero. The 'fractional cell kill' hypothesis has been shown to be true for haematological malignancies, and has been assumed to apply to solid tumours. This hypothesis states that a particular concentration of drug for a particular time will kill a fixed proportion of tumour cells, which is independent of the absolute number of cells. With each successive cycle of chemotherapy, a fixed fraction of the remaining

cells is eliminated. So the efficacy of the treatment depends on the dose of drug, the number of cycles, and the frequency of those cycles. Six cycles of treatment will reduce a tumour size of 10^{11} cells to less than one cell, if 99% of cells are killed per cycle. The timing of cycles depends on the ability of normal tissues, such as bone marrow and gastrointestinal tract, to recover, and this usually takes 3 or 4 weeks.

Most chemotherapeutic agents act only on dividing cells, and are therefore more effective in tumours with rapidly dividing cells. Others are cell-cycle specific and act only on cells in a particular phase of the cell cycle, while a few drugs act on non-dividing cells. A large reduction in tumour size by surgery, radiotherapy or chemotherapy, may increase cell division and therefore increase the growth of the tumour and also its response to chemotherapy, so-called 'Gompertzian growth'. Combination chemotherapy is used in most tumours because it reduces the effect of drug resistance, and may allow reduced toxicity without sacrificing efficacy.

2.2 Overview of side effects of chemotherapy

As stated above, the major problem with cancer chemotherapy is the unwanted side effects on normal tissues. Traditionally the bone marrow has limited the dose of chemotherapy due to toxicity, but this has been improved with the introduction of colony stimulating factors. The common bone marrow side effects are anaemia, thrombocytopaenia and neutropaenia, with recovery being required before further doses of chemotherapy can be given. G-CSF, GM-CSF and PDGF can ameliorate all of these toxicities, and since their development the doses of chemotherapeutic drugs have increased, leading to toxicity of other organ systems, particularly the gastrointestinal tract, the genitourinary system, the nervous system and the heart. The gastrointestinal crypt epithelium is particularly vulnerable to chemotherapeutic toxicity, with

symptoms including nausea and vomiting (although these are also centrally mediated), abdominal pain, distension and diarrhoea. There is currently no successful prevention nor any cure. Treatment is limited to palliation of symptoms using oral mouth washes, antifungal agents, analgesics and resting of the bowel (4).

2.3 Mucositis

Mucositis is defined as inflammation of a mucous membrane. A dictionary sub-entry is for mucositis necroticans agranulocytica, which is defined as necrotic inflammation of mucous membranes associated with agranulocytosis. However, there is no evidence of inflammation (65). The term 'mucositis' has, through usage, come to mean the damage to the oral mucosa caused by cytotoxic chemotherapy (5), which would be more correctly called oral mucositis or stomatitis. The remainder of the gastrointestinal (GI) tract may also be involved, but it is more inaccessible and consequently has often been overlooked. Certain symptoms such as abdominal pain, bloating and diarrhoea suggest gastrointestinal involvement. The prevalence of gastrointestinal mucositis is thought to be about the same as oral mucositis, which occurs in about 20-25 % of patients after chemotherapy. In this work, mucositis is acknowledged to occur in the whole of the gastrointestinal and genitourinary tracts, but interest focuses the small intestine rather than the mouth.

While oral mucositis is discussed and acknowledged in textbooks of Oncology (1), the rest of the gastrointestinal tract receives little coverage, instead having to make do with a passing reference under particular drug regimens. Nowhere is the mechanism and course of gastrointestinal toxicity described in full, because up until now other more important toxicities have curtailed treatment long before gastrointestinal toxicity became limiting. More than half

the papers on mucositis (oral) have been published since 1990 (4), with most of the remainder being published after 1980, although the number of publications in general is increasing rapidly. Small intestinal mucositis causes morbidity, and occasional mortality (6).

When gastrointestinal toxicity has been reported in the Literature (7;8), toxicities are listed as diarrhoea, nausea and vomiting, and stomatitis. No reference is made to abdominal pain nor bloating which are the indicators of small or large bowel involvement, and can be quite sinister. The drugs most often implicated in causing mucositis are methotrexate, 5-fluorouracil, actinomycin-D, adriamycin, bleomycin and vinblastine (6), however most cytotoxic drugs do have some effect on the small intestine (9).

2.4 Gastrointestinal side effects of chemotherapy

Chemotherapy has both direct and indirect effects on the gut (10). The direct effects are due to the actual injury, and depend on the drug and dose given. Diarrhoea is reported to be the primary gut symptom. The indirect effects result from the lack of enteral intake, and attenuation in secretion of gastrointestinal hormones secondary to the bowel injury. D-xylose absorption is reduced within 7 days of methotrexate administration in children with acute lymphoblastic leukaemia (11;12). Using a cellobiose/mannitol permeability test, Daniele (13) showed a correlation between change in permeability ratio and number of days with diarrhoea following 5-fluorouracil and folinic acid in sixteen patients with advanced colon cancer, implying a small intestinal origin to the diarrhoea.

The effects of chemotherapy are probably prolonged by the lack of oral intake. Anorexia, pain, mucositis, diarrhoea and the use of intravenous fluids all compromise the exposure of the gastrointestinal mucosa to food. But even if patients do eat, damage to the enteroendocrine

cell population may interfere with secretion of gastrointestinal hormonal growth factors. Small, frequent meals will provide the best trophic effect on the bowel, and as symptoms reduce, appetite and oral intake increase, and any weight lost is usually regained between cycles of chemotherapy.

In a comprehensive review of gastrointestinal injury due to drugs, Lewis (14) includes chemotherapy as a cause of injury through-out, but there is little information on any specific area. He reports that much of the nausea and vomiting due to chemotherapy arises from stimulation of the chemoreceptor trigger zone (CRTZ), rather than through direct bowel toxicity. This is true, but it is the combination of symptoms associated with bloating and diarrhoea which points to the small bowel, and tends to happen later than the CRTZ-induced problems.

2.5 Small intestinal morphology and function

The mucosa of the gastrointestinal tract has two main functions (15;16): firstly, the digestion and absorption of dietary nutrients, and secondly, defence against noxious dietary substances and bacteria. The intestinal surface area is 200-300 m^2 and is the interface between the external and the internal environment. The digestive function acts at two levels. Firstly, luminal digestive enzymes (secreted by the pancreas) reduce starch and protein to oligomeric forms, which can then be hydrolysed by brush border enzymes. Secondly, basolateral enterocyte membrane pumps control salt and water balance and absorption.

The small intestine is protected by defences that comprise physical barrier mechanisms and immunological responses. Mucus with an alkaline pH acts as a physical barrier; saliva buffers and lubricates luminal contents. Tight intercellular junctions maintain an impermeable barrier to bacteria and large molecules, while the rapid mucosal turnover quickens adaptive responses and prevents bacterial translocation. These factors comprise the mucosal barrier that modulates the passage of molecules from the lumen to the lamina propria and also from the lamina propria to the lumen. The immunological defences consist of non-selective cells such as granulocytes, macrophages and Paneth cells which phagocytose and recycle medium-sized intracellular particles. More selective activity is provided by the expression and secretion of IgA and IgM immunoglobulin in Peyer's patches or in T cell populations in intramucosal epithelium (MALT).

Crypts are the proliferative units of the small intestine, and villi are the functional units. The crypts are small flask-shaped structures, with 6-10 crypts supporting each villus (Figure 2.1). The crypt to villus ratio is 7:1 in the duodenum and 4:1 in the ileum. The epithelial monolayer comprises four cell types: columnar enterocytes, mucus-secreting goblet cells, the Paneth cells at the base, and the rare enteroendocrine cells. The cells of the mucosal epithelium derive from stem cells which reside near the bases of the crypts (level 4). There are between 4 and 16 stem cells at the base of each crypt in the small intestine (17), but there are probably a further 30-40 potential stem cells (clonogenic cells) which could take over stem cell functions in a crisis. Estimates of stem cell numbers vary from 3 to 80, but that given by Potten is most reasonable (18). Potten (17) defines stem cells as a particular population of undifferentiated cells, which is able to proliferate, self-maintain, produce large numbers of differentiated, functional progeny, regenerate the tissue after injury, and be flexible. The true stem cell has not

been isolated, but the available evidence favours its existence (19). Gap junction communication is most effective in the stem cell zone, reducing higher up the crypt. It is thought that the stem cells produce all four crypt cell types, with the Paneth cells migrating downwards, and the others migrating upwards. Paneth cells live for approximately 25 days, and differentiate when they reach the inner part of the crypt (20), the other three cell types divide in 24-96 hours. It is possible that induction of development of the various cell phenotypes may be due to the influence of various cytokines and growth factors as well as contact with the extracellular matrix.

Stem cells divide without maturation, every 12 to 32 hours. The daughter cells of the stem cells migrate up the crypt and onto the villus, moving at about 0.75 cell positions per hour, with differentiated cells confined to the top third of the villus. Cells approaching functional competence mature but do not divide. These cells are able to digest and absorb nutrients. They produce enzymes and acquire the apical microvillar brush border membrane. Transit cells are intermediate, and both divide and mature. The more severe the injury to the small intestinal mucosa, the more differentiated transit cells can be triggered to renew stem cells and regenerate a crypt. Crypts probably start off oligoclonal, but become monoclonal following injury (21), as the remaining stem cell divides. If the number of stem cells exceeds that allowed for a crypt, the crypt may undergo stochastic fission and produce a new gland (15).

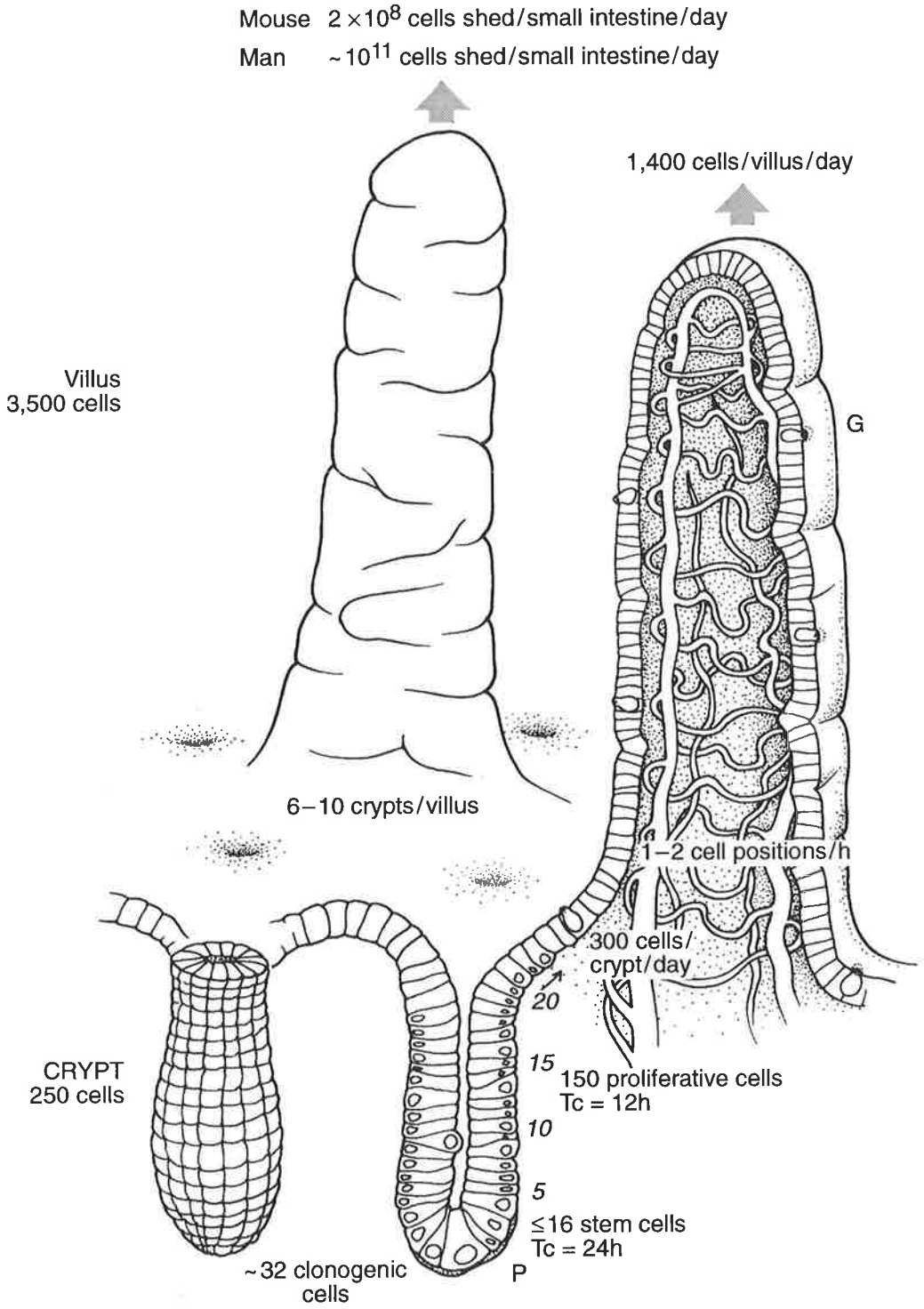


Figure 2.1 Diagrammatic representation of the three-dimensional gross architecture, histological cell organisation and cell kinetic hierarchy of the mouse ileum. Redrawn with permission from Potten (Development 110,1001–1020 1990). In longitudinal sections, the crypt cells can be identified by their position (1–20).

P = Paneth cells. Tc = cell cycle duration. G = goblet cell.

The capillary network in the villus is shown on the right.

There are many growth regulatory molecules, acting both locally and systemically. The transforming growth factors, TGF-α, TGF-β, and the insulin-like growth factors, IGF-I and IGF-II, are all thought to be important (22;23). TGF-α and TGF-β are produced by epithelial cells that express corresponding receptors on their surfaces, allowing for autocrine or paracrine regulation of cell proliferation. TGF-α is produced at the villus tip, leading to an increase in differentiated villus cells. TGF-β conversely inhibits growth. Its location however has not been so precisely defined, with both crypt and villus being possibilities. TGF-β stimulates the production of basement membrane constituents such as collagen, laminin and fibronectin. Endogenous TGF-β may play a role in promoting repair after epithelial injury. Despite its known inhibitory effects on proliferation, TGF-β has been shown to stimulate IEC-6 cell migration to reconstitute the integrity of model wounded monolayers (21), and could be important in repair after any damage. IGF-I is synthesised in the small intestine under growth hormone control, leading to proliferation of the small intestine (24). IGF-I and II receptors are found in crypt cells and on villous enterocytes (25;26). TGF-α shares a common receptor with epidermal growth factor (EGF), but TGF-α is probably the more important ligand (23).

However, the presence of food in the small intestine is actually the most important factor in maintaining the small bowel mucosa. Cessation of oral intake leads to hypoplasia. The full story still requires much investigation. Trefoil factors could also play a role in controlling epithelial cell turnover. They are increased in sites adjacent to mucosal ulceration and are thought to be produced by Goblet cells. Booth and Potten (27) developed an *in vitro* culture model using tissue obtained from the developing rat small intestine. Using this model, epithelial

growth and interaction with stromal cells can be studied. No single growth factor had overriding importance, as there were contributions from several. Insulin, IGF-I, EGF, TGF-α and PDGF were stimulating. TGF-β inhibited cell proliferation. Transferrin and glucose also stimulated the epithelium and stroma.

Proliferation in the small intestine is variable, and it is uncertain whether it is controlled more by cell loss (pull phenomenon) or by cell development in the crypt (push phenomenon). High proliferation and the high loss rate of terminally differentiated villous cells by apoptosis may protect the small intestine from malignancy. If there is a high cell mutagenicity rate, but also a high expulsion rate, there will be a low tumour rate (21)

2.6 Assessment of the structure and function of the small intestine

2.61 Historical

The small intestine has always been one of the more inaccessible areas of the body. It cannot be inspected directly, except at surgery; it does not show up on plain X-rays, and barium examinations are of low yield. The Crosby capsule allowed biopsy but not direct vision, so that yield was not particularly great, and it was not possible to tell exactly what was being biopsied.

2.62 Current 'state of the art' assessment

2.62.1 Direct

The development of upper gastrointestinal endoscopy has revolutionised our ability to inspect the small intestine, albeit only to the duodenum and sometimes the upper jejunum. It is now

possible to biopsy the duodenum under direct vision, and this correlates with results of biopsies from the jejunum.

Intestinal proliferation can be studied by metaphase arrest *in vivo*, or by uptake of tritiated thymidine and bromodeoxyuridine *in vivo and in vitro* (16). In humans, microdissection, flow cytometry, incubation with tritiated thymidine or bromodeoxyuridine, or incubation with monoclonal antibodies against antigens expressed on dividing nuclei such as Ki67 or PCNA (28) can be performed.

2.62.2 Indirect assessment of the small intestine

Barrier function is assessed by the ease of access of molecules moving from the lumen into the circulation. In health, it depends on the molecular weight and shape of the molecule. Intestinal cells have the unusual ability to gain access to nutrients both from the lumen and from the circulation. In the small intestine the lumen contributes up to half of the supply of glutamine and glucose. Reduced enteral intake leads to reduced proliferation of the small intestinal mucosa, down-regulation of digestive and absorptive enzymes, and mucosal atrophy (10). Bacterial microflora stimulates proliferation. Intestinal resection leads to a compensatory intestinal hypertrophy if the subject is allowed to feed enterally.

There are many indirect methods of measuring intestinal function, but it is not always easy to distinguish between small and large intestine using these methods. Symptoms such as abdominal pain, bloating, diarrhoea, constipation, nausea and vomiting do not distinguish between large and small intestine. Indeed, nausea and vomiting following chemotherapy are often centrally mediated. Functional measures such as xylose absorption and three-day faecal fat are either not specific or are cumbersome. Measurement of serum endotoxin is elevated if bacteria cross a malfunctioning mucosal barrier anywhere along the gastrointestinal tract, but

particularly in the colon. Small bowel bacterial overgrowth, however, may increase endotoxin crossing the small bowel mucosa.

2.62.3 Duodenal disaccharidases

The disaccharidases (lactase, sucrase and maltase) are present in the microvilli (brush border) of enterocytes. Duodenal biopsy specimens can be assayed for disaccharidase activity. A loss of disaccharidase activity indicates enterocyte damage and villous atrophy (29). Any loss of enzyme activity is usually permanent, although methotrexate has been shown to cause a significant but transient reduction in disaccharidase activity in the rat (30).

2.62.4 Intestinal permeability

Intestinal permeability measurements have been used for nearly 30 years to indirectly assess small intestinal mucosal function. To study permeability the probe must be non-toxic and metabolically inert. It must be able to be measured sensitively, accurately and easily. There are many different combinations of test sugars (31), but usually intestinal permeability is measured (32;33) by a monosaccharide (mannitol and rhamnose) and a disaccharide (lactulose). However, it does have the disadvantage of a rather sweet taste, which some subjects cannot tolerate. Other probes are polyethylene glycol (PEG), given orally and quantified in urine by gas liquid chromatography (34) and ^{51}Cr-EDTA (34). The site of increased ^{51}Cr-EDTA absorption varies with the disease under consideration: Coeliac disease is associated with increased absorption in the jejunum, Crohn's disease with the ileum, and pan-ulcerative colitis with the colon (35).

The intestinal sugar permeability test (ISPT) relies on the principle that there are two distinct pathways of aqueous permeation in the small intestinal mucosa: the high incidence "small-pore" pathway for water, urea and rhamnose, and the low incidence, large-pore pathway for larger molecules including lactulose, cellobiose, dextran, and EDTA (36). An ideal probe sugar is not metabolised, is fully excreted by the kidney, and is concentrated therefore in the urine. This is easily measured and reflects the small amount that is absorbed across the mucosa. Permeation of a test sugar depends on transit time, mucosal surface area and concentration gradient. Differences of these variables are cancelled by the use of a ratio of two probe sugars. Urine is collected for only five hours after sugar ingestion, because, after this time, colonic breakdown of lactulose will occur. The absorption ratio thus determined will be solely affected by the permeability of the small intestine.

The intestinal sugar permeability test result is expressed as a ratio of lactulose to rhamnose excreted in the urine following the ingestion of a combination sugar solution. The ratio increases in the presence of either villous atrophy or increased leakiness of the intracellular tight junctions. It was initially thought that the lactulose was absorbed via the tight junctions and that rhamnose was absorbed transcellularly, and this explains the changes seen in diseases such as coeliac disease, that is: an increased ratio in villous atrophy and with leaky tight junctions, with recovery of both permeability and morphology on treatment (37). However, there is controversy over the existence of the transcellular pathway (31;38).

2.62.5 Breath testing to assess bacterial overgrowth and oro-caecal transit time

Bacterial overgrowth has not previously been investigated in patients undergoing chemotherapy, and its role, if any, is yet to be defined. It is possible that bacterial overgrowth could increase absorption of antigens that are usually excluded, and thus set up a prolonged immune or inflammatory reaction in the small intestine. Assessment of small bowel bacterial overgrowth is problematic. The gold standard test has been bacterial culture of jejunal aspirates (39), which is difficult to do, with the more recent introduction of bacteriological analysis of mucosal biopsy specimens (40), which is also invasive. It was hoped that the use of the ^{14}C-D-xylose breath test would obviate the need for invasive procedures, but although specificity is high, sensitivity is disappointing. There is, however, also a poor sensitivity for jejunal culture (39). Riordan (40) showed a 90% sensitivity and 100% specificity for the analysis of mucosal biopsy specimens, but these are harder to do because of the need for a sterile procedure.

Using lactulose breath hydrogen to assess oro-caecal transit time is similarly problematic, with the acknowledged gold standard being a nuclear medicine test, which is invasive. This is obviously not as practical as the lactulose breath hydrogen, which is non-invasive and relatively easy to perform. The rate of hydrogen non-production is estimated to be between 30% and 60%, with less hydrogen production in adults. And in malignancy, the problem is further compounded by the fact that the malignancy itself may cause bacterial overgrowth (41).

2.63 Animal versus human

Most of the research into the small intestine has obviously been in animals (See Table2.1), because of the relative ease of access to the sight of interest. But animal work cannot be directly translated to humans, nor can one assess symptoms in animals, thus making correlation between symptoms, function and histology even more difficult.

2.7 Nutrition and malignancy

Malnutrition in cancer is common, but varies in severity from a mild abnormality to the severe form known as cachexia. Its aetiology remains uncertain (42). Weight loss at diagnosis is a poor prognostic factor, but the severity does not necessarily correlate with tumour size, indicating that protein catabolism represents a metabolic adaptation of the host rather than metabolism within the tumour. Malignancy-related malnutrition is multifactorial (43), with causes including loss of appetite, loss of taste, increased protein turnover, insulin resistance (44), the direct effects of chemotherapy and the presence of a humoral 'cachectic factor' (tumour necrosis factor[TNF] alpha). Metabolic changes are most probably mediated mainly by the cytokines TNF-α, interleukins 1 & 6 (IL-1 IL-6), and interferon gamma (IFN-γ) (45). There is increased whole body protein turnover without a change in resting energy expenditure in cancer patients with malnutrition, whereas starvation is associated with decreased resting energy expenditure. Protein-calorie malnutrition is the most important form of malnutrition in cancer patients because it leads to loss of body mass (46). Chemotherapy can cause a malabsorption syndrome by damaging rapidly dividing cells in the gut.

Cancer cachexia is most common in patients with advanced cancer, and is associated with anorexia (47). Indeed, it is a very common cause of death in cancer patients (48). Treatment of anorexia is difficult, but progestational drugs such as megestrol acetate produce some increase in appetite and food intake. Corticosteroids also have a temporary effect. If the patient has nausea as well as anorexia, then gastric emptying agents such as metoclopramide and cisapride are useful.

An increase in calories may reduce malnutrition but it doesn't treat the primary cause. Cachexia may progress in spite of adequate protein and calorie intake, without evidence of

malnutrition, and even when the tumour seems to be responding to treatment. There is no definite way of preventing this situation. Le Bricon conducted studies in tumour-bearing and healthy rats, comparing the effects of four chemotherapeutic agents given in single high dose, on weight, appetite and nitrogen balance (49). Chemotherapy caused transient weight loss, anorexia and nitrogen imbalance, but these returned to normal in all but the rats treated with cisplatin. Drug induced anorexia was worse in the tumour bearing rats, as was the nitrogen imbalance. Learned food aversions can occur in up to 48% of patients who receive drugs that are toxic to the gastrointestinal system, and the repeated association of gastrointestinal discomfort to a specific food lasts after chemotherapy is stopped.

Dewys (50) analysed the prognostic effect of weight loss prior to chemotherapy, looking at more than three thousand people enrolled in 12 ECOG trials. The frequency of weight loss ranged from 31% to 87%. Median survival was significantly reduced in the weight loss versus non-weight loss groups. Chemotherapy response rate was also lower in the weight loss groups, but only reached significance for breast cancer. Weight loss correlated with reduced ECOG performance status except for gastric and pancreatic cancer. Within the different performance status categories, weight loss was still associated with reduced median survival. The frequency of weight loss was correlated with an increase in number of metastatic sites. Big weight loss tended to be associated with tumours that present late such as gastric and pancreatic tumours. Malnutrition can affect survival by muscle wasting and increased susceptibility to pulmonary complications, or by reducing immune reactivity and therefore increasing risk of infections.

2.8 Immunology of the gastrointestinal tract and nutrition

The immunological functions of the gut are to produce secretory IgA and to provide local cellular immunity, while down-regulating the systemic reaction to antigens presented through the gut (oral tolerance) (51). Lymphoid tissue is intrinsic to the gut mucosa, and neither food nor intestinal bacterial flora is required for the acquisition of this lymphoid tissue. Indeed, the gut is the largest lymphoid organ in the body. Intestinal bacterial flora is more important than diet in expanding the size of the gut associated lymphoid population, although the exact relationship between mode of feeding, nutritional state and immunological function is not well defined. Long-term elemental diets predispose to the development of gut hypersensitivity: the high absorptive efficiency of these diets reduces caecal microflora, alters gastric acid secretion and alters small bowel motility, all of which may contribute to changing the composition of the gut flora. So alterations in luminal nutrition, increased tissue damage and increased mucosal T-cell function are all interrelated.

2.9 Response of the small intestine to injury

There is only a limited number of ways in which the small intestinal mucosa responds to injury. Small intestinal failure results in failure of digestion. Cell death results in compensatory alterations in stem cell activity with increases in frequency of cell division, first to repopulate the stem cell numbers, and second to increase daughter cell production to repopulate the crypt/villus. If a critical number of stem cells is lost, villous atrophy will occur as the remaining ones are unable to repopulate. Members of the EGF family, trefoil peptides and enteroglucagon are all involved in both normal call renewal and in repair after cell damage (52).

2.10 Apoptosis *versus* necrosis

There are two major mechanisms of cell death, namely: necrosis and apoptosis. Necrosis occurs in response to injury, and is characterised by swelling of internal and plasma membranes leading to rupture and disintegration of organised structures. There is associated inflammation. It results from injury by agents such as toxins and ischaemia, and affects groups of cells rather than individual cells (53). Apoptosis (known as programmed cell death, or cell suicide) is not associated with inflammation. It is an active process, where there is rapid condensation of the nucleus and cytoplasm, with preservation of the organelles, followed by nuclear and surface budding to produce apoptotic bodies, which are phagocytosed intact and digested by surrounding cells. It occurs in normal animals, and can be induced by certain pathological stimuli. It is more a case of active self-destruction than degeneration, and RNA and protein synthesis are required for it to occur. It was originally called shrinkage necrosis, but the term apoptosis was coined in the early 1970's by Wyllie's group (53), and it has been described as having a role that is the opposite of mitosis in tissue homeostasis. Allan (54) showed hyperthermia lead to cell death solely by apoptosis.

Apoptosis is used both to remove healthy but unwanted cells, and to remove damaged cells. It is therefore vital for tissue homeostasis. Development of malignancy may occur because of failure of homeostatic mechanisms of apoptosis. Damaged or mutated cells are normally removed by apoptosis. If this process fails then carcinogenesis may result (55). In the normal small intestine, the focus has traditionally been on the proliferative end of the crypt/villus unit. But control of cell death may actually be even more important than proliferation as a regulator of cell number, and as a preventer of malignant transformation. Potten(56) describes apoptotic cells occurring predominantly in the lower (stem cell) regions of the crypts, at the rate of about 1 apoptotic cell every five histological longitudinal crypt sections. This is about 10% of stem

cells. Following irradiation, there is an initial hypoplasia, followed by a rebound hyperplasia, and finally a decreased proliferation to reach steady state again. This second decrease apparently coincides with an increase in apoptosis at the stem cell level (55).

Hall (57) argues that the long-held belief that cell loss in the gastrointestinal tract occurs by shedding of cells from the tips of the villi into the gut lumen is not actually true, and that apoptosis along the villi (particularly towards the tip) is the major mechanism. This is, however, still controversial, as most authors have failed to find significant apoptosis towards the villus tip.

Tumours are known to undergo both apoptosis and necrosis. However, apoptosis is probably the predominant method by which tumour cells die, even without chemotherapy or radiotherapy (58). Cytotoxic chemotherapy generally causes apoptosis, but necrosis does occur at higher doses (54). Cells at different levels in the hierarchy of differentiation show varying sensitivity to the different agents (59). Radiotherapy causes an initially dose-dependent increase in apoptosis in the small intestinal crypt cells at 3 to 6 hours after exposure, acting mainly at the stem cell level. Apoptosis and necrosis can be difficult to distinguish histologically, because features such as condensation and fragmentation are shared (53). It is now possible to better identify apoptosis histochemically. Using the method of Gavrieli (60), TdT-mediated dUTP-biotin nick end labelling (TUNEL), it is now possible to measure apoptosis on formalin-fixed, paraffin-embedded tissue sections.

2.11 Comparison with coeliac disease

Moss (61) found apoptosis increased along the flattened surface of villi in untreated coeliac disease, and correlated with proliferation. After 3-6 months on a gluten-free diet, there was a reduction in apoptosis, and this almost normalised by 12 months, even though the architecture was slower to recover. Apoptosis increased to a similar degree in both sub-total and total villous atrophy, and many patients didn't return completely to normal on a gluten-free diet. Two-thirds of the apoptotic cells were found near the villus tip, 20% near the crypt base and the rest scattered in between, which gives some support to Hall's suggestion (57) that apoptosis rather than shedding is more important for cell loss into the lumen. Apoptosis was also found in the adjacent lamina propria, possibly of the intraepithelial lymphocytes. The increased epithelial apoptosis in coeliac disease may explain the paradox, that although coeliac disease is a disease of hyperproliferation, the mucosa is flat. It is the balance between proliferation and cell loss that is critical for mucosal structure, and cell loss is made up of apoptosis, necrosis and shedding in as yet uncertain proportions.

When the gut is exposed to radiation or chemotherapy, apoptosis increases at the crypt base. This suggests that apoptosis in the crypt prevents mutated cells from dividing. Apoptosis in the small intestine is very close to the stem cell level, whereas in the colon it is several positions away from the stem cell. This is thought to contribute to the fact that there is a low incidence of primary tumours in the small intestine, but a high incidence in the colon (55). Mutated stem cells in the small intestine do not survive to propagate and develop into neoplasia.

2.12 Bacterial translocation

One function of the small and large intestinal mucosa is to act as a barrier to the entry of pathogenic and commensal bacteria. The breakdown of this barrier function leads to bacterial translocation, the systemic spread of bacteria from the gut to systemic organs. Bacterial translocation is a significant cause of sepsis in critically ill patients (62). Both intact bacteria and endotoxin cause systemic infection and multi-organ failure, although there is some animal evidence that endotoxin has an anti-tumour effect which more than outweighs its negative effects (63). The causes of bacterial translocation are bacterial overgrowth, immunosuppression and loss of physical barrier (62). The route of translocation is not necessarily between the cells, and even for quite large organisms can be transcellular. Simple malnutrition alone is not sufficient to cause bacterial translocation, but parenteral nutrition and elemental diets can cause it, perhaps by inducing immunosuppression. Translocation is reduced by feeding complete enteral diets, glutamine, bombesin and by prevention of intestinal ischaemia. Chemotherapy is known to cause barrier breakdown, and therefore may increase bacterial translocation.

Berg (64) found that cyclophosphamide and prednisolone increased translocation more than did methotrexate, 5-fluorouracil and cytosine arabinoside, which is interesting because the latter drugs cause more physical damage to the mucosal barrier. This suggests that cyclophosphamide and prednisolone might act via immunosuppression.

2.13 Stem cells and regeneration of intestinal crypts after cytotoxic exposure

After exposure to cytotoxic drugs, the crypt decreases in size and proliferation (55). Minimum proliferation and crypt size values are seen from 10 to 14 hours after exposure. This cellular depletion is due to continued emigration of cells onto the villus in the presence of acute cell death and in the absence or reduction of mitosis. Thereafter there is a compensatory increase in cell cycle activity, labelling and mitosis, driven by the stem cells which starts from about 15 hours. Several authors have published work on the effects of chemotherapy on the structure and function of the small intestinal mucosa. These are summarised in tables 2.1 (structure) and 2.2 (function).

2.14 Effect of irradiation on the small intestine

Because of the difficulty in knowing the precise time at which a chemotherapeutic drug is present at a cytotoxic concentration at the intracellular targets, it is much easier to study the effects of single doses of irradiation, although they may not be exactly the same (59). Radiation leads to apoptotic cell death. Following the death of even one stem cell in a crypt, the other stem cells detect this, probably by recognition of a factor released during apoptotic death, and respond by reducing their own cycle time to 9 to 10 hours. This transiently increases their self-renewal probability in order to ensure enough stem cells are produced to repopulate the crypts (thus also tending towards monoclonality). Self-renewal is only increased transiently or there will be an unwanted reduction in differentiated cells. After five to six days there may be an overshoot in the number of stem cells and more so in the number of total cells. Radiation can also cause some cells to die through premature ageing rather than apoptosis.

Author	Drugs	Subject	Results
Trier (65;66)	MTX	human rat	hypoproliferation then
Taminiau (67)	MTX	rat	hyperproliferation
Ecknauer (68)	cyclophosphamide	human	
Perkkio (69)	ALL maintenance		
Pradja (70)	alkylating agents	rat	crypt / villus damage
Moore (71)	various	mouse	
Shaw (72)	various	human	
Gwavara (73)	MTX	human	
Smith (74)	combinations	human	
Ramadan (75)	MTX	mouse	mitochondrial changes,
Bessler (76)	oral agents	mouse	conflicting
Ijiri/Potten (77-79)	various	mouse	
Ijiri / Potten (77-79)	various	mouse	mitotic fall and apoptotic rise, variable order
Cunningham (80)	CMF	human	crypt vacuolation
Sartori (81)	platinum/etoposide	human	reduced brush border height

Table 2.1 Summary of publications concerning small intestinal morphology following chemotherapy. MTX = methotrexate. CMF = cyclophosphamide, methotrexate, 5-fluorouracil. ALL = acute lymphoblastic leukaemia.

2.15 Structure of the small intestinal mucosa following chemotherapy

Trier

In 1961, Trier presented his observations of the mucosa of the human small intestine by light and electron microscopy, following the administration of 2-5mg/kg methotrexate (65;66) Biopsies were taken before treatment and then at 1-3 hours, 3-48 hours, 48-96 hours and >4 days after treatment in 14 patients (total 48 biopsies). By light microscopy the villi were normal at all intervals. However the crypt epithelial cells showed a marked reduction in mitoses from 3 to 48 hours after methotrexate, increasing again to baseline or to slightly elevated levels by 48 to 96 hours (Figure 2.2). There was also a simultaneous appearance of discrete spherical bodies, suggestive of apoptotic bodies, within the crypt cell cytoplasm, containing nuclear DNA. These disappeared as the mitotic count returned to normal. The abnormality did not reach the villi at any stage. The mitotic count per crypt fell from 0.82 pretreatment to 0.02 at six to forty-eight hours after the drug. At beyond 96 hours, the mean mitotic count had returned to 0.87. In four patients, who had five or more biopsies, at least one of the recovery phase biopsies showed an increased mitotic count compared to baseline. Villus shortening did not occur and there was no individual epithelial cell enlargement, implying there was little cell destruction, with many epithelial cells out-living their normal life-span. It is thought that the surviving cells lived longer in order to allow time for new cells to be generated.

Gastrointestinal symptoms were mild: transient nausea, diarrhoea and vomiting only twice. Thus the human small intestine survives despite no cell replacement for at least 48 hours, with stem cells replenishing their own numbers before repopulating the crypts. In contrast, large changes were shown on electron microscopy, with patchy areas of cytoplasmic vacuolation in crypt and villus epithelium, swelling and fragmentation of the microvilli, and clumping of material within the nucleus. These had returned to normal at day 4. The damage was very

patchy, with normal cells next to severely damaged ones, indicating a marked variability in sensitivity of the cells to methotrexate.

Smith

Smith (74) studied malabsorption, and its relation to changes on jejunal biopsy in nine patients receiving chemotherapy. Absorption was measured using folate, B_{12}, carotene, calcium, prothrombin time, Schilling's test, three-day faecal fat and five-hour d-xylose absorption test. Five patients had serial biopsies under fluoroscopic control. The patients were investigated prior to treatment, 2 to 5 days into the treatment, and in the recovery phase. There were no definite abnormalities in the absorptive studies after chemotherapy. Mitotic counts in the crypts, however, fell from 0.25 before treatment to 0.05 during therapy ($p<0.01$) and rose to 0.24 in the recovery biopsy. These were lower mitotic counts with a smaller fall than Trier found. No change was seen in epithelial surface length at any time. A single patient receiving oral cyclophosphamide for 2 out of 4 weeks had no changes on biopsy. Cyclophosphamide has been shown to cause small intestinal crypt damage (77;78), as well as to have an additive immunosuppressive action. No correlation existed between degree of myelosuppression and degree of mitotic suppression in the crypts, which highlights the fact that mucositis is not associated necessarily with neutropaenia. This work confirms that of Trier, and substantiates the apparent rapid reversibility of the chemotherapeutic effect on small intestinal morphology.

