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**Pharmacogenetic association of MBL2 and CD95 polymorphisms with grade 3 infection following adjuvant therapy for breast cancer with doxorubicin and cyclophosphamide.**

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Running head

Pharmacogenetic association with infection

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**Abstract**

Life threatening infection as an adverse reaction to cytotoxic therapy of cancer remains a major problem, potentially limiting efficacy. Administration of colony-stimulation factors benefits only a minority of patients and improved stratification guidelines are needed to identify those patients likely to benefit.

We investigated single nucleotide polymorphisms (SNPs) in two genes related to immune function to identify associations with severe infection following treatment of breast cancer with doxorubicin and cyclophosphamide. CD95 mediates the extrinsic apoptosis pathway in haematopoietic cells and a *CD95* promoter SNP (rs2234767) has been shown to result in reduced expression of the receptor. MBL2 activates the classical complement pathway in the presence of pathogens and independently of antibodies. Numerous SNPs have been described including a promoter SNP (rs7096206) which results in decreased expression of the protein.

Homozygotes for the *CD95* minor allele were more likely to experience a grade 3 infection than heterozygote and homozygote wild-type patients (29%, 3% and 5% respectively $p = 0.048$). *CD95* minor allele homozygotes also had higher basal WBC and neutrophil counts compared with wild-type allele carriers, which was sustained throughout therapy. There was an allele-dose association between the *MBL2* SNP and grade 3 infection, with 2, 8 and 17% of wild-type homozygotes, heterozygotes and minor allele homozygotes, respectively, experiencing grade 3 infection ($p = 0.02$).

These associations demonstrate the utility of a pharmacogenetic approach to identify individuals more likely to acquire a life threatening infection during chemotherapy. The apparent association with a *CD95* SNP and a mild neutrophilia merits further investigation.

## Introduction

Despite recent advances in the development of targeted therapies for the treatment of cancer, most patients will still receive cytotoxic drugs as part of the management of their disease. Cytotoxic therapy is associated with serious infection, coupled with febrile neutropenia, and is a cause of morbidity and mortality. A significant minority (15%) of breast cancer patients experience at least one episode of febrile neutropenia during cytotoxic chemotherapy, in the absence of prophylactic colony stimulating factor [1]. While mortality due to infection remains low, serious therapy-induced infection may result in a dose delay or dose reduction, which may result in decreased antitumour effect and a worse survival outcome [2]. Colony stimulating factors (G-CSF or GM-CSF) during cytotoxic chemotherapy are commonly used to reduce the incidence of febrile neutropenia among those deemed at highest risk according to current guidelines [3]. However, a recent Cochrane review of trials testing efficacy and toxicity reported that less than 10% of patients benefit from prophylactic CSF, and that adverse reactions, including bone pain (43%) and injection inflammation (55%), are common [1]. Therefore there is a need to identify those patients at greatest risk of chemotherapy-related grade 3 infections, and most likely to benefit from CSF therapy or to be in need of more intense monitoring during chemotherapy.

Identification of variation in genes associated with immunological mechanisms offers one possible way of identifying patients with increased susceptibility to chemotherapy-induced infection. We therefore investigated two genes encoding proteins known to have a function in the immune response, CD95 and MBL2, both of which have functional polymorphic variants.

CD95 (FAS, APO-1, APT1), when activated by FAS ligand (CD95L), mediates cell death via the extrinsic apoptosis pathway and regulates hematopoietic homeostasis [4]. A well-studied SNP within the *CD95* gene promoter (rs2234767 -1377G>A), which disrupts an SP-1 binding site and is associated with lower expression of CD95 [5] and has been associated with increased risk of acute myeloid leukemia [5] and poorer prognosis in acute promyelocytic leukemia [6] . There are also indications that a functional CD95 pathway participates in doxorubicin-induced apoptosis in leukemia cell lines [7].

