THE UNIVERSITY of ADELAIDE

Identifying potential genetic, phenotypic and epigenetic predictive markers in the Toll-like receptor/Interleukin-1 domain innate immune signalling pathway for severe gastrointestinal toxicity risk following 5-Fluorouracil-based therapy

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by

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A PDF of this manuscript is provided in Appendix: Chapter 2

Thesis abstract

Severe gastrointestinal (GI) toxicity symptoms such as diarrhoea, mucositis, nausea and vomiting are adverse side effects following 5-fluorouracil (5-FU)-based therapy. Current supportive care measures for the treatment of severe GI toxicity are limited, with many patients requiring treatment intervention or hospitalisation to help manage and relieve symptoms. The presence of severe GI toxicity not only compromises patient clinical outcomes but, is also a financial burden on the health care system. Most importantly, the presence of severe GI toxicity symptoms decreases a patients' quality of life whilst receiving 5-FU-based therapy.

A predictive marker for severe GI toxicity risk is urgently required to identify patients at most risk of developing severe GI toxicity prior to receiving 5-FU-based therapy. This would not only allow at risk patients to be closely monitored whilst receiving 5-FU-based therapy but, improve resource utilisation and patient education, reducing the severity of GI toxicity experienced by at risk patients. Current predictive markers for severe GI toxicity, such as single nucleotide polymorphisms (SNPs) in 5-FU drug biotransformation genes, lack clinical utility, sensitivity and specificity.

As such, the gap in the knowledge is a new predictive marker(s) for severe GI toxicity risk needs to be identified. A clinical pilot study conducted in my laboratory highlighted the potential of the Toll-like Receptor/Interleukin-1 (TIR) domain innate immune signalling pathway to be predictive for severe GI toxicity risk. The TIR domain pathway is one of the key mediating pathways in the development of GI toxicity and consists of membrane bound Toll-like Receptors (TLRs), adapter proteins, transcription factors and proinflammatory cytokines such as interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α).

Based on the results of the pilot study, I firstly investigated the association of 21 SNPs within the TIR domain innate immune signalling pathway with severe GI toxicity risk in 155 participants who

received 5-FU-based therapy. Secondly, in a subset of 34 participants, I also examined TLR2- and TLR4-stimulated IL-1β and TNF-α secretion from isolated peripheral blood mononuclear cells to determine a phenotypic marker for severe GI toxicity risk. Both these studies were retrospective. Lastly, I considered whether administration of 5-FU could induce DNA methylation within the TIR domain innate immune signalling pathway *in vitro*, to identify a potential epigenetic predictive marker for severe GI toxicity risk.

The results of my thesis point to no concordant genetic, phenotypic or epigenetic predictive marker for severe GI toxicity risk within the TIR domain innate immune signalling pathway. However, observations made throughout my thesis highlight the importance of using risk prediction modelling to encompass all potential predictive factors, such as demographic, clinical, genetic, phenotypic and epigenetic, to create a truly personalised risk prediction strategy for patients receiving 5-FU-based therapy.

Nomenclature

5-FU-based therapy

5-FU-based therapy refers to chemotherapy regimens where 5-FU or capecitabine were administered as the principal chemotherapeutic agent.

GI toxicity

Throughout my thesis, GI toxicity is defined as symptoms diarrhoea, oral mucositis, nausea and vomiting.

Mild to moderate GI toxicity

Mild to moderate GI toxicity is classified as symptoms graded 1 or 2 on the National Cancer's Institute's Common Terminology for Adverse Events (NCI CTCAE v4.03 and 5.0).

NCI CTCAE

The National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI CTCAE) was the grading criteria used by clinical oncology staff to grade GI toxicity symptoms in participants. The NCI CTCAE was updated from version 4.03 to version 5.0 throughout my PhD and both versions will be referred to accordingly.

Non-toxic participants

Participants recruited to the study cohort who developed no or mild to moderate GI toxicity were categorised as non-toxic to 5-FU.

Predictive marker

Defined in my thesis as a biological marker to predict the risk of GI toxicity following 5-FU-based therapy.

Severe GI toxicity

Severe GI toxicity is classified as GI toxicity symptoms graded ≥ 3 on the NCI CTCAE v4.03 and 5.0. Additionally, GI toxicity symptoms that led to a dose reduction, treatment interruption, hospitalisation or early treatment cessation were also classified as severe.

Toxic participants

Participants recruited to the study cohort who developed severe GI toxicity were categorised as toxic to 5-FU.

List of commonly used abbreviations

5-Aza-dc 5-Aza-2'-deoxycytidine

5-FU 5-fluorouracil

BDNF Brain-derived neurotrophic factor

CASP1 Caspase-1

CASP5 Caspase-5

CRP C-Reactive protein

DAMP Damage associated molecular pattern

DNMT1 DNA methyltransferase 1, gene or enzyme

DNMT3A DNA methyltransferase 3A, gene or enzyme

DPD Dihydropyrimidine dehydrogenase enzyme

DPYD Dihydropyrimidine dehydrogenase gene

ELISA Enzyme-linked immunosorbent assay

FDR False discovery rate

FMC Flinders Medical Centre

GI Gastrointestinal

h Hours

IL-1β Interleukin 1 beta

IL10 Interleukin 10 gene

IL1B Interleukin 1 beta gene

IL2 Interleukin 2 gene

IL6 Interleukin 6 gene

LPS Lipopolysaccharide

MAF Minor allele frequency

MD2 Lymphocyte antigen 96 gene

Min Minutes

MS-HRM Methylation specific high-resolution melt

MYD88 Myeloid differentiation primary response protein 88 gene

NCI CTCAE National Cancer Institute Common Terminology Criteria for Adverse Events

OPRM1 Opioid Receptor Mu 1 gene

PAM3CSK4 Pam3CysSerLys4

PAMP Pathogen associated molecular pattern

PBMC Peripheral blood mononuclear cell

RAH Royal Adelaide Hospital

ROC AUC Receiver operator characteristic area under the curve

RT-PCR Real-time polymerase chain reaction

SAM S-adenosine methionine

Sec Seconds

SNP Single nucleotide polymorphism

TGFB Transforming growth factor beta gene

TIR Toll-like receptor/Interleukin-1

TLR Toll-like Receptor

TNF Tumour necrosis factor gene

TNF-α Tumour necrosis factor

TS Thymidylate synthase

TYMS Thymidylate synthase gene

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Chapter 1: Thesis introduction

Chapter 1 is an introductory chapter and sets the premise of my thesis.

1.1 5-FU-based therapies induce GI toxicity

5-fluorouracil (5-FU) and its prodrug, capecitabine, are staple chemotherapy agents used for the treatment of cancers of the breast, colon and upper gastrointestinal (GI) tract [1]. They are associated with improved overall survival and prognosis however, induce a range of 'off target' and dose-limiting adverse effects offsetting these benefits [1, 2]. These adverse effects are due to the non-selective nature of these chemotherapy agents targeting highly proliferative cells throughout the body [2]. One of the key dose-limiting adverse effects of 5-FU-based therapy is GI toxicity, which occurs in 20 – 50 % of patients [3]. Throughout the GI tract, 5-FU initiates DNA and non-DNA injury and death in cells of the basal epithelium and cells of the underlying tissue [1, 2]. This damage leads to the manifestation of GI toxicity symptoms including, but not limited to, mucositis, diarrhoea, nausea and vomiting [4]. Anti-metabolites such as 5-FU cause a higher degree of GI toxicity than other classes of chemotherapy agents such as taxanes or platinum-based [5]. The presence of GI toxicities at any grade throughout therapy are costly and may lead to sub-optimal therapy outcomes and decreased patient quality of life [6, 7]. Supportive care measures are recommended for the management of GI toxicity although, to date, these can only attempt to control symptoms and do not target the underlying pathology [8, 9].

1.2 Who is at risk of GI toxicity?

Not all patients are at equal risk of GI toxicity. Some patients will develop mild to moderate GI toxicity, graded as 1 or 2 on the National Cancer's Institute's Common Terminology for Adverse Events (NCI CTCAE v 4.03 and v 5.0) [10]. However, a proportion of patients will develop severe GI toxicity, graded as ≥ 3 on the NCI CTCAE, in which supportive care is inadequate at controlling

symptoms [2, 10]. Patients with severe GI toxicity will need treatment delays, dose reductions, early treatment cessation and/or hospitalisation to help manage and relieve symptoms [2, 10]. Due to the extreme interventions required to manage severe GI toxicity, which can compromise patient therapy outcomes and quality of life, is a major concern for patients receiving 5-FU-based therapy. It is unclear which factors determine the development of GI toxicity and why some patients are more susceptible to tissue injury and severe GI toxicity than others. Consequently, there is currently no way to identify patients at most risk of developing severe GI toxicity prior to receiving 5-FU-based therapy. A predictive maker would allow these 'at risk' patients to be identified prior to receiving 5-FU-based therapy. In turn, this may lead to improved resource utilisation, reducing the severity of GI toxicity experienced by 'at risk' patients and possibly minimising dose-limiting adverse effects.

1.3 Current predictive markers for severe GI toxicity risk lack clinical sensitivity and specificity

There are many controversies in the literature surrounding the identification of predictive markers for severe GI toxicity risk following 5-FU-based therapy. Research has identified traditionally-used risk prediction variables such as sex, age and 5-FU dosage to be unreliable, with many contradictory and inconsistent findings surrounding their use as predictive markers [11-15]. Candidate gene approaches, with specific interest in single nucleotide polymorphisms (SNPs) in the 5-FU rate determining enzyme dihydropyrimidine dehydrogenase (DPD, encoded by the gene *DPYD*), have been more successful in identifying consistent and reliable predictive risk markers for severe GI toxicity risk [14, 16-18]. However, these too have their own limitations, as the *DPYD* SNPs identified as predictive for severe GI toxicity risk following 5-FU-based therapy cannot account for majority of incidences due to their low minor allele frequencies, particularly amongst the Caucasian population (< 5 %) [16, 18]. The use of genome-wide association studies (GWAS) is also divisive. As GWAS analyses a genome-wide set of SNPs, the relationship between severe

GI toxicity and SNPs is broader and less restrictive, with many SNPs identified as predictive for severe GI toxicity risk found in genes with no previous underlying relationship with the development of GI toxicity [19, 20]. As such, a new approach is required to identify predictive markers for severe GI toxicity risk following 5-FU-based therapy that are not only reliable and predictive but, are also clinically sensitive and specific.

1.4 Potential predictors for severe GI toxicity risk may lie within the TIR domain innate immune signalling pathway

One of the key signalling pathways implicated in the development of severe GI toxicity is the Toll-Like Receptor/Interleukin-1 (TIR) domain innate immune signalling pathway. Therefore, predictive makers for severe GI toxicity risk may lie within this pathway. The TIR domain signalling pathway consists of membrane-bound Toll-like Receptors (TLRs), adapter proteins and transcription factors. Activation of the TIR pathway mediates the secretion of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) [21, 22]. Both preclinical and clinical studies of mucosal injury have identified an increase in tissue and circulating proinflammatory cytokine levels following 5-FU administration [5, 23, 24]. These levels correlated with cell injury, cell death and GI toxicity severity in Dark Agouti (DA) rats [5, 23]. Increased proinflammatory cytokine levels were also associated with the severity of GI toxicity experienced in patients receiving 5-FU-based therapies [24].

With preclinical and clinical studies highlighting the importance of the TIR domain innate immune signalling pathway in the development of severe GI toxicity, a small pilot study was conducted in my laboratory [25]. In 34 patients whom received 5-FU-based therapy, a general linear model of multivariate logistic regression created a risk prediction model which identified *TLR2* rs384100 and *TNF* rs1800629 SNPs in conjunction with colorectal and upper GI cancer types as predictive of severe GI toxicity risk [25]. Importantly, *TLR2* and *TNF* are key genes within the TIR domain signalling pathway.

1.5 Thesis Rationale

The risk prediction model produced in the pilot study [25] was one of the first risk prediction models to use a signalling pathway-directed approach to identify predictive markers for severe GI toxicity risk. It not only highlighted the potential of the TIR domain innate immune signalling pathway to be predictive for severe GI toxicity risk but, it also highlighted using multivariate logistic regression to build risk prediction models may be a desirable approach to accurately identify patients at most risk of severe GI toxicity. As the development of GI toxicity is multifaceted, multivariate logistic regression enables the simultaneously analysis of participant demographics, clinical data and SNPs of interest to produce a more accurate, sensitive and specific risk prediction model for severe GI toxicity. This approach was advantageous over candidate gene approach and GWAS, which focus on genetics alone and allows additional potential predictors, such as phenotypic markers and epigenetic modifications, to also be included in risk prediction modelling.

The gap in the knowledge is there were no clinically sensitive and specific predictive markers to accurately identify patients at most risk of developing severe GI toxicity prior to receiving 5-FU-based therapy. To address this gap, my thesis not only endeavoured to validate the results of the pilot study in a new, larger and independent cohort of participants but, also investigate potential phenotypic markers and epigenetic modifications within the TIR domain innate immune signalling pathway that may also be predictive for severe GI toxicity.

1.6 Thesis Hypotheses and Aims

Each research chapter is independent and was not reliant on the results of another therefore, each research chapter has its own hypotheses and aims. A timeline of study completion is provided in Figure 1.1.

1.6.1 Chapter 3

Hypotheses

1. A general linear model of multivariate logistic regression would generate a risk prediction model with similar sensitivity and specificity to the pilot study risk prediction model, identifying *TLR2* rs384100 and *TNF* rs1800629 SNPs in conjunction with colorectal and upper GI cancer types to be predictive for severe GI toxicity risk.

Aims

1. To identify which participant demographic and clinical data (5-FU-based regimen, cancer type, sex, age and number of treatment cycles) were significantly associated with severe GI toxicity using logistic regression.
2. To identify which SNPs in TIR domain pathway genes *TLR2*, *TLR4*, *IL1B*, *IL6*, *IL6R*, *IL10*, *TGFB*, *TNF*, *MYD88*, *CASP1* and *CASP5*, as well as *OPRM1* and *CRP*, were significantly associated with severe GI toxicity using logistic regression.
3. To determine the combined impact of clinical and genetic factors on severe GI toxicity risk using models of multivariate logistic regression.

Chapter 3 is my first research chapter and details the main clinical study of my thesis, the Salivary Predictors in Treatment Validation (SPiT-V) study. The SPiT-V study was formed on the basis of the pilot study [25]. I recruited a new, larger and independent cohort of participants who had received 5-FU-based therapy and built multiple risk prediction models for severe GI toxicity incorporating participant demographics, clinical data and SNPs within TIR domain innate immune signalling pathway genes.

1.6.2 Chapter 4

Hypotheses

1. Participants who reported severe GI toxicity throughout their 5-FU-based therapy would secrete decreased IL-1β and TNF-α pre- and post-stimulation with TLR2 and TLR4 agonists compared to participants who reported no or mild to moderate GI toxicity.
2. Any differences in IL-1β and TNF-α secretion identified between the two participant groups would be associated with SNPs within *TLR2*, *TLR4*, *IL1B*, *TNF*, *CASP1* and/or *CASP5* genes.

Aims

1. To determine pre-stimulation IL-1β and TNF-α secretion in participants classified as non-toxic (participants who reported no or mild to moderate GI toxicity) and toxic (participants who reported severe GI toxicity) throughout their 5-FU-based therapy.
2. To determine if TLR2- and TLR4-stimulated IL-1β and TNF-α secretion was significantly different between participants classified as non-toxic and toxic throughout their 5-FU-based therapy.
3. To identify if any significant differences in post-stimulation IL-1β and TNF-α secretion between the two participant groups were associated with *TLR2*, *TLR4*, *IL1B*, *TNF*, *CASP1* and/or *CASP5* genotypes.

Chapter 4 was an extension of the SPiT-V study. In a subset of participants recruited from the SPiT-V participant cohort, I investigated the association between pre- and post-stimulated IL-1β and TNF-α secretion, GI toxicity and SNPs within the TIR domain innate immune signalling pathway post-5-FU-based therapy.

1.6.3 Chapter 5

Hypothesis

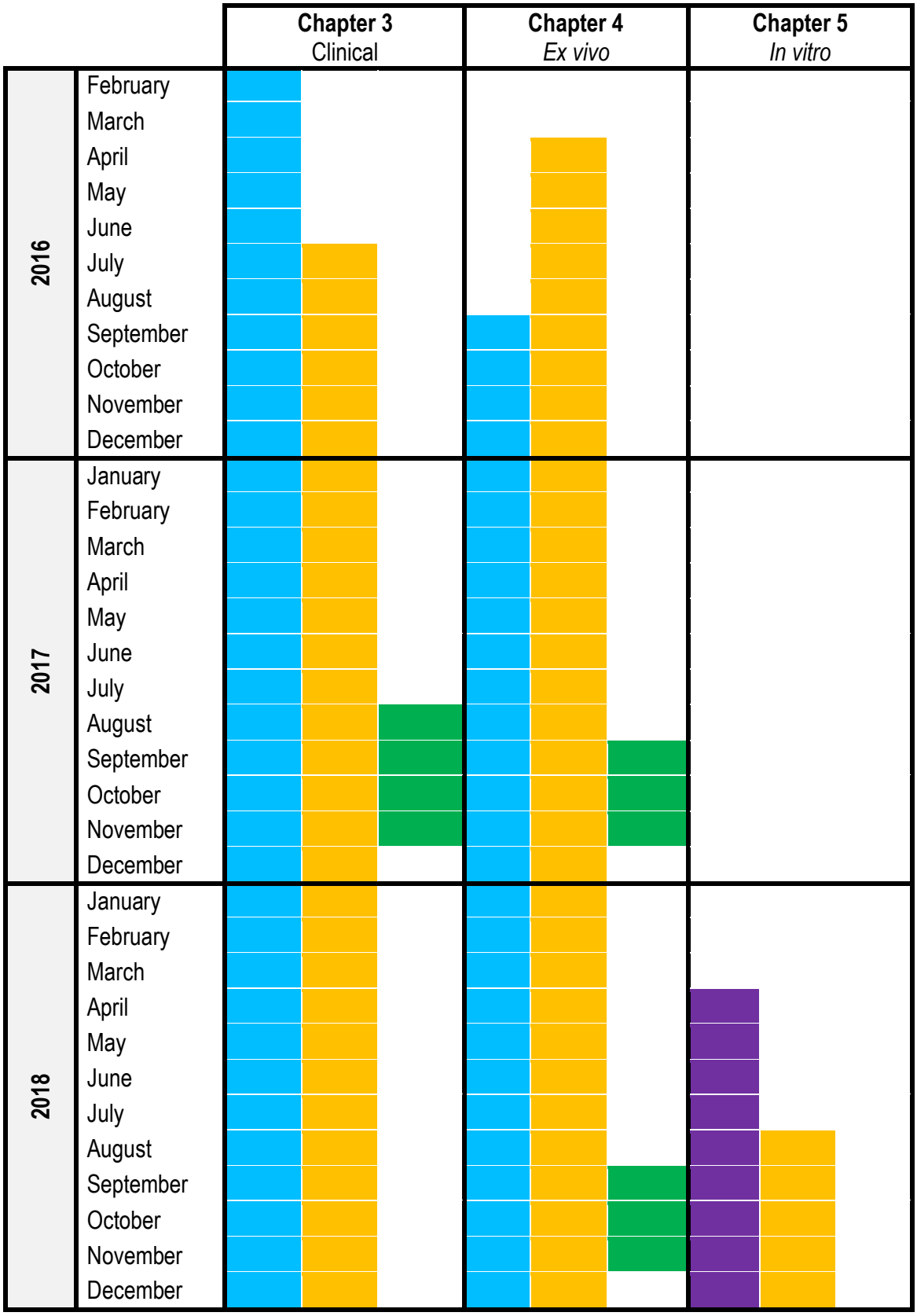
1. 5-FU reduces mRNA expression of DNA methyltransferase (DNMT)1 and DNMT3A therefore, inhibits DNA methylation at CpG sites -244, -238, -169, -163, -161, -149, -119, -72, -49 and -38 upstream of the *TNF* transcription start site in U937 cells *in vitro*.

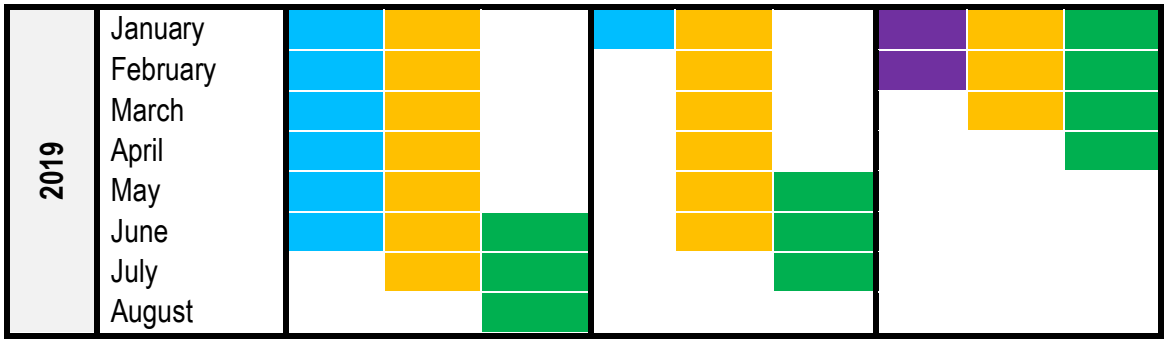
Aims

1. To optimise 5-FU and 5-Aza-2'-deoxycytidine (5-Aza-dc) concentration in the U937 cell line to determine a treatment dose that would not only induce cell injury and death but, result in a viable cell count that would allow adequate DNA and RNA extraction.
2. To determine changes in mRNA expression of TNF, DNMT1 and DNMT3A following treatment with 5-FU and/or methylation inhibitor 5-Aza-dc in U937 cells *in vitro* using real-time polymerase chain reaction (RT-PCR).
3. To determine changes in DNA methylation across CpG sites -244, -238, -169, -163, -161, -149, -119, -72, -49 and -38 upstream of the *TNF* transcription start site following treatment with 5-FU and/or methylation inhibitor 5-Aza-dc in U937 cells *in vitro* using methylation specific high-resolution melt (MS-HRM).

Chapter 5 was my final research chapter and investigated the novel idea that 5-FU reduces DNA methylation within the *TNF* gene and therefore, increases expression of TNF *in vitro*. As such, increased expression of TNF could potentially increase TNF-α secretion and be predictive for severe GI toxicity risk. As this study used experimental techniques not previously established in my laboratory, this research was conducted *in vitro*.

Figure 1.1 Timeline of research completion. Blue indicates participant recruitment, orange indicates experimental work, purple indicates cell culture and green indicates statistical analysis.

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Contribution to the Paper	First author and main contributor. I developed the concept, conducted the literature search and review, wrote the original draft, and was responsible for reviewing and editing.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Rachel Gibson		
Contribution to the Paper	Rachel was involved in conceptualisation, supervision and reviewing manuscript drafts.		
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Chapter 2: Toll-like receptor/Interleukin-1 domain innate immune signalling pathway genetic variants are candidate predictors for severe gastrointestinal toxicity risk

Chapter two is my literature review and aims to provide an in-depth summary of 5-FU-based therapy and detailed overview of the pathophysiology and clinical management of GI toxicity. The review also discusses previous literature on the influence and function of SNPs within the TIR domain innate immune signalling pathway. This chapter was published in Cancer Chemotherapy and Pharmacology. Korver SK, Gibson RJ, Bowen JM and Coller JK (2019) Toll-like receptor/Interleukin-1 domain innate immune signalling pathway genetic variants are candidate predictors for severe gastrointestinal toxicity risk. *Cancer Chemother Pharmacol* 83: 217 - 236. This chapter is presented in its **original** publication format. The referencing style and spelling have been modified to maintain consistency throughout this thesis.

2.1 Abstract

Purpose: Severe gastrointestinal (GI) toxicity is a common adverse effect following 5-fluorouracil (5-FU)-based therapy. The presence of severe GI toxicity leads to treatment revisions, sub-optimal therapy outcomes, and decreases to patients' quality of life. There are no adequate predictors for 5-FU-induced severe GI toxicity risk. The Toll-like Receptor/Interleukin-1 (TIR) domain innate immune signalling pathway is known to be a mediating pathway in the development of GI toxicity. Hence, genetic variability in this signalling pathway may alter the pathophysiology of GI toxicity and therefore, be predictive of risk. However, little research has investigated the effects of TIR domain innate immune signalling pathway single nucleotide polymorphism (SNPs) on the risk and development of severe GI toxicity.

Methods: This critical review surveyed the literature and reported on the *in vitro*, *ex vivo* and *in vivo* effects, as well as the genetic association, of selected TIR domain innate immune signalling pathway SNPs on disease susceptibility and gene functioning.

Results: Of the TIR domain innate immune signalling pathway SNPs reviewed, evidence suggests interleukin-1 beta (*IL1B*) and tumour necrosis factor alpha (*TNF*) SNPs have the greatest potential as predictors for severe GI toxicity risk. These results warrant further research into the effect of *IL1B* and *TNF* SNPs on the risk and development of severe GI toxicity.

Conclusions: SNPs of the TIR domain innate immune signalling pathway have profound effects on disease susceptibility and gene functioning, making them candidate predictors for severe GI toxicity risk. The identification of a predictor for 5-FU-induced severe GI toxicity will allow the personalisation of supportive care measures.

2.2 List of commonly used abbreviations

5-FU 5-fluorouracil

DAMPs Damage associated molecular patterns

DPD Dihydropyrimidine dehydrogenase enzyme

DPYD Dihydropyrimidine dehydrogenase gene

GI Gastrointestinal

HSCT Haematopoietic stem cell transplantation

IKK Inhibitor of NF-κB-kinase complex

IRAK1 Interleukin-1 receptor-associated kinases 1

IRAK4 Interleukin-1 receptor-associated kinases 4

MYD88 Myeloid differentiation primary response protein 88

NCI CTCAE v5.0 The National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0

PAMPs Pathogen associated molecular patterns

PBMCs Peripheral blood mononuclear cells

SNPs Single nucleotide polymorphisms

TAB1 Tak-1 binding protein 1

TAB2 Tak-1 binding protein 2

TAK1 transforming growth factor beta factor-β activated kinase 1

TIR Toll-like Receptor/Interleukin-1

TLR Toll-like receptor

TRAF6 TNF receptor associated factor 6

2.3 Introduction

5-fluorouracil (5-FU) is a commonly administered chemotherapy drug used for the treatment of breast, colorectal and upper gastrointestinal (GI) tract solid tumours [1, 26]. Although highly effective, with response rates for 5-FU-based regimens between 40 – 50 % in patients with advanced colorectal cancer [27, 28], 5-FU causes severe damage to mucosal membranes of the GI tract [2, 5, 29]. This damage results in apoptosis, altered histopathology and an increase in proinflammatory cytokine expression giving rise to inflammation [2, 5, 29]. This in turn leads to the clinical manifestation of GI toxicities including but not limited to, mucositis, diarrhoea, nausea and vomiting [4].

The development of GI toxicity is variable amongst patients, making it difficult to predict which patients will develop severe toxicity. Although supportive care measures are recommended and suggested, these are limited and are for use in highly specific patient cohorts [8]. Additionally, supportive care is often administered therapeutically not prophylactically, which in some patients is too late for the management and relief of severe GI toxicity symptoms [8, 9]. In these patients, further interventions are required to improve symptom management and relieve severe GI toxicity symptoms [30].

Currently, there are no adequate predictors for 5-FU-induced severe GI toxicity risk. It is imperative predictors are identified and translated to clinical practice to identify ‘at risk’ patients prior to 5-FU treatment. This will facilitate proactive delivery and personalisation of supportive care measures aimed at reducing the severity of GI toxicity experienced.

Following administration of 5-FU, an innate immune inflammatory response is initiated, mediated by the Toll-like Receptor/Interleukin-1 domain innate immune signalling pathway. Toll-like Receptor/Interleukin-1 is commonly abbreviated to TIR. Activation of the TIR domain innate immune signalling pathway leads to the upregulation of potent transcription factors and secretion

of proinflammatory cytokines [2, 31, 32]. Increased levels of these proinflammatory cytokines, in particular tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), have been significantly associated with severe GI toxicity in both preclinical and clinical studies [2, 5, 24]. Due to the importance of the TIR domain innate immune signalling pathway in inflammatory signalling and subsequent severe GI toxicity development, single nucleotide polymorphisms (SNPs) in this pathway may be potential predictors for severe GI toxicity risk. However, these SNPs are largely understudied in the context of GI toxicity.

This critical literature review will firstly provide an overview of 5-FU-based chemotherapy and the pathophysiology of GI toxicity; then outline and summarise previous research on the influence and function of TIR domain innate immune signalling pathway SNPs and how this can be applied to GI toxicity risk.

2.4 Background

2.4.1 5-FU Mechanism of action

5-FU is an antimetabolite drug that inhibits DNA and RNA synthesis [1]. The uracil analogue was developed in the 1950's following identification of uracil metabolism as a potential target for therapy due to the rapid use of uracil by rat hepatomas compared to normal tissues [33]. In the liver, 5-FU is metabolised to three active metabolites, fluorodeoxyuridine monophosphate (FdUMP), fluoro-deoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) [34, 35]. The oral prodrug of 5-FU, capecitabine, travels unaltered through the gut wall where it is converted to 5-FU by liver carboxylesterase (CES) 1 and CES2, and cytidine deaminase [36, 37]. The principal mechanism of action of 5-FU is inhibition of thymidylate synthase (TS, encoded by the gene *TYMS*), a critical enzyme necessary for conversion of precursor deoxyribonucleotides required for purine and pyrimidine synthesis. Its principal active metabolite, FdUMP, inhibits TS, whilst metabolites FdUTP and FUTP directly misincorporate into DNA and RNA, disrupting DNA and RNA

synthesis, RNA processing and protein synthesis [34, 38, 39]. 5-FU catabolism is governed by the rate determining enzyme dihydropyrimidine dehydrogenase (DPD, encoded by the *DPYD* gene), catabolising 80 % of administered 5-FU to dihydroflurouracil, which is then excreted in the urine [34, 40].

2.4.2 Current 5-FU regimens

5-FU is administered intravenously as either a bolus dose or continuous infusion over a 24 - 48 hour period to treat solid tumours of the breast, colon and upper GI tract [41]. In breast cancer regimens, 5-FU is generally administered in conjunction with epirubicin and cyclophosphamide [41]. For colorectal and upper GI tract cancers, 5-FU is administered as either a monotherapy in synergistic combination with leucovorin or in combination with other chemotherapeutics such as irinotecan, oxaliplatin, and cisplatin [41]. 5-FU is used in both a curative and palliative care setting. Additional therapies including radiation therapy, monoclonal antibodies (bevacizumab, cetuximab), taxanes (docetaxel and paclitaxel) and tyrosine kinase inhibitors (lapatinib) may also be administered in conjunction with or following 5-FU treatment in an attempt to further reduce tumour activity [41]. Capecitabine, the oral and more selective prodrug of 5-FU, may also be substituted in replacement of 5-FU in the before-mentioned regimens [42].

2.4.3 5-FU administration induces GI toxicity

Of all patients receiving 5-FU-based therapies, 25 – 50 % will experience GI toxicity [3]. Recording the prevalence and incidence of GI toxicity is vital for symptom management. However, this is often difficult and inconsistent due to a lack of standardised scoring criteria [43]. A number of toxicity grading scales exist, each with their own toxicity grading criteria. Among the most commonly used toxicity grading scale is The National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE v 5.0) [10]. The NCI CTCAE was developed during the late 1980's and uses clinical symptoms and number of events to grade GI toxicity on a scale of 1 to 5 [10, 44]. GI toxicity

graded as 1 or 2 is classified as mild to moderate toxicity whilst GI toxicity graded ≥ 3 is classified as severe toxicity [10]. Additionally, patients can also be categorised as suffering from a grade 3 GI toxicity on the NCI CTCAE v 5.0 if they receive a dose reduction, treatment delay, cease treatment prematurely and/or are hospitalised as a direct result of GI toxicity [10, 44].

There is currently no effective approach to prevent GI toxicity [2]. The Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) provide worldwide clinical practice guidelines for management of GI toxicity secondary to cancer therapy [8, 45, 46]. Table 2.1 lists the current MASCC/ISOO recommendations and suggestions for the management and relief of oral mucositis, diarrhoea, nausea and vomiting in adults [8, 9, 47].

Table 2.1 MASCC/ISOO clinical practice guidelines for the management of GI toxicity.

Oral Mucositis [8]
Recommended • Thirty minutes of oral cryotherapy (sucking on ice chips) to prevent mucositis in patients receiving 5-FU bolus regimens. *Suggested* • Good oral hygiene (regular tooth brushing, flossing and mouth rinsing) to prevent mucositis across all treatment modalities.
Diarrhoea [8]
Recommended • Loperamide use to treat moderate diarrhoea induced by standard or high-dose chemotherapy. • Octreotide use to treat diarrhoea induced by standard or high-dose chemotherapy if loperamide is ineffective. *Suggested* • Probiotics such as *Lactobacillus* use prior to and during chemotherapy treatment to maintain gut homeostasis and reduce diarrhoea occurrence.
Nausea and vomiting [9, 47]
Recommended • Prophylactic administration of antiemetics such as aprepitant (substance P antagonist), ondansetron (5-HT_3 receptor antagonist), dexamethasone (corticosteroid) or metoclopramide (dopamine-receptor antagonist) for patients receiving low emetogenic or high-dose chemotherapy. • No prophylactic administration of antiemetics for patients receiving minimal emetogenic chemotherapy. • Patients whom suffer vomiting should be treated as if they were receiving low emetogenic chemotherapy. • Breakthrough nausea and vomiting (categorised as nausea or vomiting that occurs within 5 days of chemotherapy administration following the use of antiemetic agents) treated with olanzapine (antipsychotic medication).

In addition to GI toxicity, 5-FU can also induce hand and foot syndrome, peripheral neuropathy and haematological toxicities including leukopenia, thrombocytopenia and neutropenia [34]. This is due to 'off-target' effects of 5-FU; 5-FU metabolites non-specifically target highly proliferative basal epithelium and haematopoietic progenitor cells, as well as highly proliferative cancer cells [2, 17]. Bolus administration of 5-FU increases the risk of haematological toxicities whilst continuous infusion of 5-FU increases the likelihood of hand and foot syndrome [34, 48]. In contrast, treatment modality does not affect the risk of GI toxicity [48]. Furthermore, patients with GI toxicity may also develop additional toxicities and experience 'toxicity clusters' [49]. In a cohort of patients with colorectal cancer receiving 5-FU-based therapy, diarrhoea was strongly linked with the presence of bloating, constipation and cystitis, whilst vomiting was strongly linked with the presence of nausea, dehydration and chills [49]. No links were identified between GI toxicities and haematological or neurological toxicities [49]. Although research is still in its infancy, symptoms grouped in clusters may share a common biological mechanism meaning that, a predictive marker for severe GI toxicity risk may also have the ability to predict the risk of additional toxicities.

2.4.4 The issue at hand – Severe GI toxicity

Of the 20 – 25 % of patients who experience GI toxicity, a subset of these patients will develop severe GI toxicity, graded as ≥ 3 on the NCI CTCAE [10]. Supportive care measures are ineffective for patients with severe GI toxicity and treatment delays, dose reductions or premature treatment cessation will be required to manage and relieve symptoms [2]. Furthermore, severe GI toxicities can induce secondary symptoms such as pain, dehydration and malnutrition requiring opioid analgesics, intravenous fluids and parenteral nutrition [2, 4]. Treatment interruption may negatively influence prognosis and potentially impact long-term survival. In addition, the presence of severe GI toxicity can decrease patient quality of life whilst receiving treatment and increase health costs, with inpatient hospitalisation as a direct result of severe GI toxicity estimated to cost the US health

care system US $15,500 per episode [6]. Currently, there is no way to identify patients at most risk of severe GI toxicity prior to treatment.

2.5 How does 5-FU induce GI toxicity?

2.5.1 Inhibition of DNA and RNA synthesis by 5-FU results in significant cell injury and death

Following inhibition of DNA and RNA synthesis by 5-FU, base-excision repair is initiated to remove precursor deoxyribonucleotides and misincorporated 5-FU metabolites, leading to excessive DNA fragmentation resulting in mucosal injury and cell apoptosis [1, 50]. Consequently, injured and apoptotic cells release damage associated molecular patterns (DAMPs) including DNA, heat shock proteins and intracellular components [2, 51]. The injury and death of intestinal epithelial cells leads to the breakdown of the mucosal barrier, allowing entry of microorganisms and the colonisation of bacteria [2]. Microorganisms and bacteria release their own endogenous danger signals such as lipopolysaccharide, lipoteichoic acid and single stranded mRNA, termed pathogen-associated molecular patterns (PAMPs) [2, 51].

The release of DAMPs and PAMPs initiates a signalling cascade mediated by Toll-like receptors (TLRs) of the TIR domain innate immune signalling pathway. Activation of the TIR domain innate immune signalling pathway leads to the recruitment of leukocytes and subsequent secretion of proinflammatory cytokines such as TNF-α and IL-1β. These molecular events, particularly TLR signalling, underlie the pathophysiology of GI toxicity [2, 23, 51-53].

2.5.2 The TIR domain innate immune signalling pathway underpins the pathophysiology of GI toxicity

TLRs are a family of highly conserved type 1 integral membrane glycoproteins containing an extracellular motif of leucine-rich repeats and cytoplasmic TIR domain (Figure 2.1) [21, 54, 55]. To date, ten TLRs have been identified in humans and are present on either the cell surface of innate immune cells (TLR1, 2, 4, 5 and 6) or localised in intracellular compartments such as the Golgi

bodies and endosomes (TLR3, 7, 8 and 9) [56, 57]. TLRs respond to a variety of PAMPs, DAMPs or synthetic compounds [22]. TLR2 and TLR4 are key TLRs which mediate mucosal destruction and protection against GI chemotoxicity [57, 58]. They are also abundantly present on a variety of cells throughout the GI tract including goblet cells, cells of the lamina propria and enterocytes [57, 58]. Activation of TLR2 and TLR4 initiates the innate immune signalling pathway via the TIR domain and leads to the subsequent activation and translocation of NF-κB [22]. The TIR domain innate immune signalling pathway is detailed in Figure 2.1.

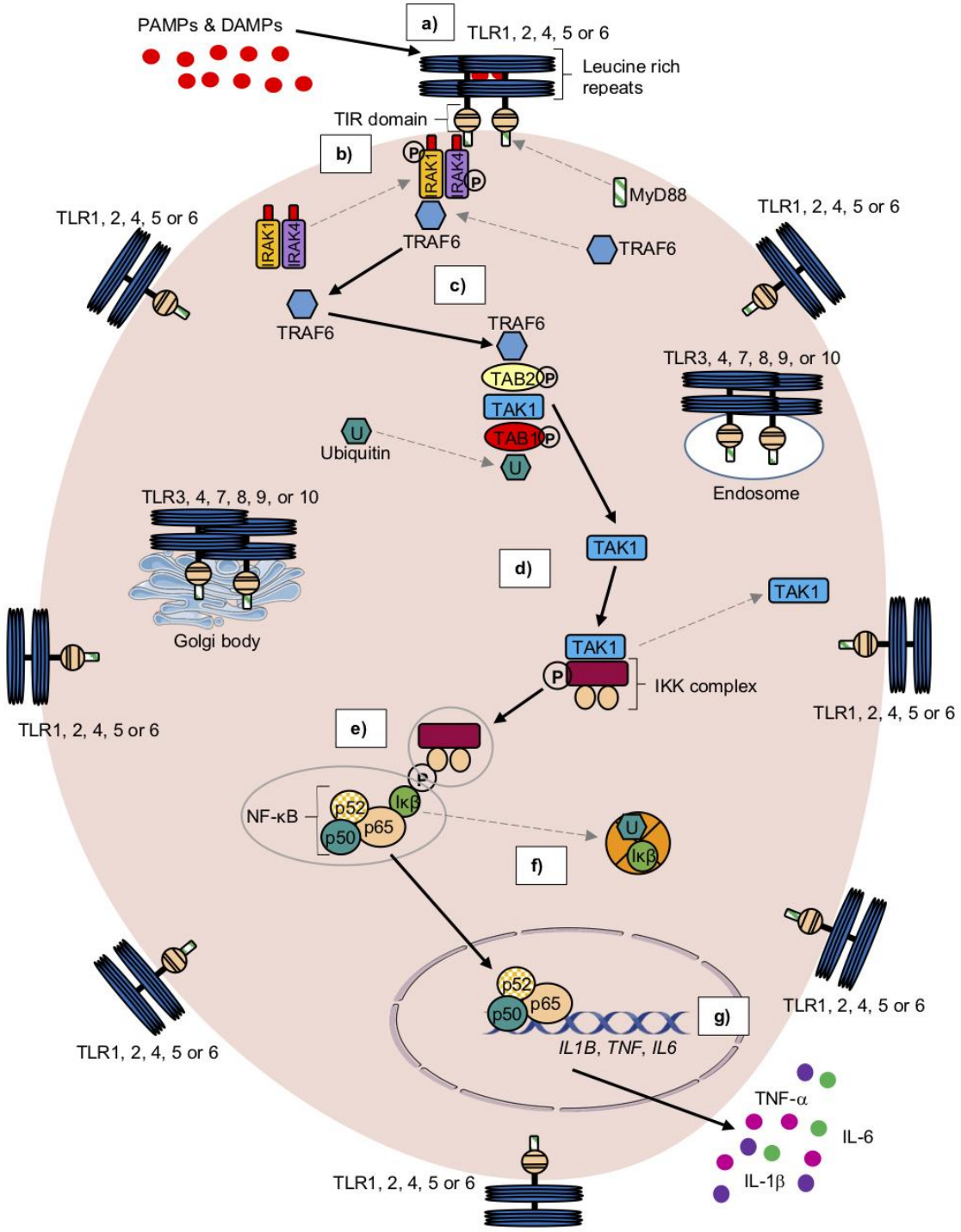
PAMPs & DAMPs
a)
TLR1, 2, 4, 5 or 6
Leucine rich repeats
TIR domain
b)
IRAK1
IRAK4
MyD88
TRAF6
TRAF6
TLR1, 2, 4, 5 or 6
TLR1, 2, 4, 5 or 6
c)
TRAF6
TRAF6
TAB2
TAK1
TAB1
Ubiquitin
U
TLR3, 4, 7, 8, 9, or 10
Endosome
TLR3, 4, 7, 8, 9, or 10
Golgi body
d)
TAK1
TAK1
TAK1
IKK complex
TLR1, 2, 4, 5 or 6
TLR1, 2, 4, 5 or 6
e)
NF-κB
p52
Iκβ
p50
p65
U
Iκβ
f)
TLR1, 2, 4, 5 or 6
TLR1, 2, 4, 5 or 6
p52
p65
p50
g)
IL1B, TNF, IL6
TNF-α
IL-6
IL-1β
TLR1, 2, 4, 5 or 6

Figure 2.1 The TIR domain innate immune signalling pathway: a) Following 5-FU administration, DAMPs are released from injured and apoptotic cells whilst PAMPs are produced from colonised bacteria and microorganisms. DAMPs and PAMPs are recognised by TLR2 and TLR4 and on binding, stimulate the homodimerisation of TLR4, and heterodimerisation of TLR2 with either TLR1 or TLR6; b) Dimerisation prompts the recruitment and binding of MyD88, which in turn recruits IRAK1 and IRAK4. Phosphorylation of IRAK1 and IRAK4 enables binding of TRAF6; c) TRAF6 disengages from the newly formed IRAK complex and forms a new complex with TAK1, TAB1 and TAB2. Phosphorylation of TAK1 and TAB2 occurs, stimulating ubiquination of TRAF6 and subsequent activation of TAK1; d) Activated TAK1 disengages and phosphorylates the IKK complex; e) Phosphorylated IKK complex disengages from TAK1 and phosphorylates the Iκβ subunit of NF-κB; f) Following phosphorylation, Iκβ is ubiquinated and degraded from NF-κB by the 26S proteosome. Activated NF-κB translocates to the nucleus; g) In the nucleus, NF-κB upregulates gene transcription and subsequent production and secretion of proinflammatory cytokines such as IL-1β, TNF-α and IL-6.

