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1 **Title page**

2 Pre-therapy mRNA expression of TNF is associated with regimen-related gastrointestinal toxicity in  
3 patients with esophageal cancer: A pilot study

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19

1 **Abstract**

2 Purpose: Esophageal cancer has a high mortality rate, and its multimodality treatment is often  
3 associated with significant rates of severe toxicity. Effort is needed to uncover ways to maximize  
4 effectiveness of therapy through identification of predictive markers of response and toxicity. As  
5 such, the aim of this study was to identify genes predictive of chemoradiotherapy-induced  
6 gastrointestinal toxicity using an immune pathway-targeted approach.

7 Methods: Adults with esophageal cancer treated with chemotherapy consisting of 5-fluorouracil and  
8 cisplatin, and 45-50 Gy radiation were recruited to the study. Pre-therapy-collected whole blood was  
9 analyzed for relative expression of immune genes using RT-PCR. Gene expression was compared  
10 between patients who experienced severe regimen-related gastrointestinal toxicity vs. those  
11 experiencing mild to moderate toxicity.

12 Results: Blood from 31 patients were analyzed by RT-PCR. Out of 84 immune genes investigated,  
13 TNF was significantly elevated (2.05-fold,  $p = 0.025$ ) in the toxic group ( $n = 12$ ) compared to the  
14 non-toxic group ( $n = 19$ ). Nausea and vomiting was the most commonly documented severe toxicity.  
15 No associations between toxicity and response, age, sex, histology or treatment were evident.

16 Conclusions: This study supports evidence of TNF as a predictive biomarker in regimen-related  
17 gastrointestinal toxicity. Confirming these findings in a larger cohort is warranted.

18

19 **Keywords**

20 Toxicity, chemoradiation, esophageal cancer, mucositis, RT-PCR

## 1 **Introduction**

2 Across the past four decades, the incidence of esophageal adenocarcinoma (EAC) has increased 6-  
3 fold throughout the Western World, particularly in men, whilst rates of squamous cell carcinoma  
4 (SCC) of the esophagus remain unchanged [1]. Esophageal cancer has one of the poorest survival  
5 rates among patients with solid tumors, and its multimodality treatment with combined chemotherapy  
6 and radiotherapy is often associated with significant rates of severe toxicity [2, 3]. Effort is needed to  
7 uncover ways to maximize the effectiveness of therapy through identification of predictive markers of  
8 response and toxicity.

9 Acute toxicity affecting the gastrointestinal (GI) mucosa is a major impediment to optimal cancer  
10 treatment and is particularly problematic in cancers of the esophagus. Patients with locally advanced  
11 tumors are typically managed with neoadjuvant or definitive chemoradiotherapy (CRT) [4, 5], which  
12 results in up to 43% of patients experiencing severe (grade  $\geq 3$ ) GI toxicity (including oral mucositis,  
13 esophagitis, nausea and vomiting and diarrhea) depending on the protocol [6]. Of significant clinical  
14 importance, GI toxicity can lead to up to 45% of patients not receiving full dose chemotherapy, and  
15 up to 30% having interrupted radiotherapy [3, 7], and this can negatively impact on treatment success.  
16 Complete pathological response to CRT is associated with better long term survival [8]. However,  
17 25% of patients do not respond to CRT and hence undergo this treatment and its toxicities  
18 unnecessarily [9].

19 Reliable predictive markers of severe GI toxicity risk are currently unavailable. One potential  
20 approach is the use of gene expression signatures [10-12]. Numerous lines of evidence support the  
21 role of basal gene expression in establishing a pre-treatment risk profile, and a number of studies have  
22 found associations between genetic factors and regimen-related toxicity [13-15]. Recently, attention  
23 has moved away from pharmacogenetic risk gene such as dihydropyrimidine dehydrogenase (*DPYD*)  
24 due to the rarity of informative variants and consequent failure to account for the majority of toxicity  
25 seen [16]. Instead, increased focus has been placed on the role of immune determinants of mucosal  
26 inflammation. This is borne out of the knowledge that GI toxicity (most notably mucositis) is