MBL2 is a secreted hepatic protein component of the innate immune system. The primary structure contains a carbohydrate recognition domain and a collagen-like domain comprising of 19 Gly-Xaa-Yaa repeats [8]. The triple helix formation of the collagen motif results in the formation of a homotrimer. These structures in turn form a higher order polymeric structure with multiple mannan-binding domains per single quaternary structure [9]. This higher order structure can recognise pathogens and initiate the classical complement pathway independently of antibodies [10] and facilitate phagocytosis [11]. Five common polymorphisms of the *MBL2* gene have been extensively studied [9] (Figure S1a). Three of these are located on exon 1, encoding the collagen-like motif. Two are non-synonymous SNPs, termed *MBL2* B (rs1800450) and C (rs1800451), that result in substitution of glycine residues and disrupt the formation of the tertiary structure [12, 13]. The third exon 1 SNP (*MBL2* D, rs5030737) codes for a cysteine to arginine substitution in the collagen-like domain. This does not disrupt the canonical motif sequence, but may contribute to loss of higher order structure [14], although the effect of the D allele on the concentration of functional MBL in plasma is less than that seen with the other two coding SNPs [15, 16]. There have been suggestions that the exon 1 SNPs, in addition to the loss of

the collagen-like structure, may also result in decreased protein expression compared to wild type. However a greater effect on expression of the MBL2 monomer of the protein may result from two promoter polymorphisms [17]. We chose for this study to investigate polymorphisms that had the greatest magnitude effect on expression of the monomer.

We therefore studied the impact of functional SNPs in the *MBL2* and *CD95* genes on risk of chemotherapy-induced infection in breast cancer patients treated with doxorubicin (Adriamycin) and cyclophosphamide (AC) therapy and subject to uniform follow up.

## Methods

*Patient populations*

A total of 364 women with breast cancer were recruited to this study from medical oncology outpatient clinics in the Newcastle upon Tyne NHS foundation trust. A whole blood sample for extraction of genomic DNA was collected from all 364 patients and a plasma sample was taken from 101 (30%) patients.

Two hundred and sixty five of the patients were treated with a chemotherapy regimen of 60 mg/m$^2$ doxorubicin and 600 mg/m$^2$ cyclophosphamide, administered intravenously on day 1 of each 21 day cycle for a maximum of six cycles. Clinical data were collected from patient notes and from the Trust laboratory and Patient Administration Service (PAS) databases. Median follow-up time was 74 months (range 2 to 120 months) and this follow up was uniform with patients attending clinic every three months from the time of treatment for the first two years, every six months from two to five years and thereafter annually for up to ten years. Grade 3 infections in this study are defined as those where the patient required hospital admission and treatment during therapy. The “any infection” category includes all self-reported incidences not requiring treatment. These 265 samples are homogenous for AC therapy and follow-up and represent the main cohort for the assessment of associations between genotype, infection and haematopoietic parameters. Pharmacogenetic studies based on 227 of the patients who received AC adjuvant therapy have previously been published[18-20].

All patients gave written informed consent and the study was given ethical approval by the Newcastle and North Tyneside Research Ethics Committee I.

*MBL2 ELISA*

Circulating MBL2 concentration in plasma was measured using a commercial ELISA kit (Human MBL ELISA kit, R&D, Abingdon UK) as per the manufacturer's instructions. Briefly, 96-well plates were incubated overnight at room temperature (RT) with 0.8 µg/ml capture antibody in PBS. Plates were then washed three times with 0.2 µm filtered wash buffer of 0.05% Tween20 (Sigma, Poole, UK) in PBS (Life, Paisley, UK) and incubated for 1 hour at RT in a 0.2 µm filtered solution of 1% BSA (Sigma, Poole, UK) in PBS. Patient plasma samples were diluted 1:750, 1:1750 or 1:4000 in 1% BSA solution and added to the plate in triplicate and incubated for two hours at RT. After further washing, the plates were incubated with a Biotinylated anti-MBL antibody (R&D Systems) for two hours at RT, washed and incubated for 20 minutes at room temperature with a Streptavidin conjugated HRP solution (R&D Systems). After a final wash the plates were incubated for 20 minutes at room temperature in a 1:1 solution of $H_2O_2$ and tetramethylbenzidine (Thermo Scientific, Loughborough, UK). The reaction was stopped with the addition of 2N Sulphuric acid and the absorbance read at 450 and 540 nm on a Fluostar omega spectrophotometer (BMG, Offenburg, Germany). The $A_{450}$-$A_{540}$ value was used to determine the concentration by interpolation of a seven point standard curve and corrected for dilution.

