

Biomarkers of chemotherapy-induced diarrhoea: a clinical study of intestinal microbiome alterations, inflammation and circulating matrix metalloproteinases

Andrea M. Stringer · Noor Al-Dasooqi ·
Joanne M. Bowen · Thean H. Tan · Maryam Radzuan ·
Richard M. Logan · Bronwen Mayo ·
Dorothy M. K. Keefe · Rachel J. Gibson

Received: 19 September 2012 / Accepted: 29 January 2013 / Published online: 10 February 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract

Purpose A common side effect of chemotherapy treatment is diarrhoea. Unfortunately, the underlying mechanisms of chemotherapy-induced diarrhoea (CD) are poorly understood. We aimed to determine if faecal microbes of CD patients were displaced, if faecal calprotectin increased during CD and if there were alterations in circulating matrix metalloproteinases, nuclear factor kappa B (NF- κ B), IL-1 β and TNF.

Patients and methods Twenty-six cancer patients receiving chemotherapy were enrolled and requested to provide stool samples and blood samples at various times during their chemotherapy cycle. Stool samples were analysed using conventional culture techniques and qRT-PCR. ELISA kits determined faecal calprotectin levels, levels of circulating

matrix metalloproteinases and circulating NF- κ B, IL-1 β and TNF.

Results The majority of patients with CD showed decreases in *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp. and *Enterococcus* spp. Increases were observed in *Escherichia coli* and *Staphylococcus* spp. Methanogenic archaea were also quantified, with all patients except one showing a decrease. Faecal calprotectin levels were increased in 81.25 % of patients with CD. Circulating MMP-3 and MMP-9 significantly increased following chemotherapy. Circulating levels of NF- κ B, IL-1 β and TNF were increased following chemotherapy, although this did not reach significance.

Conclusions We demonstrated that CD is associated with marked changes in intestinal microflora, methanogenic archaea, matrix metalloproteinase and serum levels of NF- κ B, IL-1 β and TNF. These changes may result in diminished bacterial functions within the gut, altering gut function and initiating intestinal damage, resulting in the onset of diarrhoea. More importantly, these changes may provide clinicians with a possible new target for biomarkers of toxicity.

Keywords Biomarkers · Mucositis · Microbiome · Matrix metalloproteinases

A. M. Stringer · M. Radzuan
School of Pharmacy and Medical Sciences, University of South
Australia, Adelaide, Australia

A. M. Stringer · N. Al-Dasooqi · B. Mayo · D. M. K. Keefe
Discipline of Medicine, University of Adelaide,
Adelaide, Australia

A. M. Stringer · N. Al-Dasooqi · J. M. Bowen · R. J. Gibson (✉)
School of Medical Sciences, University of Adelaide,
North Terrace, Adelaide, 5005,
South Australia, Australia
e-mail: rachel.gibson@adelaide.edu.au

T. H. Tan · D. M. K. Keefe
Cancer Centre, Royal Adelaide Hospital,
Adelaide, South Australia, Australia

R. M. Logan
Oral Pathology, School of Dentistry, University of Adelaide,
Adelaide, Australia

Introduction

Chemotherapy-induced gastrointestinal mucositis presents a major oncological problem. Clinically, mucositis is characterised by a wide spectrum of symptoms including ulceration, nausea and vomiting, bloating, diarrhoea and constipation [1]. Although the pathobiology of mucositis is complex, much progress has been made in recent years in

terms of our understanding of the pathological [2, 3] and signalling alterations [4] occurring in the gut subsequent to chemotherapy treatment. Briefly, these include clonogenic cell death [5, 6], the induction of inflammatory responses [3], changes in the gut microbiome [7, 8] and activation of submucosal signalling and damage of submucosal structures [5]. The gene expression changes which occur in the gut following chemotherapy treatment have also been investigated. The pathways that govern mucositis development include mitogen-activated protein kinase signalling, nuclear factor kappa B (NF- κ B) signalling, Fos/Jun signalling and Wnt signalling [4]. Furthermore, the downstream mediators of this damage include cytokines, matrix metalloproteinases, ceramide and cyclooxygenase-2 [3, 9].

Despite these advances, the mechanisms contributing to mucositis diarrhoea remain poorly understood [10]. Regimens, for colorectal cancer in particular, illustrate that the diarrhoea component of mucositis is one of the most common and clinically important aspects of toxicity. The absolute percentage of patients that have chemotherapy-induced diarrhoea (CD) as a result of their treatment has yet to be fully defined, although general estimates place 50 % of all cancer patients [10] and 10 % of advanced cancer patients being afflicted [10]. This represents a significant clinical and, importantly, economic burden in oncology as the presence of any mucositis during a cycle of chemotherapy significantly increases the risk of dose reduction, the frequency of infections and bleeding and increases the length and cost of hospitalisation [11]. Reductions in treatment doses may also lead to reduced survival [12].

Although mucositis and CD is highly prevalent, there are currently no diagnostic biomarkers and the only method by which it can be diagnosed is through assessment of symptoms according to clinical mucositis grading scales. However to date, there is no established scale that is universally accepted or meets all the criteria a clinical mucositis scoring system should achieve [13]. Furthermore, accurate evaluation of mucositis development is made impossible due to the difficulty of obtaining sequential biopsies before, during and after treatment and the inaccessibility of important segments of the alimentary tract [13]. This has great implications when conducting clinical evaluation of mucositis interventions, where accurate evaluation of mucositis is required.

Calprotectin has been used for a number of years as a biomarker for intestinal inflammation in inflammatory bowel diseases. It is a member of the S100 family and is a 36-kDa abundant calcium-binding protein [14], and members of this family are able to be detected within faecal samples [15]. In brief, calprotectin is released when leukocytes become activated as a direct consequence of pathology in varying organs, leading to increased levels in plasma,

cerebrospinal fluid, urine or faeces [15]. Previously, calprotectin has been shown to be increased in inflammatory intestinal diseases, including inflammatory bowel disease, Crohn's disease and ulcerative colitis [16]. More recently, calprotectin has been used with mixed results in the oncology arena [17, 18].