Following recognition and binding of DAMPs and PAMPs, TLR2 dimerises with TLR1 or 6, whilst TLR4 homodimerises [22]. TLR dimerisation prompts recruitment and binding of adapter protein myeloid differentiation primary response protein 88 (MyD88) to the TIR domain [22]. Binding of MyD88 in turn recruits interleukin-1 receptor-associated kinases (IRAK) 1 and IRAK4, resulting in phosphorylation of IRAK4 and subsequent activation and auto-phosphorylation of IRAK1 [22]. Phosphorylation of IRAK1 and IRAK4 enables binding of TNF receptor associated factor (TRAF) 6 to the TLR/MyD88/IRAK complex. Once bound, TRAF6 disengages from the TLR/MyD88/IRAK complex and associates with transforming growth factor beta factor-β activated kinase (TAK) 1, TAK1-binding protein (TAB) 1 and TAB2 to form a new complex in the cytosol [22]. TAB1 enhances TAK1 kinase activity whilst TAB2 is an adapter protein responsible for linking TAK1 with TRAF6. Phosphorylation of TAB2 and TAK1 then occurs and the remaining complex associates with ubiquitin enzymes leading to the ubiquitylation of TRAF6 and activation of TAK1 [22]. TAK1 then phosphorylates the IKK complex (inhibitor of NF-κβ-kinase complex).

Transcription factor NF-κB exists as a heterodimer of three subunits, NF-κβ1 (p50/p105), NF-κβ2 (p52/p100) and p65 (Rel A) bound to the inhibitor kappa beta (Iκβ) subunit, responsible for maintaining NF-κB in an inactive state [22, 59, 60]. Once phosphorylated, the IKK complex phosphorylates the Iκβ subunit of NF-κβ, leading to its ubiquitination and subsequent degradation from the NF-κB complex by the 26S proteasome. Degradation of Iκβ permits the translocation of activated NF-κB to the nucleus (Figure 2.1) [59].

In the nucleus, NF-κB regulates nearly 200 target genes, many of which are implicated in mucosal injury and cell death [61]. Of particular importance are the upregulation of genes including interleukin-1 beta (*IL1B*) and tumour necrosis factor alpha (*TNF*) encoding the proinflammatory cytokines IL-1β and TNF-α, respectively [31]. Inactive precursor IL-1β is cleaved by caspase1/IL-1β-converting enzyme (ICE) to its active form and is released from peripheral blood mononuclear cells and tissue macrophages [62]. TNF-α is a pleiotropic cytokine produced by

tissue macrophages, natural killer cells and T lymphocytes [63, 64]. The production of IL-1β and TNF-α mediates an inflammatory response reducing epithelial oxygenation, initiating mesenchymal-epithelial signalling and stimulating further injury and death in cells of the epithelium and submucosa [2, 32, 52]. In addition, these cytokines can amplify primary damage by degrading Iκβ, further activating NF-κB and instigating its translocation to the nucleus [2, 32, 52].

These changes have been reported in both preclinical and clinical studies following 5-FU administration. For example, in Dark Agouti rats given 5-FU, TNF-α levels were highly elevated in the oral mucosa, jejunum and colon whilst IL-1β levels were highly elevated in the oral mucosa [5]. Additionally, TNF-α and IL-1β levels were associated with severe GI damage including reduced epithelial thickness, blunting and fusion of villi and obliteration of crypts [5]. Elevated TNF-α and IL-1β are also observed in the peripheral blood of patients whom experience GI toxicity following chemotherapy [2, 24].

To summarise, following damage instigated by 5-FU administration, the TIR domain innate immune signalling pathway is activated initiating a signalling cascade leading to the activation of proinflammatory cytokines such as IL-1β and TNF-α. These proinflammatory cytokines are consistently elevated in severely damaged mucosal tissue and associated with GI toxicity symptoms such as diarrhoea and mucositis.

2.6 Have any predictors for severe GI toxicity risk been identified?

2.6.1 Sex and age

Patient characteristics such as sex and age are important facets that must be considered when identifying patients at risk of severe GI toxicity [15]. However, evidence defining the relationship between sex, age and severe GI toxicity is highly contradictory, with sex and age more adequately serving as co-contributors rather than predictors for severe GI toxicity risk. In patients receiving 5-FU-based therapy for colorectal, gastric and upper GI tract cancers, women have been identified

at being of higher risk for developing mucositis (P = 0.04) [14]. No significant relationship was identified between patients of advanced age and GI toxicity risk in the cohort [14]. Likewise, in patients receiving 5-FU and leucovorin for colorectal cancer, females developed higher counts of severe diarrhoea (P < 0.01) and vomiting (P = 0.03) compared to their male counterparts [11]. Univariate analysis identified sex (P < 0.0001) as an independent predictor for severe GI toxicity in addition to advanced age (P = 0.001) [14], contradictory to the findings by Schwab *et al* [14]. In similar studies, no significant relationship between the development of GI toxicity and sex was identified in patients receiving 5-FU-based therapy [13], whilst advanced age was identified as protective against severe GI toxicity, with decreases in diarrhoea and nausea/vomiting episodes in advanced age patients (P = 0.01) [12]. Although sex and age cannot solely predict severe GI toxicity risk and the utility of these associations is not understood [15], it is known that certain SNPs, such as those found in dihydropyrimidine dehydrogenase (*DPYD*), are more commonly identified in one sex than the other [14] and, this may be true for other SNPs that are identified as predictors for severe GI toxicity risk. Therefore, factors such as sex and age are important co-contributors for severe GI toxicity risk prediction.

2.6.2 SNPs in the 5-FU rate determining enzyme

SNPs in *DPYD*, responsible for encoding the 5-FU rate determining enzyme DPD, have been thoroughly investigated for their role in the development of severe GI toxicity. During 5-FU-based therapies, 60 – 100 % of patients carrying one or multiple *DPYD* SNPs develop grade 3 to 4 toxicities (non-haematological and haematological) compared to 10 – 20 % of patients carrying no *DPYD* SNPs [14, 30, 65, 66]. In particular, two recent meta-analyses identified the presence of *DPYD* SNPs, IVS14+1 G > A, 2846 A > T, 1679 T > G, and 1236 G > A, increased the risk of grade 3 GI toxicities such as mucositis and diarrhoea (P ≤ 0.05) [16, 18]. In addition, a clinical study of patients with colorectal cancer receiving FOLFOX (fluorouracil and oxaliplatin) or FOLFIRI (fluorouracil and irinotecan), showed *DPYD* SNPs D949V and 2*A increased the risk of diarrhoea

(P = 0.003) and nausea/vomiting (P = 0.007), respectively [17]. However, all of these aforementioned SNPs occurred in less than 5 % of the populations studied and did not account for the majority of severe GI toxicity events [16-18]. Consequently, the clinical usefulness of routine genotyping in Caucasians for these *DPYD* SNPs prior to 5-FU treatment hasn't been established and current literature regarding the clinical sensitivity and specificity of diagnostic testing of the *DPYD* gene in this population is yet to be demonstrated [16]. This is further complicated by inconsistencies in the literature regarding the association of particular *DPYD* variants and GI toxicity between different ethnic populations [67, 68], such that there is the possibility that a rare variant in one ethnicity may be more frequent in another ethnicity and therefore, have a greater impact on GI toxicity prevalence. Nevertheless, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) warns about the increased risk of severe toxicity in patients carrying at least one decreased function *DPYD* allele on 5-FU and capecitabine drug labels [69-71] and, the Clinical Pharmacogenomics Implementation Consortium recommends altered 5-FU and capecitabine dosing based on *DPYD* genotype and the resulting phenotype [15]. Interestingly though, neither agency requires genetic testing for *DPYD* SNPs prior to treatment [17, 72]. As a result, new genetic predictors for severe GI toxicity risk need to be identified.

2.7 Candidate predictors for severe GI toxicity may lie within the TIR domain innate immune signalling pathway

2.7.1 SNPs in key TIR domain innate immune signalling pathway genes are uniquely positioned to influence gene functioning

The TIR domain innate immune signalling pathway is known to play a pivotal role in the development of GI toxicity, therefore, SNPs in key TIR domain innate immune signalling pathway genes may alter the pathophysiology and subsequent severity of GI toxicity. Hence, these mutations are candidate predictors for severe GI toxicity risk. A subset of mutations in TIR domain

innate immune signalling pathway genes *TLR2*, *TLR4*, *MYD88*, *IRAK1*, *IRAK4*, *TRAF6*, *NFKB*, *IL1B* and *TNF* are summarised in Table 2.2.

Table 2.2 Summary information about TIR domain innate immune signalling pathway SNPs [73][a].

ID	Type of variant	Base pair change	Amino acid change	MAF
TLR2				
rs11938228	intronic	C > A	-	CEU: 0.30, HCB: 0.35, JPT: 0.48, YRI: 0.10
rs1898830	intronic	A > G	-	CEU: 0.31, HCB: 0.36, JPT: 0.49, YRI: 0.08
rs3804099	cds-synon	T > C	-	CEU: 0.45, HCB: 0.37, JPT: 0.27, YRI: 0.63
rs3804100	cds-synon	T > C	-	CEU: 0.08, HCB: 0.33, JPT 0.22, YRI: 0.06
rs4696480	intronic	T > A	-	CEU: 0.00, HCB: 0.00, JPT 0.00, YRI: 0.00
rs5743708	missense	G > A	arginine > glutamine	CEU: 0.05, HCB:0.00, JPT:0.00, YRI:0.00
TLR4				
rs10759930	upstream	C > T	-	CEU: 0.37, HCB: 0.62, JPT: 0.65, YRI: 0.05
rs10759932	upstream	T > C	-	CEU: 0.14, HCB: 0.26, JPT: 0.25, YRI: 0.25
rs4986790	missense	A > G	aspartate > glycine	CEU: 0.04, HCB: 0.00, JPT: 0.00, YRI: 0.04
rs4986791	missense	C > T	threonine > isoleucine	CEU: 0.05, HCB: 0.01, JPT: 0.00, YRI: 0.00
rs5030710	cds-synon	T > C	-	CEU: 0.00, HCB: 0.00, JPT: 0.00, YRI: 0.18
rs7044464	upstream	T > A	-	CEU: 0.14, HCB: 0.07, JPT: 0.07, YRI: 0.29
rs7856729	upstream	G > T	-	CEU: 0.15, HCB: 0.05, JPT: 0.06, YRI: 0.41
MYD88				

rs6853	3'-UTR	A > G	-	CEU: 0.12, HCB: 0.03, JPT: 0.01, YRI: 0.33
rs7744	3'-UTR	A > G	-	CEU: 0.15, HCB: 0.41, JPT: 0.28, YRI: 0.01
IRAK1				
rs1059701	cds-synon	C > T	-	HCB: 0.14, JPT: 0.18, YRI: 0.15
rs1059702	missense	T > C	phenylalanine > serine	CEU: 0.79, HCB: 0.15, JPT: 0.24, YRI: 0.98
rs1059703	missense	C > T	serine > leucine	CEU: 0.77, HCB: 0.17, JPT: 0.20, YRI: 0.62
rs2239673	intronic	C > T	-	GMAF: 0.48
rs3027898	downstream	C > A	-	CEU: 0.74, HCB: 0.12, JPT: 0.22, YRI: 0.52
rs5945174	intronic	G > A	-	GMAF: 0.48
rs7061789	intronic	G > A	-	GMAF: 0.48
rs731642	intronic	G > A	-	GMAF: 0.44
IRAK4				
rs1141168	3'-UTR	A > G	-	CEU: 0.53, HCB: 0.47, JPT: 0.63
rs1461567	intronic	C > T	-	CEU: 0.03, HCB: 0.41, JPT: 0.54, YRI: 0.02
rs3794262	intronic	T > A	-	CEU: 0.93, HCB: 0.83, JPT: 0.91, YRI: 0.38
rs4251429	intronic	G > C	-	CEU: 0.02, HCB: 0.07, JPT: 0.06, YRI: 0.35
rs4251431	intronic	G > T	-	CEU: 0.05, HCB: 0.14, JPT: 0.14, YRI: 0.16
rs4251466	intronic	C > T	-	CEU: 0.08, HCB: 0.09, JPT: 0.06, YRI:0.22
rs4251513	intronic	C > G	-	CEU: 0.50, HCB: 0.42, JPT: 0.32, YRI: 0.05

rs4251532	intronic	C > T	-	CEU: 0.07, HCB: 0.16, JPT: 0.09, YRI: 0.62
rs4251545	missense	G > A	alanine > threonine	CEU: 0.08, HCB: 0.09, JPT: 0.06, YRI: 0.31
rs4251569	5'-UTR	C > T	-	CEU: 0.09, HCB: 0.17, JPT: 0.12, YRI: 0.00
TRAF6				
rs16928973	intronic	C > T	-	CEU: 0.19, HCB: 0.01, JPT: 0.12, YRI: 0.00
rs331449	intronic	T > C		CEU: 0.00, HCB: 0.00, JPT: 0.00, YRI: 0.20
rs3740961	3'-UTR	A > G	-	CEU: 0.11, HCB: 0.51, JPT: 0.55, YRI: 0.01
rs5030411	intronic	C > T	-	CEU: 0.60, HCB: 0.71, JPT: 0.72, YRI: 0.06
rs5030416	intronic	A > C	-	CEU: 0.17, HCB: 0.10, JPT: 0.14, YRI: 0.16
rs5030445	intronic	A > T		GMAF: 0.24
NFKB				
rs28362491	insertion/ deletion	- > ATTG	delete codon	GMAF: 0.42
IL1B				
rs1143623	upstream	C > G	-	CEU: 0.67, HCB: 0.61, JPT: 0.67, YRI: 0.94
rs1143627	promoter	T > C	-	CEU: 0.37, HCB: 0.47, JPT: 0.46, YRI: 0.64
rs1143634	cds-synon	T > C	-	CEU: 0.02, HCB: 0.01, JPT: 0.05, YRI: 0.09
rs16944	promoter	C > T	-	CEU: 0.35, HCB: 0.45, JPT: 0.47, YRI: 0.58
rs4848306	promoter	G > A	-	CEU: 0.46, HCB: 0.52, JPT: 0.46, YRI: 0.30
TNF				

rs1799964	downstream	T > C	-	CEU: 0.21, HCB: 0.23, JPT: 0.14, YRI: 0.12
rs1800629	promoter	G > A	-	CEU: 0.17, HCB: 0.03, JPT: 0.02, YRI: 0.09
rs1800750	promoter	G > A	-	CEU: 0.01, HCB:0.00, JPT: 0.00, YRI: 0.01
rs361525	promoter	G > A	-	GMAF: 0.06
rs4248158	downstream	C > T	-	GMAF: 0.02

[a] >: change; cds-synon: coding synonymous mutation; CEU: Caucasian population Utah, USA; GMAF: global minor allele frequency; HCB: Asian population Beijing, China; JPT: Asian population Tokyo, Japan; MAF: minor allele frequency; UTR: untranslated region; YRI: Sub-African population Yoruba, Nigeria

The *TLR2* and *TLR4* genes are located on chromosomes 4q31.3 and 9q33.1, respectively, with many *TLR* SNPs resulting in either synonymous or missense mutations [73]. Synonymous mutations do not alter the primary amino acid sequence. However, they may have indirect effects on gene functioning by influencing mRNA splicing and subsequent mRNA translation [74]. The *MYD88*, *IRAK1*, *IRAK4* and *TRAF6* genes are located on chromosomes 3q22.2, Xq28, 12q12, and 11p12, respectively [73]. Many of the *MYD88*, *IRAK1, IRAK4* and *TRAF6* mutations are located within the intronic regions of their respective genes. It is currently thought intronic mutations influence mRNA stability and translation, and may cause alternative splicing sites [21, 75, 76].

The *NFKB* gene is located on chromosome 4q24, with the *NFKB* mutation rs38362491 resulting in a premature stop codon [73]. Dependent on location in the gene, premature stop codons can lead to early termination of gene transcription and subsequent changes in protein function [77, 78]. The *IL1B* and *TNF* genes are located on chromosomes 2q14.1 and 6q21.33, respectively [73]. Key *IL1B* and *TNF* mutations lie within the promoter region therefore, are in prime location to alter transcription factor binding and subsequent gene transcription [73].

2.7.2 Evidence suggests TIR domain innate immune signalling pathway SNPs alter gene functioning and influence disease susceptibility

The *in vitro*, *ex vivo* and *in vivo* effects of key TIR domain innate immune signalling pathway SNPs as well as their association with disease susceptibility, is summarised in Table 2.3. It should be noted that for some SNPs, there is a lack of knowledge regarding functional impacts; a current limitation that warrants further investigation.

Table 2.3 *In vitro*, *ex vivo*, *in vivo* and genetic association studies investigating the effects of TIR domain innate immune signalling pathway SNPs on disease susceptibility, gene transcription, protein binding and cytokine secretion[b].

Gene	SNPs	Study details	Number of Participants	Impact of SNPs	Ref
TLR2	***Ex vivo***				
	rs4696480	Stimulated TNF-α, IL-6 and IL-10 secretion from isolated PMNs in patients with cirrhosis	n = 114	• ↓ stimulated TNF-α secretion in HE and HM vs WT (P < 0.01) • ↓ stimulated IL-6 secretion in HE and HM vs WT (P < 0.01) • ↑ stimulated IL-10 secretion in HE and HM vs WT (P < 0.01)	[79]
	In vivo				
	rs3804099 rs3804100	Association of SNPs with measles vaccine-induced immune responses	n = 745	Changes in antibody titer: • rs3804100 ↓ HE and HM vs WT (P = 0.002) Changes in cytokine secretion: • rs3804099 ↓ IFNλ-1 HE (P = 0.002) and HM (P = 0.009) vs WT	[80]
	rs4696480	Circulating LTA, LPS, TNF-α and IL-6 levels; stimulated secretion of TNF-α, IL-16 and IL-10 from cultured PMNs from patients with cirrhosis	n = 114	• ↓ circulating TNF-α in HM vs WT (P < 0.05) • ↓ circulating IL-6 in HE and HM vs WT (P < 0.05)	[79]

	Genetic association				
	rs3804100	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	n = 34	Predictive of severe CIGT incidence in conjunction with *TNF* rs1800629 and colorectal and gastric cancer types (P = 0.033, ROC AUC = 87.3 %)	[25]
	rs5743708	Association of SNPs with the severity and course of sepsis in critically ill patients	n = 145	• ↑ sepsis HE vs WT (P = 0.03) • ↑ number of infections HE vs WT (P = 0.012) • ↑ difficult-to-treat pathogens HE patients vs WT (P = 0.045)	[81]
	rs5743708	Association of SNPs with sepsis and pneumonia in patients with AML following induction chemotherapy	n = 155	↑ pneumonia HE vs WT (P = 0.006, OR: 10.78, 95 % CI = 2 - 23)	[82]
	rs11938228 rs1898830 rs3804099 rs3804100	Association of SNPs with HCC susceptibility	n = 443	rs3804099 and rs3804100: • in LD (R^2 > 0.9) • ↓ HCC rs3804099 HE vs WT (P < 0.001, OR = 0.49, 95 % CI = 0.3-0.7) • rs3804100 HE vs WT (P < 0.001, OR = 0.509, 95 % CI = 0.3 - 0.8) rs11938228 & rs1898830: • no significant associations	[83]

Gene	SNPs	Study details	Number of Participants	Impact of SNPs	Ref
TLR4	***In vitro***				
	rs4986790 rs4986791	FEV1 decline following inhalation of LPS	n = 83	• SNPs were in LD (data not provided) • Dose-response decline in FEV1: WT 1.86 % vs HE 0.59 % (P = 0.037)	[84]
	Ex vivo				
	rs4986790	Stimulated TNF-α, IL-6 and IL-10 secretion from isolated PMNs in patients with cirrhosis	n = 114	• ↓ stimulated IL-6 secretion in HE vs WT (P < 0.01) • ↑ stimulated IL-10 secretion in HE vs WT (P < 0.01)	[79]
	rs4986790 rs4986791	LPS-stimulated response in isolated airway epithelial cells	n = 83	• SNPs were in LD (data not provided) • ↓ IL-1α secretion by airway epithelial cells of HE vs WT patients (P < 0.01)	[84]
	In vivo				
	rs10759930 rs10759932 rs16906053 rs5030710 rs7044464	Association of SNPs with measles vaccine-induced immune responses	n = 745	Changes in antibody titer: rs5030710 and rs16906053 • ↑ HE (P = 0.001) and HM (P = 0.005) vs WT rs16906053 • ↓ IFNλ-1 HE (P = 0.002) and HM (P = 0.009) vs WT	[80]

	rs7856729			rs7044464 and rs7856729 • ↓ IL-10 HE and HM vs WT (P = 0.001) rs10759932 • ↑ IFN-α HE and HM vs WT (P = 0.001) rs10759930 • ↓ IFN-γ HE and HM vs WT (P = 0.001) • ↑ IFNλ-1 HE and HM vs WT (P = 0.006)	
	rs4986790	Circulating LTA, LPS, TNF-α and IL-6 levels; stimulated secretion of TNF-α, IL-16 and IL-10 from cultured PMNs from patients with cirrhosis	n = 114	• ↓ stimulated IL-6 secretion in HE vs WT (P < 0.01) • ↑ stimulated IL-10 secretion in HE vs WT (P < 0.01) • No significant differences in circulating TNF-α, IL-6 and IL-10 levels between WT, HE and HM (P > 0.05)	[79]
	rs4986790 rs4986791	LPS-stimulated response in THP-1 cells	-	↓ NF-κB activation by THP-1 cells with rs4986790 allele vs WT (P < 0.01)	[84]
	Genetic association				
	rs4986790	Association of SNPs with the severity and course of sepsis in critically ill patients	n = 145	• ↑ endocarditis HE vs WT (P < 0.05) • ↑ bloodstream infections HE vs WT (P < 0.05) • ↑ progression of sepsis to septic shock HE vs WT (P = 0.014)	[81]

	rs4986790 rs4986791	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	n = 34	No significant associations	[25]
	rs4986790 rs4986791	Association of SNPs with sepsis and pneumonia in patients with AML following induction chemotherapy	n = 155	• SNPs were in LD (data not provided) • ↑ sepsis development (P = 0.021, OR: 3.6, 95 % CI = 1.2 - 10.4) • ↑ pneumonia HE vs WT (P = 0.014, OR: 3.6, 95 % CI = 1.3 - 9.9)	[82]
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**
MYD88	***Ex vivo***				
	rs6853 rs7744	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	n = 90	No significant associations	[76]
	Genetic association				
	rs6853	Association of SNPs with severe CIGT incidence	n = 34	No significant associations	[25]

		following 5-FU-based treatment			
	rs6853 rs7744	Association of SNPs with T2DM and its vascular complications	n = 1106	rs6853: • ↑ T2DM HE and HM vs WT (P = 0.01, OR: 2.9, 95 % CI = 1.3 - 6.7)	[85]
	rs6853 rs7744	Association of SNPs with sepsis-induced ALI susceptibility	n = 548	No significant associations	[76]
	rs6853 rs7744	Association of SNPs with SIPD risk and 2^{o} SIPD symptoms	n = 200	rs6853 genotype analysis: • ↑ SIPD WT vs HE and HM (P < 0.0001, OR = 2.1, 95 % CI = 1.8 - 2.5) • ↑ death from SIPD HE and HM vs WT (P = 0.005, OR = 16.1, 95 % CI = 3.3 - 77.6) rs6853 allele analysis: • ↑ SIPD in patients carrying variant allele (P < 0.0001, OR = 1.9, 95 % CI = 1.5-2.6) • ↑ association of death in patients carrying variant allele (P = 0.0064, OR = 8.4, 95 % CI = 2.5-28.5) rs7744 genotype and allele analysis:	[86]

				• No significant associations	
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**
IRAK1	***Ex vivo***				
	rs1059703	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	n = 90	No significant associations	[76]
	Haplotype: rs1059701 rs1059702 rs1059703 rs2239673 rs3027898 rs5945174 rs7061789 rs731642	Association of IRAK-1 haplotype with stimulated NF-κB activation from peripheral blood neutrophils	n = 30	↑ NF-κB nuclear accumulation post-LPS in HE and HM vs WT (P = 0.001)	[87]
	Genetic association				

	rs1059703	Association of SNPs with sepsis-induced ALI susceptibility	n = 548	No significant associations	[76]
	rs1059702 rs1059703	Association of SNPs with SIPD risk and 2º SIPD symptoms	n = 200	rs1059701: • ↑ SIPD WT vs HE and HM (P = 0.0067, OR = 1.4, 95 % CI = 1.1 - 1.8) rs1059702: • ↑ leucocytosis HE and HM vs WT (P = 0.046, OR = 7.5, 95 % CI = 1.9 - 30.2)	[86]
	Haplotype: rs1059701 rs1059702 rs1059703 rs2239673 rs3027898 rs5945174 rs7061789 rs731642	Association of IRAK-1 haplotype with 2º symptoms in patients with sepsis	n = 30	• ↑ likelihood of developing septic shock in HE and HM vs WT (P = 0.047) • 2.6 ↑ likelihood of death from sepsis in HE and HM vs WT (P = 0.05)	[87]
	Meta-analysis				

	rs1059702 rs1079703 rs3027898	Susceptibility of ADs in patients with IRAK1 SNPs	-	rs1059702: • ↑ ADs WT vs HE, HM (P = 0.000, OR = 0.8, 95 % CI = 0.7 - 0.8) • ↑ SLE WT vs HE (P = 0.000, OR = 0.7, 95 % CI = 0.6 - 0.7) • ↑ SSc WT vs HE and HM (P = 0.032, OR = 0.8, 95 % CI = 0.6 - 0.9) rs1079703: • ↑ SLE variant vs WT (P = 0.000, OR = 1.5, 95 % CI = 1.3 - 1.6) rs3027898: • ↑ ADs WT vs HE, HM (P = 0.034, OR = 0.7, 95 % CI = 0.6 - 0.9) • ↑ SLE WT vs HE (P = 0.001, OR = 0.8, 95 % CI = 0.6 - 0.9) • ↑ RA WT vs HE (P = 0.021, OR = 0.8, 95 % CI = 0.7 - 0.9)	[88]
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**
IRAK4	***Ex vivo***				
	rs1461567 rs3794262 rs4251429 rs4251431 rs4251466 rs4251513 rs4251545	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	n = 90	No significant associations	[76]

	rs4251569				
	Genetic association				
	rs1141168 rs1461567 rs4251513	Association of SNPs with SIPD risk and 2º SIPD symptoms	n = 200	rs4251513 genotype analysis: • ↑ SIPD WT vs HE and HM (P < 0.0001, OR = 2.2, 95 % CI = 1.6 - 3.0) • ↑ SIPD sequelae HE and HM vs WT (P = 0.001, OR = 7.1, 95 % CI = 2.6 - 18.9) rs4251513 allele analysis: • ↑ presence of SIPD in patients carrying variant allele (P < 0.0001, OR = 1.5, 95 % CI = 1.4 - 1.5) rs1461567: • ↑ SIPD variant allele vs WT (P = 0.016, OR = 1.5, 95 % CI = 1.1 - 1.9) rs1141168: • no significant associations	[86]
	rs1461567 rs4251513 rs4251532 rs4251569	Association of SNPs with T2DM and its vascular complications	n = 1106	1.66 ↑ T2DM HE vs WT and HM (P = 0.03, OR = 1.7, 95 % CI = 1.1 - 2.6)	[85]

	rs1461567 rs3794262 rs4251429 rs4251431 rs4251466 rs4251513 rs4251545 rs4251569	Association of SNPs with sepsis-induced ALI susceptibility	n = 548	No significant associations	[76]
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**
TRAF6	***Ex vivo***				
	rs4755453 rs5030493 rs540386	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	n = 90	rs4755453: • ↑ TRAF6 baseline mRNA expression WT vs HE (P = 0.012) and vs HM (P = 0.003) • ↑ TRAF6 post-LPS mRNA expression WT vs HE (P = 0.009) and vs HM (P = 0.005) • ↑ TNF-α post-LPS secretion WT vs HE and HM (P = 0.015) • ↑ IL-6 post-LPS secretion WT vs HE and HM (P = 0.009) rs5030493 & rs540386:	[76]

				• no significant associations	
	In vivo				
	rs331449	Association of SNPs with measles vaccine-induced immune responses	n = 745	↑ HE and HM vs WT (P = 0.007)	[80]
	Genetic association				
	rs16928973 rs5030445	Association of SNPs with T2DM and its vascular complications	n = 1106	No significant associations	[85]
	rs4755453 rs5030493 rs540386	Association of SNPs with sepsis-induced ALI susceptibility	n = 548	↓ % variant allele in sepsis-induced ALI vs sepsis alone groups (P = 0.003 ,OR: 0.5, 95 % CI = 0.4 - 0.7)	[76]
	rs3740961 rs5030411 rs5030416 rs5030445	Association of SNPs with susceptibility and severity of sepsis	n = 510	No significant associations	[89]
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**
NFKB	***In vitro***				

	rs28362491	Transient transfection luciferase reporter gene assay; and EMSA to determine protein binding in HeLA and HT-29 cells	-	• ↓ activity at baseline with variant vs WT allele in HeLA cells (P = 0.05) • ↓ activity following stimulation with variant vs WT allele in HeLA cells (P = 0.02) • ↓ activity following stimulation with variant vs WT allele in HT-29 cells (P = 0.02)	[78]
	Genetic association				
	rs28362491	Association of SNP with IBD risk	n = 822	No significant associations	[90]
	rs28362491	Association of SNPs with pathological response in patients with rectal cancer treated with PCRT	n = 159	↑ pathological response HE and HM vs WT (P = 0.03, OR = 6.4, 95 % CI = 0.8 - 52.7)	[91]
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**
IL1B	***In vitro***				
	rs1143634	Association of SNP with IL-1β serum concentration following infliximab treatment	n = 47	↑ IL-1β with variant vs WT allele (P = 0.026)	[92]

	rs1143623 rs1143627 rs16944 rs4848306	Transient transfection reporter gene assay and gel mobility shift assay in THP-1 cells	-	rs4848306: • ↓ nuclear protein binding with variant vs WT allele (P < 0.01) rs1143623: • ↑ nuclear protein binding with variant vs WT allele (P < 0.01) • ↓ transcriptional activity variant vs WT allele (P < 0.01) rs1143627: • ↑ nuclear protein binding of complex 1 with variant vs WT allele (P < 0.01) • ↓ binding of complex 2 and 3 with variant vs WT allele (P < 0.01) • ↓ transcriptional activity with variant vs WT allele (P < 0.01) rs16944: • ↑ transcriptional activity with variant vs WT allele (P < 0.05)	[93]
	Ex vivo				
	rs1143627 rs1143634 rs16944	Stimulation of isolated human monocytes and EMSA of nuclear extracts	n = 442	rs1143627: • rs1143627 and rs16944 in LD (no data given) • 5-fold ↑ DNA binding post-LPS stimulation in HE vs WT	[94]
	Genetic association				

	rs16944	Association of SNPs with toxicity in patients undergoing 5-FU and cisplatin chemotherapy	n = 100	• ↑ thrombocytopenia HE and HM vs WT (P = 0.015, OR = 2.9, 95 % CI = 1.2-7.0) • Predictive of stomatitis (P < 0.01) and thrombocytopenia (P = 0.02) in conjunction with *TNF* rs1799964	[95]
	rs1143627 rs16944	Association of SNPs with pathological response in patients with rectal cancer treated with PCRT	n = 159	No significant associations	[91]
	rs1143627 rs1143634 rs16944	Association of SNPs with risk of gastric cancer	n = 442	rs1143627: • rs1143627 and rs16944 in LD (0.99) • HE associated with gastric cancer risk (OR = 1.9, 95 % CI = 1.5 - 2.6) rs16944: • No significant association	[94]
	rs1143627 rs1143634 rs16944	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	n = 34	No significant associations	[25]
Gene	**SNPs**	**Study details**	**Number of Participants**	**Impact of SNPs**	**Ref**

TNF	***In vitro***				
	rs1800629	Functional analysis of SNP using CAT reporter gene in Jurkat and Raji cells	-	No significant associations	[96]
	rs1800629	Transient transfection luciferase reporter gene assay in Jurkat and U937 cells	-	2-fold ↑ transcription with variant vs WT allele in both Jurkat and U937 cells ($P < 0.05$)	[97]
	Ex vivo				
	rs1800629 rs1800750 rs361525	Association of SNPs with endotoxin-induced TNF-α secretion from PBMCs	n = 129	No significant differences	[98]
	rs1800629 rs1800750 rs361525	Stimulated TNF-α secretion from whole blood	n = 179	No significant associations	[99]
	rs1800629 rs1800750 rs361525 rs4248158	Association of SNPs with TNF-α serum concentration following infliximab treatment	n = 47	No significant associations	[92]
	Genetic association				

	rs1800629	Association of SNP with clinical outcome, incidence and severity of toxic complications and GvHD in HSCT patients	n = 70	↑ severe toxicity HE vs WT (P = 0.014, OR = 17.2, 95 % CI = 1.8 - 168.1)	[77]
	rs1800629	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	n = 34	*TLR2* and *TNF* SNPs were predictive of severe CIGT incidence in conjunction with colorectal and gastric cancer types (P = 0.033, ROC AUC = 87.3 %)	[25]
	rs1799964	Association of SNPs with toxicity in patients undergoing 5-FU and cisplatin chemotherapy	n = 100	• ↑ stomatitis HE and HM vs WT (P = 0.02, OR = 3.1, 95 % CI = 1.2 - 8.3) • Predictive of stomatitis (P < 0.01) and thrombocytopenia (P = 0.02) in conjunction with *IL1B* 16944	[95]
	rs1800629 rs1800750 rs361525	Association of SNPs with MS	n = 179	No significant associations	[99]

[b] ↑: increase; ↓: decrease; 2º: secondary; AD: autoimmune disease; ALI: acute lung injury; AML: acute myeloid leukaemia; CAT: chloramphenicol acetyltransferase; EMSA: electromobility shift assay; GvHD: graft vs. host disease; HCC: hepatocellular carcinoma; HE; heterozygous; HM: homozygous; HSCT: haemtopoietic stem cells; IBD: irritable bowel syndrome; LD: linkage disequilibrium; LPS: lipopolysaccharide; LTA: lipoteichoic acid; MS: multiple sclerosis; OR: odds ratio; PCRT: primary chemoradiation therapy; PBMCs: peripheral blood mononuclear cells; PMNs: peripheral polymorphonuclear cells; RA: rheumatoid arthritis; ROC AUC: receiver operator characteristic, area under the curve; SLE: systemic lupus erythaemtosus; SSC: systemic sclerosis; SIPD: invasive pneumococcal disease; T2DM: type 2 diabetes; WT: wild-type.

A pilot study conducted by Coller *et al.* was the first to investigate the association between innate immune receptor genetic variability and severe GI toxicity risk following 5-FU-based therapy [25]. *TLR2* rs384100 and *TNF* rs1800629 SNPs (in conjunction with colorectal and gastric cancer types) were identified to be predictive of severe GI toxicity risk [25]. However, no relationship was identified with *TLR4*, *MYD88* and *IL1B* SNPs [25].

Other studies have focussed on association of SNPs with disease susceptibility and impact of the SNPs on protein expression. Patients with *TLR2* and *TLR4* SNPs were identified to have a higher risk of developing bloodstream infections and sepsis, and higher circulating levels of PAMPs such as lipoteichoic acid (as described in Table 2.3) [79, 81]. On a molecular level, *TLR2* and *TLR4* SNPs have been linked to altered interferon gamma secretion, altered specific antibody responses and reduced NF-κB circulating serum levels and activation (as described in Table 2.3) [79, 80, 84]. No relationship was identified between *MYD88* SNPs and TNF-α and IL-6 concentrations post-stimulation of *ex vivo* PBMCs and the effect of *MYD88* SNPs on MyD88 protein expression is variable (as described in Table 2.3) [76, 100]. *IRAK1* and *IRAK4* SNPs have been associated with an increased risk of autoimmune disease, with *IRAK1* SNPs also identified to increase NF-κB activation (as described in Table 2.3) [87, 88]. *TRAF6* SNPs were found to increase TNF-α and IL-6 secretion at baseline and post-stimulation of *ex vivo* PBMCs, but no association was identified between *TRAF6* SNPs with sepsis susceptibility and severity (as described in Table 2.3) [76, 89]. *NFKB* SNP rs28362491 was associated with severe toxic complications in haematopoietic stem cell transplantation (HSCT) patients and decreased NF-κB activity (as described in Table 2.3) [77, 78].

2.7.3 IL1B and TNF SNPs show the greatest potential as predictors for severe GI toxicity risk

Of the TIR domain innate immune signalling pathway SNPs discussed in Table 2.3, *IL1B* and *TNF* SNPs show the greatest potential as predictors for severe GI toxicity as they have been identified

to not only alter gene function but also increase cancer risk and predict toxic side effects following chemotherapy. The *IL1B* SNP rs16944 was shown to increase transcriptional activity of IL-1β and was identified as being predictive of stomatitis (in conjunction with *TNF* 1799964) in patients receiving 5-FU and cisplatin chemotherapy treatment [93, 95] (Table 2.3). *IL1B* rs1143634 was identified to increase the risk of gastric cancer, with an increase in nuclear protein binding identified in reporter gene assays [93, 94] (Table 2.3). This, in addition to further evidence presented in Table 2.3, demonstrates *IL1B* SNPs are candidate predictors for severe GI toxicity risk. However, using individual *IL1B* SNPs as predictors for severe GI toxicity risk may be complicated, as many SNPs in *IL1B* are in linkage disequilibrium therefore, SNPs with opposing effects may 'cancel' one another out [94]. With regards to *TNF* SNPs, rs1800629 influences gene functioning, with a 2-fold increase in transcriptional activity identified in patients carrying the variant [97]. In addition, a small pilot study of patients receiving 5-FU-based therapy, *TNF* rs1800629 was found to be predictive of severe GI toxicity risk in a multivariate logistic regression model [25].

2.8 Conclusion

Severe GI toxicity is a debilitating side effect following 5-FU-based therapy. It is essential predictors for severe GI toxicity risk are identified to allow patients at most risk of severe GI toxicity to be identified prior to treatment, allowing the personalisation of supportive care measures to reduce the risk of developing severe GI toxicity. This would not only improve clinical outcomes and long-term prognosis, but also improve patient quality of life whilst on otherwise life-saving chemotherapy treatment. This critical review has provided evidence to suggest TIR domain innate immune signalling pathway SNPs are suitable candidate predictors for severe GI toxicity risk following 5-FU-based therapy.

However, further investigation is required to thoroughly understand the effect of these TIR domain innate immune signalling pathway SNPs on the mechanisms underlying the development of GI

toxicity. To allow the 'bench to beside' translation of TIR domain innate immune signalling pathway SNPs as clinical predictors for severe GI toxicity risk, it is critical to not only associate, but identify mechanisms by which these SNPs influence the development of severe GI toxicity in addition to understanding the exact functional impact of the SNPs themselves.

Chapter 3: The Salivary Predictors in Treatment Validation (SPiT-V) Study

Chapter 3 is my first research chapter and is presented as a traditional thesis chapter. This clinical study was named the Salivary Predictors in Treatment Validation (SPiT-V) Study and was undertaken over a four year period. The SPiT-V study built on previous research conducted in my laboratory.