1 fundamentally underpinned by mucosal inflammatory damage. Substantial preclinical and clinical  
2 research has shown that many of the key mediators of regimen related mucosal injury are derived  
3 from the innate immune response pathway [17]. In particular, activation of the transcription factor  
4 NF- $\kappa$ B, and up-regulation of its pro-inflammatory cytokine target genes including TNF- $\alpha$ , IL-1 $\beta$  and  
5 IL-6 are implicated in modulating injury [18, 19]. Furthermore, the severity of mucosal injury  
6 correlates with the intensity of pro-inflammatory cytokine production, and interference with cytokine  
7 production favorably modifies the course of experimental mucositis [20]. In clinical studies, increased  
8 levels of TNF- $\alpha$  and IL-6 measured in peripheral blood correlates with mucositis severity [21, 22].  
9 These findings implicate pro-inflammatory cytokines as central mediators in the pathogenesis of  
10 cancer therapy-induced GI toxicity and promote their further investigation as risk modifiers. In  
11 support of observations from mRNA and animal models, genetic variants in mucosal injury mediators,  
12 such as TNF- $\alpha$ , NF- $\kappa$ B, IL-1 $\beta$  and IL-6, have been linked to toxicity [13, 23, 24].

13 Based on these previous studies, we hypothesized that pre-therapy expression of innate immune genes  
14 would be measurably different in patients that go on to suffer from severe GI toxicity compared to  
15 those that do not. As such, this proof of concept pilot study used an immune pathway-targeted, multi-  
16 array approach to prospectively analyze pre-therapy gene expression profiles in patients with  
17 esophageal cancer treated with CRT.

## 1 **Methods**

### 2 *Patient identification and sample collection*

3 This study was approved by the Royal Adelaide Hospital Research Ethics Committee and complied  
4 with the Declaration of Helsinki. All study participants gave written informed consent. Eligible  
5 participants were identified at the South Australian Statewide Upper Gastrointestinal Cancer  
6 Multidisciplinary Team Meeting or by endoscopy surgical lists, and approached for inclusion at the  
7 hospital or by mailed study information sheets. Patients with cancer of the esophagus (including  
8 adenocarcinoma, squamous cell carcinoma and gastroesophageal junction) scheduled to receive  
9 concurrent CRT were eligible for inclusion. Chemotherapy entailed 2 cycles of 5-fluorouracil (1000  
10 mg/m<sup>2</sup>/day for 96 h by ambulatory pump) and cisplatin (75 mg/m<sup>2</sup>) spaced 4 weeks apart. Radiation  
11 consisted of 45 Gy, if given before surgical resection, and 50 Gy if given as definitive treatment,  
12 administered as 25 equal fractions across 5 weeks. Pretreatment supportive medication was standard  
13 and consisted of 165mg aprepitant, 12mg dexamethasone and 10mg ondansetron / 250mcg  
14 palonosetron. Post infusion medication included 165mg aprepitant, 10-20mg metoclopramide (as  
15 required for nausea), 8mg dexamethasone (2 tablets daily x 3 days) and 2-4mg loperamide (as  
16 required for diarrhea). Patients who had received prior chemotherapy or radiation, had a serious  
17 concomitant medical or psychiatric illness, or had active mucosal ulceration were excluded. Patients  
18 were prospectively recruited and gave 2.5 mL of blood, collected into PaxGene RNA tubes for  
19 stabilization of RNA (PreAnalytiX, Qiagen) prior to therapy.

20 Clinical records were reviewed for collection of clinical information, including patient demographics,  
21 chemotherapy and radiation protocol, tumor histology, and toxicity. Two specialist esophageal  
22 surgeons independently reviewed the clinical records to determine response to CRT, as summarized in  
23 table 1. All toxicity data was graded according to the National Cancer Institute Common Terminology  
24 Criteria for Adverse Events version 4.0. To maintain uniformity, a single investigator [IW] was  
25 responsible for data extraction and final toxicity scoring as directed by physician reporting in case  
26 notes. For the purpose of analysis, patients were categorized as either toxic or non-toxic. Patients

1 with any reported GI toxicity grade  $\geq 3$  were assigned as toxic, whereas patients with grade  $\leq 2$  toxicity  
2 were assigned as “non-toxic”. Any patient that required a dose reduction, treatment break or  
3 hospitalization due to GI toxicity was automatically assigned to the toxic group. The Fisher’s exact  
4 test was used to compare patient characteristics between groups.