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases which participate in extracellular matrix turnover [19]. Recent research within our laboratory has identified these proteases as major mediators of chemotherapy-induced gut damage in rats [9]. Hence, MMPs can be used as tissue-specific markers of chemotherapy-induced gut injury and specifically CD. However, it is not known whether these changes in MMPs are translated systemically and hence whether or not they could be useful in identifying impending toxicity.

The purpose of this small clinical study in patients receiving chemotherapy regimens containing 5-fluorouracil, capecitabine or irinotecan was threefold. Firstly, we determined if the faecal microbes of patients with CD were displaced from that of healthy controls; secondly, we determined if faecal calprotectin was increased during CD; and thirdly, we investigated the potential of MMPs and pro-inflammatory cytokines as systemic biomarkers to reflect the development of gut toxicity.

Patients and methods

Patient cohorts

Cohort 1 (proof-of-concept)

All patients recruited for cohort 1 were hospital inpatients due to mucositis manifesting as diarrhoea (CD). Sixteen patients (6 males and 10 females) with a median age of 71 years (range 36–82) receiving chemotherapy provided informed consent. Patients receiving concurrent radiotherapy were excluded from the study to eliminate radiotherapy-induced diarrhoea. All other medications were recorded for each patient. The malignancies (number of patients) included colorectal cancer (11), breast cancer (two), laryngeal cancer (one), oesophageal cancer (one), and melanoma (one). There were not enough subjects to perform a subset analysis based on the individual chemotherapy agents, as many different chemotherapy regimens were used for these patients including capecitabine, cisplatin/5-FU, FOLFOX, 5-FU/folinic acid, COFF and paclitaxel, carboplatin and gemcitabine. In addition to these subjects, stool samples from two healthy volunteers (one male and one female) were obtained for the study (Table 1). This study was approved by the Ethics of Human Research Committee of the Royal Adelaide Hospital and was carried out in

Table 1 Characteristics of patient cohorts 1 and 2

Patient characteristics	Chemotherapy-treated patients	Healthy controls
Cohort 1		
Number of patients	16	2
Age	71 (36–82)	n/a
Gender (M/F)	6:10	1:1
Cancer diagnosis		
Colon	8	n/a
Rectal	3	
Breast	2	
Melanoma	1	
Other	2	
Toxicities		
Diarrhoea	16	n/a
Cohort 2		
Number of patients	10	5
Age	63 (40–77)	n/a
Gender (M/F)	7:3	1:4
Cancer diagnosis		
Colon	2	n/a
Rectal	6	
Breast	2	
Toxicities		
Oral tenderness	1	n/a
Diarrhoea	3	
Vomiting	3	
Abdominal cramps	1	
Other	1	

accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study.

Cohort 2

Ten patients (seven males and three females) with a mean age of 62 years (range 40–77) receiving FOLFOX4, FOLFOX6, FOLFIRI or capecitabine for breast cancer (two patients) as well as various gastrointestinal tumours (eight patients) were recruited from The Royal Adelaide Hospital Cancer Centre (Table 1). Patients were excluded if they were receiving concurrent radiotherapy. None of the patients presented with conditions associated with a known increase in serum MMPs (including rheumatoid arthritis, pulmonary fibrosis and liver cirrhosis). Stool and blood samples were collected before a chemotherapy cycle commenced and on days 2, 5 and 10 of one chemotherapy cycle. Stool samples were used for determination of calprotectin levels. Blood samples were used for serum collection for determination of circulating MMPs and pro-inflammatory cytokines TNF, IL-

1B and IL-6. Five healthy, cancer-naïve, control subjects with a mean age of 26 years (range 23–30) were also enrolled in this study as cancer patients are known to have elevated serum MMP levels [20]. This study was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital and was carried out in accordance with the Declaration of Helsinki.

Analysis of cohort medical records

To identify the toxicities associated with anti-cancer regimens, both cohorts of patients had case note reviews undertaken. The toxicities recorded from patient case notes were those identified by the National Cancer Institute common gastrointestinal tract toxicity criteria following anti-cancer treatment. These included oral symptoms, stomatitis, nausea and vomiting, diarrhoea, constipation, abdominal pain and bloating. Institutional reporting guidelines for toxicity collection were changed between cohort recruitment, meaning toxicities for patients in cohort one were not as comprehensively reported as toxicities for patients in cohort 2.

Sample analysis

Culture of faecal samples

To determine which bacteria were present in the faeces of patients with CD, a variety of selective and non-selective media (Oxoid, Adelaide, Australia) were used in an attempt to identify as many bacteria as possible from the gastrointestinal tract using our established methods [7]. The level of growth of each bacterium was graded using a qualitative assessment technique using the following criteria: 0, no growth; 1, very light growth (less than 10 cfu); 2, light growth (growth in the original inoculation zone only); 3, moderate growth (growth in the first streak line); and 4, heavy growth (growth in the second streak line or greater). All gradings were conducted in a blinded fashion (AMS). This grading system had previously been validated in our laboratory using quality control organisms and sample organisms to ensure consistent results [7].

DNA extraction from bacteria

DNA was extracted from standard bacterial samples using a DNeasy Tissue Mini Kit (Qiagen, Doncaster, Australia). Briefly, bacteria were grown to log phase in nutrient broth. Cells were harvested by centrifuging for 10 min at 5,000 × g. Gram-positive bacteria were lysed in enzymatic lysis buffer (20 mM Tris-Cl, pH8.0, 2 mM sodium EDTA, 1.2 % Triton® X-100, 20 mg/ml lysozyme) at 37 °C, followed by proteinase K at 70 °C. Gram-negative bacteria were lysed in provided buffer and proteinase K at 55 °C, followed by further lysis in

buffer at 70 °C. For both Gram-positive and Gram-negative bacteria, DNA was bound to the membrane in the provided spin column. The membrane was washed with two buffer solutions before DNA was eluted. DNA concentration was determined using a Nanodrop spectrophotometer (BioRad, Gladesville, NSW, Australia) and diluted to a final working concentration of 10 ng/μl.