3.1 Introduction

3.1.1 5-fluorouracil induces gastrointestinal toxicity

5-fluorouracil (5-FU) and its prodrug, capecitabine, are highly prescribed antimetabolite chemotherapy agents used for the treatment of solid tumours of the breast, colon and upper gastrointestinal (GI) tract, among others [1]. Despite being highly effective, due to their non-selective nature, 5-FU and capecitabine may lead to severe damage to the mucosal membranes of the GI tract [2]. This damage may in turn lead to the manifestation of GI toxicities including, but not limited to, mucositis, diarrhoea, nausea and vomiting [4]. Of patients receiving 5-FU-based therapy, 20 – 50 % will experience GI toxicity [3]. These patients will be administered supportive care measures, such as antiemetics and antidiarrhoeals, to help manage and relieve symptoms [8, 9]. However, a subset of these patients will develop severe GI toxicity graded as ≥ 3 on the National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI CTCAE v 4.03 and v 5.0) [10]. Patients who require dose reductions, treatment delays, early treatment cessation and/or hospitalisation to relieve and manage GI toxicity symptoms are also reported as developing severe GI toxicity [2]. Current supportive care measures and practices are ineffective at managing and relieving severe GI toxicity symptoms [2], with as many as 14 – 25 % of patients receiving 5-FU-based therapy requiring a dose delay or reduction as a direct result of severe GI toxicity symptoms [14, 101, 102]. The incidence of all severe GI toxicity-related hospitalisations is

as high as 30 % in some instances [14, 101, 102]. In addition, secondary symptoms including, but not limited to, dehydration and malnutrition develop, requiring intravenous fluids and parenteral nutrition [2]. Treatment interruptions negatively influence prognosis and potentially impact long-term survival, leading to poorer clinical outcomes. The presence of any grade of GI toxicity can also decrease patient quality of life and increase health care costs [6, 7]. There is currently no effective approach to identify which patients will develop severe GI toxicity prior to receiving 5-FU-based therapy.

3.1.2 Current predictive markers for severe GI toxicity lack clinical sensitivity and specificity

There is a lack of clinically sensitive and specific predictive markers available to predict the risk of severe GI toxicity following 5-FU-based therapy. Traditional variables such as sex, age and 5-FU dosage are unreliable, with many contradictory and inconsistent findings surrounding their use as predictive markers for severe GI toxicity risk in patients following 5-FU-based therapy [11-14]. Single nucleotide polymorphisms (SNPs) in the dihydropyrimidine dehydrogenase (*DPYD*) gene, responsible for encoding the rate determining 5-FU metabolic enzyme DPD, are also inadequate predictive markers for severe GI toxicity risk [14, 30, 65, 66]. Although the US Food and Drug Administration (FDA) warn of the increased likelihood of adverse effects for patients carrying one or multiple *DPYD* SNPs [71], these SNPs occur in < 5 % of the Caucasian population [70, 71, 73]. Therefore, they are not adequate, sensitive or specific predictive markers for severe GI toxicity risk.

3.1.3 The Salivary Predictors in Treatment (SPiT) pilot study identified new potential predictive markers for severe GI toxicity risk

In 2014, my laboratory conducted a pilot study investigating the association between SNPs in key mediating genes of the Toll-like receptor/interleukin-1 (TIR) domain innate immune signalling pathway with the risk of severe GI toxicity following 5-FU-based therapy [25]. The TIR domain pathway is a key mediating pathway in the development of GI toxicity [2, 22]. Its activation following

5-FU-induced mucosal injury mediates the transcription and secretion of proinflammatory cytokines tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6 through TLR signalling [2, 22]. These proinflammatory cytokines further facilitate mucosal damage and subsequent GI toxicity development and have been consistently associated with the severity of GI toxicity in preclinical and clinical studies [5, 23, 24, 53]. Briefly, this small pilot study recruited 34 participants (10 with severe GI toxicity graded > 3 on the NCI CTCAE v 4.03 [10] or received a dose reduction, dose delay, ceased 5-FU-based therapy early or were hospitalised as a result of GI toxicity) from the Flinders Medical Centre in South Australia, Australia. A general linear model of multivariate logistic regression created a risk prediction model identified *TLR2* rs384100 and *TNF* rs1800629 SNPs, in conjunction with colorectal and upper GI cancer types, to be predictive for severe GI toxicity risk, with a receiver operator characteristic area under the curve (ROC AUC) of 87 % [25]. When colorectal and upper GI cancer types were removed from the risk prediction model, the predictive power of the model decreased, with a ROC AUC of 77 % [25]. This demonstrated the inclusion of cancer type gave a more informative, sensitive and specific risk prediction model for GI toxicity compared to using SNPs alone. This was one of the first studies associating genetic variability within innate immune signalling genes with the risk of severe GI toxicity [25]. These results suggest SNPs within the TIR domain innate immune signalling pathway may be potential predictive markers for severe GI toxicity risk. However, like any pilot study, the results need to be validated in a new, larger and independent cohort of participants.

3.2 Hypothesis and aims

The overall objective of this study was to validate the results of the pilot study in a larger independent cohort of participants.

This study hypothesised:

1. A general linear model of multivariate logistic regression would generate a risk prediction model with similar sensitivity and specificity to the pilot study risk prediction model, identifying *TLR2* rs384100 and *TNF* rs1800629 SNPs, in conjunction with colorectal and upper GI cancer types, to be predictive for severe GI toxicity risk.

Aims

1. To identify which participant clinical data (5-FU-based regimen, type of cancer, sex, age and number of treatment cycles) were significantly associated with severe GI toxicity using logistic regression ($P < 0.05$).

2. To identify which SNPs in TIR domain pathway genes *TLR2*, *TLR4*, *IL1B*, *IL2, IL6*, *IL6R*, *IL10*, *TGFB*, *TNF*, *MYD88*, *MD2, CASP1* and *CASP5* as well as *OPRM1* and *CRP* were significantly associated with severe GI toxicity using logistic regression ($P < 0.05$).

3. To determine the combined impact of clinical and genetic factors on severe GI toxicity using models of multivariate logistic regression.

A target sample size of 150 participants (45 who reported severe GI toxicity) was determined as providing 99 % power at alpha = 0.05 to predict severe GI toxicity risk. Participants for this study were recruited from the Flinders Medical Centre (FMC) in addition to a new recruitment site, the Royal Adelaide Hospital (RAH), both located in Adelaide, South Australia, Australia.

3.3 Materials and Methods

3.3.1 Reagents

Ethanol (100 %) was obtained from Chem-Supply Pty Ltd (SA, Australia). Ethanol (75 %) was achieved by diluting ethanol (100 %) with Milli-Q® obtained from Merck (Darmstadt, Germany).

Sodium acetate was obtained from Merck (Darmstadt, Germany) and diluted to 3 M with Milli-Q®. Chloroform (stabilised with ethanol, Multisolvent® HPLC grade) was obtained from Scharlab (Barcelona, Spain).

3.3.2 Ethics approval

This study was approved by both the Southern Adelaide Clinical Human Research Ethics Committee (HREC/12/SAC/519) and the Royal Adelaide Hospital Human Ethics Committee (SSA/14/RAH/519). All participants provided informed written consent prior to participating in accordance with the Declaration of Helsinki.

3.3.3 Participant recruitment

This was a retrospective cohort study. 2185 potential participants who received 5-FU-based or capecitabine chemotherapy regimens between January 2012 and January 2018 were identified from pharmacy dispensary records obtained from FMC and RAH (Figure 3.1). 243 potential participants were mailed a letter of invitation to participate in the study. 170 responded to the invitation and were subsequently telephone screened to ensure they met eligibility criteria and fully understood the study. Exclusion criteria included; not fluent in the spoken English language, having a pre-existing medical condition associated with GI damage (i.e. Crohn's Disease or Ulcerative Colitis), still receiving chemotherapy treatment, unwilling to consent to providing a saliva sample or having their clinical records reviewed. Following this check, 168 eligible participants were enrolled in the study and mailed a saliva collection kit (DNA Genotek Inc., Ontario, Canada) and study consent forms. 155 participants collected their own saliva and returned the sample and consent forms in a reply-paid envelope. Following receipt of the saliva sample, participants' clinical medical records were reviewed to obtain clinical demographics, participant comorbidities, treatment and toxicity data. Participants were further screened for exclusion from the study if they were not Caucasian, had received concurrent radiation therapy or received a blood transfusion

throughout their 5-FU-based therapy. However, none were excluded on these criteria.

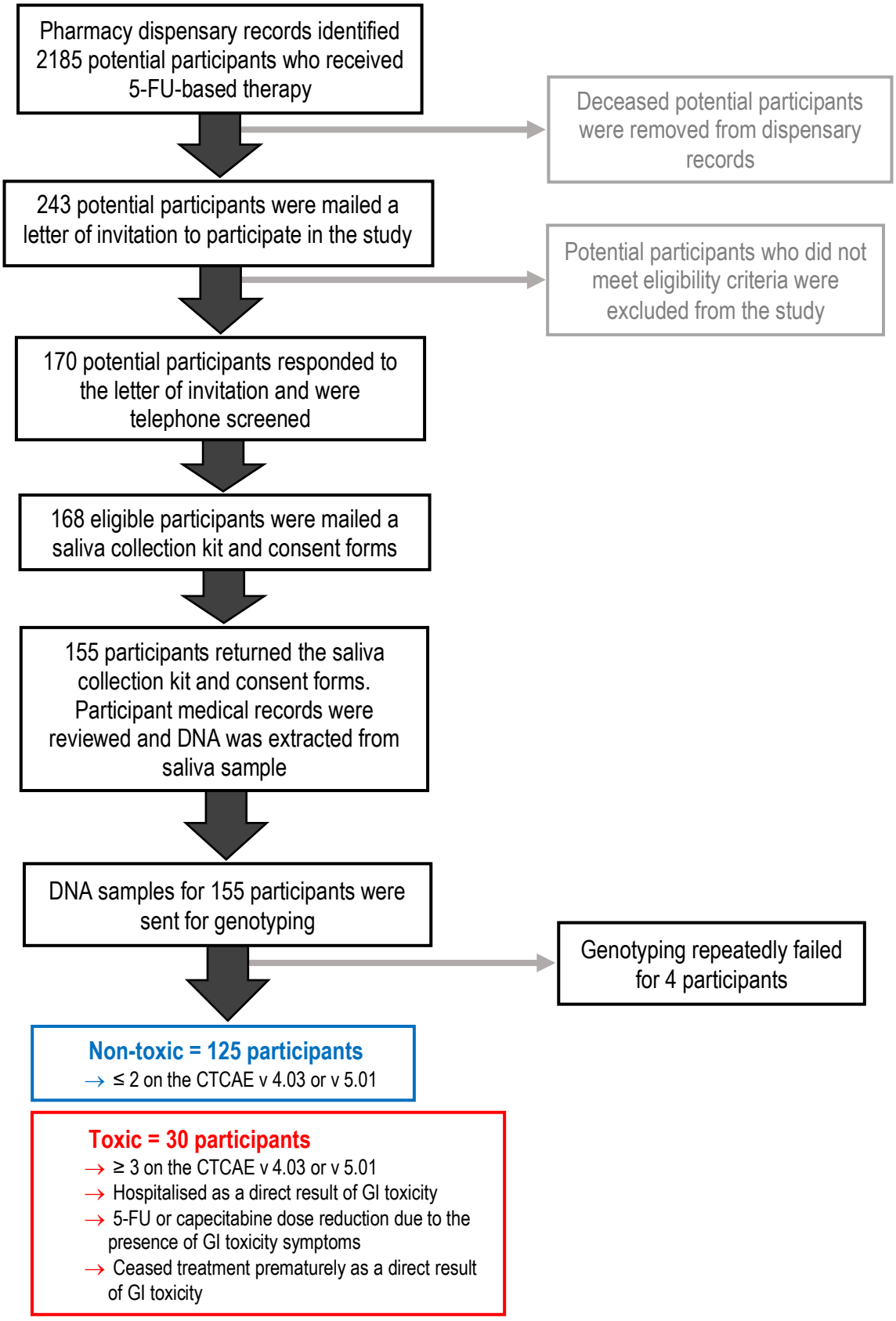

Figure 3.1 Flow diagram of the recruitment process. Grey text indicates the exclusion of participants.

3.3.4 GI toxicity classification

Clinical oncology staff at the FMC and RAH used the NCI CTCAE v 4.03 or v 5.0 to report and grade adverse events experienced by participants during chemotherapy treatment; this is recorded in participants' clinical medical records (Table 3.1) [10]. Participants were classified into two GI toxicity groups for analysis. Participants classified as 'non-toxic' reported no GI toxicity (grade 0) or, mild to moderate GI toxicity (diarrhoea, mucositis, nausea and/or vomiting graded as 1 or 2 on the CTCAE v 4.03 or 5.0) (Table 3.1) [10]. Participants classified as 'toxic' reported severe GI toxicity (diarrhoea, mucositis, nausea and/or vomiting graded as ≥ 3 on the CTCAE v 4.03 or v 5.0) or, were hospitalised, received a 5-FU or capecitabine dose reduction or, ceased treatment prematurely as a direct result of GI toxicity. This classification of GI toxicity has previously been used in clinical studies conducted in my laboratory [25, 103].

Table 3.1 NCI CTCAE v 4.03 and v 5.0 classification of GI toxicity during chemotherapy treatment [10]. Blue highlighted boxes indicate mild to moderate GI toxicity symptoms whereas red highlighted boxes indicate severe GI toxicity symptoms.

Gastrointestinal Disorders						
Adverse Event	**Grade 0**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Diarrhoea	No symptoms	Increase of < 4 stools per day over baseline. Mild increase in ostomy output compared to baseline.	Increase of 4-6 stools per day over baseline. Moderate increase in ostomy output compared to baseline.	Increase of ≥ 7 stools per day over baseline. Incontinence, hospitalisation indicated, severe increase in ostomy output compared to baseline.	Life-threatening consequences. Urgent intervention indicated.	Death
Vomiting		1-2 episodes (separated by 5 min) in 24 h.	3-5 episodes (separated by 5 min) in 24 h.	≥ 6 episodes (separated by 5 min) in 24 h. Tube feeding, hospitalisation.	Life-threatening consequences: Urgent intervention indicated.	Death
Nausea		Loss of appetite without alteration in eating habits.	Oral intake decreased without significant weight loss, dehydration or malnutrition.	Inadequate oral caloric or fluid intake, tube feeding or hospitalisation indicated.		
Oral Mucositis (Ulceration of the oral mucosa)		Asymptomatic or mild symptoms. Intervention not indicated.	Moderate pain. Not interfering with oral intake, modified diet indicated.	Severe pain. Interfering with oral intake. Hospitalisation indicated.	Life-threatening consequences. Urgent intervention indicated.	Death

3.3.5 DNA extraction from participant saliva samples

Genomic DNA was isolated and purified from a 500 μL sample of participants' saliva using the prepIT-L2P DNA extraction kit according to manufacturer's instructions (DNA Genotek Inc., ON Canada). Prior to extraction, saliva was incubated at 72°C for at least 2 h. Following incubation, 500 μL of saliva was added to 25 μL of PT-L2P, mixed by vortex and incubated on ice for 10 min. Following incubation, the sample was centrifuged at room temperature for 15 min at 15,000 x g. Supernatant was collected in a new microcentrifuge tube and 600 μL of 100 % ethanol was added. The sample was mixed by inversion 10 times and left to stand at room temperature for 10 min to allow DNA precipitation. The sample was centrifuged at room temperature for 2 min at 15,000 x g. Supernatant was carefully removed, leaving the DNA pellet, and a further 250 μL of 75 % ethanol was added. The sample was left to stand at room temperature for 1 min prior to further centrifugation at room temperature for 2 min at 15,000 x g. Supernatant was carefully removed and the DNA pellet was left overnight at room temperature to allow the evaporation of any residual ethanol. The DNA pellet was resuspended in 100 μL nuclease free water and DNA concentration and purity were quantified by spectrophotometry using the Synergy™ Mx Microplate Reader (BioTek Instruments, VT USA).

3.3.6 Genotyping

Following spectrophotometry, DNA was diluted to a concentration of 10 ng.μL^{-1} and sent to the Australian Genome Research Facility (AGRF) for genetic analysis. Genotyping for 21 SNPs was conducted by a previously established [25] customised Sequenom MassArray (iPLEX GOLD) assay. 19 SNPs in genes within TIR domain innate immune signalling pathway as well as, 2 SNPs in genes encoding the mu opioid receptor (*OPRM1*) and C-reactive protein (*CRP*) were also analysed (Table 3.2).

Table 3.2 SNPs analysed in the Sequenom MassArray assay [25, 73][a].

Gene	rs ID	Type of variant	Base pair change	MAF
IL1B	rs16944	Upstream variant	A > G	0.65
	rs1143627	5'-UTR	G > A	0.64
	rs1143634	Synonymous	G > A	0.24
IL2	rs2069762	Upstream variant	A > C	0.29
IL6	rs10499563	Not reported	T > C	0.22
IL10	rs1800871	Upstream variant	A > G	0.76
	rs1800896	Upstream variant	T > C	0.45
IL6R	rs8192284	Missense	A > C	0.36
TGFB	rs1800469	Upstream variant	A > G	0.68
	rs11466314	Upstream variant	C > T	0.00
TNF	rs1800629	Upstream variant	G > A	0.13
TLR2	rs3804100	Synonymous	T > C	0.06
TLR4	rs4986790	Missense	A > G	0.05
	rs4986791	Missense	C > T	0.05
MYD88	rs6853	3'-UTR	A > G	0.13
MD2	rs11466004	Missense	C > G	0.02
CASP1	rs580253	Synonymous	G > A	0.17
CASP5	rs554344	Upstream variant	G > C	0.17
OPRM1	rs1799971	Missense	A > G	0.16
CRP	rs2794521	Upstream Variant	C > T	0.71
BDNF	rs6265	Missense	C > T	0.19

[a] >: change; 3'-UTR: 3' untranslated region; 5'-UTR: 5' untranslated region; MAF: MAF sourced from 1000 genomes European Caucasian population as reported on dbSNP [73].

3.3.7 Chloroform clean-up of DNA that failed genotyping

For DNA samples that failed genotyping due to low DNA quality (n = 20), DNA was re-extracted from participant saliva samples and chloroform cleaned to improve DNA quantity and quality. Following DNA extraction (as previously described), 5 μL of chloroform was added to the resuspended DNA pellet, vortexed for 30 sec and centrifuged at room temperature for 15 min at 15,000 x g. The aqueous layer was carefully transferred to a new microcentrifuge tube and, 2.1 times the volume of 20:1 100 % ethanol: 3 M sodium acetate solution was added. The microcentrifuge tube was inverted 3 times, vortexed for 10 sec and centrifuged for 5 min at 15,000 x g. Supernatant was removed without disturbing the DNA pellet and 250 μL of 70 % ethanol was added. The sample was vortexed at 2/3 power for 30 sec and centrifuged for a further 5 min at 15,000 x g. Supernatant was carefully removed and the DNA pellet was left overnight to allow the evaporation of any residual ethanol. The DNA pellet was resuspended in 100 μL nuclease free water and DNA concentration and purity were quantified by spectrophotometry using the Synergy Mx Microplate Reader. DNA was diluted to a concentration of 10 ng.μL^{-1} and sent to the AGRF for repeat genetic analysis.

3.4 Statistical analysis

3.4.1 Participant demographics and clinical data

Chi-square or Fisher's exact tests were used to examine differences in sex, type of cancer and 5-FU-based regimen between participants classified as non-toxic or toxic within the pilot [25], interim and final participant cohorts. In addition, Mann-Whitney U tests examined differences in age and number of treatment cycles between the two participant groups for each cohort. Additionally, Chi-square and Mann-Whitney U tests also determined differences in the non-toxic and toxic groups between the pilot [25], interim and final participant cohorts.

3.4.2 Genetic analysis

Hardy-Weinberg equilibrium analysis was used to ascertain whether the observed allele frequencies in the participant cohorts differed from expected ($P > 0.05$). Chi-square tests compared the MAFs within the pilot [25], interim and final participant cohorts to the global Caucasian population [73]. Due to multiple comparisons, P-values were adjusted for false discovery rate (FDR) and considered significant if the FDR-corrected P-values was ≤ 0.05. The MAF for SNPs identified as predictive in the pilot [25], interim and final risk prediction models were also compared between non-toxic and toxic participants in the pilot [25], interim and final participant cohorts using Chi-square tests (FDR-corrected $P \leq 0.05$).

3.4.3 Interim risk prediction model

Due to the large amount of time taken to reach the target sample size of 150, an interim risk prediction model was produced with 105 participants. The interim risk prediction model was built using multivariate logistic regression on the statistical program R [104]. The model was built with step-wise addition of individual covariates (SNPs, 5-FU-based regimen, type of cancer, sex, hospital, age and number of treatment cycles) to the risk prediction model. If the covariate improved the model, determined by ANOVA analysis ($P < 0.05$), it remained in the model and was identified as predictive for severe GI toxicity risk.

3.4.4 Final risk prediction models

For the final risk prediction models, a more comprehensive statistical modelling method was used to build the risk prediction models in comparison to the interim risk prediction model. General linear models of multivariate logistic regression were used once again to build multiple risk prediction models for GI toxicity risk also using R [104].

As well as building a model of multivariate logistic regression producing a final risk prediction model

for severe GI toxicity in the final participant cohort, several additional models were also investigated to further explore the data. These were:

- Risk of severe GI toxicity in participants who received 5-FU-based therapy for either colorectal or upper GI cancer (83 participants were treated for colorectal and upper GI cancer, n = 83). As colorectal and upper GI cancer types were identified as predictive for severe GI toxicity risk in the pilot risk prediction model [25], this model was investigated to determine significant predictors for severe GI toxicity in participants who were treated for colorectal or upper GI cancer.

- Risk of severe GI toxicity risk in the participant cohort with the exclusion of participants who reported grade 2 GI toxicity (155 participants minus 37 participants with grade 2 GI toxicity, n = 118). As grading of GI toxicity can be ambiguous and in some medical records the grade of GI toxicity reported was not clear, it was possible some participants were reported as developing grade 2 instead of grade 3 GI toxicity. This model was investigated excluding participants who developed grade 2 GI toxicity to determine if any potential ambiguity would lead to the production of a risk prediction model that is different to that produced in the final participant cohort.

- Risk of severe GI toxicity between participants who received 5-FU-based therapy at FMC (n = 107) or the RAH (n = 48). As GI toxicity reporting procedures differed across both hospitals, it was investigated if these differences had any significant effect on identifying significant predictors for severe GI toxicity.

Firstly, for each model, individual predictors (such as SNPs, treatment regimen, type of cancer, sex, age and number of treatment cycles) were identified using logistic regression. Due to multiple comparisons, P-values were corrected using the false discovery rate (FDR-corrected $P < 0.05$). Any significant predictors were included in the model of multivariate logistic regression, with

step-down removal of predictors that did not improve the model with the final risk prediction model having the lowest Akaike information criterion score. The ROC AUC assessed the ability of the risk prediction models to identify participants with different grades of toxicity in comparison to the observed number of participants with that grade of toxicity. A ROC curve is the plot of sensitivity (true positive rate) against 100-specifcity (false positive rate) for different cut-off points of a parameter [105]. The AUC measured the overall fit of the model [105]. If no significant predictors were identified in the first instance, then no model of multivariate logistic regression and risk prediction model was built.

3.5 Participant results

3.5.1 Diarrhoea was the most frequent severe GI toxicity symptom experienced in both interim and final participant cohorts

A breakdown of GI toxicity symptoms experienced by participants in both interim and final participant cohorts are presented in Table 3.3. Some participants reported more than one GI toxicity symptom throughout their 5-FU-based therapy. Of the 105 participants in the interim cohort, 24 (23 %) reported severe GI toxicity and were classified as toxic. With the addition of 50 participants (44 non-toxic and 6 toxic) the final participant cohort was n = 155; the overall percentage of participants classified as toxic reducing by 4 % (Table 3.3). In both participant cohorts, diarrhoea was the most commonly experienced severe GI toxicity symptom (Table 3.3).

Table 3.3 GI toxicity symptoms reported by participants in the interim (n = 105) and final (n = 155) cohorts throughout their 5-FU-based therapy. Data is presented as n (%). Some participants reported more than one GI toxicity symptom throughout their 5-FU-based therapy.

Grade [10]		Highest GI toxicity grade reported by participants		Mucositis		Nausea/ vomiting		Diarrhoea	
Cohort		Interim	Final	Interim	Final	Interim	Final	Interim	Final
Non-toxic	0	12 (11%)	22 (14%)	49 (47%)	74 (48%)	26 (25%)	44 (28%)	54 (51%)	81 (52%)
	1	48 (46%)	66 (43%)	40 (38%)	58 (37%)	46 (44%)	62 (40%)	30 (29%)	44 (28%)
	2	21 (20%)	37 (24%)	11 (10%)	16 (10%)	22 (21%)	36 (24%)	6 (6%)	11 (8%)
Toxic	3	24 (23%)	30 (19%)	5 (5%)	7 (5%)	11 (10%)	13 (8%)	15 (14%)	19 (12%)

3.5.2 Clinical data was significantly different between non-toxic and toxic participants in the interim and final cohorts

In both interim and final participant cohorts, cancer type (P = < 0.001 and P = 0.002, respectively) and 5-FU-based regimen (P = 0.002 and P < 0.001, respectively) were significantly different between non-toxic and toxic participants (Table 3.4). Toxic participants in both interim and final cohorts were more likely to have had colorectal cancer compared to non-toxic participants, who were more likely to have had breast cancer. The significant differences in 5-FU-based regimen between non-toxic and toxic participants in both participant cohorts was likely a direct result of cancer type.

Sex was identified as significantly different between non-toxic and toxic participants in the interim cohort (P = 0.01) however, was not significantly different between the two participant groups in the final participant cohort (Table 3.4). In contrast, with the addition of 50 participants to the final participant cohort, significant differences were identified in age (P = 0.002) and number of treatment cycles (P = 0.032) between non-toxic and toxic participants (Table 3.4).

Table 3.4 Participant demographics and clinical data from the pilot (n = 34), interim (n = 105) and final (n = 155) cohorts. Data is presented as n (%) or median (range)[b].

		Pilot cohort [25]			Interim cohort			Final cohort		
		Non-toxic (n = 24)	**Toxic** (n = 10)	**P-value**	**Non-toxic** (n = 83)	**Toxic** (n = 22)	**P-value**	**Non-toxic** (n = 125)	**Toxic** (n = 30)	**P-value**
Sex	Female	18 (75%)	4 (40%)	0.110	61 (73%)	10 (45%)	0.010*	82 (66%)	16 (53%)	0.061
	Male	6 (25%)	6 (60%)		22 (27%)	12 (55%)		43 (34%)	14 (47%)	
Age	Median (range)	57 (39 – 80)	66 (48 – 78)	0.780	61 (32 – 86)	68 (28 – 78)	0.080	61 (32 – 78)	68 (28 – 86)	0.002*
Cancer type	Breast	12 (50%)	1 (10%)	0.057	53 (64%)	4 (18%)	< 0.001*	66 (52.8%)	6 (20%)	< 0.001*
	Colorectal	11 (46%)	7 (70%)		28 (34%)	15 (68%)		52 (41.6%)	21 (70%)	
	Upper GI	1 (4%)	2 (20%)		2 (2%)	3 (14%)		7 (5.6%)	3 (10%)	
5-FU-based regimen	5-FU monotherapy	4 (16.7%)	2 (20%)	0.800	10 (12%)	5 (23%)	0.002*	17 (14%)	6 (20%)	< 0.001*
	Mayo	4	2		10	5		17	6	
	5-FU combination	17 (70.8%)	6 (60%)		67 (81%)	10 (45%)		99 (79%)	15 (50%)	
	DECO	0	0		0	0		1	0	
	ECF	0	2		1	1		2	1	

	EOF	0	0		0	1		0	1	
5-FU-based regimen	FEC	12	1		53	5		65	6	
	FOLFOX4	2	1		8	2		14	4	
	FOLFOX 6	3	2		5	1		17	3	
	Capecitabine	3 (12.5%)	2 (20%)		6 (7%)	7 (32%)		9 (7%)	9 (30%)	
	Capecitabine	3	2		4	6		7	6	
	Xelox	0	0		2	1		2	3	
Cycles	Median (range)	5 (3 – 12)	5 (2 – 30)	0.180	5 (2 – 30)	8 (2 - 30)	0.076	6 (2 – 30)	8 (3 – 30)	0.037*
Hospital	FMC	24 (100%)	10 (100%)	-	54 (65%)	15 (68%)	> 0.999	86 (69%)	21 (70%)	> 0.999
	RAH	-	-		29 (35%)	7 (32%)		39 (31%)	9 (30%)	

[b] 5-FU monotherapy: 5-FU administered with folinic acid as part of the Mayo regimen; 5-FU combination: 5-FU administered as part of a regimen such as DECO (docetaxel, cisplatin, 5-FU), ECF (epirubicin, cisplatin, 5-FU), EOF (epirubicin, oxaliplatin, 5-FU), FEC (5-FU, epirubicin, cyclophosphamide) or FOLFOX (5-FU, oxaliplatin, folinic acid); Capecitabine: Capecitabine monotherapy or XELOX (capecitabine and oxaliplatin); Cycles: Number of treatment cycles received; FMC: Flinders Medical Centre; RAH: Royal Adelaide Hospital.

3.5.3 Comparison of demographic and clinical characteristics of the non-toxic and toxic participant cohorts

There were no significant differences in non-toxic participants between the pilot [25], interim and final cohorts ($P > 0.05$) (Table 3.5) [25]. However, there were significant differences in 5-FU-based regimen between toxic participants in the pilot, interim and final cohorts ($P < 0.001$) (Table 3.5). 5-FU-based combination regimens were the most commonly administered in the toxic population across all three participant cohorts (Table 3.4). However, the percentage of toxic participants who received capecitabine regimens compared to 5-FU monotherapy was increased in the interim and final participant cohorts in comparison to the pilot participant cohort (Table 3.4).

Table 3.5 Comparison of demographic and clinical data between non-toxic and toxic participants in the pilot (n = 34), interim (n = 105) and final (n = 155) cohorts.

	Sex		Age	Cancer type		5-FU-based regimen		Cycles
	Chi-square	P-value	P-value	Chi-square	P-value	Chi-square	P-value	P-value
Non-toxic (Pilot vs Interim vs Final)	2.240	0.326	0.411	5.061	0.281	3.534	0.473	0.310
Toxic (Pilot vs Interim vs Final)	3.406	0.182	0.303	6.794	0.142	55.350	< 0.001*	0.550

3.6 Genetic analysis

Hardy-Weinberg equilibrium analysis revealed no deviation between observed and expected MAF in the interim or final participant cohorts therefore, the results of the SNP assays were reliable ($P > 0.05$). Hardy-Weinberg equilibrium was also not significant for SNP assays in the pilot participant cohort [25].

3.6.1 The MAF for SNPs in IL1B, TGFB and CRP were not reflective of the general Caucasian population

Chi-square tests identified the MAF for *IL1B* rs16944 and rs1143627, *TGFB* rs1800469 and *CRP* rs2794521 SNPs were lower in the pilot, interim and final participant cohorts in comparison to the general 1000 genomes and GnomAD European Caucasian population (FDR-corrected $P < 0.001$) (Table 3.6). The MAF of remaining SNPs was reflective of the general 1000 genomes and GnomAD European Caucasian population.

Table 3.6 MAFs of investigated SNPs in the pilot (n = 34), interim (n = 105) and final (n = 155) cohorts compared to the European Caucasian population from 1000 Genomes and GnomAD [73][c].

Gene	rs ID	MAF					Chi-square	FDR-corrected P-value
		1000 Genomes [73]	GnomAD [73]	Pilot cohort (n = 34)	Interim cohort (n = 105)	Final cohort (n = 155)		
IL1B	rs16944	0.65	0.65	0.29	0.37	0.37	47.07	< 0.001*
	rs1143627	0.65	0.65	0.29	0.38	0.37	46.30	< 0.001*
	rs1143634	0.24	0.24	0.24	0.23	0.24	0.044	0.998
IL2	rs2069762	0.29	0.30	0.34	0.30	0.28	0.987	0.998
IL6	rs10499563	0.22	0.19	0.25	0.28	0.30	4.225	0.998
IL10	rs1800871	0.24	0.23	0.25	0.25	0.23	0.219	0.998
	rs1800896	0.45	0.47	0.46	0.49	0.49	0.513	0.998
IL6R	rs8192284	0.36	0.38	0.38	0.43	0.43	1.723	0.998
TGFB	rs1800469	0.68	0.70	0.34	0.28	0.29	73.190	< 0.001*
	rs11466314	0.00	0.00	0.00	0.00	0.00	-	-

TNF	rs1800629	0.13	0.16	0.19	0.16	0.17	1.385	0.998
TLR2	rs3804100	0.06	0.06	0.09	0.04	0.04	3.075	0.998
TLR4	rs4986790	0.06	0.06	0.01	0.06	0.05	3.493	0.998
	rs4986791	0.05	0.07	0.03	0.07	0.06	2.119	0.998
MD2	11466004	0.02	0.02	0.01	0.02	0.01	0.762	0.998
MYD88	rs6853	0.13	0.12	0.10	0.15	0.16	1.990	0.998
CASP1	rs580253	0.17	0.17	0.21	0.20	0.17	1.012	0.998
CASP5	rs554344	0.17	0.17	0.21	0.19	0.15	1.422	0.998
OPRM1	rs1799971	0.16	0.14	0.14	0.12	0.12	0.953	0.998
CRP	rs2794521	0.71	0.74	0.35	0.34	0.36	68.74	< 0.001*
BDNF	rs6265	0.20	0.18	0.21	0.19	0.19	0.3326	0.998

[c] - : no chi-square calculation possible. A single Chi-square test examined the difference between the MAFs reported by 1000 genomes, GnomAD and the pilot, interim and final cohorts for each SNP with the P-values obtained FDR-corrected to account for multiple comparisons.

3.6.2 MAFs for TNF rs1800629, TLR2 rs384100 and IL1B rs16944 were compared between the pilot, interim and final participant cohorts.

SNPs in *TNF r*s1800629 and *TLR2* rs384100 were identified as significantly predictive for severe GI toxicity risk in the pilot study risk prediction model [25] whilst, SNPs in *IL1B* rs1143634 and rs16944 SNPs were identified as significantly predictive for severe GI toxicity in the interim risk prediction model. Therefore, the MAFs for these *TNF*, *TLR2* and *IL1B* SNPs were compared between the pilot, interim and final participant cohorts.

The MAF for *TNF r*s1800629 and *TLR2* rs384100 was significantly increased in toxic participants of the pilot cohort compared to toxic participants of the interim and final cohort (FDR-corrected P = 0.016 and 0.004, respectively) (Table 3.7). Similarly, the MAF for *IL1B* rs16944 was significantly increased in toxic participants of the interim cohort compared to the pilot and final cohorts (FDR-corrected P = 0.004) (Table 3.7).

There were no significant differences in the MAF for *TNF r*s1800629, *TLR2* rs384100 and *IL1B* rs16944 in non-toxic participants between the pilot, interim and final cohorts (Table 3.7).

Table 3.7 Comparison of MAFs for SNPs identified as significantly predictive in the pilot study risk prediction model and the interim risk prediction model between the pilot, interim cohort and final cohorts.

Gene	rs ID	MAF for Non-toxic participants			Chi-square	FDR-corrected P-value
		Pilot cohort (n = 24)	Interim cohort (n = 83)	Final cohort (n = 125)		
TNF	rs1800629	0.15	0.16	0.17	0.149	0.928
TLR2	rs3804100	0.04	0.05	0.04	0.1608	0.928
IL1B	rs16944	0.27	0.35	0.35	1.950	0.928
	rs1143634	0.25	0.19	0.24	1.179	0.928

Gene	rs ID	MAF for Toxic participants			Chi-square	FDR-corrected P-value
		Pilot cohort (n = 10)	Interim cohort (n = 22)	Final cohort (n = 30)		
TNF	rs1800629	0.30	0.13	0.20	8.800	0.016*
TLR2	rs3804100	0.20	0.02	0.04	24.59	0.004*
IL1B	rs16944	0.25	0.47	0.45	3.386	0.004*
	rs1143634	0.20	0.34	0.27	1.365	0.078

3.7 Risk prediction models

3.7.1 The interim risk prediction model identified IL1B SNPs in conjunction with cancer type to be predictive for severe GI toxicity

In the interim cohort, a general linear model of multivariate logistic regression built a risk prediction model for severe GI toxicity identifying *IL1B* SNPs rs16944 and rs1143634 in conjunction with colorectal and upper GI cancer types as predictive for severe GI toxicity, with a ROC AUC of 82 % ($P < 0.001$) (Figure 3.2). Individually, cancer type had a ROC AUC of 73 % ($P = 0.012$) and *IL1B* SNPs rs16944 and 1143634 had a ROC AUC of 69 % ($P = 0.016$) (Figure 3.2). As seen, the inclusion of *IL1B* SNPs rs16944 and 1143634 and cancer type gave a more informative, sensitive and specific risk prediction model for severe GI toxicity compared to using SNPs alone or cancer type alone (Figure 3.2).

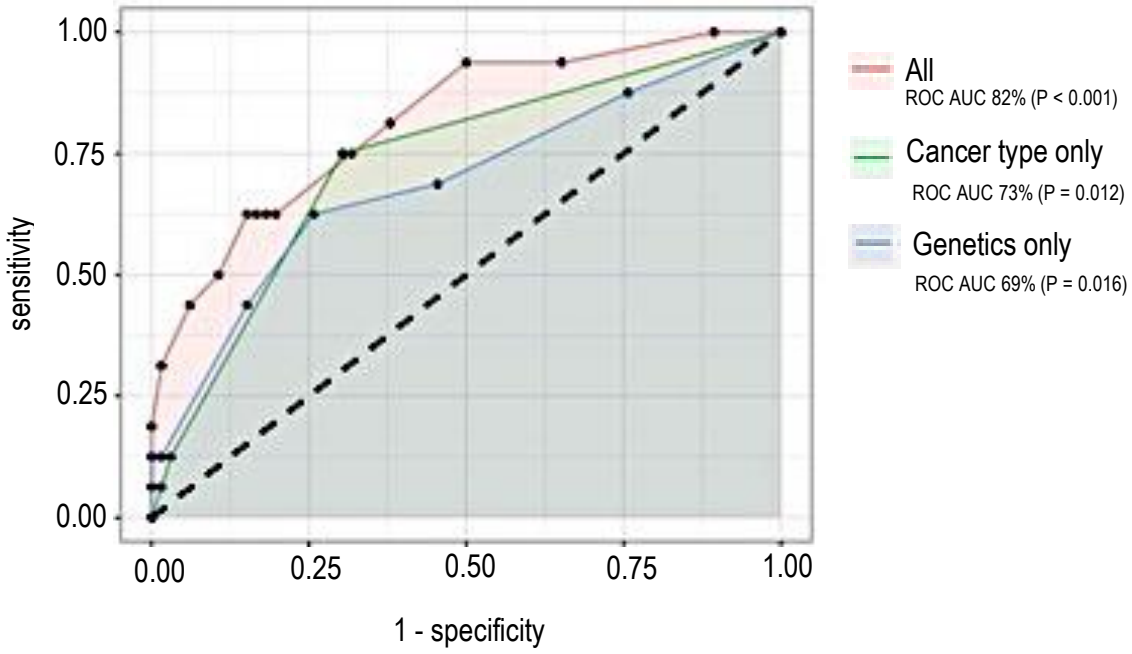

Figure 3.2 The interim risk prediction model for severe GI toxicity. The model identified *IL1B* rs16944 and rs1143634 SNPs (blue) and colorectal and upper GI cancer types (green) to be predictive for severe GI toxicity. Using both genetics and cancer type as predictors improved the risk prediction model (red).

3.7.2 The final risk prediction model identified 5-FU-based regimen to be predictive for severe GI toxicity

At the end of the recruitment period, a final risk prediction model was completed encompassing all 155 participants. Firstly, logistic regression identified individual predictors cancer type (FDR-corrected P = 0.047) and 5-FU-based regimen (FDR-corrected P = 0.047) to be associated with severe GI toxicity. No SNPs were identified as individual predictors. These significant predictors were then both included in the model of multivariate logistic regression. The inclusion of cancer type was not significant (FDR-corrected P > 0.05). Subsequently, this was removed from the risk prediction model, leaving 5-FU-based regimen with the lowest Akaike information criterion score. The final risk prediction model produced identified 5-FU-based regimen as predictive for severe GI toxicity, with a ROC AUC of 66 % (P = 0.028) (Figure 3.3).

A separate risk prediction model including predictors identified to be associated with severe GI toxicity from the pilot study (cancer type, *TLR2* rs3804100 and *TNF* rs1800629) was also built (Figure 3.3) [25]. The inclusion of *TLR2* rs3804100 and *TNF* rs1800629 was not significant (FDR-corrected P > 0.05) in the final participant cohort and therefore, were removed from the risk prediction model, leaving cancer type with the lowest Akaike information criterion score. The risk prediction model produced identified cancer type as predictive for severe toxicity, with a ROC AUC of 66 % ($P = 2.35 \times 10^{-8}$) (Figure 3.3). Participants who had colorectal cancer were at a higher risk of developing severe GI toxicity compared to participants who had breast cancer (P = 0.003). No associations were determined with upper GI cancer.

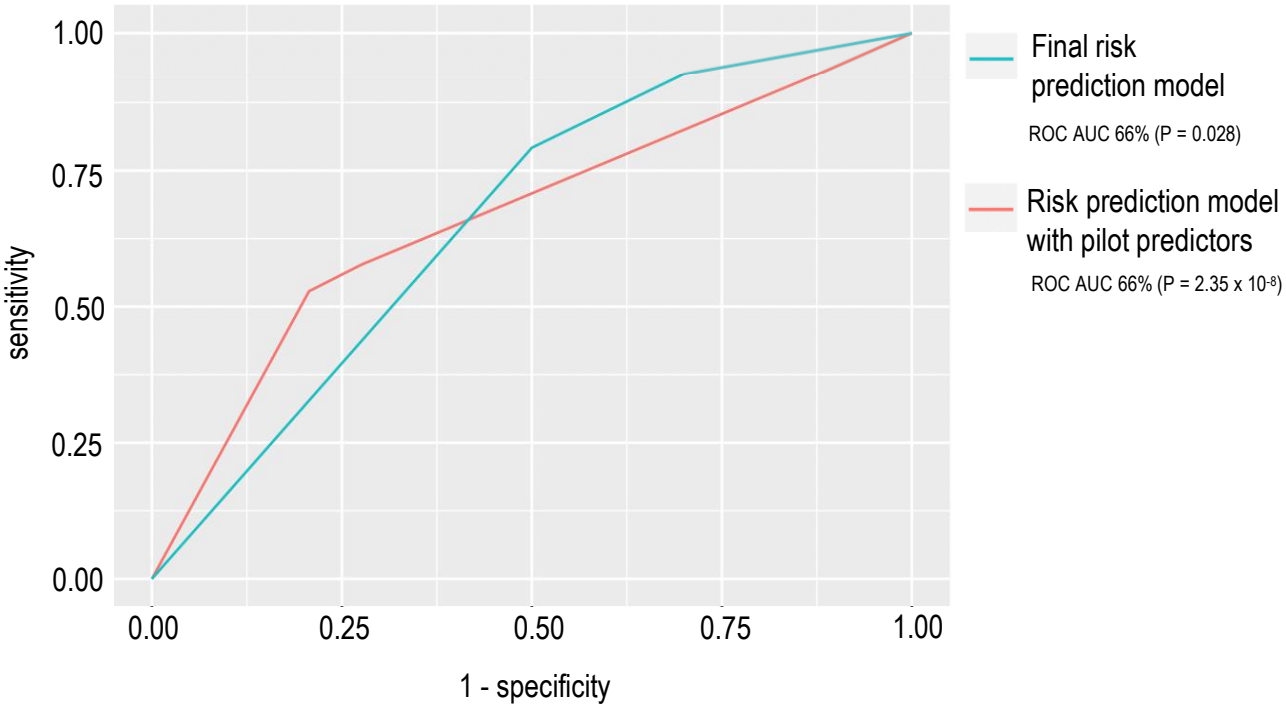

Figure 3.3 Risk prediction models for severe GI toxicity in the final participant cohort (n = 155). The validation risk prediction model (green) identified 5-FU-based regimen to be predictive for severe GI toxicity. A risk prediction model (red) including the significant predictors (*TNF* rs1800629, *TLR2* 384100 and cancer type) identified in the pilot study was also produced.