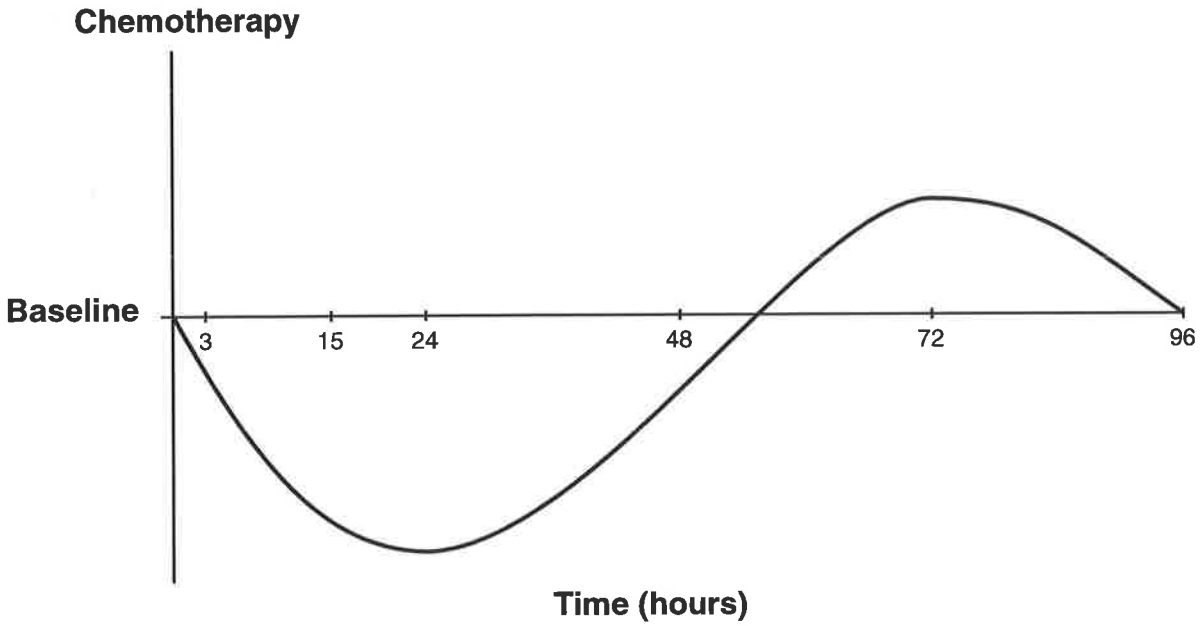


Figure 2.2 Diagram to show the proliferation cycle in the small intestine in humans following chemotherapy. Hypoproliferation was present by 3 hours, with a nadir at 24 hours, followed by a rebound hyperplasia at approximately 72 hours, and normalisation by 96 hours.

Mitchell

Mitchell (6) reports that epithelial changes persist during chemotherapy, but resolve within two weeks of stopping, slightly longer than other work would suggest. The sequence of events is: 1). Initial injury during the first three days, 2). progressive injury over the next seven days, and 3). regeneration and repair starting a few days after the therapy is stopped. This is in contrast to the work by Trier (65;66) and especially Potten (77;78) which show evidence of injury occurring in the first 24 hours.

2.16 Histology of the small intestine following chemotherapy

Ijiri and Potten

Much of the major work on the effect of chemotherapy on the small intestine has been done in animals by Ijiri and Potten (9;59). They studied the effects of different drugs on intestinal crypts and the cell hierarchy in mice. Not all chemotherapeutic agents act on the stem cells (level 4 of the crypt cell hierarchy), so that the picture is more complex than the simple idea of destruction at the stem cell level followed by regeneration from there with increased cell division and self-regeneration. Irradiation, bleomycin and adriamycin act at positions 4-6, ie the stem cells. Actinomycin D and cyclophosphamide act at positions 6-8, and mechlorethamine, vincristine, 5-fluorouracil, hydroxyurea and methotrexate act at positions 8-11 in the crypt (Figure 2.1). Antibiotics and radiation injure cells at the lower end of the crypt, antimetabolites and alkylating agents injure higher cell positions. Apoptosis and mitosis in the crypts were scored over time after chemotherapy, and largely mirrored each other, with an initial drop in mitosis coinciding with or just preceding the rise in apoptosis after most cytotoxic drugs. A minority showed mitosis decreased after apoptosis increased, and vincristine caused metaphase

arrest so that all cells died in mitosis rather than by apoptosis. This general pattern is followed by a decrease in apoptosis and rise in mitosis, as recovery begins. It is further postulated that diurnal variations in cell susceptibility to chemotherapy may be important. It may be that some of the drugs that affect cells higher up the crypts damage the stem cells if given at a different time of day, when for example more of the stem cells are in S-phase. However, the results of altering the time of administration are not yet known. 0300 is the best time for irradiation (82).

Potten also reports (2) that stem cells respond to damage by an immediate reduction in cell cycle duration and increase in self-renewal probability. If the stem cell numbers are still low after this division, they continue with the rapid cell cycles, but the self-renewal probability has to reduce a little, or not enough differentiated cells would develop.

There may be a related differential in potential for killing tumour stem cells. Drugs that kill cells in the upper crypt might have good palliative effects but not be curative, whereas drugs that affect the crypt stem cells may be able to cure cancers. However, the drugs may not affect tumour cell hierarchies in the same way as crypt cell hierarchies.

Al-Dewachi

Al-Dewachi (83) showed that hyperproliferation starts in the stem cell region of the crypt, then extends into the mid- and upper crypts. It is associated with a reduction in cell cycle time, particularly in the G_1 and S-phases.

Perkkio

Perkkio (69) biopsied the jejunum in nine children at the end of their 3 year maintenance treatment for acute lymphoblastic leukaemia. The biopsies were taken several days after the last chemotherapy dose, and were compared to normal controls. He measured disaccharidase activity in the specimens as well as morphometry and intra-epithelial lymphocytes. Villus height, crypt depth and mitotic activity were increased. These findings are consistent with those of Trier (84), with biopsies being taken in the rebound hyperplastic phase. There is a suggestion that the suppression of the intestinal mucosa may last for longer after prolonged cytostatic chemotherapy. Disaccharidases were reduced in three of nine patients. Intraepithelial lymphocyte numbers were reduced, as were IgA cells. A few patients also had reduced serum IgA and IgM. It is suggested that the defect in the mucosal barrier may allow more intestinal micro-organisms to penetrate into the blood stream.

Cunningham

Cunningham (80)studied six patients receiving CMF chemotherapy for breast cancer, performing biopsies via Crosby capsule before and 48 hours after chemotherapy. There was no change in brush border disaccharidases after chemotherapy. Pre-treatment biopsies were normal, even though all patients had received previous cycles of chemotherapy, (day 1 and day 8, every 28 days), thus implying normalisation within 21 days. Post-treatment biopsies showed a normal villus pattern, but there were focal changes in the crypts, and occasionally on the lowest parts of the villi. There were occasional vacuolated cells. Under electron microscopy the cells were seen to contain large secondary lysosomes containing cell debris. The vacuoles were membrane-limited and contained variable amounts of partially degraded cellular debris suspended in electron lucent background material, again suggestive of apoptotic bodies. No

significant abnormalities were seen in the mature enterocyte population of the post-chemotherapy villus. Five of the six patients developed vomiting, two also had diarrhoea and one had oral mucositis, but there was no correlation between the development of diarrhoea and biopsy changes. There was no significant reduction in absorption of water, sodium nor chloride. It would be expected that function should be abnormal later than morphology, because of migration of functionally immature enterocytes onto the villi. These findings again agree with those of Trier.

In the small intestine of the adult, cell proliferation occurs in the crypt region, the cells then migrating to the villus tip. The cell cycle takes approximately 48 hours and the migration some 5 days. There are therefore two potential periods of mature enterocyte dysfunction after chemotherapy, first a direct effect on the non-dividing cells of the villous region, evolving within hours, and second an effect incurred in the crypt but only evident after villous migration, ie, delayed for 5 to 7 days. However, no changes were seen in the villus even at days 5 to 7, as the unaffected cells remain longer in order to compensate, with increased mitoses, thus giving the appearance of rebound hyperplasia in the villi, without individual cells growing larger.

Gwavava

Gwavava (73) compared jejunal biopsies in ten children with acute lymphoblastic leukaemia on methotrexate, with those from ten children being investigated for failure to thrive or diarrhoea. The time from chemotherapy administration in the methotrexate group varied from 3 to 96 hours. The major abnormalities were between 24 and 72 hours after methotrexate, and consisted of cellular atrophy in the upper and middle regions of the villi, with marked enlargement of the lateral basal intercellular spaces. There was also extensive vacuolar degeneration, and some basement membrane blebbing. There was a relationship between the

time since methotrexate and severity of damage, up to 72 hours, and between dose of methotrexate and severity of damage. In the upper and middle regions of the villi there was most damage between 24 and 72 hours, and in the lower villi there was less damage at 3 and 96 hours than in the middle of the villi. This contrasts with Trier's work in that the changes are occurring in the villi rather than the crypts. The timing however is still consistent with the two-stage hypothesis above.

Sartori

Sartori (81) studied the effects of cisplatin and etoposide (in ten patients with lung cancer) on the duodenal brush border before chemotherapy, 8 days after its start, and one month after the third cycle of treatment. No significant light microscopic changes were seen, but brush border height was reduced on electron microscopy at eight days after chemotherapy, and there was villous rarefaction and variable villous heights. Damage caused to the small intestine resolved one month after chemotherapy, and there was no difference in number of episodes of vomiting between patients with or without changes to the brush border.

Shaw

Shaw (72) took biopsies before chemotherapy and again 2 days before the expected white count nadir. He saw no abnormalities prior to chemotherapy, but there was villous blunting in post treatment biopsies. There was also swelling and dilation of the mitochondria and endoplasmic reticulum, with shortened microvilli. There were no problems with absorption.

Smit

In a paper by Smit (85), seventeen patients with metastatic melanoma were studied while receiving bleomycin, dacarbazine, vindesine and dactinomycin. All had enteral tube feeding starting from 5 to 11 days before chemotherapy and lasting at least 3 weeks. Nine patients were given normal alimentation and eight were given hyperalimentation. Peroral jejunal biopsies were taken before treatment and 5 to 9 days after chemotherapy. In six patients on the normal alimentation, a third biopsy was taken 5 to 6 weeks after treatment. After chemotherapy, diarrhoea of grade 2 or greater occurred in three out of nine patients on normal alimentation and one of eight on hyperalimentation. Disaccharidases were reduced more in the hyperalimentation group, and they also had increased crypt depth in contrast to the normal alimentation group. Both groups showed reduced villus height and mitotic indices. There was no correlation between disaccharidases and morphology, and in the six biopsies post treatment, morphology had returned to normal.

There was no correlation between disaccharidases and diarrhoea, nor between mucosal morphology and diarrhoea, so the diarrhoea is probably not due to the loss of mucosal tissue, rather perhaps to an increase in small intestinal fluid and electrolyte secretion by the damaged intestine. Hyperalimentation increased the loss of maltase, sucrase and palatinase, but not lactase. This could be due to the lysosomal lactase contribution. Thus hyperalimentation caused increased migratory activity from the crypt to the villus, and it is postulated that the immature cells reaching the villus cannot produce the normal amounts of brush border enzymes. Overall there was not shown to be any benefit from hyperalimentation by the elemental diet.

Taminiau

Taminiau (67) studied jejunal epithelial structure and function after methotrexate administration in the rat. Thymidine kinase is an enzyme confined to intestinal crypt cells. Acutely, at 24 and 48 hours, there were reduced mitoses in crypt cells, shortened villi, and decreased thymidine kinase activity. At 96 hours, with methotrexate no longer detectable in serum, there was increased proliferation characterised by increased crypt mitoses, accelerated migration of enterocytes along the villi, and the presence on the villi of cells with the enzyme profile of crypt cells (decreased disaccharidase, alkaline phosphatase and sodium/potassium-ATPase, with increased thymidine kinase). Crypts depth was increased as was villus height. Villus cell numbers were decreased from 24 to 96 hours, and crypt cell numbers from 24 to 48 hours with an increase at 96 hours. Pair fed controls had similar weight losses as nutrient intake decreased, and regained weight at the same rate, indicating that malnutrition is not an important cause of the abnormalities. Occasional loose stools were seen on days 2 to 4 post methotrexate in the treatment group only. All the above studies are consistent with a pattern of transient suppression of proliferation in the crypt from 3 to somewhere between 10 and 48 hours, depending on the author, followed at 48 hours by the onset of a hyperproliferative phase. This is earlier than the changes seen in humans by Trier.

Ecknauer

Ecknauer administered cyclophosphamide to rats at a dose of 100 mg/kg (68), and then sacrificed them at various times. At 24 hours, there was a reduction in cell number per crypt, but not in crypt depth. There was a reduction in mitotic index and villus size. At 48 hours, the morphology had returned to normal with a slight increase in crypt cell count and mitotic index, but the villi were smaller then controls. At 48 hours, he also found an increase in thymidine

kinase, protein and DNA, which had not been present at 24 hours. There was no change in disaccharidases throughout.

Ramadan

Ramadan (75) found a variable reduction in size and number of mitochondria in the small intestine of mice following methotrexate, which is in direct contrast to the work by Trier.

2.17 Oral chemotherapy

Bessler (76) studied three oral chemotherapeutic agents and their effects on the small intestinal mucosa under electron microscopy. Unfortunately, no direct comparison with intravenous chemotherapy was made. Methotrexate, cyclophosphamide and 5-fluorouracil all increased mitochondrial size, and amount of rough endoplasmic reticulum, with 5-fluorouracil having the greatest effect and methotrexate the least. Methotrexate had the smallest effect on mitochondrial size, but the biggest effect on the small intestinal cell as a whole. In mice injected with methotrexate, DNA synthesis was suppressed by 90% at 3 hours. Cyclophosphamide acts via induction of immunosuppression or by a direct effect on the cell, and 5-fluorouracil acts via inhibition of thymidilate synthetase. It is possible that all three drugs affect mitochondrial DNA synthesis. Fluid retention in the small intestine due to the cytotoxic drugs was suggested as a mechanism. A single large dose of methotrexate (40 mg/m^2) given to rats induced fluid retention in the small intestine with subsequent prostaglandin E_2 (PGE_2) formation. PGE_2 has an entero-pooling effect, so that fluid accumulation may cause diarrhoea. In his electron microscopy work, Trier (66), using intravenous methotrexate, reported swelling and fragmentation of the internal structure of some of the mitochondria, suggesting that the change seen by Bessler may not be due to the oral administration of the drug.

2.18 Dose of chemotherapy and crypt survival

Moore (71) constructed dose-survival curves for different chemotherapeutic agents in the mouse, and assessed regeneration of jejunal crypts. He constructed a mathematical model to calculate the surviving fraction of crypts after a single dose of the agent. He showed that cyclophosphamide, vincristine, and hydroxyurea do not kill any crypts, whereas mechlorethamine and BCNU caused a 3-log reduction in crypts over the dose ranges used. He also constructed a table of comparison of doses required to ablate marrow versus those required to kill via gut toxicity. This calculates the gastrointestinal toxicity safety margin when using marrow ablative chemotherapy, assuming it is applicable to humans. Cyclophosphamide has a high gastrointestinal safety margin, but mechlorethamine is very gut toxic.

Thus the intestinal changes seen following chemotherapy consist of an initial increase in apoptosis and reduction in mitosis, leading to crypt hypoplasia. Regeneration follows with stem cells dividing to repopulate the crypt and then the villi, leading to a hyperplastic phase before homeostasis is restored. In man this takes four to seven days, but in rodents the cycle is only four days.

2.19 Bone marrow transplantation and the small intestine

In 1995, Forbes published two papers on gastrointestinal changes in patients undergoing bone marrow transplantation (86;87). He showed that screening upper gastrointestinal endoscopy is safe at 30 days after bone marrow transplantation. Fifty-one percent of the forty-one patients had an abnormality requiring a change in treatment either before or after transplantation. A higher number of patients had abnormalities requiring an alteration of therapy at day 30 (31%) and at day 120 (32%) than pre-transplant (24%). These findings supported those of

Williams (88). The majority of the patients was asymptomatic or had only minor symptoms at the time of the biopsies, and would not normally have had endoscopies. The abnormalities found included graft versus host disease (GVHD), infection, and erosive or ulcerative disease. Twenty-two patients also had sigmoid mucosal biopsies before and 30 days after transplantation (11 allogeneic and 11 autologous transplants). Both groups had a rise in lamina propria CD16+ mononuclear cells post transplantation, but the rise was higher in the allogeneic group. These cells appeared histologically like tissue macrophages, but there was no rise in total macrophage numbers (CD14+). In patients with acute GVHD, the lamina propria CD4+:CD8+ lymphocyte ratio fell with a decrease in CD4+ lymphocytes. There was also a fall of intraepithelial lymphocyte (IEL) numbers. Thus, lamina propria tissue macrophages were upregulated after transplantation, which may be related to the development of acute GVHD. These changes were present 30 days after chemotherapy, and are therefore likely related to bone marrow transplantation itself. However, GVHD is rare in the autologous setting as bone marrow cells are 'self', and so there may be some prolonged effect of chemotherapy/ radiotherapy involved.

In a study by Johansson (89), disruption of barrier function, as measured by ^{51}Cr-EDTA, preceded clinical findings in eighteen patients undergoing bone marrow transplantation. Permeability was significantly increased 2 days after starting chemotherapy, and remained high 10 days post stem cell infusion, while peak clinical toxicity was delayed until 7 days after stem cell reinfusion. Gastrointestinal but not oral toxicity was correlated with abnormal ^{51}Cr-EDTA permeability, which confirms the view that the oral mucosa has a poor correlation with gastrointestinal mucositis. Permeability changes were, however, unable to predict the severity of the gastrointestinal toxicity. The correlation between translocation of endotoxin and increased ^{51}Cr-EDTA absorption was shown by Ferry (90).

2.20 Irinotecan: A special case?

Irinotecan (91) is a semi-synthetic derivative of camptothecin, which is derived from an oriental tree. It is a topoisomerase-I inhibitor, and acts by blocking the rejoining of broken DNA. One of its major dose-limiting effects is diarrhoea, and it is thought to have a different mechanism from other agents. Following the administration of irinotecan to the mouse (91), the morphological changes in the ileum included epithelial vacuolation, dilation of blood vessels, polymorphonuclear leucocyte cell infiltration, and villous shortening. However, there was also goblet cell hyperplasia and excess sulphomucin in the caecum, probably leading to hypersecretion of mucin, as well as malabsorption of water and electrolytes presumably due to epithelial vacuolation and villous shortening. Mucin hypersecretion was postulated by the authors to be the major cause of irinotecan-induced diarrhoea, in contrast to cisplatin, which in the same study did not produce goblet cell hyperplasia, although it produced the same mucosal atrophy. Cisplatin also produced similar changes throughout the bowel whereas irinotecan affected the ileum more than the jejunum, and both more than the colon. High dose loperamide controls the diarrhoea induced by irinotecan in most cases. Hypersecretion of mucin, however, has not previously been thought to cause diarrhoea.

2.21 Functional changes in the small intestine following chemotherapy

The literature is summarised in Table 2.2.

Mizuno

Mizuno (92) studied the effects of mitomycin C (MMC), 5-fluorouracil (5FU) and cyclophosphamide on the small intestine by treating rats with a single dose of the agent and then assessing absorption of sulphanilamide and l-tryptophan. Thymidine kinase activity was also measured. MMC and 5FU significantly reduced the absorption of both drugs whereas cyclophosphamide had no effect. The absorption was decreased in parallel to a reduction in wet weight of the intestine. With MMC, thymidine kinase activity fell by 80% at 24 hours and a further 50% by 48 hours, returning to normal by 96 hours. 5FU effect was only measured at 48 hours and both alkaline phosphatase and thymidine kinase were depressed. Again, cyclophosphamide had no effect. In parallel to the reduction in wet weight with MMC and 5FU, there was also a reduction in total phospholipid and more so in free cholesterol. Interestingly the use of a prodrug for MMC with the same antitumour activity did not produce the same reduction in absorption of sulphanilamide nor salicylic acid.

The lack of effect of cyclophosphamide is probably due to poor distribution of the drug to the small intestine. MMC and 5FU both have effects in intravenous as well as oral forms, which implies that the systemic administration of the drug can have small intestinal effects. The plasma half-lives of the drugs are short, being minutes only, so only a small amount of drug should remain in the intestinal tissue at 48 hours when the drug absorption of sulphanilamide is most depressed. This implies that the action of MMC is not a direct toxic effect on mature enterocytes, but an indirect effect secondary to the mitotic inhibition of dividing cells. The reduction of thymidine kinase supports this theory, as thymidine kinase is related to cell

proliferation and is found mostly in the crypts. Alkaline phosphatase was less depressed, and is associated with the differentiated intestinal cells. The authors also measured the release rate of D-glucose from mucosal lipid liposomes as a measure of mucosal permeability, which increased.

Mitchell

Mitchell (6) measured absorptive function of small intestine, before, during and after chemotherapy with B12, carotene, calcium, prothrombin, Schilling's test, three day faecal fat and 5 hour D-xylose, and biopsies were also performed in five patients. Mitotic counts reduced during chemotherapy and returned to normal on recovery. There were no changes in absorptive function nor any cumulative toxicity at 6 months.

Kralovanszky

Kralovanszky (93) studied the effects of different platinum drugs on the small intestine in rats. Thymidine kinase was used as the marker of crypt cell proliferative activity, and alkaline phosphatase, sucrase and maltase were used as digestive function markers. Once again, they showed that thymidine kinase activity reached a nadir 24 hours before the other enzymes, suggesting that platinum acted via the crypt cells, and that the decrease in functional activity was only secondary. Due to inhibition of disaccharidase function, osmotically active substances can remain in the lumen thus causing diarrhoea. Thymidine kinase rebounded rapidly on day 3 implying compensatory cell proliferation, although complete recovery took more than four days. There was some difference in severity between the different platinum analogues, with iproplatin being most toxic, cisplatin being intermediate, and carboplatin being least toxic. Allan (94) showed similar results but with a longer time course in the mouse.

Author	Drugs	Subject	Results
Smith (74)	various	human	no change in absorption
Perkkio (69)	combinations	human	reduced disaccharidases
Cunningham (80)	CMF	human	no change in disaccharidases
Taminiau (67)	MTX	rat	reduced thymidilate synthetase
Lifschitz (95)	MTX	human	increased PEG recovery
Dyduch (30)	MTX	rat	reduced disaccharidases
Phelan (96)	MTX	human	reduced xylose excretion
Prajda (70)	alkylating agents	rat	reduced enzyme activity
Mizuno (92)	MMC / 5FU cyclophosphamide	rat	reduced absorption no effect
Kralovansky (93)	platinum	rat	reduced enzyme activity
Allan (94)	platinum	mouse	reduced enzyme activity
Ecknauer (68)	cyclophosphamide	rat	increased enzyme activity
Shaw (72)	various	human	no change in absorption

Table 2.2 Summary of publications concerning small intestinal function following chemotherapy. MTX = methotrexate. CMF = cyclophosphamide, methotrexate, 5-fluorouracil. MMC = mitomycin C. 5FU = 5-fluorouracil.

Prajda

Prajda (70) studied the effects of two alkylating agents on the functional and proliferating zones of the small intestine by measuring activity of thymidine kinase, xanthine oxidase, alkaline phosphatase, sucrase and maltase in isolated rat gut cell homogenates. He showed a transient decline in protein content to approximately 60% at 48 hours, which returned to normal by 5 days. Enzyme activity was also reduced, particularly for thymidine kinase (80% reduction), with the nadir at 24 hours. Xanthine oxidase was reduced less with the nadir at 48 hours, and the others much less so. Again, the timing implies that the damage to the villi was secondary to damage to crypt cells. Morphologically, there was erosion of the epithelium at the top of the villi on day 1, along with crypt degeneration and coalescence, in parallel with reduction of thymidine kinase activity. On day 2, there was a progressive destruction of the epithelium with denuding of the villi. These changes are more pronounced than those seen elsewhere in reports in the literature. Brush border enzymes decreased in parallel. On day 7 the biochemical changes had resolved but the morphology was still abnormal, particularly in the villi. This contrasts with the work by Trier, where mucosal recovery began at 48 hours, and others where it began even earlier.

2.22 The effect of chemotherapy on small intestinal permeability

Low-dose continuous methotrexate given on a weekly basis to children with acute lymphoblastic leukaemia (95) was studied by intestinal permeability as measured by polyethylene glycol (PEG) recovery after oral administration. There was a significant increase in PEG recovery in the post-dose tests, implying increased transmucosal passage of the PEG.

In an animal study (30), methotrexate was found to reduce the activity of the brush border disaccharidases lactase and maltase in rats after oral or intramuscular methotrexate. The reduction was only transient, however, and returned to normal by one to two months. Phelan (96) investigated the cumulative effects of low dose methotrexate on xylose permeability in patients with rheumatoid arthritis receiving weekly methotrexate for at least 1 year. There was a significant reduction in the urinary xylose excretion ratio in patients on methotrexate compared with controls, but there were no changes on biopsies taken 72 to 96 hours after methotrexate. This is perhaps explained by either the dose being too small, or more likely, by the biopsies being taken too late in the recovery phase. Koninkx (97) found a change in the mucins produced by the goblet cells following methotrexate, with a predominance of neutral mucin and sialomucin during the hyperproliferative phase, and neutral and sulphomucin at recovery (144 hours). This contrats with the excess sulphomucin following irinotecan (32).

Siber (98) measured urinary excretion of ^{14}C polyvinyl pyrrolidone and tobramycin, given orally to assess gastrointestinal permeability to large molecules, in ten patients having 5-fluorouracil (days 1-5 every 28) for metastatic colorectal carcinoma. He found a dose-related increase in permeability, but there was a range of responses from 2 to 20-fold. The maximum increase in permeability was 8 to 15 days after the start of treatment, which correlated with gastrointestinal symptoms. However, there wasn't a very good correlation with severity. The prevalence of infections correlated with neutropaenia during the period 17 to 24 days after treatment, but did not correlate with the peak of intestinal permeability, which makes bacterial translocation an unlikely cause of febrile neutropaenia.

Pegues (99) in 1984 reported a study where patients receiving intensive chemotherapy for acute leukaemia had D-xylose absorption measured before and immediately after chemotherapy. A big fall in excretion (implying dysfunction of the jejunal mucosal barrier) was associated with risk of severe infection, but again the timing of infection did not always correspond the the nadir of reduced D-xylose absorption. Gastrointestinal symptoms were only moderate and didn't predict well for reduced absorption.

2.23 Treatment of chemotherapy-induced small intestinal damage

Most agents act as potential preventers of mucosal damage, or protectors, rather than as cures for damage once it has occurred. Many different remedies have been tried in an attempt to cure the gastrointestinal side effects of chemotherapy, but none has so far been effective. See Table 2.3. It is hard to compare trials because of the multitude of different toxicity grading scales, but the most commonly used are the NCI Common Toxicity Criteria, or the WHO scales which are very similar (4). Each potential remedy has its devoted followers, but there is no evidence in randomised controlled trials, that any is of any benefit. Some treatments for oral mucositis are sucralfate (100;101), chamomile (102), and GM-CSF mouth wash (103). A recent overview by Sonis (4) showed that there was no proven treatment, and that all were still palliative, using pain relief, anti-fungal agents and good oral hygiene. There is, however, much interest in such agents as keratinocyte growth factor (KGF), interleukin-11 (IL-11) and transforming growth factor beta (TGF-β) for prevention of both oral and small intestinal mucositis. These will be discussed below.

Successful chemoprotectants do exist for other organ systems such as bone marrow, bladder, nervous system and heart (104). Nothing yet exists for the gastrointestinal tract. All current agents being investigated in mucositis are preventive agents. Any agent is unlikely to quickly reverse apoptosis or necrosis of cells once they were committed to that pathway.

Agent	Site of action	Result
Azelastine (105)	oral mucosa	effective in small study
Benzydamine HCl (106)	oral mucosa	success not reproducible
Sucralfate (100;101)	oral mucosa	no benefit in controlled trials
GM-CSF (103;107-109)	oral mucosa	no benefit and poorly tolerated
Elemental diet (110)	small intestine	no benefit
Glutamine (111-117)	oral mucosa and small intestine	conflicting evidence
Parenteral nutrition (118;119)	small intestine	no effect
Interleukin-11 (120-124)	small intestine	effective
Keratinocyte growth factor (KGF) (125-129)	oral mucosa and small intestine	effective
TGF-β (82;130-132)	small intestine	effective
Whey growth factor extract (WGFE) (133)	oral mucosa and ?small intestine	effective
Amifostine (134-136)	small intestine	no evidence
Misoprostol (137)	small intestine	no effect
Bombesin (138)	small intestine	promotes tumour growth

Steroids (139)	small intestine	no effect
Thymostimulin (140)	oral mucosa and small intestine	warrants further investigation
Epidermal growth factor (EGF) (10)	small intestine	warrants further investigation
GH / IGF-1 (10)	small intestine	possibly effective
Vitamin A (141;142)	small intestine	need human trials

Table 2.3. Agents reported to be useful in the prevention/treatment of mucositis.

2.24 Trials of oral and small intestinal mucositis prevention

2.24.1 Glutamine

Glutamine is the most abundant amino acid in the blood and the free amino acid pool (143;144). It is the principal fuel used by the gastrointestinal tract acting both as an ammonia scavenger and as a precursor to DNA synthesis. It is thought to be required for normal intestinal mucosal growth and function. It is also a principal fuel used for growth by most rapidly dividing tumours, and the stealing of glutamine from the host by the tumour may have a role in the development of cachexia. Supplementing glutamine appears to increase the host's glutamine metabolism without increasing that of the tumour, and work by Klimberg (111) in the rat has suggested that tumour shrinkage to methotrexate is greater in the presence of glutamine supplementation, and morbidity to the host is decreased. Glutamine also protects against radiation damage (144), and promotes healing of small intestinal mucosa after radiation (145). Glutamine stimulates the proliferative response of enterocytes (10), increases nutrient transport and increases enteral absorption of glucose in normal animals. It increases the absorption of water, sodium and chloride in animals with experimental diarrhoea. Glutamine may also be rate-limiting for synthesis of glutathione, the most important intracellular antioxidant in the body.

Intestinal mucosal epithelial cells obtain glutamine from two sources: the arterial blood across the basolateral cell membrane, and after food from the intestinal lumen across the brush border. Glutamine in the lumen depresses the rate of utilisation from the blood, but the combination is still greater than that from the arterial supply alone. Glutamine suppresses the use of glucose. It is metabolised locally to prevent it reaching neurotoxic levels in the blood. Glutamine

utilisation decreases in sepsis and increases in thermal injury. Ziegler (112) has shown that adding glutamine to total parenteral nutrition (TPN) improves nitrogen balance and decreases clinical infection. If given before damage occurs, it prevents deterioration of gut permeability and preserves mucosal structure. It reduces hospital stay which has marked effects on cost. Van der Hulst added glutamine to TPN and showed that it prevented an increase in gastrointestinal permeability and preserved mucosal structure (118). Reduction of the muscle free glutamine pool appears to be the hallmark of response to injury (146). The extent and duration of this reduced glutamine source are proportional to the severity of the illness. Shloerb (147) reported a randomised double-blind study of TPN with or without glutamine in bone marrow transplantation. The length of hospital stay was reduced 5.8 days in the glutamine arm, but there was no difference in the number of positive bacterial cultures, infection rate nor mortality. Unfortunately there was no control arm not receiving TPN. Growth hormone increases the uptake of glutamine from the small intestine, and therefore increases it actions (148).

However, high unphysiological doses of intravenous glutamine have been associated with neurotoxicity. It is postulated that a lower oral dose would be beneficial if it is delivered directly to the small intestine, where it would be metabolised locally. Recent work in humans using oral glutamine has been contradictory. Skubitz has reported a reduction in oral mucositis in 13 out of 14 patients given oral glutamine as a 'swish and swallow' preparation (113), but Jebb (114) found no effect of oral glutamine in the prevention of oral mucositis in bone marrow transplantation. In the small intestine, however, Bozzetti has found no reduction in chemotherapy induced diarrhoea in patients taking glutamine supplements (115), whereas

Elia (114) reports a possible beneficial effect on small intestinal mucositis. A recent phase III, placebo-controlled trial failed to show any benefit from glutamine (117) in reducing mucositis, but the glutamine was given concurrently and not as pretreatment.

2.24.2 Transforming growth factor-β (TGF-β)

TGF-β plays an important role in the control of cellular proliferation, and inhibits lymphocyte proliferation (149). Among the earliest events detectable in human lymphocyte stimulation are an increase in intracellular calcium and the activation and translocation of protein kinase C, both of which can be induced by anti-CD3 antibodies. Porcine TGF-β1 and -β2, and milk growth factor significantly inhibit proliferation of human lymphocytes induced by anti-CD3 antibodies (150) without inhibiting the increase of intracellular calcium nor the activation of protein kinase C. TGF-β1 is a potent growth inhibitor of cultured jejunal epithelial cells (151). It is found at highest concentration in the crypt cells but also in lesser amounts at the villus tips, and expression in the epithelium may arrest growth of cells emerging from the crypt, and induce or maintain the terminally differentiated state. In contrast, TGF-α is found at the villus tips, and not in the crypts. TGF-α and-β may play significant roles in the regulation of the balance between proliferative and differentiated cell compartments in the intestinal epithelium. In an epithelial barrier model (130), TGF-β is able to decrease the capacity of IFN-γ to disrupt epithelial barrier function. This protective effect lasted for several days after a single dose of TGF-β, and worked better if it was applied to the basolateral rather than the apical epithelial membrane. TGF-β also reduced the barrier dysfunction caused by *Cryptosporidium parvum* which implies that it is able to maintain and/or improve the barrier function of human enterocytes. Potten (82) showed that TGF-β_3 given over 24 hours prior to irradiation,

increased the number of crypts surviving the irradiation by 4 to 6 fold. After 14.5 Gy, only 35% of animals survived 12 days, whereas 95% survived >30 days when pretreated with TGF-β_3. TGF-β_3 inhibits cell cycle progression through G1 by inhibition of the cdk-/cyclin-dependent kinase activity, but 24 hour exposure is necessary in order to protect all the cells. Giving the TGF-β_3 after the irradiation abolished the effect. There is some evidence from Potten that the stem cells in the crypts have a very strong circadian rhythm compared with the other crypt cells. The stem cells have their peak DNA synthesis at 0300h with a cell cycle duration of 24 hours. When the irradiation was given at 0300h there was better protection than when it was given at 0900 or 1200h. The effects of circadian rhythms on the tumour are thought to be less, but are not very well defined.

Sonis (152) showed that the administration of TGF-β_3 to hamsters before chemotherapy reduced the incidence and severity of cheek pouch mucositis, reduced weight loss and increased survival. Theoretically it would not be advantageous to suppress proliferation during the recovery phase after injury, as this would delay healing rather than promote it. Puolakkainen developed a delivery system using alginate beads (153), in order to deliver the TGF-β directly to the gastrointestinal mucosa. There was no release in the acid stomach, but good release and good effect in the small bowel, with stem cell quiescence. As mentioned previously, epithelial cells secrete TGF-β and have TGF-β receptors, which implies there might be an inhibitory autocrine loop. TGF-β inhibits the proliferation of T and B lymphocytes, but stimulates fibrosis and angiogenesis. It is possible that secretion by tumours could be important for angiogenesis and development of tumour stroma. Thus the inhibitory effects of TGF-β on immune surveillance and the stimulatory effects on angiogenesis might promote an environment that is conducive to tumour cell growth. Rowe (154) reviewed the diverse and

often contradictory actions of TGF-β's$_{1-3}$. Suppression of breast cancer by tamoxifen has been attributed to induction of TGF-β secretion in pre-cancerous mammary duct epithelium. TGF-β's$_{1-3}$ are endogenous mediators of growth, maintenance and repair processes. They are expressed by most cells and have a wide range of autocrine, paracrine and endocrine functions via interaction with a signalling receptor complex on the cell surface. It is not certain why TGF-β_3 would be better than TGF-β_1 and TGF-β_2, which also show chemoprotective effects, both for the gastrointestinal and bone marrow stem cells. The role of TGF-β in tumour promotion/ suppression is still not resolved and will need proper safety studies, although TGF-β_3 has entered phase I studies in humans (132).

2.24.3 Interleukin-11

Interleukin-11 is a stroma derived multifunctional cytokine (123), which acts by limiting cell death. It shares many of its biological properties with IL-6. The gene is on chromosome 19. It may be up-regulated by TGF-β and IL-1, and promotes colony growth and differentiation in haemopoetic cells, plasmacytoma proliferation, and T-cell dependent development of immunoglobulin producing B cells. It would be interesting to consider the opposite approach to the one we consider with most of the chemopreventive agents, that is: are there any stimulatory factors which speed up the cell cycle following exposure to cytotoxics, and that could therefore speed up regeneration, and be administered after the injury?. IL-11 may perhaps qualify (124).

Interleukin-11 is becoming increasingly popular as a potential chemoprotectant, which reduces gut toxicity without reducing cytotoxicity on tumour in several animal models. Du (121)

showed that recombinant human IL-11 increased survival of mice treated with previously lethal doses of 5-fluorouracil and irradiation. It reduced the incidence of sepsis, and increased recovery of intestinal epithelium. The same group (120) showed that IL-11 reduced apoptosis and increased mitosis, while increasing villous length and reducing the crypt to villus ratio after irradiation.

Peterson (122) assessed IL-11's ability to reduce cellular proliferation on a rat intestinal cell line (IEC-6), because reduction of proliferation can be cytoprotective. He found that IL-11 reduced proliferative rate as measured by cell counts and [^{3}H]thymidine incorporation, and that was not mediated through TGF-β. There was delayed entry into S-phase of the cell cycle and suppression of retinoblastoma protein phosphorylation.

Potten (123)showed that IL-11 protects mouse small intestine from radiation damage, when administered prior to and during radiation therapy, by increasing the survival of intestinal clonogenic stem cells, although the mechanism of protection has not been elucidated. It was not however due to reduced proliferation. An intact epithelium can be regenerated from one cell surviving every two to five crypts. There would appear to be a hierarchy of stem cells in the crypts: firstly, six steady state stem cells which are highly radiosensitive, secondly another six more resistant cells the can regenerate the crypt (clonogenic cells), and thirdly 24 even more resistant reserve clonogenic cells. Potten reports that it is the latter group which is protected by the IL-11. Sonis (124) treated hamsters given chemotherapy and buccal irritation with IL-11 for 12 days after treatment, and showed a reduction in incidence, severity and duration of mucositis in the IL-11 treated animals, along with less weight loss, morbidity and mortality. Thus IL-11 may also treat established mucositis. IL-11 has now reached phase I testing in humans, for its platelet elevating effect (155).