DNA extraction from faecal samples

DNA was extracted from patients' faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen). Briefly, 200 mg faeces was homogenised in lysis buffer and heated at 95 °C. Samples were centrifuged and the supernatant collected. Inhibitors were adsorbed to InhibitEX tablets before samples were centrifuged again and the supernatant collected. Proteins were digested with proteinase K and buffer. DNA was then bound to the membrane in the provided spin column. The membrane was washed with two wash buffers before DNA was eluted. DNA concentration was determined using a spectrophotometer and diluted to a final working concentration of 5 ng/μl.

Real-time PCR

Real-time PCR was carried out on a Corbett Rotorgene 3000 rotary cycler (Corbett Research, USA). Primers used in this study have been published previously [21–25]. PCR mixtures consisted of 1× Quantitect® SYBR Green Mastermix (Qiagen), 2.5 ng/μl of each primer and 10 ng of DNA in a volume of 10 μl. The cycling parameters consisted of enzyme activation at 95 °C followed by cycles of melting at 95 °C for 15 s; annealing for the specified times and temperatures; and extension at 72 °C for a specified time, based on optimisation of the cycling conditions for each primer set. The SYBR green fluorescent signals were acquired at 72 °C. Standard curves were constructed from PCR reactions using 10-fold serial dilutions of known bacterial DNA. Data were analysed using the Rotor-gene 6 software (Corbett Research, USA).

Faecal calprotectin assay

Faecal calprotectin was measured from stool samples using the Human Calprotectin ELISA kit (Hycult Biotech, Uden, Netherlands). Briefly, samples were extracted using extraction buffer. Standards and extracted samples were transferred (in duplicate) into the appropriate wells and incubated at room temperature for 1 h. The wells were emptied, and the plate washed four times with wash buffer, repeated following each incubation step. Diluted tracer was added to the wells and incubated for 1 h at room temperature, followed by incubation with streptavidin-peroxidase for 1 h at room

temperature. Substrate solution was added to the wells and incubated for 20 min in darkness before the reaction stop solution was added. The plate was read at 450 nm.

ELISA detection of serum matrix metalloproteinase-2, metalloproteinase-3 and metalloproteinase-9

Whole blood samples were drawn from controls and patients (cohort 2) receiving anti-cancer treatment at various time points during one chemotherapy cycle. Serum samples were separated by centrifugation and stored at –80 °C until use. Serum levels of MMP-2, MMP-3 and MMP-9 were measured using quantikine immunoassay ELISA kits specific to the MMP of interest (RnD Systems, MN, USA) according to the manufacturer's instructions (catalog numbers DMP2F0, DMP300 and DMP900). All samples were tested in duplicates.

ELISA detection of serum NF-κB and proinflammatory cytokines IL-1β and TNF

Whole blood samples were drawn from controls and patients (cohort 2) receiving anti-cancer treatment at various time points during one chemotherapy cycle. Serum samples were separated by centrifugation and stored at –80 °C until use. Serum levels of NF-κB, IL-1β and TNF were measured using quantikine immunoassay ELISA kits specific to each transcription factor or proinflammatory cytokine (Imgenex, NFκB/p65 active; catalog number IMK-503; eBioscience IL-1β and TNF; catalog numbers 88-7010-22 and 88-7346-22, respectively) according to the manufacturer's instructions. All samples were tested in duplicates.

Statistical analysis

All significance levels were set at $p < 0.05$. Linear regression analysis was applied to determine if changes to faecal microbes were significant. Repeated measures analyses were applied to analyse the changes over time in MMP-2, MMP-3 and MMP-9 levels. This utilised a linear mixed effects model where time was included as a categorical predictor (fixed effect), while patient was included as a random effect. A one-way ANOVA was used to determine significance for ELISA detection of serum NF-κB and proinflammatory cytokines IL-1β and TNF. Correlations between changes in serum MMPs and GI toxicity were investigated using Fisher's exact test.

Results

Toxicities induced following cytotoxic chemotherapy (cohort 1)

Institutional reporting guidelines for toxicity collection were changed between cohort recruitment, meaning toxicities for patients in cohort 1 were not as comprehensively reported as toxicities for patients in cohort 2. However, all patients recruited for cohort 1 were hospitalised for chemotherapy-induced diarrhoea.

Toxicities induced following cytotoxic chemotherapy (cohort 2)

The most prevalent gastrointestinal toxicities were nausea and vomiting (3/10 patients—30 %) and diarrhoea (3/10 patients—30 %). Oral tenderness was also reported by one patient (10 %).

Faecal microflora analysis (cohort 1)

The overall culture analysis revealed that 75 % of patients had a decreased anaerobic component of their microflora, with respect to both the level of growth and diversity of species present (data not shown). No marked differences between gender were observed. One patient presented with no detectable bacteria in culture, and another patient exhibited the presence of *Staphylococcus* spp. only in the faecal samples. There was a large difference in the diversity of bacteria present in the microflora between patients experiencing CD and healthy controls and also between individuals (data not shown).

Quantitative real-time PCR demonstrated differences in the amount of bacteria present in the faecal samples of patients with CD and healthy controls. The majority of patients experiencing CD showed a decrease compared with controls in *Lactobacillus* spp. (with some exceptions) and *Bacteroides* spp. (all patients) (Fig. 1). Decreases were also seen with *Bifidobacterium* spp. (one exception) and *Enterococcus* spp. (all patients) (data not shown). Increases were observed in *Escherichia coli* (Fig. 1) and *Staphylococcus* spp. (with some exceptions) (data not shown). A decrease compared with controls in methanogenic archaea was seen in the majority of patients with CD (one exception) (Fig. 1).