*DNA and Plasma extraction*

A 10 ml volume of whole blood was taken from each patient into an EDTA tube and stored at -20$^{o}$C prior to extraction of DNA. DNA was extracted using a QIAmp Maxi Blood kit (Qiagen, UK) as per manufacturer's instructions. The quantity and quality of DNA was

determined by measuring absorbance between 220 and 750 nm using a Nanodrop ND-1000 (Thermo scientific, Loughborough, UK). Samples from which plasma was isolated were centrifuged at 2000 g for 10 minutes and the plasma removed. Cellular and plasma fractions were stored at -20$^{o}$C priors to DNA extraction and analysis.

*Genotyping*

Genotyping was performed using Taqman Assays on demand as per the manufacturer's instructions (Applied Biosystems). The primer/probe kits were:- rs7096206, C_27858274_10; rs5030737, C_2336610_10; rs1800450, C_2336609_20; rs1800451, C233608-20, all in the MBL2 gene and rs2234767, C_12123966_10 for the CD95 SNP.

For *MBL2* rs1103125 the following primers and probes were designed using the Taqman custom assays algorithms; Primer 1 GATCAACCTCAACCTTAGTCACCAA, Primer 2 CACTCTGCCAGGGCCAA Probe 1 CAAGCCTGTCTAAAACA, Probe 2 CAAGCCTGTGTAAAACA. The custom assay PCR was run under standard Taqman conditions.

## Results

*MBL2 Genotype-phenotype relationship*

Before investigating the impact of *MBL2* SNPs on clinical parameters we wanted to determine whether individual SNP or haplotype status was associated with plasma MBL2 protein levels measured by ELISA. The distribution frequency of each of the *MBL2* SNPS was determined in 363 of the patients from whom germline DNA was extracted (Table 1). All SNPs 3' of the promoter G-550C SNP were in linkage disequilibrium (LD) and two of the exonic SNPs were in LD with the G-221C promoter SNP. The exonic SNPs were not in LD with each other (Figure S1b).

The impact of each of the SNPs on the plasma concentration of MBL2 was measured in 101 breast cancer patients for whom both genomic DNA and plasma samples were available. All the SNPs, other than the exonic G170A SNP (rs1800451, *MBL2* C), demonstrated an allele dose response, with lower concentrations of MBL2 being associated with alleles predicted from the literature to be associated with low expression or function (figure S1c). The largest magnitude effect was seen with the -221 SNP with 7/7 patients homozygous for this SNP having less than 100 ng/ml plasma MBL2, compared with 2/63 homozygous WT and 6/31 heterozygous patients ($p < 0.0001$, Fisher's exact test). The seven homozygous patients for the -221 variant had a mean MBL2 concentration of 32 ± 15 ng/ml and were WT homozygotes for all other *MBL2* SNPs investigated. Combining SNPs into haplotypes as previously described did not increase the magnitude of the effect [21]. Therefore, the rs7096206 SNP was used to investigate the impact of *MBL2* genotype on infection and survival.

*SNP genotype status and association with clinical and demographic features.*

Genomic DNA samples from 265 breast cancer patients, with comprehensive and standardised follow-up data after receiving AC chemotherapy, were genotyped for the *CD95* G-1377A and *MBL2* G-221C promoter SNPs (264 genotypes for each SNP, due to failed genotyping in 1 case each). There was no evidence of deviation from Hardy-Weinberg equilibrium (HWE) for either SNP (p = 0.405 and 0.598 respectively, $\chi^2$ test), and the frequency distributions were broadly in agreement with 1000 genomes phase 3 European cohort data [22]. The mean age of the cohort at recruitment was 58 years and there was no association between age and genotype for any of the variants investigated (data not shown). There was no association between *MBL2* genotype and tumour hormone receptor status, stage, grade, focality, nodal involvement or metastatic disease. (Table 2). Likewise, there was no association between *CD95* genotype for any of the clinical parameters investigated, with the exception of focality (p=0.045, Table 2), although this was not reflected in a clear gene dose response.