#### 5 *RNA isolation*

6 Total RNA was isolated from whole blood using the PreAnalytiX RNA extraction kit as per  
7 manufacturers’ instructions (Qiagen, CA, USA). Briefly, silica-membrane spin column technology is  
8 used, where RNA  $>18$  nucleotides (including miRNA) binds to the membrane, is subjected to DNase  
9 digestion to remove genomic DNA contamination and washed before final elution in proprietary  
10 buffer solution. Integrity of eluted RNA was tested with the Agilent Bioanalyzer at the Adelaide  
11 Microarray Centre (University of Adelaide).

#### 12 *PCR arrays and RT-PCR*

13 The RT<sup>2</sup> Profiler™ PCR Array Human Innate & Adaptive Immune Responses in 100 disk format  
14 (Qiagen, CA, USA) was used to compare relative transcript expression between groups. All arrays  
15 were conducted according to manufacturers’ instructions, including cDNA synthesis using the RT<sup>2</sup>  
16 HT First Strand Kit, and use of SYBR Green ROX FAST Mastermix for the Rotor-Gene cyclers.  
17 Cycle threshold values were analyzed by  $2^{-\Delta\Delta CT}$  to determine expression fold changes and unpaired t-  
18 test used to detect significance between groups, respectively.

19 For real-time polymerase chain reaction (RT-PCR) validation of differentially expressed transcripts,  
20 RNA was converted to cDNA using the iScript cDNA synthesis kit (Biorad, CA, USA) as per  
21 manufacturers’ instructions. Primers were purchased from Qiagen (QuantiTect Primer Assays) with  
22 expression normalized to housekeeping gene, UBC, which has been extensively used in our laboratory  
23 [25], although not present on the PCR array. All RT-PCR reactions contained 1  $\mu$ l (10 ng) cDNA, 5  
24  $\mu$ l Sybr Green, 3  $\mu$ l RNase-free water and 1  $\mu$ l of primer mix. Using the Rotor-Gene Q (Qiagen, CA,  
25 USA) each run consisted of 40 cycles; 95°C (15 seconds), 55°C (30 seconds) and 72°C (30 seconds).

1 Cycle threshold cutoff was set whilst viewing fluorescent readings in log scale. A melt curve analysis  
2 was conducted to ensure amplification of specific products. Normalized expression values were  
3 assessed by Wilcoxon Sign Rank test, with a p value of  $<0.05$  considered significant.

4

## 1 **Results**

### 2 *Patient Toxicity*

3 Between December 2009 and March 2013 a total of 31 blood samples were collected from eligible  
4 patients (Figure 1). These were classified as toxic (n = 12) or non-toxic (n= 19) and included in the  
5 molecular analysis (Full description in supplementary table 1). Patients were well balanced across the  
6 two groups for age, tumor histology, clinical stage and treatment (Table 2). Grade  $\geq 3$  nausea or  
7 vomiting occurred in 8/31 (26%) patients, making it the most common severe GI toxicity  
8 documented. This was as expected given that the regimen is highly emetogenic. All GI toxicities that  
9 occurred with frequency of 10% or greater are shown in Table 3.

### 10 *PCR array*

11 Initially, RNA from 4 toxic and 4 non-toxic patients was compared in the PCR array analysis which  
12 included 84 innate and adaptive immune response genes, and 5 housekeeping genes. A filter was  
13 applied so that genes with less than 75% detection rate (3 out of 4 arrays in each group) were  
14 classified as absent. This limit resulted in 25 genes being excluded from analysis (Supplementary  
15 table 2). Three housekeeping genes were stable and used for normalization; B2M (-1.11-fold), ACTB  
16 (-1.05-fold) and GAPDH (1.17-fold). Two housekeeping genes, RPL13A and HPRT1, were excluded  
17 due to a significant difference in expression (average CT value difference greater than 2) between the  
18 two groups. Three innate immunity genes were significantly upregulated in the toxic group compared  
19 to the non-toxic group; NFKB1 (1.67-fold, p = 0.01), IL1B (2.19-fold, p = 0.029) and TNF (2.14-fold,  
20 p = 0.042). No genes were significantly downregulated in the toxic group.