3.7.3 The risk prediction model excluding participants with grade 2 GI toxicity identified 5-FU-based regimen and cancer type to be predictive for severe GI toxicity

To determine if the ambiguity of GI toxicity grading was masking significant predictors for severe GI toxicity, another risk prediction model was produced excluding participants who reported grade 2 GI toxicity form the analysis (n = 118). Similarly, logistic regression identified individual predictors cancer type (FDR-corrected P = 0.027) and 5-FU-based regimen (FDR-corrected P = 0.027) to be associated with severe GI toxicity. No SNPs were identified as individual predictors. These significant predictors were then included in the model of multivariate logistic regression and together, they produced the lowest Akaike information criterion score. The risk prediction model produced a ROC AUC of 74 % (P = 0.005) (Figure 3.4). Participants who had colorectal and upper GI cancer were at a higher risk of developing severe GI toxicity compared to participants who had breast cancer (P = 0.045 and P = 0.024, respectively). Likewise, participants who received a capecitabine regimen were at higher risk of developing severe GI toxicity compared to participants whom had received a 5-FU monotherapy regimen. No significant associations were determined with 5-FU-based combination regimens.

Once again, a separate risk prediction model including the predictors identified to be associated with severe GI toxicity from the pilot study (cancer type, *TLR2* rs3804100 and *TNF* 1800629) was also built with the exclusion of participants who reported grade 2 GI toxicity (Figure 3.4) [25]. The inclusion of *TLR2* rs3804100 and *TNF* 1800629 was not significant (FDR-corrected P > 0.05) and therefore, was removed from the risk prediction model, leaving cancer type with the lowest Akaike information criterion score. The risk prediction model produced identified cancer type as predictive for severe GI toxicity, with a ROC AUC of 68 % ($P = 1.2 \times 10^{-6}$) (Figure 3.4). Participants whom had colorectal cancer were at a higher risk of developing severe GI toxicity compared to participants whom had breast cancer (P = 0.002).

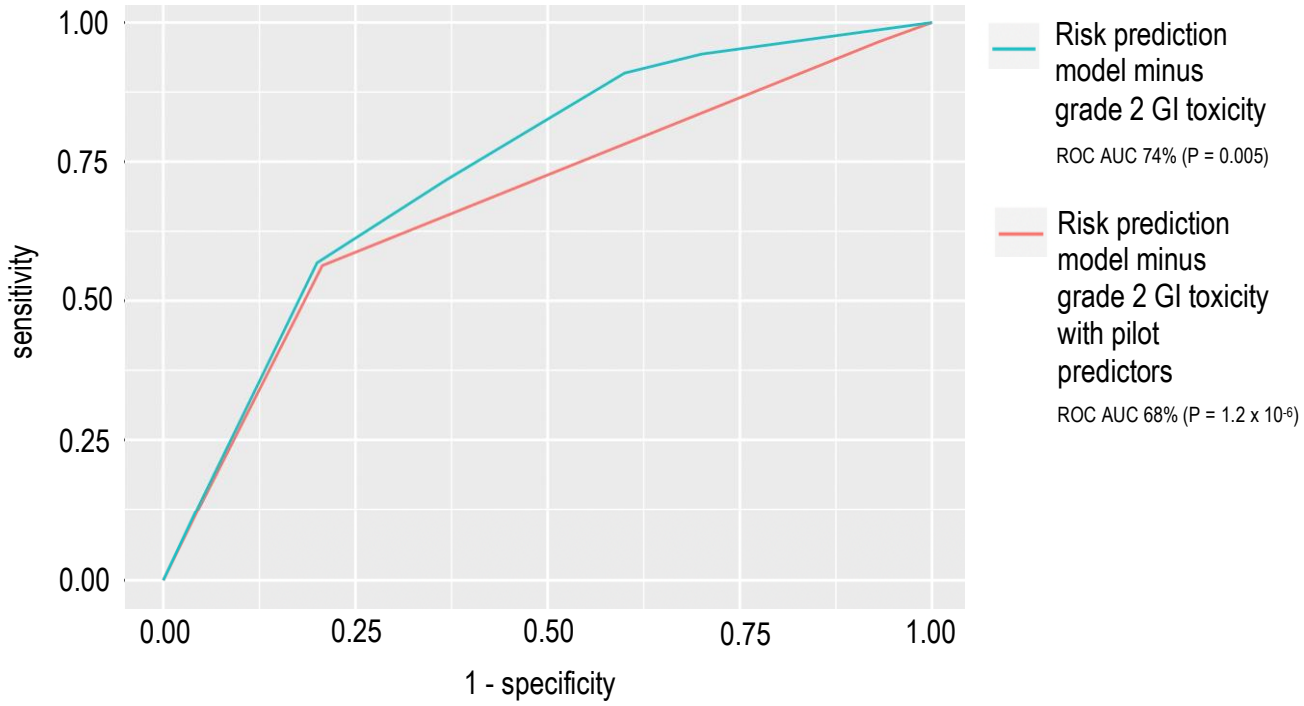

Figure 3.4 Risk prediction models for severe GI toxicity with the exclusion of participants who reported grade 2 GI toxicity (n = 118). The risk prediction model (green) identified 5-FU-based regimen and cancer type to be predictive for severe GI toxicity. A risk prediction model (red) including the significant predictors (*TNF* rs1800629, *TLR2* 384100 and cancer type) identified in the pilot study was also produced.

3.7.4 No significant predictors were identified for severe GI toxicity in participants who were treated for colorectal or upper GI cancer

To determine if there were any significant predictors for severe GI toxicity risk in participants with colorectal and upper GI cancer, another risk prediction model was investigated for participants who were treated for these cancer types (n = 83). Logistic regression did not identify individual predictors to be associated with severe GI toxicity (FDR-corrected P > 0.05). Therefore, no model of multivariate logistic regression and final risk prediction model was built.

3.7.5 No significant predictors were identified for severe GI toxicity in participants treated at FMC or the RAH

To determine if there were any significant predictors for each hospital site, another two risk prediction models were investigated in participants recruited from FMC (n = 107) or the RAH (n = 48). Logistic regression did not identify individual predictors to be associated with severe GI toxicity (FDR-corrected P > 0.05). Therefore, no model of multivariate logistic regression and final risk prediction model was built.

3.8 Discussion

Genetic variation within the TIR domain innate immune signalling pathway has previously been identified as predictive for severe GI toxicity risk following 5-FU-based therapy [25]. The objective of this study was to validate the results of a pilot study [25], that identified *TLR2* rs384100 and *TNF* rs1800629 SNPs in conjunction with colorectal and upper GI cancer types to be predictive for severe GI toxicity risk in a larger independent cohort of participants. It is imperative a predictive marker for identifying patients at most risk of severe GI toxicity prior to receiving 5-FU-based therapy is identified. This would allow patients at most risk of severe GI toxicity to have their supportive care measures personalised, which may reduce the severity of GI toxicity experienced and may improve their quality of life throughout their 5-FU-based therapy.

This current study compared participant demographics, clinical data, genotype and, risk prediction models for severe GI toxicity within and between the interim and final participant cohorts, as well as, the participant cohort recruited in the pilot study [25]. Several significant differences exist between the pilot, interim and final participant cohorts, which may indicate reasons as to why the pilot, interim and final risk prediction models produced for severe GI toxicity were vastly different.

Participant demographics and clinical data were identified to be either significantly different between toxic and non-toxic participants or, identified as predictive for severe GI toxicity risk in the pilot [25], interim or final participant cohorts. Cancer type, in particular colorectal and upper GI cancer types, were identified as predictive for severe GI toxicity risk in both the pilot and interim risk prediction models. Although, significant differences in cancer type between non-toxic and toxic participants were only identified in the interim and final participant cohorts. Likewise, 5-FU-based regimen was also significantly different between non-toxic and toxic participants in the interim and final participant cohorts yet, was only identified as predictive for severe GI toxicity risk in the final risk prediction model.

The 5-FU-based regimen administered to participants in this study was specific to cancer type. Therefore, it was not surprising both cancer type and 5-FU-based regimen were consistently associated with severe GI toxicity risk in the pilot, interim and final participant cohorts. Due to the dosage and frequency of administration, 5-FU-based regimens administered to treat colorectal and upper GI cancers are known to increase the risk of severe GI toxicity [1, 41]. 5-FU monotherapy in addition to 5-FU combination regimens administered for colorectal and upper GI cancers include a single bolus dose as well as a continuous intravenous infusion (usually over a 24 - 48 h period) of 5-FU with one cycle spanning either 7 or 14 days [41]. In comparison, 5-FU-based combination regimens administered for breast cancer include a single bolus dose of 5-FU with one cycle spanning 21 days [41]. The higher exposure to 5-FU in regimens administered for colorectal and

upper GI cancers is likely to increase the risk of severe GI toxicity [1, 41]. However, when participants who received treatment for colorectal or upper GI cancer from the final participant cohort were analysed in a separate risk prediction model, no significant predictors for severe GI toxicity risk were identified. This suggests predictors for severe GI toxicity risk are highly specific for patient cohorts, particularly across cancer types and 5-FU-based regimens.

Additional participant demographics sex, age and number of treatment cycles were significantly different between non-toxic and toxic participants in the interim (sex) and final (age and number of treatment cycles) participant cohorts. However, sex, age and number of treatment cycles were not identified as predictive for severe GI toxicity risk in either the interim or final risk prediction models. Contradictory and inconsistent findings have previously surrounded the association of age and sex with severe GI toxicity risk [11-14]. In a large cohort of patients receiving 5-FU-based therapy for colorectal and upper GI cancer, no significant relationship was identified between severe GI toxicity symptoms and advanced age or sex [14]. In comparison, in a similar size cohort of patients receiving 5-FU-based therapy, increasing age was identified as an independent predictor for severe GI toxicity risk ($P = 0.001$) and, women developed higher counts of diarrhoea ($P < 0.01$) and vomiting ($P = 0.03$) than their male counterparts [11]. With regards to the impact of the number of treatment cycles in this current study, participants classified as toxic in the final participant cohort received significantly more treatment cycles compared to non-toxic participants. However, participants recruited in the final participant cohort reported GI toxicity symptoms within the first three cycles of treatment. Therefore, the relevance of this significant difference between the two toxicity groups is unclear.

When comparing participant demographics and clinical data between the three participant cohorts, only a significant difference in 5-FU-based regimen between toxic participants was identified. Over the course of recruitment, some of the 5-FU-based regimens administered were discontinued

(i.e. Mayo) or superseded (i.e. FOLFOX4 to FOLFOX6) as it was consensus these regimens were 'too toxic' for patients [41]. As a result, a shift towards prescribing other 5-FU-based regimens, such as those containing capecitabine, could possibly explain why capecitabine was the second most administered regimen amongst toxic participants in the interim and final participant cohorts whereas, only one toxic participant was administered capecitabine in the pilot participant cohort.

When analysis shifted to genotype, significant differences in the MAF of *TLR2* rs384100, *TNF* rs1800629, *IL1B* rs16944 and *IL1B* rs1143634 SNPs between toxic participants in the pilot, interim and final participant cohorts were identified. The *in vitro* and *ex vivo* effect of these *TLR2, TNF* and *IL1B* SNPs on gene expression and proinflammatory cytokine secretion has been discussed in Chapter 2 [106]. The interim risk prediction model was the first to associate *IL1B* rs16944 and rs1143436 SNPs with severe GI toxicity risk. However, following the addition of 50 participants (40 non-toxic and 10 toxic) to produce the final participant cohort, the MAF of *IL1B* rs16944 and rs1143436 SNPs decreased in the toxic population (0.47 vs 0.45 and 0.34 vs 0.27, respectively). This could be a possible explanation for why the predictive nature of individual SNPs for severe GI toxicity risk change between analyses.

The genetic findings above demonstrate there was no consistent TIR domain SNP identified in the non-toxic or toxic groups across the three participant cohorts. Different SNPs were identified by the pilot and interim risk prediction models. It was observed as the genetic composition changed between studies or with the addition of new participants, the predictive nature of the SNPs also changed. Additionally, the MAF of *IL1B* rs16944, *IL1B* rs1143627, *TGFB* rs1800469 and *CRP* rs2794521 SNPs across all three participant cohorts was lower than what is observed in the Caucasian population [73]. This is especially relevant for *IL1B* rs16944, as the MAF in the interim participant cohort was almost half of what is observed in the Caucasian population.

Aside from the pilot, interim and final risk prediction models identifying different predictors of severe

GI toxicity risk, their ROC AUCs were also different from one another. This indicates the pilot, interim and final risk prediction models were not equally sensitivity and specific. The closer the ROC AUC is to 100 %, the more sensitive and specific the risk prediction model is in determining which participants are at risk of severe GI toxicity [105]. The pilot risk prediction model reported the highest ROC AUC of 87 % [25], followed by the interim and final risk prediction models of 83 % and 66 %, respectively. As the number of participants included in the model increased, it became more difficult for the risk prediction model to accurately determine which participants were at risk of severe GI toxicity based on the predictors identified, signified by a lower ROC AUC. Although the pilot risk prediction model was more sensitive and specific compared to the interim and final risk prediction models, in a clinical setting, the pilot model may not be accurate in identifying patients at risk of severe GI toxicity as only 34 participants were included in the risk prediction model.

Additional risk prediction models were also produced based on observations made throughout participant recruitment. As participants were recruited from two hospital sites, different medical oncologists were prescribing 5-FU-based regimens to participants. Therefore, it was possible bias was present if the prescribing medical oncologist preferred one 5-FU-based regimen over another. In addition, GI toxicity reporting procedures were different at both hospital sites. However, when participants in the final cohort were analysed based on hospital site, no significant predictors for severe GI toxicity risk at each hospital site were identified.

Furthermore, a risk prediction model was built with the removal of participants who reported grade 2 GI toxicity from the final participant cohort. As GI toxicity was graded by clinical oncology staff by comparing participant reported GI toxicity symptoms to the NCI CTCAE scale, GI toxicity grading may have been ambiguous. Potentially, some participants recruited to this study who were reported as developing severe GI toxicity (grade 3) may have been incorrectly reported as developing moderate GI toxicity (grade 2). These participants would have been wrongly classified as non-toxic

and analysed in the incorrect toxicity group. Additionally, some studies such as Wolff *et al.*, classified grade 2 toxicity as severe due to the substantial impact grade 2 toxicity had on patient quality of life [107]. As my study was retrospective and toxicity data was collected from participant clinical medical records, it was difficult to determine if quality of life was substantially affected in participants who reported grade 2 GI toxicity. Therefore, grading classification was maintained with previous clinical work conducted in my laboratory [25, 103].

The risk prediction model built with the exclusion of participants who reported grade 2 GI toxicity identified both cancer type and 5-FU-based regimen to be predictive for severe GI toxicity risk. It was further identified capecitabine and, colorectal and upper GI cancers, increased participants' risk of severe GI toxicity. In comparison to the final risk prediction model, only 5-FU-based regimen was identified as significantly predictive for severe GI toxicity. No individual regimens or cancer types were determined to be more toxic than another. The ROC AUC was higher in this risk prediction model compared to the final risk prediction model (74 % vs 66 %), suggesting the removal of participants who reported grade 2 toxicity produces a more sensitive and specific predictive marker for accurately identifying participants at risk of severe GI toxicity.

In this current study, there were a number of limitations which may have influenced the identification of a predictive marker for severe GI toxicity risk. Firstly, a sample size of 150 participants (45 who reported severe GI toxicity) was initially determined as providing 99 % power at alpha = 0.05 to predict severe GI toxicity risk. Although the target sample size of 150 was exceeded, the breakdown between non-toxic (n = 125) and toxic (n = 30) participants did not adequately power the study. In addition, the calculated sample size of 150 participants was not adequate to ensure the MAF of all TIR domain SNPs of interest was reflective of the Caucasian population, with the MAF of some SNPs well below what is observed in the Caucasian population.

The clinical makeup of the participant cohort was also variable, with mixed cancer types and multiple 5-FU-based regimens included in analysis. This may have also influenced the identification of a predictive marker for severe GI toxicity risk as this risk is not equal across cancer types and 5-FU-based regimens. This was evident when cancer type (colorectal and upper GI) was identified as predictive for severe GI toxicity risk in the pilot and interim risk prediction models yet, when participants with these cancer types were analysed separately, no predictors for severe GI toxicity risk were identified. This highlights the necessity for future research to investigate predictors for severe GI toxicity risk in individual cancer types. Participants were not able to be analysed based on cancer type or 5-FU-based regimen as to reach the target sample size and ensure the final risk prediction model was accurately powered, participants had to be analysed as a whole.

The retrospective nature of this study was also a constraint and limits the clinical relevance of the predictive markers for severe GI toxicity identified in both the interim and final risk prediction models. In particular, some of the regimens administered to participants recruited to this study are no longer used in clinic today. Prospective recruitment and analysis would allow a clinically relevant and more accurate predictive marker to be identified for severe GI toxicity risk. A prospective study would also allow consistent grading of GI toxicity symptoms, eliminating any potential ambiguity in grading, as well as, enable participants to be screened for genotype prior to study enrolment, ensuring the MAF of the participant cohort is reflective of the Caucasian population. Additionally, participants could also be selected based on cancer type and the 5-FU-based regimens received in a prospective study would be currently used in clinic. A future study design for this research should be modelled off the QUASAR study [108], a prospective multi-site study recruiting 5,500 patients receiving 5-FU and folinic acid for colorectal cancer to determine survival of patients following 5-FU-based therapy.

3.9 Conclusion

To conclude, both the interim and final risk prediction models for severe GI toxicity produced in this validation study did not identify the same predictors for severe GI toxicity risk as the pilot risk prediction model [25]. The participant cohorts analysed in the pilot, interim and final risk prediction models were both clinically and genetically different to one another, which was reflected in the risk prediction models produced for each participant cohort. This confirms predictors for severe GI toxicity are reflective of the participant cohort analysed and emphasised predictive markers for severe GI toxicity risk are required for specific patient cohorts.

As the development of severe GI toxicity is multifaceted, the results of the pilot, interim and final participant cohort analysis suggests using an approach combining clinical data and SNPs may be required to identify a predictive marker for severe GI toxicity risk. Additional data such as tumour genetics, participant comorbidities and other 5-FU-induced toxicities should also be included in risk prediction modelling to accurately reflect what a patient would look like in the consultation room.

A major roadblock with identifying a predictive marker for severe GI toxicity risk is a poor classification of a phenotypic marker for severe GI toxicity. If a phenotypic marker for severe GI toxicity, such as increased proinflammatory cytokine secretion, was included in the risk prediction model, this would allow the model to identify either a clinical factor or SNP responsible for the change in phenotype observed. This is the focus of Chapter 4 of my thesis.

Chapter 4: Stimulated IL-1β secretion in participants post-5-FU-based therapy is predictive for severe GI toxicity risk

Chapter 4 is my second research chapter and is unpublished and unsubmitted work written in manuscript style. It is intended to be submitted to the *British Journal of Clinical Pharmacology*. This chapter is presented in its intended publication format with referencing style modified to maintain consistency throughout this thesis.

4.1 Abstract

Aims: Severe gastrointestinal (GI) toxicity following 5-fluorouracil (5-FU)-based therapy is highly prevalent and negatively affects solid tumour therapy. The TIR domain innate immune signalling pathway mediates proinflammatory cytokine secretion following 5-FU-based therapy. Genetic variation within the TIR domain pathway has been identified as predictive for severe GI toxicity. However, a predictive phenotypic marker for severe GI toxicity risk is yet to be determined.

Methods: Thirty-two participants were recruited to this study. Participant demographics, 5-FU-based regimen, GI toxicity and genetic variation within TIR domain pathway genes were known. *Ex vivo* quantification of TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β and TNF-α secretion from isolated PBMCs was determined by ELISA. IL-1β and TNF-α secretion was natural log-transformed and compared between participants with no or mild to moderate (grade 1 and 2) GI toxicity and participants with severe (grade ≥ 3) GI toxicity and, between *TLR2*, *TLR4*, *IL1B*, *TNF*, *CASP1* and *CASP5* genotypes using t-tests and one-way ANOVA. In addition, ANCOVA modelling identified predictors significantly associated with log IL-1β and TNF-α secretion.

Results: ANCOVA determined increased log IL-1β secretion was associated with no or mild to moderate GI toxicity and, *CASP1* rs580253 (G > A) and *CASP5* rs554344 (G > C) SNPs following

stimulation with TLR2 (PAM3CSK4) and TLR4 (LPS) agonists ($P < 0.05$) post-5-FU-based therapy. No predictors were associated with log TNF-α secretion.

Conclusions: TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion was significantly different post-5-FU-based therapy between participants who reported no or mild to moderate GI toxicity and severe GI toxicity. Therefore, the IL-1β secretory response is a potential predictive phenotypic marker for severe GI toxicity risk.

4.2 Statement 1: What is already known on this subject

- The TIR domain innate immune signalling pathway is a key mediating pathway in the development of severe GI toxicity.
- Activation of the TIR domain pathway leads to increased proinflammatory cytokine secretion.
- Genetic variation within the TIR domain pathway is predictive for severe GI toxicity following 5-FU-based therapy.

4.3 Statement 2: What this study adds

- TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion was significantly higher post-5-FU-based therapy in participants who reported no or mild to moderate GI toxicity.
- *CASP1* rs580253 (G > A) and *CASP5* rs554344 (G > C) genotypes were associated with TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion post-5-FU-based therapy.

4.4 Introduction

There are currently no clinically sensitive and specific predictive markers available for severe gastrointestinal (GI) toxicity risk following 5-fluorouracil (5-FU)-based therapy. GI toxicity symptoms develop in 25 – 50 % of patients receiving 5-FU-based therapy [3], with a subset of these patients developing severe GI toxicity, graded as $\geq$ 3 on the National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI CTCAE v 4.03 and v 5.0) [3, 10]. Patients with severe GI toxicity require treatment delays, dose reductions, early treatment cessation and hospitalisation to help manage and relieve symptoms [2, 3]. This not only results in sub-optimal therapy outcomes and decreases patient quality of life whilst on treatment but, is also an economic burden on the health care system [2, 6, 7, 109]. The identification of a predictive marker for severe GI toxicity risk may allow the personalisation of supportive care measures for patients at most risk of developing severe GI toxicity prior to receiving 5-FU-based therapies. Not only will this decrease the severity of GI toxicity experienced by the patient but, will also decrease the likelihood of their 5-FU-based therapy being compromised by the presence of severe GI toxicity symptoms.

Previous research has identified proinflammatory cytokine secretion contributes to the development of severe GI toxicity and, genetic variation within the Toll-like Receptor/Interleukin-1 (TIR) domain innate immune signalling pathway is predictive for severe GI toxicity risk. Administration of 5-FU initiates an innate immune response mediated by the TIR domain signalling pathway [5, 22, 53, 58]. Activation of the TIR domain pathway is mediated by Toll-like Receptors (TLRs) and upregulates potent transcription factors and secretion of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) [2, 23, 110].

Secretion of TNF-α and IL-1β has been consistently associated with severe mucosal injury and cell death in the oral mucosa, jejunum and colon following 5-FU administration in preclinical models [5, 23]. Likewise, clinical studies have also demonstrated increased TNF-α and IL-1β levels in

peripheral blood of patients with GI toxicity symptoms included, but not limited to, diarrhoea, mucositis, nausea and vomiting, following 5-FU-based therapy [2, 4, 24]. Genetic association identified the single nucleotide polymorphism (SNP) in tumour necrosis factor (*TNF*) -1031T/C (rs1799964) was predictive for stomatitis (also referred to as oral mucositis) in patients receiving 5-FU-based therapy [95]. Similarly, my laboratory demonstrated in a small clinical study *TLR2* rs3804100 and *TNF* rs1800629 SNPs were predictive for severe GI toxicity risk following 5-FU-based therapy in conjunction with colorectal and gastric cancer types (P = 0.033, ROC AUC = 87 %) [25].

Although research consistently demonstrates the potential of the TIR domain innate immune signalling pathway to be predictive for severe GI toxicity risk, no phenotypic marker for severe GI toxicity has yet been identified. Furthermore, it has not yet been determined if IL-1β and TNF-α secretion significantly differs between patients who develop no (grade 0), mild to moderate GI toxicity (graded as 1 or 2 on the NCI CTCAE v 5.0 [10]) or severe GI toxicity (graded as ≥ 3 on the NCI CTCAE v 5.0 [10], or patients that have a dose reduction, hospitalisation or cease treatment prematurely as a direct result of GI toxicity). If secretion of IL-1β and TNF-α does differ between the two toxicity groups and, is associated with one of multiple SNPs within the TIR domain pathway, this would be a strong genotype-phenotype predictive marker for severe GI toxicity risk that could potentially be translated to clinic.

This study is the first to my knowledge to investigate the innate immune response in participants post-chemotherapy. Changes in long-term proinflammatory cytokine secretion following exposure to either damage associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) is believed to occur as a result of innate immune memory [111]. On secondary challenge with either DAMPs or PAMPs, proinflammatory cytokine secretion may be diminished in comparison to secretion patterns that experienced pre-exposure (or pre-chemotherapy) [111]. DAMPs and PAMPs play a significant role in 5-FU-induced mucosal injury. Therefore, toxic

participants may be tolerant to a greater extent than non-toxic participants as a result of the high exposure to DAMPs and PAMPs that occurs during severe GI toxicity.

This study firstly hypothesised participants who reported severe GI toxicity throughout their 5-FU-based therapy would secrete decreased IL-1β and TNF-α pre- and post-stimulation with TLR2 and TLR4 agonists compared to participants who reported no or mild to moderate GI toxicity. Secondly, it was hypothesised any differences in IL-1β and TNF-α secretion identified between the two participant groups would be associated with SNPs within the *TLR2*, *TLR4*, *IL1B*, *TNF*, *CASP1* and/or *CASP5* genes.

These hypotheses were investigated by the following aims:

1. To determine pre-stimulation IL-1β and TNF-α secretion in participants classified as non-toxic (participants who reported no or mild to moderate GI toxicity) and toxic (participants who reported severe GI toxicity) throughout their 5-FU-based therapy.
2. To determine if TLR2- and TLR4-stimulated IL-1β and TNF-α secretion was significantly different between participants classified as non-toxic and toxic throughout their 5-FU-based therapy.
3. To identify if any significant differences in post-stimulation IL-1β and TNF-α secretion between the two participant groups was associated with *TLR2*, *TLR4*, *IL1B*, *TNF*, *CASP1* or *CASP5* genotypes.

4.5 Materials and Methods

4.5.1 Ethics

This study was approved by the Southern Adelaide Clinical Human Research Ethics Committee (HREC/15/SAC/231) and the Royal Adelaide Hospital Human Ethics Committee

(SSA/15/RAH/397). All participants provided informed written consent prior to participating in accordance with the Declaration of Helsinki.

4.5.2 Study participants

107 participants who had participated in previous [25] and current immune genetic studies were invited to further participate in this study. Briefly, participants had received 5-FU-based therapy from the Flinders Medical Centre or Royal Adelaide Hospital in South Australia, Australia between January 2012 and June 2018. In the first instance, invitation letters were mailed exclusively to participants who reported no, grade 1 or grade 3 GI toxicity. However, to meet the target sample size of 46 (to adequately power the study to detect a 2-fold change in IL-1β and TNF-α secretion between non-toxic and toxic participants), the invitations were extended to participants who developed grade 2 GI toxicity. 42 participants (39 %) responded to the letter of invitation and 32 participants (30 %) were subsequently recruited to this study.

Participant demographics, chemotherapy regimen, GI toxicity and SNPs within TIR domain pathway genes *TLR2* (rs3804100), *TLR4* (rs4986790 and rs4986791), *IL1B* (rs16944, rs1143634 and rs1143627), *TNF* (rs1800629), *CASP1* (rs580253) *and CASP5* (rs554344) were collated from the immune genetic studies [25]. The SNPs had previously been determined using a customised Sequenom MassArray (iPLEX GOLD) assay [25].

4.5.3 GI toxicity classification

All study participants had previously been classified into two GI toxicity groups for analysis. Participants assigned as 'non-toxic' had reported either no GI toxicity (grade 0) or, mild to moderate GI toxicity (graded as 1 or 2 on the NCI CTCAE v 4.03 and v 5.0 [10]) throughout their 5-FU-based therapy. Participants assigned as 'toxic' had reported severe GI toxicity (graded as ≥ 3 on the NCI CTCAE v 4.03 [10]), or required a dose reduction, treatment break or hospitalisation due to GI

toxicity throughout their 5-FU-based therapy. This classification of GI toxicity has been previously used in clinical studies conducted in my laboratory [25, 103].

4.5.4 Whole blood collection and peripheral blood mononuclear cell (PBMC) isolation

It was confirmed participants were not ill, had not recently received a vaccination or were not currently taking immunosuppressant medication prior to blood draw to limit outside influences on IL-1β and TNF-α secretion. 27 mL of whole blood was collected from participants into EDTA tubes via venepuncture between 10 am and 11 am on the study day to ensure circadian rhythm did not influence secretion. PBMCs were immediately isolated to investigate proinflammatory cytokine secretion using the iodixanol mixer technique [112, 113]. Briefly, 2 mL of Optiprep™ (Sigma-Aldrich, NSW, Australia) was added to whole blood and mixed by repeated inversion. Following inversion, 1 mL of enriched media containing RPMI 1640 + L-glutamine, 10% foetal bovine serum and 1 % penicillin/streptomycin (Thermo Fisher Scientific, MA, USA) was layered on top and the sample was centrifuged at 1500 x g for 30 min with no deceleration. Following centrifugation, PBMCs were collected from the mononuclear layer and washed with 5 mL of RPMI 1640 + L-glutamine. PBMCs were diluted to a working concentration of 1 x 10^6 cells.mL^{-1} using enriched media and plated into Costar® 96-well plates (Corning, NY, USA).

4.5.5 TLR2 (PAM3CSK3) and TLR4 (LPS) stimulated IL-1β and TNF-α secretion

TLR2 agonist synthetic triacylated lipoprotein (PAM3CSK4) (Sigma Aldrich, NSW, Australia) was added to isolated PBMCs at concentrations of 0.00005, 0.01 and 1 μg.mL^{-1}. TLR4 agonist lipopolysaccharide (LPS, *E.coli*, Sigma Aldrich, NSW, Australia) was added to isolated PBMCs at concentrations 0.00005, 0.1 and 100 μg.mL^{-1}. Agonist treatments were performed in triplicate to stimulate an IL-1β and TNF-α secretory response [112]. Appropriate negative controls minus TLR agonists were also included. Plates were incubated for 20 h at 37 °C with 5 % CO_2. Following

incubation, supernatant from triplicate wells was collected, pooled and stored at -20 °C until further analysis.

4.5.6 Quantification of IL-1β and TNF-α secretion

IL-1β and TNF-α concentrations from PBMC supernatants pre- and post-stimulation were determined by commercially available human ELISA kits (eBioscience, CA, USA). Manufacturer's instructions were followed, including generation of standard curves from 2.34 to 150 $pg.mL^{-1}$ (IL-1β) and 7.81 to 500 $pg.mL^{-1}$ (TNF-α). UV absorbance was quantified using the Synergy Mx Microplate Reader™ (BioTek Instruments, VT, USA) at 450 nm. Individual ELISA assay results were accepted if the standard curve coefficient was $R^2 \geq 0.99$. When absorbance exceeded the standard curve, samples were diluted with ELISA diluent and repeated absorbance measurements taken.

4.5.7 Statistical analysis

Hardy-Weinberg equilibrium analysis was used to ascertain whether the observed allele frequencies in the participant cohort differed from expected ($P > 0.05$). Age, number of treatment cycles and number of months (between last treatment cycle and PBMC isolation) were tested for normality using the D'Agostino & Pearson test. Chi-square or Fisher's exact tests and Mann-Whitney U tests compared clinical data between non-toxic and toxic participants as data was not normally distributed (D'Agostino & Pearson $P < 0.05$). As IL-1β and TNF-α secretion was highly skewed, secretion was natural log-transformed for each participant. Unpaired parametric t-tests compared log IL-1β and TNF-α secretion between non-toxic and toxic participants at each TLR2 (PAM3CSK4) and TLR4 (LPS) agonist concentration. Likewise, unpaired parametric t-tests and one-way ANOVA compared log IL-1β and TNF-α secretion between *TLR2*, *TLR4*, *IL1B*, *TNF*, *CASP1 and CASP5* genotypes at each TLR2 (PAM3CSK4) and TLR4 (LPS) agonist concentration.

Due to multiple comparisons, P-values were adjusted for the false discovery rate (FDR) and considered significant if the FDR-corrected P-value was ≤ 0.05.

4.5.8 Analysis of covariance (ANCOVA) modelling

An ANCOVA is a general linear model which blends ANOVA and regression. ANCOVA modelling was used to identify covariates significantly associated with log IL-1β and TNF-α secretion. A base model ANCOVA determined the association of log agonist concentration, TLR2 (PAM3CSK4) and TLR4 (LPS) stimulation, GI toxicity and the interaction between these covariates, on log IL-1β and TNF-α secretion using the software program R [104]. All covariates and their interaction terms were examined in the ANCOVA model with step-wise removal of the covariate or interaction term of least significance until only significant covariates or interaction terms remained in the model. The covariates or interaction terms remaining in the model were identified as significantly associated with log IL-1β and/or TNF-α secretion.

Additionally, the association of participant clinical data such as sex, 5-FU-based regimen, cancer type, age, number of treatment cycles and; *TLR2* rs3804100, *TLR4* rs4986790 and rs4986791, *IL1B* rs16944, rs1143634 and rs1143627, *TNF* rs1800629, *CASP1* rs580253 *and CASP5* rs554344 genotypes, were also examined in an ANCOVA model. Firstly, participant clinical data or genotype considered individually associated with log IL-1β or TNF-α secretion were identified using a likelihood ratio test (FDR corrected P-values ≤ 0.05). These were included in the ANCOVA model using a step-up approach starting with the covariate with the lowest FDR-corrected P-value. If the clinical data and/or SNPs improved the ANCOVA model, it was retained in the model. If they did not improve the ANCOVA model, it was removed.

4.6 Results

4.6.1 Participant clinical data

Participant clinical data is provided in Table 4.1. 32 participants were recruited to the study with 19 classified as non-toxic and 13 as toxic following 5-FU-based therapy. There were no significant differences in clinical data between the two participant groups ($P > 0.05$) (Table 4.1). All genotypes conformed with Hardy-Weinberg equilibrium ($P > 0.05$). Genotype results for one participant were not available.

Table 4.1 Participant clinical data presented as n (%) or median (range)[a].

Total Participants n = 32	**Non-toxic** (n = 19)	**Toxic** (n = 13)	**P-value**
Sex			
Female	9 (47%)	8 (62%)	0.430
Male	10 (53%)	5 (38%)	
Age			
Median (range)	60 (40 – 73)	68 (40 – 73)	0.172
Cancer type			
Breast	8 (42%)	2 (15%)	0.227
Colorectal	10 (53%)	9 (70%)	
Upper GI	1 (5%)	2 (15%)	
5-FU-based regimen			
5-FU monotherapy	3 (16%)	3 (23%)	0.841
5-FU combination	12 (63%)	7 (54%)	
Capecitabine	4 (21%)	3 (23%)	
Number of treatment cycles			
Median (range)	6 (3 – 30)	6 (2 – 16)	0.780
Months between last treatment cycle and PBMC isolation			
Median (range)	45 (11 – 77)	40 (7 – 90)	0.826

[a] 5-FU monotherapy*:* 5-FU administered with folinic acid; 5-FU combination*:* 5-FU administered as part of a regimen such as DECO (docetaxel, cisplatin, 5-FU), ECF (epirubicin, cisplatin, 5-FU), EOF (epirubicin, oxaliplatin, 5-FU), FEC (5-FU, epirubicin, cyclophosphamide) or FOLFOX (5-FU, oxaliplatin, folinic acid); Capecitabine: Capecitabine monotherapy or XELOX (capecitabine and oxaliplatin).

4.6.2 No significant differences in IL-1β and TNF-α secretion were identified between non-toxic and toxic participants from univariate analysis

No significant differences in pre-stimulated log-transformed IL-1β secretion was identified between non-toxic and toxic participants (P = 0.833, Table 4.2). No significant differences were also identified in pre-stimulated log-transformed TNF-α secretion (P = 0.649, Table 4.2).

No significant differences in TLR2 (PAM3CSK4) (0.00005, 0.01 and 1 $\mu g.mL^{-1}$) or TLR4 (LPS) (0.00005, 0.1 and 100 $\mu g.mL^{-1}$) stimulated log-transformed IL-1β and TNF-α secretion between non-toxic and toxic participants were also identified (P > 0.05, Table 4.2).

Table 4.2 Natural log-transformed IL-1β and TNF-α -secretion (pg.mL^{-1}) at pre-stimulation and post TLR2 (PAM3CSK4) or TLR4 (LPS) stimulation between non-toxic (n = 19) and toxic (n = 13) participants[b].

		Non-toxic (n = 19)	**Toxic** (n = 13)	
	μg.mL^{-1}	**Mean** (range)	**Mean (**range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	2.0 (0.5 – 3.4)	1.8 (0.8 – 3.5)	0.833
	0.00005	2.5 (0.9 – 3.6)	2.1 (1.6 – 3.6)	0.544
	0.1	2.5 (1.5 – 3.9)	1.7 (2.3 – 3.7)	0.438
	100	2.7 (2.4 – 4.0)	1.8 (2.2 – 3.8)	0.160
PAM IL-1β	Pre-stimulation	2.0 (0.1 -2.6)	1.8 (1.0 – 3.0)	0.833
	0.00005	2.1 (0.2 – 3.0)	2.1 (1.5 – 3.0)	0.833
	0.01	2.6 (1.4 – 3.1)	2.2 (1.7 – 3.0)	0.350
	1	2.5 (1.9 – 3.1)	2.3 (1.7 – 2.9)	0.160
LPS TNF-α	Pre-stimulation	2.4 (1.2 – 3.8)	2.0 (1.4 – 3.8)	0.649
	0.00005	2.6 (0.9 – 3.9)	2.1 (1.4 – 3.3)	0.322
	0.1	2.4 (1.6 – 4.1)	2.5 (1.6 – 3.6)	0.350
	100	2.9 (1.4 – 4.5)	2.5 (2.0 – 3.7)	0.435
PAM TNF-α	Pre-stimulation	2.4 (1.0 – 3.7)	2.0 (1.2 – 3.9)	0.649
	0.00005	2.4 (1.0 – 3.7)	2.3 (0.4 – 2.8)	0.322
	0.01	2.5 (1.4 – 3.6)	2.2 (1.5 – 3.1)	0.322
	1	2.6 (1.3 – 3.8)	2.4 (1.6 – 3.0)	0.322

[b]LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; LPS TNF-α: TLR4 (LPS) stimulated TNF-α secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; PAM TNF-α: TLR2 (PAM3CSK4) stimulated TNF-α secretion.

4.6.3 *Significant differences in TLR2 (PAM3CSK4) stimulated IL-1β secretion between CASP1 and CASP5 genotypes were identified from univariate analysis*

One-way ANOVA determined significant differences in log IL-1β secretion pre- and post-stimulation with TLR2 (PAM3CSK4) concentration of 0.00005 $\mu g.mL^{-1}$ between participants with homozygous wild-type, heterozygous or homozygous variant genotype for *CASP1* rs580253 and *CASP5* rs554344 (P = 0.031, Table 4.3). The genotype for *CASP1* rs580253 and *CASP5* rs554344 was equivalent for each participant. Linkage disequilibrium analysis was not conducted.

No significant differences in TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated log IL-1β and TNF-α secretion between carriers of the homozygous wild-type, heterozygous or homozygous variant genotype for *TLR2*, *TLR4*, *IL1B* or *TNF* were identified (Appendix: Chapter 4).

Table 4.3 Natural log-transformed IL-1β secretion (pg.mL^{-1}) pre- and post-TLR2 (PAM3CSK4) or TLR4 (LPS) stimulation for carriers of the homozygous wild-type, heterozygous or homozygous variant genotypes for *CASP1* rs580253 and *CASP5* rs554344. The genotype for *CASP1* rs580253 and *CASP5* rs554344 was equivalent for each participant[c].