2.24.4 Keratinocyte growth factor (KGF)

Keratinocyte growth factor is a member of the heparin-binding fibroblast growth factor family. It acts by binding to the KGF receptor, which is a variant of fibroblast growth factor (FGF) factor 2. KGF receptor and KGF mRNA are found within the entire gastrointestinal tract, suggesting the entire gut both synthesises and responds to KGF. KGF acts predominantly on epithelial cells, and produces various effects such as increased proliferation, migration and morphogenesis (156). Among other actions, it has been shown to promote healing of the oral mucosa, gastric mucosa (125;126), and small intestinal mucosa (127), and has begun clinical trials in humans to prevent mucositis following chemotherapy. Farrell (128;129) has shown that KGF given prior to treatment but not after treatment, increased mouse survival following 5-fluorouracil. It reduced hepatic ulcers and weight loss, and increased oral intake. The effect does not seem to be confined to 5-fluorouracil, but probably holds for most chemotherapeutic agents.

Playford (157) showed that KGF reduces basal acid secretion but does not prevent indomethacin-induced gastric damage. There is also a possibility that it can increase growth of certain tumours, as it increases CEA production by a colon cancer cell line (126).

2.24.5 GM-CSF and G-CSF

GM-CSF mouthwash has had some recent popularity after anecdotal reports that patients receiving GM-CSF suffered less mucositis. However, this was not confirmed in clinical trials (103;107) and indeed it was poorly tolerated by patients, who had less than 50% compliance with either the treatment or placebo arm in one study (107). However, a recent study (109) suggests that GM-CSF mouthwash can reduce severity and duration of oral mucositis in non-

neutropaenic cancer patients if given once mucositis has occurred. There is also a report that G-CSF can work after the event (158). This would be a very useful effect if proven.

2.24.6 Whey growth factor extract (WGFE)

Whey growth factor extract is a by-product from the manufacture of cheese. It is a very rich source of growth factors, but its contents have not yet been fully characterised. However, it is known to contain TGF-β, IGF-I and II, and fibroblast growth factor. The growth factor extract is prepared by ion exchange chromatography of cheese whey, that removes 99% of whey protein, but enriches the growth factor extract. Howarth has shown that WGFE ameliorates small intestinal mucositis in the rat (133). WGFE or vehicle was fed to the rats, for 5 to 12 days, starting on the first of three methotrexate treatment days. Weight gain, food intake, and small intestinal weight were not affected by the WGFE, but there was a reduction in severity of villous atrophy by about 50%, particularly in the mid-jejunum and proximal ileum. Sucrase was higher in the ileum of the WGFE fed rats, and bacterial translocation was significantly reduced. There are no results in humans yet, although phase I and II studies are under way.

2.24.7 Total Parenteral Nutrition (TPN)

The role of TPN itself in reducing the side effects of chemotherapy is controversial. There is no difference in side effects of chemotherapy between normally nourished and malnourished patients with respect to myelotoxicity and gastrointestinal toxicity. There is also no change in nutrition of normally nourished patients with chemotherapy. But in patients who are malnourished to start with, prealbumin, retinal-binding protein and nitrogen balance are

improved when TPN is given with chemotherapy. TPN, however, causes mucosal atrophy in rats (10); glutamine reduces this atrophy. In inflammatory bowel disease in humans, glutamine maintained the mucosa and prevented the increased permeability induced by TPN (118). It also improved survival in rats undergoing abdominal irradiation (58;159), or chemotherapy (116).

In an unpublished study, we (160) studied 60 patients undergoing high dose chemotherapy and autologous peripheral blood stem cell transplantation. The first twenty patients received TPN, the second twenty received an electrolyte solution equivalent to TPN minus the intralipid, and the final twenty received simply normal saline with electrolyte supplements as required. There was no difference in outcome between the three groups with respect to grade of oral mucositis, days in hospital, neutropaenic days nor infective episodes.

Cummins (161) described partial villous atrophy and rhamnose malabsorption in patients receiving enteral feeding via percutaneous endoscopic gastrostomy, due to poor 'luminal drive' associated with the liquid enteral feeding. Luminal drive is the stimulation to small intestinal growth and integrity that is provided by normal dietary intake.

2.24.8 Other special dietary modifications

Kehoe (162) studied the effect of a chemically defined liquid diet on rats treated with methotrexate compared to normal chow. The rationale was that both chemotherapy and diet influence the intestinal epithelium, and that controversy existed as to whether they each had the same or opposite effects. The rats received one of the two diets for 14 days, and were then given intraperitoneal methotrexate 25-50 mg/kg, and continued on the same diets. Autopsies were performed on rats that died, and survivors were followed for late toxicity. After the

methotrexate, the chemically defined liquid diet-fed rats stopped eating by day 3, developed diarrhoea, and died between 60 and 90 hours after methotrexate. The normal chow fed rats continued normal consumption in the next 3 days and maintained weight. None of them died within a two week period. At autopsy of the chemically defined liquid diet rats, the small bowel and colon appeared attenuated and almost translucent. There was evidence of intraluminal blood with pooling in the caecum. The lining of the gastrointestinal tract showed varying degrees of focal necrosis and sloughing.

In a second study, rats were fed the same two diets, and were given methotrexate or saline on day 14. Blood was taken for aerobic and anaerobic culture, small intestinal luminal contents were aspirated for culture, and segments of small and large bowel were biopsied. The chow fed rats had more positive small bowel cultures than the liquid diet fed rats both in the methotrexate and saline groups. The flora changed in the rats fed liquid diet, with increased *E.coli*, whereas the normally fed rats grew mostly Lactobacillus species and non-haemolytic Streptococci. There was no difference in either group between cultures from methotrexate or saline treated rats. The small bowel of the liquid treated rats that received methotrexate showed severe enteritis. The mucosa was thin and villi were flattened. Multiple mucosal erosions and areas of focal haemorrhage were seen. There was a polymorphonuclear leucocyte infiltrate in the crypts, and the inflammation extended through the muscularis mucosae. The colons of the same animals had only very mild mucosal erosions with disruption of the normal colonic architecture. Small areas of focal inflammation were seen in the crypts. The small bowel of the regular diet fed rats showed only minor changes, with a few flattened villi and minimal destruction of architecture. There was no difference in histology of the intestine between the rats given the two different diets and not given methotrexate, so diet alone had no effect on the mucosa, whereas methotrexate affected the mucosa dependent on the type of diet

fed. In summary, feeding a chemically defined diet to rats receiving methotrexate was lethal, and produced severe small bowel toxicity. It could be that the altered bowel flora caused bacteraemia of more toxic organisms in the presence of the severe enteritis, but there was no increase in bacteria cultured from the blood of these animals, either from the portal vein nor the inferior vena cava, and any bacteria that were cultured in all groups were lactobacilli, not the more toxic coliforms. The autopsies showed that the rats died from intraluminal bowel haemorrhage secondary to severe inflammation and destruction of small bowel mucosa after methotrexate in chemically defined liquid diet-fed rats, indicating more severe mucositis. A further study showed that the methotrexate levels in the serum remained high for longer (48 versus 24 hours) in the chemically defined liquid diet-fed rats, and this may be the cause of the increased toxicity, as methotrexate toxicity is directly related to a tissue's duration of exposure, once a critical threshold level is reached.

In contrast, Malhotra (110) has recently presented work in patients undergoing bone marrow transplantation who were fed an elemental diet. The diet (EN9444) is composed of protein (67g/l), fat (23g/l), carbohydrates (198g/l) and short chain fatty acids (EPA/DHA) at a calorific concentration of 1.3 Kcal/ml. Patients either received an ad libitum diet, or EN9444 as the sole source of calories from 72 hours before the start of treatment and throughout therapy. Forty-five patients were randomised. Two weeks after the bone marrow transplant, the elemental diet group had lower permeability than the normal diet group. The elemental diet increased early nausea and diarrhoea but reduced severity of post-bone marrow transplantation mucositis defined symptomatically.

2.24.9 Epidermal Growth Factor (EGF)

EGF is a peptide hormone secreted by the salivary glands and the specialised enteroendocrine cells in the small intestine. It is a potent mitogen for mucosa (163), but shares a receptor with TGF-α, which may be the more important cytokine for growth of the small intestine. Glutamine is an essential nutrient for EGF-stimulated intestinal cell proliferation, and the trophic effects of glutamine and EGF are additive (164). However there have been no human trials reported and the results in animals have been contradictory (165;166).

2.24.10 Growth Hormone and Insulin-like growth factor-I (IGF-1).

Growth hormone causes mucosal hypertrophy after resection, and increases amino acid transport in the small intestine due to an increased number of functional carriers in the brush border membrane (148;167). It regulates water and sodium transport in the small intestine and colon. IGF-1 is produced in a number of tissues after stimulation by growth hormone. It mediates most of the anabolic effects of growth hormone. It increases the weight and length of the small and large intestines (24), reduces the mucosal atrophy associated with TPN and maintains bowel integrity. IGF-1 and glutamine combined produce much more protein deposition in resected bowel than either IGF-1 or glutamine alone (10).

2.24.11 Other potential agents to prevent or treat mucositis

Azelastine

Azelastine, a cell membrane stabiliser and leucocyte suppressor, has been tested as a treatment for mucositis in humans by Osaki (105). Subjects, who were about to receive combined chemo-radiotherapy for head and neck cancer, were given either a mouthwash containing

Azelastine plus Vitamin C, vitamin E and glutathione, or the same mixture without Azelastine. In the Azelastine group, there were fewer cases of mucositis, with later onset and shorter duration of symptoms. However the numbers were small, with only twenty-six in the treatment group; there was no group receiving no treatment, and further studies are needed. In 1985, Sonis reported a pilot study of benzydamine hydrochloride mouth wash (106), with a beneficial effect on oral mucositis. Unfortunately the results have not been reproduced in randomised studies. Sucralfate initially showed promise, but a randomised controlled trial showed no benefit (101).

Amifostine

Amifostine is a chemoprotectant that has been shown to protect against organ toxicities of chemotherapy (134-136), particularly kidney and bone marrow, while also offering protection from radiotherapy induced damage. It is an organic thiophosphate derivative of cysteamine. While it is thought to protect the small intestine from radiotherapy-induced damage, there is no evidence that it protects the small intestine from chemotherapy-induced damage. It is thought to act as a free radical scavenger and also by attacking charged carbonium ions of activated alkylating agents, so protecting critical nucleic acids. It is accumulated more slowly and less efficiently by tumour cells, allowing it to protect the normal cells during exposure to chemotherapy or radiotherapy.

Misoprostol

Misoprostol is a prostaglandin E1 analogue. It has been used to prevent duodenal ulcers induced by non-steroidal anti-inflammatory drugs (NSAIDs) or alcohol, and therefore was investigated for a potential role in preventing the damage induced by chemotherapy (137). Eighteen patients undergoing intrahepatic arterial infusion of 5-FUDR and mitomycin C for

metastatic colorectal carcinoma were randomised to receive either misoprostol or placebo, to protect against gastrointestinal mucosal injury. Endoscopy-proven mucosal injury developed in four of ten patients on misoprostol, and three of eight patients on placebo, showing no protective effect ($p>0.1$). The high incidence of this toxicity (between 8 and 40%) was due to the inadvertent infusion of part of the stomach due to aberrant blood vessels (168). The toxic effects were confined to the duodenum, and ranged from solitary ulcers to inflammation of the entire mucosa.

Steroids

Single injections of prednisolone (139) reduce thymidine labelling and mitotic index in rat jejunal mucosa due to reduced cell production rate. Recovery occurs over 7 days. If continued on a daily basis, there is a more sustained reduction in proliferation. This would make the use of steroids to protect the gut very unlikely to work, unless a much shorter acting agent were available. Allan (94)compared the toxicity of cisplatin and its analogues, and the effect of dexamethasone on cisplatin-induced toxicity. Platinum is an extremely emetogenic drug, and acts both centrally and locally on the small intestinal mucosa. Dexamethasone, however, had no protective effect on the mucosal damage, with even a suggestion that it might worsen the damage induced by cis-platin in particular.

Hyperthermia and glutathione depletion

Laskowitz (169) assessed the effect of melphalan, hyperthermia and glutathione depletion on tumour killing and toxicity. Both hyperthermia and glutathione depletion have previously been shown to enhance melphalan effect and toxicity. The mortality rate for the mice given melphalan plus glutathione depletion plus hyperthermia was higher (53%) than for any combination of treatments or single treatment (highest mortality 13.5%). There was marked

increase in small intestinal damage with the triple therapy, consisting of crypt necrosis and epithelial denudement, whereas neither hyperthermia nor glutathione depletion alone caused increased small intestinal toxicity. Gavage of sterile water twice daily completely prevented mortality in the triple treated mice and these latter mice also showed the biggest delay in tumour growth, but the mechanism of action of this phenomenon is not understood.

Cyclophosphamide priming prior to high-dose melphalan

The use of high dose melphalan for multiple myeloma is limited by the occurrence of severe oral mucositis and diarrhoea. Priming with a low dose of cyclophosphamide, given approximately one week prior to melphalan, has been used in some centres to protect the gut from damage (170-172). The exact timing of the dose varies between centres, but there is agreement that only one dose is beneficial whereas multiple doses are deleterious. However, the use of cyclophosphamide is controversial (173), and has not been confirmed in randomised controlled trials. The mechanism is also not known, although it may relate to aligning the cell cycle in gastrointestinal mucosal cells, so that they are not susceptible to melphalan when it is administered.

Bombesin

Bombesin (138) is a tetradecapeptide originally isolated from the skin of the frog *Bombina bombina*. It is analogous to mammalian gastrin-releasing peptide. Among other actions, it stimulates growth of the gut and pancreas in neonatal rats. In adult rats it prevents the mucosal atrophy of the small intestine which is produced by a liquid elemental diet. Fox (116) showed that glutamine in an elemental diet significantly improved survival after methotrexate. The effects of bombesin and glutamine are due to their trophic effects on the small intestinal mucosa. Maintenance of gut mucosal structure is probably crucial to survival from the

enterocolitis produced by methotrexate. The actual mechanism of action of bombesin is unknown, but it may be acting through the release of other gut hormones. Unfortunately, however, bombesin also stimulates the growth of small cell lung cancer, breast cancer, prostate cancer, stomach cancer, gastrinoma and some colon and pancreas cancers. It is therefore of no use in man.

Vitamin A

There is some evidence that vitamin A protects the small intestine from methotrexate-induced damage in rodents (141;142), but no human trials are reported.

Thymostimulin

Thymostimulin (extracted thymic hormone) was used to try to prevent febrile neutropaenia (140). It failed in that regard but the patients on the thymostimulin had fewer episodes of mucositis and diarrhoea, which should be further investigated.

2.24.12. Combinations of growth factors to prevent mucositis

There is a variety of peptides associated with regulation of the small intestinal mucosa, and the challenge is to find the best strategy for chemoprevention. Glucagon-like peptide-2 is derived from pro-glucagon and is synthesised in the L cells of the small and large intestine. It increases bowel weight and villus growth in the jejunum and ileum, and shows promise as part of a possible strategy. Interactions between the different growth factors are very complex. IL-12, for example protects the bone marrow from radiotherapy, but makes gastrointestinal toxicity lethal therefore precluding its use (174). Subcutaneous GM-CSF may protect the oral mucosa

from radiation-induced mucositis (108), but it has no effect on chemotherapy-induced mucositis when given orally (107).

Gut protection during chemotherapy was reviewed by Wilmore (10). He suggested a mixture of various factors in an attempt to protect the gut. Glutamine was suggested due to its ability to stimulate gut growth and promote gut health. The other components were growth hormone, IGF-I, glucagon-like peptide-2, and IL-11, and one should consider WGFE and KGF. Byrne (175) describes a new treatment for patients with short bowel syndrome, using a combination of low-fat, complex carbohydrates, moderate protein, glutamine and growth hormone. This combination has had very promising results with increased mucosal growth, increased absorption across the remnant bowel and a reduced need for TPN. It may lead to further developments in mucosal protection.

2.25 Safety issues in mucositis prevention

Of critical importance with any agent that might potentially prevent or treat mucosal damage from chemotherapy, is that it should not adversely effect the way that chemotherapy acts on the tumour. Therefore studies of new agents must also assess tumour growth, as well as mucosal protection. Animal studies obviously must precede human studies. Although glutamine is preferentially taken up by the tumour, it is thought to enhance the effect of methotrexate on the tumour, but this has not previously been tested in a model that can assess the small bowel mucosa and the tumour simultaneously.

2.26 Summary

As can be seen from the above review of the literature, there have been many small studies of various aspects of small intestinal reactions to chemotherapy, and large amounts of data have been collected. However, there is no definitive account that covers all aspects of this problem. The above studies provide much information about oral and small intestinal mucosal damage following cytotoxic chemotherapy, particularly in rodent models, but to some extent in humans. We know that chemotherapeutic agents act on the stem cells and some other cells in the small intestinal mucosa to cause hypoproliferation followed by a rebound hyperplasia, which overcompensates for the defect before settling to normal. We do not know, however, the exact time course of these changes in humans following chemotherapy, nor how these changes relate to the symptoms of small intestinal and oral mucositis that patients experience after treatment, nor yet to the nutritional and other functional changes that occur.

This project, therefore, set out to assess symptoms, function and morphology in a large group of patients undergoing cytotoxic chemotherapy, in an attempt to provide answers, and then to set up an animal model that would enable us to simultaneously assess the effect of any chemoprotective agent on both tumour and small bowel, so that human studies would only be attempted with agents that could be shown not to promote tumour growth.

CHAPTER THREE

THE EFFECT OF HIGH-DOSE CHEMOTHERAPY ON SMALL INTESTINAL SUGAR PERMEABILITY IN HUMANS

3.1 INTRODUCTION

Mucositis is a clinical term describing a syndrome defined by mucosal ulceration and gastrointestinal symptoms (1), which is a common side effect of many forms of cancer chemotherapy. It is uncertain whether inflammation is present. Most of the limited research carried out on mucositis has been confined to the oral mucosa, as examination is easy and results of treatment are evident. Despite its common occurrence in patients having chemotherapy, the exact mechanism is unclear and there is no definitive treatment. It has become the main factor that limits higher doses of chemotherapy as bone marrow toxicity is reduced and recovery improved by the use of colony stimulating factors. Some symptoms such as nausea, abdominal pain and particularly diarrhoea would suggest an intestinal origin.

Intestinal function has been traditionally measured by xylose absorption. This monosaccharide sugar undergoes passive mediated absorption in the jejunum (37;176;177). An alternative monosaccharide test sugar is rhamnose which is passively absorbed throughout the whole of the small intestine. In this respect, it is a better measure of intestinal function. A further development has been to combine rhamnose with the disaccharide sugar lactulose. The ratio of lactulose to rhamnose is termed 'sugar permeability'.

By using the ratio of the two sugars, effects such as altered gastrointestinal transit time or mild renal impairment are cancelled out. Neither sugar is metabolised except by bacteria. Rhamnose is believed to be absorbed transcellularly by passive diffusion across enterocytes with little intestinal reserve, and thus absorption reflects total intestinal absorptive capacity (176). Thus, rhamnose is a better measure of carbohydrate absorption than xylose. There is, however, some controversy about the exact path of the monosaccharide absorption (38). Lactulose is normally excluded (<2% absorbed) with any absorption that does occur being paracellular, and presumed to involve leakiness of tight epithelial junctions between enterocytes. The advantages of this double sugar test are that it is well-tolerated, non-invasive, and that it may be repeated sequentially. In the future it could be used to assess possible treatments of mucositis. See figure 3.11.

Our aims in this study were to assess the severity and time-course of changes in intestinal permeability after high-dose chemotherapy and autologous blood stem cell transplantation, to assess ease of administration and patient tolerance of the test, and to assess incidence and severity of mucositis.

Intestinal sugar permeability

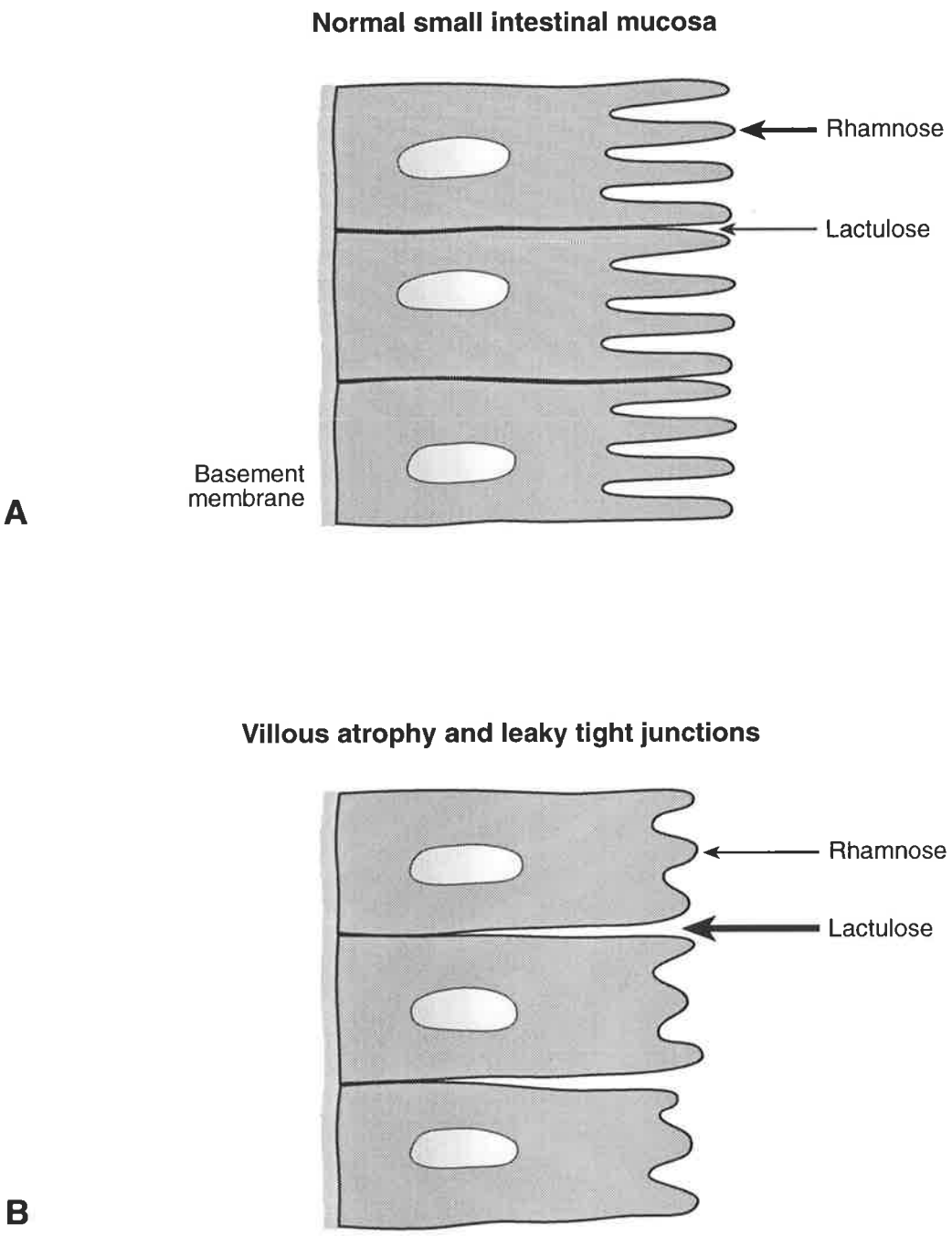


Fig. 3.1.1 Principle of the small intestinal sugar permeability test.

A. shows the normal small bowel mucosa with rhamnose being passively absorbed through the microvilli (up to 20% of an oral dose) but lactulose passing between the cells (<2% of an oral dose).

B. Shows the situation with villous atrophy (reduced rhamnose absorption) and "leaky" tight junctions (increased lactulose absorption). Diagrams are not to scale.

3.2 METHODOLOGY

Subjects

All patients receiving high dose chemotherapy and autologous stem cell transplantation at The Queen Elizabeth Hospital were eligible, and the study was approved by the Ethics of Human Research Committee at The Queen Elizabeth Hospital. The study was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study. Patients were excluded if they had pre-existing small bowel disease (such as coeliac disease, inflammatory bowel disease or small bowel malignancy), or were unwilling to participate in the study. Insulin dependent diabetes was also an exclusion criterion as the sugar solution contained glucose. The characteristics of the thirty-five patients enrolled in the study are given in Table 3.1. Four patients underwent the procedure twice. The chemotherapy involved combinations of drugs in marrow ablative doses given over several days. Most patients received combinations of drugs such as busulphan, cyclophosphamide, epirubicin and melphalan (Table 3.2). Patients were studied prior to receiving chemotherapy, and at 7, 28, 60 and 90 days after stem cell infusion. They underwent an intestinal sugar permeability test (ISPT) and an assessment of gastrointestinal toxicity on each occasion.

SEX	TREATMENT EPISODES
Male	13 (10 patients)
Female	26 (25 patients)
AGE (Years)	
median (range)	39 (16-61)

DISEASE	TREATMENT EPISODES
Breast cancer	18 (17 patients)
Acute myeloid leukaemia	6
Non-Hodgkin's lymphoma	5
Sarcoma	5 (3 patients)
Germ cell tumour	2 (1 patient)
Multiple myeloma	1
Hodgkin's disease	1
Small cell lung cancer	1
TOTAL	39 (35 patients)

Table 3.1. Patient characteristics and type of malignancy present

Drug combination	Number of episodes
cyclophosphamide carboplatin melphalan	18
carboplatin melphalan etoposide	8
cyclophosphamide busulphan methotrexate	6
carboplatin etoposide	5
etoposide	1
epirubicin cyclophosphamide	1

Table 3.2. High dose chemotherapy regimens used prior to stem cell rescue

Intestinal Permeability

After an overnight fast (water was allowed throughout), the patient was given a solution to drink comprising 5g lactulose, 1g rhamnose and 22.6g glucose in 100 ml water. The glucose acts as an osmotic stressor on the tight junctions. All urine was collected for the next 5 hours, the total volume was recorded and a 10 ml aliquot was stored for later analysis by high performance thin layer chromatography (37). Rhamnose was measured in urine using a method originally designed for plasma but with a modification to correct for urinary urea which co-elutes with rhamnose in this system (37). A quadratic equation was derived for rhamnose versus urea concentrations which had been previously established in the laboratory. Standards were regularly applied to the chromatograph plates in the analysis. Intestinal permeability was expressed as the mg ratio of urinary lactulose to rhamnose, with each expressed as the percentage of ingested dose.

The National Cancer Institute common toxicity criteria were used for assessing oral mucositis, diarrhoea, nausea and vomiting. Toxicities are graded from 1 to 4, following the convention that 1 = mild, 2 = moderate, 3 = severe and 4 = life-threatening (178). See also Table 4.21.2.

Statistics

Results were analysed using Peritz' F test, which is a robust measure of differences in the group means, where the group sizes are not identical (179). Permeability ratios and both lactulose and rhamnose absorption values were transformed to $\log_{10}(x+1)$ to normalise the data and stabilise the variance before analysis. A p value of <0.05 was used across Peritz' analysis for significance testing, but an adjusted p value was calculated for pairwise comparison. For ease of presentation, median values were also calculated as the measure of central tendency.

3.3 RESULTS

Forty-four courses of high-dose chemotherapy and autologous blood stem cell transplantation were performed in forty patients. Two patients declined to be enrolled, one patient was withdrawn for medical reasons, and two were excluded because they only had the test prior to treatment. Thus thirty-five patients were available for the study, with four undergoing the treatment twice, giving thirty-nine treatment episodes. Their characteristics are shown in Table 3.1. The female preponderance was due to a higher proportion of patients being treated for breast cancer. Thirty-four of the patients received chemotherapy as priming prior to stem cell harvesting from peripheral blood. Only one patient did not have chemotherapy prior to enrolment.

Patient acceptability

Four patients found the test sugar solution unacceptably sweet and were unable to swallow the solution on day 7. They were able to swallow it by day 9. No other adverse effects were experienced from the test.

Oral mucositis

Oral mucositis occurred in all patients, with toxicity reaching common toxicity criteria grade 3-4 in 50%. Diarrhoea with a grading of 3-4 occurred in 41% and grade 3-4 nausea and vomiting in 16%. These symptoms peaked at 7 days after chemotherapy. Table 3.3 shows the percentage of patients reaching each mucositis grade.

Toxicity/Grade	Stomatitis	Nausea/Vomiting	Diarrhoea
0	0	3	9
1	12	25	9
2	28	56	41
3	44	16	38
4	16	0	3

Table 3.3. Percentage of patients reaching a maximum of each grade of toxicity

Intestinal permeability

Intestinal permeability is shown in Figure 3.3.1. The median lactulose to rhamnose milligram excretion ratio prior to high dose chemotherapy was 0.09 (n=39). Thirteen patients had a mild abnormality prior to treatment. There was a 6.8-fold increase to a peak of 0.62 (n=36) on day 7, and this decreased thereafter to 0.12 by day 28 (n=27), to 0.08 by day 60 (n=17) and to 0.06 by day 90 (n=13).

The percentage absorptions of rhamnose are shown in figure 3.3.2. Median rhamnose absorption was 5.53% prior to treatment, and decreased 6.1-fold to 0.90% on day 7, and improved thereafter to pre-treatment levels. This indicated a reduction in monosaccharide absorption following high-dose chemotherapy.

The percentage absorptions of lactulose are shown in Figure 3.3.3. Median lactulose absorption was 0.42% prior to treatment. It increased to 0.68% on day 7, and subsequently decreased again to pretreatment levels, indicating a transient increase in disaccharide absorption following high-dose chemotherapy.

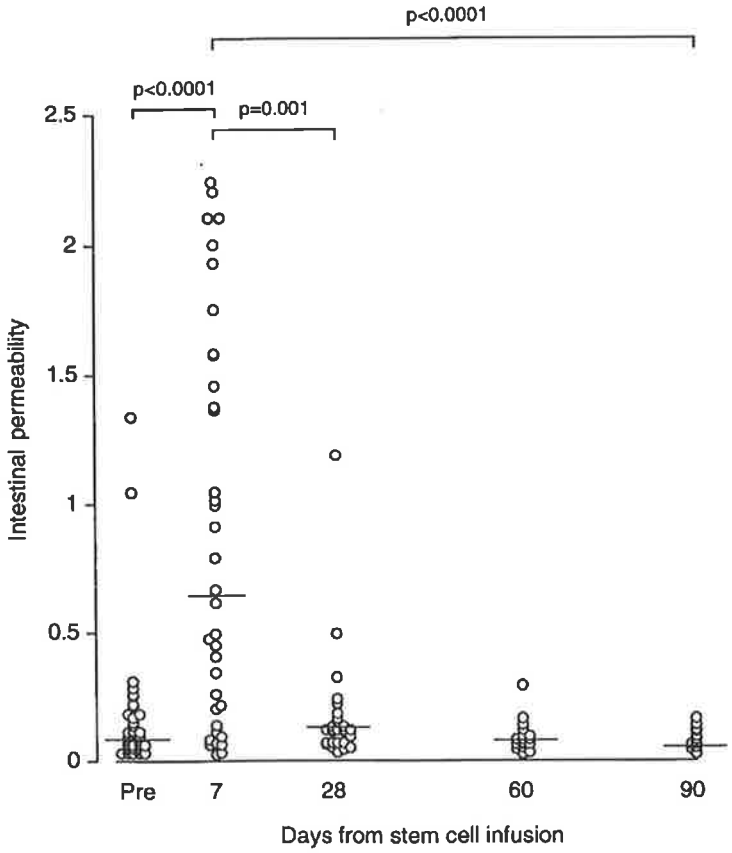


Fig. 3.3.1 Urinary lactulose to rhamnose permeability ratio in patients receiving high-dose chemotherapy and autologous blood stem cell transplantation. Data are given as individual values and the median value is indicated by a bar. P values are shown for the differences between pre and post chemotherapy values.

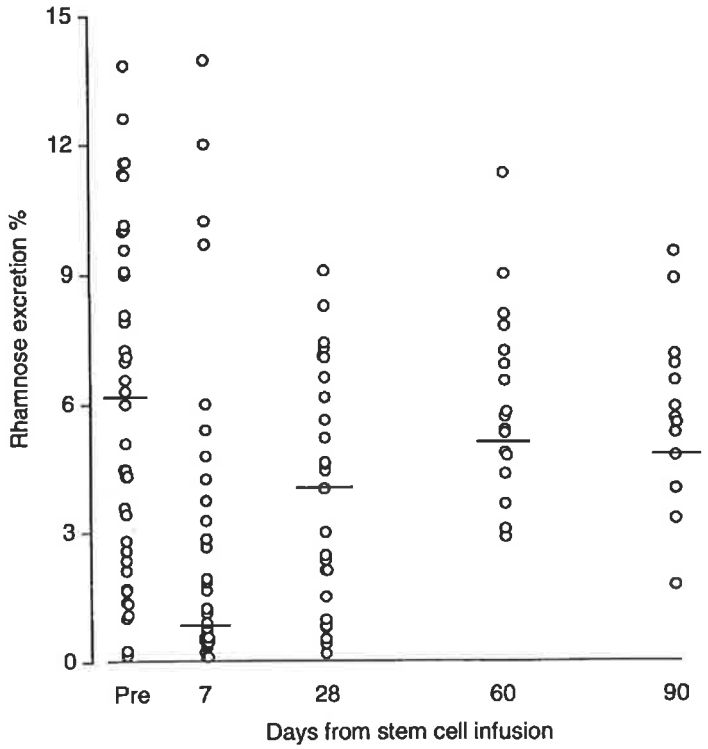


Fig. 3.3.2 Urinary rhamnose excretion in patients receiving high-dose chemotherapy and autologous blood stem cell transplantation. Data are given as individual values and the median value is indicated by a bar.

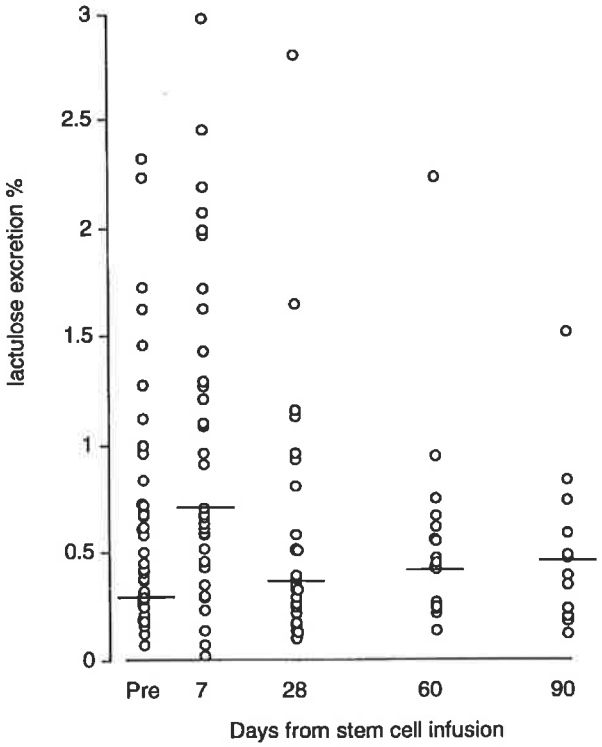


Fig. 3.3.3 Urinary lactulose excretion in patients receiving high-dose chemotherapy and autologous blood stem cell transplantation. Data are given as individual values and the median value is indicated by a bar.

3.4 DISCUSSION

The gastrointestinal tract is particularly vulnerable to the side-effects of chemotherapy presumably because of high physiological cell proliferation and turn-over (180;181). Intestinal mucosal damage is traditionally documented by biopsy either by upper gastrointestinal endoscopy or by a Crosby capsule (74). Both of these procedures may be hazardous in patients after chemotherapy and autologous blood stem cell transplantation because of low platelet and white cell counts. However, Forbes (86) was able to safely perform upper endoscopy at 30 days after transplantation, and found a large number of unsuspected gastrointestinal lesions. This and another study (182) investigated allogeneic bone marrow transplantation, but graft versus host disease or irradiation may confound interpretation of any findings. We were interested in this study in applying the non-invasive test of sugar permeability as a way of assessing intestinal damage in patients receiving high-dose chemotherapy and autologous blood stem cell transplantation.

We showed that the non-invasive intestinal sugar permeability test was well tolerated. The only problem was a transient taste aversion in some patients. In part this could have been due to the hyperosmolar test solution containing glucose. A mild abnormality of intestinal permeability was present in some patients prior to high-dose chemotherapy. This could be due to the effects of the malignancy, subclinical malnutrition, or due to prior chemotherapy. One patient had high-dose chemotherapy as first ever chemotherapy, and, interestingly, she had normal permeability before treatment. The other patients had all had at least one cycle of chemotherapy before the present high dose treatment.

The maximum sugar permeability abnormality occurred at 7 days after stem cell infusion (which is 14 days after the start of chemotherapy), and returned to normal by 28 days after stem cells (35 days after chemotherapy). The ratio continued to decline until day 90. This abnormality corresponded with the period that patients were unwell from anorexia, nausea and other gastrointestinal symptoms.

Analysis of the two components of the sugar permeability test elucidated the cause of the heightened permeability. One reason was a 62% increase in median lactulose permeability presumably through loss of integrity of the mucosal barrier constituted by tight junctions between epithelial cells. However, the second reason for increased permeability was an 84% decrease in median rhamnose permeation implying a lowered intestinal surface area for nutrient absorption. As permeability is a ratio of lactulose to rhamnose absorption, it will be increased by either an increased lactulose absorption or a decreased rhamnose absorption.

The prolonged period of increased permeability suggested that the damage was not purely due to a direct toxic effect of the chemotherapy on the mucosa, but rather there was also an indirect, prolonged component. Direct enterocyte damage would be expected to be present for the life-cycle of one cohort of enterocytes, and thus should have resolved by 48-72 hours. Studies in the rat after methotrexate have shown that there is a direct toxic effect resulting in mucosal hypoplasia, but also that this is followed by a rebound hyperplasia (67), indicating an indirect component in this animal model. Studies of the effects of methotrexate on the human small intestine by Trier (65), showed a transient reduction in crypt cell mitoses for 48 hours after methotrexate administration, followed by a return to baseline or higher by 96 hours.