Faecal microflora analysis (cohort 2)

Real-time PCR analysis revealed that *E. coli* increased over time (0.034 ± 0.011 to 0.174 ± 0.086 ng/reaction, control to day 10, respectively). *Lactobacillus* spp. was shown to rise gradually until day 5 (0.0669 ± 0.412 to 0.936 ± 0.0541 ng/reaction), before decreasing at day 10 (0.402 ± 0.212 ng/reaction) (Fig. 2). Other bacterial genera tested did not show a noticeable difference between these time points (data not shown). Values are expressed as mean \pm standard error of mean. Statistical analysis showed no statistical significance due to low subject number.

Antibiotics

A number of patients received antibiotics prior to collection of a faecal sample. These patients generally did not exhibit any marked difference to the changes in microflora, demonstrated with either culture techniques or quantitative real-time PCR, compared with patients who did not receive antibiotics.

Calprotectin

Cohort 1

Faecal calprotectin was increased in 81.25 % of patients (13 of 16) with CD, when compared to healthy controls (data not shown). The healthy control volunteers ($n=2$) were measured at 0.54 ± 0.54 ng/ml (mean \pm SEM), whereas the mean for the patients with CD was 6.82 ± 1.20 ng/ml. Eleven of the patients in this cohort were diagnosed with gastrointestinal cancers, which has previously been indicated as being a confounding factor in this type of experiment [26].

Cohort 2

In patients with gastrointestinal cancers, faecal calprotectin was significantly increased in comparison to healthy controls (10.98 ± 1.69 vs 1.82 ± 0.94 , $p < 0.05$). Calprotectin levels remained significantly elevated compared to healthy controls at 2, 5 and 10 days following treatment (data not shown). However, there was no significant change in calprotectin levels pre- and post-treatment (data not shown) indicating the difficulty of using calprotectin as a mucosal injury marker in patients with gastrointestinal cancers [26]. Two of the 10 patients in this cohort had breast cancer, and although extremely small numbers, there was no significant difference seen in faecal calprotectin levels in pre- and post-treatment (data not shown).

Serum levels of matrix metalloproteinase-2, metalloproteinase-3 and metalloproteinase-9

MMP-2 The change in MMP-2 levels over time was assessed using a linear mixed effects model. According to this model, there was no significant difference in mean MMP-2 levels across the three post-treatment time points in comparison to the pre-treatment serum levels ($p=0.64$) (Table 2).

MMP-3 A significant change in mean MMP-3 levels was noted in the serum of patients post-chemotherapy treatment ($p=0.0002$) (Table 2, Fig. 3). Pre-treatment MMP-3 levels were measured to be 0.3 ± 0.1 ng/ml. MMP-3 levels were significantly increased at 2 and 5 days post-treatment in comparison to pre-treatment. At 2 days following treatment,