		WT/WT (n = 22)	**WT/Var** (n = 7)	**Var/Var** (n = 2)	
	μg.mL^{-1}	**Mean** (range)	**Mean** (range)	**Mean** (range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	2.0 (0.5 – 3.5)	1.4 (0.7 – 2.5)	2.6 (1.7 – 3.4)	0.254
	0.00005	2.4 (1.2 – 3.5)	1.8 (0.8 – 3.2)	2.6 (2.3 – 2.9)	0.291
	0.1	2.4 (1.5 - 3.9)	2.0 (1.9 – 3.1)	2.7 (2.4 – 3.0)	0.291
	100	2.5 (1.8 – 4.0)	2.4 (2.1 – 3.6)	2.6 (2.3 – 2.8)	0.644
PAM IL-1β	Pre-stimulation	2.0 (1.1 – 3.0)	1.1 (0.1 – 1.9)	2.2 (2.0 – 2.4)	0.008*
	0.00005	2.2 (1.6 – 3.0)	1.5 (0.2 – 2.4)	2.2 (2.1 – 2.2)	0.013*
	0.01	2.5 (1.7 – 3.1)	2.1 (1.4 – 2.7)	2.7 (2.4 - 2.9)	0.055
	1	2.5 (1.9 – 3.1)	2.2 (1.7 – 2.5)	2.7 (2.5 – 2.9)	0.055

[c] LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; WT/WT: homozygous wild-type genotype; WT/Var: Heterozygous genotype; Var/Var: Homozygous variant genotype.

4.6.4 The base ANCOVA model identified TLR2 (PAM3CSK4) and TLR4 (LPS) stimulation in conjunction with GI toxicity was predictive for log-transformed IL-1β secretion

The final ANCOVA model identified two covariates significantly associated with log IL-1β secretion ($P < 2.2 \times 10^{-16}$, Figure 4.1). The linear equations produced by the base ANCOVA model are below:

TLR2 (PAM3CSK4) stimulated log IL-1β secretion (toxic participants): $y = 0.10x + 5.45$

TLR2 (PAM3CSK4) stimulated log IL-1β secretion (non-toxic participants): $y = 0.16x + 6.21$

TLR4 (LPS) stimulated log IL-1β secretion (toxic participants): $y = 0.05x + 5.46$

TLR4 (LPS) stimulated log IL-1β secretion (non-toxic participants): $y = 0.11x + 6.21$

Firstly, the model identified stimulation with both TLR2 (PAM3CSK4) and TLR4 (LPS) agonists was significantly associated with log IL-1β secretion (Figure 4.1a). For both agonists, there was a concentration-dependent increase in log IL-1β secretion with a greater increase over the concentrations used for TLR2 (PAM3CSK4) as indicated by the steeper slopes of the association (Figure 4.1a).

The model also identified GI toxicity was significantly associated with log IL-1β secretion (Figure 4.1b). TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated log IL-1β secretion was higher in non-toxic participants, with a greater increase as indicated by the steeper slope of the association, compared to toxic participants (Figure 4.1b).

The final ANCOVA model identified no significant associations between TLR2 (PAM3CSK4) and TLR4 (LPS) stimulation, GI toxicity and the interaction between these covariates with log TNF-α secretion ($P > 0.05$).

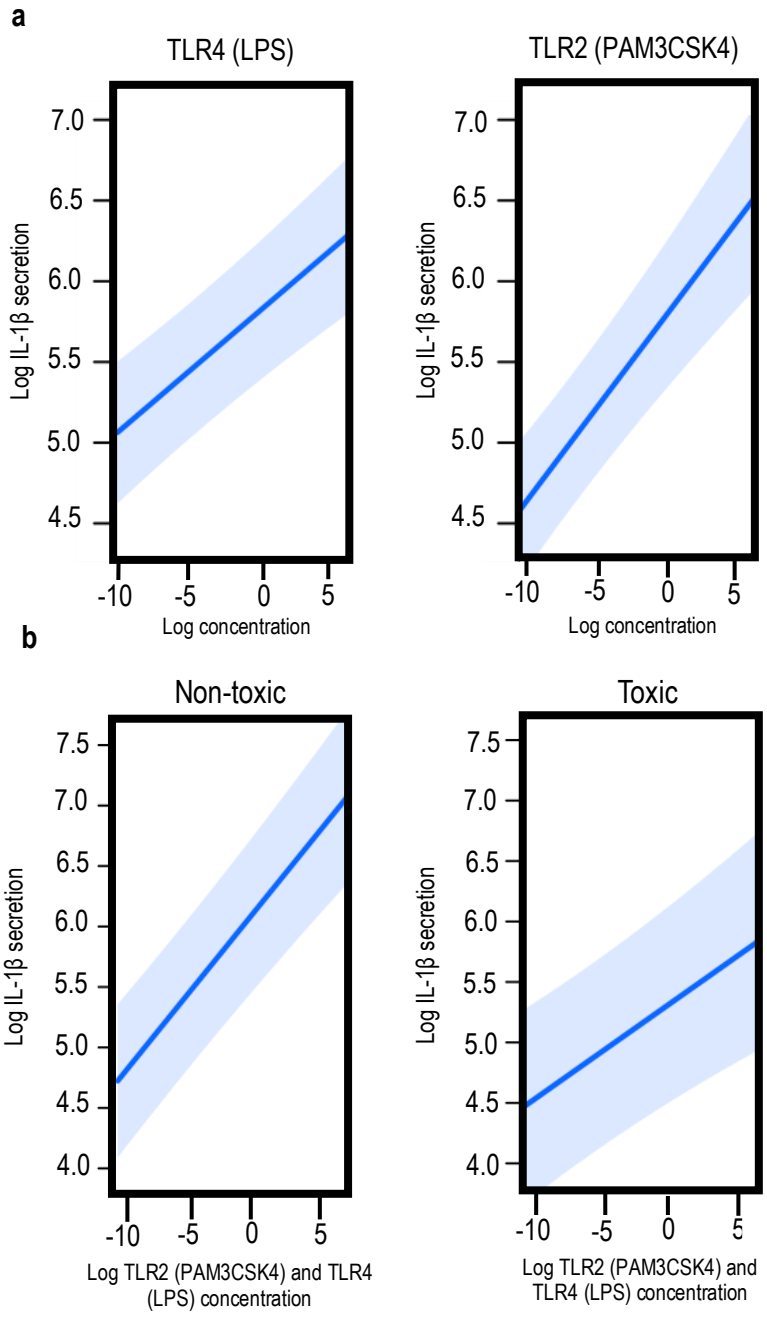

Figure 4.1 Linear regression models of natural log-transformed IL-1β secretion between a) TLR4 (LPS) and TLR2 (PAM3CSK4) stimulation and, b) non-toxic and toxic participants following TLR4 (LPS) and TLR2 (PAM3CSK4) stimulation. Blue shaded regions indicate the 95 % confidence intervals of the associations.

4.6.5 Addition of CASP1 and CASP5 SNPs improved the base ANCOVA model

Likelihood ratio tests identified *CASP1* rs580253 and *CASP5* rs554344 SNPs were associated with TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated log IL-1β secretion (P = 0.003, Figure 4.2). Following addition of *CASP1*/*CASP5* SNPs into the base ANCOVA model, the model identified these SNPs as predictive for TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated log IL-1β secretion. An overall P-value and linear equations for this ANCOVA could not be obtained.

Linear regression identified TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated log IL-1β secretion was significantly different between carriers of the homozygous wild-type, heterozygous and homozygous variant genotypes (Figure 4.2). Carriers of the heterozygous genotype (Figure 4.2b) demonstrated the steepest slope, followed by carriers of the homozygous wild-type genotype (Figure 4.2a) and finally, carriers of the homozygous variant genotype (Figures 4.2c). This indicates log IL-1β secretion increased at a greater rate over the TLR2 (PAM3CSK4) and TLR4 (LPS) concentrations in carriers of the heterozygous genotype compared to a gradual increase in secretion in carriers of the homozygous wild-type or homozygous variant genotypes.

Likelihood ratio tests identified no significant associations with participant clinical data (sex, 5-FU-based regimen, cancer type, age and number of treatment cycles) or SNPs for *TLR2, TLR4, IL1B or TNF* with TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated log TNF-α secretion (P > 0.05).

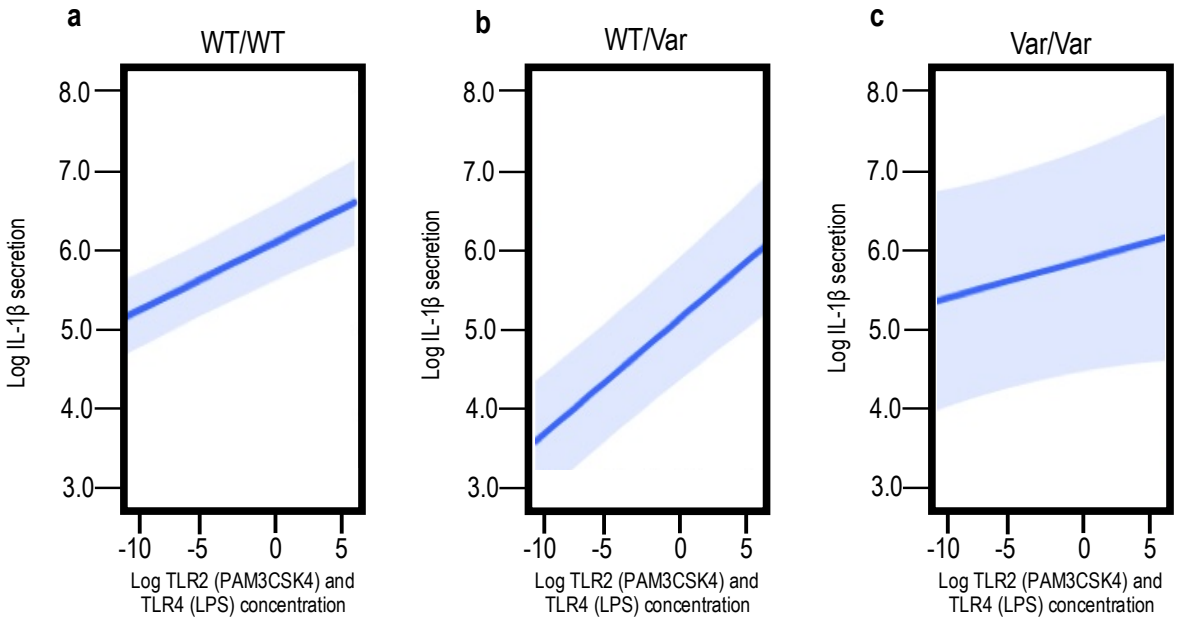

Figure 4.2 Linear regression models of natural log-transformed IL-1β secretion following TLR2 (PAM3CSK4) and TLR4 (LPS) stimulation between carriers of *CASP1* and *CASP5* a) homozygous wild-type (WT/WT), b) heterozygous (WT/Var) or c) homozygous variant (Var/Var) genotypes. Blue shaded regions indicate the 95% confidence intervals of the associations.

4.7 Discussion

Severe GI toxicity is a common and debilitating side effect following 5-FU-based therapy leading to sub-optimal therapy outcomes and decreased patient quality of life [2, 6]. It is crucial to identify predictive markers for severe GI toxicity to allow personalisation of supportive care measures for these patients. This retrospective study identified significant differences in TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion. Firstly, non-toxic participants (participants who developed no or mild to moderate GI toxicity) demonstrated increased stimulated IL-1β secretion over the concentrations investigated in comparison to toxic participants (participants who developed severe GI toxicity). Secondly, homozygous wildtype, heterozygous and homozygous variant genotypes of *CASP1* and *CASP5* demonstrated significantly different stimulated IL-1β secretion patterns independent of GI toxicity. As a result, this study identified the IL-1β secretory response as a candidate predictive phenotypic marker for severe GI toxicity risk.

This current study is the first to investigate stimulated IL-1β and TNF-α cytokine secretion post-5-FU-based therapy. Previous *ex vivo* analysis is limited to circulating IL-1β and TNF-α levels in plasma pre- and post-5-FU-based therapy and, have not associated circulating proinflammatory cytokine levels with the presence of GI toxicity. In patients with breast cancer receiving FEC (5-FU, epirubicin and cyclophosphamide), pre- and post-therapy circulating IL-1β and TNF-α levels in plasma were not significantly different [114]. Of note, baseline IL-1β and TNF-α levels in plasma were only detectable in 21 % and 19 % of patients, respectively [114]. In contrast, a similar study reported mean pre-therapy TNF-α plasma levels were significantly increased in an independent breast cancer cohort (n = 20) also receiving FEC ($P < 0.001$) compared to healthy controls [115]. However, this result was not compared to post-therapy TNF-α plasma levels [115].

It is difficult to compare pre- and post-stimulation levels of IL-1β and TNF-α from PBMCs to pre- and post-therapy circulating IL-1β and TNF-α levels in plasma. However, this current study

demonstrates measuring IL-1β and TNF-α secretion from pre- and post-stimulated PBMCs is more consistent in comparison to measuring circulating IL-1β and TNF-α levels. For example, pre-stimulated IL-1β and TNF-α were detectable in all participants in this current study compared to a previous study measuring IL-1β and TNF-α levels in plasma serum [114]. Additionally, on average, pre-stimulated IL-1β and TNF-α levels were log 1.44 and log 0.67 ($pg.mL^{-1}$) higher compared to the reported pre-therapy circulating IL-1β and TNF-α levels in plasma [115], respectively.

It is not surprising the pre-stimulation results presented were higher than the IL-1β and TNF-α pre-therapy plasma serum levels as participants in my study were not chemotherapy naïve and, innate immune memory may have influenced stimulated IL-1β and TNF-α secretion. Innate immune memory can occur following significant innate immune signalling (such as that experienced during 5-FU-induced mucosal injury) and leads to a change in reactivity in innate immune cells previously exposed to stimuli such as DAMPs or PAMPs [111, 116]. Post-exposure to DAMPs and PAMPs, innate immune cells such as monocytes and macrophages can become tolerant, resulting in a diminished innate immune response on secondary exposure [111, 116]. Innate immune memory has been reported in animal models, where challenges with microbial ligands prior to infection with *Escherichia Coli* (3×10^3 to 5.5×10^3 colony forming units) or *Pseudomonas aeruginosa* (4×10^5 colony forming units) resulted in diminished proinflammatory cytokine response and protection against sepsis and mortality [117-119].

The results of this present study support the hypothesis that innate immune memory diminishes the innate immune response to a greater extent in toxic participants. This is most likely a direct result of the increased exposure to DAMPs and PAMPs during their severe 5-FU-induced mucosal injury. Linear regression analysis produced a steeper slope for IL-1β secretion for non-toxic participants, indicating a higher response rate to stimulation with TLR2 (PAM3CSK4) and TLR4 (LPS) compared to toxic participants. Similar clinical studies investigating TLR2 (PAM3CSK4) and

TLR4 (LPS) stimulated IL-1β secretion from concentrations of 0.00005 to 100 μg.mL^{-1} between patients with chronic pain (another condition with an inflammatory basis) and healthy patients, also identified significant differences in TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion between the two patient groups [112, 120].

Although stimulated IL-1β secretion was significantly different between non-toxic and toxic participants, the degree of IL-1β secretion was also significantly different between TLR2 (PAM3CSK4) and TLR4 (LPS) stimulation. The response rate to TLR2 (PAM3CSK4) was higher than that of TLR4 (LPS), indicated by a steeper slope of association. This finding suggests dampening of TLR4 mediated IL-1β secretion may be more prominent than TLR2 mediated IL-1β secretion as a result of innate immune memory post-5-FU-based therapy. In comparison to chronic inflammatory conditions, such as chronic pain, TLR4 (LPS) stimulated IL-1β secretion has been reported to be higher than TLR2 (PAM3CSK4) stimulated IL-1β secretion [112, 120]. This comparison suggests TLR2 and TLR4 stimulated IL-1β secretion differs between acute inflammatory conditions, such as severe GI toxicity, and chronic inflammatory conditions, such as chronic pain, following innate immune memory.

Following completion of 5-FU-based treatment, GI toxicity symptoms, such as diarrhoea, may not be completely resolved. Up to 49 % of patients whom received chemotherapy (not specific to 5-FU) for colorectal cancer reported frequent and/or ongoing bouts of diarrhoea 4 - 5 years post-chemotherapy [121, 122]. This suggests pre- and/or post-stimulation of IL-1β and TNF-α secretion following 5-FU-based therapy may also be a predictive phenotypic marker of current GI inflammation and may assist in managing ongoing GI symptoms that persist post-5-FU-based therapy. However, in this study, it was not reported whether participants were experiencing GI symptoms such as diarrhoea post-5-FU-based therapy.

A potential association between IL-1β and genotype was also identified, further highlighting the importance of IL-1β secretion. The ANCOVA model also identified *CASP1* and *CASP5* genotypes were associated with TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion. *CASP1* encodes caspase-1, also known as interleukin-1β-converting enzyme, and is responsible for cleaving inactive precursor IL-1β to its active form [123]. *CASP5* encodes caspase-5 which plays an important role in apoptosis, inflammation and proliferation [124, 125]. *CASP1* rs580253 is a synonymous G > A mutation whilst *CASP5* rs554344 is an upstream gene variant [73]. The functional impact of these SNPs and their effect on IL-1β secretion is not reported [73]. Homozygous wild-type carriers secreted lower TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β over the concentration ranges compared to heterozygous and homozygous variant carriers. However, no consistent response of TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β secretion was identified as the presence of wild-type alleles decreased. In addition, in this study only two participants were homozygous variant carriers for *CASP1* rs580253 and *CASP5* rs554344, which is not reflective of the observed minor allele frequency (0.17) in the Caucasian population [73]. Therefore, the exact nature of association is unclear and future studies need to be conducted with an adequate number of participants with each genotype.

In comparison to other TIR domain genes of interest, no associations were identified between *TLR2*, *TLR4*, *IL1B* and *TNF* SNPs and stimulated TLR2 (PAM3CSK4) and TLR4 (LPS) IL-1β and TNF-α secretion. This is contrary to the hypothesis and despite *TLR2* rs384100 and *TNF* rs1800629 SNPs previously being identified as strong predictors for severe GI toxicity [25]. One explanation of this unexpected finding could be PBMCs were stimulated with TLR2 (PAM3CSK4) and TLR4 (LPS) agonists in place of stimulation of different cell populations that make up PBMCs. The PBMC population contains macrophages, monocytes, dendritic, B, T and Natural Killer cells, with the number of these cells highly variable across individuals [126, 127]. The secretion profile of these cells is also known to differ from one another, with macrophages and monocytes in

particular predominately responsible for IL-1β and TNF-α secretion [126, 127]. Therefore, it is possible some wells may have had a different population of cells from another, leading to variability in TLR2 (PAM3CSK4) and TLR4 (LPS) stimulated IL-1β and TNF-α secretion, limiting the ability to detect an impact of genotypes on secretion. This was a key limitation of this study. In future, isolating monocytes and macrophages by cell sorting methods such as fluorescence-activated cell sorting (FACS) and flow cytometry, similar to other studies [128], and stimulating identical numbers of these individual cells types with TLR2 (PAM3CSK4) and TLR4 (LPS) agonists may give a more accurate IL-1β and TNF-α secretion profile for each participant.

Another limitation of this study was the target sample size of 46 participants (n = 23 non-toxic and n = 23 toxic) was not reached. This sample size was selected to ensure the study was adequately powered to detect a 2-fold change in IL-1β and TNF-α secretion between non-toxic and toxic participants. However, only 32 participants (19 non-toxic and 13 toxic) were recruited within the time constraints of the study. In addition, ELISA assays on some supernatant samples needed to be repeated due to failure of the standard curves (R^2 < 0.99). As a result, some supernatant samples required multiple freeze-thaws. This meant degradation of IL-1β and TNF-α during the thawing process may have occurred and on repeat ELISA analysis, a lower concentration of IL-1β and TNF-α may have been detected in comparison to what was initially present. An optimisation experiment was conducted comparing fresh and multiple freeze thaw samples of supernatant following stimulation with TLR2 (PAM3CSK4) at 0.00005, 0.01 and 1 $\mu g.mL^{-1}$ and TLR4 (LPS) at 0.00005, 0.1 and 100 $\mu g.mL^{-1}$. It was identified IL-1β and TNF-α concentration did decrease, though this was only significant after 3 freeze-thaws (P = 0.031) (data not shown). In contrast, samples were only freeze-thawed a maximum of twice in this study. Therefore, although IL-1β and TNF-α may have been decreased following thawing, this was not likely to be significant and impact the results.

The results of this study need to be validated free of the limitations discussed. Furthermore, investigation in a prospective cohort following a similar study design is also required to determine when changes in IL-1β and TNF-α secretion occur as a result of innate immune memory post-5-FU-based therapy. If these changes are evident following the first cycle of 5-FU-based therapy, then pre- and post-stimulated IL-1β and TNF-α secretion may be predictive for severe GI toxicity from cycle two of therapy onwards. This would allow patients at most risk of severe GI toxicity to be identified and have their supportive care measures personalised from cycle 2 of therapy. This may not only decrease their risk of either developing or further developing severe GI toxicity but, also improve their quality of life whilst receiving 5-FU-based therapy and their long-term therapy outcomes.

4.8 Conclusion

To conclude, this retrospective study was the first to identify a significant difference in stimulated IL-1β secretion between non-toxic (participants who developed no or mild to moderate GI toxicity) and toxic (participants who developed severe GI toxicity) participants post-5-FU-based therapy. This indicates the IL-1β secretory response may be a potential predictive phenotypic marker for severe GI toxicity risk and highlights the importance of long-term changes in IL-1β secretion linked to GI toxicity incidence.

Chapter 5: 5-FU does not significantly inhibit DNA methylation within the TNF promoter region following treatment *in vitro*

Chapter 5 is presented as a traditional thesis chapter. As well as genetic variation, epigenetic modifications can also influence gene expression within the TIR domain innate immune signalling pathway. Therefore, epigenetic modifications may be potential predictive markers for severe GI toxicity risk. DNA methylation was selected as the epigenetic modification to be investigated in this chapter and tumour necrosis factor (*TNF*) was selected as the gene of interest. The reasoning behind this selection will be discussed in the chapter.

5.1 Introduction

Severe gastrointestinal (GI) toxicity, graded as ≥ 3 on the National Cancer Institute's Common Terminology for Adverse Effects (NCI CTCAE v 4.03 and v 5.0) [10], is a debilitating adverse effect of 5-FU-based therapy, with patients requiring dose reductions, treatment delays, early treatment cessation and/or hospitalisation to help manage and relieve symptoms [2, 30]. Not only does this compromise optimal therapy outcomes but, more importantly, decreases patient quality life whilst receiving therapy [6].

Proinflammatory cytokine tumour necrosis factor alpha (TNF-α), encoded by the gene *TNF*, is known to play a significant role in the development of severe GI toxicity following 5-FU-based therapy [5, 24]. 5-FU is an antimetabolite drug most commonly used for the treatment of solid tumours in the breast, oesophagus and colon [1, 41]. Administration of 5-FU initiates an innate immune response [2, 110], mediated by the Toll-like Receptor/Interleukin-1 (TIR) domain innate immune signalling pathway [22, 53, 58], subsequently leading to the production of TNF-α from peripheral monocytes and tissue macrophages [63, 64]. Secretion of TNF-α mediates an inflammatory response, reducing epithelial cell oxygenation, initiating mesenchymal-epithelial cell

signalling and further stimulating injury and death in cells of the epithelium and sub-mucosa, particularly in the vulnerable GI tract [2]. Cell injury and death throughout the GI tract can result in the manifestation of severe GI toxicity symptoms such as nausea, vomiting, mucositis and diarrhoea [2].

Increasing levels of TNF-α in the peripheral blood of patients receiving 5-FU-based therapy has been associated with the severity of GI toxicity [2, 24]. A single nucleotide polymorphism (SNP) in the *TNF* gene, rs1800629, has been identified as being predictive of severe GI toxicity symptoms (diarrhoea, mucositis, nausea and vomiting) in patients following 5-FU-based therapy [25]. Additionally, a *TNF* SNP, rs1799964, was also associated with the occurrence of severe stomatitis (also referred to as oral mucositis), also in patients receiving 5-FU-based therapies [95].

A study by Huizinga *et al* in whole blood cultures identified a relationship between lipopolysaccharide (LPS)-stimulated TNF-α secretion and a *TNF* SNP, rs361525, at a low LPS concentration (10 ng.mL^{-1}) in healthy participants ($P < 0.05$) [99]. However, at a high LPS concentration (1000 ng.mL^{-1}), no significant associations were identified between LPS-stimulated TNF-α secretion and the *TNF* SNPs rs1800629, 1800750 and rs361525 ($P > 0.05$) [99]. de Jong *et al* also identified in healthy individuals no associations between LPS-stimulated (1000 ng.mL^{-1}) TNF-α secretion and the *TNF* SNPs rs1800629, 1800750, rs361525 and rs80267959 ($P > 0.05$) [98]. The *in vitro* and *ex* vivo effect of these *TNF* SNPs on gene expression and TNF-α secretion has been discussed in Chapter 2 [106].

These results and, those previously discussed in Chapters 2 [106], 3 and 4 of my thesis, demonstrate genetic variation within *TNF* is not necessarily the only factor responsible for the change in TNF-α secretion observed during GI toxicity.

The increase in TNF-α secretion observed following 5-FU-based therapy may possibly be explained by epigenetic modifications, which influence gene expression without altering the DNA

sequence [129]. DNA methylation is a well-known epigenetic modification characterised by the addition of a methyl group by a family of enzymes known as DNA methyltransferases (DNMT), from S-adenosine methionine (SAM) to the 5-position of the cytosine ring [129]. DNA methylation predominately occurs at CpG dinucleotides, many of which are present throughout the promoter region of genes, including *TNF* [130]. Hypermethylated DNA results in inactive chromatin, leading to a decrease in gene transcription and subsequent decline in gene expression [129]. DNA methylation is reversible and can be easily influenced by an array of exogenous factors [129]. Inhibition of thymidylate synthase (TS) by 5-FU also simultaneously prevents the production of a key folinic acid derivative required for DNA methylation [1, 35]. Therefore, DNA methylation was selected as the epigenetic modification to be investigated in this thesis chapter.

5-FU is an uracil analogue [35]. The principal active metabolite of 5-FU, fluorodeoxyuridine monophosphate (FdUMP) forms a covalent complex with TS, a critical enzyme necessary for precursor deoxyribonucleotide synthesis cycle [1, 35]. TS also plays an important role in the folate cycle [1, 35]. Inhibition of TS by FdUTP prevents the conversion of dUMP to dTMP and blocks the simultaneous conversion of 5, 10-methylene tetrahydrofolate to dihydrofolate, a key folinic acid derivative [1, 35]. Dihydrofolate recycles methyl groups and is the source of carbon donors required for methionine synthesis used for SAM generation [129]. SAM is the primary methyl donor required by DNMT1 and DNMT3A for DNA methylation [129]. With inhibition of TS and consequently, no production of dihydrofolate, intracellular levels of methionine and SAM are depleted [131]. Depleted levels of methionine and SAM can result in decreased DNA methylation which can subsequently upregulate gene transcription and overall gene expression [131].

In vitro, 5-FU has been identified to influence DNA methyltransferase activity [132, 133]. In a human lung cancer cell line, DNMT1 and DNMT3A protein as well as DNMT1 and DNMT3A mRNA expression was decreased following 5-FU treatment (2 - 200 µM). However, this was restored when 5-FU was administered in combination with SAM [132]. In another study using DNA-based

hybridisation chain reaction to determine activity of methyltransferase (M.ssl MTase, isolated from *E.coli*), Xu *et al* identified 5-FU (0 - 400 μM) inhibited M.ssl MTase and, inhibition was proportional to the logarithmic value of M.ssl MTase concentration [133]. These results demonstrate the ability of 5-FU to inhibit DNA methylation.

The effect of 5-FU on DNA methylation throughout the *TNF* promoter gene region is unknown. However, differences in methylation at CpG sites throughout the *TNF* promoter gene region have been identified in conditions with a similar underlying inflammatory basis as GI toxicity. Stephens *et al* identified at CpG sites -350, -344 and -342 (also referred to as -169, -163 and -161, respectively, upstream of the *TNF* transcription start site), DNA methylation was increased in patients with mild breast pain following breast cancer surgery compared to patients with no breast pain following surgery ($P < 0.05$) [134]. Logistic regression also analysed breast cancer pain with multiple covariates (such as DNA methylation, SNPs (including *TNF* rs1800610), pain group, presence of preoperative pain, genomic estimates of self-reported race/ethnicity and mastectomy six months post-surgery). The model determined DNA methylation at CpG sites -350 (-169) and -344 (-163) was significantly associated with mild breast pain following breast cancer surgery compared to other covariates ($P = 0.024$ and $P = 0.033$, respectively) [134]. Similarly, dental patients with periodontitis showed increased DNA methylation at CpG sites -163 and -161 compared to patients with no periodontitis ($P = 0.02$ and $P = 0.04$, respectively) [135]. An inverse correlation between DNA methylation at CpG site -163 and TNF mRNA expression was also identified ($R^2 = 0.16$, $P = 0.018$) [135]. The findings of Stephens *et al*. and Zhang *at al*. support the concept that DNA methylation within the *TNF* promoter region may differ between patients who develop severe GI toxicity (grade ≥ 3 on the NCI CTCAE v 4.03 and v 5.0) compared to patients who develop no (grade 0) or, moderate to mild GI toxicity (grade 1 or 2 on the NCI CTCAE v 4.03 and v 5.0) following 5-FU-based therapy.

A decrease in DNA methylation has been identified to increase *TNF* transcriptional expression *in vivo*, suggesting administration of potential DNA methylation inhibitor 5-FU, will increase *TNF* transcriptional expression. In addition to associating DNA methylation of selected *TNF* CpG sites with the presence of periodontitis, Zhang *at al*. [135] also investigated the effect of DNA methylation inhibitor 5-Aza-2'-deoxycytidine (5-Aza-dc) on *TNF* transcriptional expression in the monocytic-like THP-1 cell line. At 24 h post-5-Aza-dc treatment, messenger RNA level of *TNF* was 1.6-fold higher ($P = 0.03$) and, 3-fold higher 96 h post-5-Aza-dc treatment ($P = 0.003$) in comparison to mock treated cells [135]. This finding implies that although inflammatory conditions such as breast pain and periodontitis have been associated with increased DNA methylation at selected *TNF* CpG sites, during severe GI toxicity induced by 5-FU, DNA methylation at these CpG sites may be reduced. Therefore, increasing *TNF* transcriptional activity, TNF mRNA expression and TNF-α secretion.

The results discussed above led to the hypothesis 5-FU administration may reduce DNA methylation throughout the *TNF* promoter gene region and potentially increase *TNF* gene transcription. This in turn may increase TNF-α secretion and therefore, increase the risk of severe GI toxicity. *TNF* was selected as the gene of interest for this thesis chapter as it has been consistently implicated with severe GI toxicity [25, 95] and, contains multiple CpG sites within the promoter region prone to DNA methylation [136]. In comparison, other TIR domain innate immune signalling pathway genes such as *TLR2*, *TLR4* and *IL1B* do not have multiple CpG sites throughout their promoter regions [136]. Therefore, DNA methylation would not be significant within the promoter region to influence gene expression. This current study was designed to investigate the concept that 5-FU can inhibit DNA methyltransferase mRNA expression and therefore, reduce DNA methylation across CpG sites throughout the *TNF* promoter gene region in monocytes.

5.2 Hypotheses and aims

This study hypothesised:

1. 5-FU reduces mRNA expression of DNMT1 and DNMT3A therefore, inhibits DNA methylation across CpG sites -244, -238, -169, -163, -161, -149, -119, -72, -49 and -38 upstream of the *TNF* transcription start site in U937 cells *in vitro*.

Aims

1. To optimise 5-FU and 5-Aza-2'-deoxycytidine (5-Aza-dc) concentration in the U937 cell line to determine a treatment dose that would not only induce cell injury and death but, result in a viable cell count that would allow adequate DNA and RNA extraction.
2. To determine changes in mRNA expression of TNF, DNMT1 and DNMT3A following treatment with 5-FU and/or methylation inhibitor 5-Aza-dc in U937 cells *in vitro* using real-time polymerase chain reaction (RT-PCR).
3. To determine changes in DNA methylation at CpG sites -244, -238, -169, -163, -161, -149, -119, -72, -49 and -38 upstream of the *TNF* transcription start site following treatment with 5-FU and/or methylation inhibitor 5-Aza-dc in U937 cells *in vitro* using methylation specific high-resolution melt (MS-HRM).

Chapter 5 is the only non-clinical study presented in my thesis. This novel study was conducted *in vitro* and, designed to investigate the concept that 5-FU can inhibit DNMT mRNA expression and therefore, reduce DNA methylation across selected CpG sites throughout the *TNF* promoter region. DNA methylation is an epigenetic modification that can be influenced by 5-FU. Differences in DNA methylation within genes such as *TNF* following 5-FU administration may lead to increased TNF-α and potentially, increase the risk of mucosal injury and the development of GI toxicity. Therefore, DNA methylation patterns may be potential predictive marker for severe GI toxicity risk. Depending

on the results of this chapter, future research may be conducted *ex vivo* using peripheral blood mononuclear cells (PBMCs) isolated from participants.

In addition, although there are 22 CpG sites throughout the *TNF* promoter region, CpG sites -244, -238, -169, -163, -161, -149, -119, -72, -49 and -38 were selected for analysis in this thesis chapter. All these sites have previously been investigated and alterations in DNA methylation at these CpG sites have previously been reported [134, 135].

5.3 Methods

5.3.1 Reagents

RPMI + L-glutamine cell culture media, foetal bovine serum (FBS), penicillin streptomycin (Pen Strep), Dulbecco's phosphate buffer solution (DPBS), SYTO9™ green fluorescent nucleic acid stain and trypan blue stain were obtained from Thermo Fisher Scientific (MA, USA). Dimethyl sulfoxide (DMSO), 5-Aza-2'-deoxycytidine (5-Aza-dc), sterile distilled H_2O (Milli-Q®), 100 % methylated DNA and 100 % unmethylated DNA were obtained from Merck (Darmstadt, Germany). 5-FU was obtained from Hospira (IL, USA). Quantitect SYBR® green polymerase chain reaction (PCR) master mix, 10 X PCR buffer, RNase-free water, magnesium chloride (MgCl) and HotStarTaq™ DNA polymerase were obtained from QIAGEN (CA, USA). Deoxyribonucleotide (dNTP) solution mix was obtained from New England Biolabs (MA, USA). Enriched media was prepared using RPMI + L-glutamine, 10 % FBS and 1 % Pen Strep.

5.3.2 Culture of U937 cells

5.3.2.1 Establishing cell culture

Cells of the human myeloid cell line U937 were obtained from the European Collection of Authenticated Cell Culture (ECACC 85011440). U937 cells were selected for this study as they exhibit monocytic-like properties [137]. TNF-α can be secreted from peripheral monocytes [63, 64]

therefore, it was essential the cell line selected for this study exhibited monocytic-like properties. To establish cell culture, cells were removed from liquid nitrogen and rapidly thawed in a water bath at 36 °C. Once thawed, cells were transferred to a 15 mL Falcon® tube containing 4 mL of pre-warmed enriched media. Cells were centrifuged at 300 x g for 5 min and supernatant was removed and discarded. The cell pellet was resuspended in 4 mL of DPBS and cells were centrifuged again at 300 x g for 5 min to remove residual cryoprotectant. Supernatant was removed and discarded, and cells resuspended in 10 mL of pre-warmed enriched media. Viable cell count was determined using trypan blue staining. Briefly, 10 µL of cells were mixed thoroughly with 10 µL of trypan blue in a 96-well plate. 10 µL of the 1:1 mixture was loaded into a counting slide and placed in a T20 automated cell counter (Bio-Rad Laboratories Inc., CA, USA). The total cell count was 4.96 x 10^4 with 92 % cell viability. Cells were subsequently seeded in a Corning® T25 vented flask (Corning Incorporated, NY, USA) and placed in a humified incubator (Sanyo, Japan) at 37 °C and 5 % CO_2. Cell growth was assessed daily using trypan blue staining as well as under a phase contrast microscope (Nikon, Japan). After 72 h, cells were confluent with a total cell count was 1.37 x 10^6 and 87 % cell viability. Cells were centrifuged at 300 x g for 5 min and supernatant was removed and discarded. The cell pellet was resuspended in 2 mL of pre-warmed enriched media and cells were split, with 1 mL of cells added to two 50 mL Falcon® tubes containing 24 mL of pre-warmed enriched media. Cells were mixed by inversion and seeded in two Corning® T75 vented flasks (Corning Incorporated, NY, USA) and incubated at 37 °C and 5 % CO_2.

5.3.2.2 Maintaining cell culture

U937 cells were grown and maintained at a cell count of 1 x 10^6 cells in 25 mL. Cells were checked every 48 h. Firstly, viable cell count was determined using trypan blue staining as previously described. When viable cell count was less than 1 x 10^6 cells, cells were centrifuged at 300 x g for 5 min, supernatant was removed and the cell pellet was resuspended in 25 mL of pre-warmed enriched media. Cells were returned to their respective Corning® T75 flasks and incubated at 37

°C and 5 % CO_2 for a further 48 h. When viable cell count was greater than 1 x 10^6 cells, cells were split or cryopreserved. To split cells, cells were transferred to a 50 mL Falcon® tube and centrifuged at 300 x g for 5 min. Supernatant was removed and the cell pellet was resuspended in 2 mL of pre-warmed enriched media. 1 mL of cells were added to two 50 mL Falcon® tubes each containing 24 mL of pre-warmed enriched media. Cells were mixed by inversion, seeded in new Corning® T75 vented flasks and incubated at 37 °C and 5 % CO_2 for 48 h. For cryopreservation, cells were centrifuged at 300 x g for 5 min and supernatant was removed. The cell pellet was resuspended in 7.5 mL of RPMI + L-glutamine supplemented with 20 % DMSO and 10 % FBS and cryovials were filled with 1.5 mL of resuspended cells. Cryovials were cooled slowly overnight at -80 °C using a CoolCell® (Biocision, CA, USA) and then transferred to liquid nitrogen storage the following day. The above procedures were repeated until cells reached passage 10, at which time they were used for treatment 5-Aza-dc, 5-FU or 5-Aza-dc in combination with 5-FU.

5.3.3 5-FU and 5-Aza-dc optimisation

5-Aza-dc is a cytidine analogue and potent methylation inhibitor irreversibly binding DNMT1, leading to a decrease in global DNA methylation during DNA replication [138]. 5-Aza-dc was included in treatment as a positive control. Based on previous literature using a THP-1 cell line, a monocyte-like cell line with similar properties to U937, it was determined cells would be treated with 5-Aza-dc at a concentration of 5 µM [135]. As the combined effect of 5-Aza-dc and 5-FU was unknown, 5-FU concentration needed to be optimised. This was to ensure 5-FU would induce cell death in combination with 5-Aza-dc and, cell viability following treatment would be adequate to extract DNA and RNA from cells.

Prior to drug treatment, cells were serum starved for 24 h to induce cell cycle arrest in the G1 phase. As both 5-FU and 5-Aza-dc are antimetabolites, adding these drugs to cells in G1 arrest increases the likelihood of these antimetabolites becoming incorporated in newly synthesised DNA.

Briefly, cells were centrifuged at 300 x g for 5 min and supernatant was removed and replaced with RPMI + L-glutamine supplemented with 1 % Pen Strep. Cells were incubated at 37 °C and 5 % CO_2 for 24 h. Following serum starvation, viable cell count was determined using trypan blue staining. Cells were centrifuged at 300 x g for 5 min and supernatant was removed and replaced with pre-warmed enriched media, diluting the cells to a working viable cell count of 2 x 10^5 cells.

In a Corning® 24-well plate, 1.2 mL of 2 x 10^5 viable cells were plated and 5-FU was added in triplicate to give a final concentration of 0, 10, 30, 40 and 50 µM in combination with 5 µM 5-Aza-dc. 50 mg 5-Aza-dc was dissolved in 1 mL of Milli-Q® prior to treatment and diluted to a working concentration of 50 µM using Milli-Q®. Aliquots were stored at -80 °C. 5-FU was supplied at a concentration of 1 g in 10 mL sterile water and was diluted to working concentrations prior to treatment using Milli-Q®. Working drug concentrations were made 10-fold higher than the desired concentration to reflect the 1/10 dilution that occurred when added to cell medium. Cells were treated with 5-FU and 5-Aza-dc at 0, 24 and 48 h and, cell viability was measured using trypan blue staining prior to each treatment time point. Total cell count and live cell count were averaged between the replicates and percentage cell viability was calculated for each 5-FU concentration by: average live cell count/average total cell count x 100. Optimisation was repeated twice over a two-week period. The optimisation results are presented in Figure 5.1 and will be discussed in the results.

5.3.4 5-FU and 5-Aza-dc treatment

From the optimisation experiments (Figure 5.1), it was determined cells would be treated in triplicate on six separate occasions to produce six biological replicates and six sets of independent results as follows: vehicle control (Milli-Q®), 5 µM 5-Aza-dc, 40 µM 5-FU or, 5 µM 5-Aza-dc in combination with 40 µM 5-FU. Cells were treated at 0 and 24 h time points, with cell viability measured at each time point. Prior to treatment, cells were serum starved for 24 h and diluted to a

working viable cell count of 2 x 10^5 as described previously. At 48 h, well contents were collected, pooled for each treatment group and percentage cell viability was calculated for each treatment group by: average live cell count/average total cell count x 100. Well contents were then stored at -20 °C until RNA or DNA isolation. Cells were treated in triplicate on six separate occasions to produce six biological replicates and six sets of independent results.