Smith (74) also showed a transient reduction in crypt cell mitoses after chemotherapy using various cytotoxic drugs. The abnormal permeability lasts for longer than these changes.

The severity of the permeability defect was similar to that seen in untreated coeliac disease, where the increased lactulose/rhamnose milligram excretion ratio correlates with villous atrophy on duodenal biopsy. This suggests there may be a similar abnormality of villous atrophy at least transiently in the small intestine after chemotherapy. The flow rate of fluid through the small intestinal mucosa may also be important in determining the permeability as a high fluid flow rate leads to reduced permeability ratio (183). Chemotherapy could have transiently decreased fluid flow through the small intestinal mucosa.

The advantages of the sugar permeability test are that it is non-invasive, well tolerated, and can be repeated sequentially. It is an objective measure of small intestinal permeability, and could be used to test the efficacy of future interventions.

CHAPTER FOUR

AN INVESTIGATION INTO ABDOMINAL AND ORAL SYMPTOMS AND SMALL INTESTINAL FUNCTION FOLLOWING CYTOTOXIC CHEMOTHERAPY

4.1 INTRODUCTION

There is an increasing body of literature concerning mucositis following cytotoxic chemotherapy, although much of this pertains to the oral mucosa. The relative frequency and duration of oral and gastrointestinal symptoms has not been properly investigated, nor have these symptoms been related to functional nor histological changes in the small intestine. The first study of mucositis after high-dose chemotherapy showed a peak abnormality of symptoms and permeability after 14 days, but only weekly and monthly intervals were investigated. The question also arises of any effect of standard dose chemotherapy, and whether the tumour itself causes abnormalities in the mucosa. In contrast to the previous study, an isosmolar sugar solution was used to increase palatability.

The aims of this second study were to assess the frequency, duration and severity of oral and gastrointestinal symptoms, and nutritional changes, following chemotherapy; to measure functional changes in the small intestine using serum endotoxin levels and sugar permeability; to assess bacterial overgrowth and small intestinal transit time using a combined ^{14}C-D-xylose and lactulose breath hydrogen breath test; to compare all these parameters in a group of patients before and after chemotherapy, thus using each subject as his own control; and to analyse the subjects for any age, sex, disease or treatment effects.

4.2 METHODOLOGY

Patient selection

All patients referred to the Department of Haematology/Oncology at The Queen Elizabeth Hospital in Woodville, South Australia, with newly diagnosed malignancy, were eligible as were all patients undergoing high dose chemotherapy and autologous peripheral blood stem cell transplantation. The study was approved by the Ethics of Human Research Committee at The Queen Elizabeth Hospital and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study. A total of sixty patients was recruited, with ten of these undergoing breath testing.

There was no age exclusion and both males and females were eligible. Patients with pre-existing benign or malignant gastrointestinal disease were excluded. This included patients with inflammatory bowel disease, coeliac disease, infectious enteropathies, gastric or small bowel malignancy. Colon cancer, however, was not an exclusion criterion. Patients were excluded if they were unwilling to participate. Patients were withdrawn from the study at their own request, or if they were unable to comply with the trial protocol. Unlike the previous study (Chapter 3), insulin dependent diabetes was not an exclusion criterion, as the sugar solution no longer contained glucose.

Subjects were initially split into three groups according to the intensity of dose of chemotherapy given. The groups were

1. standard dose chemotherapy.
2. high dose cyclophosphamide priming (for stem cell harvesting).
3. high-dose chemotherapy and autologous blood stem cell transplantation.

In the final analysis, however, the two higher dose groups were combined, as there were no significant differences between them, and their combined number was half of the study population. Diseases were grouped into solid tumours *versus* haematological malignancies. Age was split into <45 *versus* >45, as the median age was 45 years.

The patient population here contrasts with the previous study (Chapter 3), where all patients were having high-dose chemotherapy with stem cell transplant, and all patients but one had had previous chemotherapy. The current group of patients includes those receiving standard dose chemotherapy as first ever chemotherapy, which enables the effects of chemotherapy to be distinguished from those due to the disease itself.

Methods

Patients were studied prior to receiving chemotherapy, and at 3, 7, 10, 14 and 28 days after starting chemotherapy. All patients underwent a nutritional assessment, completed a symptom questionnaire, performed an intestinal sugar permeability test (ISPT), and underwent blood testing for serum endotoxin on six occasions. A subset of patients had breath tests performed prior to chemotherapy, and 10 and 28 days after chemotherapy. See Table 4.2.1.

Time	*Pre*	*3*	*7*	*10*	*14*	*28*
Questionnaire	✓	✓	✓	✓	✓	✓
ISPT	✓	✓	✓	✓	✓	✓
Nutrition	✓	✓	✓	✓	✓	✓
Endotoxin	✓	✓	✓	✓	✓	✓
Breath Test	✓			✓		✓

TABLE 4.2.1 Summary of tests performed. All sixty patients underwent the first four tests, with a subset of ten undergoing breath tests.

4.21 Assessment of nutrition and symptoms

Nutritional assessment

The patients underwent nutritional assessments on six occasions, corresponding with the other tests, namely: before chemotherapy and 3, 7, 10, 14 & 28 days after chemotherapy. The tests performed were body mass index (BMI), mid-arm muscle circumference, lymphocyte count, albumin, serum transferrin, and serum and red blood cell folate. This selection of tests was chosen because no single measure gives a good representation of nutritional status. BMI can be affected by fluid gains or losses. Lymphocyte count falls due to loss of production in the bone marrow following chemotherapy. Transferrin is an acute phase protein, and albumin can be affected by protein losing states. Folate can be affected by dietary intake.

Symptoms

At each of the study times, the patients filled in a symptom questionnaire, which was designed to assess oral and gastrointestinal mucositis. The first twenty-seven patients filled in the bowel-only questionnaire, but the second thirty-three patients had the added oral-relevant questions. The questionnaire is shown in Figure 4.21.1. As there was no suitable questionnaire available in the published literature, a questionnaire was devised to provide the specific information that was required. Patients were either free to answer the questionnaire by themselves, or with the assistance of one of the trial investigators. The questions concerning normal consistency and normal frequency of bowel motion were repeated at each visit, thus providing information regarding the reliability and repeatability of answers. There was very high repeatability. The questionnaires enabled determination of grade of mucositis according to the NCI common toxicity criteria. See Table 4.21.2. An overall score was allocated to each patient for 'gastrointestinal toxicity', and each individual toxicity was recorded.

Please answer the following questions.

Name: **Unit Record Number:**

Date of chemotherapy:

In the last 24 hours have you had any of the following?

1. Abdominal Pain ☐Yes ☐No.

If yes, was it ☐sharp ☐dull

☐continuous ☐intermittent

☐relieved by opening bowels ☐not relieved.

Pain lasted ☐seconds ☐minutes ☐hours

2. Abdominal Bloating ☐Yes ☐No

3. Blood in bowel motion ☐Yes ☐No

4. Mucus in bowel motion ☐Yes ☐No

How many times have you had your bowels open in the last 24 hours?

☐0 ☐1-3 ☐4-6 ☐7-9 ☐>9

How many times per day is normal for you?

☐<1 ☐1-3 ☐4-6 ☐7-9 ☐>9

What was the consistency?

☐watery ☐loose ☐semi-formed ☐formed ☐hard

What is normal for you?

☐watery ☐loose ☐semi-formed ☐formed ☐hard

Have you been up at night to have your bowels open? ☐Yes ☐No

Have you had any accidents/incontinence? ☐Yes ☐No

(added after patient 27)

IN THE LAST 24 HOURS:

Have you had any mouth pain? ☐Yes ☐No

If yes, was it ☐mild ☐moderate ☐severe

Were you able to ☐eat + drink ☐drink only ☐not eat nor drink

Do you have any mouth ulcers? ☐Yes ☐No

Have you had any nausea? ☐Yes ☐No

If yes, were you able to eat ☐normally ☐a small amount ☐not eat

Have you vomited in the last 24 hours? ☐Yes ☐No

If yes, how many times?
☐1 ☐2-5 ☐6-10 ☐>10

Figure 4.21.1. Chemotherapy and the small intestine: symptom questionnaire. All sixty patients answered the first page, with the last 33 patients answering the second page.

Grade	1	2	3	4
Nausea	able to eat reasonable intake	intake significantly decreased, but can eat	no significant intake	
Vomiting episodes per 24 hours	1	2-5	6-10	>10 or needs parenteral support
Diarrhoea	increase of 2-3 stools per day over pretreatment	increase of 4-6 or nocturnal stools or moderate cramping	increase of 7-9 or incontinence or severe cramping	increase of >9 or gross blood or need for parenteral support
Stomatitis	painless ulcers, erythema/ mild soreness	painful erythema, oedema or ulcers but can eat	painful erythema, oedema or ulcers and cannot eat	requires parenteral or enteral support

Table 4.21.2 Assessment of mucositis using the NCI common toxicity criteria. These criteria measure toxicity on a scale of 0 to 4, with 1 being mild and 4 being life-threatening.

Where common toxicity criteria do not apply, or in case of concomitant diseases use the following score:

score 1	**score 2**	**score 3**	**score 4**
mild	moderate	severe	life-threatening

4.22 Intestinal sugar permeability test

A double sugar permeability test with lactulose and rhamnose was used in this study. A third sugar, glucose, was used in the high-dose chemotherapy study (Chapter 3) in order to place an osmotic stress on the tight junctions, which increases the sensitivity of the test, by increasing the likelihood of an abnormal lactulose absorption being detected. It also, however, made the solution very sweet, and some patients found this unpleasant after chemotherapy. In this study, therefore, an isotonic solution, without the glucose, was used. This was better tolerated, but had the disadvantage of reducing sensitivity (see Result 4.32 below).

After an overnight fast, with water being allowed throughout, the patient was given a solution of sugars to drink. This comprised 5g lactulose and 1g rhamnose in 100 ml water (isotonic sugar solution). All urine was collected for the next 5 hours, the total volume was recorded, and a 10 ml aliquot was stored for later analysis, by high performance liquid chromatography. In the high-dose chemotherapy study, we used a method of HPTLC to assay the urine for sugars, as described in Chapter 3, but in this study, we changed to the modified HPLC method as described by Miki (32).The change occurred due to an improvement in technique in the laboratory. The methodology of Miki uses an amine-modified silica column and refractive index detection. The normal range for a paediatric and adolescent population was 0.047 +/- 0.018. A group of healthy adult controls had a similar range. The patient preparation, sugar ingestion and urine collection procedures were the same as for the HPTLC.

4.23 Endotoxin assay

Endotoxin, which is produced by gram negative bacteria, is normally present in the colon, and possibly in the small intestine in the presence of bacterial overgrowth. It is generally unable to cross an intact mucosal barrier to enter the blood stream. Small amounts of endotoxin manage to enter the blood, are cleared in the liver and are not normally detectable. However, in the presence of mucosal barrier breakdown and malfunction, sufficient endotoxin may cross the intestinal barrier to overwhelm the hepatic clearance mechanisms and circulate in the peripheral blood. This could be a potential source for the increased systemic infection which occurs in the presence of mucosal barrier failure.

Serum was collected on all patients prior to chemotherapy and then at 3, 7, 10, 14 and 28 days after chemotherapy. Blood was collected into sterile, pyrogen-free tubes. It was centrifuged at 2000 revolutions per minute for 10 minutes, and the supernatant was transferred in a sterile manner into pyrogen-free glass tubes. Specimens were frozen at -20° Celsius until assayed. They were batched for ease of assay.

Specimens were assayed using the Bio Whittaker QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) test kit (Bio Whittaker, Walkersville, MD, USA). The use of LAL for the detection of endotoxin is a result of the discovery by Bang (184) that a gram-negative infection of *Limulus polyphemus*, the horseshoe crab, resulted in fatal intravascular coagulation. The LAL method utilises the initial part of the LAL endotoxin reaction to activate an enzyme which in turn releases p-nitroaniline from a synthetic substrate, producing a yellow colour.

Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyses the splitting of p-nitroaniline (pNA) from the colourless substrate Ac-ile-Glu-Ala-Arg-pNA. The pNA released is measured photometrically at 405-410 nm after the reaction is stopped. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU/ml range. The concentration of endotoxin in a sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standard. So the chromogenic LAL test is a quantitative test for gram-negative bacterial endotoxin. Prior to assaying, serum specimens were diluted 1 in 10 and then heated to 70°C to remove the non-specific inhibition that occurs in serum. Ten specimens from untreated volunteers were also assayed as controls. After the calculation of endotoxin concentration from a standard curve, results were multiplied by 10 to correct for the initial dilution.

4.24 Breath testing: combined lactulose breath hydrogen and ^{14}C-D-xylose breath test

A subset of ten patients underwent breath testing prior to chemotherapy, and 10 and 28 days after chemotherapy. The breath tests comprised a combined lactulose breath hydrogen test and a ^{14}C-D-xylose breath test, looking for changes in oro-caecal time and for the presence of bacterial overgrowth.

4.24.1 Test principles

Lactulose breath hydrogen

The hydrogen (H_2) breath test is based on the bacterial fermentation of unabsorbed carbohydrate within the body. Hydrogen is one of several gases produced by bacterial carbohydrate fermentation, and a portion of this hydrogen is absorbed into the blood stream and expired through the lungs. It is measured using a Quintron Microlyzer (Endomed Pty Ltd) which measures hydrogen in parts per million in expired and dried air. A positive result for the lactulose breath test is a rise in hydrogen of >10 ppm between 66 and 132 minutes post loading. Lactulose is not normally broken down in the small intestine, and an early rise in expired hydrogen following ingestion of a lactulose load is either due to abnormal fermentation of lactulose within the small intestine due to bacterial overgrowth, or to the arrival of the lactulose at the caecum, as lactulose is broken down in the large bowel. In the absence of bacterial overgrowth, an early rise in expired hydrogen implies a reduction in oro-caecal transit time. Small bowel bacterial overgrowth is indicated by a high baseline H_2, two H_2 peaks due to lactulose being fermented before it reaches the colon, or one peak in which the H_2 produced from overgrowth within the distal ileum may merge with H_2 produced by colonic flora. If no rise in H_2 occurs within 3 hours, either the patient has not been properly prepared for the test, or the patient is not colonised by H_2-producing bacteria, or the patient has a very slow oro-caecal transit time. Oro-caecal transit time is important for drug absorption (185). Longer transit times are associated with later peak methotrexate concentration and a more erratic profile with two peaks. Both fast and slow transit reduce peak methotrexate concentration.

^{14}C-D-xylose breath test

The ^{14}C-D-xylose breath test is based on the catabolism of xylose by intestinal bacteria, which are normally found only in the large, but not the small, intestine. The catabolism products of ^{14}C-D-xylose include ^{14}C-labelled bicarbonate, which is absorbed into the blood stream and expired as ^{14}Carbon dioxide ($^{14}CO_2$). Increased levels of $^{14}CO_2$ over a 4 hour period indicate small bowel bacterial overgrowth or reduced oro-caecal time.

4.24.2 Methodology

Patient preparation: The patients were asked to refrain from taking oral antibiotics and bowel clean outs for one month prior to the test. For 24 hours before the test they refrained from eating food high in carbohydrates, food containing bacterial culture or food with yeast. They fasted from midnight the night before the test, taking no medication on the morning of the test. Smoking was not allowed for 2 hours before the test, nor during it.

Test Procedure: At the start of the test, the patient was asked to exhale into a collection bag through the mouthpiece assembly, which discarded the dead-space air. This collected air was then used for hydrogen analysis. The patient was then asked to exhale deeply through a polyethylene tube into a collection mixture, which turned from blue to clear when the required amount (2 mmol) of carbon dioxide was collected. This was then used for the ^{14}C-D-xylose estimation.

The patients drank the test solution, and this time was recorded as time zero. The test solution comprised 90 ml distilled water, 10 ml lactulose, 5 μCu ^{14}C-D-xylose, and 1 g D-xylose. Samples were then collected at 30, 60, 90, 120, 180 and 240 minutes after ingestion of test solution, and analysed together at the conclusion of the test. The patients remained at rest throughout the procedure, and were free to eat and drink when it was complete.

4.24.3 Result analysis

Lactulose breath hydrogen: After calibration of the Microlyzer, 50 ml samples of expired air were withdrawn from the sample bags, via a closed system, and injected, via a drying column, into the Microlyzer. Hydrogen concentration in ppm was then read from the display.

^{14}C-D-xylose breath test: At the conclusion of the test, 4 mls of PCS (phase combining system) was added to each vial, and the vials were left in the dark overnight, to reduce chemiluminescence. Vials were placed in the scintillation counter, and the ^{14}C was counted. The quench correction was applied to yield sample activity in disintegrations per minute (DPM). DPM were expressed as a percentage of administered dose per mmol CO_2 by 10^{-4}. The sum of the individual time points represents the cumulative excretion over the 4 hour period, and the normal cumulative result is <70. For an example of typical bacterial overgrowth and normal curves, see Appendix (I)

4.25 STATISTICAL ANALYSIS

The statistical analysis was carried out using a repeated measures analysis of variance, because there was a sequence of observations from the same person over time: pre-chemotherapy, day 3, day 7, day 10, day 14 and day 28. Because there was a pre-treatment measure, the data were transformed by subtracting the initial value from each of the post-treatment values (new value at time t = old value at time t minus value at t0) so that each subject acted as his own control, and this differencing operation removed the person effect, leaving a measure of what the treatment alone did. All variables have been analysed as a function of age, sex, diagnosis, treatment group and time. Treatment group, diagnosis, and sex were used as grouping factors. Time course is a within-factor subset of time, for which we used a linear representation. Age is a covariate. The presence of a sequence of numbers gives a potential for correlation to occur, so that the measurement at day 7 is likely to be related to that at day 3, and the measurement at day 10 is likely to be related to that at day 3, but less so. This increased distance with time is known as an autoregressive error structure, and this has been taken into account during the analysis.

Only significant differences, and important negative results are presented. All other results are not significant at the $p=0.02$ level. A p value of <0.02 has been used throughout this analysis because of the large number of tests overall, the large number of analyses, and the need for estimation around missing values. A p value of < 0.02 is strong evidence of significance.

The statistical program used was Program 5.V (unbalanced repeated measures) from the BMDP statistical software package (186). Statistical analysis was carried out by Mr. Phil Leppard, Statistical Consultant, Department of Statistics, University of Adelaide.

Analysis of individual cases has not been performed so that we cannot predict whether the presence of symptoms in a particular case implies the presence of functional changes. The same applies for histological changes (See Chapter 5). Symptoms, function and histology do however appear to be related because they each have the same pattern of change with time. In this analysis group effects have been assessed.

4.3 RESULTS

4.30 Patient demographics

Sixty patients were recruited for this study. Ten patients underwent Breath Testing. The diseases were as shown in Table 4.30.1

There were 31 males and 29 females, with 19 haematological malignancies and 41 solid tumours. The age range was 18 to 77 with a median of 45 years. There were 29 patients in the standard chemotherapy dose group, 12 in the high dose cyclophosphamide priming group, and 19 in the high dose chemotherapy and stem cell transplant. Thus the patients were evenly divided between standard and high dose chemotherapy (which included high dose cyclophosphamide priming). No patients in the standard dose treatment group had had prior chemotherapy, so that any abnormalities found on pre-treatment evaluations were likely due to the malignancy itself. The staging was as shown in Table 4.30.2

DIAGNOSIS	NUMBER OF PATIENTS
Breast Cancer	22
Small Cell Lung Cancer	4
Non-Small Cell Lung Cancer	4
Non-Hodgkins Lymphoma	9
Testicular carcinoma	2
Hodgkins disease	3
Oesophageal carcinoma	2
Colorectal carcinoma	4
Chronic myeloid leukaemia	2
Bladder carcinoma	1
Acute Myeloblastic leukaemia	3
Chronic lymphocytic leukaemia	1
Osteosarcoma	2
Multiple Myeloma	1
TOTAL	60

Table 4.30.1 Number of patients by diagnosis

Stage	Number of patients
1	3
2	21
3	9
4	20
unknown	7
TOTAL	60

Table 4.30.2 Number of patients by stage of disease. All malignancies are staged from 1 to 4, with stage 1 being localised disease, and stage 4 being metastatic disease. Leukaemias are all stage 4 by definition.

4.31 Nutrition and symptoms

Nutrition

Ninety-four percent of potential nutritional results were available prior to chemotherapy, dropping to 80% on day 3, 77% on day 7, 72% on day 10, 67% on day 14 and 65% on day 28. No patient was malnourished prior to starting chemotherapy in this study. Anthropomorphic measurements revealed a difference with dose of chemotherapy: patients receiving standard dose chemotherapy showed no changes, but patients on high dose chemotherapy showed a loss of weight and consequently of body mass index (BMI) following treatment. See Figures 4.31.1. and 4.31.2. Weight fell from a mean of 73.7 kg pre-treatment by 0.6 kg on day 3 ($p=0.004$) and by 0.9 kg on day 14 ($p=0.0004$), and returned to normal by day 28. There was no age, sex nor diagnosis effect. BMI fell from a mean of 25.8 pre treatment by 0.2 at day 3 and by 0.3 at day 14 before recovering by day 28. Triceps skin fold thickness had a negative displacement of 0.2 from a baseline of 1.58 ($p=0.002$), and this did not recover. See Figure 4.31.3. However, there was no corresponding change in mid-arm muscle circumference. Once again there was no age, sex, diagnosis nor treatment group effect. The lines shown on the graphs of the analysis of repeated measures, are the lines of best fit, representing the closest linear approximation to the actual results. They convey an overall impression rather than give each individual result for the sixty patients.

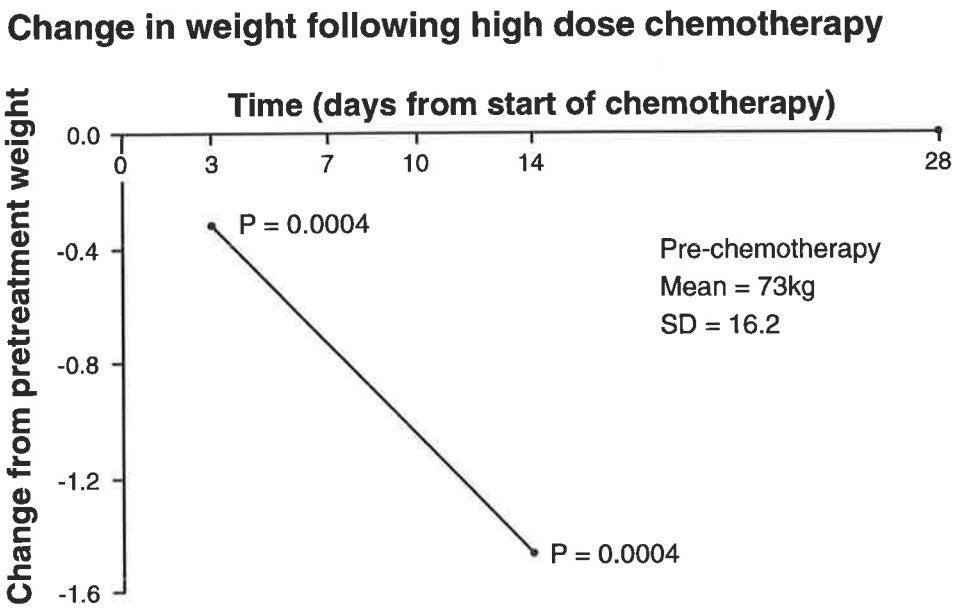


Fig. 4.31.1 Analysis of repeated measures. Change in weight following high dose chemotherapy. Weight fell significantly by day +3, continued falling until day +14, but recovered fully to baseline value by day +28. Standard dose chemotherapy had no effect on weight.

Change in body mass index following high dose chemotherapy

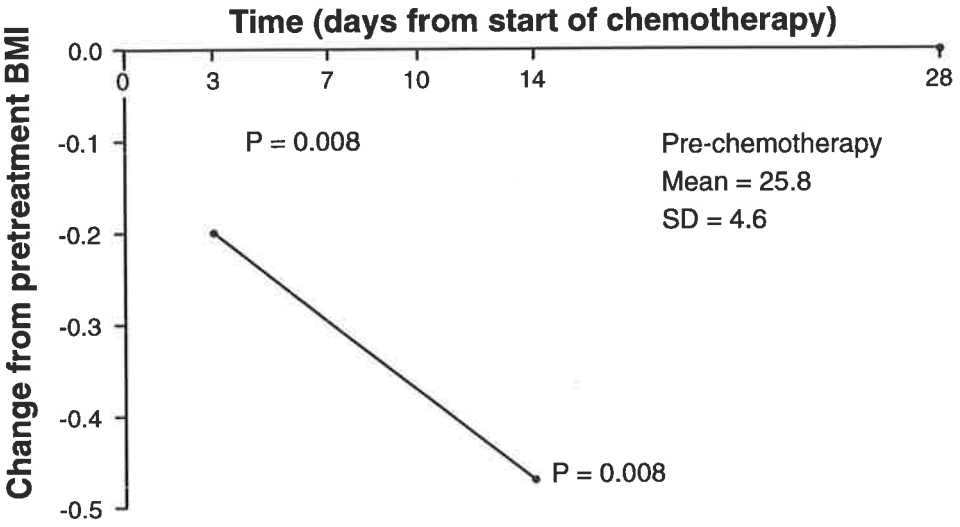


Fig. 4.31.2 Analysis of repeated measures. Change in body mass index (BMI) following high dose chemotherapy. BMI fell significantly by day +3, continued falling until day +14, but recovered fully to baseline value by day +28. Standard dose chemotherapy had no effect on BMI.

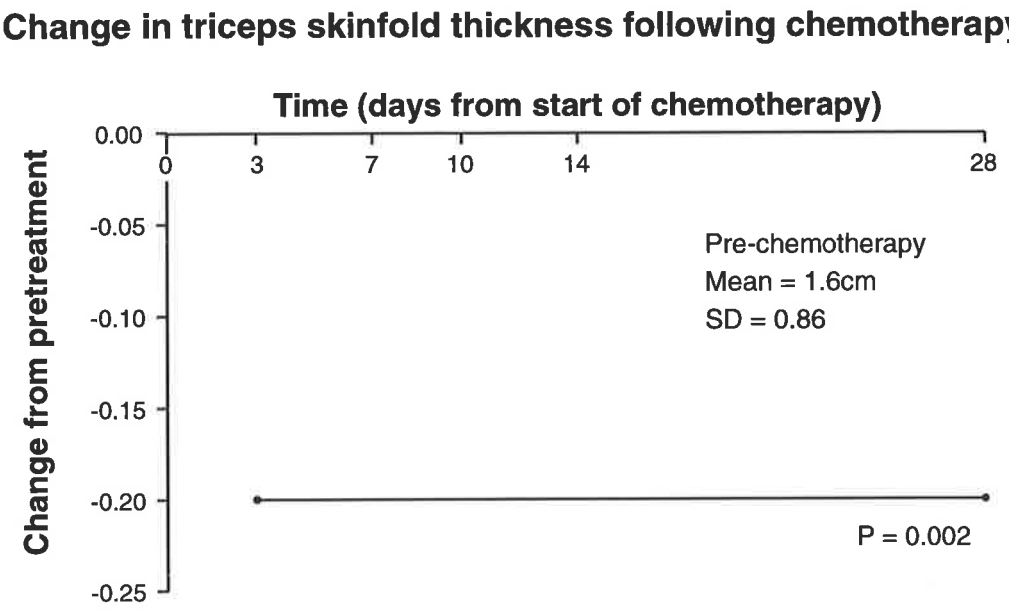


Fig. 4.31.3 Analysis of repeated measures. Change in triceps skinfold thickness (TST) following chemotherapy. TST is significantly reduced by day +3 and fails to recover by +28. There is no dose effect.

All patients showed a drop in lymphocyte count (See Figure 4.31.4), which did not return to normal during the study. This occurred at both dose levels, in both sexes, with all diagnoses and at all ages. A fall in lymphocyte count following chemotherapy is very common, as chemotherapy affects the bone marrow directly. This fall in lymphocyte count therefore is unlikely to reflect malnutrition, but rather reflects a direct effect on lymphocyte production by the bone marrow. However, it would normally have been expected to have recovered by day 28. The continuing low value is probably a reflection of the wide range of values of lymphocyte count (range 110-38,390 prior to treatment).

Albumin values fell in all treatment groups following treatment ($p=0.000$), with no dose difference, and returned to baseline at day 28. There was no subgroup effect. (See Figure 4.31.5) Serum transferrin decreased in both treatment dose groups, but the fall was more intense and more prolonged in the high dose chemotherapy group, with the standard dose chemotherapy group returning to baseline by day 14 and the high dose chemotherapy group only normalising by day 28. (See Figure 4.31.6.) Red blood cell folate fell in all groups on day 3, by 78 units, and failed to recover by day 28 (See Figure 4.31.7). There was a sex difference in serum folate that was maintained throughout, with males showing a rise following chemotherapy, and females showing no change. (See figure 4.31. 8).

In summary there were only mild, transient changes in nutrition, with more prominence in the high-dose treatment group. There were no prolonged significant effects, nor any significant malnutrition due to the tumours.

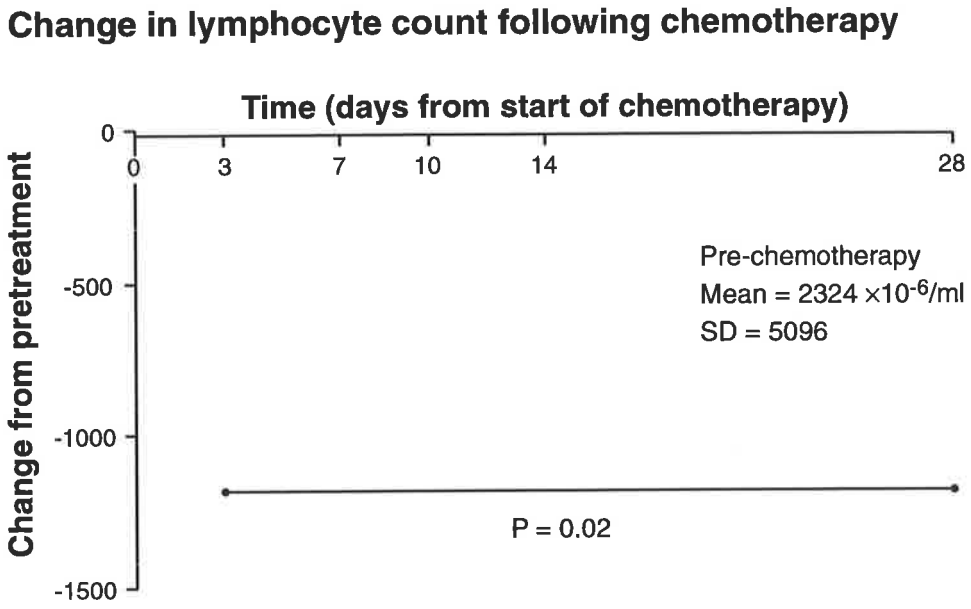


Fig. 4.31.4 Analysis of repeated measures. Change in lymphocyte count following chemotherapy. Lymphocyte count is significantly reduced by day +3 and fails to recover by day +28. There is no dose effect.

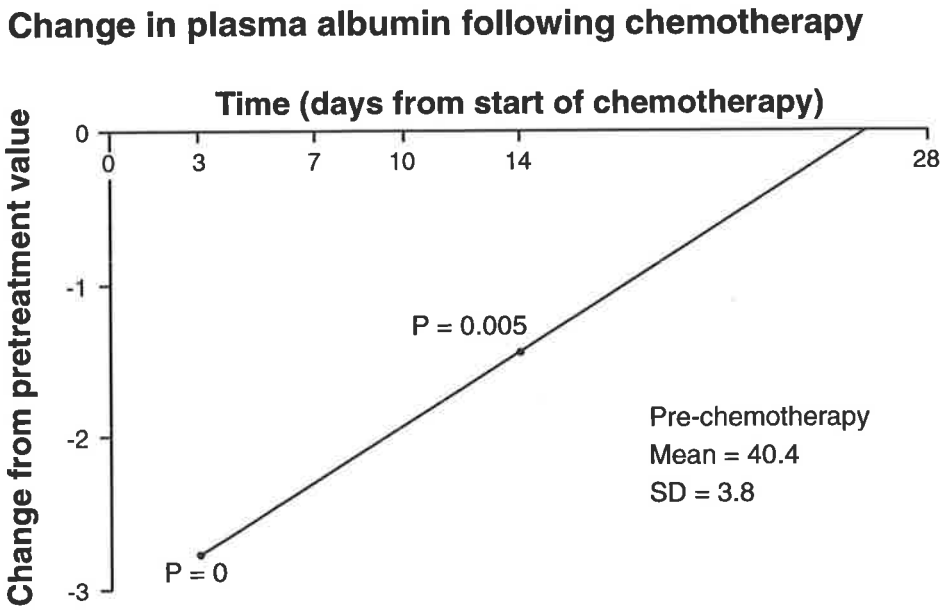


Fig. 4.31.5 Analysis of repeated measures. Change in plasma albumin following chemotherapy. Albumin falls significantly by day +3, begins to recover by day +14 and has fully normalised by day +28. There is no dose effect.

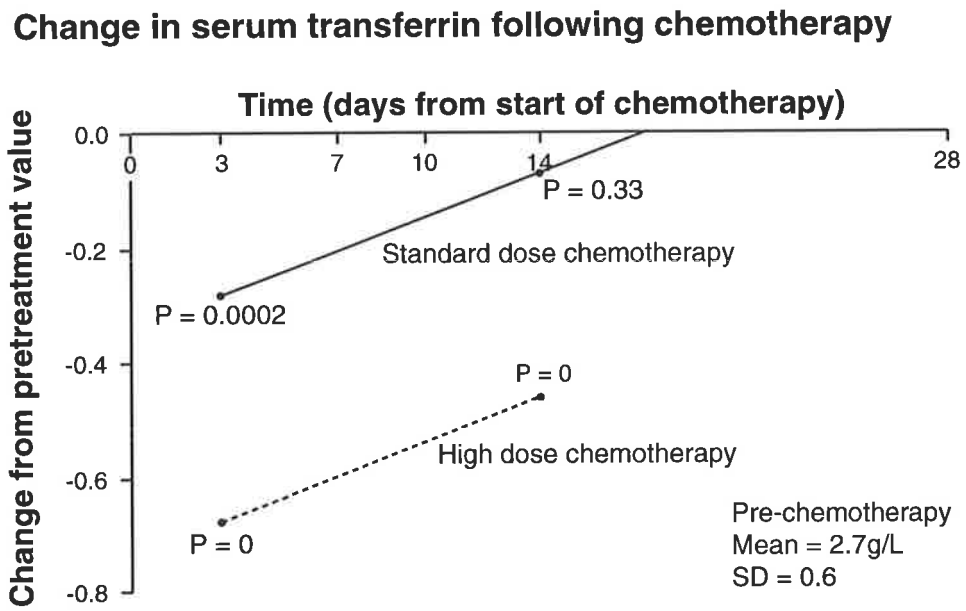


Fig. 4.31.6 Analysis of repeated measures. Change in serum transferrin following chemotherapy. Serum transferrin falls significantly by day +3. In the standard dose group it has normalised by day +14, but in the high dose group it remains significantly abnormal at day +14, normalising by day +28.

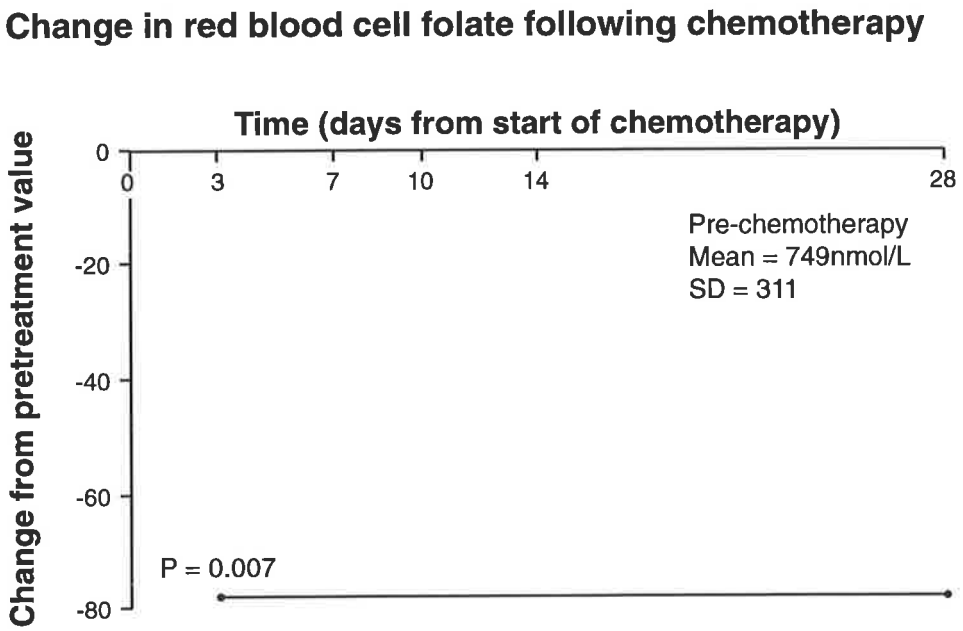


Fig. 4.31.7 Analysis of repeated measures. Change in red blood cell (rbc) folate following chemotherapy. Rbc folate is significantly reduced by day +3 and fails to recover by day +28. There is no dose effect.