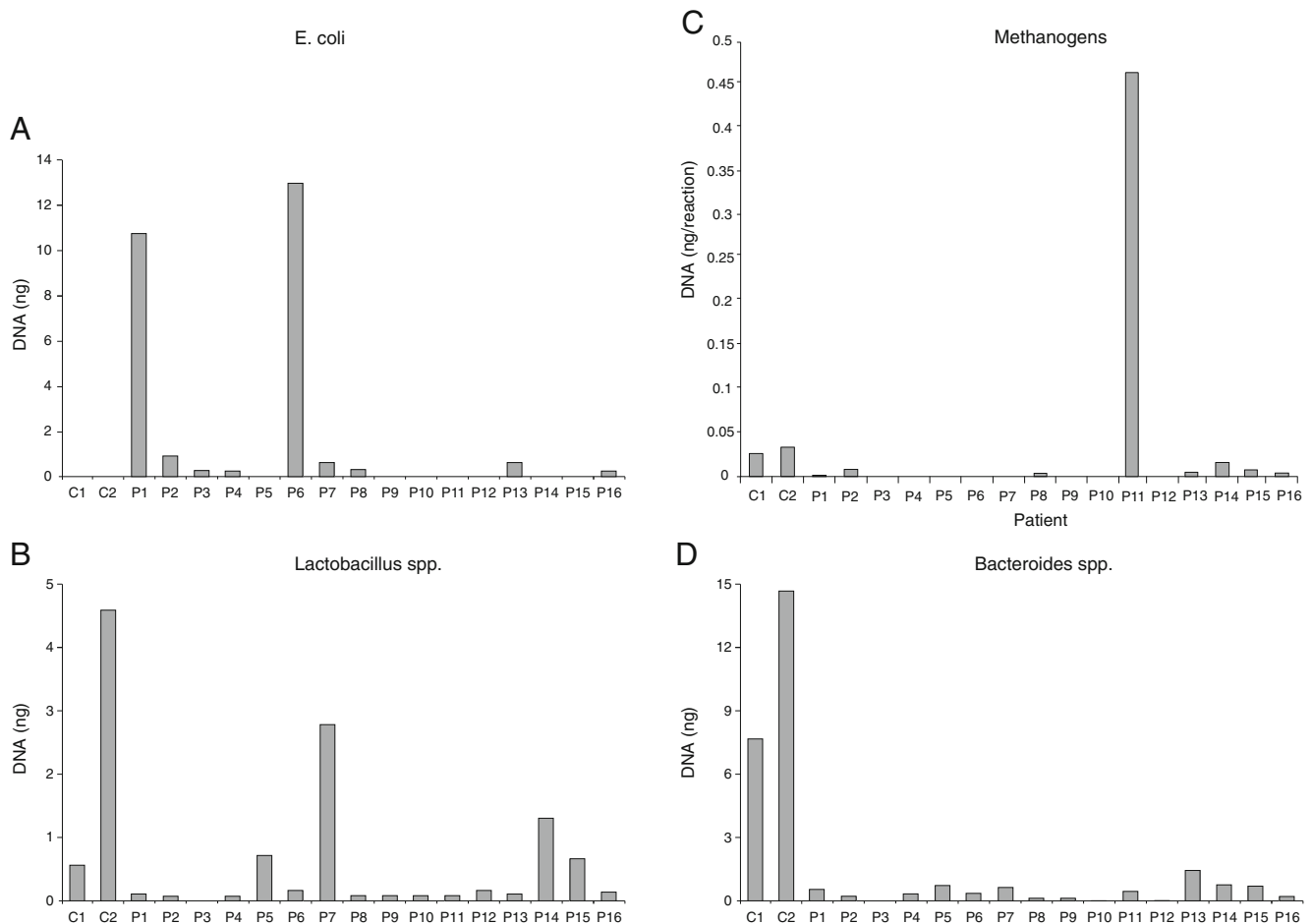


Fig. 1 a–d Microflora composition of patients in cohort 1 with CD (P1–16) and healthy controls (C1–2). The majority of patients with CD demonstrated a decrease in *Lactobacillus* spp. and *Bacteroides* spp. and an increase in *E. coli*

MMP-3 levels increased to 1.74 ± 0.6 ng/ml ($p=0.0001$). At 5 days, circulating MMP-3 levels were 1.03 ± 0.3 ng/ml ($p=$

0.01). MMP-3 levels returned to pre-treatment levels at 10 days following treatment (MMP-3 = 0.48 ± 0.13 ng/ml).

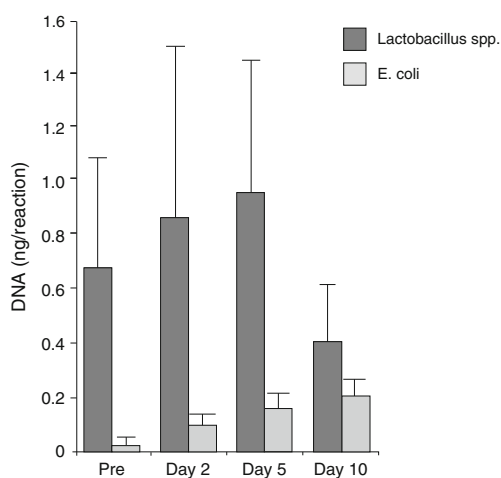


Fig. 2 Microflora composition of patients in cohort 2 with CD. *E. coli* increased over time following chemotherapy, whereas *Lactobacillus* spp. rose gradually until day 5, before decreasing at day 10 after chemotherapy. Statistical analysis showed no statistical significance due to low patient number

MMP-9 A significant change in mean MMP-9 levels was noted in the serum of patients post-chemotherapy treatment ($p=0.02$) (Fig. 3). Pre-treatment MMP-9 levels were measured to be 6 ± 1.4 ng/ml. MMP-9 levels were significantly increased at 2 days post-treatment (Table 2). At 2 days following treatment, MMP-9 levels significantly increased to 11.30 ± 2.0 ng/ml ($p=0.02$). There was no significant change in MMP-9 at 5 and 10 days post-treatment in comparison to pre-treatment samples (Table 2).

Serum levels of NF- κ B and proinflammatory cytokines IL-1 β and TNF

NF- κ B Mean levels of serum NF- κ B were increased from pre-treatment samples at all time points investigated post-chemotherapy, although this did not reach significance (Fig. 4). Levels increased at day 2 and day 5 post-treatment before decreasing, although still not returning to pre-treatment values at day 10 post-therapy.

Table 2 Mean changes in serum MMP levels (nanogramme per millilitre) post-treatment

	Healthy control	Pre-treatment	2 days post-treatment	5 days post-treatment	10 days post-treatment
MMP-3	0.1345	0.30325	1.740125*	1.035688**	0.479938
MMP-9	9.487	6.00694	11.2975***	7.770438	4.168313
MMP-2	18.38	25.6961	27.12806	28.54081	26.68544

* $p=0.0001$; ** $p=0.01$;
*** $p=0.02$

IL-1 β Mean levels of serum IL-1 β did not show any significant change at any time point investigated ($p=0.05$) (data not shown).

TNF Mean serum levels of TNF showed increases at days 2 and 10 post-treatment although this did not reach significance (Fig. 4). Levels of serum TNF at day 5 post-therapy were very similar to the levels seen in pre-treatment samples ($p=0.05$).

Discussion

Chemotherapy-induced diarrhoea is a common, and potentially life-threatening, side effect of cancer treatment [10]. Unfortunately, there has been very little research conducted on the underlying mechanisms. Of the total number of patients undergoing chemotherapy, approximately 20–40 % are reported to experience severe diarrhoea [7, 8,

27]. The pathophysiology of CD is extensive, complex and regulated by a number of mechanisms [27] which are yet to be completely understood. The chemotherapeutic agents known to commonly cause diarrhoea are 5-fluorouracil, irinotecan, methotrexate, taxanes, monoclonal antibodies and hormonal agents among others [10]. In our clinical study, many of these agents were the causative agents for CD. Previous *in vivo* research has also indicated that the intestinal microflora are involved in chemotherapy-induced mucositis [7, 8, 27]. This is the first clinical study to examine potential biomarkers of CD including assessment of faecal microflora levels, methanogenic archaea and matrix metalloproteinases.