5.3.5 Gene expression of TNF, DNMT1 and DNMT3A using Real-Time Polymerase Chain Reaction (RT-PCR)

5.3.5.1 RNA isolation

Total RNA was isolated from 2 mL of cells at a minimum concentration of 2 x 10^5 cells using the NucleoSpin® RNA Isolation Kit as per the manufacturer's instructions (Macherey-Nagel, Germany). Cells were centrifuged at 15,000 x g for 2 min and supernatant was discarded. To lyse cells, the cell pellet was resuspended in 350 µL of RA1 buffer and 3.5 µL of β-mercaptoethanol was added and vortexed vigorously. Following vortexing, 350 µL of 70 % ethanol was added, the sample was again vortexed and loaded into a NucloSpin® RNA column placed in a 2 mL collection tube. To bind RNA to the silica membrane, the column was centrifuged at 11,000 x g for 30 sec and flow-through was discarded from the collection tube. To desalt the silica membrane, 350 µL of membrane desalting buffer was added and the column was centrifuged at 11,000 x g for 1 min. To remove genomic DNA, 95 µL of rDNase reaction mixture was added to the matrix of the column and incubated at room temperature for 15 min. Following incubation, 200 µL of buffer RAW2 was added and the column was centrifuged at 11,000 x g for 30 sec. The column was placed in a new 2 mL collection tube and 600 µL of buffer RA3 was added to the column and centrifuged again at 11,000 x g for 30 sec. The flow through was discarded and 250 µL of buffer RA3 was added to the column and centrifuged at 11,000 x g for 30 sec. The column was placed in a 1.5 mL microcentrifuge tube and RNA was eluted by adding 60 µL of RNase-free water and centrifuged

at 11,000 x g for 1 min. RNA was quantified using the Synergy™ Mx Microplate Reader (BioTek Instruments, VT, USA) and was stored at -20 °C until cDNA conversion.

5.3.5.2 cDNA synthesis

Isolated RNA was converted to cDNA using the iScript cDNA synthesis kit as per manufacturer's instructions (Bio-Rad Laboratories Inc). Briefly, 4 µL of 5 x iScript and 1 µL of iScript reverse transcriptase was added to 15 µL of RNA in a 0.65 mL PCR tube. The following incubation was run on an FTS320 Thermal Sequencer (Corbett Research, NSW, Australia): 25 °C for 5 min, 46 °C for 20 min, 95 °C for 1 min. Following incubation, cDNA was quantified using the Synergy™ Mx Microplate Reader. cDNA was immediately diluted to 100 ng/µL using RNase-free water and stored at -20 °C until RT-PCR analysis.

5.3.5.3 RT-PCR

Primers used for RT-PCR were designed using Primer3 [139] and purchased from Integrated DNA Technologies (IA, USA) (Table 5.1). Gene expression was normalised to the housekeeping gene GAPDH. GAPDH has previously been used as a housekeeper gene in RT-PCR following 5-FU treatment [132, 140, 141]. RT-PCR reactions contained 1 µL cDNA sample (100 ng.µL^{-1}), 5 µL Quantitect SYBR® Green PCR Master Mix, 0.5 µL of each forward and reverse primer (50 µM) and 3 µL RNase-free water to make a total volume of 10 µL. All samples were run in triplicate and all assay runs contained a non-template control. Using the Rotor-Gene Q (QIAGEN), each PCR consisted of a hold at 95 °C for 15 min followed by 40 cycles of 95 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec. Cycle threshold (C_t) values were calculated and C_t cut-off was set while viewing fluorescent readings in log scale. Melt curve analysis was conducted to ensure no amplification of non-specific products.

Table 5.1 a) RT-PCR and b) MS-HRM primers.

a RT-PCR primers

Gene of interest	Primer sequence	Melting temperature (°C)	Product size (base pairs)
TNF	Forward: 5'-AATCAGTCAGTGGCCCAGAAG-3'	57	95
	Reverse: 3'-AAAGTTGGGGACACACAAGC-5'	56	
DNMT1	Forward: 5'-ACTGCTGGGTTTCAAATGCC-3'	56	78
	Reverse: 3'-TGAAACACCAAGGCACCAAG-5'	56	
DNMT3A	Forward: 5'-TTTGCTTGACAGTGGTTGCG-3'	57	83
	Reverse: 3'-TGCAGCGGCTTTTCTATAGC-5'	56	
GAPDH	Forward: 5'-CTCTCTGCTCCTCCTGTTCGAC-3'	59	69
	Reverse: 3'-TGAGCGATCTGGCTCGGCT-5'	61	

b MS-HRM primers

CpG sites included within the primer region	Primer sequence	Melting temperature (°C)
-244, -238	Forward: 5'-TAGGTTTTGAGGGGTATGGG-3'	54
	Reverse: 5'-TCAAAAATACCCCTCACACTCC-3'	55
-169, -163, -161	Forward: 5'-GAGTGTGAGGGGTATTTTTGATG-3'	54
	Reverse: 5'-GCAACCATAATAAACCCTACACCTTC-3'	56
-149, -119	Forward: 5'-GCAACCATAATAAACCCTACACCTTC-3'	54
	Reverse: 5'-CAACCAACCAAAAACTTCCTTAAT-3'	52
-72, -49, -38	Forward: 5'-GAGGGGTATTTTTGATGTTTGTGT-3'	54
	Reverse: 5'-CCAACAACTACCTTTATATATCCC-3'	51

5.3.6 DNA methylation analysis of the TNF gene using methylation-specific high-resolution melt (MS-HRM)

5.3.6.1 DNA isolation

DNA was isolated from 2 mL of cells at a minimum concentration of 2 x 10^5 cells using the DNeasy® Blood and Tissue Kit (QIAGEN). Cells were centrifuged at 15,000 x g for 2 min and supernatant was discarded. To lyse cells, the cell pellet was resuspended in 200 µL DPBS and, 20 µL of protein kinase K and 200 µL of Buffer AL was added. The sample was vortexed vigorously and incubated at 56 °C for 10 min. Following incubation, 200 µL of 100 % ethanol was added, the sample was vortexed and loaded into a DNeasy® Mini spin column placed in a 2 mL collection tube. The column was centrifuged at 6,000 x g for 1 min and transferred to a new 2 mL collection tube. 500 µL of Buffer AW1 was added and the column was centrifuged at 6,000 x g for 1 min. The flow through was discarded and 500 µL of Buffer AW2 was added. The column was centrifuged for at 15,000 x g for 3 min and transferred to a new 1.5 mL microcentrifuge tube. DNA was eluted by adding 200 µL Buffer AE to the matrix of the column membrane, incubating the column for 1 min at room temperature and centrifuged at 6000 x g for 1 min. DNA concentration was quantified using the Synergy™ Mx Microplate Reader and stored at 4 °C until bisulfite modification.

5.3.6.2 Bisulfite modification of DNA

Isolated DNA was diluted to 300 ng.µL^{-1} using RNase-free water and bisulfite modified using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA). In addition, 300 ng.µL^{-1} of 100 % methylated DNA and 100 % unmethylated DNA were also bisulfite modified using the beforementioned kit. 100 % methylated DNA and 100 % unmethylated DNA were used as positive and negative controls, respectively, in MS-HRM. 130 µL of CT conversion reagent was added to 20 µL of diluted DNA in a 0.65 mL PCR tube and mixed by vortexing. The following incubation was run on an FTS-320 Thermal Sequencer: 98 °C for 10 min and 64 °C for 2.5 h. Immediately after

incubation, the sample was loaded into a Zymo-Spin™ IC Column containing 600 µL of M-Binding Buffer placed in a collection tube. The sample was mixed in the column by inversion, and the column was centrifuged at 10,000 x g for 30 sec. The flow through was discarded and 100 µL of M-Wash Buffer was loaded into the column. The column was centrifuged again at 10,000 x g for 30 sec and flow through was discarded. 200 µL of M-Desulphonation Buffer was loaded onto the matrix of the column and incubated at room temperature for 20 min. Following incubation, the column was centrifuged at 10,000 x g for 30 sec. 200 µL of M-Wash Buffer was added to the column and centrifuged again at 10,000 x g for 30 sec. This step was repeated twice. Bisulfite modified DNA was eluted by placing the column in a new 1.5 mL microcentrifuge tube, adding 10 µL of M-Elution Buffer directly to the column matrix and centrifuged at 10,000 x g for 30 sec. The concentration of bisulfite modified DNA was quantified using the Synergy™ Mx Microplate Reader. Bisulfite modified DNA was immediately diluted to 100 ng.µL^{-1} and stored at -20°C until MS-HRM analysis.

5.3.6.3 MS-HRM

Primers used for MS-HRM were from previous literature and purchased from Integrated DNA technologies (IA, USA) (Table 5.1) [135]. Each primer set included a number of CpG sites (Table 5.1). MS-HRM reactions contained 1 µL bisulfite modified DNA (100 ng.µL^{-1}), 2.5 µL 10 x PCR buffer, 1.5 µL MgCl (25 mM), 2 µL dNTP solution mix (2.5 mM), 1.5 µL of each forward and reverse primer (50 µM), 0.75 µL SYTO™ 9, 0.25 µL HotStarTaq™ DNA Polymerase and 9 µL RNase-free water to make a total volume of 20 µL. All samples were run in duplicate and for each primer pair, a reaction containing bisulfite modified 100 % methylated and 100 % unmethylated DNA was included as a positive and negative control, respectively. Using the Rotor-Gene 600 (Corbett Research, NSW, Australia) each MS-HRM consisted of a hold at 95 °C for 15 min followed by 50 cycles of 95 °C for 5 sec, 60 °C for 10 sec and 72 °C for 10 sec. Following cycling, a hold at 50 °C

for 30 sec occurred before HRM was run for 1 cycle of 0.1 °C temperature increases every 12 sec rising from 65 °C to 95 °C. Fluorescence values were calculated by Rotor-Gene Q analysis software (Version 2.3, QIAGEN).

5.3.7 Statistical Analysis

Data was tested for normality using the D'Agostino & Pearson test. In the optimisation experiment, data was normally distributed therefore, differences in cell viability between 5-FU concentrations was determined using t-tests. Correlation analysis was performed using Pearson correlation between 5-FU concentration alone or in combination with 5-Aza-dc and percentage cell viability. Differences in viability between cells treated with vehicle control, 5-Aza-dc, 5-FU or 5-Aza-dc in combination with 5-FU was determined using either the Friedman or Wilcoxon test with Dunn's multiple comparison test as data was not normally distributed. For RT-PCR analysis, C_t values were analysed using the ΔC_t method [142] to calculate relative expression. Relative expression was then compared between cells treated with vehicle control, 5-Aza-dc, 5-FU or 5-Aza-dc in combination with 5-FU using the Friedman test and Dunn's multiple comparison test as data was not normally distributed. For MS-HRM analysis, fluorescence values for the four treatment groups (vehicle control, 5-Aza-dc, 5-FU or 5-Aza-dc in combination with 5-FU) as well as for the 100 % methylated DNA positive control, were normalised to the 100 % unmethylated DNA negative control. Normalisation corresponded to the percentage of DNA methylation for each sample and this was then compared between cells from each treatment group using the Friedman test and Dunn's multiple comparison test as data was not normally distributed.

5.4 Results

5.4.1 Optimisation of 5-FU and 5-Aza-dc in U937 cells

Percentage cell viability was highly correlated with 5-FU concentration alone (R^2 = 0.939, P = 0.007) and, 5 μM 5-Aza-dc in combination with 5-FU concentration (R^2 = 0.924, P = 0.009) following 48 h exposure (Figure 5.1).

5-FU at concentrations of 30 μM (P = 0.024), 40 μM (P = 0.019) and 50 μM (P = 0.002) induced significant cell death compared to untreated cells (Figure 5.1). 5 μM 5-Aza-dc alone did not induce significant cell death compared to untreated cells (P = 0.051) (Figure 5.1). However, 5 μM 5-Aza-dc treated in combination with 5-FU at either 30 μM (P < 0.001), 40 μM (P < 0.001) or 50 μM (P < 0.001) induced significant cell death compared to untreated cells (Figure 5.1).

When comparing 5-FU treated alone or in combination with 5 μM 5-Aza-dc, only a significant difference in cell viability between cells treated with 40 μM 5-FU alone or 5 μM 5-Aza-dc in combination with 40 μM 5-FU was determined (P < 0.001). No significant differences between cells treated with i) 10 μM 5-FU alone or 5 μM 5-Aza-dc in combination with 10 μM 5-FU (P = 0.068), ii) 30 μM 5-FU alone or 5 μM 5-Aza-dc in combination with 30 μM 5-FU (P = 0.213) and iii) 50 μM 5-FU alone or 5 μM 5-Aza-dc in combination with 50 μM 5-FU (P = 0.051) were identified.

From the optimisation experiment, it was determined cells would be treated with 5 μM 5-Aza-dc, 40 μM 5-FU or 5 μM 5-Aza-dc in combination with 40 μM 5-FU. Median cell viability for cells treated with 40 μM 5-FU alone or with 5 μM 5-Aza-dc in combination with 40 μM 5-FU was 68 % and 56 %, respectively (Figure 5.1). This confirmed the concentration of 40 μM 5-FU was sufficient to induce DNA damage and cell death as well as, result in adequate cell viability necessary for DNA and RNA extraction from cells.

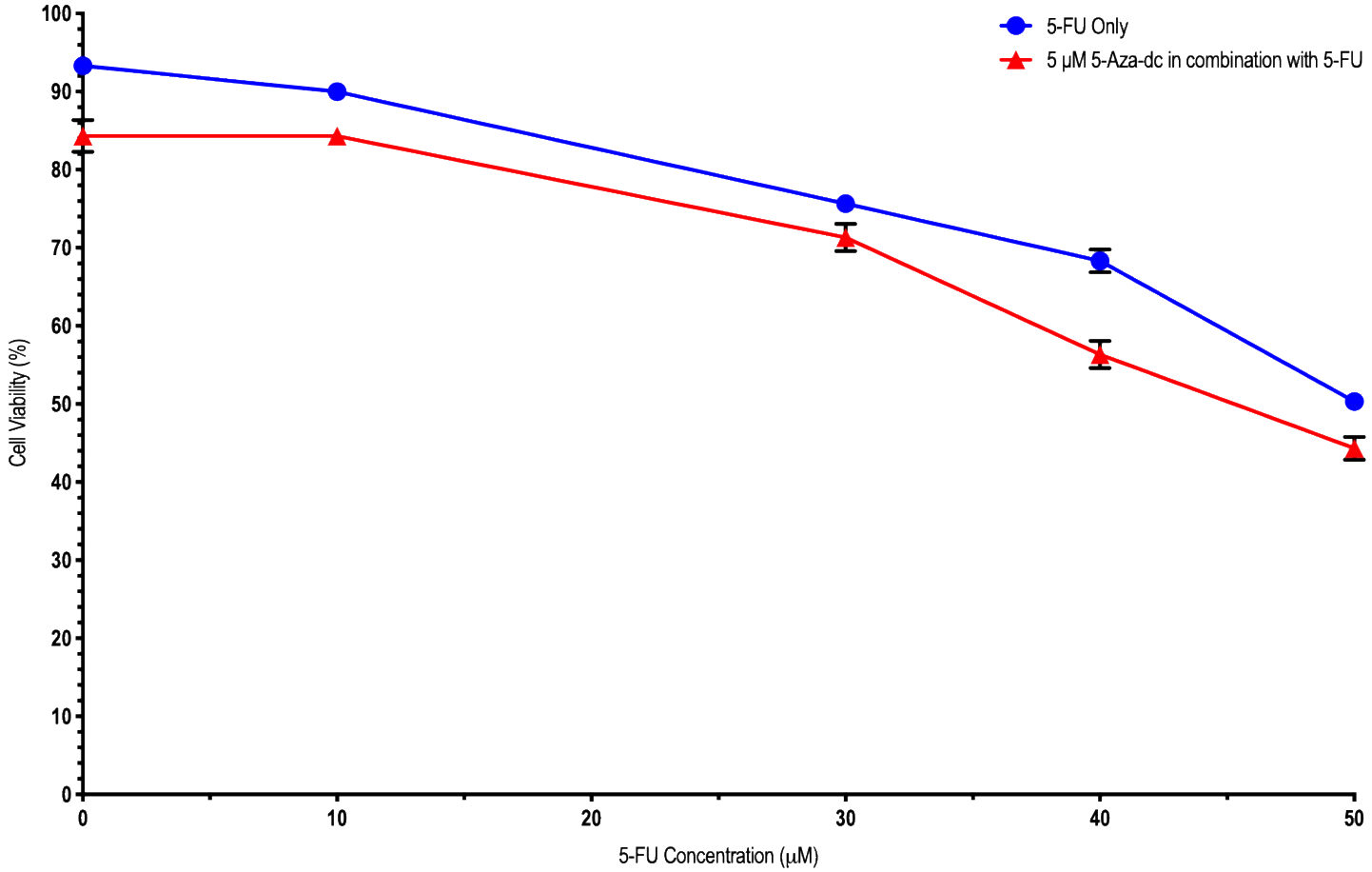

Figure 5.1 Optimisation of 5-FU and 5-Aza-dc in U937 cells to determine a treatment dose that will not only induce cell injury and death but, result in a viable cell count that will allow adequate DNA and RNA extraction. Data is presented as mean +/- SEM (n = 6).

5.4.2 5-FU and 5-Aza-dc induce cell death

At 24 h following treatment, median viability of cells treated with 5-FU was 77 % (P = 0.044) and those treated with 5-Aza-dc in combination with 5-FU was 73 % (P = 0.007), significantly decreased compared to cells treated with vehicle control at 91 % median cell viability (Figure 5.2a). At 48 h (48 h post-first treatment and 24 h post-second treatment), median viability of cells treated with 5-FU was 70 % (P = 0.022) and those treated with 5-Aza-dc in combination with 5-FU was 57 % (P < 0.001), which was also significantly decreased compared to cells treated with vehicle control at 93 % median cell viability (Figure 5.2b). No significant differences in median cell viability were identified between cells treated with vehicle control and 5-Aza-dc at 24 h and 48 h (P = 0.202 and P > 0.999, respectively). The significant cell death observed in cells treated with 5-FU or 5-Aza-dc in combination with 5-FU, compared to untreated cells at 48 h, is consistent with the results of the optimisation experiment (Section 5.4.1).

Wilcoxon tests determined there were no significance differences in median cell viability between 24 and 48 h (48 h post-first treatment and 24 h post-second treatment) for cells treated with vehicle control (P = 0.875), 5-Aza-dc (P = 0.656), 5-FU (P = 0.219) and 5-Aza-dc in combination with 5-FU (P = 0.563).

Images of cells were taken at 24 and 48 h (48 h post-first treatment and 24 h post-second treatment) following treatment under a phase contrast microscope at 50 x magnification (Figure 5.3). Morphological changes were obvious following treatment with 5-Aza-dc, 5-FU and 5-Aza-dc in combination with 5-FU. A clear decrease in cell number was observed, cells lost their spherical shape and cell granularity became increasingly evident.

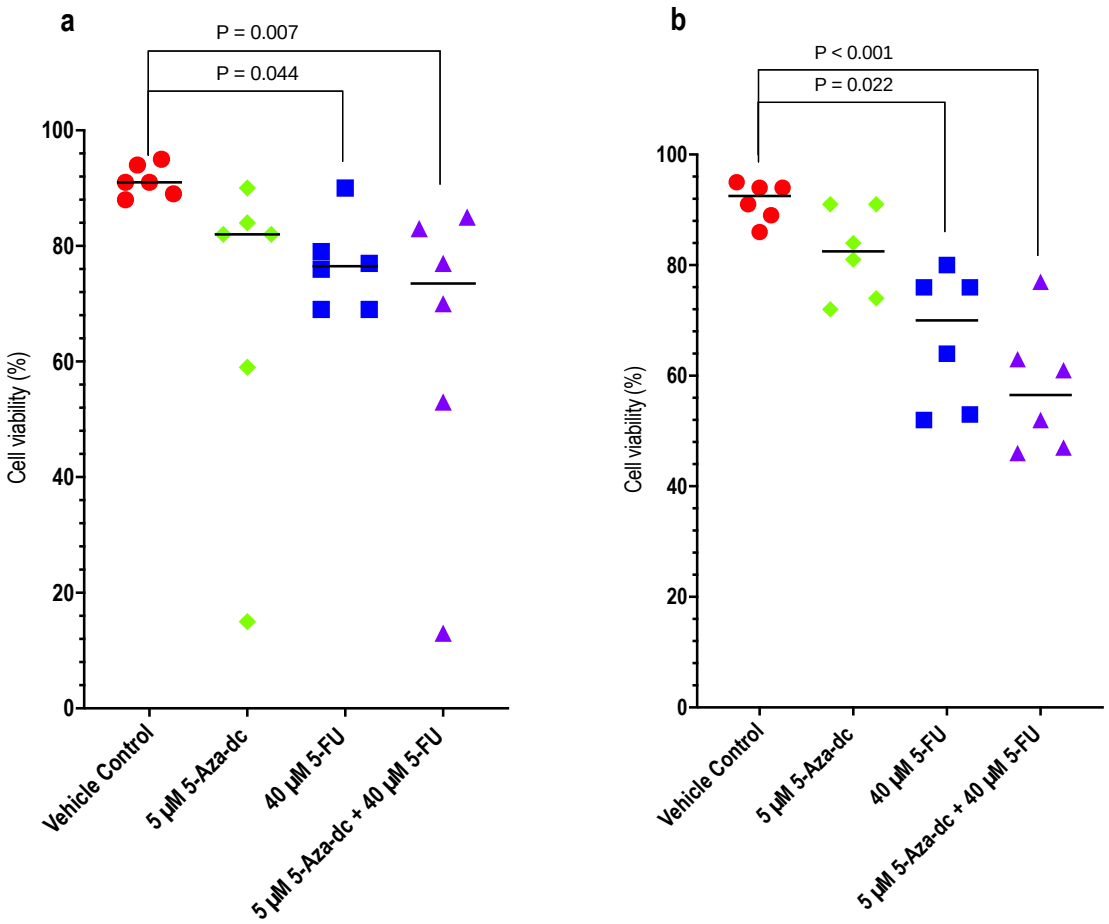

Figure 5.2 U937 cell viability at a) 24 and b) 48 h following treatment with vehicle control (Milli-Q®), 5 µM 5-Aza-dc, 40 µM 5-FU or, 5 µM 5-Aza-dc in combination with 40 µM 5-FU. Line indicates median (n = 6).

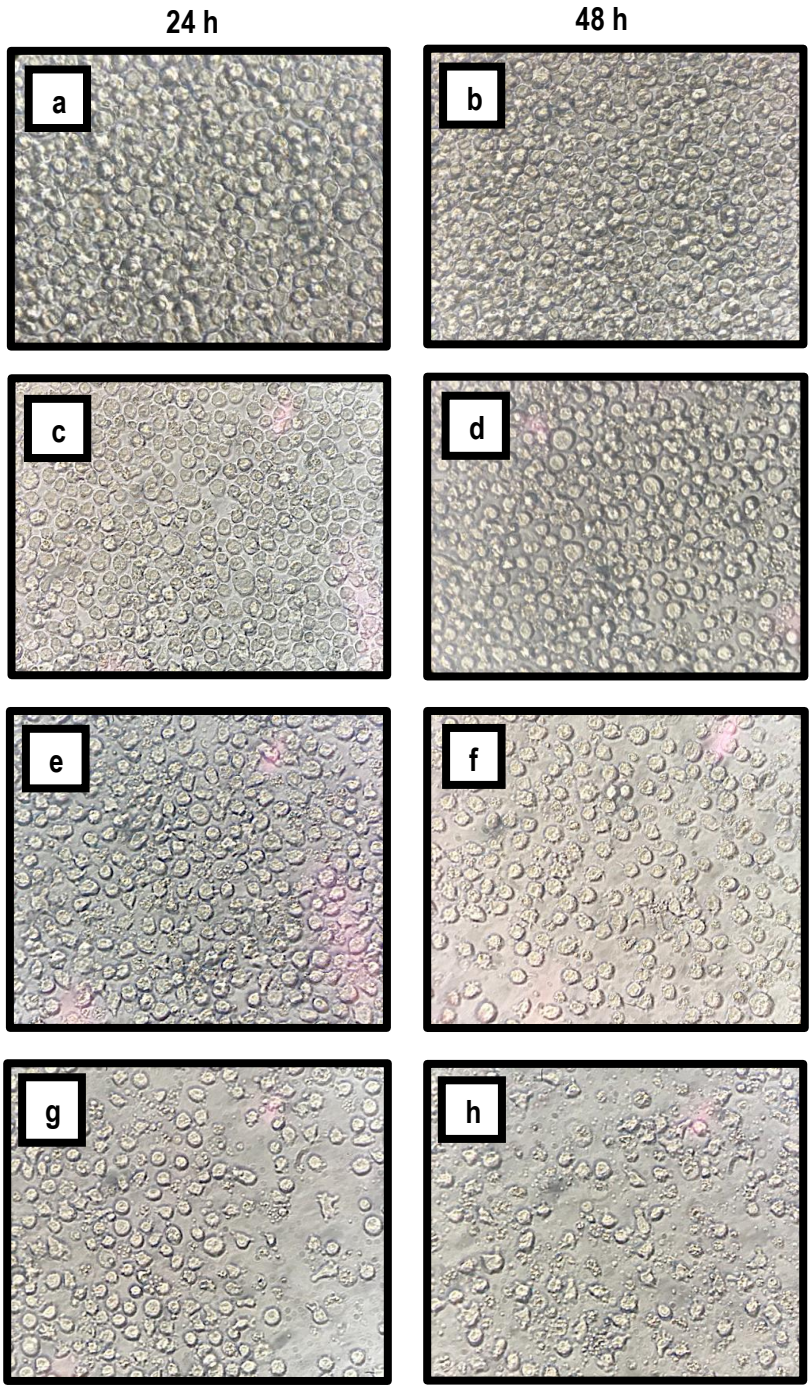

Figure 5.3 Morphological characteristics of U937 cells at 24 and 48 h following treatment with a and b) vehicle control (Milli-Q®), c and d) 5 µM 5-Aza-dc, e and f) 40 µM 5-FU or, g and h) 5 µM 5-Aza-dc in combination with 40 µM 5-FU. Images were taken under a phase contrast microscope at 50 x magnification.

5.4.3 Gene expression of TNF is significantly increased following treatment with 5-FU and 5-FU in combination with 5-Aza-dc

Cells treated with vehicle control had significantly lower relative expression of TNF compared to cells treated with 5-FU or 5-Aza-dc in combination with 5-FU (Figure 5.4a). The median expression ratio of TNF in cells treated with 5-FU was 0.187 compared to 0.070 in cells treated with vehicle control ($P = 0.005$). Likewise, the median expression ratio of TNF for cells treated with 5-Aza-dc in combination with 5-FU was 0.171 compared to vehicle control ($P = 0.044$). There were no significant differences in relative expression of TNF between cells treated with 5-Aza-dc and cells treated with vehicle control ($P = 0.265$).

Cells treated with 5-Aza-dc had significantly lower relative expression of DNMT1 compared to cells treated with vehicle control, with the median expression ratio of DNMT1 in cells treated with 5-Aza-dc being 0.075 compared to 0.500 in cells treated with vehicle control ($P < 0.001$) (Figure 5.4b). There were no significant differences in relative expression of DNMT1 between cells treated with 5-FU or 5-Aza-dc in combination with 5-FU and cells treated with vehicle control ($P > 0.999$ and $P = 0.152$, respectively).

There were no significant differences in relative expression of DNMT3A between cells treated with vehicle control, 5-Aza-dc, 5-FU or 5-Aza-dc in combination with 5-FU ($P > 0.05$) (Figure 5.4c).

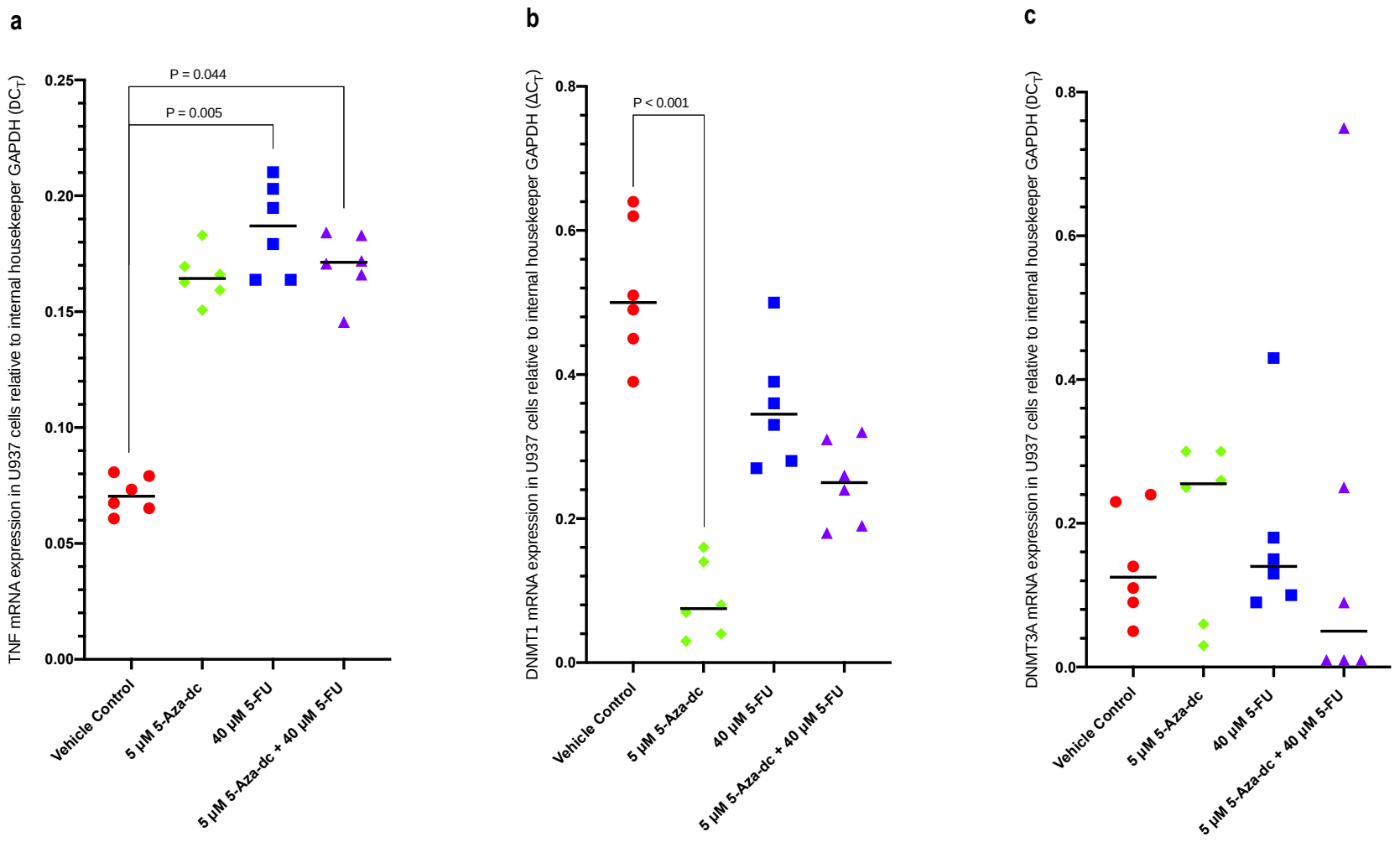

Figure 5.4 Relative gene expression of a) TNF, b) DNMT1 and c) DNMT3A in U937 cells treated with vehicle control (Milli-Q®), 5 µM 5-Aza-dc, 40 µM 5-FU or, 5 µM 5-Aza-dc in combination with 40 µM 5-FU. Line indicates median (n = 6).

5.4.4 DNA Methylation at TNF CpG Sites

Across CpG sites -244 and -238, DNA methylation was significantly lower in cells treated with 5-Aza-dc and 5-Aza-dc in combination with 5-FU compared to cells treated with vehicle control (Figure 5.5a). Median DNA methylation of -244 and -238 in cells treated with 5-Aza-dc was 53 % compared to 78 % in cells treated with vehicle control (P = 0.0005). Median DNA methylation across these CpG sites in cells treated with 5-Aza-dc in combination with 5-FU was 55 % compared to cells treated with vehicle control (P = 0.031). There were no significant differences in DNA methylation across CpG sites -244 and -238 between cells treated with i) 5-FU and vehicle control (P > 0.999), ii) 5-FU and 5-Aza-dc (P = 0.061), iii) 5-FU and 5-Aza-dc in combination with 5-FU (P = 0.877) and, iv) 5-Aza-dc and 5-Aza-dc in combination with 5-FU (P > 0.999).

Across CpG sites -169, -163 and -161, DNA methylation was also significantly lower in cells treated with 5-Aza-dc and 5-Aza-dc in combination with 5-FU compared to cells treated with vehicle control (Figure 5.5b). Median DNA methylation of -169, -163 and -161 in cells treated with 5-Aza-dc was 54 % compared to 84 % in cells treated with vehicle control (P = 0.044). Median DNA methylation across these CpG sites in cells treated with 5-Aza-dc in combination with 5-FU was 43 % compared to cells treated with vehicle control (P < 0.001). There were no significant differences in DNA methylation across CpG sites 169, -163 and -161 between cells treated with i) 5-FU and vehicle control (P > 0.999), ii) 5-FU and 5-Aza-dc (P > 0.999), iii) 5-FU and 5-Aza-dc in combination with 5-FU (P = 0.054) and, iv) 5-Aza-dc and 5-Aza-dc in combination with 5-FU (P > 0.999).

MS-HRM primers amplifying CpG sites -149 and -119, and -72, -49 and -38 upstream of the *TNF* transcription start site were not analysed as these primers amplified non-specific products and could provide any results (data not shown). Therefore, DNA methylation across these CpG sites could not be investigated.

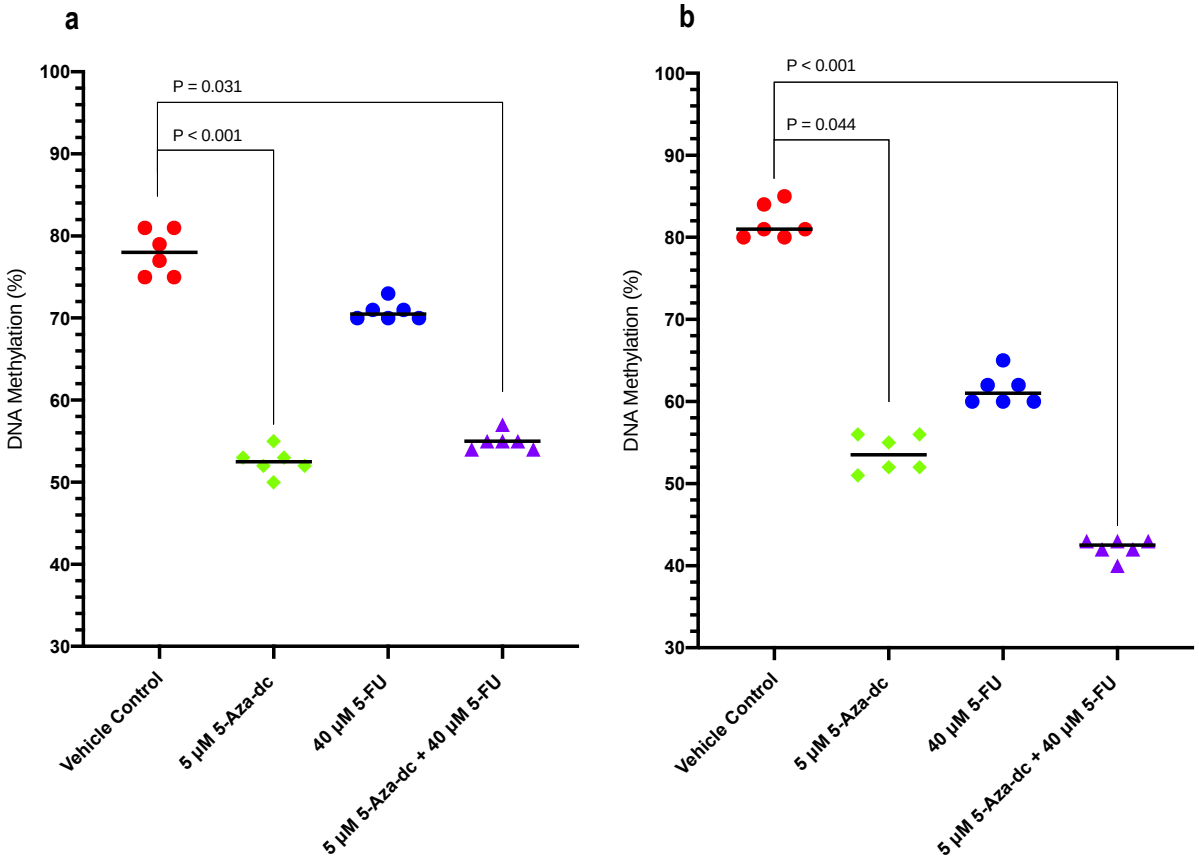

Figure 5.5 DNA methylation was compared in U937 cells treated with vehicle control (Milli-Q®), 5 µM 5-Aza-dc, 40 µM 5-FU or, 5 µM 5-Aza-dc in combination with 40 µM 5-FU across CpG sites a) -244 and -238 and, b) -169, -163 and -161 upstream of the *TNF* transcription start site. Line indicates median (n = 6).

5.5 Discussion

The findings of this study indicate 5-FU does not inhibit DNA methyltransferase activity in monocytic-like cells and does not reduce DNA methylation at CpG sites throughout the *TNF* promoter region. The aim of this study was to investigate if 5-FU could inhibit mRNA expression of DNMT1 and DNMT3A and therefore, reduce DNA methylation across CpG sites -244, -238, 169, -163, -161, -149, -119, -72, -49 and -38 upstream of the *TNF* transcription start site in U937 cells. It was identified only treatment with DNA methylation inhibitor 5-Aza-dc was able to significantly decrease DNMT1 mRNA expression and reduce DNA methylation across CpG sites -244, -238, -169, -163 and -161. This demonstrates 5-FU is not a potent DNA methylation inhibitor and therefore, may not increase *TNF* gene expression and subsequent TNF-α secretion that is characteristic of mucosal injury experienced during severe GI toxicity.

This was one of the first studies investigating the effect of 5-FU in combination with 5-Aza-dc on mRNA expression and DNA methylation within the *TNF* gene in the U937 cell line. It was determined treatment with 5 μM 5-Aza-dc did not significantly decrease cell viability in U937 cells, suggesting the antimetabolite action of 5-Aza-dc as a cytidine analogue [138] and, its effect as a potent DNA methylation inhibitor [143], did not induce significant cell death in the U937 cell line. Significant cell death was determined to be a direct consequence of 5-FU, with median cell viability inversely proportional to 5-FU concentration. Following treatment with 5-Aza-dc and/or 5-FU at 24 and 48 h, cell viability was significantly different between cells treated with 5-FU and 5-Aza-dc in combination with 5-FU compared to cells treated with vehicle control. This decrease in cell viability was expected based on the results of the optimisation experiment.

DNA methylation is mediated by DNMT1 and DNMT3A which both catalyse the transfer of methyl groups to CpG sites within the DNA sequence. To determine if 5-FU inhibits DNA methylation, it was investigated if treatment with 5-FU inhibited mRNA expression of DNMT1 and DNMT3A. No such inhibition of DNMT1 and DNMT3A was observed following treatment with 5-FU, with a

significant decrease in DNMT1 only observed following treatment with 5-Aza-dc alone. These results are contradictory to previous literature which demonstrated 5-FU (2 – 200 μM) *in vitro* significantly decreased DNMT1, DNMT3A and overall methyltransferase expression [132]. Ham *et al.* investigated the effect of 5-FU on DNMT1 and DNMT3A expression in a human lung cancer cell line (A549) [132] compared to the monocytic-like U937 cell line used in this study. Cancer cells are highly methylated in comparison to monocytes and as a result, the degree of inhibition of expression of DNMT1 and DNMT3A following the addition of a DNA methylation inhibitor or drug that inhibits DNA methylation, may differ between the two cell types. Cancer cells may have a greater degree of DNA methylation inhibition due to their already highly methylated state, explaining why Ham *et al* identified significant differences in DNMT1 and DNMT3A expression following 5-FU treatment whereas, no significant differences in DNMT1 and DNMT3A following 5-FU treatment were identified in this study. This suggests 5-FU may not inhibit DNA methylation in monocytic-like cells to the same degree as cancer cell types.

To determine if inhibition of DNA methylation increases *TNF* expression, TNF mRNA expression was also investigated in this study. TNF mRNA expression was significantly increased only in cells treated with 5-FU alone or with 5-FU in combination with 5-Aza-dc. This result is in agreement with previous preclinical research demonstrating increased TNF expression and TNF-α serum levels following 5-FU treatment [110]. However, TNF mRNA expression was not significantly different following treatment with 5-Aza-dc, contrary to the hypothesis that cells treated with 5-Aza-dc would show a significant increase in TNF mRNA expression. This demonstrated DNA methylation did not influence TNF mRNA expression, contradictory to the hypothesis for this current study which was based on previous results by Zhang *et el.* [135] who identified THP-1 cells treated with 5 μM 5-Aza-dc exhibited an increase in TNF expression. Zhang *et al.* [135] investigated DNA methylation in the THP-1 cell line, a human monocytic leukaemia cell line [144], with similar properties to the U937 human myeloid leukaemia cell line [137] used in this current study. However, a possible

explanation for the discrepancy in results between this current study and the study by Zhang *et al.* is that TNF mRNA expression for cells treated with 5-Aza-dc was determined at 48 h in this study compared to 24 h in the study by Zhang *et al.* [135]. Although cell culture media was replaced and cells were re-treated at 24 h, ideally TNF mRNA expression should have also been investigated at the 24 h time point (24 h following first treatment) in order to confirm cell death did not significantly impact the TNF mRNA expression. When 5-Aza-dc and 5-FU were first administered, cells were in cell cycle arrest at the G1 phase. This was to increase the likelihood of these antimetabolites becoming incorporated in newly synthesised DNA. When re-treated with 5-Aza-dc and 5-FU at 24 h, cells were not necessarily in the same cell cycle phase and cell death was prominent, particularly in cells already treated with 5-Aza-dc, 5-FU, and 5-Aza-dc in combination with 5-FU. Therefore, the inhibitory effect of 5-FU on DNMT1 and DNMT3A mRNA expression as well as the effect of 5-Aza-dc on TNF mRNA expression may have been significant at 24 h but, lost significance at 48 h due to cell death and variation in cell cycle phases.