*CD95 and MBL2 genotype in relation to risk of Infection or survival*

There was a significant association between *CD95* SNP status and infection subsequent to chemotherapy (Figures 1a & b). Specifically, 2 of 7 (29%) homozygotes for the minor allele experienced a grade 3 infection, compared to 2/65 (3%) heterozygotes and 10/192 (5%) homozygotes for the common allele (p=0.048, Fisher's exact test). Likewise, 5 of 7 (71%) homozygotes for the minor allele experienced an infection (of any grade), compared to 21/65 (32%) heterozygotes and 60/192 (31%) homozygotes for the major allele (p=0.047,

Fisher's exact test). Although not significant when corrected for multiple testing, these data suggest a recessive genetic model, whereby homozygosity for the minor allele is associated with increased risk of infection post-chemotherapy.

There was a significant association between the *MBL2* -221 allele dose and grade 3 infection during chemotherapy with frequencies of 3, 8 and 17% (p= 0.02, Fisher's exact test) for wild type homozygotes, heterozygotes and minor allele homozygotes, respectively (Figure 1c), suggesting an additive genetic model between this variant and incidence of high-grade infection. However, there was no apparent association between *MBL2* genotype and risk of infection considering all grades (p = 0.404, Fisher's exact test, Figure 1d)).

In addition to the increased risk of infection associated with homozygosity for the *CD95* minor allele, the same individuals had significantly higher white blood cell (WBC) count (p <0.0001, 2-way ANOVA), neutrophil count (p <0.0001, 2-way ANOVA) and hemoglobin (p = 0.0002, 2-way ANOVA) prior to chemotherapy and following each subsequent cycle of chemotherapy (Figure 2). A statistically significant higher WBC count in *MBL2* -221 minor allele homozygotes (p = 0.01, 2-way ANOVA) was not sustained over the course of therapy and was not accompanied by a difference in neutrophil count. (Supplementary figure 2).

Despite clear evidence for an association with high grade infection, there was no significant association between either the *MBL2* -221 or *CD95* genotype and progression-free or overall survival (Figure 3).

## Discussion

### *MBL2 genotype and risk of infection*

MBL2 has a well characterised role in the innate immune response, facilitating the classical complement pathway independently of antibodies [10, 23]. The importance of this pathway has been illustrated by the immune deficiency reported in individuals unable to opsonise saccharomyces cerevisiae [12, 24], a condition which may be treatable by MBL2 replacement therapy [25]. It seems likely that this pathway is even more essential during periods of compromised cellular immunity, where in the absence of neutrophils, MBL2-mediated complement activation can cause lysis of pathogens. Numerous studies in pediatric and leukemic populations receiving cytotoxic therapy have examined the potential association between low circulating higher order polymeric structure MBL2 or *MBL2* genotype and frequency of infection. While some studies have revealed an association with MBL2 phenotype and infection [26, 27] others have not [28, 29] and this inconsistency may be due to population heterogeneity and use of different markers of MBL2 function. There has been only one study into the association of *MBL2* genotype with infection following cytotoxic therapy in solid tumours, in which febrile neutropenia in irinotecan-treated patients was associated with the -550 G variant. [21]

*MBL2* genetics are frequently reported as seven common haplotypes, encompassing the three structural and two promoter polymorphisms. In practice, the presence of any one of the three exonic SNPs (B,C and D) is frequently reported as O and any phenotypic effect of promoter SNPs is considered only in individuals who are homozygous WT for the exonic SNPs [9]. While these haplotypes are associated with high, intermediate and low circulating levels of MBL2 of a higher order structure, they may not have any effect on the plasma

concentration of the monomer. For example the D allele has minimal effect on higher order structure in heterozygotes and no effect on expression of the primary structure, compared with the B allele which has a large effect on quaternary structure and is associated with lower circulating levels of the monomer [16, 17, 30]. We therefore chose to take an unbiased approach to the *MBL2* genotyping, aiming to establish which SNP in our population was associated with lower circulating levels of the MBL2 monomer. The -221 promoter polymorphism had the largest effect on circulating MBL2 monomers, based on a polyclonal ELISA assay, in the patients in our population from whom we had plasma samples. In contrast, the compound exonic haplotype (A/O and O/O vs AA) had less effect on circulating MBL2 monomer than the -221 promoter SNP alone and the effect was not enhanced by inclusion of the -221 or -550 SNPs in A/A individuals.