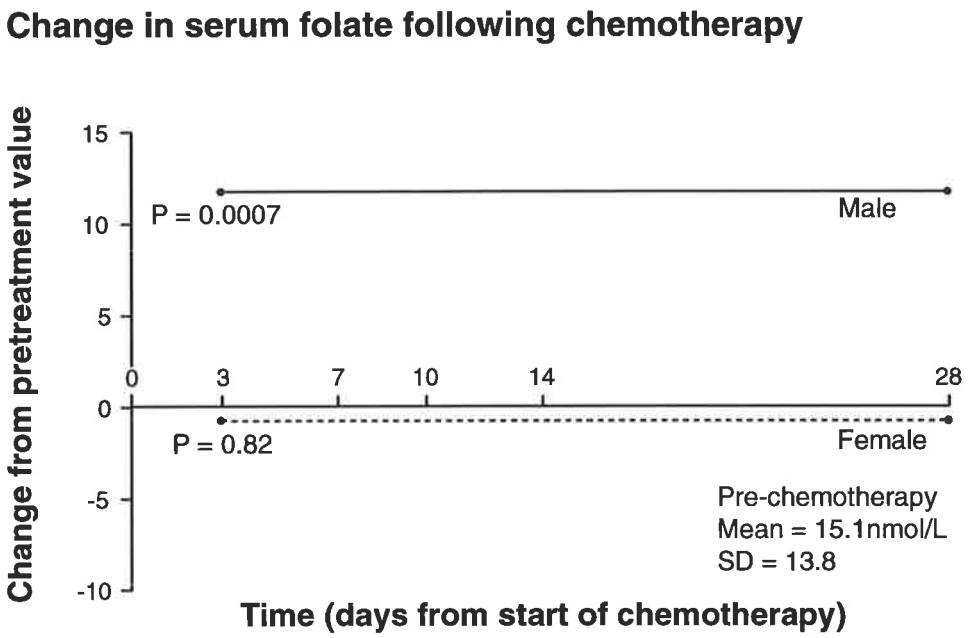


Fig. 4.31.8 Analysis of repeated measures. Change in serum folate following chemotherapy. There is a sex related increase in serum folate following chemotherapy. The male patients show a significant increase in serum folate at day +3 that does not recover by day +28. There is no change in females.

Symptoms

The percentage of patients filling in the questionnaire varied from 100% pre-treatment to 83% at day 7 and 62% at day 28. In order to analyse the symptom questionnaire, symptoms were split into two groups, namely those relating to the gastrointestinal tract such as abdominal pain, bloating and diarrhoea, and those relating to the mouth such as mouth pain and ulcers. Mouth symptoms were only recorded for the latter 32 patients. Patients were given a score for the proportion of potential symptoms which they had prior to treatment, this was combined for the group, and the change in this score with time was measured. The combined score gave a measure of 'symptom burden' for the group. The gastrointestinal symptom burden for the group prior to starting chemotherapy was 8%, and there was no difference between the group that was chemotherapy naive (standard dose group), and the group that had already had prior chemotherapy (priming and high-dose group) (See Figure 4.31.9). Thus the tumour itself was most likely causing symptoms. Symptom burden increased to 14.1% at day 3, 14.5% at day 7, and reduced to 10.7% at day 10, 9.8% at day 14 and 6.2% at day 28. The significant increase in gastrointestinal symptoms occurred at day 3, thereafter reducing to baseline again at day 28. (See figure 4.31.10). Breaking gastrointestinal symptoms down into the individual components shows that the most common symptoms were nausea, which peaked at 52%, pain which peaked at 28%, and bloating at 24%. (See Figures 4.31.11 and 4.31.12). Nausea had a large peak of 52% on day 3, whereas actual vomiting had a more gradual course, remaining in the 10 to 15% range for most of the time. Abdominal pain, bloating and nocturnal defecation were all increased from days 3 to 10. Diarrhoea, however, was quite prominent before treatment (18%), peaked on day 7 at 24% and then slowly dropped. (See Figure 4.31.13). The individual symptom peaks were higher than the group peaks, because of the lower denominator.

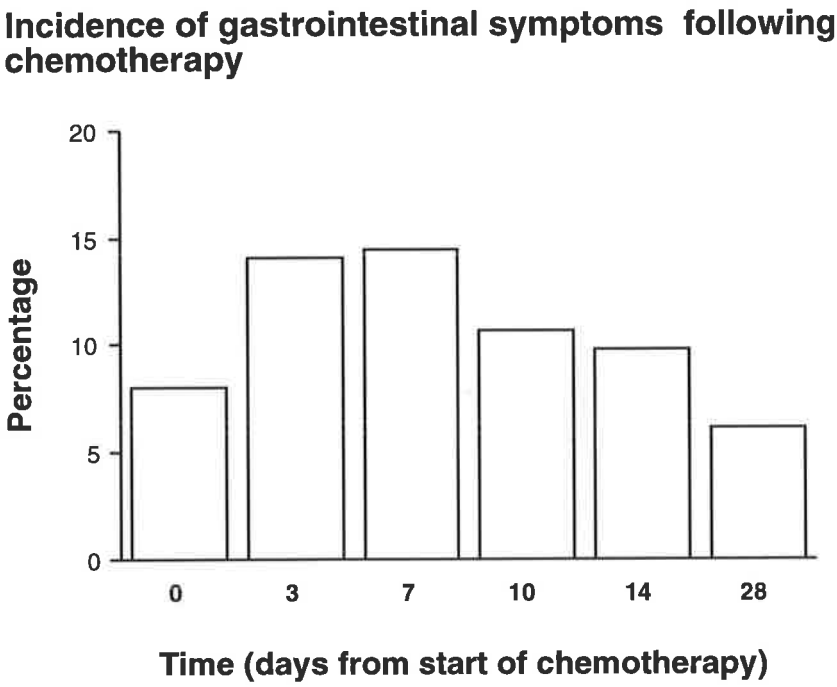


Fig. 4.31.9 Analysis of repeated measures. Burden of gastrointestinal symptoms as a function of time from starting chemotherapy.

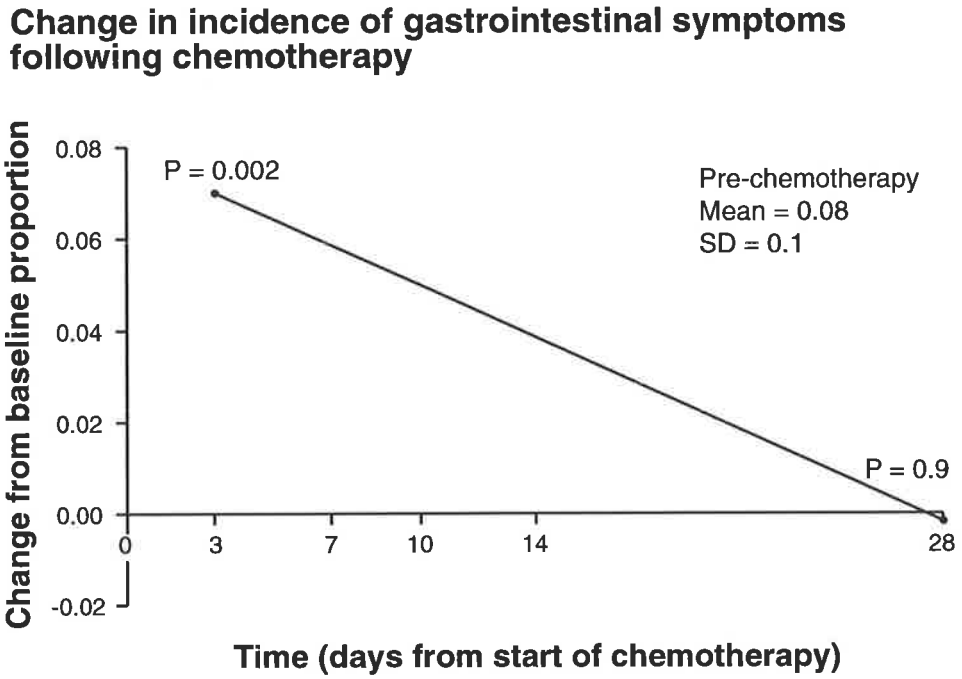


Fig. 4.31.10 Analysis of repeated measures. Change in incidence of overall gastrointestinal symptoms with time after chemotherapy. Each value is shown as a difference from the pretreatment baseline, with the line of best fit showing the trend, in this case showing a peak difference at day +3, returning to baseline by day +28.

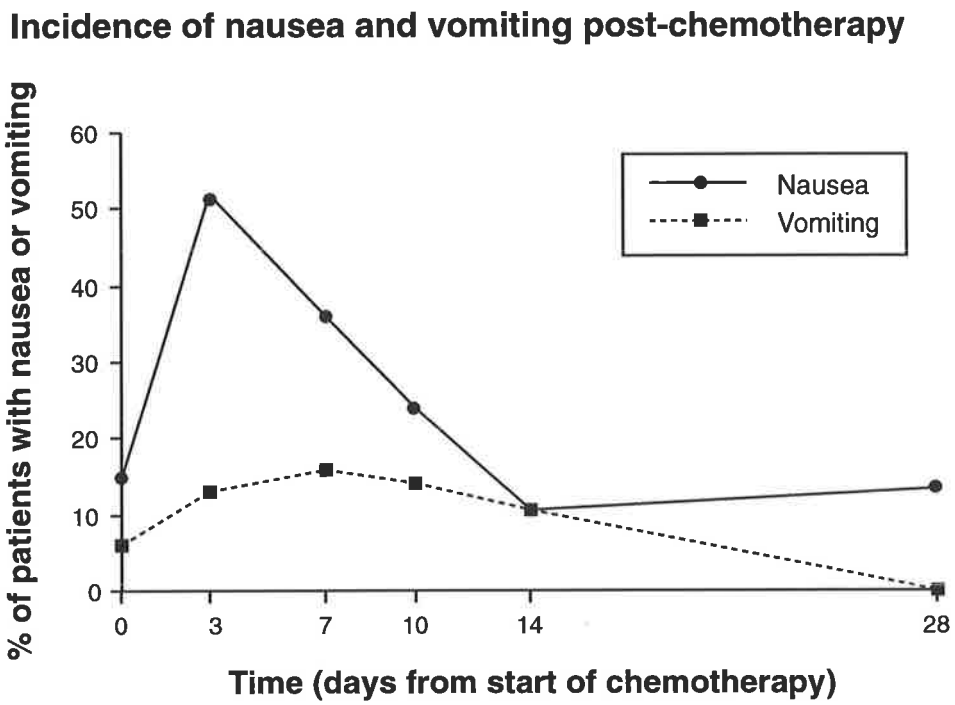


Fig. 4.31.11 Incidence of nausea and vomiting following chemotherapy.

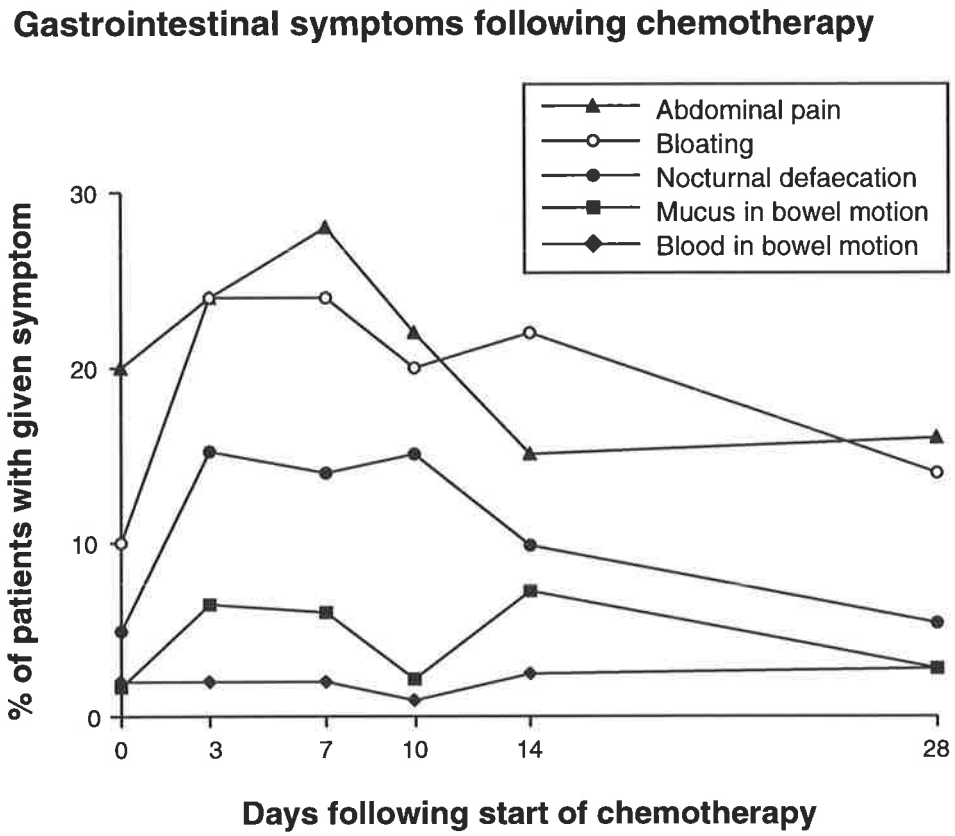


Fig. 4.31.12 Incidence of individual gastrointestinal symptoms as a function of time following chemotherapy.

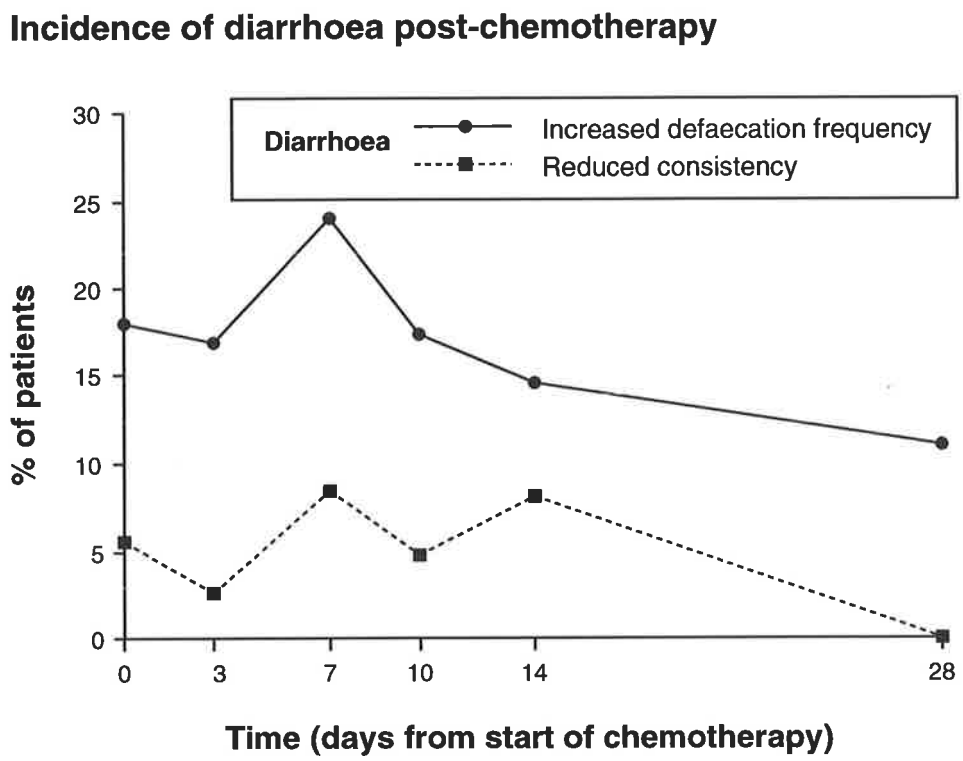


Fig. 4.31.13 Change in bowel habit following chemotherapy.

Mouth symptoms were recorded in the latter thirty three patients, and showed a different profile over time. Only 4% of patients complained of mouth symptoms before treatment, with a more gradual increase to 11% on day 3, 38% on day 7 and 45% on day 10. Symptoms decreased to 21% on day 14 and 13% on day 28. (See Figure 4.31.14). The change in oral symptoms from baseline (See Figure 4.31.15) was the only parameter that did not follow the trend to a peak abnormality at day 3, returning to normal by day 14 or 28, which was seen in all the other areas of symptoms, function and morphology. The oral symptoms comprised mouth pain and mouth ulcers, and as can be seen from Figure 4.31.16, mouth pain was more prominent, rising to greater than 60% on day 10 before falling to 20% on day 28, still high when most other parameters had normalised. Actual ulceration was much less common, peaking at 30%, but, interestingly, we did not see a chemotherapy dose effect in this study, which we would have expected.

In summary, gastrointestinal symptoms peaked from days 3 to 10, and recovered by day 28, whereas oral symptoms did not increase significantly until day 7, then remained high until day 14, before returning to normal by day 28.

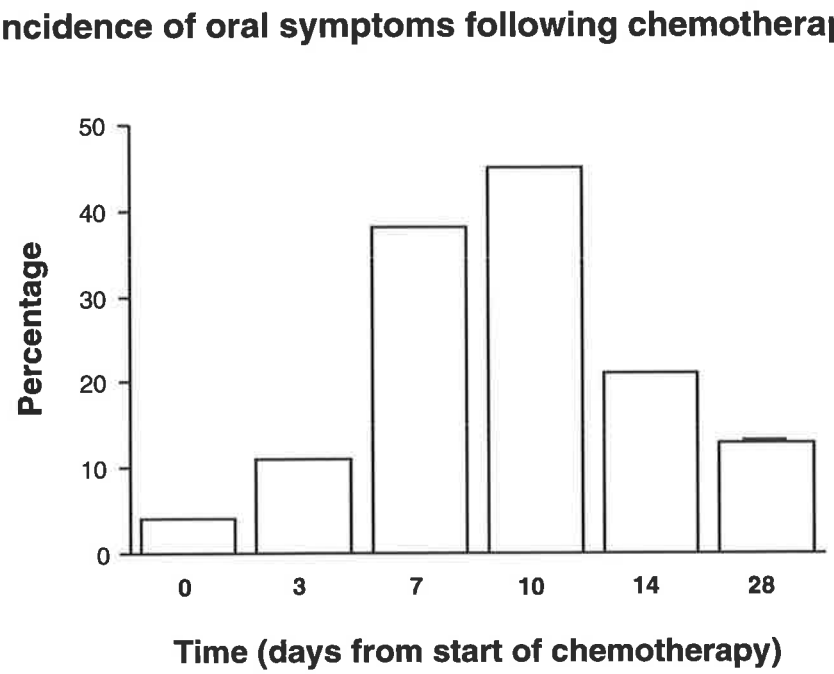


Fig. 4.31.14 Incidence of oral symptoms (mouth pain & ulcers) as a function of time from starting chemotherapy.

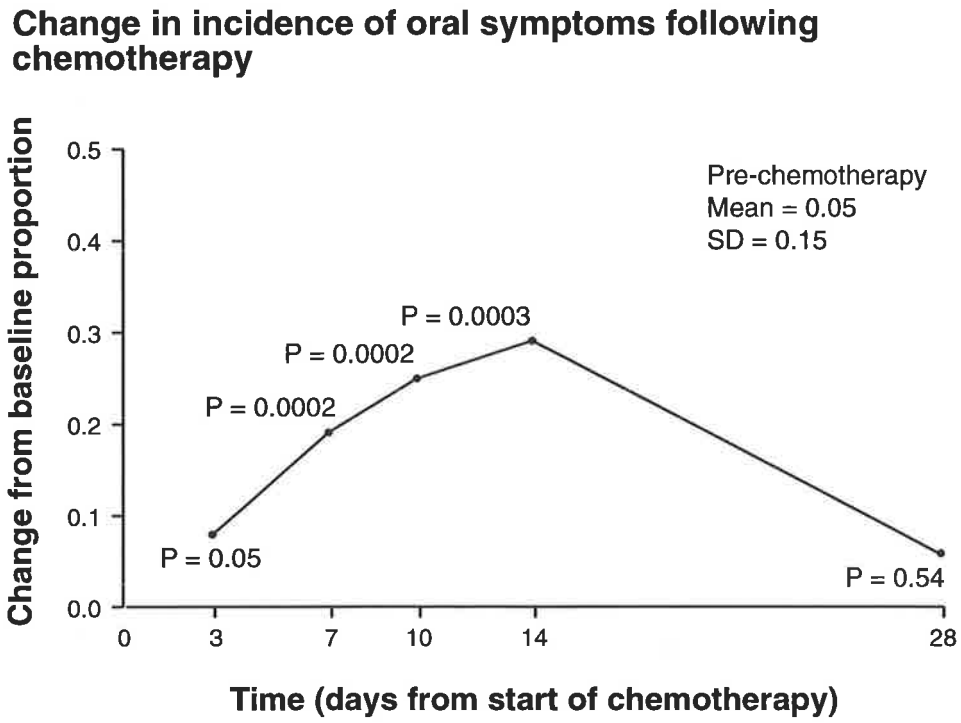


Fig. 4.31.15 Analysis of repeated measures. Change in incidence of overall oral symptoms with time after chemotherapy. Each value is shown as a difference from the baseline value, with that difference continuing to increase up to day +14, before returning to baseline by day +28.

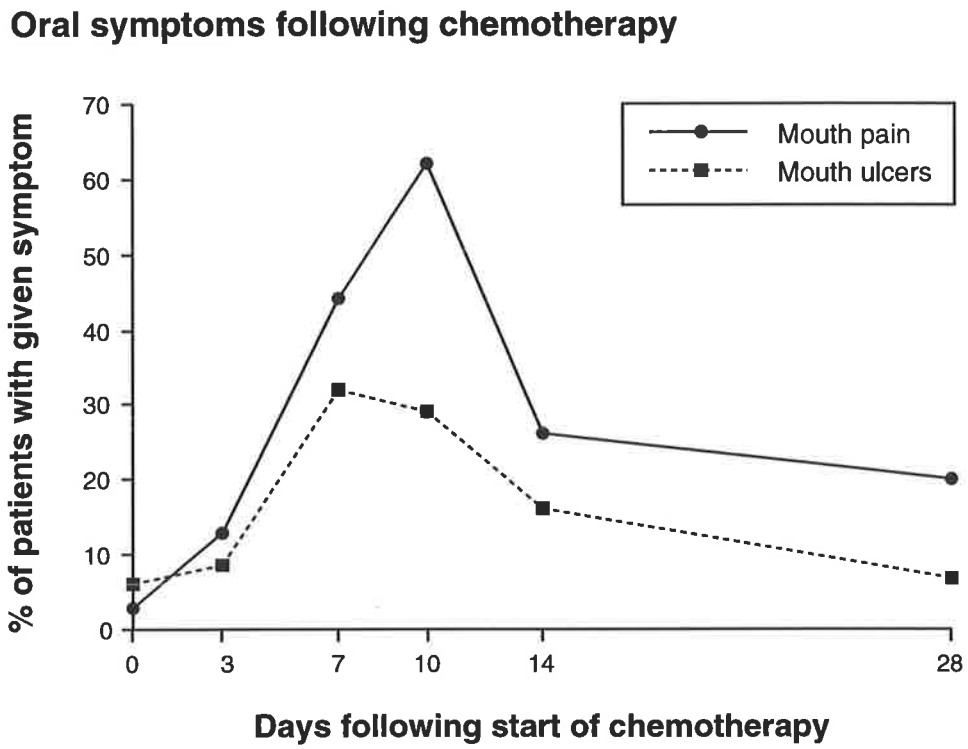


Fig. 4.31.16 Incidence of individual oral symptoms as a function of time following chemotherapy.

Symptom Severity

Patients were given an overall mucositis score at each assessment. Diarrhoea alone was assessed in the first 27 patients, and nausea, vomiting, stomatitis and diarrhoea in another 33 patients. Table 4.31.1 shows the maximum mucositis scores reached during the study. The individual maxima are given in appendix III.

Fifty percent of patients suffered from at least grade 1 diarrhoea, indicative of small intestinal mucositis. 22% had grade 1, 15% had grade 2, 11% had grade 3 and only 2% had grade 4 diarrhoea. This was assessed in 54 patients, as 6 were unevaluable.

Nausea, vomiting and stomatitis were assessed in 33 patients, of whom 27 were evaluable. Vomiting only occurred in 33% of patients, with a maximum grade of 2. The peak day of vomiting post chemotherapy, however, is usually day +1 when no assessments were performed. Thirty percent and 26% of patients suffered grade 3 nausea and stomatitis respectively, which were the most problematic toxicities.

Toxicity/ Grade	Nausea	Vomiting	Stomatitis	Diarrhoea	Overall
0	33	67	33	50	35
1	22	18	4	22	15
2	15	15	37	15	24
3	30		26	11	24
4				2	2

Table 4.31.1 Percentage of patients for whom mucositis grade reached a given level.

Summary

Sixty patients filled in the symptom questionnaire on a maximum of six occasions. There was a small amount of drop-off as the study proceeded, with 42 completed questionnaires on day 14 and 39 completed on day 28. This fall-off in numbers was taken into account during the analysis. Oral symptoms, although measured in a smaller number of patients, were more common than gastrointestinal symptoms. Nausea was more common and more severe than vomiting, but the peak of vomiting had most likely been missed. Mouth pain was the single most prominent symptom, occurring in 62% of patients on day 10, which was a delayed peak compared to the other gastrointestinal symptoms, which peaked on day 3. Abdominal pain (28%) was more prominent than other gastrointestinal symptoms, with diarrhoea occurring in 24%. There were no subgroup effects. Analysis of the group symptom burden as a whole showed lower values for symptoms than the individual scores, because each value in the analysis was a proportion of potential symptoms rather than a sum of individual symptoms.

4.32 Intestinal sugar permeability

Lactulose excretion, rhamnose excretion and lactulose to rhamnose milligram excretion ratio were all measured. There was no change in lactulose excretion with time, nor with any of the covariables. Rhamnose excretion however, fell in the high-dose chemotherapy group after treatment and did not recover with time ($p=0.000$). (See Figure 4.32.1). There was no change with standard dose chemotherapy ($p=0.53$). This fall in rhamnose excretion led to an increase in lactulose to rhamnose milligram excretion ratio, seen in both groups on day 3 ($p=0.0012$) with no recovery by day 28. (See Figure 4.32.2). This result is disappointing as it does not confirm the results of the earlier high-dose chemotherapy study (see Chapter 3). The reduction in sensitivity of the test due to the removal of glucose led to a loss of significance. The normal ranges are for lactulose excretion <0.7%, rhamnose excretion 8-20%, and lactulose/rhamnose milligram excretion ratio 0.023-0.074, using the HPLC method. Pre-treatment lactulose excretion was 0.62%, rhamnose was 7.8% and the lactulose/rhamnose milligram excretion ratio was 0.079. Both lactulose and rhamnose excretions were normal, but the ratio was elevated. The lactulose/rhamnose milligram excretion ratio rose 2.5-fold to 0.20 on day 3, and then fell to 0.13 on day 7. It remained elevated at 0.14 on day 28. (See Figure 4.32.3).

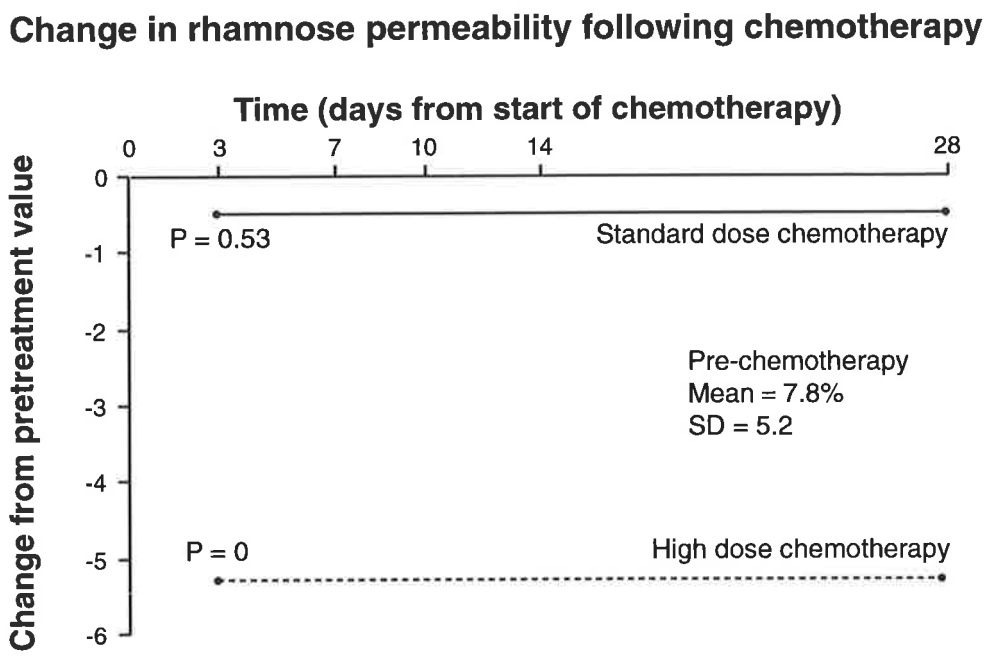


Fig. 4.32.1 Analysis of repeated measures. Change in rhamnose permeability following chemotherapy, with patients being split into high dose and standard dose chemotherapy groups. There was no change in rhamnose permeability with standard dose chemotherapy. The decrease in rhamnose permeability with high dose chemotherapy did not recover by day +28. For definitions of standard & high dose chemotherapy see text.

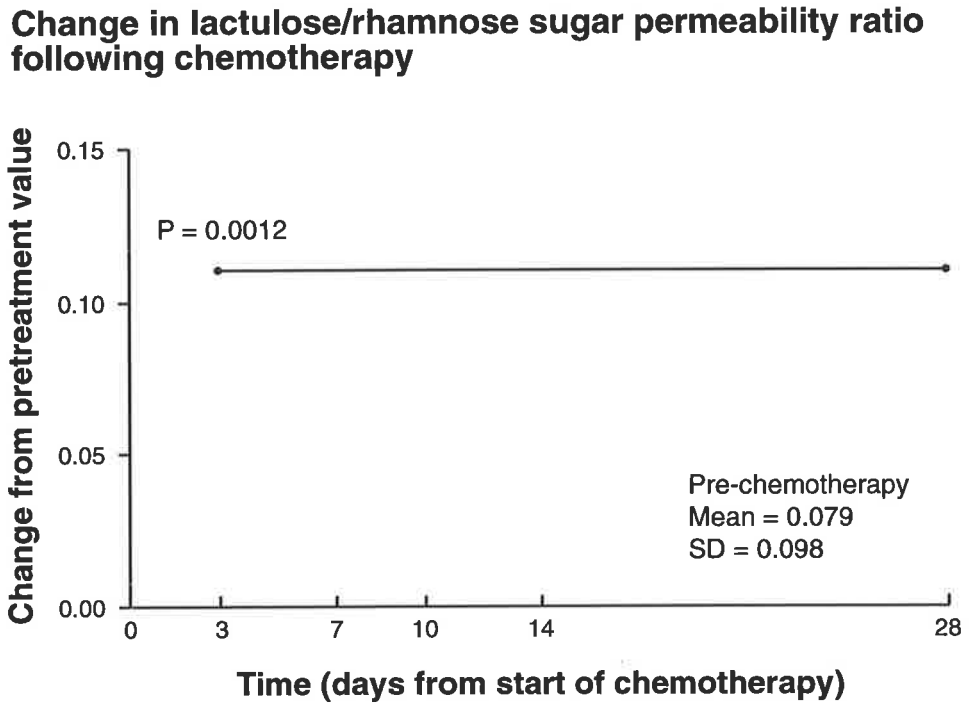


Fig. 4.32.2 Analysis of repeated measures. Change in sugar permeability ratio following chemotherapy. There is no dose effect evident nor any recovery by day +28.

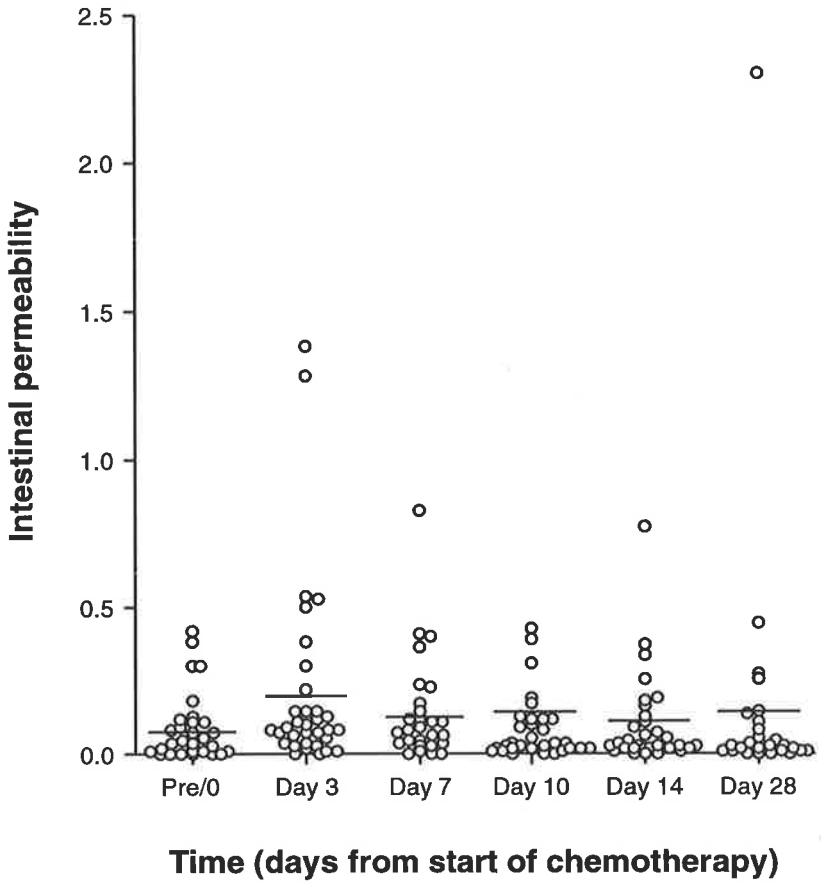


Fig. 4.32.3 Urinary lactulose to rhamnose permeability following chemotherapy. The bars represent the mean values.

4.33 Serum endotoxin

There was no change in detectable serum endotoxin level throughout the time course, the estimated change with time being 0.041 with p=0.73. There were no covariable effects, particularly no chemotherapy dose effect. A small amount of endotoxin was detectable throughout.

4.34 Assessment of bacterial overgrowth

Breath testing was performed on ten patients, but was not analysed as part of the overall analysis because the results were not satisfactory. See Table 4.34.

It was not possible to prevent patients from taking antibiotics, as they were quite commonly needed after chemotherapy. Nor were patients keen to adhere to the dietary restrictions. In the ten patients studied, we did not find any convincing evidence of bacterial overgrowth either before or after chemotherapy. The major effect seen was of an accelerated oro-caecal transit. Thus two patients who were thought not to be hydrogen producers on the basis of no hydrogen detection at 4 hours in the pre-chemotherapy sample, were found to produce hydrogen 2 hours after lactulose ingestion, after chemotherapy. The only two patients who appeared to have positive tests for bacterial overgrowth had that before chemotherapy, with no change after 10 days, and a normalisation at day 28 in one. Greater than 50% of the results were equivocal due to poor dietary compliance, which made the test impossible to interpret. The breath testing exercise was abandoned after the first 10 patients as there were too many variables.

Patient	Pre-chemotherapy	Day 10	Day 28	Comments
1	equivocal	equivocal	equivocal	poor compliance
2	equivocal	equivocal	normal	
3	equivocal	normal	equivocal	poor compliance
4	equivocal			
5	positive	positive		
6	normal	normal	normal	
7	equivocal	positive	equivocal	poor compliance
8	equivocal	equivocal		
9	positive	positive	normal	
10	equivocal	equivocal	equivocal	

Table 4.34. Summary of combined ^{14}C-D-xylose and lactulose breath hydrogen breath test results. For graphic representation see Appendix (I).

4.4 DISCUSSION

The hypothesis of this study was that chemotherapy causes a transient, dose-dependent abnormality in the small intestinal mucosa which is clinically manifest by abdominal symptoms, abnormal small intestinal functional studies, and changes in histomorphometry. The histomorphometry results are discussed in Chapter 5. The symptoms were as expected, but the functional studies did not show much abnormality, nor was the dose-dependent effect as large nor as consistent as we expected. We did not see any effect of age, sex, disease nor stage, and it may be that the numbers of patients were not big enough to show them. In this sort of study, which is exploratory, rather than seeking a particular level of difference, it is not feasible to do power calculations to determine the number of patients required, rather it is important to balance a realistic number for recruitment purposes, with a large enough number that will detect a significant difference (187). It was thought that sixty patients was a reasonable compromise. A longitudinal study, however, has other advantages, in that it is possible to use each subject as his own control over the time-course of the intervention.

Demographics

The age spread, mixture of diagnoses and stages of disease, give a broadly typical picture of patients treated at one institution. The results showed that small intestinal mucositis was not affected by such factors as age, sex, diagnosis nor disease stage. Less expected, however, was the small nature of the dose effect, which was not seen at all in the biopsy group. It is postulated that this small effect is due to the nature of the chemotherapy, and the fact that even with the high dose chemotherapy, it was always an autologous peripheral blood stem cell transplant that was done, not a bone marrow transplant (autologous nor allogeneic). Bone marrow transplantation is associated with more severe mucositis, due to a combination of higher doses of chemotherapy, more prolonged bone marrow suppression, the added

complication of graft versus host disease, and in some cases the use of total body irradiation (TBI) as part of the conditioning regimen. There was no TBI used in this study, indeed radiotherapy was an exclusion criterion, and graft versus host disease is rare with autologous transplantation. A larger study might show dose-dependent effects, but would be much harder to perform, especially at a single institution. The mixture of drugs used was large, as it would not have been possible to recruit the numbers of patients required if only one drug combination were chosen. While it has been commonly thought that only certain drugs actually caused mucositis, Ijirri and Potten (9) showed that each one of 18 chemotherapeutic agents they tested did affect the small intestinal crypt, albeit at different levels in the hierarchy. The full list of drug combinations used is shown in appendix II, and the majority is known to cause mucositis.

The study aims will be addressed in turn, to consider whether each has been fulfilled, and what could be changed in future work.

1. To assess the prevalence, severity and duration of gastrointestinal symptoms and nutritional changes in patients receiving cancer chemotherapy.

Nutritional assessment

We did not demonstrate any evidence of malnutrition prior to chemotherapy. This is probably due to a bias in the selection of patients who received chemotherapy. Poor performance status and weight loss at diagnosis are poor prognostic signs for response to treatment and for survival. Patients who are malnourished are therefore not as likely as well nourished patients to respond to treatment, and are therefore less likely to be offered chemotherapy. We did find, however, evidence of a transient reduction in some parameters of nutrition following a single

cycle of cytotoxic chemotherapy. Weight and body mass index fell after chemotherapy and remained significantly reduced on day 14, but normalised by day 28, which would usually be the start of the next cycle of chemotherapy. Thus, it would be expected that patients would tolerate chemotherapy by regaining any lost weight towards the end of each cycle. Albumin and transferrin also fell transiently after chemotherapy. Transferrin is an acute phase protein and could be expected to fall following chemotherapy. It is interesting that it had one of the very few dose-related effects. The fall in albumin is less easy to explain as it has a longer half-life, but it may be due to altered tissue distribution following the chemotherapy. Both are recovered by day 28 and the next cycle of treatment.