Key findings from our study indicated marked changes in the microflora of patients with CD and a change in circulating MMP-3 and MMP-9 following chemotherapy treatment. Mucositis, including CD, remains a major clinical problem, affecting 40 % of patients receiving standard dose chemotherapy and 100 % of patients receiving high-dose chemotherapy [1]. However, there is currently no prophylactic intervention available for this condition [1]. Furthermore, there are no validated grading systems for the assessment of mucositis development in patients [13]. This has prompted the need for the identification of a biomarker of gut toxicity

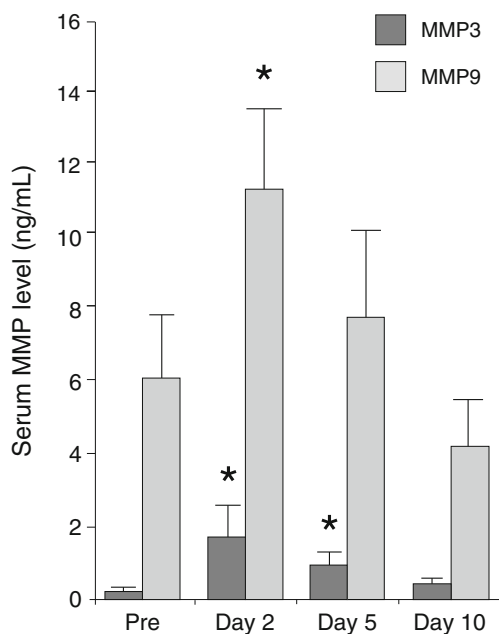


Fig. 3 MMP-3 and MMP-9 serum levels in patients from cohort 2. MMP-3 levels significantly increased at 2 ($p=0.0001$) and 5 days ($p=0.01$) post-treatment in comparison to pre-treatment. MMP-9 levels were significantly increased at 2 days post-treatment ($p=0.02$), but no significant change was seen at 5 and 10 days post-treatment in comparison to pre-treatment samples

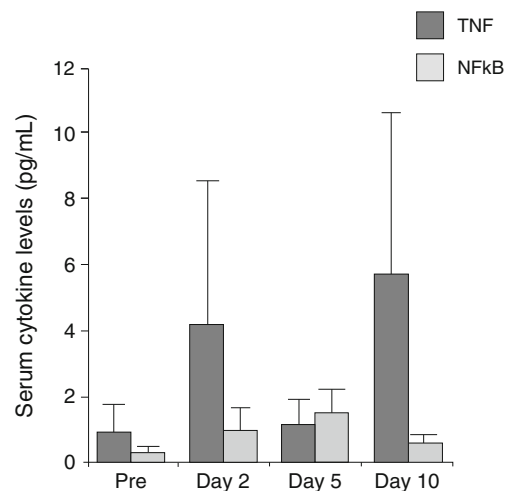


Fig. 4 NF- κ B and TNF serum levels in patients from cohort 2. Mean levels of serum NF- κ B were increased from pre-treatment samples at all time points investigated post-chemotherapy, although this did not reach significance. Mean serum levels of TNF showed increases at days 2 and 10 post-treatment although this did not reach significance

in response to cytotoxic anti-cancer treatment. To date, a wide range of biomarkers have been investigated in relation to gut toxicity including citrulline [28], calprotectin [16] and the proinflammatory cytokines [29]. Findings from such studies have highlighted the difficulties in identifying a biomarker for mucositis. This is due in part to (1) our lack of understanding its pathobiology and (2) the fact that mucositis is multifactorial, often arising from a combination of pathologies.

The present study demonstrated marked changes in the microflora of patients with CD. It is highly likely that these changes play a critical role in the development of CD in these patients, with numerous studies indicating that intestinal microflora may be involved in chemotherapy-induced diarrhoea and mucositis [7, 8, 27]. The changes seen in this study varied between patients, and this may be due to a variety of reasons, including but not limited to natural variation between subjects, due to age, gender, race and dietary influence [30]. However, when we differentiated gender, we found no differences. There were not enough enrolled patients in our study to further differentiate into age groups, and race and dietary influence were unknown. Antibiotic use was shown not to influence the results in CD patients, with patients who received antibiotics showing similar changes to patients who did not receive antibiotics.

The individual effects of each chemotherapy agent may be another reason for the variability seen in our study. Previously, animal studies have demonstrated changes to intestinal microflora with a variety of different chemotherapy agents [7, 8, 27], with different changes observed with each chemotherapy agent used. We propose that any differences seen may be due to the different classes and subsequent different mechanisms of action of chemotherapy agents. Irinotecan is an example of one agent where the intestinal flora are hypothesised to play a role in the actual metabolism of the drug [7]. Whilst the metabolism of irinotecan has been published in detail previously [31], in brief, the intestinal microflora are thought to produce β -glucuronidase, which is able to hydrolyse SN-38G (the less toxic metabolite from irinotecan), once transported to the intestine, to SN-38 (the toxic metabolite) resulting in the characteristic damage and diarrhoea [31].

Whilst alteration to microflora is unlikely to be the sole foundation of CD, it is likely to substantially contribute. The intestinal bacteria provide a variety of functions that mammalian species have not yet evolved in exchange for residence and access to nutrients in the luminal contents [32]. Protection is one such function, where the intestinal microbiome is able to prevent colonisation by pathogens [33]. Metabolism of bilirubin, intestinal mucins, pancreatic enzymes, fatty acids, bile acids, cholesterol, carbohydrates and steroid hormones are also important features required for normal gut function [34]. Other functions of gastrointestinal

bacteria include nutrient processing, regulation of intestinal angiogenesis and immune functions [34]. *Bifidobacterium* spp. is known to colonise the gastrointestinal tract soon after birth [35] and plays a pivotal role in maintaining the microbial balance in the healthy gastrointestinal tract [36].

Methanogenic archaea remove hydrogen (H_2) gas from the intestine by producing methane (CH_4) [37]. A recent study has shown that serotonin (a neurotransmitter regulating gut peristalsis) is linked to CH_4 [38]. A reduction in CH_4 would lead to a reduction in serotonin, leading to decreased control of gut peristalsis which may contribute to CD. Methanogens are also slow-growing organisms and may be displaced during the rapid gut transit associated with diarrhoea [39]. Previous studies have suggested the functionality of methanogens may be dependent on the presence and/or activity of other microbial groups [40], for example, those with the fermentation capacity to produce adequate H_2 to support methanogenesis in a competitive environment. In the present study, we hypothesise that the decrease in other bacteria may have resulted in the decrease in methanogens and hence may have contributed to CD.