The effect of 5-FU on DNA methylation within the *TNF* gene, particularly at CpG sites -244 and -238, and -169, -163 and -161 upstream of the *TNF* transcription start site, was also determined. Treatment with 5-Aza-dc or 5-Aza-dc in combination with 5-FU significantly decreased DNA methylation across these CpG sites, while there was no impact of 5-FU. This is contradictory to previous research identifying differences in DNA methylation across these CpG sites in other inflammatory-based conditions [134, 135]. These studies investigated DNA methylation in gingival tissue, containing a range of cell types including fibroblasts, macrophages, osteoblasts and inflammatory cells (such as neutrophils and eosinophils) [135] and; DNA methylation in DNA isolated from peripheral blood mononuclear cells, comprised of lymphocytes, monocytes and dendritic cells [134]. In my study, as DNA methylation was solely investigated in monocytic-like U937 cells, this further suggests DNA methylation varies across cell types.

MS-HRM primers amplifying CpG sites -149 and -119, and -72, -49 and -38 upstream of the *TNF* promoter region were not included in analysis and, DNA methylation could not be investigated at these sites. Following multiple MS-HRM runs, these primers consistently amplified non-specific regions (determined from melt curve analysis following each MS-HRM run) and PCR products could not be detected using agarose gel electrophoresis (data not shown). These primers were used previously in THP-1 cells [135], a cell line with similar properties to U937 cells [137]. However, it is possible in U937 cells, the regions of interest for these primers may contain a low frequency of DNA methylated sites. Consequently, as the primers were specific to methylated regions it is possible they would not amplify the region containing the CpG sites of interest due to low DNA methylation frequency. Additionally, with the consistent amplification of non-specific products, there may have been high background within the regions of interest, making it difficult to distinguish changes in DNA methylation across the selected CpG sites.

A key limitation of this study was TLR agonists such as lipopolysaccharide (LPS) were not included in treatment with 5-Aza-dc and/or 5-FU. As discussed in Chapter 2, TLRs mediate an innate immune response via the TIR domain signalling pathway, leading to the secretion of proinflammatory cytokines such as TNF-α, which is characteristic of 5-FU-induced GI toxicity. The inclusion of LPS or a similar TLR agonist would have investigated an alternate hypothesis; namely the inflammatory response induced by 5-FU is responsible for inhibition of DNA methylation of the *TNF* gene, rather than 5-FU itself. This could have possibly explained why DNA methylation was not significantly different throughout the *TNF* promoter region in this study following 5-FU treatment.

As this was a pilot study aimed to investigate the concept that 5-FU reduces DNA methylation within the *TNF* gene promoter region, MS-HRM was selected to detect DNA methylation over pyrosequencing. Pyrosequencing is a high-cost, outsourced DNA methylation detection assay which determines the exact DNA methylation percentage at individual CpG sites. In comparison,

although MS-HRM is low cost and provides fast and sensitive detection of DNA methylation, it does so across multiple selected CpG sites which is its biggest pitfall. As a result, the methylation of combined CpG sites may hide the fact that methylation at one CpG site may have been significantly different after treatments. In addition, with missing data from CpG sites -149 and -119, and -72, -49 and -38 upstream of the *TNF* promoter region, it was not determined if 5-FU was able to significantly reduce DNA methylation at these CpG sites.

5.6 Conclusion

In conclusion, 5-FU did not reduce mRNA expression of DNMT1 and DNMT3A or, reduce DNA methylation across *TNF* promoter CpG sites -244 and -238, and -169, -163 and -161. Although the hypothesis was not supported, the limitations of this study prevent a concrete conclusion that DNA methylation within the *TNF* gene following 5-FU-based therapy is not a potential predictive marker for severe GI toxicity. This study needs to be repeated addressing the limitations discussed, notably, treating not only U937 cells but a cancer cell line as a positive control with a variety of 5-FU and/or 5-Aza-dc concentrations in addition to TLR agonists such as LPS. Once the predictive potential of DNA methylation within the *TNF* promoter gene region is identified following 5-FU treatment, *ex vivo* analysis can then occur in isolated PBMCs to determine if the results are clinically translatable and DNA methylation of the TNF gene is viable predictive marker for severe GI toxicity risk.

Chapter 6: Thesis discussion

This thesis presents results of studies investigating the potential of the TIR domain innate immune signalling pathway to predict severe GI toxicity risk following 5-FU-based therapy. The major findings of this thesis are divided into retrospective clinical, *ex vivo* and *in vitro* observations. It must be noted the design and conduct of each study was independent from other studies.

In Chapter 3, multivariate logistic regression built a final risk prediction model which identified 5-FU-based regimen to be predictive for severe GI toxicity risk in participants classified as non-toxic (no or moderate to mild GI toxicity) and toxic (severe GI toxicity) to 5-FU. This was the first time logistic regression modelling has demonstrated 5-FU-based regimen to be predictive for severe GI toxicity risk. This result is contradictory to the pilot study [25], which identified *TLR2* rs384100 and *TNF* rs1800629 SNPs in conjunction with cancer type (colorectal and upper GI) to be predictive for severe GI toxicity risk.

Throughout this current study as the demographic, clinical and genetic composition of the participant cohort changed, so did the predictive nature of demographic, clinical and genetic variables as identified by risk prediction modelling. This was most evident when the interim risk prediction model identified SNPs within *IL1B* (rs16944 and rs1143434), in conjunction with cancer type (colorectal and upper GI), to be predictive for severe GI toxicity risk. However, these identified predictors lost their predictive nature in the final risk prediction model.

Risk prediction models are becoming increasingly prominent in clinical practice and therefore, consistent with similar research examining patients receiving chemotherapy and/or radiotherapy for advanced cancers [145-147], multivariate logistic regression modelling was chosen for statistical analysis. However, due to the small and highly variable clinical make-up of the participant cohorts, Bayesian Networking (BN), another form of risk prediction modelling, may have been a more appropriate modelling method. BN has also been used extensively in patient cohorts

receiving chemotherapy and/or radiotherapy [148, 149]. Compared to the multivariate logistic regression method used for statistical analysis in Chapter 3 (discussed in Section 3.4.4), BN can outperform multivariate logistic regression models as less data is required to reach peak model performance [150]. Additionally, BN determines the relationship between the covariates included in the model and their dependency on each other, depicted by a directed acyclic graph [150]. Therefore, BN may provide a more informative risk prediction model for severe GI toxicity following 5-FU-based therapy. Multivariate logistic regression and BN models have been compared within clinical patient cohorts previously but, one has not consistently been identified as more reliable or predictive than the other [151, 152]. Due to time constraints, BN was not modelled with the final participant cohort and compared to the final risk prediction model built using the multivariate logistic regression method. Nonetheless, the sensitivity and specificity of a risk prediction model to accurately identify patients at most risk of severe GI toxicity would be greatly improved with the addition of a phenotypic marker. However, there is a lack of phenotypic marker for severe GI toxicity. This led to the *ex vivo* study in Chapter 4.

In Chapter 4, investigation of an *ex vivo* phenotypic marker for severe GI toxicity in a subset of participants recruited from Chapter 3 was undertaken. It was determined stimulated IL-1β secretion post-5-FU-based therapy was significantly increased in participants classified as having a non-toxic response to 5-FU at the time of therapy. Additionally, *CASP1* rs580253 and *CASP5* rs554344 genotypes were also associated with stimulated IL-1β secretion post-5-FU-based therapy however, there was no consistent change in secretion of stimulated IL-1β secretion with the increasing presence of the variant allele. The association between *CASP1* rs580253 and *CASP5* rs554344 genotypes and stimulated IL-1β secretion was independent of GI toxicity risk. To the best of my knowledge, this was the first study investigating stimulated TIR domain innate immune response in participants post-5-FU-based therapy.

Overall, no three-way association with GI toxicity, genotype or stimulated IL-1β secretion was identified. This suggests IL-1β as a phenotypic marker for severe GI toxicity risk is not dependent on genotype. This is consistent with previous research which has identified many SNPs in *IL1B* are in linkage disequilibrium therefore, those with opposing effects on gene functioning and IL-1β secretion [94] may 'cancel' one another out, which could explain why no relationship between stimulated IL-1β secretion and SNPs within *IL1B* was not identified in my study. The results of Chapter 4 indicate differences in stimulated IL-1β secretion post-5-FU-based therapy and, potentially general IL-1β secretion, may not be heavily influenced by genotype but more so, the innate immune cells, such as monocytes and macrophages, from which IL-1β is secreted. Additionally, no significant associations were determined between stimulated TNF-α secretion and GI toxicity, suggesting any potential differences in TNF-α secretion between the two toxicity groups may only occur during treatment with 5-FU itself and not following TLR2 or TLR4 stimulation. Future studies could examine proinflammatory cytokine responses in PBMCs collected from participants prior to receiving 5-FU-based therapy to determine when changes in IL-1β and TNF-α secretion occur as a result of innate immune memory induced by 5-FU-based therapy.

The results of the interim predictive risk model in Chapter 3 and, secretion results from Chapter 4, implicated *IL1B* genotype and stimulated IL-1β secretion, respectively, with GI toxicity in participants recruited to both study cohorts. However, due to the limitations of both studies, it is difficult to conclude if SNPs within *IL1B*, stimulated IL-1β secretion and SNPs within *CASP1* and *CASP5* genes (*CASP1* and *CASP5* are both responsible for cleaving inactive IL-1β to its active form) are potential genetic and phenotypic predictive markers for severe GI toxicity risk. The MAF of SNPs within *IL1B* in participants recruited in Chapter 3 was not reflective of the Caucasian population and, as more participants were added to the study cohort, *IL1B* rs16944 and rs1143634 were no longer identified to be predictive for severe GI toxicity risk in the final risk prediction model. In Chapter 4, whilst stimulated IL-1β secretion was significantly different between non-toxic and

toxic participants, secretion was observed in PBMCs isolated from participants post-5-FU-based therapy. Consequently, due to the presence of innate immune memory, this secretion may not be reflective of the stimulated IL-1β secretion profile observed in PBMCs pre- and throughout 5-FU-based therapy. Therefore, IL-1β secretion may potentially have no significance as a phenotypic marker for severe GI toxicity risk pre- and throughout 5-FU-based therapy. Additionally, although stimulated IL-1β secretion was also significantly different between SNPs within *CASP1* (rs580253) and *CASP5* (rs554344), only 2 participants were carriers of the homozygous variant genotype for both SNPs. This may explain why no consistent decrease in stimulated IL-1β secretion pattern was observed with changes in genotype.

Neither Chapters 3 or 4 identified a significant relationship between SNPs within *TNF* and severe GI toxicity risk or, stimulated TNF-α secretion post 5-FU-based therapy with GI toxicity risk, respectively. This formed the basis for investigating whether DNA methylation induced by 5-FU was an alternate genetic modification within *TNF* that may be predictive for severe GI toxicity in Chapter 5. This was the only non-clinical study in my thesis and investigated the effect of 5-FU on DNA methylation within the *TNF* gene. Although 5-FU did increase TNF mRNA expression following treatment as expected, treatment with the DNA methylation inhibitor 5-Aza-dc did not significantly increase TNF mRNA expression. This demonstrated inhibition of DNA methylation was not necessarily responsible for increased TNF mRNA expression. This was contradictory to previous research by Zhang *et al.* upon which the methodology of Chapter 5 was based [135]. Furthermore, treatment with 5-FU did not significantly decrease DNMT1 mRNA expression compared to 5-Aza-dc, suggesting 5-FU is not a potent DNA methylation inhibitor. At the selected CpG sites within the *TNF* gene promoter region, treatment with 5-FU also did not significantly reduce DNA methylation compared to untreated cells and cells treated with 5-Aza-dc. This further supports the suggestion 5-FU does not reduce DNA methylation within the *TNF* gene in a

monocytic-like cell line. But, further research is needed in PBMCs isolated from participants pre- and throughout 5-FU-based therapy to more closely resemble clinic.

It is difficult to compare the results of Chapter 5 to previous research as to my knowledge, this is the first study to investigate the influence of 5-FU treatment on DNA methylation within *TNF in vitro.* The hypotheses for this study was based upon an additional inhibitory effect of 5-FU on the enzyme thymidylate synthase (TS). As well as inhibiting DNA synthesis, 5-FU also prevents the conversion of a key precursor folinic acid derivative required for DNA methylation. The results in Chapter 5 suggest this inhibitory effect is not sufficient to prevent DNA methylation of the *TNF* gene therefore, this mechanism cannot explain increased TNF mRNA expression following 5-FU treatment. However, the results of this study must be considered in the context of its limitations, which prevents a concrete conclusion to be made. Most notably, missing data for 5 of the 10 CpG sites selected for analysis and, not treating cells with a variety of 5-FU and 5-Aza-dc concentrations. In future, DNA methylation investigations could occur simultaneously in a study like that presented in Chapter 4. The results would potentially carry greater clinical relevance as analysis would occur in PBMCs isolated from participants who received 5-FU-based therapy.

Future directions

The research conducted in Chapters 3 and 4 was retrospective in nature. However, to counteract the limitations discussed and identify clinically translatable genetic, phenotypic and epigenetic predictors for severe GI toxicity risk, prospective studies would need to occur. Retrospective analysis allowed identification of 2185 potential participants who were treated with 5-FU-based therapy from 2012 to 2018. However, even with these 2185 potential participants, it still took over 3½ years to recruit the target sample size of 150 participants. Prospective analysis was unable to occur due to high expense of conducting such a study, funding that was not able to be obtained during my PhD.

Prospective analysis would allow accurate collection of GI toxicity data and selection of participants based on genotype to ensure the MAF of the cohort is reflective of the Caucasian population. For *ex vivo* analysis, a prospective study would allow pre- and post-5-FU-based stimulation of PBMCs to determine changes in the IL-1β and TNF-α secretory response that may occur during 5-FU-based therapy. Simultaneously, DNA methylation analysis could also occur on PBMCs isolated from participants directly after receiving 5-FU. However, as severe GI toxicity risk is an under represented area of medical oncology, the best chance of investigating severe GI toxicity in a prospective cohort would be to conduct research in combination with a clinical trial.

Significance of results

Although severe GI toxicity is mediated by the TIR domain innate immune signalling pathway, the development of severe GI toxicity is multifaceted. Therefore, the more information included in a risk prediction model for severe GI toxicity, such as demographics, clinical data, genotype, proinflammatory secretory response and DNA methylation percentage, the greater the chance of accurately identifying patients at risk of severe GI toxicity.

As discussed throughout this thesis, predictive risk modelling gives the most accurate method of correctly identifying patients at risk of severe GI toxicity prior to receiving 5-FU-based therapy. However, the logistics of translating a predictive risk model for severe GI toxicity into clinic is not straight forward. Evidently, statisticians would be required to input patient data, run the risk prediction model as well as, report and translate the results of the model to the medical oncologist in charge of the patients' care. More importantly, extra clinical staff would also be required to collect the necessary demographic data, clinical data and patient biological samples necessary for the model on behalf of already time poor medical oncologists. If assays on patient biological samples are required to be undertaken, laboratory staff would also need to be employed. Before translating risk prediction models into clinic, it would also need to be considered if the financial cost of

employing additional staff at every hospital site to conduct the risk prediction modelling outweighs not only the financial benefit on the health care system and for patients receiving 5FU-based therapy but, also outweighs improved patient quality of life whilst receiving treatment. This type of economic evaluation has not been previously conducted.

Identifying patients at most risk of severe GI toxicity is imperative to allow the personalisation of their supportive care measures to reduce the severity of GI toxicity experienced. Not only would this improve patient quality of life during 5-FU-based therapy but, will also positively influence their immediate and long term prognosis. Although the observations made throughout this thesis point to no concordant predictive marker for severe GI toxicity risk, my thesis has shown that GI toxicity can be interrogated by demographic, genetic, phenotypic and epigenetic approaches. Further work can be used to model these factors together as well as identify deeper relationships with personal characteristics to move to a truly personalised therapy age.

Appendix: Chapter 2

Korver SK, Gibson RJ, Bowen JM & Coller JC (2019) Toll-like receptor/interleukin-1 domain innate immune signalling pathway genetic variants are candidate predictors for severe gastrointestinal toxicity risk following 5-fluorouracil-based chemotherapy. *Cancer Chemother Pharmacol* 83: 217 - 236.

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REVIEW ARTICLE

Toll-like receptor/interleukin-1 domain innate immune signalling pathway genetic variants are candidate predictors for severe gastrointestinal toxicity risk following 5-fluorouracil-based chemotherapy

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Abstract

Purpose Severe gastrointestinal (GI) toxicity is a common adverse effect following 5-fluorouracil (5-FU)-based chemotherapy treatment. The presence of severe GI toxicity leads to treatment revisions, sub-optimal therapy outcomes, and decreases to patients' quality of life. There are no adequate predictors for 5-FU-induced severe GI toxicity risk. The Toll-like receptor/interleukin-1 (TIR) domain innate immune signalling pathway is known to be a mediating pathway in the development of GI toxicity. Hence, genetic variability in this signalling pathway may alter the pathophysiology of GI toxicity and, therefore, be predictive of risk. However, little research has investigated the effects of TIR domain innate immune signalling pathway single nucleotide polymorphism (SNPs) on the risk and development of severe GI toxicity.

Methods This critical review surveyed the literature and reported on the in vitro, ex vivo and in vivo effects, as well as the genetic association, of selected TIR domain innate immune signalling pathway SNPs on disease susceptibility and gene functioning.

Results Of the TIR domain innate immune signalling pathway SNPs reviewed, evidence suggests interleukin-1 beta (*IL1B*) and tumour necrosis factor alpha (*TNF*) SNPs have the greatest potential as predictors for severe GI toxicity risk. These results warrant further research into the effect of *IL1B* and *TNF* SNPs on the risk and development of severe GI toxicity.

Conclusions SNPs of the TIR domain innate immune signalling pathway have profound effects on disease susceptibility and gene functioning, making them candidate predictors for severe GI toxicity risk. The identification of a predictor for 5-FU-induced severe GI toxicity will allow the personalization of supportive care measures.

Keywords 5-Fluorouracil (5-FU) · Gastrointestinal (GI) toxicity · Toll-like receptors (TLRs) · Proinflammatory cytokines · Single nucleotide polymorphisms (SNPs) · Genetic variant

Abbreviations

5-FU	5-fluorouracil
DAMPs	Damage associated molecular patterns
DPD	Dihydropyrimidine dehydrogenase enzyme
DPYD	Dihydropyrimidine dehydrogenase gene
GI	Gastrointestinal
HSCT	Hematopoietic stem cell transplantation
IKK	Inhibitor of NF-κB-kinase complex
IRAK1	Interleukin-1 receptor-associated kinases 1
IRAK4	Interleukin-1 receptor-associated kinases 4
MYD88	Myeloid differentiation primary response protein 88
NCI CTCAE v5.0	The National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0

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PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
SNPs	Single nucleotide polymorphisms
TAB1	Tak-1 binding protein 1
TAB2	Tak-1 binding protein 2
TAK1	Transforming growth factor beta factor-β activated kinase 1
TIR	Toll-like receptor/interleukin-1
TLR	Toll-like receptor
TRAF6	TNF receptor associated factor 6

Introduction

5-fluorouracil (5-FU) is a commonly administered chemotherapy drug used for the treatment of breast, colorectal and upper gastrointestinal (GI) tract solid tumours [1, 2]. Although highly effective, with response rates for 5-FU-based regimens between 40 and 50% in patients with advanced colorectal cancer [3, 4], 5-FU causes severe damage to mucosal membranes of the GI tract [5–7]. This damage results in apoptosis, altered histopathology and an increase in proinflammatory cytokine expression giving rise to inflammation [5–7]. This in turn leads to the clinical manifestation of GI toxicities including but not limited to, mucositis, diarrhea, nausea and vomiting [8].

The development of GI toxicity is variable amongst patients, making it difficult to predict which patients will develop severe toxicity. Although supportive care measures are recommended and suggested, these are limited and are for use in highly specific patient cohorts [9]. Additionally, supportive care is often administered therapeutically not prophylactically, which in some patients is too late for the management and relief of severe GI toxicity symptoms [9, 10]. In these patients, further interventions are required to improve symptom management and relieve severe GI toxicity symptoms [11].

Currently, there are no adequate predictors for 5-FU-induced severe GI toxicity risk. It is imperative predictors are identified and translated to clinical practice to identify "at risk" patients prior to 5-FU treatment. This will facilitate proactive delivery and personalization of supportive care measures aimed at reducing the severity of GI toxicity experienced.

Following administration of 5-FU, an innate immune inflammatory response is initiated, mediated by the Toll-like receptor/interleukin-1 domain innate immune signalling pathway. Toll-like receptor/interleukin-1 is commonly abbreviated to TIR. Activation of the TIR domain innate immune signalling pathway leads to the upregulation of potent transcription factors and secretion of proinflammatory cytokines [7, 12, 13]. Increased levels of these proinflammatory cytokines, in particular tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), have been significantly associated with severe GI toxicity in both preclinical and clinical studies [5, 7, 14]. Due to the importance of the TIR domain innate immune signalling pathway in inflammatory signalling and subsequent severe GI toxicity development, single nucleotide polymorphisms (SNPs) in this pathway may be potential predictors for severe GI toxicity risk. However, these SNPs are largely understudied in the context of GI toxicity.

This critical literature review will firstly provide an overview of 5-FU-based chemotherapy and the pathophysiology of GI toxicity; then outline and summarize previous research on the influence and function of TIR domain innate immune signalling pathway SNPs and how this can be applied to GI toxicity risk.

Background

5-FU mechanism of action

5-FU is an antimetabolite drug that inhibits DNA and RNA synthesis [1]. The uracil analogue was developed in the 1950s following identification of uracil metabolism as a potential target for therapy due to the rapid use of uracil by rat hepatomas compared to normal tissues [15]. In the liver, 5-FU is metabolised to three active metabolites, fluorodeoxyuridine monophosphate (FdUMP), fluoro-deoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) [16, 17]. The oral prodrug of 5-FU, capecitabine, travels unaltered through the gut wall where it is converted to 5-FU by liver carboxylesterase (CES) 1 and CES2, and cytidine deaminase [18, 19]. The principal mechanism of action of 5-FU is inhibition of thymidylate synthase (TS, encoded by the gene *TYMS*), a critical enzyme necessary for conversion of precursor deoxyribonucleotides required for purine and pyrimidine synthesis. Its principal active metabolite, FdUMP, inhibits TS, whilst metabolites FdUTP and FUTP directly misincorporate into DNA and RNA, disrupting DNA and RNA synthesis, RNA processing and protein synthesis [16, 20, 21]. 5-FU catabolism is governed by the rate determining enzyme dihydropyrimidine dehydrogenase (DPD, encoded by the *DPYD* gene), catabolising 80% of administered 5-FU to dihydroflurouracil, which is then excreted in the urine [16, 22].

Current 5-FU regimens

5-FU is administered intravenously as either a bolus dose or continuous infusion over a 24–48 h period to treat solid tumours of the breast, colon and upper GI tract [23]. In breast cancer regimens, 5-FU is generally administered in

conjunction with epirubicin and cyclophosphamide [23]. For colorectal and upper GI tract cancers, 5-FU is administered as either a monotherapy in synergistic combination with leucovorin or in combination with other chemotherapeutics such as irinotecan, oxaliplatin and cisplatin [23]. 5-FU is used in both a curative and palliative care setting. Additional therapies including radiation therapy, monoclonal antibodies (bevacizumab, cetuximab), taxanes (docetaxel and paclitaxel) and tyrosine kinase inhibitors (lapatinib) may also be administered in conjunction with or following 5-FU treatment in an attempt to further reduce tumour activity [23]. Capecitabine, the oral and more selective prodrug of 5-FU, may also be substituted in replacement of 5-FU in the before-mentioned regimens [24].

5-FU administration induces GI toxicity

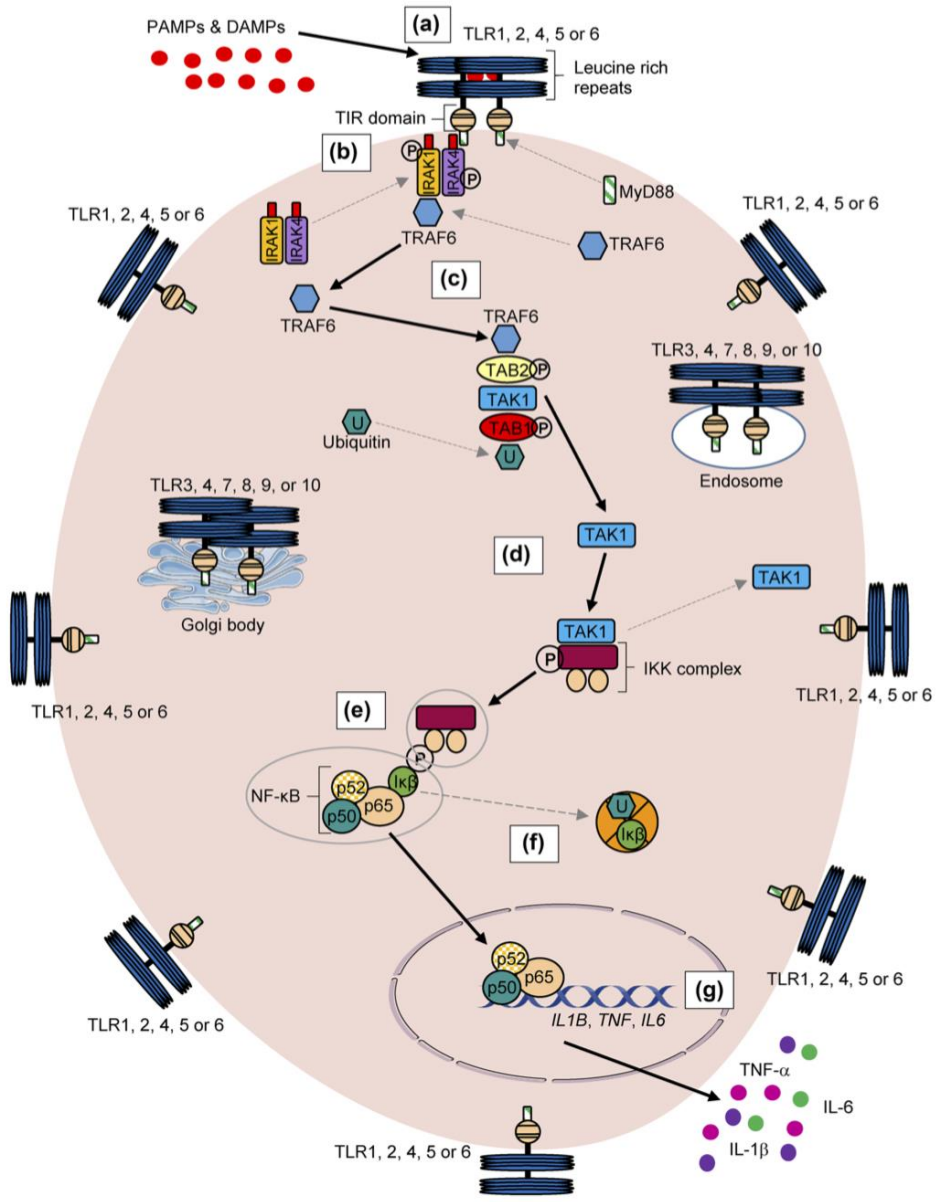
Of all patients receiving 5-FU-based therapies, 25–50% will experience GI toxicity [25]. Recording the prevalence and incidence of GI toxicity is vital for symptom management. However, this is often difficult and inconsistent due to a lack of standardised scoring criteria [26]. A number of toxicity grading scales exist, each with their own toxicity grading criteria. Among the most commonly used toxicity grading scale is The National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE v5.0) [27]. The NCI CTCAE was developed during the late 1980s and uses clinical symptoms and number of events to grade GI toxicity on a scale of 1 to 5 [27, 28]. GI toxicity graded as 1 or 2 is classified as 'mild' toxicity whilst GI toxicity graded 3 or 4 is classified as 'severe' toxicity [27]. Additionally, patients can also be categorized as suffering from a grade 3 or 4 GI toxicity on the NCI CTCAE v5.0 if they receive a dose reduction, treatment delay, cease treatment prematurely and/or are hospitalized as a direct result of GI toxicity [27, 28].

There is currently no effective approach to prevent GI toxicity [7]. The Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) provide world-wide clinical practice guidelines for management of GI toxicity secondary to cancer therapy [9, 29, 30]. Table 1 lists the current MASCC/ISOO recommendations and suggestions for the management and relief of oral mucositis, diarrhea, nausea and vomiting in adults [9, 10, 31].

In addition to GI toxicity, 5-FU can also induce hand and foot syndrome, peripheral neuropathy and hematological toxicities including leukopenia, thrombocytopenia and neutropenia [16]. This is due to off-target effects of 5-FU; 5-FU metabolites non-specifically target highly proliferative basal epithelium and hematopoietic progenitor cells, as well as highly proliferative cancer cells [7, 32]. Bolus administration of 5-FU increases the risk of hematological toxicities whilst continuous infusion of 5-FU increases the likelihood of hand and foot syndrome [16, 33]. In contrast, treatment modality does not affect the risk of GI toxicity [33]. Furthermore, patients with GI toxicity may also develop additional toxicities and experience 'toxicity clusters' [34]. In a cohort of patients with colorectal cancer receiving 5-FU-based treatment, diarrhea was strongly linked with the presence of bloating, constipation and cystitis, whilst vomiting was strongly linked with the presence of nausea, dehydration and chills [34]. No links were identified between GI toxicities and hematological or neurological toxicities [34]. Although research is still in its infancy, symptoms grouped in

Table 1 MASCC/ISOO clinical practice guidelines for the management of GI toxicity

Oral Mucositis [9]
Recommended
Thirty min of oral cryotherapy (sucking on ice chips) to prevent mucositis in patients receiving 5-FU bolus regimens
Suggested
Good oral hygiene (regular tooth brushing, flossing and mouth rinsing) to prevent mucositis across all treatment modalities
Diarrhea [9]
Recommended
Loperamide use to treat moderate diarrhea induced by standard or high-dose chemotherapy
Octreotide use to treat diarrhea induced by standard or high-dose chemotherapy if loperamide is ineffective
Suggested
Probiotics such as *Lactobacillus* use prior to and during chemotherapy treatment to maintain gut homeostasis and reduce diarrhea occurrence
Nausea and vomiting [10, 31]
Recommended
Prophylactic administration of antiemetics such as aprepitant (substance P antagonist), ondansetron (5-HT_3 receptor antagonist), dexamethasone (corticosteroid) or metoclopramide (dopamine-receptor antagonist) for patients receiving low emetogenic or high-dose chemotherapy
No prophylactic administration of antiemetics for patients receiving minimal emetogenic chemotherapy
Patients whom suffer vomiting should be treated as if they were receiving low emetogenic chemotherapy
Breakthrough nausea and vomiting (categorized as nausea or vomiting that occurs within 5 days of chemotherapy administration following the use of antiemetic agents) treated with olanzapine (antipsychotic medication)


PAMPs & DAMPs
(a)
TLR1, 2, 4, 5 or 6
Leucine rich repeats
TIR domain
(b)
MyD88
IRAK1
IRAK4
TRAF6
TLR1, 2, 4, 5 or 6
(c)
TAB2
TAK1
TAB1
Ubiquitin
TLR3, 4, 7, 8, 9, or 10
Endosome
Golgi body
(d)
IKK complex
(e)
NF-κB
p52
p65
p50
Iκβ
(f)
(g)
IL1B, TNF, IL6
TNF-α
IL-6
IL-1β

◂**Fig. 1** The TIR domain innate immune signalling pathway: **a** following 5-FU administration, DAMPs are released from injured and apoptotic cells whilst PAMPs are produced from colonized bacteria and microorganisms. DAMPs and PAMPs are recognized by TLR2 and TLR4 and on binding, stimulate the homodimerization of TLR4, and heterodimerization of TLR2 with either TLR1 or TLR6; **b** dimerization prompts the recruitment and binding of MyD88, which in turn recruits IRAK1 and IRAK4. Phosphorylation of IRAK1 and IRAK4 enables binding of TRAF6; **c** TRAF6 disengages from the newly formed IRAK complex and forms a new complex with TAK1, TAB1 and TAB2. Phosphorylation of TAK1 and TAB2 occurs, stimulating ubiquination of TRAF6 and subsequent activation of TAK1; **d** activated TAK1 disengages and phosphorylates the IKK complex; **e** phosphorylated IKK complex disengages from TAK1 and phosphorylates the Iκβ subunit of NF-κB; **f** following phosphorylation, Iκβ is ubiquinated and degraded from NF-κB by the 26S proteosome. Activated NF-κB translocates to the nucleus; **g** in the nucleus, NF-κB upregulates gene transcription and subsequent production and secretion of proinflammatory cytokines such as IL-1β, TNF-α and IL-6

clusters may share a common biological mechanism meaning that, a predictive marker for severe GI toxicity risk may also have the ability to predict the risk of additional toxicities.

The issue at hand: severe GI toxicity

Of the 20–25% of patients who experience GI toxicity, a subset of these patients will develop severe GI toxicity, graded as ≥ 3 on the NCI CTCAE [27]. Supportive care measures are ineffective for patients with severe GI toxicity and treatment delays, dose reductions or premature treatment cessation will be required to manage and relieve symptoms [7]. Furthermore, severe GI toxicities can induce secondary symptoms such as pain, dehydration and malnutrition requiring opioid analgesics, intravenous fluids and parenteral nutrition [7, 8]. Treatment interruption may negatively influence prognosis and potentially impact long-term survival. In addition, the presence of severe GI toxicity can decrease patient quality of life whilst receiving treatment and increase health costs, with inpatient hospitalization as a direct result of severe GI toxicity estimated to cost the US health care system US$15,500 per episode [35]. Currently, there is no way to identify patients at most risk of severe GI toxicity prior to treatment.

How does 5-FU induce GI toxicity?

Inhibition of DNA and RNA synthesis by 5-FU results in significant cell injury and death

Following inhibition of DNA and RNA synthesis by 5-FU, base-excision repair is initiated to remove precursor deoxyribonucleotides and misincorporated 5-FU metabolites, leading to excessive DNA fragmentation resulting in mucosal injury and cell apoptosis [1, 36]. Consequently, injured and apoptotic cells release damage associated molecular patterns (DAMPs) including DNA, heat shock proteins and intracellular components [7, 37]. The injury and death of intestinal epithelial cells leads to the breakdown of the mucosal barrier, allowing entry of microorganisms and the colonization of bacteria [7]. Microorganisms and bacteria release their own endogenous danger signals such as lipopolysaccharide, lipoteichoic acid and single-stranded mRNA, termed pathogen-associated molecular patterns (PAMPs) [7, 37].

The release of DAMPs and PAMPs initiates a signalling cascade mediated by Toll-like receptors (TLRs) of the TIR domain innate immune signalling pathway. Activation of the TIR domain innate immune signalling pathway leads to the recruitment of leukocytes and subsequent secretion of proinflammatory cytokines such as TNF-α and IL-1β. These molecular events, particularly TLR signalling, underlie the pathophysiology of GI toxicity [7, 37–40].

The TIR domain innate immune signalling pathway underpins the pathophysiology of GI toxicity

TLRs are a family of highly conserved type 1 integral membrane glycoproteins containing an extracellular motif of leucine-rich repeats and cytoplasmic TIR domain (Fig. 1) [41–43]. To date, ten TLRs have been identified in humans and are present on either the cell surface of innate immune cells (TLR1, 2, 4, 5 and 6) or localized in intracellular compartments such as the Golgi bodies and endosomes (TLR3, 7, 8 and 9) [44, 45]. TLRs respond to a variety of PAMPs, DAMPs or synthetic compounds [46]. TLR2 and TLR4 are key TLRs which mediate mucosal destruction and protection against GI chemotoxicity [44, 47]. They are also abundantly present on a variety of cells throughout the GI tract including goblet cells, cells of the lamina propria and enterocytes [44, 47]. Activation of TLR2 and TLR4 initiates the innate immune signalling pathway via the TIR domain and leads to the subsequent activation and translocation of NF-κB [46]. The TIR domain innate immune signalling pathway is detailed in Fig. 1.

Following recognition and binding of DAMPs and PAMPs, TLR2 dimerizes with TLR1 or 6, whilst TLR4 homodimerizes [46]. TLR dimerization prompts recruitment and binding of adapter protein myeloid differentiation primary response protein 88 (MyD88) to the TIR domain [46]. Binding of MyD88 in turn recruits interleukin-1 receptor-associated kinases (IRAK) 1 and IRAK4, resulting in phosphorylation of IRAK4 and subsequent activation and auto-phosphorylation of IRAK1 [46]. Phosphorylation of IRAK1 and IRAK4 enables binding of TNF receptor associated factor (TRAF) 6 to the TLR/MyD88/IRAK complex. Once bound, TRAF6 disengages from the TLR/MyD88/IRAK complex and associates with

transforming growth factor beta factor-β activated kinase (TAK) 1, TAK1-binding protein (TAB) 1 and TAB2 to form a new complex in the cytosol [46]. TAB1 enhances TAK1 kinase activity whilst TAB2 is an adapter protein responsible for linking TAK1 with TRAF6. Phosphorylation of TAB2 and TAK1 then occurs and the remaining complex associates with ubiquitin enzymes leading to the ubiquitylation of TRAF6 and activation of TAK1 [46]. TAK1 then phosphorylates the IKK complex (inhibitor of NF-κβ-kinase complex).

Transcription factor NF-κB exists as a heterodimer of three subunits, NF-κβ1 (p50/p105), NF-κβ2 (p52/p100) and p65 (Rel A) bound to the inhibitor kappa beta (Iκβ) subunit, responsible for maintaining NF-κB in an inactive state [46, 48, 49]. Once phosphorylated, the IKK complex phosphorylates the Iκβ subunit of NF-κβ, leading to its ubiquitination and subsequent degradation from the NF-κB complex by the 26S proteasome. Degradation of Iκβ permits the translocation of activated NF-κB to the nucleus (Fig. 1) [48].

In the nucleus, NF-κB regulates nearly 200 target genes, many of which are implicated in mucosal injury and cell death [50]. Of particular importance is the upregulation of genes including interleukin-1 beta (*IL1B*) and tumour necrosis factor alpha (*TNF*) encoding the proinflammatory cytokines IL-1β and TNF-α, respectively [12]. Inactive precursor IL-1β is cleaved by caspase1/IL-1β-converting enzyme (ICE) to its active form and is released from peripheral blood mononuclear cells and tissue macrophages [51]. TNF-α is a pleiotropic cytokine produced by tissue macrophages, natural killer cells and T lymphocytes [52, 53]. The production of IL-1β and TNF-α mediates an inflammatory response reducing epithelial oxygenation, initiating mesenchymal-epithelial signalling and stimulating further injury and death in cells of the epithelium and submucosa [7, 13, 38]. In addition, these cytokines can amplify primary damage by degrading Iκβ, further activating NF-κB and instigating its translocation to the nucleus [7, 13, 38].

These changes have been reported in both preclinical and clinical studies following 5-FU administration. For example, in Dark Agouti rats given 5-FU, TNF-α levels were highly elevated in the oral mucosa, jejunum and colon whilst IL-1β levels were highly elevated in the oral mucosa [5]. Additionally, TNF-α and IL-1β levels were associated with severe GI damage including reduced epithelial thickness, blunting and fusion of villi and obliteration of crypts [5]. Elevated TNF-α and IL-1β are also observed in the peripheral blood of patients whom experience GI toxicity following chemotherapy [7, 14].

To summarise, following damage instigated by 5-FU administration, the TIR domain innate immune signalling pathway is activated initiating a signalling cascade leading to the activation of proinflammatory cytokines such as IL-1β and TNF-α. These proinflammatory cytokines are consistently elevated in severely damaged mucosal tissue and associated with GI toxicity symptoms such as diarrhea and mucositis.

Have any predictors for severe GI toxicity risk been identified?

Sex and age

Patient characteristics such as sex and age are important facets that must be considered when identifying patients at risk of severe GI toxicity [54]. However, evidence defining the relationship between sex, age and severe GI toxicity is highly contradictory, with sex and age more adequately serving as co-contributors rather than predictors for severe GI toxicity risk. In patients receiving 5-FU-based chemotherapy for colorectal, gastric and upper GI tract cancers, women have been identified at being of higher risk for developing mucositis ($P = 0.04$) [55]. No significant relationship was identified between patients of advanced age and GI toxicity risk in the cohort [55]. Likewise, in patents receiving 5-FU and leucovorin for colorectal cancer, females developed higher counts of severe diarrhea ($P < 0.01$) and vomiting ($P = 0.03$) compared to their male counterparts [56]. Univariate analysis identified sex ($P < 0.0001$) as an independent predictor for severe GI toxicity in addition to advanced age ($P = 0.001$) [55], contradictory to the findings by Schwab et al. [55]. In similar studies, no significant relationship between the development of GI toxicity and sex was identified in patients receiving 5-FU-based chemotherapy [57], whilst advanced age was identified as protective against severe GI toxicity, with decreases in diarrhoea and nausea/vomiting episodes in advanced age patients ($P = 0.01$) [58]. Although sex and age cannot solely predict severe GI toxicity risk and the utility of these associations is not understood [54], it is known that certain SNPs, such as those found in dihydropyrimidine dehydrogenase (*DPYD*), are more commonly identified in one sex than the other [55] and, this may be true for other SNPs that are identified as predictors for severe GI toxicity risk. Therefore, factors such as sex and age are important co-contributors for severe GI toxicity risk prediction.