We therefore chose to use the -221 SNP alone to test for association with infection in the patients in our cohort who received uniform therapy and follow-up. The clear gene-dose effect between this polymorphism and grade 3 infection indicates that measurement of the MBL2 monomer rather than direct measurement of the higher order structures per se may have a role in predicting likelihood of serious infection in response to cytotoxic therapy.

*The CD95 rs2234767 SNP*

We report here an association between CD95 genotype (rs2234767) and risk of infection in breast cancer patients post-chemotherapy. Our data also identify an association between CD95 genotype and WBC, neutrophil count and haemoglobin levels in cancer patients. Following activation by ligand binding, the CD95 receptor initiates the assembly of a

signalling complex that promotes cell death [31]. The CD95 signalling pathway plays a key role in cellular homeostasis, including elimination of self-reactive B-lymphocytes [32], megakaryocyte cell death [33], and regulating T-lymphocyte homeostasis [34]. CD95 also mediates neutrophil cell death [35]. Redundancy in pro-apoptotic stimuli of neutrophil apoptosis is likely and apoptosis independent of CD95L:CD95 interaction does occur [35]. However, CD95L induced apoptosis is not seen in CD95 deficient mice [36] and a CD95 blocking antibody has been shown to abrogate CD95L induced apoptosis of human neutrophils *ex-vivo* [37]. As such, putatively low expression of the receptor conferred by homozygosity for the rs2234767 minor allele may result in a failure to eliminate neutrophils and concomitantly higher baseline numbers of this cell type, as seen in the breast cancer patients in our study. Congenital disorders in neutrophil proliferation and function are associated with infant morbidity and mortality, and are typically characterised by neutropenia and immunodeficiency [38]. In contrast, CD95 deficient mice have an autoimmune phenotype [39] and mutations in CD95 or CD95L cause a rare human pathology, autoimmune lymphoproliferative disorder (ALPS), although a proportion of ALPS patients are neutropenic[40]. Given the allelic frequency of the rs2234767 SNP, homozygosity is unlikely to be associated with the severe congenital syndromes seen with other neutrophil dysfunction phenotypes. The novel observation that the *CD95* rs2234767 SNP is associated with a basal and sustained mild neutrophilia in this cohort is consistent with the functional impact of the SNP and the function of the protein and needs confirmation and characterisation in further cohorts. Although the clinical significance of this observation requires further validation, it is tempting to speculate that the higher WBC count in patients with the variant allele is also associated with impaired activity of these neutrophils, leading to a greater risk of infection.

CD95 also plays a role in mediating apoptosis induced by the cytotoxic chemotherapeutic agents, including cyclophosphamide and doxorubicin, used to treat many cancers[41]. Compromised efficacy of chemotherapy as a result of CD95 dysfunction (or low receptor expression) can confer poor prognosis[42, 43] and susceptibility to infections concomitant with progressive late-stage cancer. Sunter and colleagues (2012) reported significantly lower overall and progression-free survival in APL patients carrying at least one copy of the minor rs2234767 allele, and risk of infection-related death was significantly higher in this patient group[6]. Functional analysis demonstrated that the SNP affects an SP1 transcription factor binding site and that the variant allele is associated with low transcription factor binding and lower CD95 transcription, which is predicted to render cells resistant to chemotherapy-induced apoptosis. The absence of an impact on survival of the rs2234767 minor allele in the breast cancer cohort reported here is in contrast to the APL findings, although the current study is relatively underpowered. Nevertheless, it remains possible that the minor rs2234767 allele may also have a direct impact on immune function and subsequent risk of infection, independent of any effect on infection risk due to underlying progressive cancer. It should also be acknowledged that the results of this retrospective study require validation in an independent dataset.

*Conclusion*

We have identified two SNPs in two immune related genes that have an association with severe and potentially life-threatening infection following doxorubicin and

cyclophosphamide therapy for breast cancer. It should be acknowledged that susceptibility to infection is multi factorial, and that any relationship between a gene and a single phenotype could be affected by confounding variables. Nevertheless, these data suggest CD95 and MLB2 genotype could aid in the identification of patients at high risk of serious infection, and who may benefit from prophylactic CSF therapy.