A recent study has proposed that abrupt changes in the microbiome may result in excessive generation of reactive oxygen species (ROS) in the epithelium [41]. The previously proposed five-phase model of mucositis involves an up-regulation and message generation phase, whereby ROS are strongly implicated in the up-regulation of NF- κ B, and subsequent up-regulation of pro-inflammatory cytokines, inducing inflammation, apoptosis and tissue injury [42]. We have demonstrated in the present study increases in patient in serum levels of the transcription factor NF- κ B after chemotherapy, and these had still not returned to pre-treatment levels by day 10. Further, we showed increases in serum pro-inflammatory cytokines, IL-1 β and TNF following cytotoxic therapy. Although these increases did not reach significance, this was most probably due to the small cohort size. We further hypothesise that the changes to intestinal microflora, as reported in this study, may be partly responsible for initiating the NF- κ B pathway, resulting in increases in serum levels of NF- κ B. This in turn leads to up-regulation of pro-inflammatory cytokine, extensive intestinal damage and subsequent diarrhoea. Studies now need to focus on further exploring this relationship in larger cohorts of patients.

The present study also demonstrated a significant increase in circulating MMP-3 and MMP-9 levels following chemotherapy treatment. Past studies have demonstrated that MMPs play a key role in the pathogenesis of mucositis [9, 43]. Hence, it is plausible to suggest that an increase in circulating MMPs may potentially serve as an excellent marker of the inflammatory response induced by chemotherapeutic agents. To date, no studies have been conducted to explore whether tissue changes in MMP levels are

translated systemically. Although an increase in serum MMPs was noted in this study, no association was found between MMP levels and the observation of GI toxicity. These findings suggest that (1) there is an inflammatory response after treatment which is not confined to the gastrointestinal tract or (2) the symptoms reported following treatment are a poor indicator of underlying pathology [44]. The latter is a more plausible explanation for the lack of association observed in this small study. Confirmation of these findings in a larger patient cohort is now required as biomarker studies require adequate sample size calculation above all else in order to rule out false negative or false positive results.

Past studies have illustrated the complexity of mucositis pathogenesis and emphasised the importance of assaying a combination of biomarkers rather than just one when assessing mucositis [44]. Furthermore, Lutgens and colleagues have identified two criteria for a biomarker of gastrointestinal toxicity which are (1) that it is easily accessible to clinicians and (2) that it must work independently of each patient's medication and metabolic activities such as individual diet and nutrition [28]. The latter of these criteria means that any one biomarker would require extensive validation for us to be able to make an association with gut injury as not all physiological changes occurring after cytotoxic treatment are reflective of tissue damage [44]. In the present study, patients were on various treatment regimens, at different cycle numbers and not controlled for age or gender. All of these factors are crucial when investigating the relation between pathological and physiological responses. Hence, larger, multi-institutional studies are now required to follow-up on these preliminary findings.

In conclusion, we have demonstrated that CD is associated with marked changes in the intestinal microflora, methanogenic archaea, circulating matrix metalloproteinases and serum levels of NF- κ B, IL-1 β and TNF. These changes may result in diminished bacterial functions within the gut, altering gut function and initiating intestinal damage, resulting in the onset of diarrhoea. More importantly, these changes may provide clinicians with a possible new target for biomarkers of toxicity.

Acknowledgments Funding for this project was provided by a Cure Cancer/Cancer Australia Research Grant awarded to Dr Rachel Gibson; Dr Andrea Stringer is the recipient of an NHMRC Post-Doctoral Training Fellowship; Dr Noor Al-Dasooqi is the recipient of a Clinical Centre of Research Excellence Post-Doctoral Fellowship; Dr Joanne Bowen is the recipient of an NHMRC Post-Doctoral Training Fellowship; and Professor Dorothy Keefe is the Professor of Cancer Medicine.

Conflict of interest None of the authors listed on this manuscript have any financial relationship with the funding bodies that provided money for this research. Further, all authors have full control of all primary data and we consent to allowing the journal to review any/all data if requested.

References

1. Keefe DM, Schubert MM, Elting LS, Sonis ST, Epstein JB, Raber-Durlacher JE, Migliorati CA, McGuire DB, Hutchins RD, Peterson DE (2007) Updated clinical practice guidelines for the prevention and treatment of mucositis. *Cancer* 109(5):820–831
2. Gibson RJ, Bowen JM, Cummins AG, Keefe DM (2005) Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat. *Clin Exp Med* 4(4):188–195
3. Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST, Keefe DM (2008) Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: implications for the pathobiology of mucositis. *Cancer Chemother Pharmacol* 62(1):33–41
4. Bowen JM, Gibson RJ, Cummins AG, Tyskin A, Keefe DM (2007) Irinotecan changes gene expression in the small intestine of the rat with breast cancer. *Cancer Chemother Pharmacol* 59(3):337–348
5. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, Haimovitz-Friedman A, Cordon-Cardo C, Kolesnick R (2001) Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* 293:293–297
6. Gibson RJ, Bowen JM, Inglis MR, Cummins AG, Keefe DM (2003) Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. *J Gastroenterol Hepatol* 18(9):1095–1100
7. Stringer AM, Gibson RJ, Logan RM, Bowen JM, Yeoh AS, Burns J, Keefe DM (2007) Chemotherapy-induced diarrhea is associated with changes in the luminal environment in the DA rat. *Exp Biol Med (Maywood)* 232(1):96–106
8. Stringer AM, Gibson RJ, Logan RM, Bowen JM, Yeoh AS, Hamilton J, Keefe DM (2009) Gastrointestinal microflora and mucins may play a critical role in the development of 5-fluorouracil-induced gastrointestinal mucositis. *Exp Biol Med (Maywood)* 234(4):430–441
9. Al-Dasooqi N, Gibson RJ, Bowen JM, Logan RM, Stringer AM, Keefe DM (2010) Matrix metalloproteinases are possible mediators for the development of alimentary tract mucositis in the dark agouti rat. *Exp Biol Med (Maywood)* 235(10):1244–1256
10. Gibson RJ, Keefe DM (2006) Cancer chemotherapy-induced diarrhoea and constipation: mechanisms of damage and prevention strategies. *Support Care Cancer* 14(9):890–900
11. Elting LS, Cooksley C, Chambers M, Cantor SB, Manzullo E, Rubenstein EB (2003) The burdens of cancer therapy. Clinical and economic outcomes of chemotherapy-induced mucositis. *Cancer* 98(7):1531–1539
12. Savarese DM, Hsieh C, Stewart FM (1997) Clinical impact of chemotherapy dose escalation in patients with hematologic malignancies and solid tumors. *J Clin Oncol* 15(8):2981–2995
13. Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, Bekele BN, Raber-Durlacher J, Donnelly JP, Rubenstein EB (2004) Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. *Cancer* 100(9 Suppl):1995–2025
14. Costa F, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M (2005) Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 54(3):364–368
15. Foell D, Wittkowski H, Ren Z, Turton J, Pang G, Daebritz J, Ehrchen J, Heidemann J, Borody T, Roth J, Clancy R (2008) Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. *J Pathol* 216(2):183–192