Symptoms

Symptoms peaked over several days, with gastrointestinal scores increased from days 3 to 10, and oral scores increased from days 7 to 14. This delay in onset of oral symptoms is consistent with previous experience which indicates that they manifest from days 10 to 14 following chemotherapy. It is also worth noting that subjective (patient dependent) symptoms were more common than those which could be assessed by objective signs. For example, nausea was more common than vomiting, and mouth pain was more common than mouth ulcers. There was no difference detected for age, sex, diagnosis, nor stage, which is not surprising, but surprisingly no dose effect was seen. This is probably related partly to the number of patients, although it may be that even in the high-dose group, the doses were not sufficiently great to show an effect.

Criticisms of the symptom questionnaire include the fact that the oral symptoms were not added until the second half of the study. This could have led to some bias, and oral symptoms will be included in further work.

2. To measure functional changes in the small intestine using intestinal sugar permeability and associated changes in serum endotoxin.

Intestinal sugar permeability

Intestinal sugar permeability was minimally elevated prior to chemotherapy, and increased 2.5-fold at 3 days after chemotherapy. This increased permeability was entirely due to decreased rhamnose absorption rather than any increased lactulose absorption, despite the fact that we have shown an increase in open tight junctions by electron microscopy (see Chapter 5). It is interesting that the permeability defect persisted until at least day 28, when intestinal morphometry had recovered. The first study (Chapter 3) also showed increased intestinal permeability after high-dose chemotherapy until 35 days after starting treatment. These two studies therefore show a prolonged functional abnormality that persists in spite of histological recovery. An immunological/inflammatory mechanism is possible to explain these data, but endotoxin is not the foreign antigen leading to a prolonged reaction.

The major criticism of the permeability measurements in this study is that the use of an isotonic sugar solution, rather than the hypertonic solution used in the first study, resulted in reduced sensitivity. Since lactulose absorption was affected, this could be explained by reduced stress on tight junctions. However, what was lost in sensitivity was partly made up for by improved patient tolerability with fewer patients refusing to drink the isotonic sugar solution. Future studies are being performed with the hypertonic solution in an attempt to increase sensitivity.

Endotoxin

Serum endotoxin estimation was used as one measure of integrity of the gastrointestinal mucosal barrier, particularly that of the colon, where endotoxin forming bacteria reside. Small bowel bacterial overgrowth could also increase endotoxaemia, but this was not confirmed. The serum endotoxin assay detected a small amount of endotoxin present prior to chemotherapy, which persisted without change after chemotherapy. This small detectable amount was not due to contamination, as similar low levels of endotoxin were present in sera of healthy controls. We conclude that there was no significant increase in endotoxin absorption following chemotherapy.

3. To assess small bowel bacterial overgrowth using a combined lactulose breath hydrogen and ^{14}C-D-xylose breath test

The assessment of small bowel bacterial overgrowth is problematic. The traditional method has been by culture of jejunal aspirates, but breath testing has frequently been used (39), and more recently, culture of sterile biopsy specimens has been investigated (40). Unfortunately the results are dependent on proper patient preparation, namely fasting overnight before the test, and complying with a relatively strict diet for the 24 hours leading up to the test. Patients are also not allowed to smoke, and have to avoid oral antibiotics for up to one month prior to testing. Obviously it is hard for patients undergoing cancer chemotherapy to adhere to all these restrictions, as was seen in our results, which showed a predominance of inadequate preparation. The only two patients in whom we found convincing evidence of bacterial overgrowth, already had this prior to treatment, so that it cannot be used to explain symptoms occurring after chemotherapy. The most interesting observation from the ten patients

undergoing breath tests, was that two patients who were thought not to be hydrogen producers in the pre-chemotherapy test, did produce hydrogen after chemotherapy. This is probably explained by shortened oro-caecal transit time. The negative endotoxin results, and the inadequate breath testing results mean that we have not been able to fully explore the role, if any, of small bowel bacterial overgrowth in the development and prolongation of small intestinal mucositis following cancer chemotherapy. Further studies would obviously need to be done to clarify the situation.

CHAPTER 5

SMALL INTESTINAL MORPHOLOGICAL CHANGES AFTER CHEMOTHERAPY

5.1 INTRODUCTION

The aim of this study was to further characterise the changes occurring in the human small intestinal mucosa following chemotherapy by measuring morphological changes using apoptotic cell count, histomorphometry and electron microscopy. As the study was carried out in conjunction with the studies in Chapter 4, results of symptoms, nutrition and functional studies were available for most of the patients, allowing comparison of symptoms, function and morphology. Patients had upper gastrointestinal endoscopy and duodenal biopsies performed prior to and 1, 3, 5, and 16 days after chemotherapy. Each patient had a maximum of two endoscopies. Eighteen patients were part of the main study, and had biopsies pre-chemotherapy and either on day 3 or day 16 post chemotherapy. The remaining six patients were recruited to have biopsies on day 1 (n=5) and day 5 (n=2).

The safety of upper gastrointestinal endoscopy in patients having had chemotherapy is of utmost importance, but most of the work in this area is concerned with patients who have had bone marrow transplantation (188). Our patient group all received chemotherapy, either in standard doses, as high-dose cyclophosphamide priming or as marrow-ablative chemotherapy prior to autologous stem cell rescue. The marrow recovery times after stem cell transplantation are much shorter than after bone marrow transplantation, and patients are not at as high a risk of bleeding and infection for so long. Following Forbes' paper (86), we initially performed upper gastrointestinal endoscopies at 16 days after chemotherapy, but changed this to 3 days after the first eight patients. The platelet and white cell counts had not fallen to unsafe levels by

3 days. In a study by Kaw (182), it was shown that the incidence of bacteraemia in bone marrow transplantation patients undergoing endoscopy was low, and that routine antibiotic prophylaxis was not required in all cases.

5.2 METHODOLOGY

Test procedure

Patients were prepared in the normal manner for endoscopy. They fasted for 4 hours prior to the procedure, gargled an anaesthetic mouth-wash, and were given fentanyl and midazolam intravenously. The upper gastrointestinal tract was inspected, and multiple biopsies were taken from the third part of the duodenum. Tissue was taken for disaccharidases analysis, and tissue was fixed in Clarke's fixative for morphometry. Villus area, crypt length and mitotic count were measured by a microdissection technique. This technique works well in human and animal biopsy specimens (28). Further biopsies were taken for electron microscopy (to measure tight gap junction width and brush border height), in Carnoy's fixative to assess enterocyte height, and in formalin for TUNEL (tdt-mediated dUTP-biotin nick end labelling) to assess apoptosis.

5.21 Disaccharidases

Tissue for disaccharidase assay was sent on ice to the Institute of Medical and Veterinary Science, Adelaide, for analysis, and disaccharidases were expressed as micromoles disaccharide hydrolysed per minute per gram wet weight of tissue at 37°C. The normal ranges were lactase: 3-14, sucrase: 6-26, and maltase: 13-44 (90% confidence intervals).

5.22. Light microscopy

Intestinal morphometry

Duodenal biopsies were fixed in Clarke's fixative overnight and then transferred to 70% alcohol until microdissection was performed. They were stained with Feulgen reagent and microdissected, using the method of Goodlad (28). Villus area was calculated using a trapezoid approximation as described by Cummins (189) See Figure 5.2.1. Crypt length and mitotic count per crypt were also recorded, with a minimum of 10 crypts per sample being averaged.

Apoptotic count by TUNEL method

Formalin fixed, paraffin embedded, 5μm sections were stained by the TUNEL method of Gavrieli (60), apoptotic cells per crypt were counted in at least ten crypts, and expressed as apoptotic bodies per crypt. These studies were undertaken by Mr. Gary Goland, Technical Officer, in the Department of Gastroenterology.

Enterocyte height

Biopsies were fixed in Carnoy's fixative overnight, and then transferred into 100% alcohol prior to paraffin embedding, sectioning and haematoxylin and eosin staining. 5 μm sections were examined under light microscopy at a magnification power of x40, with a graduated eyepiece. Enterocyte height was measured at approximately the mid-villus position for at least ten cells and the average calculated.

Measurement of villus length and area and crypt length of the small intestine

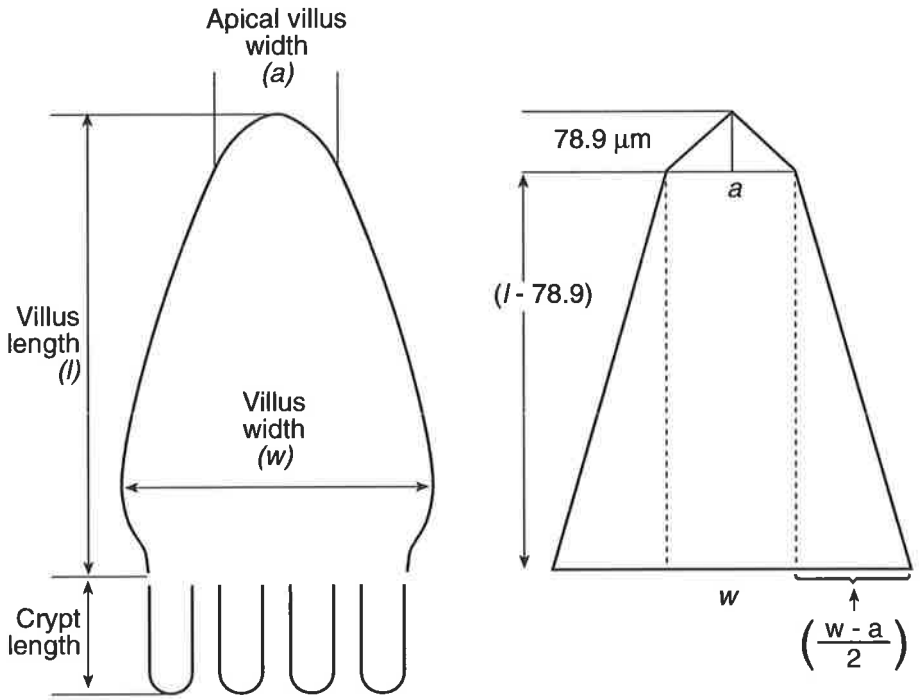


Fig. 5.22.1 Trapezoid approximation used for calculation of villus area at microdissection.

5.23 Electron Microscopy

Electron microscopic studies were performed by Mr. John Brealey at The Queen Elizabeth Hospital.

Fixation: Duodenal tissue pieces (approximately 0.5 mm^2) were fixed for at least 1 hour in a mixture of 1% glutaraldehyde and 10% formalin in 0.1M sodium cacodylate buffer.

Processing: Tissue was post-fixed in 2% osmium tetroxide and stained *en bloc* in 2% uranyl acetate. Tissue was dehydrated through a graded series of alcohols (70%, 90%, 100%) and further processed through epoxypropane (100%), a 50/50 mixture of epoxypropane and Procure 812 resin (Electron Microscopy Sciences, Pennsylvania, USA), and two changes of Procure 812 resin. The tissue pieces were embedded in polythene capsules and cured overnight at 90°C.

Microtomy: Survey sections were cut at approximately 2 μm and stained with toluidine blue. Duodenal villi were considered suitable for ultramicrotomy if they were clearly attached to lamina propria. Thin sections were cut at silver-gold colour (approximately 100 nm) and mounted on copper grids.

Staining: Thin sections were stained with 2% uranyl acetate and Reynold's lead citrate

Ultrastructural Examination: Thin sections were examined with a Hitachi H-600 transmission electron microscope (TEM) at a potential difference of 75 kV. Tight junctions were examined at a final magnification of 80,000X. All specimens were randomised by a co-worker such that the examiner was effectively blinded to the history of each specimen. Tight junctions were examined at sites where the luminal microvilli were considered perpendicular or near perpendicular to the axis of the electron beam. One hundred tight junctions per specimen

were examined and scored as closed (normal), open (leaky), or oblique (unable to comment on the status of the tight junction due to obliquity of the junction in the plane of section) at the luminal surface. A tight junction was considered morphologically normal if there was no evidence of a gap between apposing cells at the luminal surface, and there was no visible gap between apposing leaflets of the tight junction. Due to the subjective nature of the scoring system it was felt that a large number of junctions (100) needed to be examined to achieve an adequate base for data comparison.

Brush border height

Brush border height was measured on electron micrographs photographed at a magnification of x18,000. Only intact microvilli were measured, and at least 10 separate microvilli were measured in order to calculate an average height. Measurements were made on biopsies taken prior to treatment and at 3 and 16 days after chemotherapy.

5.24 Statistics

Biopsies from the eighteen patients who were part of the main study were analysed together with their symptoms and function. A description of the statistics is given in section 4.25. Additional biopsies were performed at days 1 or 5 after chemotherapy. Differences between morphometry and apoptotic counts were analysed using Peritz' F-test (179) which is a robust test of comparison of group means, where the group sizes are not identical.

Eighteen patients underwent endoscopy and biopsy pre-treatment, five on day 1, eight on day 3, two on day 5, and seven on day 16. One patient only had a pre-treatment biopsy, and then withdrew from the study.

5.3 RESULTS

5.31 Disaccharidases

The disaccharidases, lactase, sucrase and maltase, showed no significant change over time, and remained within normal limits. All three disaccharidase results increased slightly with time, being lowest, and in the case of lactase below the normal range, before treatment, being higher at day 3, and even higher on day 16. The reason for this trend is unclear.

5.32 Light microscopy

Histomorphometry

Villus area ($p=0.002$), crypt length ($p=0.0001$) and mitotic index ($p=0.000$) decreased on day 3. Both crypt length ($p=0.01$) and mitotic index ($p=0.005$) rebounded above baseline on day 16, whereas villus area returned to baseline ($p=0.4$). See figures 5.32.1, 2 and 3.

However, when the day 1 and day 5 biopsies were added, the analysis had to be changed. A simple comparison of means was performed using Peritz' F-test, and this is shown in Figures 5.32.4 and 5.32.5. Villus area was not found to show any significant change. Crypt length however was reduced from a pre-treatment mean value of 191 μm to a value of 141 μm on day 3 ($p = 0.0009$). There was also a significant difference between crypt lengths on day 3 and day 16 (210μm), $p<0.0001$, and between day 5 (147μm) and day 16, $p=0.0123$, but the difference between day 3 and day 5 was insignificant (See figure 5.32.4). The low number of day 5 biopsies ($n=2$) makes this particular figure less certain. Mitotic index fell from a mean of 2.5 counts per crypt pre-treatment to 0.7 on day 3 ($p=0.0043$), and increased again to 3.1 on day 16 ($p=0.0008$). See figure 5.32.5.

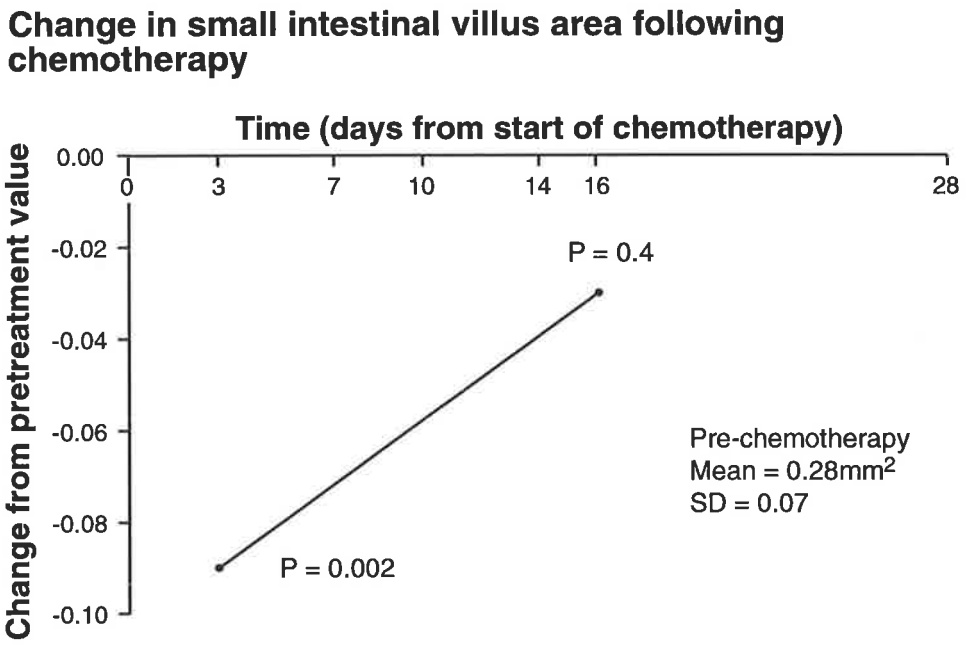


Fig. 5.32.1 Analysis of repeated measures. Change in small intestinal villus area following chemotherapy. Villus area fell significantly by day +3 and rebounded to baseline value by day +16.

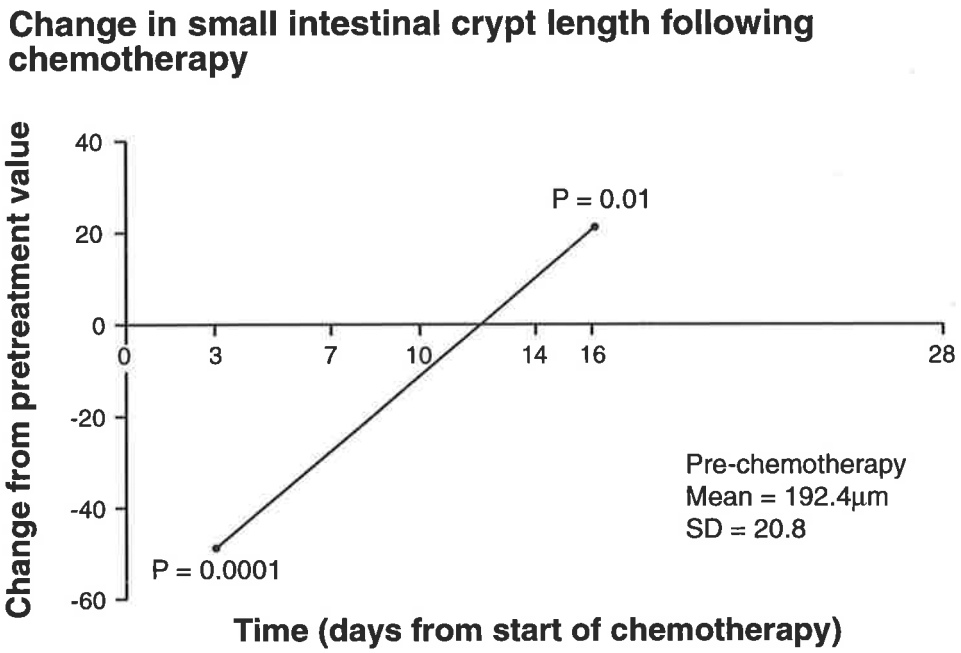


Fig. 5.32.2 Analysis of repeated measures. Change in small intestinal crypt length following chemotherapy. Crypt length fell significantly by day +3 and rebounded to significantly greater than baseline value by day +16.

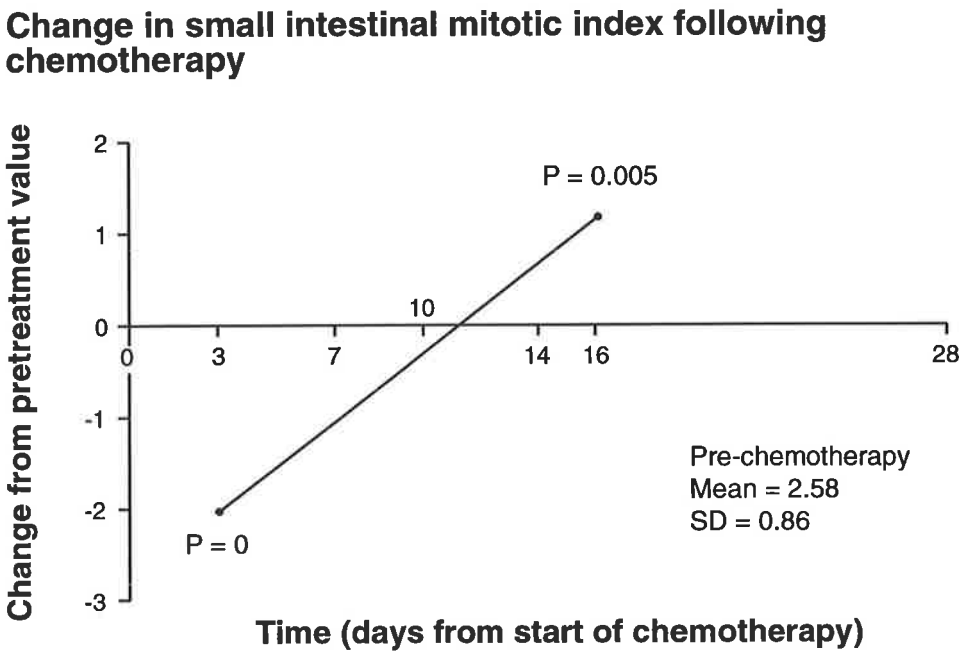


Fig. 5.32.3 Analysis of repeated measures. Change in small intestinal mitotic index following chemotherapy. Mitotic index fell significantly by day +3 and rebounded to significantly greater than baseline value by day +16.

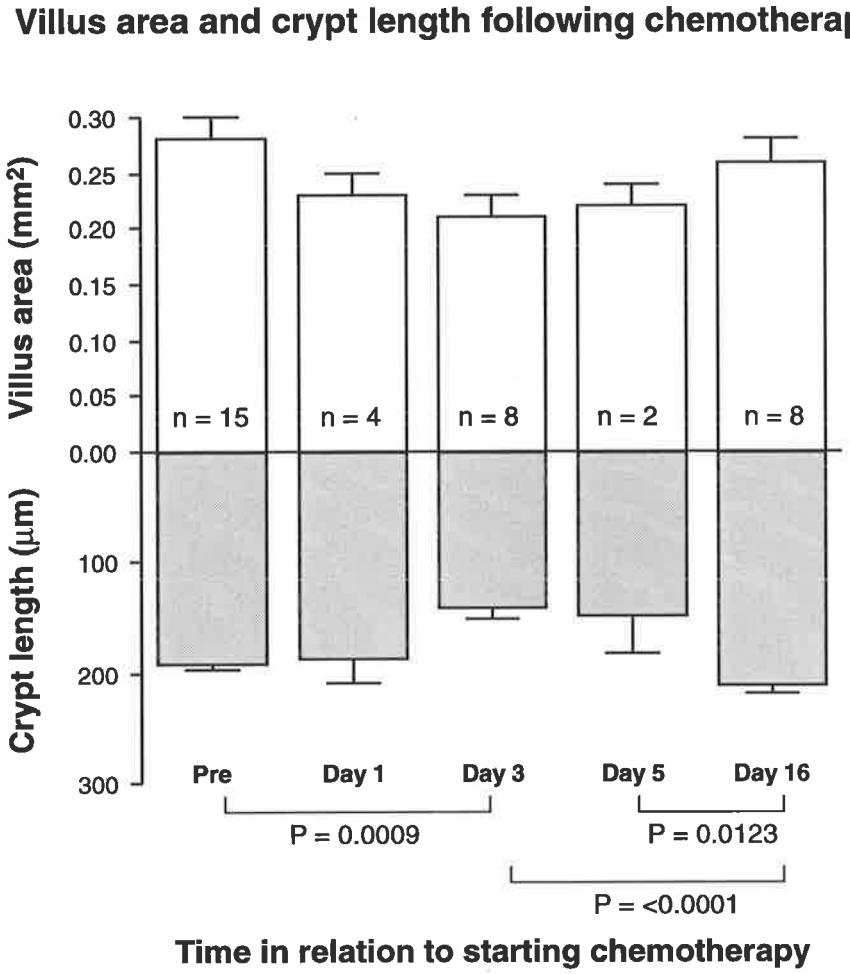


Fig. 5.32.4 Villus area and crypt length following chemotherapy. Using Peritz' F-test, there was a significant reduction in crypt length at day +3, which normalised with an increase by day +16. Changes in villus area did not reach statistical significance. Data are given as mean + standard error.

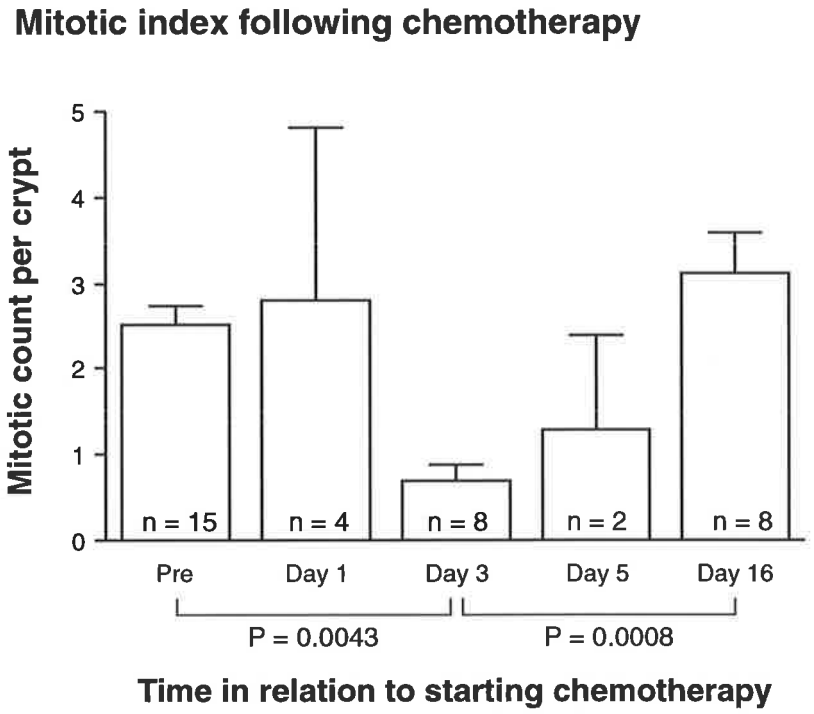


Fig. 5.32.5 Mitotic index following chemotherapy. Using Peritz' F-test, there was a significant reduction in mitotic index at day +3, increasing again by day +16. Data are given as mean + standard error.

Apoptosis

This was also analysed using Peritz' F-test. The mean apoptotic cell count per crypt increased from 0.18 pre-treatment to a peak of 1.3 on day 1 ($p<0.0001$), before falling to 0.41 on day 3, to 0.37 on day 5 and to 0.10 on day 16. Data for apoptosis of intestinal crypts are given in Figure 5.32.6. Thus, the first histological effect of chemotherapy was early induction of apoptosis in intestinal crypts that preceded hypoproliferation. This normalised by day 16. However, we had only two patients at day 5 after chemotherapy. Figure 5.32.7 shows an electron micrograph of an apoptotic epithelial cell on day 1 after chemotherapy.

Enterocyte height

The analysis for enterocyte height is shown in Figure 5.32.8. Enterocyte height at day 3 decreased 4.32 μm from a mean of 31.9 μm pre-treatment ($p=0.018$) with a return to normal by day 16 ($p=0.56$). This is consistent with the results above.

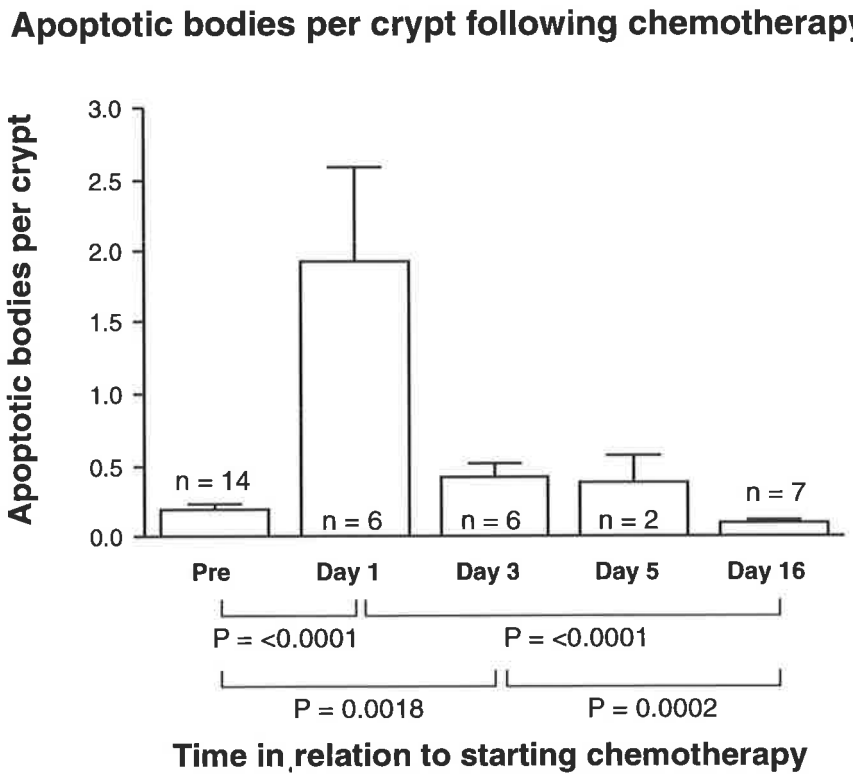


Fig. 5.32.6 Number of apoptotic bodies per crypt following chemotherapy. There is a significant rise in apoptosis on day +1 following chemotherapy, which gradually falls to baseline by day +16. This increase precedes other changes in the crypt/villus. Data are given as mean + standard error.

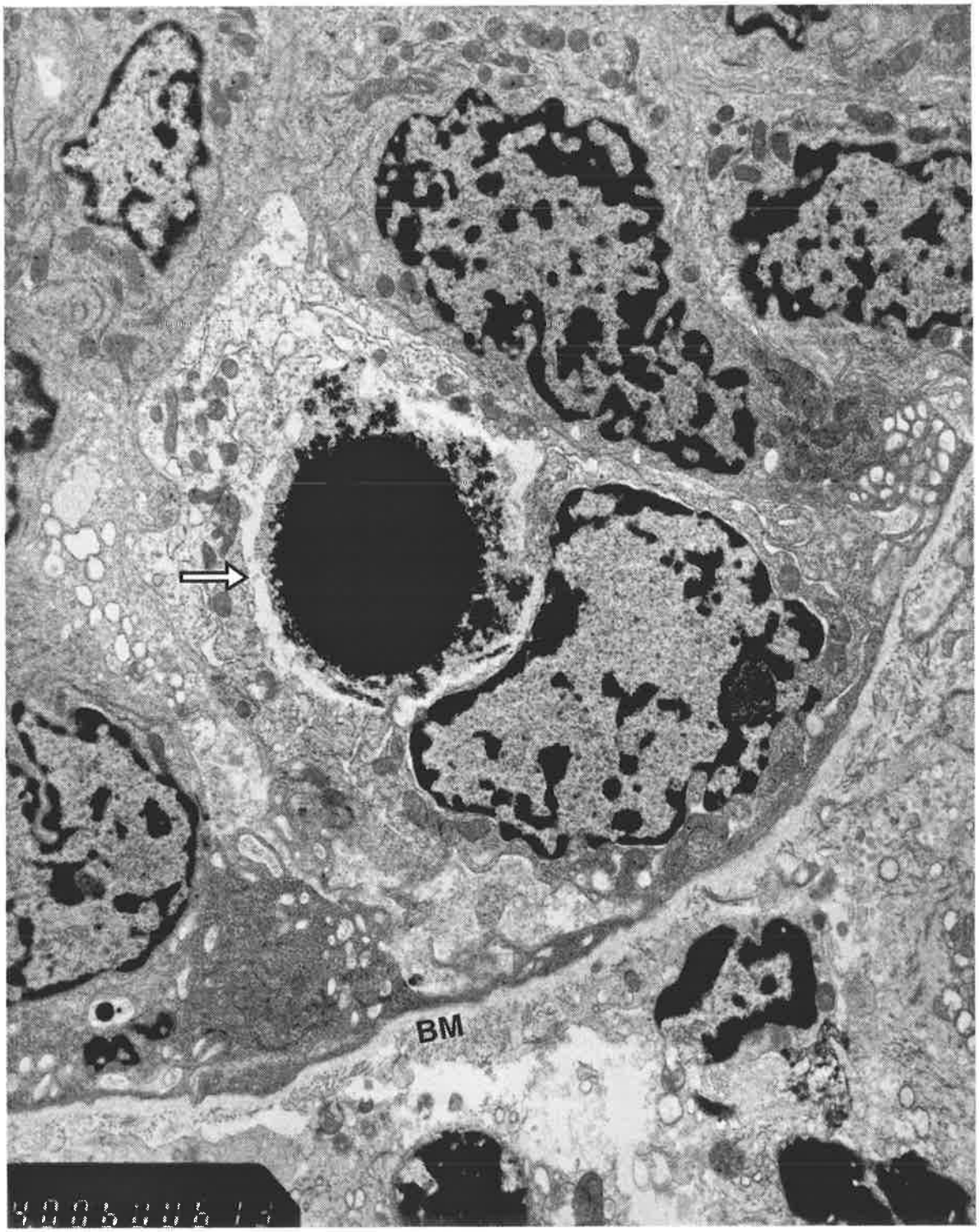


Fig. 5.32.7 An electron micrograph of an apoptotic cell in the epithelial layer of the small intestinal crypt on day 1 post chemotherapy. The arrow points to the pyknotic nucleus. BM = basement membrane. Magnification × 12,000.

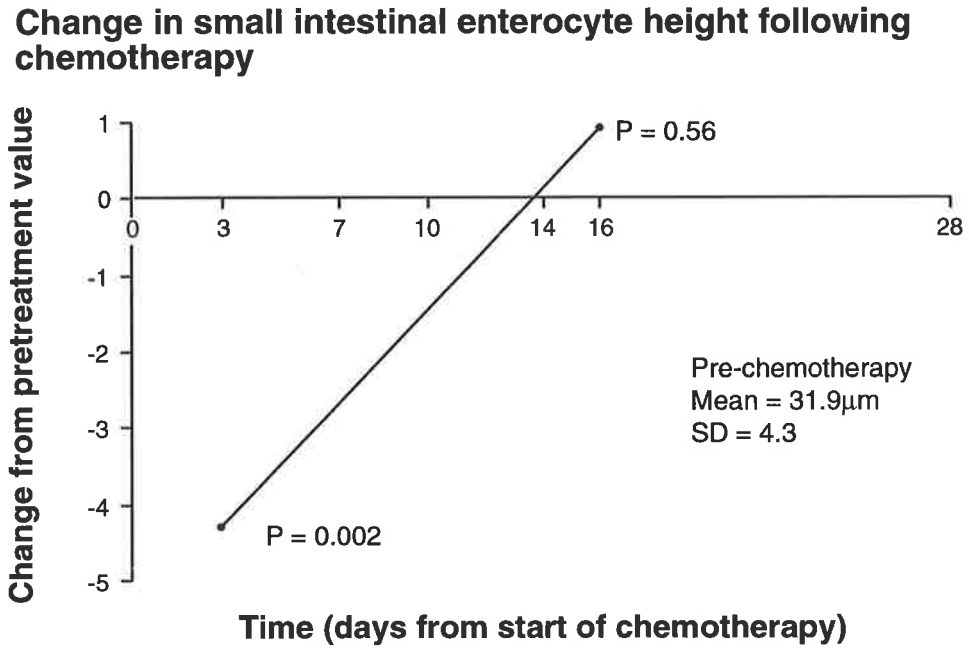


Fig. 5.32.8 Analysis of repeated measures. Change in small intestinal enterocyte height following chemotherapy. Enterocyte height fell significantly by day +3 and recovered to baseline value by day +16.

5.33 Electron microscopy

Figure 5.33.1. shows an electron micrograph of an open and of a closed tight junction. The percentage of leaky tight junctions increased from 38.9 pre-treatment to 54.6 on day 1 and 63.3 on day 3. It fell again to 46.6 on day 16. (See figure 5.33.2). In the overall analysis of repeated measures, the percentage had increased significantly by day 3, ($p = 0.0000$), and returned to baseline on day 16 ($p = 0.46$). (See Figure 5.33.3). These changes are similar to, but less severe than, those seen in cholera (190). We found no increase in lactulose permeability associated with the increase in open tight junctions, implying that the size of tight junction opening is not the only important factor in lactulose absorption. Indeed, it is also possible that open junctions may be a manifestation of immaturity of the enterocytes, which is consistent with repopulation of the crypt/villus with immature cells. Brush border height, as measured on electron microscopy, did not show any significant change with time. (See Figure 5.33.4).

As each patient had a maximum of only two biopsies, it is not possible to say that the day 16 results are definitely related to the day 3 results, in the same way that one can compare the symptom and function results in Chapter 4. However, the biopsy changes do follow a similar pattern to symptom and function changes.