Conflict of Interest:

None declared.

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Table 1)

Table 1) *MBL2* genotype frequency in breast cancer patients

| | SNP | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs# | rs11003125 | | | rs7096206 | | rs5030737 | | | rs1800450 | | | rs1800451 | |
| Nucleotide | C-550G | | | G-221C | | C154T | | | G161A | | | G170A | |
| Protein | | | | | | R52C | | | G54D | | | G57E | |
| Nomenclature | L/H | | | Y/X | | A/D | | | A/B | | | A/C | |
| Function | Increased Expression | | | Decreased Expression | | Decreased oligormerisation | | | Decreased oligormerisation | | | Decreased oligormerisation | |
| | | No. | (%) | | No. | (%) | | No. | (%) | | No. | (%) | | No. | (%) |
| Genotype | CC | 150 | (41.3) | GG | 215 | (59.2) | CC | 321 | (88.4) | GG | 277 | (76.3) | GG | 342 | (94.2) |
| | CG | 167 | (46.0) | GC | 123 | (33.9) | CT | 42 | (11.6) | GA | 81 | (22.3) | GA | 21 | (5.8) |
| | GG | 46 | (12.7) | CC | 25 | (6.9) | | | | AA | 5 | (1.4) | | | |
| | total | 363 | | | 363 | | | 363 | | | 363 | | | 363 | |
| MAF (%) | | 35.7 | | | 23.8 | | | 5.8 | | | 12.5 | | | 2.9 | |
| HWE (*p* for $\chi 2$) | | 0.964 | | | 0.205 | | | 0.242 | | | 0.736 | | | 0.570 | |

Table 2

Table 2) Frequency distribution of *MBL2* promoter and *CD95* SNPs in clinical parameters.

| | | *MBL2* -221 (rs7096206) | | | $\chi$2 (p) | *CD95* (rs2234767) | | | $\chi$2 (p) |
|---|---|---|---|---|---|---|---|---|---|
| | Total | wt (GG) | het (GC) | var (CC) | | wt (GG) | het (GA) | var (AA) | |
| All | 264* | 154 | 92 | 18 | 0.405[c] | 192 | 65 | 7 | 0.598[c] |
| ER Negative | 262 | 37 | 25 | 6 | 0.551[a] | 51 | 15 | 1 | 0.909[a] |
| ER Positive | | 116 | 67 | 11 | | 141 | 49 | 5 | |
| PR Negative | 250 | 52 | 35 | 8 | 0.306[b] | 74 | 19 | 1 | 0.392[a] |
| PR Positive | | 97 | 51 | 7 | | 110 | 41 | 4 | |
| Size | | | | | | | | | |
| < 20.0mm | 261 | 62 | 37 | 9 | 0.661[a] | 80 | 25 | 3 | 0.956[a] |
| 20.0-49.9mm | | 74 | 48 | 8 | | 93 | 33 | 4 | |
| 50.0mm+ | | 16 | 6 | 1 | | 16 | 7 | 0 | |
| Grade | | | | | | | | | |
| Grade I | 260 | 14 | 6 | 0 | 0.271[a] | 16 | 4 | 0 | 0.977[a] |
| Grade II | | 71 | 35 | 7 | | 82 | 29 | 3 | |
| Grade III | | 65 | 51 | 11 | | 90 | 32 | 4 | |
| Focality | | | | | | | | | |
| Unifocal | 262 | 132 | 75 | 16 | 0.723[a] | 167 | 48 | 7 | 0.045[a] |
| Multifocal | | 21 | 16 | 2 | | 24 | 16 | 0 | |
| Nodes | | | | | | | | | |
| 0 | 260 | 86 | 58 | 12 | 0.222[b] | 112 | 39 | 4 | 0.931[a] |
| 1 or more | | 66 | 32 | 6 | | 78 | 24 | 3 | |
| Metastases | | | | | | | | | |
| Detected | 264 | 3 | 3 | 1 | 0.354[a] | 4 | 2 | 1 | 0.174[a] |
| Not Detected | | 151 | 89 | 17 | | 188 | 63 | 6 | |

[a]Fishers exact test. [b]Pearson Chi-square. [c]HWE Chi-square. *263 patients were common to both genotypings, the 264 sample in both cohorts is not the same person.