### 21 *RT-PCR validation*

22 The three genes identified as significantly upregulated by PCR array were then investigated in all  
23 toxic and non-toxic samples by semi-quantitative RT-PCR. TNF expression was significantly  
24 increased in the toxic group (2.05-fold, p = 0.025), whereas IL1B and NFKB1 expression was similar  
25 between groups (Figure 2). The housekeeping gene, UBC, was stable (-1.27-fold).

1 *Response to CRT*

2 Response data was available 29 patients. Complete or near complete response was 31%, partial  
3 response (including minimal, moderate, good partial and poor partial) was 62%, and poor or no  
4 response was 7%. Severe GI toxicity was not associated with the response of the tumor to CRT  
5 treatment (Table 4).

## 1 **Discussion**

2 GI toxicity is a collection of adverse effects of cancer treatment that seriously impact on patient  
3 quality of life and treatment compliance. This study included the most commonly experienced acute  
4 GI symptoms associated with 5-FU, cisplatin and radiation therapy, namely nausea and vomiting,  
5 diarrhea and mucositis/esophagitis [3, 6, 7], to uncover genes that would act as pre-therapy predictive  
6 markers of GI toxicity. We found that severe GI toxicity occurred frequently within our cohort, with  
7 39% of patients experiencing at least one of the symptoms at a severe level within the first cycle of  
8 treatment. Importantly, each of the GI toxicity symptoms occurred within the wide range of  
9 frequency expected for the regimen [3]. Most patients did not experience toxicities singularly, but  
10 rather two or more toxicities were reported within the first cycle of therapy. This is consistent with  
11 previous studies that have shown that toxicities do not occur in isolation and are likely to be  
12 biologically linked, perhaps through systemic cytokine production and release [26, 27]. In regards to  
13 potential underpinning mechanisms between TNF and nausea and vomiting, this has not been studied  
14 directly to date. However, in a phase I clinical trial of systemic TNF for solid tumors, nausea and  
15 vomiting were of the most common toxicities, indicating a possible relationship between TNF levels  
16 and this symptom [28]. In the present study, toxicity that developed only within the first 4 weeks of  
17 treatment was included in analysis. As such, late onset radiation esophagitis may have been under  
18 reported. This is a serious and dose-limiting toxicity for patients receiving thoracic radiation and is  
19 associated with fibrotic changes that can present months to years following completion of  
20 radiotherapy [29]. Acute radiation esophagitis has also been reported to occur during or just after the  
21 completion of therapy and is highly dose-dependent [15], although the etiology is difficult to separate  
22 from mucosal injury in other areas including the oral cavity and pharynx. In response to this, previous  
23 clinical trials have classified mucositis and acute esophagitis as a single entity [30,31] and shown  
24 incidence rates similar to that seen in our study.

25 In our cohort of patients we found that pre-therapy elevated expression of pro-inflammatory genes  
26 was associated with toxicity. In particular, TNF was consistently elevated in patients that experienced  
27 severe CRT-induced GI toxicity. In contrast, IL1B and NFKB1 were only elevated in the PCR array.