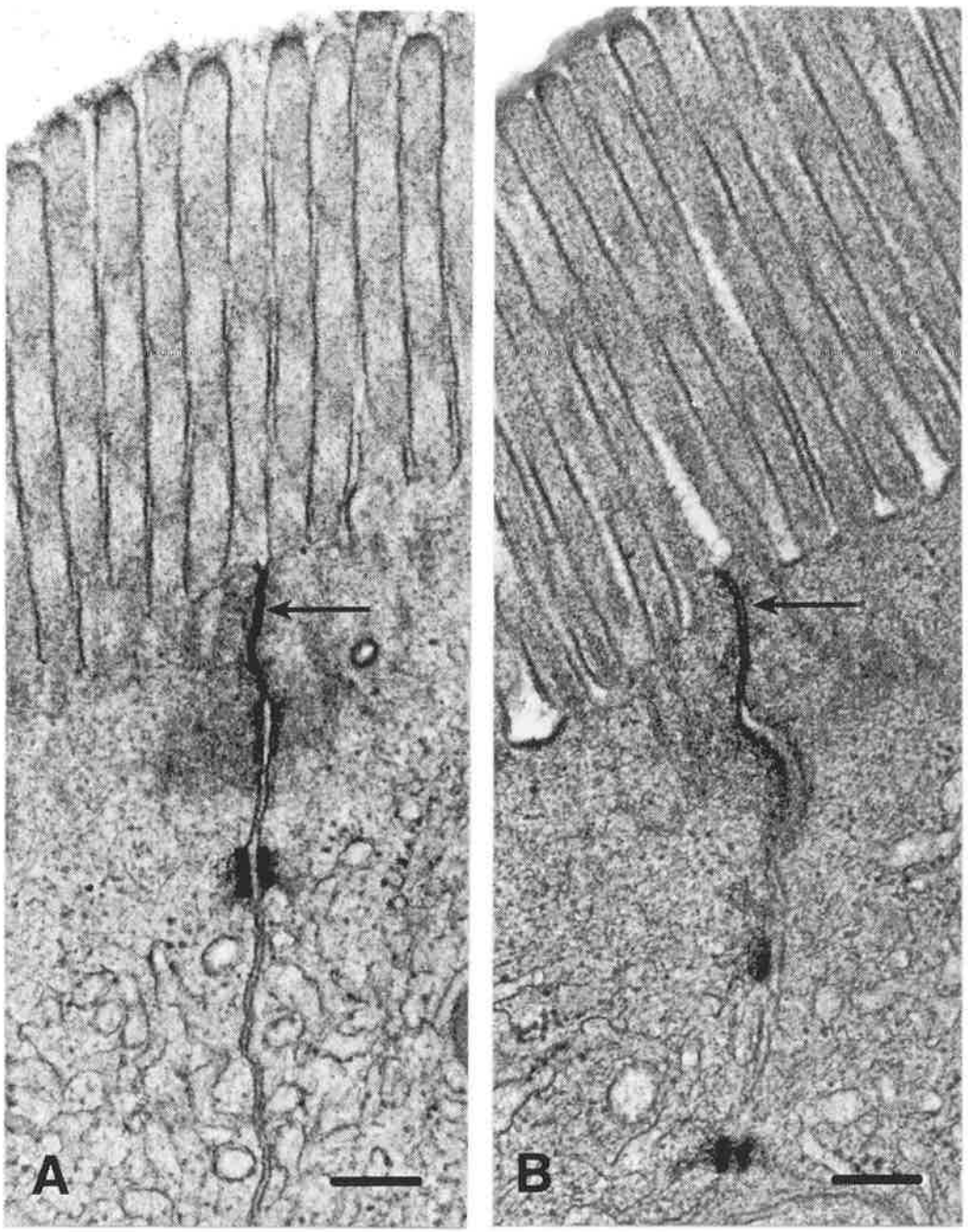


Fig. 5.33.1 Electronmicrographs of two duodenal biopsies; both depict the ultrastructure of apposing epithelial cells at the luminal surface.
A. Pre-chemotherapy. Junctional complex with normal tight junction (arrow).
B. Post-chemotherapy. Junctional complex with open (leaky) tight junction (arrow).
Oral magnification: 66,200X. Bar represents 200nm.

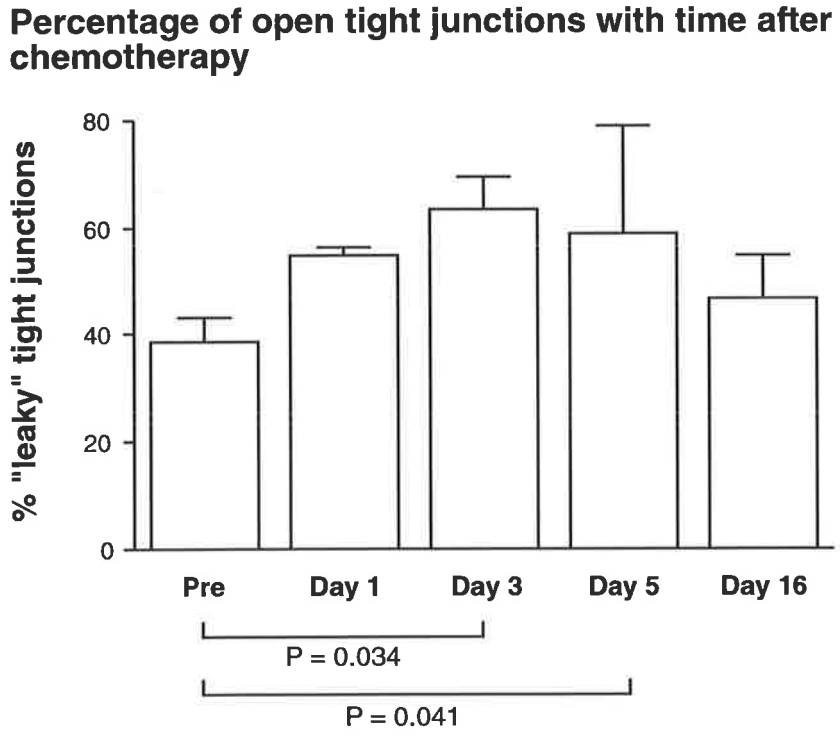


Fig. 5.33.2 Percentage of open epithelial tight junctions following chemotherapy. There is a significant increase in the number of open tight junctions at day +3 and day +5, with a reduction to baseline by day +16.
Data are given as mean + standard error.

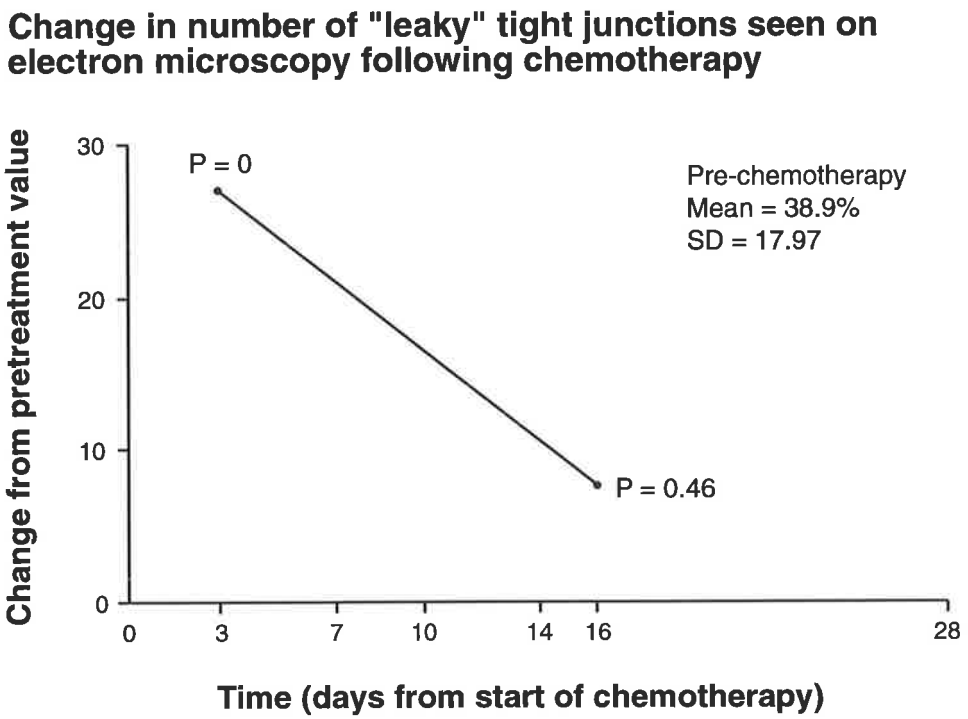


Fig. 5.33.3 Analysis of repeated measures. Change in number of open epithelial tight junctions following chemotherapy. There is a significant increase in open tight junctions at day +3 with a return to baseline by day +16.

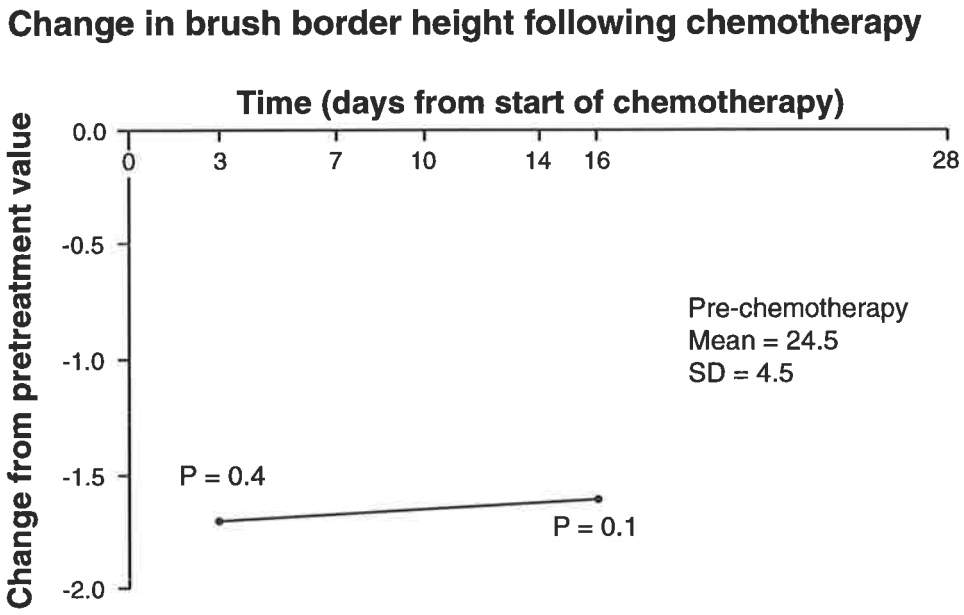


Fig. 5.33.4. Analysis of repeated measures. Brush border height following chemotherapy. There is no significant difference with time.

5.4 Discussion

The aim of this study was to measure histomorphometry, apoptosis, and tight junction integrity in the small intestine of patients receiving chemotherapy. The earliest event visible after chemotherapy in these patients was an increase in apoptotic cell count per crypt at 1 day after treatment. This is followed after three days by a reduction in villus area, crypt length and mitotic index. At this time apoptosis decreased but to still elevated levels. In a very small number at day 5, we showed a return towards normal, and by day 16 there was no significant change from pre-treatment levels. The new finding of this study was the early induction of apoptosis of intestinal crypts. Interestingly, Trier did see discrete spherical bodies, suggestive of apoptosis in crypts, although at that time true apoptosis was not recognised as a pathophysiological process. Cunningham also found pyknotic material focally in crypts which could have been apoptotic bodies. These studies confirm the hypoproliferative changes seen previously by Trier, Smith, Cunningham, Potten, Taminiau and others (See Table 2.1). These apoptotic changes preceded small intestinal symptoms, but the histomorphometrical changes were present at the same time as symptoms. The gastrointestinal symptoms and morphological changes were decreasing as mouth symptoms began.

The morphological changes occurred in a non dose-dependent manner, ie. there was no delineation between standard and high-dose chemotherapy. We have therefore shown a dose-dependent, prolonged change in permeability, but a dose-independent, short-lived change in histomorphometry. This implies two separate mechanisms. It would have been preferable to have the patients who had day 1 or day 5 biopsies as part of the whole study, so that their symptoms and functional changes could have been investigated. However, that was not possible, and despite that draw back, they do provide important information, especially with respect to apoptosis.

The change in enterocyte height parallels the changes in villus area, crypt length and mitotic index, showing that a reduction in individual cell size contributed to the abnormality. The lack of change in brush border height is harder to explain. The trend in disaccharidases to increase following chemotherapy is very unusual, and might be explained by the release of lysosomal enzymes following chemotherapy. The other explanation would be that the cancer itself has a mild effect on the small intestinal mucosal enzyme function which is corrected by chemotherapy, which would obviously imply that the chemotherapy was having some effect on the tumour and its ability to affect other organs. Further investigation will be required to elucidate the cause.

The fifth aim of the study was to correlate symptoms, function and morphology. These data did not indicate whether one particular symptom or functional change predicted any other, and similarly for morphological changes. However, peak changes in gastrointestinal symptoms, function and morphology did all occur at day 3 after chemotherapy, and these generally returned to normal values between days 14 and 28. Dose-dependent changes occurred only with decreased body weight, body mass index, transferrin and rhamnose absorption, otherwise patients across all groups experienced symptoms and morphological changes regardless of the dose of chemotherapy. It is probable therefore that small intestinal symptoms, function and morphology are related following chemotherapy. Oral symptoms were not apparently related, as these were more common than gastrointestinal symptoms, and were delayed in onset.

5.5 Summary

This study has shown that the first change in the small intestine following chemotherapy is an increase in apoptosis on day 1, followed by crypt hypoplasia on day 3, which is accompanied by the onset of abdominal pain, bloating and diarrhoea, along with functional changes such as increased sugar permeability, and minor nutritional changes. The small intestinal symptoms began to recover when mouth pain and ulcers occurred from day 7 to 10. However, the functional changes persist, with nutritional changes only normalising by day 14 or 28, but sugar permeability remaining abnormal until at least day 28. Morphological changes recovered by day 16. This is the first time that symptoms, function and morphology have been so extensively studied in humans in a prospective manner following chemotherapy. Gastrointestinal side effects were confirmed as a significant problem following chemotherapy, that needs to be addressed either by prevention or active treatment.

Not surprisingly, there remain many unanswered questions. For example, why does the functional defect persist after recovery of the physical mucosal barrier? How can we protect the small intestine from the effects of chemotherapy without compromising anti-tumour effect? How can we successfully assess small bowel bacterial overgrowth in chemotherapy patients? All these are areas for further study. Our results agree with the literature, expand our knowledge of the sequence of events, and provide a platform for further research.

The next step is to move into work on an animal model for initial prevention studies, before human prevention trials. Successful treatment of already developed mucositis would be very difficult, as the stem cells of the small intestinal crypts are already stimulated, self-replicating and providing increased daughter cells to repopulate the damaged crypts. It would be far better to prevent the chemotherapy from doing the damage in the first place, rather than to try to catch up when it already too late.

CHAPTER SIX

DEVELOPMENT OF THE DARK AGOUTI RAT MAMMARY ADENOCARCINOMA MODEL TO ASSESS SMALL INTESTINAL AND TUMOUR EFFECTS OF CHEMOPREVENTION

6.1 GENERAL INTRODUCTION

There are now many agents available, such as TGF-β, IL-11, KGF, glutamine, and WGFE, that might, either alone or in combination, help to protect the gastrointestinal tract from the unwanted toxicities of chemotherapy (See Table 2.3). The major problem with any new agent that will be used in humans is proving that it is safe. In trials of chemoprevention, there is the added necessity of proving that the agent which protects the gastrointestinal tract from chemotherapy doesn't also protect the tumour from the cytotoxicity of the chemotherapy. There are theoretical reasons why a chemoprotectant might protect the tumour. Glutamine, for example, is the favoured fuel of the small intestine. It is also, however, a favoured fuel for tumours, so that it might theoretically help them to grow. TGF-β acts by maintaining cells in resting phase so that they are not damaged by the cell-cycle specific agents. If it also keeps tumour cells in resting phase, it will stop the chemotherapy from acting. KGF is a growth factor for the gut which might theoretically also promote tumour growth.

There is a need for an animal model which would allow simultaneous assessment of the effects of chemotherapy and chemoprotectants on both the small intestine and the tumour.

The Dark Agouti Rat Mammary Adenocarcinoma model (DAMA)

A model was therefore developed using subcutaneously implanted isogeneic rat breast cancer in the female dark agouti (DA) rat. The mammary adenocarcinoma arose spontaneously in the 1970's, and has been propagated ever since by passage through female rats. Female rats are used because the tumour passages more effectively through females than males. The tumour is injected (as a cell suspension) subcutaneously into both flanks, and is harvested after two weeks. Tissue can be stored frozen in liquid nitrogen, or used immediately. This model has previously been used for studies of malnutrition following chemotherapy by Rofe (191;192); and for studies of neuroprotection by glutamate by Boyle (193). It has not previously been used to assess the small intestine nor gastrointestinal protection after chemotherapy. The model allows an attempt at ameliorating small intestinal toxicity to proceed while guarding against paradoxically increasing tumour growth. The following studies were performed at the Child Health Research Institute, at The Women's and Children's Hospital in Adelaide, South Australia. Ethics Committee approval was granted by the Research Ethics Committee of the Hospital.

6.2 A STUDY TO FIND THE OPTIMUM DOSE OF METHOTREXATE TO CAUSE NON-LETHAL SMALL INTESTINAL MUCOSITIS IN THE DARK AGOUTI RAT

6.21 INTRODUCTION

The aims of this study were:

1. To find the optimal dose of methotrexate (MTX) to administer intramuscularly to the DA rat in order to produce significant, non-lethal, small intestinal mucositis, and

2. To determine the optimal days for tissue collection in relation to small intestinal damage and healing, so that we could proceed to further experiments.

In his studies using the DAMA model, Rofe used 0.5 mg/kg MTX intramuscularly on 2 consecutive days. However, based on his food intake data, it was felt that this dose would be insufficient to produce significant mucositis. This first experiment, however, was carried out in DA rats that did not have the mammary adenocarcinoma, as it was purely a dose finding study. Once the dose had been established, all further studies were to occur in tumour bearing rats.

6.22 METHODOLOGY

Twenty-eight female DA rats of 150g ± 20g were purchased from the Institute of Medical and Veterinary Science (IMVS), Adelaide. Animals were fed a casein diet from 5 days before treatment with methotrexate (MTX), and settled in metabolism cages two days before the MTX for the duration of the study. The rats were each randomised to one of four treatment groups:

Group 1	MTX 0.50 mg/kg/d	2 consecutive days (days 0 to 1)	8 rats
Group 2	MTX 0.75 mg/kg/d	2 consecutive days (days 0 to 1)	8 rats
Group 3	MTX 1.00 mg/kg/d	2 consecutive days (days 0 to 1)	8 rats
Group 4	saline control	2 consecutive days (days 0 to 1)	4 rats

MTX (or saline) was injected into the muscle on the dorsal surface of the thigh. The syringes were drawn back prior to injecting to ensure the MTX was not injected intra-venously. Four rats from each of groups 1-3 were killed on day +2, that is 24 hours after the second MTX injection. The remaining rats in all four groups were killed on day +5, that is 96 hours after the second MTX injection. Food intake and body weights of the rats were measured daily from the day before the first MTX injection.

The rats were sacrificed using CO_2, and 2 cm samples of small intestine were collected at sites corresponding to 25% and 75% of the jejuno-ileum, into formalin and Clarke's fixative. Samples were transferred into 70% ethanol after 24 hours. Formalin fixed tissues were paraffin embedded for haematoxylin and eosin staining and for subsequent TUNEL analysis of apoptosis. Clarke's fixed tissues were examined by stereo microscopy for villus area, crypt length and mitotic index.

6.23 RESULTS

Body weight and food intake

The data for body weight and food intake for different MTX doses are shown in Figures 6.23.1 and 6.23.2. The rats were smaller than anticipated on arrival, with rats weighing an average of 142g. Body weights and food intakes fell with increasing doses of MTX, compared with saline-injected controls. Thus rats given 1.0 mg/kg MTX weighed a mean of 8 grams less than those given saline at day 2, but regained their weights by day 5. Food intake also decreased more in rats treated with MTX, being 6.8 g in the 1.0 mg/kg group on day 2 compared with 10.4 g in the saline controls. On day 5, this had reversed, with the 1.0 mg/kg MTX rats increasing their intake to 10.2 g on day 5, compared with 8.5 g in controls.

Histomorphometry

The histomorphometry results are shown in Figures 6.23.3 and 6.23.4. These show a progressive decrease in crypt length and proliferation at day 2, but these returned to normal values by day 5. Overall these changes were modest as seen by only small changes in villus area.

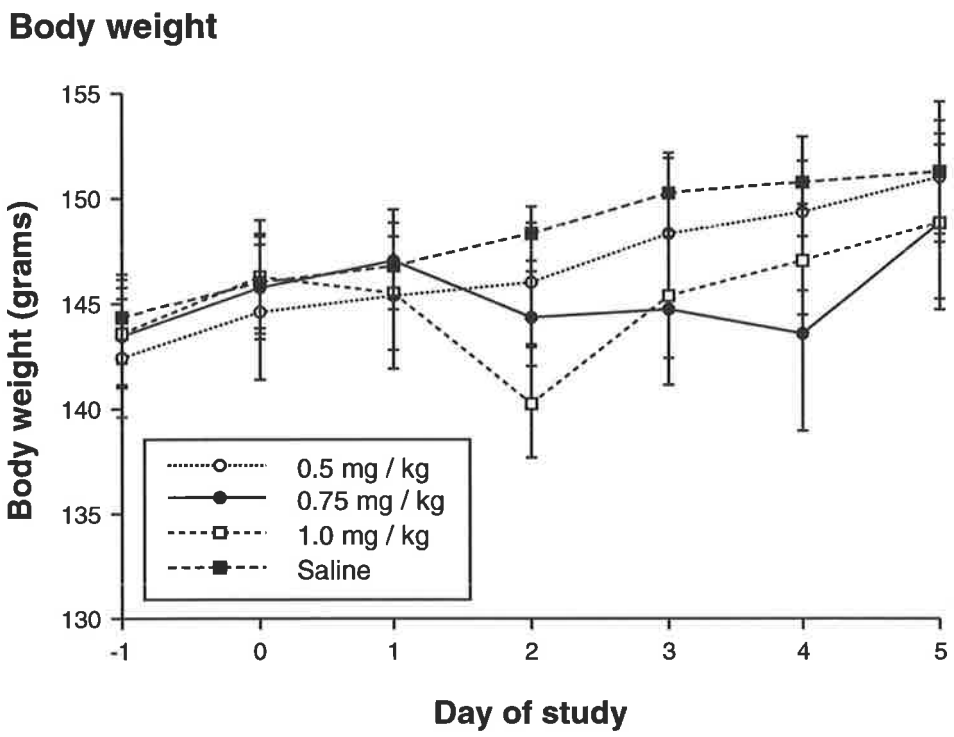


Fig. 6.23.1 Body weights of dark agouti rats treated with increasing doses of MTX versus saline on days 0 and +1. Rats were killed on day +5. Values shown are means and standard errors.

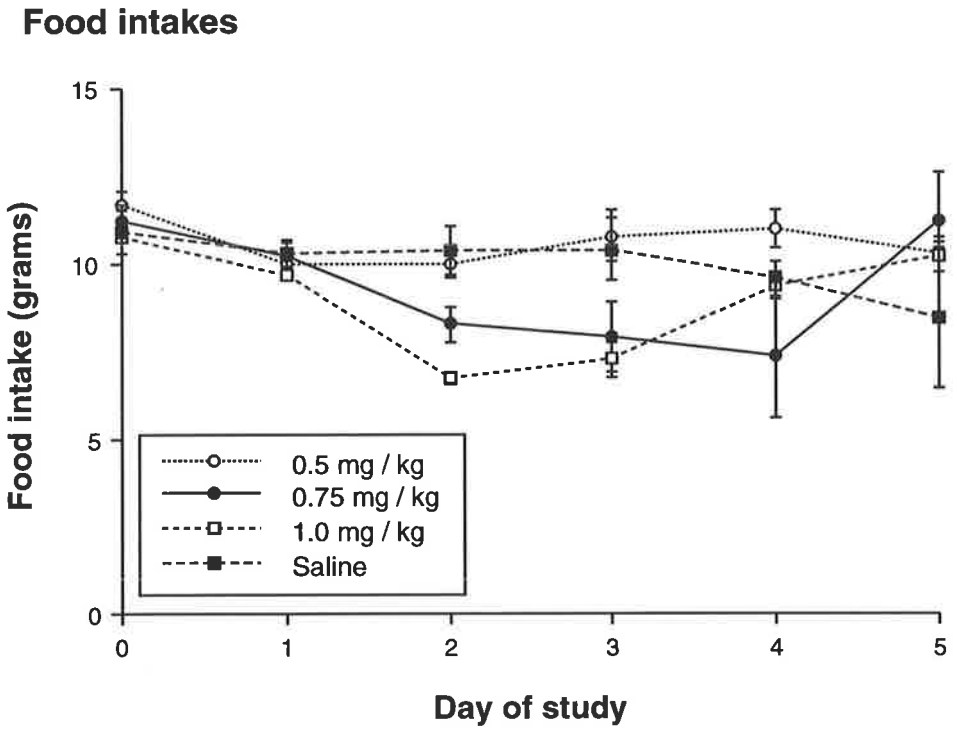


Fig. 6.23.2 Daily food intake of dark agouti rats treated with increasing doses of MTX versus saline on days 0 and +1. Rats were killed on day +5. Values shown are means and standard errors.

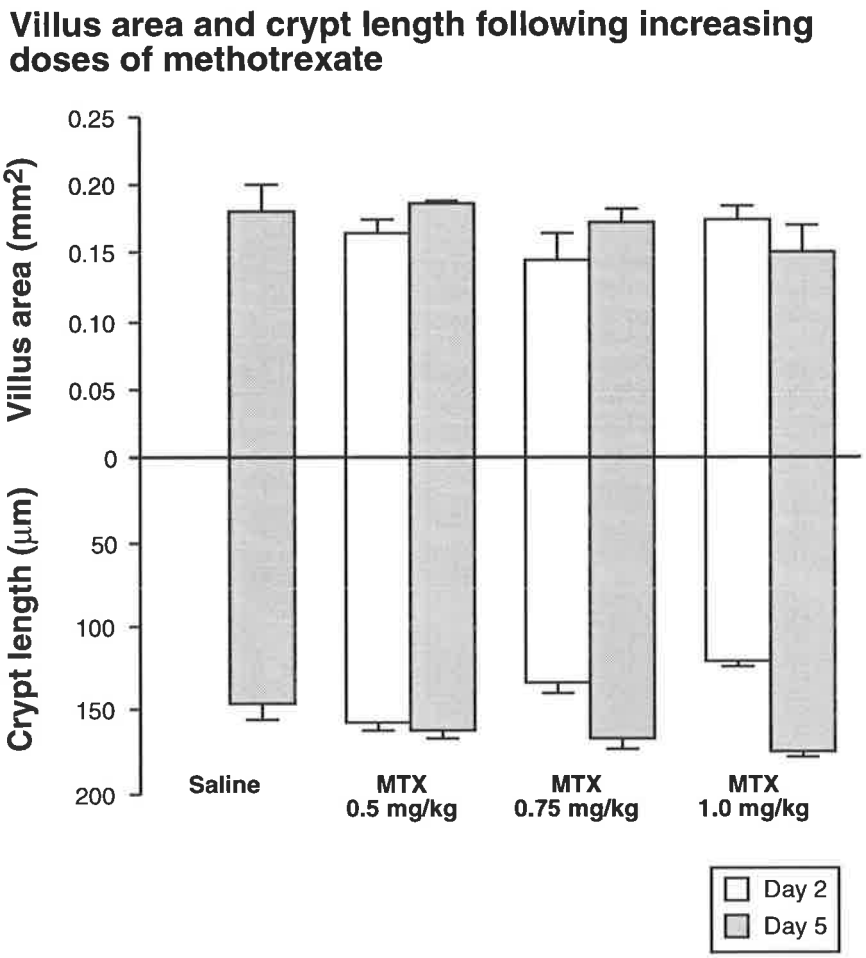


Fig. 6.23.3 Villus area and crypt length in the dark agouti rat small intestine following increasing doses of im methotrexate (MTX). Increasing doses of MTX lead to reduced crypt length on day +2, but there has been full recovery by day +5 even at the highest dose.

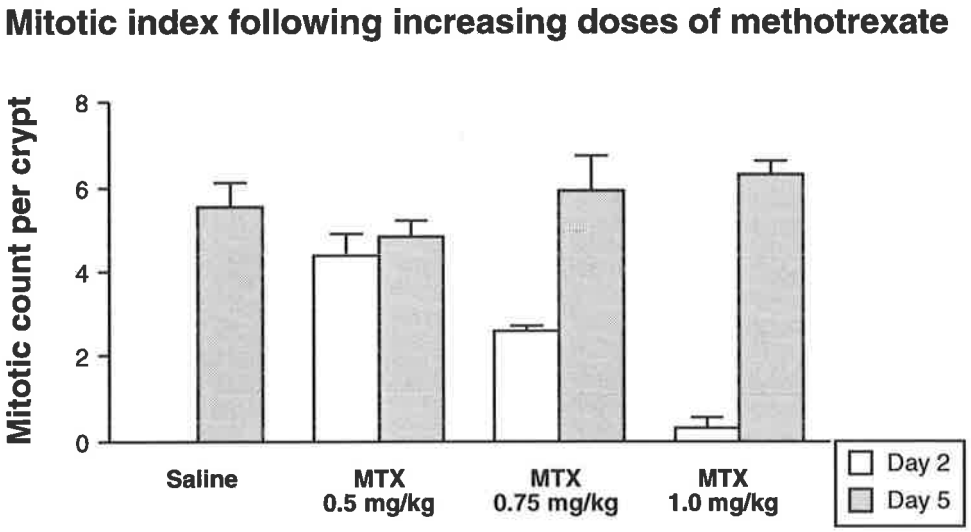


Fig. 6.23.4 Mitotic count per crypt in the dark agouti rat small intestine following increasing doses of im MTX. Increasing doses of MTX lead to a progressive reduction in mitotic count at day +2, with full recovery at day +5.

6.24 DISCUSSION

The dose that produced the most significant morphometrical abnormalities was 1.0 mg/kg. No dose, however, produced a particularly severe result, and all the animals had recovered by day 5. It was therefore decided to proceed with a higher dose of 1.5 mg/kg/d MTX for 2 days for the further experiments, and to kill the rats on day 2, in order to see a significant effect.

6.3 THE EFFECT OF GLUTAMINE IN PREVENTING SMALL INTESTINAL MUCOSITIS IN THE DARK AGOUTI RAT MAMMARY ADENOCARCINOMA MODEL

6.31 INTRODUCTION

A dose of 1.5 mg/kg/d MTX im for two doses was chosen from the previous experiment, as a dose likely to cause significant non-lethal small intestinal mucositis. Day 2 was selected as the best single day to kill the rats: that is twenty-four hours after the second dose of methotrexate, at the estimated peak of small intestinal damage. The aim of this study was to determine the effects of glutamine on mammary adenocarcinoma growth, and on small intestinal damage and repair in the methotrexate-treated DA rat.

6.32 METHODOLOGY

Twenty-four female DA rats were used for this study. Tumour suspension was supplied by Tanya Ellis at the IMVS, at a dose of 0.2 ml of a 25% cell suspension, and 0.1 ml was injected subcutaneously into each flank of anaesthetised rats, using a 23 gauge needle (194). The skin over each flank was shaved prior to injection for ease of measurement of the tumour. See Figure 6.32.1.

Fig. 6.32.1 A tumour-bearing DA rat. The tumour was implanted subcutaneously into each flank, and allowed to grow for 10 days.

Rats were transferred to a casein based diet 11 days before the first methotrexate injection, which was taken as day zero. The study outline is given in Figure 6.32.2. Animals were placed into metabolism cages on day -6, and stabilised there for 4 days before starting their trial gavages on day -2, the latter being continued until sacrifice on day +2. Animals were randomised to receive gavages of either glutamine or BSA (bovine serum albumin) solution, 2 ml twice daily. The aim was to provide 10% of protein intake as glutamine, and this was calculated as 0.2 g glutamine per day, given as 2 ml of a 50 mg/ml solution twice daily. The rats receiving BSA received 2 ml twice daily of a solution containing 55 mg/ml BSA. Each rat received 1.5 mg/kg MTX on days 0 and +1, intramuscularly into the quadriceps muscle.

Study monitoring

1. Food intake and body weight were recorded daily from day -3.
2. Tumour size (length and width) was measured with callipers alternate daily from day -5. The size of the tumour as a percentage of body weight was calculated using the formula: [(width x length)/2=g of tumour]/body weight of rat.
3. General condition of the rats and ulceration of the tumour were recorded daily.

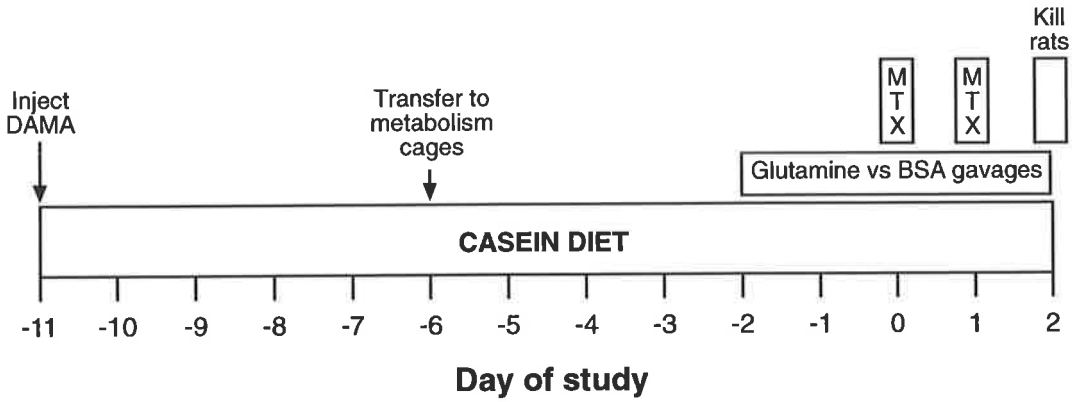


Fig. 6.32.2 Schematic diagram of protocol for dark agouti rat mammary adenocarcinoma model, glutamine prevention study. Female rats were injected with tumour cell suspension on day -11, transferred to metabolism cages on day -6, and started on glutamine or BSA gavages on day -2. MTX 1.5mg /kg/d im was injected on days 0 and +1, with rats being killed on day +2.

Tissue collection

On day +2, the rats were anaesthetised with halothane, and a terminal blood sample was collected into heparin. Two 2 cm samples of small intestine were collected, at sites corresponding to 25% and 75% of the jejuno-ileum, into Clarke's fixative and formalin. The tumour was weighed and two cross-sections across the centre of the tumour (approximately 5g) were collected into formalin and methacarn. Samples in both formalin and Clarke's were transferred to 70% alcohol at 24 hours. The Clarke's fixed small intestinal specimens were examined under stereo microscopy for villus area, crypt length and mitotic index. The formalin fixed tissue, from both small intestine and breast tumour, was paraffin embedded; 5 μm sections were cut, and stained with haematoxylin and eosin or prepared for TUNEL assay for apoptosis. Tumour tissue was also assessed for mitotic count as a measure of proliferative activity in the tumour (195).

Statistics

Results were analysed with the assistance of Mr. Phil Leppard, Department of Statistics, University of Adelaide. A two-way analysis of variance was used with factors of diet at two levels (casein versus glutamine), and methotrexate at two levels (none versus some). The program used was program 7D from BMDP Statistical Software Inc.

6.33 RESULTS

Food intake

The food intakes for the DA rats with tumours are shown in Figure 6.33.1. Food intake fell slightly in the control group over time, from 12.5g on day -3 to 10g on day +2, but the fall was more pronounced in MTX treated animals. The casein MTX group fell from 7.5g on day +1 to 6g on day +2, while the glutamine MTX group maintained its intake on day +1 before a fall to less than 6g on day +2.

Body weight

The body weights are shown in Figure 6.33.2. All animals increased their body weights over time, but in the MTX groups, there was a fall-off in gain on day +1, and then a fall on day +2. This was slightly more pronounced in the casein fed animals. At day 2 the no MTX groups had gained weight to >175g, whereas the MTX animals had fallen to 160g. The glutamine did not have a protective effect.

Tumour size

The change in tumour size is shown in Figure 6.33.3, and Figure 6.33.4 shows the calculated tumour size as a percentage of body weight. The actual tumour size as a percentage of body weight at animal sacrifice is also shown. Tumour weights at death are shown in Figure 6.33.5 and Table 6.33.5. There was no diet effect, but MTX reduced tumour weight compared with control ($p=0.004$) in both groups.

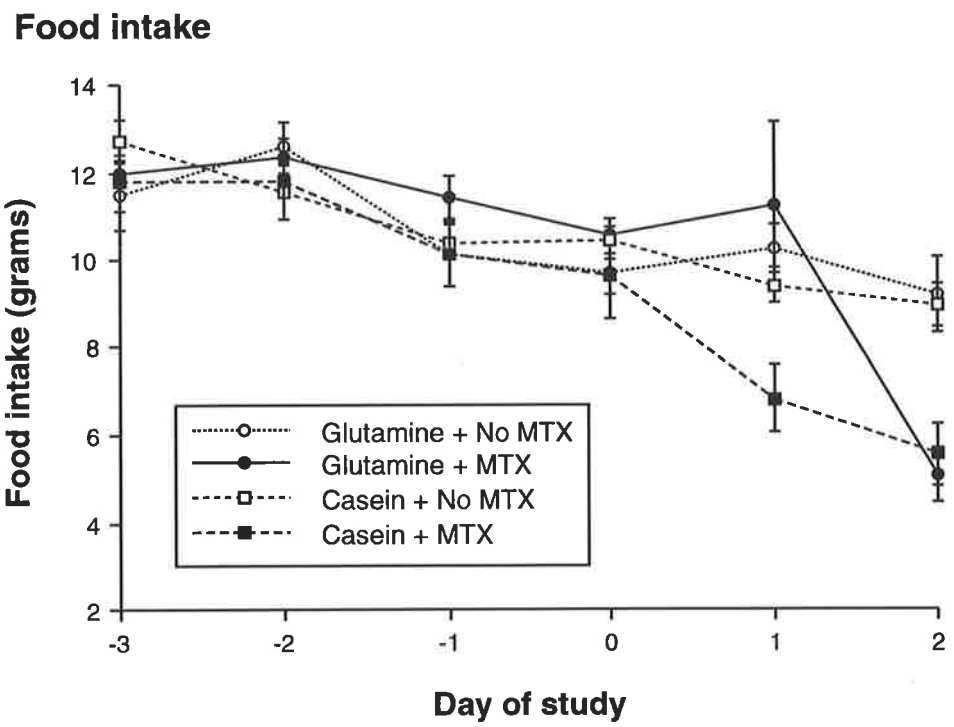


Fig. 6.33.1 Food intakes for tumour-bearing dark agouti rats fed a standard casein diet versus glutamine-supplemented diet and treated with MTX 1.5mg/m^2/d versus placebo on day 0 and +1. Data points shown are means and standard errors.