16. Roseth AG (2003) Determination of faecal calprotectin, a novel marker of organic gastrointestinal disorders. *Dig Liver Dis* 35(9):607–609
17. Wedlake L, McGough C, Hackett C, Thomas K, Blake P, Harrington K, Tait D, Khoo V, Dearnaley D, Andreyev HJ (2008) Can biological markers act as non-invasive, sensitive indicators of radiation-induced effects in the gastrointestinal mucosa? *Aliment Pharmacol Ther* 27(10):980–987
18. van Vliet MJ, Tissing WJ, Rings EH, Koetse HA, Stellaard F, Kamps WA, de Bont ES (2009) Citrulline as a marker for chemotherapy induced mucosal barrier injury in pediatric patients. *Pediatr Blood Cancer* 53(7):1188–1194
19. Page-McCaw A, Ewald AJ, Werb Z (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 8(3):221–233
20. Stankovic S, Konjevic G, Gopcevic K, Jovic V, Inic M, Jurisic V (2010) Activity of MMP-2 and MMP-9 in sera of breast cancer patients. *Pathol Res Pract* 206(4):241–247
21. Edwards KJ, Kaufmann ME, Saunders NA (2001) Rapid and accurate identification of coagulase-negative staphylococci by real-time PCR. *J Clin Microbiol* 39(9):3047–3051
22. Layton A, McKay L, Williams D, Garrett V, Gentry R, Saylor G (2006) Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl Environ Microbiol* 72(6):4214–4224
23. Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol* 70(12):7220–7228
24. Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE (2005) Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett* 243(1):141–147
25. Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 97(6):1166–1177
26. Loitsch SM, Shastri Y, Stein J (2008) Stool test for colorectal cancer screening—it's time to move! *Clin Lab* 54(11–12):473–484
27. Stringer AM, Gibson RJ, Bowen JM, Logan RM, Yeoh AS, Keefe DM (2007) Chemotherapy-induced mucositis: the role of gastrointestinal microflora and mucins in the luminal environment. *J Support Oncol* 5(6):259–267
28. Lutgens LC, Blijlevens NM, Deutz NE, Donnelly JP, Lambin P, de Pauw BE (2005) Monitoring myeloablative therapy-induced small bowel toxicity by serum citrulline concentration: a comparison with sugar permeability tests. *Cancer* 103(1):191–199
29. Logan RM, Stringer AM, Bowen JM, Yeoh AS, Gibson RJ, Sonis ST, Keefe DM (2007) The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: pathobiology, animal models and cytotoxic drugs. *Cancer Treat Rev* 33(5):448–460
30. Heller F, Duchmann R (2003) Intestinal flora and mucosal immune responses. *Int J Med Microbiol* 293(1):77–86
31. Smith NF, Figg WD, Sparreboom A (2006) Pharmacogenetics of irinotecan metabolism and transport: an update. *Toxicol In Vitro* 20(2):163–175
32. Wexler HM (2007) Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev* 20(4):593–621
33. Neish AS (2002) The gut microflora and intestinal epithelial cells: a continuing dialogue. *Microbes Infect* 4(3):309–317
34. Cassel SL, Sutterwala FS, Flavell RA (2008) The tiny conductor: immune regulation via commensal organisms. *Cell Host Microbe* 3(6):340–341
35. McCartney AL, Wenzhi W, Tannock GW (1996) Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. *Appl Environ Microbiol* 62(12):4608–4613
36. Masco L, Van Hoorde K, De Brandt E, Swings J, Huys G (2006) Antimicrobial sensitivity of *Bifidobacterium* strains from humans, animals and probiotic products. *J Antimicrob Chemother* 58:85–94
37. Gibson GR, Cummings JH, Macfarlane GT (1988) Competition for hydrogen between sulphate-reducing bacteria and methanogenic bacteria from the human large intestine. *J Appl Bacteriol* 65(3):241–247
38. Pimentel M, Kong Y, Park S (2004) BS subjects with methane on lactulose breath test have lower postprandial serotonin levels than subjects with hydrogen. *Dig Dis Sci* 49(1):84–87
39. Scanlan PD, Shanahan F, Marchesi JR (2008) Human methanogen diversity and incidence in healthy and diseased colonic groups using *mcrA* gene analysis. *BMC Microbiol* 8:79
40. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci U S A* 105(43):16767–16772
41. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS (2007) Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. *EMBO J* 26(21):4457–4466
42. Sonis ST (2004) The pathobiology of mucositis. *Nat Rev Cancer* 4(4):277–284
43. Al-Dasooqi N, Gibson RJ, Bowen JM, Keefe DM (2009) Matrix metalloproteinases: key regulators in the pathogenesis of chemotherapy-induced mucositis? *Cancer Chemother Pharmacol* 64(1):1–9
44. Gibson RJ, Bowen JM (2011) Biomarkers of regimen-related mucosal injury. *Cancer Treat Rev* 37(6):487–493