1 PCR arrays were not conducted on the full cohort of patients , and the PCR array cohort was more  
2 homogeneous than the full cohort because it only included males and a maximum of grade 1 toxicity  
3 in the non-toxic group. This may have led to a much wider separation in gene expression signatures  
4 compared to the full cohort, which included females and grade 2 toxicity. The observation of elevated  
5 TNF supports findings by other investigators that have shown evidence of inflammatory markers as  
6 risk predictors of treatment toxicity. An association between the *TNFA*-1031T>C promoter  
7 polymorphism, which is known to alter protein levels, and toxicity has been shown in Japanese  
8 patients with esophageal cancer treated by CRT [13]. Investigators used a multivariate logistic  
9 regression model to show that *TNFA*-1031T>C was significantly associated with oral mucositis and  
10 this was the only significant independent risk factor identified. Furthermore, patients heterozygous  
11 for the *TNFA*-308G>A promoter polymorphism (known to increase expression of TNF- $\alpha$ ) are at  
12 significantly increased risk of severe toxicity affecting the mucosa following myeloablative  
13 chemotherapy for HSCT [23]. In a study investigating genetic variation and risk of radiation  
14 esophagitis in patients with non-small cell lung cancer, investigators identified the *TNFA*-857C>T  
15 promoter polymorphism [24]. They found no significant differences between patients who developed  
16 severe esophagitis and those who did not with regard to age, sex, smoking status, histology, clinical  
17 stage and performance status. In addition, previous research using samples collected from patients  
18 with esophageal cancer treated with neoadjuvant chemotherapy, found monocytes with increased  
19 capacity to secrete IL-12 and reduced IL-10 in response to lipopolysaccharide and interferon were  
20 predictive of severe mucositis [32]. Collectively, these studies strongly support a role for elevated  
21 TNF signaling as an important risk factor for CRT-induced GI toxicity. However, it is important to  
22 note the limitation of our current approach, which is the use of housekeeping genes for normalization  
23 of data. Future research will utilize a quantitative approach to improve accuracy and reproducibility  
24 of results.

25 With regard to toxicity severity, grade  $\geq 3$  GI toxicities are reported to occur at a rate of anywhere  
26 between 6 and 50% [3, 33-37], showing high inter-study and interpatient variability. This may be  
27 partially explained by the different regimens in use as well as the difficulty of assessing GI toxicities,

1 which rely on the subjective description given by the patients and lack of quantitative tests for the  
2 assessment of nausea, vomiting and diarrhea. There is currently no effective tool to stratify patients  
3 for toxicity, and traditional treatment-based and patient-based factors are poor predictors [38]. Our  
4 study found no associations between toxicity and age, sex, tumor histology, stage or treatment, which  
5 is in line with previous studies [39-41]. However it should be noted that we did not look specifically  
6 for associations between gene expression and these same variables separate to toxicity. Given that  
7 gene expression profiles have been used previously to generate predictive models of patient response  
8 to CRT [42-44], this approach may be equally able to generate predictive models of toxicity from  
9 CRT in patients. We chose the arbitrary cut off of CTCAE grade 3 toxicity to categorize patients as  
10 toxic. As such, the non-toxic group contained a mixture of no (grade 0), mild (grade 1) and moderate  
11 (grade 2) GI toxicities, which may have caused reduced separation in marker expression. Comparison  
12 of our findings to other studies is also made difficult by the use of different toxicity scoring systems  
13 and variation in group allocation thresholds.

14 Finally, we found no association between severe GI toxicity and response to CRT, although our study  
15 size was underpowered to detect this. The roughly 30% complete or near complete response rate seen  
16 in our study is in line with previous clinical studies for esophageal cancer [45]. Our findings are in  
17 contrast to a recent study that found acute toxicity may be a significant prognostic factor for response  
18 and overall survival in patients with esophageal cancer [41]. Investigators showed that patients with  
19 worsening odynophagia (described as mucositis of the esophagus) during CRT were more likely to  
20 have tumor regression grade 1 and improved 5-year survival. Importantly, this was the only  
21 independent prognostic factor identified. As such, there is merit in investigating the relationship  
22 between acute tissue toxicity and tumor response in clinical trials to help delineate supportive care  
23 approaches for patients with esophageal cancer.

24 In conclusion, this study has provided supporting evidence for TNF as a gene associated with GI  
25 toxicity risk. Use of molecular, blood-based biomarkers such as gene expression is a quick and non-  
26 invasive method for obtaining toxicity risk information and could be easily translated to a diagnostic

1 tool. Although these initial results are positive, the interpretation of our findings is limited given the  
2 small sample size of this pilot study which will require confirmation in a larger cohort.

3

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5

6 **Disclosures**

7 The authors have no conflict of interest associated with publication of this manuscript

8

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1 **Figure notes**

2 Figure 1. Study workflow.

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4 Figure 2. Relative mRNA expression in toxic vs non-toxic samples. Data shown is fold difference in  
5 expression where the average non-toxic delta CT value was used as the comparator. Box and whisker  
6 plot gives median with range for TNF, NFKB1 and IL1B. Only TNF was found to be elevated in the  
7 toxic group.

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