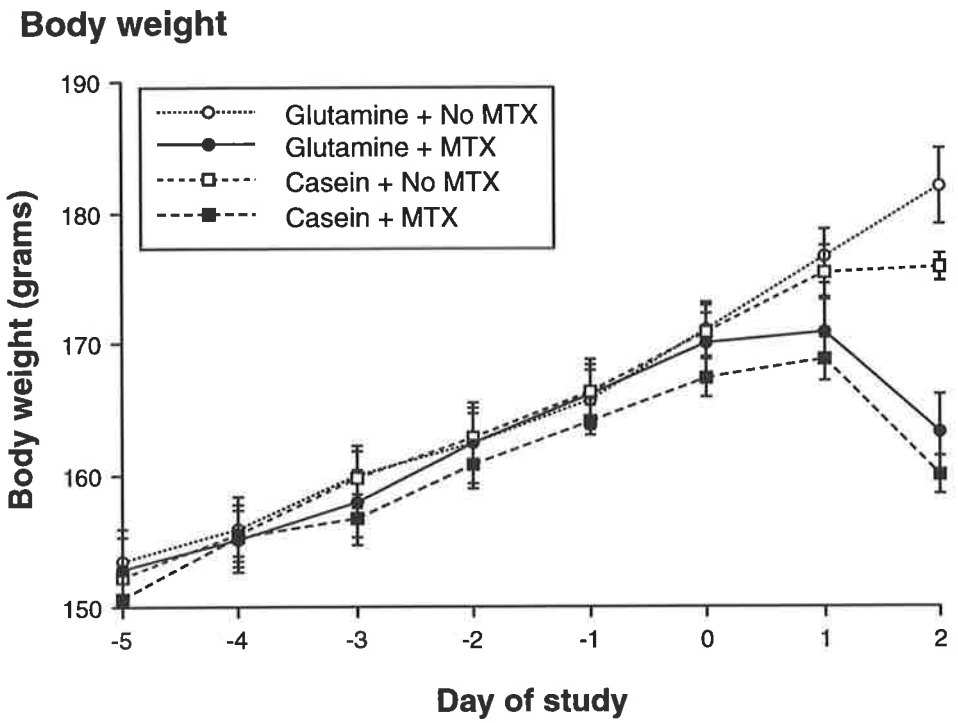


Fig. 6.33.2 Body weights of tumour-bearing dark agouti rats fed a casein diet versus glutamine-supplemented diet and treated with MTX 1.5mg/m^2/d versus placebo on days 0 and +1. Data points shown are means and standard errors.

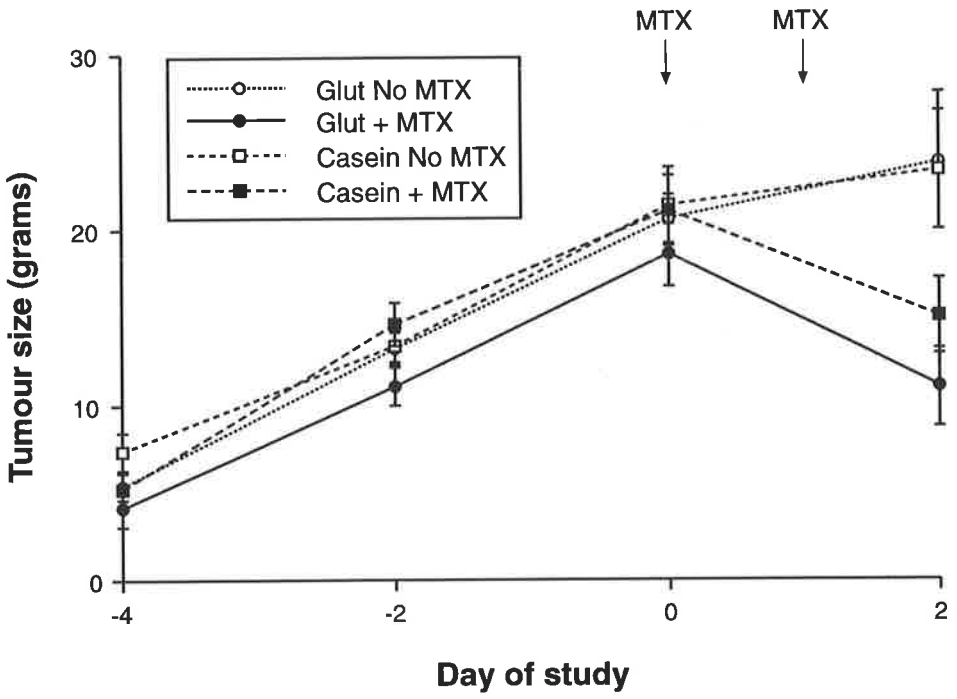


Fig. 6.33.3 Calculated tumour size in grams in MTX treated rats fed glutamine versus casein diet. Data are given as mean ± standard error.

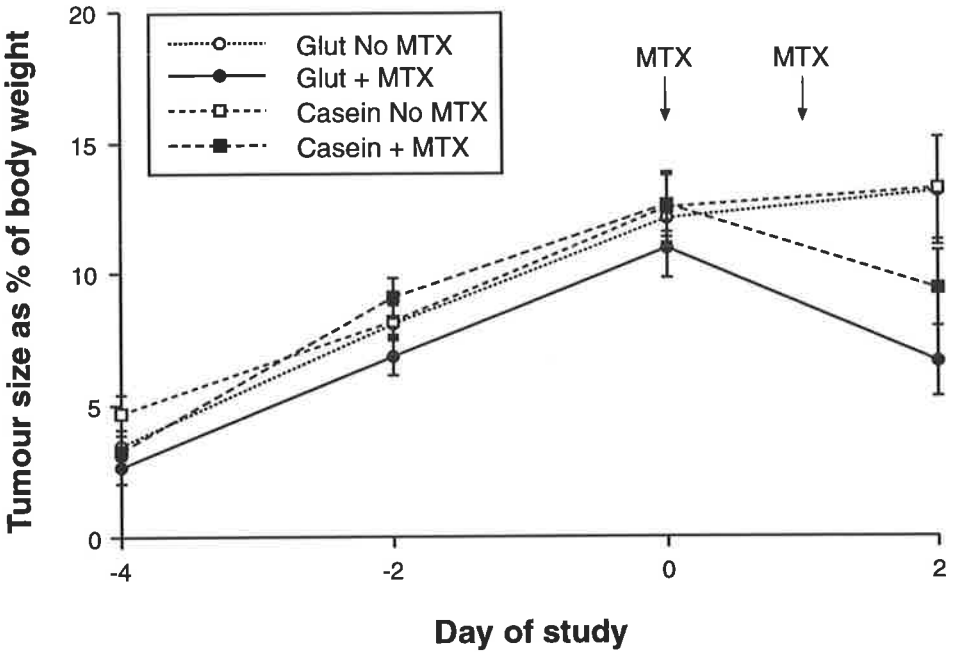


Fig. 6.33.4 Tumour size as a percentage of body weight in DA rats treated with MTX on a casein versus glutamine supplemented diet. Data are given as mean ± standard error.

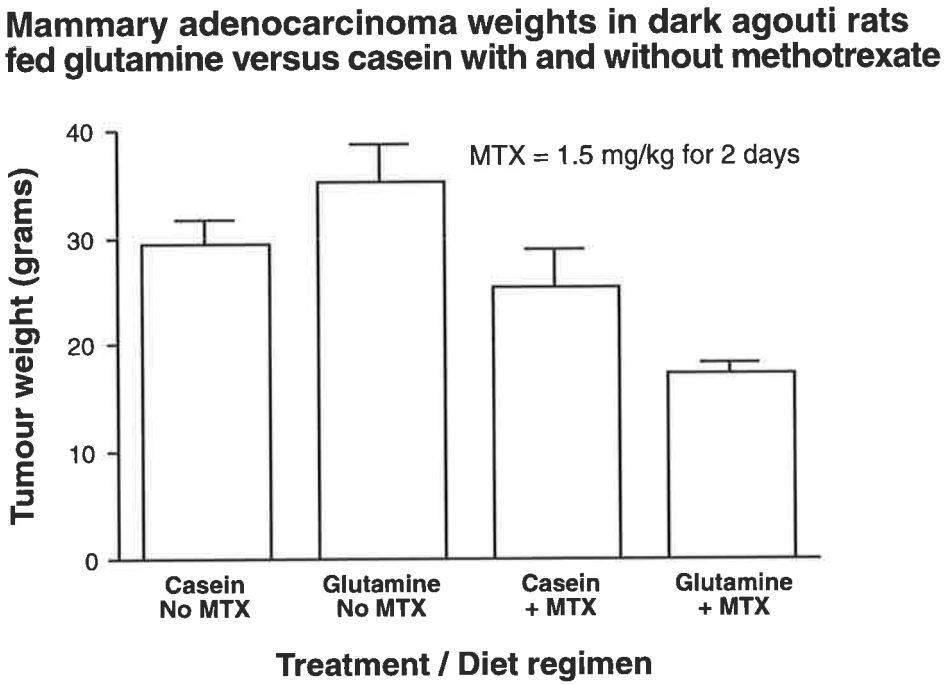


Fig. 6.33.5 Final mammary tumour weights at kill on day +2 in rats fed glutamine versus casein and treated with and without MTX. Data are given as mean + standard error.

Treatment	Rat #	Weight at day +2	Treatment	Rat #	Weight at day +2
Glutamine	35	27.91	Glutamine	31	18.54
&	41	31.87	&	38	17.83
No MTX	45	27.91	MTX	42	14.33
	57	31.58		43	19.49
	58	46.79		46	15.12
	63	44.91		67	19.44
mean		35.16			17.46
sem		3.46			0.91
Treatment	Rat #	Weight at Day +2	Treatment	Rat #	Weight at Day +2
Casein	33	25.11	Casein	32	18.86
&	36	23.60	&	44	17.47
No MTX	50	30.69	MTX	52	23.46
	54	35.11		53	18.08
	65	32.56		64	22.02
	68			66	17.81
mean		29.41			25.43
sem		2.19			3.5

Table 6.33.5. Tumour weights at sacrifice on day +2.

Histomorphometry

There is no diet effect throughout, but MTX has a significant effect on all parameters except villus area. The results are shown in Table 6.33.6. Figure 6.33.6 shows villus area and crypt length, and Figure 6.33.7 shows mitotic index. As can be seen, there is no change in villus area. The reduction in crypt length with MTX is significant (p=0.000) as is the reduction in mitotic index (p=0.000). The mitotic index in the glutamine and MTX treated rats is double that in the casein and MTX rats, but the protection is not clinically significant given the large reduction in scores due to the chemotherapy. The apoptotic counts per crypt are shown in Table 6.33.8, for both jejunum and ileum. MTX increased apoptotic counts in the ileum (p=0.0001) as shown in Figure 6.33.8, and in the jejunum (p=0.003), as shown in Figure 6.33.9. There was no difference between the casein and glutamine fed rats.

Tumour indices

Tumour proliferation, as measured by mitotic counts per unit area, is shown in Figure 6.33.10. Tumour proliferation was higher in glutamine fed rats, and showed no significant reduction after MTX (p=0.51).The apoptotic count per unit area for the tumours is shown in Figure 6.33.11. MTX produced an increase in apoptosis on day +2 (p=0.008), but again there was no significant diet effect.

Treatment	Rat #	Villus area	Crypt length	mitotic index	Treatment	Rat #	Villus area	Crypt length	mitotic index
Glutamine & No MTX	35	0.183	123	5.6	Glutamine & MTX	31	0.09	80	0.1
	41	0.122	112	3.4		38	0.148	93	0.2
	45	0.116	130	4.1		42	0.142	75	0.1
	57	0.154	153	4.0		43	0.121	80	0.3
	58	0.132	125	3.0		46	0.124	78	0.3
	63	0.123	133	2.4		67	0.136	72	0.0
mean		0.140	129.33	3.75			0.13	79.67	0.17
sem		0.01	5.58	0.45			0.01	2.95	0.05
Treatment	Rat #	Villus area	Crypt length	mitotic index	Treatment	Rat #	Villus area	Crypt length	mitotic index
Casein & No MTX	33		112	3.4	Casein & MTX	32	0.114	83	0.1
	36		110	2.9		44	0.088	75	0.1
	50	0.196	105	4.5		52	0.143	79	0.1
	54	0.131	127	2.8		53	0.126	72	0.1
	65	0.135	130	3.3		64	0.115	87	0
	68					66	0.160	76	0.1
mean		0.15	116.8	3.38			0.12	78.67	0.08
sem		0.02	4.93	0.3			0.01	2.26	0.02

Table 6.33.6. Histomorphometry results in glutamine fed rats

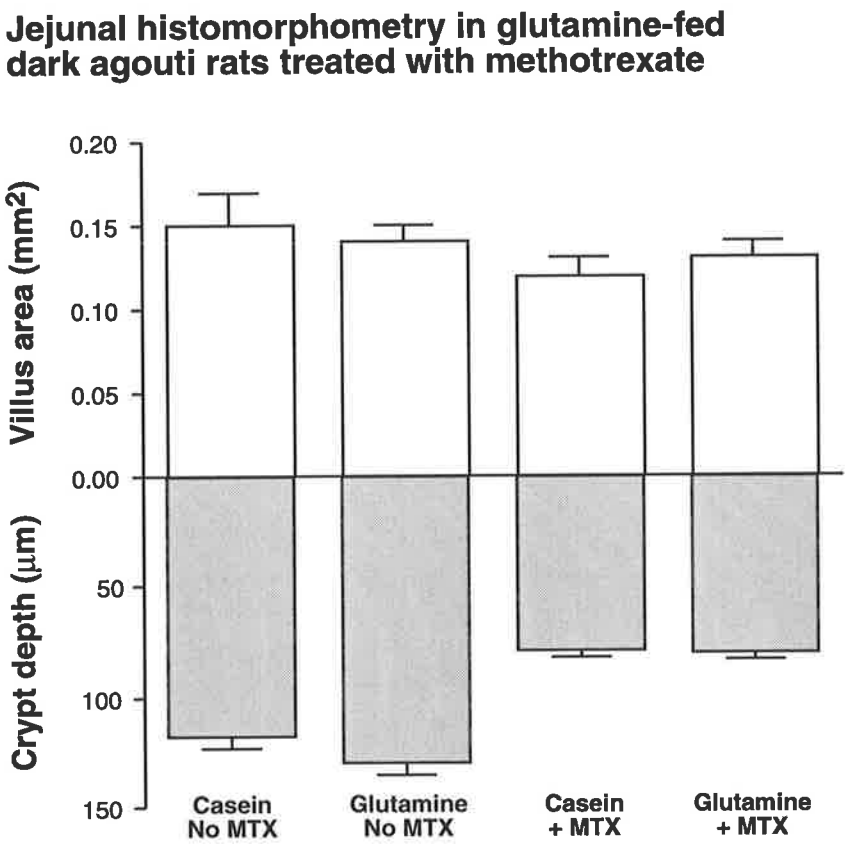


Fig. 6.33.6 Villus area and crypt length on day +2 in small intestine of tumour-bearing DA rats fed glutamine versus casein and treated with and without MTX. Data are given as mean + standard error.

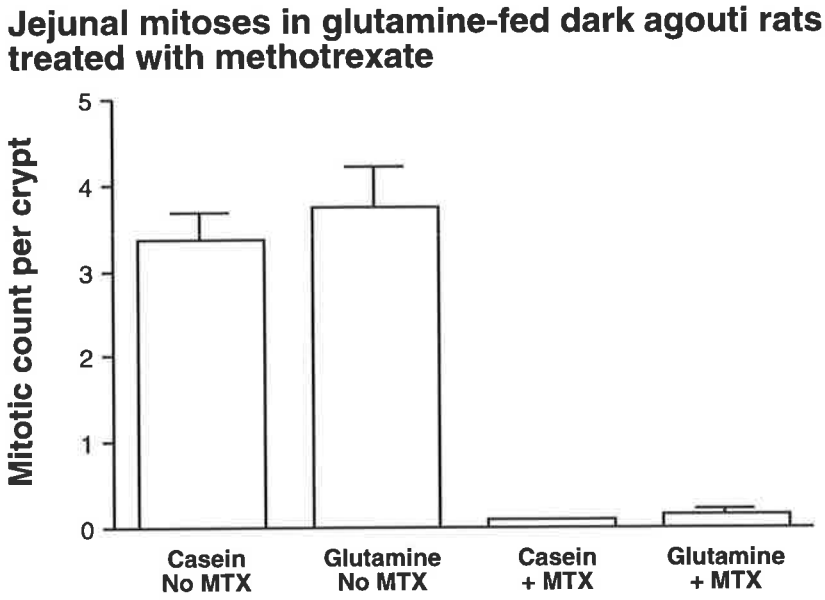


Fig. 6.33.7 Mitotic count per jejunal crypt on day +2 in tumour-bearing DA rats fed glutamine versus casein and treated with and without MTX. Data are given as mean + standard error.

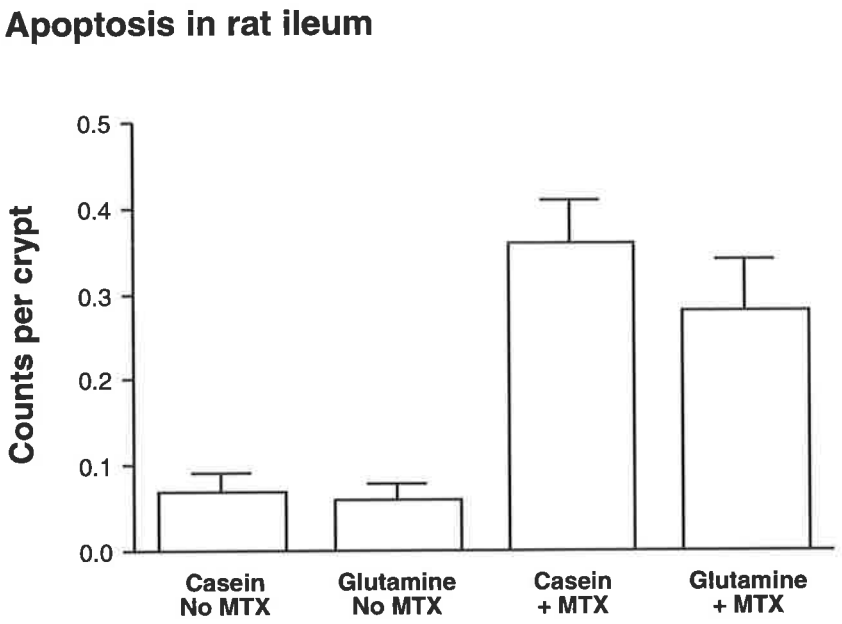


Fig. 6.33.8 Apoptotic counts per crypt in dark agouti rat ileum on day +2 following treatment with either MTX or saline. Data are given as mean + standard error.

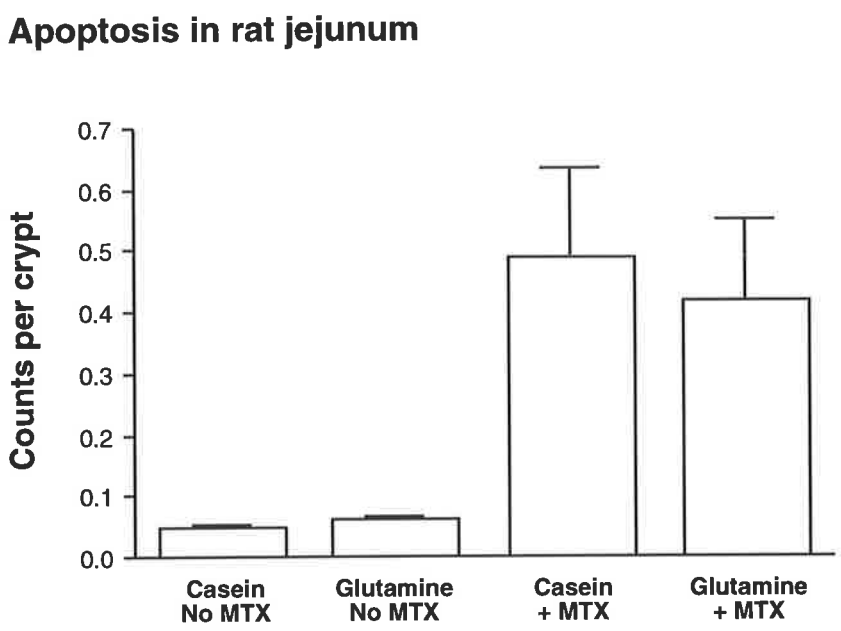


Fig. 6.33.9 Apoptotic counts per crypt in rat jejunum on day +2 following treatment with either MTX or saline. Data are given as mean + standard error.

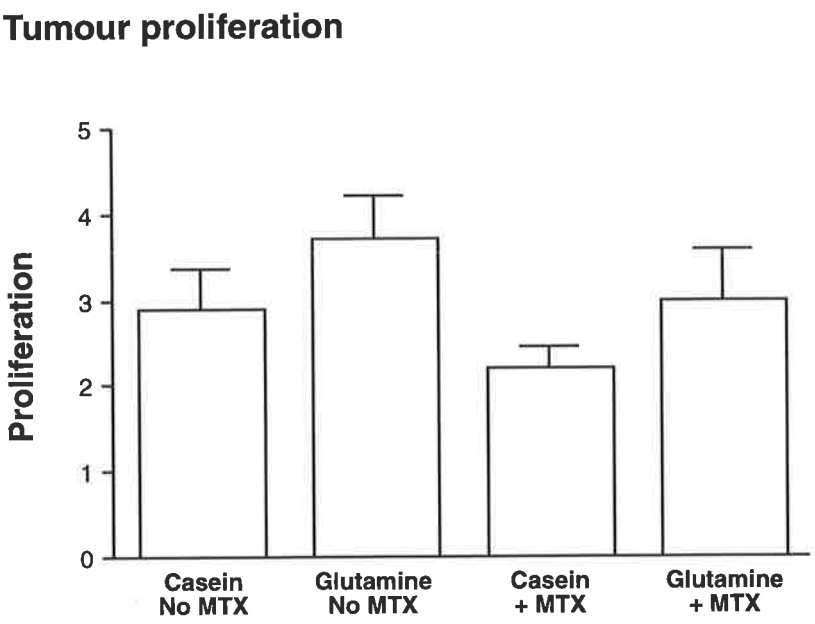


Fig. 6.33.10 Proliferation as measured by mitotic count per unit area in breast tumour in DA rats. Data are given as mean + standard error.

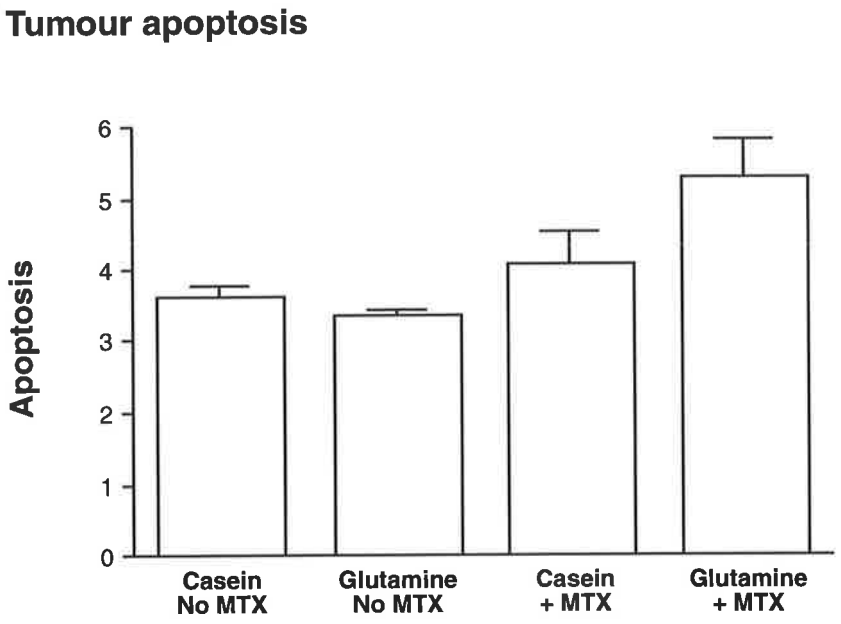


Fig. 6.33.11 Tumour apoptosis in DA rat breast carcinoma. Apoptosis is measured per unit area. Data are given as mean + standard error.

6.34 DISCUSSION

Our studies showed a trend for glutamine to increase tumour growth, and also to increase the ability of MTX to kill the tumour, however this did not reach statistical significance, in contrast to Klimberg's report (111). Thus glutamine would be safe for use in human cancer trials. However, it did not significantly ameliorate the effects of MTX on the small intestinal mucosa. From the results of recent publications (117), it would seem that glutamine alone is unlikely to be of value in chemoprotection, but it may well have a role as part of a combination of factors for the future (10).

The tumour burden in this study may have played a role in the lack of success of the glutamine. We had intended to administer the chemotherapy when the rats had a tumour burden of approximately 5% of body weight, but the tumour grew faster than anticipated, so that tumour burden at MTX injection was already approximately 10%. This is a very heavy tumour burden, and can itself be associated with malnutrition, cachexia and non-specific small bowel histological changes. Rofe in this model (191) found that MTX reduced the glucose requirements of the tumour at the same time as inhibiting tumour progression.

Future work will be carried out at a lower tumour burden. The dose of methotrexate of 1.5 mg/kg for two consecutive days proved to be successful in causing non-lethal small intestinal mucositis, and also reducing tumour growth. The same dose will be used in future studies.

CHAPTER 7

SUMMARY OF INVESTIGATIONS INTO SMALL INTESTINAL EFFECTS OF CYTOTOXIC CHEMOTHERAPY

7.1 Intestinal permeability after high-dose chemotherapy

High-dose chemotherapy caused a transient increase in intestinal sugar permeability, which reached a peak 14 days after the start of chemotherapy and recovered by 35 days after the start of chemotherapy. It was characterised by both a decrease in monosaccharide absorption, implying villous atrophy, and an increase in disaccharide absorption, implying opening of the intraepithelial tight junctions. It was further accompanied by oral mucositis in all patients, and small intestinal mucositis in 41% of patients. This indicated that small intestinal permeability is likely to be useful in assessing intestinal mucositis.

7.2 Small intestinal symptoms, function and morphology following chemotherapy

Symptoms, function and histological changes all paralleled one another (see Table 7.1). The earliest change was an increase in apoptosis in small intestinal crypts at 1 day after chemotherapy, followed by crypt hypoplasia at day 3 and normalisation by day 16. Gastrointestinal symptoms peaked at days 3 to 7, while oral mucositis was slower to peak at days 10 to 14. All recovered by day 28. Nutritional changes were small, with transient weight loss, albumin and transferrin reduction, which all normalised by day 28. Functional permeability changes were less pronounced with the isotonic sugar solution, and remained abnormal for longer, as these did not recover by day 28. There was also a dose-effect in permeability, with changes being confined to the high-dose chemotherapy group. Transferrin reduction, weight

and body mass index reduction also showed a dose effect, but all other changes occurred both in patients on standard and high dose chemotherapy. Electron microscopy confirmed the opening of the tight gap junctions, but this reversed by day 16.

7.3 The DA rat mammary adenocarcinoma and the role of glutamine in mucositis prevention.

The DA rat mammary adenocarcinoma model is a useful model for the study of small intestinal mucositis. A dose of 1.5 mg/kg im for two consecutive days reliably caused severe, non-lethal mucositis. The mammary tumours implanted subcutaneously were able to be studied simultaneously with the small intestine and allow assessment of safety of cytoprotectants. Glutamine had no significant effect in reducing small intestinal mucositis caused by MTX.

7.4 These results in the context of the published literature

These results agree with the published literature, which has shown small intestinal hypoproliferation followed by hyperproliferation in the small intestine after chemotherapy. We confirmed the work of Trier and others, and showed that these changes occurred over three to seven days in humans, in contrast with published rodent work by Potten and others which showed a shorter time course (See Table 2.1). These results expand our knowledge of the sequence of events in human small intestinal mucositis, by showing that apoptosis is the first event in the crypts. They also show that histological changes occur at the same time as gastrointestinal symptoms. The timing of oral mucositis, however, coincides neither with gastrointestinal symptoms nor small intestinal mucositis, confirming that oral mucositis is not a good indicator of changes in the small intestine.

Day	Pre	1	3	5	7	10	14	16	28
GIT symptoms			X	X	X	X			
Oral symptoms					X	X	X		
Nutrition			X	X	X	X	X		
Permeability			X	X	X	X	X	X	X
Morphology		X	X	X					

Table 7.1. An overview of changes found with time after chemotherapy. An X indicates the presence of changes.

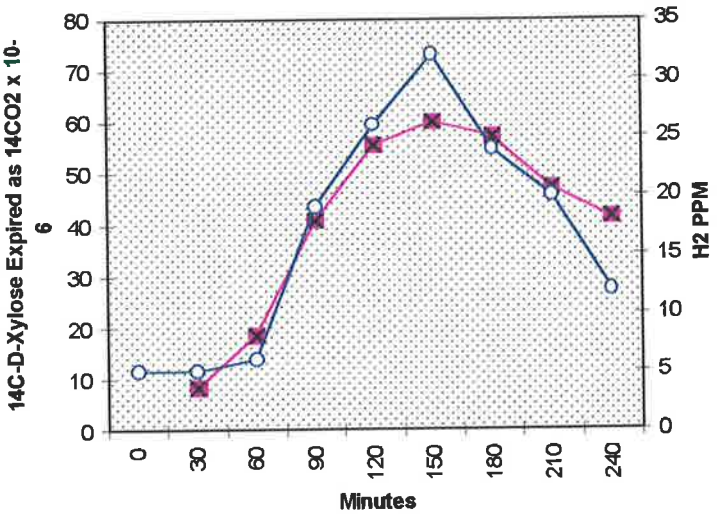
We have demonstrated that the DA mammary adenocarcinoma model is a useful model for further research that will be required before testing potential cytoprotectants in humans. A dose of 1.5 mg/kg reliably produces small intestinal mucositis, and the same histological techniques can be applied to the small intestine as in humans, with the tumour also being evaluable.

7.5 A platform for future research

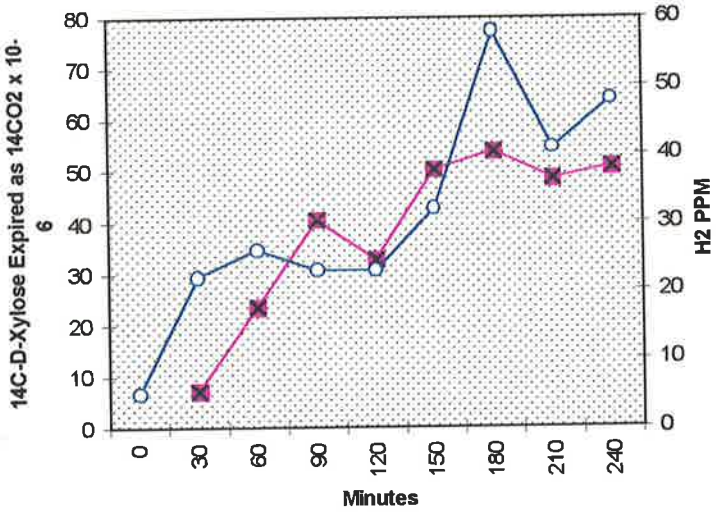
Further work planned includes expansion of the DA rat model to assess other agents and compare them with glutamine. Potential candidate agents include TGF-β, IL-11, WGFE and KGF. In the future, a mixture of agents may be required, and this would be an excellent model in which to test the effect on small intestinal mucositis. Any agents that showed response in this model without promoting tumour growth could then be considered for human clinical trials, either singly or in combination.

APPENDIX 1

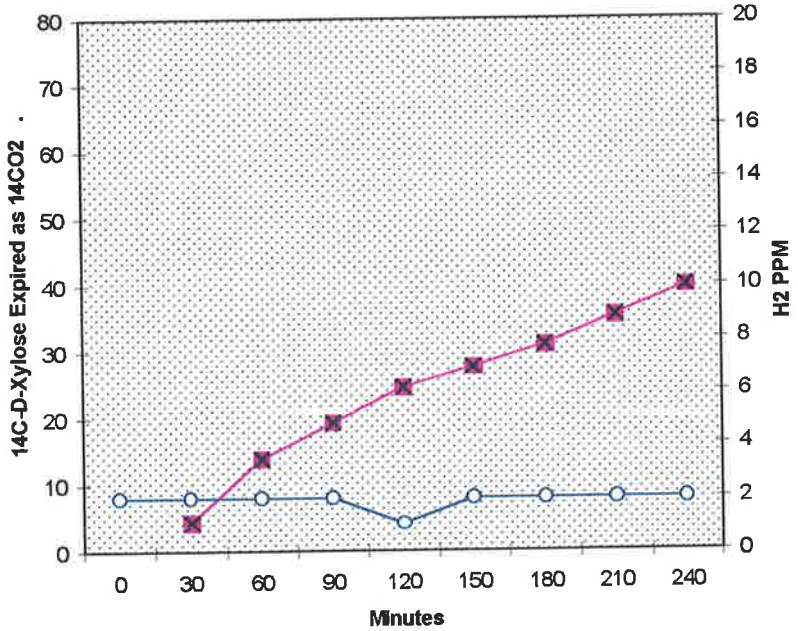
EXAMPLES OF BREATH TEST RESULTS GRAPHS



1. Normal breath test. No evidence of small bowel bacterial overgrowth. Oro-caecal time is 90 minutes. The pink line indicates xylose and the blue line hydrogen.



2. A positive response indicating small bowel bacterial overgrowth. The pink line indicates xylose and the blue line hydrogen.



3. An equivocal result, with no rise in breath hydrogen, nor a peak in ^{14}C-D-xylose. The pink line indicates xylose and the blue line hydrogen.

APPENDIX II

CHEMOTHERAPY REGIMENS RECEIVED BY PATIENTS AS PART OF STUDY

1. Standard dose chemotherapy regimens

Regimen	Drugs and doses	Number of patients
AC	doxorubicin 60 mg/m^2 cyclophosphamide 600 mg/m^2	2
Platinum/etoposide	cisplatin 75 mg/m^2 d1 etoposide 100 mg/m^2/d d1-3	7
MOPP	mustine 6 mg/m^2 vincristine 1.4 mg/m^2 procarbazine 100 mg/m^2 prednisolone 40 mg/m^2	2
5-FU	5-fluorouracil 425 mg/m^2 /d d1-5	3
PCAB	prednisolone 60 mg/m^2 d1-5 cyclophosphamide 600 mg/m^2 d1 doxorubicin 30 mg/m^2 d1 BCNU 30 mg/m^2 d1	1
5-FU/Lobaplatin	5-fluorouracil 1000 mg/m^2/d d1-5 lobaplatin 50 mg/m^2 d1	1

M-VAP	methotrexate 30 mg/m^2 vinblastine 3 mg/m^2 doxorubicin 30 mg/m^2 carboplatin AUC dose of 4-5 mg/ml min	1
MVC	mitomycin 8 mg/m^2 vinblastine 6 mg/m^2 cisplatin 50 mg/m^2	1
KISH	cisplatin 100 mg/m^2 d1 5-fluorouracil 200 mg/m^2 d d1-5	3
COP	cyclophosphamide 750 mg/m^2 vincristine 1.4 mg/m^2 prednisolone 100 mg/m^2 d1-5	2
CMF	cyclophosphamide 750 mg/m^2 methotrexate 60 mg/m^2 5-fluorouracil 750 mg/m^2	3
CHOP	cyclophosphamide 750 mg/m^2 vincristine 1.4 mg/m^2 doxorubicin 50 mg/m^2 prednisolone 100 mg/m^2 d1-5	1
AC	doxorubicin 25 mg/m^2/d d1-3 cisplatin 100 mg/m^2 d1	3

2. Regimen for cyclophosphamide priming for stem cell harvesting

Regimen	Drug and dose	Number of patients
Cyclophosphamide prime	cyclophosphamide 4 g/m^2	12

3. High-dose chemotherapy regimens

Drugs and doses	Number of patients
ifosphamide 3g/m^2/d d1-5 etoposide 600 mg/m^2/d d1-3	1
carboplatin 250 mg/m^2/d d1-5 etoposide 600 mg/m^2/d d1-3 ifosphamide 3g/m^2/d d1-5	1
melphalan 95 mg/m^2 d1 carboplatin 300 mg/m^2/d d2-4 cyclophosphamide 35 mg/kg/d d2-4	5
epirubicin 200 mg/m^2 cyclophosphamide 1g/m^2	5
melphalan 140 mg/m^2/d d1 carboplatin 400 mg/m^2/d d2-3 etoposide 300 mg/m^2/d d4-7 cytarabine 200 mg/m^2/d d4-7	4
busulphan 4 mg/kg/d d1-4 cyclophosphamide 60 mg/kg/d d5-6	1

APPENDIX III

INDIVIDUAL PATIENT ORAL AND GASTROINTESTINAL TOXICITY GRADING USING THE NCI COMMON TOXICITY CRITERIA

Patient number	Nausea	Vomiting	Diarrhoea	Stomatitis	Overall
1			0		0
2			1		1
3			0		0
4			1		1
5			0		0
6			0		0
7			0		0
8			3		3
9			0		0
10			0		0
11			3		3
12			0		0
13			2		2
14			3		3
15			0		0
16			1		1
17			3		3

Patient number	Nausea	Vomiting	Diarrhoea	Stomatitis	Overall
18			3		3
19			0		0
20			0		0
21			0		0
22			0		0
23			0		0
24			0		0
25			1		1
26			2		2
27			0		0
28	0	0	0	0	0
29	3	1	2	2	2
30	0	0	0	3	3
31	N/A				
32	1	0	2	2	2
33	2	0	0	2	2
34	N/A				
35	N/A				
36	N/A				
37	3	2	1	3	3
38	3	0	0	0	3
39	1	0	0	0	1

Patient number	Nausea	Vomiting	Diarrhoea	Stomatitis	Overall
40	0	0	2	0	2
41	3	2	0	3	3
42	1	0	2	0	2
43	0	0	1	2	2
44	3	2	3	3	3
45	0	0	0	0	0
46	3	1	1	3	3
47	0	0	0	0	0
48	1	1	1	3	3
49	2	1	2	2	2
50	N/A				
51	1	0	0	2	2
52	0	1	1	1	1
53	2	0	2	2	2
54	2	0	1	2	2
55	3	0	0	2	3
56	0	0	1	0	1
57	0	0	1	0	1
58	3	2	4	3	4
59	N/A				
60	1	0	0	2	2

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PUBLICATIONS ARISING FROM THIS THESIS

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