SNPs in the 5-FU rate determining enzyme

SNPs in *DPYD*, responsible for encoding the 5-FU rate determining enzyme DPD, have been thoroughly investigated for their role in the development of severe GI toxicity. During 5-FU-based therapies, 60–100% of patients

carrying one or multiple *DPYD* SNPs develop grade 3–4 toxicities (non-hematological and hematological) compared to 10–20% of patients carrying no *DPYD* SNPs [11, 55, 59, 60]. In particular, two recent meta-analyses identified the presence of *DPYD* SNPs, IVS14 + 1G > A, 2846A > T, 1679T > G***, and 1236G > A, increased the risk of grade 3 GI toxicities such as mucositis and diarrhoea ($P \leq 0.05$) [61, 62]. In addition, a clinical study of patients with colorectal cancer receiving FOLFOX (fluorouracil and oxaliplatin) or FOLFIRI (fluorouracil and irinotecan), showed *DPYD* SNPs D949V and 2*A increased the risk of diarrhea ($P = 0.003$) and nausea/vomiting ($P = 0.007$), respectively [63]. However, all of these aforementioned SNPs occurred in less than 5% of the populations studied and did not account for the majority of severe GI toxicity events [61–63]. Consequently, the clinical usefulness of routine genotyping in Caucasians for these *DPYD* SNPs prior to 5-FU treatment hasn't been established and current literature regarding the clinical sensitivity and specificity of diagnostic testing of the *DPYD* gene in this population is yet to be demonstrated [61]. This is further complicated by inconsistencies in the literature regarding the association of particular *DPYD* variants and GI toxicity between different ethnic populations [64, 65], such that there is the possibility that a rare variant in one ethnicity may be more frequent in another ethnicity and, therefore, have a greater impact on GI toxicity prevalence. Nevertheless, the US Food and Drug Administration (FDA) and The European Medicines Agency (EMA) warns about the increased risk of severe toxicity in patients carrying at least one decreased function *DPYD* allele on 5-FU and capecitabine drug labels [66–68] and, the Clinical Pharmacogenomics Implementation Consortium recommends altered 5-FU and capecitabine dosing based on *DPYD* genotype and the resulting phenotype [54]. Interestingly though, neither agency requires genetic testing for *DPYD* SNPs prior to treatment [63, 64]. As a result, new genetic predictors for severe GI toxicity risk need to be identified.

Candidate predictors for severe GI toxicity may lie within the TIR domain innate immune signalling pathway

SNPs in key TIR domain innate immune signalling pathway genes are uniquely positioned to influence gene functioning

The TIR domain innate immune signalling pathway is known to play a pivotal role in the development of GI toxicity; therefore, SNPs in key TIR domain innate immune signalling pathway genes may alter the pathophysiology and subsequent severity of GI toxicity. Hence, these mutations are candidate predictors for severe GI toxicity risk. A subset of mutations in TIR domain innate immune signalling pathway genes *TLR2, TLR4, MyD88, IRAK1, IRAK4, TRAF6, NFKB, IL1B* and *TNF* are summarized in Table 2.

The *TLR2* and *TLR4* genes are located on chromosomes 4q31.3 and 9q33.1, respectively, with many *TLR* SNPs resulting in either synonymous or missense mutations [69]. Synonymous mutations do not alter the primary amino acid sequence. However, they may have indirect effects on gene functioning by influencing mRNA splicing and subsequent mRNA translation [70]. The *MYD88, IRAK1, IRAK4* and *TRAF6* genes are located on chromosomes 3q22.2, Xq28, 12q12, and 11p12, respectively [69]. Many of the *MYD88, IRAK1, IRAK4* and *TRAF6* mutations are located within the intronic regions of their respective genes. It is currently thought intronic mutations influence mRNA stability and translation, and may cause alternative splicing sites [41, 71, 72].

The *NFKB* gene is located on chromosome 4q24, with the *NFKB* mutation rs38362491 resulting in a premature stop codon [69]. Dependent on location in the gene, premature stop codons can lead to early termination of gene transcription and subsequent changes in protein function [73, 74]. The *IL1B and TNF* genes are located on chromosomes 2q14.1 and 6q21.33, respectively [69]. Key *IL1B* and *TNF* mutations lie within the promoter region and, therefore, are in prime location to alter transcription factor binding and subsequent gene transcription [69].

Evidence suggests TIR domain innate immune signalling pathway SNPs alter gene functioning and influence disease susceptibility

The in vitro, ex vivo and in vivo effects of key TIR domain innate immune signalling pathway SNPs as well as their association with disease susceptibility, are summarized in Table 3. It should be noted that for some SNPs, there is a lack of knowledge regarding functional impacts: a current limitation that warrants further investigation.

A pilot study conducted by Coller et al. was the first to investigate the association between innate immune receptor genetic variability and severe GI toxicity risk following 5-FU-based chemotherapy [77]. *TLR2* rs384100 and *TNF* rs1800629 SNPs (in conjunction with colorectal and gastric cancer types) were identified to be predictive of severe GI toxicity risk [77]. However, no relationship was identified with *TLR4, MyD88* and *IL1B* SNPs [77].

Other studies have focussed on association of SNPs with disease susceptibility and impact of the SNPs on protein expression. Patients with *TLR2* and *TLR4* SNPs were identified to have a higher risk of developing bloodstream infections and sepsis, and higher circulating levels of PAMPs such as lipoteichoic acid (as described in Table 3) [75, 78]. On a

Table 2 Summary information about TIR domain innate immune signalling pathway SNPs [69]

ID	Type of variant	Base pair change	Amino acid change	MAF
TLR2				
rs11938228	Intronic	C > A	–	CEU: 0.30, HCB: 0.35 JPT: 0.48, YRI: 0.10
rs1898830	Intronic	A > G	–	CEU: 0.31, HCB: 0.36 JPT: 0.49, YRI: 0.08
rs3804099	cds-synon	T > C	–	CEU: 0.45, HCB: 0.37 JPT: 0.27, YRI: 0.63
rs3804100	cds-synon	T > C	–	CEU: 0.08, HCB: 0.33 JPT 0.22, YRI: 0.06
rs4696480	Intronic	T > A	–	CEU: 0.00, HCB: 0.00 JPT 0.00, YRI: 0.00
rs5743708	Missense	G > A	Arginine > glutamine	CEU: 0.05, HCB:0.00 JPT:0.00, YRI:0.00
TLR4				
rs10759930	Upstream	C > T	–	CEU: 0.37, HCB: 0.62 JPT: 0.65, YRI: 0.05
rs10759932	Upstream	T > C	–	CEU: 0.14, HCB: 0.26 JPT: 0.25, YRI: 0.25
rs4986790	Missense	A > G	Aspartate > glycine	CEU: 0.04, HCB: 0.00 JPT: 0.00, YRI: 0.04
rs4986791	Missense	C > T	Threonine > isoleucine	CEU: 0.05, HCB: 0.01 JPT: 0.00, YRI: 0.00
rs5030710	cds-synon	T > C	–	CEU: 0.00, HCB: 0.00 JPT: 0.00, YRI: 0.18
rs7044464	Upstream	T > A	–	CEU: 0.14, HCB: 0.07 JPT: 0.07, YRI: 0.29
rs7856729	Upstream	G > T	–	CEU: 0.15, HCB: 0.05 JPT: 0.06, YRI: 0.41
MYD88				
rs6853	3′-UTR	A > G	–	CEU: 0.12, HCB: 0.03 JPT: 0.01, YRI: 0.33
rs7744	3′-UTR	A > G	–	CEU: 0.15, HCB: 0.41 JPT: 0.28, YRI: 0.01
IRAK1				
rs1059701	cds-synon	C > T	–	HCB: 0.14, JPT: 0.18, YRI: 0.15
rs1059702	Missense	T > C	Phenylalanine > serine	CEU: 0.79, HCB: 0.15 JPT: 0.24, YRI: 0.98
rs1059703	Missense	C > T	Serine > leucine	CEU: 0.77, HCB: 0.17 JPT: 0.20, YRI: 0.62
rs2239673	Intronic	C > T	–	GMAF: 0.48
rs3027898	Downstream	C > A	–	CEU: 0.74, HCB: 0.12 JPT: 0.22, YRI: 0.52
rs5945174	Intronic	G > A	–	GMAF: 0.48
rs7061789	Intronic	G > A	–	GMAF: 0.48
rs731642	Intronic	G > A	–	GMAF: 0.44
IRAK4				
rs1141168	3′-UTR	A > G	–	CEU: 0.53, HCB: 0.47 JPT: 0.63
rs1461567	Intronic	C > T	–	CEU: 0.03, HCB: 0.41 JPT: 0.54, YRI: 0.02
rs3794262	Intronic	T > A	–	CEU: 0.93, HCB: 0.83 JPT: 0.91, YRI: 0.38
rs4251429	Intronic	G > C	–	CEU: 0.02, HCB: 0.07 JPT: 0.06, YRI: 0.35

Table 2 (continued)

ID	Type of variant	Base pair change	Amino acid change	MAF
rs4251431	Intronic	G > T	–	CEU: 0.05, HCB: 0.14 JPT: 0.14, YRI: 0.16
rs4251466	Intronic	C > T	–	CEU: 0.08, HCB: 0.09 JPT: 0.06, YRI:0.22
rs4251513	Intronic	C > G	–	CEU: 0.50, HCB: 0.42 JPT: 0.32, YRI: 0.05
rs4251532	Intronic	C > T	–	CEU: 0.07, HCB: 0.16 JPT: 0.09, YRI: 0.62
rs4251545	Missense	G > A	Alanine > threonine	CEU: 0.08, HCB: 0.09 JPT: 0.06, YRI: 0.31
rs4251569	5′-UTR	C > T	–	CEU: 0.09, HCB: 0.17 JPT: 0.12, YRI: 0.00
TRAF6				
rs16928973	Intronic	C > T	–	CEU: 0.19, HCB: 0.01 JPT: 0.12, YRI: 0.00
rs331449	Intronic	T > C		CEU: 0.00, HCB: 0.00 JPT: 0.00, YRI: 0.20
rs3740961	3′-UTR	A > G	–	CEU: 0.11, HCB: 0.51 JPT: 0.55, YRI: 0.01
rs5030411	Intronic	C > T	–	CEU: 0.60, HCB: 0.71 JPT: 0.72, YRI: 0.06
rs5030416	Intronic	A > C	–	CEU: 0.17, HCB: 0.10 JPT: 0.14, YRI: 0.16
rs5030445	Intronic	A > T		GMAF: 0.24
NFKB				
rs28362491	Insertion/deletion	– > ATTG	Delete codon	GMAF: 0.42
IL1B				
rs1143623	Upstream	C > G	–	CEU: 0.67, HCB: 0.61 JPT: 0.67, YRI: 0.94
rs1143627	Promoter	T > C	–	CEU: 0.37, HCB: 0.47 JPT: 0.46, YRI: 0.64
rs1143634	cds-synon	T > C	–	CEU: 0.02, HCB: 0.01 JPT: 0.05, YRI: 0.09
rs16944	Promoter	C > T	–	CEU: 0.35, HCB: 0.45 JPT: 0.47, YRI: 0.58
rs4848306	Promoter	G > A	–	CEU: 0.46, HCB: 0.52 JPT: 0.46, YRI: 0.30
TNF				
rs1799964	Downstream	T > C	–	CEU: 0.21, HCB: 0.23 JPT: 0.14, YRI: 0.12
rs1800629	Promoter	G > A	–	CEU: 0.17, HCB: 0.03 JPT: 0.02, YRI: 0.09
rs1800750	Promoter	G > A	–	CEU: 0.01, HCB:0.00 JPT: 0.00, YRI: 0.01
rs361525	Promoter	G > A	–	GMAF: 0.06
rs4248158	Downstream	C > T	–	GMAF: 0.02

> change, *cds-synon* coding synonymous mutation, *CEU* Caucasian population Utah, USA, *GMAF* global minor allele frequency, *HCB* Asian population Beijing, China, *JPT* Asian population Tokyo, Japan, *MAF* minor allele frequency, *UTR* untranslated region, *YRI* Sub-African population Yoruba, Nigeria

molecular level, *TLR2* and *TLR4* SNPs have been linked to altered interferon gamma secretion, altered specific antibody responses and reduced NF-κB circulating serum levels and activation (as described in Table 3) [75, 76, 81]. No relationship was identified between *MYD88* SNPs and TNF-α and IL-6 concentrations post-stimulation of ex vivo PBMCs

Table 3 In vitro, ex vivo, in vivo and genetic association studies investigating the effects of TIR domain innate immune signalling pathway SNPs on disease susceptibility, gene transcription, protein binding and cytokine secretion

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
TLR2	Ex vivo				
	rs4696480	Stimulated TNF-α, IL-6 and IL-10 secretion from isolated PMNs in patients with cirrhosis	$n = 114$	↓ stimulated TNF-α secretion in HE and HM vs WT ($P < 0.01$) ↓ stimulated IL-6 secretion in HE and HM vs WT ($P < 0.01$) ↑ stimulated IL-10 secretion in HE and HM vs WT ($P < 0.01$)	[75]
	In vivo				
	rs3804099 rs3804100	Association of SNPs with measles vaccine-induced immune responses	$n = 745$	Changes in antibody titer rs3804100 ↓ HE and HM vs WT ($P = 0.002$) Changes in cytokine secretion rs3804099 ↓ IFNλ-1 HE ($P = 0.002$) and HM ($P = 0.009$) vs WT	[76]
	rs4696480	Circulating LTA, LPS, TNF-α and IL-6 levels; stimulated secretion of TNF-α, IL-16 and IL-10 from cultured PMNs from patients with cirrhosis	$n = 114$	↓ circulating TNF-α in HM vs WT ($P < 0.05$) ↓ circulating IL-6 in HE and HM vs WT ($P < 0.05$)	[75]
	Genetic association				
	rs3804100	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	$n = 34$	Predictive of severe CIGT incidence in conjunction with *TNF* rs1800629 and colorectal and gastric cancer types ($P = 0.033$, ROC AUC = 87.3%)	[77]
	rs5743708	Association of SNPs with the severity and course of sepsis in critically ill patients	$n = 145$	↑ sepsis HE vs WT ($P = 0.03$) ↑ number of infections HE vs WT ($P = 0.012$) ↑ difficult-to-treat pathogens HE patients vs WT ($P = 0.045$)	[78]
	rs5743708	Association of SNPs with sepsis and pneumonia in patients with AML following induction chemotherapy	$n = 155$	↑ pneumonia HE vs WT ($P = 0.006$, OR: 10.78 95% CI = 2–23)	[79]
	rs11938228 rs1898830 rs3804099 rs3804100	Association of SNPs with HCC susceptibility	$n = 443$	rs3804099 and rs3804100 were in LD ($R^2 > 0.9$) ↓ HCC rs3804099 HE vs WT ($P < 0.001$, OR = 0.49, 95% CI = 0.3–0.7) and rs3804100 HE vs WT ($P < 0.001$, OR = 0.509, 95% CI = 0.3–0.8) rs11938228 & rs1898830: no significant associations	[80]
TLR4	In vitro				
	rs4986790 rs4986791	FEV1 decline following inhalation of LPS	$n = 83$	SNPs were in LD (data not provided) Dose–response decline in FEV1: WT 1.86% vs HE 0.59% ($P = 0.037$)	[81]
	Ex vivo				
	rs4986790	Stimulated TNF-α, IL-6 and IL-10 secretion from isolated PMNs in patients with cirrhosis	$n = 114$	↓ stimulated IL-6 secretion in HE vs WT ($P < 0.01$) ↑ stimulated IL-10 secretion in HE vs WT ($P < 0.01$)	[75]
	rs4986790 rs4986791	LPS-stimulated response in isolated airway epithelial cells	$n = 83$	SNPs were in LD (data not provided) ↓ IL-1α secretion by airway epithelial cells of HE vs WT patients ($P < 0.01$)	[81]

Table 3 (continued)

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
	In vivo				
	rs10759930 rs10759932 rs16906053 rs5030710 rs7044464 rs7856729	Association of SNPs with measles vaccine-induced immune responses	$n=745$	Changes in antibody titer rs5030710 and rs16906053 ↑ HE ($P=0.001$) and HM ($P=0.005$) vs WT rs16906053 ↓ IFN-λ-1 HE ($P=0.002$) and HM ($P=0.009$) vs WT rs7044464 and rs7856729 ↓ IL-10 HE and HM vs WT ($P=0.001$) rs10759932 ↑ IFN-α HE and HM vs WT ($P=0.001$) rs10759930 ↓ IFN-γ HE and HM vs WT ($P=0.001$) ↑ IFN-λ-1 HE and HM vs WT ($P=0.006$)	[76]
	rs4986790	Circulating LTA, LPS, TNF-α and IL-6 levels; stimulated secretion of TNF-α, IL-16 and IL-10 from cultured PMNs from patients with cirrhosis	$n=114$	↓ stimulated IL-6 secretion in HE vs WT ($P<0.01$) ↑ stimulated IL-10 secretion in HE vs WT ($P<0.01$) No significant differences in circulating TNF-α, IL-6 and IL-10 levels between WT, HE and HM ($P>0.05$)	[75]
	rs4986790 rs4986791	LPS-stimulated response in THP-1 cells	–	↓ NF-κB activation by THP-1 cells with rs4986790 allele vs WT ($P<0.01$)	[81]
	Genetic association				
	rs4986790	Association of SNPs with the severity and course of sepsis in critically ill patients	$n=145$	↑ endocarditis HE vs WT ($P<0.05$) ↑ bloodstream infections HE vs WT ($P<0.05$) ↑ progression of sepsis to septic shock HE vs WT ($P=0.014$)	[78]
	rs4986790 rs4986791	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	$n=34$	No significant associations	[77]
	rs4986790 rs4986791	Association of SNPs with sepsis and pneumonia in patients with AML following induction chemotherapy	$n=155$	SNPs were in LD (data not provided) ↑ sepsis development ($P=0.021$, OR: 3.6 95% CI = 1.2–10.4) ↑ pneumonia HE vs WT ($P=0.014$, OR: 3.6 95% CI = 1.3–9.9)	[79]
MyD88	Ex vivo				
	rs6853 rs7744	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	$n=90$	No significant associations	[71]
	Genetic association				
	rs6853	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	$n=34$	No significant associations	[77]
	rs6853 rs7744	Association of SNPs with T2DM and its vascular complications	$n=1106$	rs6853 2.9 ↑ T2DM HE and HM vs WT ($P=0.01$, OR: 2.9 95% CI = 1.3–6.7)	[82]
	rs6853 rs7744	Association of SNPs with sepsis-induced ALI susceptibility	$n=548$	No significant associations	[71]

Table 3 (continued)

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
	rs6853 rs7744	Association of SNPs with SIPD risk and 2° SIPD symptoms	$n = 200$	rs6853 Genotype analysis ↑ SIPD WT vs HE and HM ($P < 0.0001$, OR = 2.1 95% CI = 1.8–2.5) ↑ death from SIPD HE and HM vs WT ($P = 0.005$, OR = 16.1, 95% CI = 3.3–77.6) Allele analysis ↑ SIPD in patients carrying variant allele ($P < 0.0001$, OR = 1.9, 95% CI = 1.5–2.6) ↑ association of death in patients carrying variant allele ($P = 0.0064$, OR = 8.4, 95% CI = 2.5–28.5) rs7744: no significant associations	[83]
IRAK1	Ex vivo				
	rs1059703	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	$n = 90$	No significant associations	[71]
	Haplotype rs1059701 rs1059702 rs1059703 rs2239673 rs3027898 rs5945174 rs7061789 rs731642	Association of IRAK-1 haplotype with stimulated NF-κB activation from peripheral blood neutrophils	$n = 30$	↑ NF-κB nuclear accumulation post-LPS in HE and HM vs WT ($P = 0.001$)	[84]
	Genetic association				
	rs1059703	Association of SNPs with sepsis-induced ALI susceptibility	$n = 548$	No significant associations	[71]
	rs1059702 rs1059703	Association of SNPs with SIPD risk and 2° SIPD symptoms	$n = 200$	rs1059701 ↑ SIPD WT vs HE and HM ($P = 0.0067$, OR = 1.4 95% CI = 1.1–1.8) rs1059702 ↑ leukocytosis HE and HM vs WT ($P = 0.046$, OR = 7.5, 95% CI = 1.9–30.2)	[83]
	Haplotype rs1059701 rs1059702 rs1059703 rs2239673 rs3027898 rs5945174 rs7061789 rs731642	Association of IRAK-1 haplotype with 2° symptoms in patients with sepsis	$n = 30$	2.9 ↑ likelihood of developing septic shock in HE and HM vs WT ($P = 0.047$) 2.6 ↑ likelihood of death from sepsis in HE and HM vs WT ($P = 0.05$)	[84]

Table 3 (continued)

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
	Meta-analysis				
	rs1059702	Susceptibility of ADs in patients with *IRAK1* SNPs	–	rs1059702	[85]
	rs1079703			↑ ADs WT vs HE and HM ($P = 0.000$, OR = 0.8, 95% CI = 0.7–0.8)	
	rs3027898			↑ SLE WT vs HE ($P = 0.000$, OR = 0.7, 95% CI = 0.6–0.7)	
				↑ SSc WT vs HE and HM ($P = 0.032$, OR = 0.8, 95% CI = 0.6–0.9)	
				rs1079703	
				↑ SLE variant vs WT ($P = 0.000$, OR = 1.5, 95% CI = 1.3–1.6)	
				rs3027898	
				↑ ADs WT vs HE and HM ($P = 0.034$, OR = 0.7, 95% CI = 0.6–0.9)	
				↑ SLE WT vs HE ($P = 0.001$, OR = 0.8, 95% CI = 0.6–0.9)	
				↑ RA WT vs HE ($P = 0.021$, OR = 0.8, 95% CI = 0.7–0.9)	
IRAK4	Ex vivo				
	rs1461567	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	$n = 90$	No significant associations	[71]
	rs3794262				
	rs4251429				
	rs4251431				
	rs4251466				
	rs4251513				
	rs4251545				
	rs4251569				
	Genetic association				
	rs1141168	Association of SNPs with SIPD risk and 2[yr] SIPD symptoms	$n = 200$	rs4251513	[83]
	rs1461567			Genotype analysis	
	rs4251513			↑ SIPD WT vs HE and HM ($P < 0.0001$, OR = 2.2, 95% CI = 1.6–3.0)	
				↑ SIPD sequelae HE and HM vs WT ($P = 0.001$	
				OR = 7.1, 95% CI = 2.6–18.9)	
				Allele analysis	
				↑ presence of SIPD in patients carrying variant allele ($P < 0.0001$, OR = 1.5, 95% CI = 1.4–1.5)	
				rs1461567	
				↑ SIPD variant allele vs WT ($P = 0.016$, OR = 1.5, 95% CI = 1.1–1.9)	
				rs1141168: no significant associations	
	rs1461567	Association of SNPs with T2DM and its vascular complications	$n = 1106$	1.66 ↑ T2DM HE vs WT and HM ($P = 0.03$, OR = 1.7, 95% CI = 1.1–2.6)	[82]
	rs4251513				
	rs4251532				
	rs4251569				
	rs1461567	Association of SNPs with sepsis-induced ALI susceptibility	$n = 548$	No significant associations	[71]
	rs3794262				
	rs4251429				
	rs4251431				
	rs4251466				
	rs4251513				
	rs4251545				
	rs4251569				

Table 3 (continued)

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
TRAF6	Ex vivo				
	rs4755453 rs5030493 rs540386	TRAF6 expression and stimulated TNF-α and IL-6 secretion from PBMCs of patients with sepsis-induced ALI	*n* = 90	rs4755453 ↑ TRAF6 baseline mRNA expression WT vs HE ($P = 0.012$) and vs HM ($P = 0.003$) ↑ TRAF6 post-LPS mRNA expression WT vs HE ($P = 0.009$) and vs HM ($P = 0.005$) ↑ TNF-α post-LPS secretion WT vs HE and HM ($P = 0.015$) ↑ IL-6 post-LPS secretion WT vs HE and HM ($P = 0.009$) rs5030493 & rs540386: no significant associations	[71]
	In vivo				
	rs331449	Association of SNPs with measles vaccine-induced immune responses	*n* = 745	↑ HE and HM vs WT ($P = 0.007$)	[76]
	Genetic association				
	rs16928973 rs5030445	Association of SNPs with T2DM and its vascular complications	*n* = 1106	No significant associations	[82]
	rs4755453 rs5030493 rs540386	Association of SNPs with sepsis-induced ALI susceptibility	*n* = 548	↓ % variant allele in sepsis-induced ALI vs sepsis alone groups ($P = 0.003$,OR: 0.5, 95% CI = 0.4–0.7)	[71]
	rs3740961 rs5030411 rs5030416 rs5030445	Association of SNPs with susceptibility and severity of sepsis	*n* = 510	No significant associations	[86]
NFKB	In vitro				
	rs28362491	Transient transfection luciferase reporter gene assay; and EMSA to determine protein binding in HeLA and HT-29 cells	–	↓ activity at baseline with variant vs WT allele in HeLA cells ($P = 0.05$) ↓ activity following stimulation with variant vs WT allele in HeLA cells ($P = 0.02$) ↓ activity following stimulation with variant vs WT allele in HT-29 cells ($P = 0.02$)	[74]
	Genetic association				
	rs28362491	Association of SNP with IBD risk	*n* = 822	No significant associations	[87]
	rs28362491	Association of SNPs with pathological response in patients with rectal cancer treated with PCRT	*n* = 159	↑ pathological response HE and HM vs WT ($P = 0.03$, OR = 6.4, 95% CI = 0.8–52.7)	[88]
IL1B	In vitro				
	rs1143634	Association of SNP with IL-1β serum concentration following infliximab treatment	*n* = 47	↑ IL-1β with variant vs WT allele ($P = 0.026$)	[89]

Table 3 (continued)

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
	rs1143623 rs1143627 rs16944 rs4848306	Transient transfection reporter gene assay and gel mobility shift assay in THP-1 cells	–	rs4848306 ↓ nuclear protein binding with variant vs WT allele (P <0.01) rs1143623 ↑ nuclear protein binding with variant vs WT allele (P <0.01) ↓ transcriptional activity variant vs WT allele (P <0.01) rs1143627 ↑ nuclear protein binding of complex 1 with variant vs WT allele (P <0.01) ↓ binding of complex 2 and 3 with variant vs WT allele (P <0.01) ↓ transcriptional activity with variant vs WT allele (P <0.01) rs16944 ↑ transcriptional activity with variant vs WT allele (P <0.05)	[90]
	Ex vivo				
	rs1143627 rs1143634 rs16944	Stimulation of isolated human monocytes and EMSA of nuclear extracts	n = 442	rs1143627 and rs16944 in LD (no data given) rs1143627 Fivefold ↑ DNA binding post-LPS stimulation in HE vs WT	[91]
	Genetic association				
	rs16944	Association of SNPs with toxicity in patients undergoing 5-FU and cisplatin chemotherapy	n = 100	↑ thrombocytopenia HE and HM vs WT (P = 0.015, OR = 2.9, 95% CI = 1.2–7.0) predictive of stomatitis (P <0.01) and thrombocytopenia (P = 0.02) in conjunction with *TNF* rs1799964	[92]
	rs1143627 rs16944	Association of SNPs with pathological response in patients with rectal cancer treated with PCRT	n = 159	No significant associations	[88]
	rs1143627 rs1143634 rs16944	Association of SNPs with risk of gastric cancer	n = 442	rs1143627 and rs 16944 in LD (0.99) rs1143627 HE associated with gastric cancer risk OR = 1.9 (95% CI = 1.5–2.6) rs16944: no significant association	[91]
	rs1143627 rs1143634 rs16944	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	n = 34	No significant associations	[77]
TNF	In vitro				
	rs1800629	Functional analysis of SNP using CAT reporter gene in Jurkat and Raji cells	–	No significant associations	[93]
	rs1800629	Transient transfection luciferase reporter gene assay in Jurkat and U937 cells	–	Twofold ↑ transcription with variant vs WT allele in both Jurkat and U937 cells (P <0.05)	[94]
	Ex vivo				
	rs1800629 rs1800750 rs361525	Association of SNPs with endotoxin-induced TNF-α secretion from PBMCs	n = 129	No significant differences	[95]

Table 3 (continued)

Gene	SNPs	Study details	Number of participants	Impact of SNPs	Refs.
	rs1800629 rs1800750 rs361525	Stimulated TNF-α secretion from whole blood	$n=179$	No significant associations	[96]
	rs1800629 rs1800750 rs361525 rs4248158	Association of SNPs with TNF-α serum concentration following infliximab treatment	$n=47$	No significant associations	[89]
	Genetic association				
	rs1800629	Association of SNP with clinical outcome, incidence and severity of toxic complications and GvHD in HSCT patients	$n=70$	↑ severe toxicity HE vs WT ($P=0.014$, OR = 17.2 95% CI = 1.8–168.1)	[73]
	rs1800629	Association of SNPs with severe CIGT incidence following 5-FU-based treatment	$n=34$	*TLR2* and *TNF* SNPs were predictive of severe CIGT incidence in conjunction with colorectal and gastric cancer types ($P=0.033$, ROC AUC = 87.3%)	[77]
	rs1799964	Association of SNPs with toxicity in patients undergoing 5-FU and cisplatin chemotherapy	$n=100$	↑ stomatitis HE and HM vs WT ($P=0.02$, OR = 3.1 95% CI = 1.2–8.3) predictive of stomatitis ($P<0.01$) and thrombocytopenia ($P=0.02$) in conjunction with *IL1B* 16,944	[92]
	rs1800629 rs1800750 rs361525	Association of SNPs with MS	$n=179$	No significant associations	[96]

↑ increase, ↓ decrease, *2°* secondary, *AD* autoimmune disease, *ALI* acute lung injury, *AML* acute myeloid leukaemia, *CAT* chloramphenicol acetyltransferase, *EMSA* electromobility shift assay, *GvHD* graft vs host disease, *HCC* hepatocellular carcinoma, *HE* heterozygous, *HM* homozygous, *HSCT* hematopoietic stem cells, *IBD* irritable bowel syndrome, *LD* linkage disequilibrium, *LPS* lipopolysaccharide, *LTA* lipoteichoic acid, *MS* multiple sclerosis, *OR* odds ratio, *PCRT* primary chemoradiation therapy, *PBMCs* peripheral blood mononuclear cells, *PMNs* peripheral polymorphonuclear cells, *RA* rheumatoid arthritis, *ROC AUC* receiver operator characteristic, area under the curve, *SLE* systemic lupus erythematosus, *SSC* systemic sclerosis, *SIPD* invasive pneumococcal disease, *T2DM* type 2 diabetes, *WT* wild-type

and the effect of *MYD88* SNPs on MyD88 protein expression is variable (as described in Table 3) [71, 97]. *IRAK1* and *IRAK4* SNPs have been associated with an increased risk of autoimmune disease, with *IRAK1* SNPs also identified to increase NF-κB activation (as described in Table 3) [84, 85]. *TRAF6* SNPs were found to increase TNF-α and IL-6 secretion at baseline and post-stimulation of ex vivo PBMCs, but no association was identified between *TRAF6* SNPs with sepsis susceptibility and severity (as described in Table 3) [71, 86]. *NFKB* SNP rs28362491 was associated with severe toxic complications in hematopoietic stem cell transplantation (HSCT) patients and decreased NF-κB activity (as described in Table 3) [73, 74].

IL1B and *TNF* SNPs show the greatest potential as predictors for severe GI toxicity risk

Of the TIR domain innate immune signalling pathway SNPs discussed in Table 3, *IL1B* and *TNF* SNPs show the greatest potential as predictors for severe GI toxicity as they have been identified to not only alter gene function but also increase cancer risk and predict toxic side effects following chemotherapy. The *IL1B* SNP rs16944 was shown to increase transcriptional activity of IL-1β and was identified as being predictive of stomatitis (in conjunction with *TNF* 1799964) in patients receiving 5-FU and cisplatin chemotherapy treatment [90, 92] (Table 3). *IL1B* rs1143634 was identified to increase the risk of gastric cancer, with an increase in nuclear protein binding identified in reporter gene assays [90, 91] (Table 3). This, in addition to further evidence presented in Table 3, demonstrates *IL1B* SNPs are candidate predictors for severe GI toxicity risk. However, using individual *IL1B* SNPs as predictors for severe GI toxicity risk may be complicated, as many SNPs in *IL1B* are in linkage disequilibrium; therefore, SNPs with opposing effects may 'cancel' one another out [91]. With regards to *TNF* SNPs, rs1800629 influences gene functioning, with a twofold increase in transcriptional activity identified in patients carrying the variant [94] In addition, a small pilot study of patients receiving 5-FU-based chemotherapy, rs1800629 was found to be predictive of severe GI toxicity risk in a multivariate logistic regression model [77].

Conclusion

Severe GI toxicity is a debilitating side effect following 5-FU-based chemotherapy. It is essential predictors for severe GI toxicity risk are found to allow patients at most risk of severe GI toxicity to be identified prior to treatment, allowing the personalization of supportive care measures to reduce their risk of developing severe GI toxicity. This would not only improve clinical outcomes and long-term prognosis, but also improve patient quality of life whilst on otherwise life-saving chemotherapy treatment. This critical review has provided evidence to suggest TIR domain innate immune signalling pathway SNPs are suitable candidate predictors for severe GI toxicity risk following 5-FU-based chemotherapy.

However, further investigation is required to thoroughly understand the effect of these TIR domain innate immune signalling pathway SNPs on the mechanisms underlying the development of GI toxicity. To allow the 'bench to beside' translation of TIR domain innate immune signalling pathway SNPs as clinical predictors for severe GI toxicity risk, it is critical to not only associate, but also identify mechanisms by which these SNPs influence the development of severe GI toxicity in addition to understanding the exact functional impact of the SNPs themselves.

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Compliance with ethical standards

Conflict of interest Samantha Korver declares she has no conflict of interest. Rachel Gibson is a consultant for Kaleido Biosciences, Mundipharma and Onyx Pharmaceuticals, and has received research funding from Onyx Pharmaceuticals and AstraZeneca. Joanne Bowen has received research funding from AstraZeneca, Helsinn, Pfizer and Puma Biotechnology Inc. Janet Coller declares she has no conflict of interest.

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Appendix: Chapter 4

Chapter 4, Appendix Natural log-transformed IL-1β secretion ($pg.mL^{-1}$) pre- and post- TLR2 (PAM3CSK4) or TLR4 (LPS) stimulation for carriers of homozygous wild-type, heterozygous or homozygous variant genotypes for a) *TLR2* rs3804100, b) *TLR4* rs4986790 and rs4986791, c) *IL1B* rs16944 and rs1143627, d) *IL1B* rs1143634 and e) *TNF* rs1800629.

a *TLR2* rs3804100

		WT/WT (n = 28)	**WT/Var** (n = 3)	
	µg.ml^{-1}	**Mean** (range)	**Mean** (range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	1.9 (0.5 – 3.5)	2.1 (0.8 – 2.1)	0.665
	0.00005	0.4 (0.1 – 2.3)	0.4 (0.2 – 0.4)	0.747
	0.1	2.4 (1.5 – 3.9)	2.4 (2.1 – 2.8)	0.765
	100	2.5 (1.8 – 4.0)	2.3 (2.1 – 2.4)	0.665
PAM IL-1β	Pre-stimulation	1.9 (0.1 – 3.0)	1.4 (1.3 – 2.0)	0.747
	0.00005	2.1 (0.2 – 3.0)	2.0 (1.9 – 2.1)	0.747
	0.01	2.4 (1.4 – 3.1)	2.2 (1.9 – 2.4)	0.665
	1	2.5 (1.7 – 3.1)	2.2 (1.9 – 2.2)	0.665
LPS TNF-α	Pre-stimulation	2.1 (1.2 – 3.8)	3.4 (1.6 – 3.5)	0.665
	0.00005	2.4 (1.1 – 4.1)	3.2 (2.1 – 3.5)	0.665
	0.1	2.4 (1.6 – 3.8)	3.6 (2.3 – 3.8)	0.665
	100	2.5 (1.4 – 4.0)	3.7 (2.0 – 3.8)	0.747
PAM TNF-α	Pre-stimulation	2.0 (0.8 – 3.5)	2.8 (1.2 – 2.9)	0.665
	0.00005	2.3 (1.0 – 3.7)	2.6 (0.4 – 3.1)	0.665
	0.01	2.3 (1.4 – 3.6)	3.1 (2.9 – 3.3)	0.665
	1	2.5 (1.2 – 3.8)	3.0 (2.3 – 3.4)	0.665

LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; WT/WT: homozygous wild-type genotype; WT/Var: Heterozygous genotype.

b *TLR4* rs4986790 and rs4986791

		WT/WT (n = 28)	**WT/Var** (n = 3)	
	µg.ml^{-1}	**Mean** (range)	**Mean** (range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	1.9 (0.5 – 3.5)	3.1 (1.7 – 3.1)	0.801
	0.00005	0.4 (0.08-2.5)	0.5 (0.2 – 0.5)	0.966
	0.1	2.4 (1.5 – 3.9))	3.2 (1.7 – 3.7)	0.801
	100	2.5 (1.9 – 4.0))	3.3 (1.8 – 3.6)	0.801
PAM IL-1β	Pre-stimulation	1.8 (0.1 -3.0)	2.5 (1.6 – 2.6)	0.801
	0.00005	2.1 (0.2 – 3.0)	2.5 (1.7 – 2.5)	0.866
	0.01	2.4 (1.4 – 3.0)	2.9 (1.7 – 3.1)	0.801
	1	2.5 (1.7 – 3.1)	2.8 (2.0 – 3.0)	0.801
LPS TNF-α	Pre-stimulation	2.3 (1.2 – 3.8)	1.9 (1.6 – 2.9)	0.801
	0.00005	2.5 (1.1 – 4.1)	2.3 (2.3 – 3.2)	0.966
	0.1	2.4 (1.6 – 3.8)	2.4 (2.3 – 3.2)	0.966
	100	2.6 (1.4 – 4.0)	2.5 (2.3 – 3.2)	0.866
PAM TNF-α	Pre-stimulation	2.2 (0.8 – 3.5)	1.7 (1.4 – 2.1)	0.801
	0.00005	2.3 (0.4 – 3.7)	1.8 (1.8 – 2.5)	0.801
	0.01	2.3 (1.4 – 3.6)	1.5 (1.4 – 2.3)	0.400
	1	2.5 (1.2 – 3.8)	2.1 (1.8 – 2.5)	0.801

LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; WT/WT: homozygous wild-type genotype; WT/Var: Heterozygous genotype.

c *IL1B* rs16944 and rs1143627

		WT/WT (n = 13)	**WT/Var** (n = 15)	**Var/Var** (n = 3)	
	µg.ml^{-1}	**Mean** (range)	**Mean** (range)	**Mean** (range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	1.9 (0.5 – 3.4)	2.1 (0.7 – 3.5)	1.8 (11 – 1.8)	0.622
	0.00005	0.4 (0.2 – 0.6)	0.4 (0.08 – 0.6)	0.3 (0.3 – 0.3)	0.547
	0.1	2.4 (1.9 – 3.9)	2.5 (1.5 – 3.7)	2.1 (2.0 – 2.4)	0.622
	100	2.5 (2.1 – 4.0)	2.4 (1.8 – 3.8)	2.1 (2.1 – 2.4)	0.622
PAM IL-1β	Pre-stimulation	1.9 (0.11 – 2.6)	2.0 (1.1 – 1.8)	1.4 (0.1 – 1.8)	0.547
	0.00005	2.1 (1.2 – 3.0)	2.1 (0.2 – 3.0)	2.0 (1.9 – 2.1)	0.92
	0.01	2.5 (1.9 – 3.0)	2.4 (1.4 – 3.1)	2.1(2.0 – 3.1)	0.622
	1	2.5 (2.2 – 3.1)	2.4 (1.7 – 3.0)	2.2 (2.0 – 2.4)	0.547

LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; WT/WT: homozygous wild-type genotype; WT/Var: Heterozygous genotype; Var/Var: Homozygous variant genotype.

d *IL1B* rs1143634

		WT/WT (n = 17)	**WT/Var** (n = 9)	**Var/Var** (n = 5)	
	µg.ml^{-1}	**Mean** (range)	**Mean** (range)	**Mean** (range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	1.8 (0.5 – 3.4)	2.1 (0.7 – 3.2)	2.5 (1.7 – 3.5)	0.881
	0.00005	0.4 (0.2 – 0.6)	0.4 (0.08 – 0.5)	0.5 (0.2 – 0.6)	0.881
	0.1	2.5 (2.0 – 4.0)	2.4 (1.5 – 3.3)	2.4 (1.7 – 3.7)	0.881
	100	2.5 (1.9 – 4.0)	2.4 (2.1 – 3.3)	2.8 (1.8 – 3.8)	0.881
PAM IL-1β	Pre-stimulation	1.6 (0.1 – 2.6)	2.0 (0.2 – 2.6)	1.8 (1.6 – 3.0)	0.881
	0.00005	2.1 (1.2 – 3.0)	2.1 (0.2 – 2.9)	2.3 (1.7 – 3.0)	0.881
	0.01	2.4 (1.9 – 3.1)	2.4 (1.3 – 2.9)	2.6 (1.7 – 3.0)	0.898
	1	2.4 (1.7 – 3.1)	2.5 (1.9 – 2.9)	2.5 (2.0 – 2.9)	0.881

LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; WT/WT: homozygous wild-type genotype; WT/Var: Heterozygous genotype; Var/Var: Homozygous variant genotype.

e *TNF* rs1800629

		WT/WT (n = 21)	**WT/Var** (n = 7)	**Var/Var** (n = 3)	
	µg.ml^{-1}	**Mean** (range)	**Mean** (range)	**Mean** (range)	**FDR-corrected P-value**
LPS IL-1β	Pre-stimulation	2.8 (1.6 – 3.8)	1.8 (1.4 – 3.5)	1.6 (1.2 – 3.5)	0.360
	0.00005	2.6 (1.1 – 4.1)	2.1 (1.5 – 3.5)	2.4 (2.1 – 2.4)	0.513
	0.1	2.8 (1.6 – 3.8)	2.3 (1.5 – 3.8)	2.0 (1.9 – 2.3)	0.400
	100	3.0 (1.4 – 4.0)	2.4 (1.9 – 3.8)	2.3 (2.0 – 2.4)	0.400
PAM IL-1β	Pre-stimulation	2.4 (0.8 – 1.3)	1.8 (1.3 – 2.9)	1.5 (1.2 – 1.8)	0.400
	0.00005	2.4 (1.0 – 3.7)	2.4 (2.3 – 3.1)	1.9 (0.3 – 2.2)	0.360
	0.01	2.5 (1.4 – 3.6)	2.3 (1.5 – 3.3)	2.2 (2.2 – 2.3)	0.738
	1	2.6 (1.3 – 3.8)	2.5 (1.6 – 3.4)	2.4 (2.3 – 2.5)	0.739

LPS IL-1β: TLR4 (LPS) stimulated IL-1β secretion; n: number of participants; PAM IL-1β: TLR2 (PAM3CSK4) stimulated IL-1β secretion; WT/WT: homozygous wild-type genotype; WT/Var: Heterozygous genotype; Var/Var: Homozygous variant genotype.

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