Figure legends

Figure 1

Frequency (percentage) of Grade 3 infection (a & c) and any (b & d) infection following the first three cycles of doxorubicin and cyclophosphamide chemotherapy for breast cancer. Patients are categorised according to genotype for CD95 rs2234767 (a&b) or MBL2-221 rs7096206 (c&d) genotypes (wt = wild-type, Het = heterozygote, v = variant).

Figure 2

Blood counts (White Blood Cells, Neutrophils, Haemoglobin and Platelets) in breast cancer patients during doxorubicin and cyclophosphamide chemotherapy according to CD95 genotype (wt = wild-type, Het = heterozygote, v = variant).

Figure 3

Kaplan-Meier curves for Overall Survival (a & c) and Progression Free Survival (b & d) of breast cancer patients treated with doxorubicin and cyclophosphamide chemotherapy according to MBL2-221 rs7096206 genotype (a & b) or CD95 rs2234767 genotype (c & d).

Figure 1

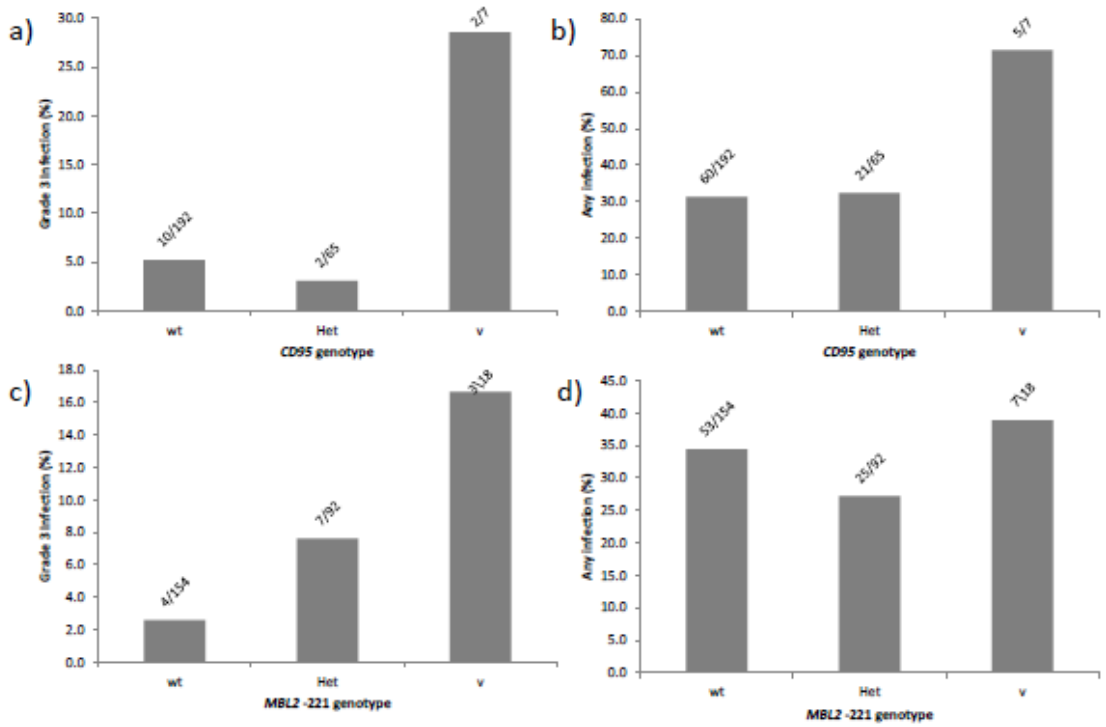

a)
Grade 3 Infection (%)
10/192
2/65
2/7
wt
Het
v
CD95 genotype
b)
Any Infection (%)
60/192
21/65
5/7
wt
Het
v
CD95 genotype
c)
Grade 3 Infection (%)
4/154
7/92
3/18
wt
Het
v
MBL2 -221 genotype
d)
Any Infection (%)
53/154
25/92
7/18
wt
Het
v
MBL2 -221 genotype

Figure 2

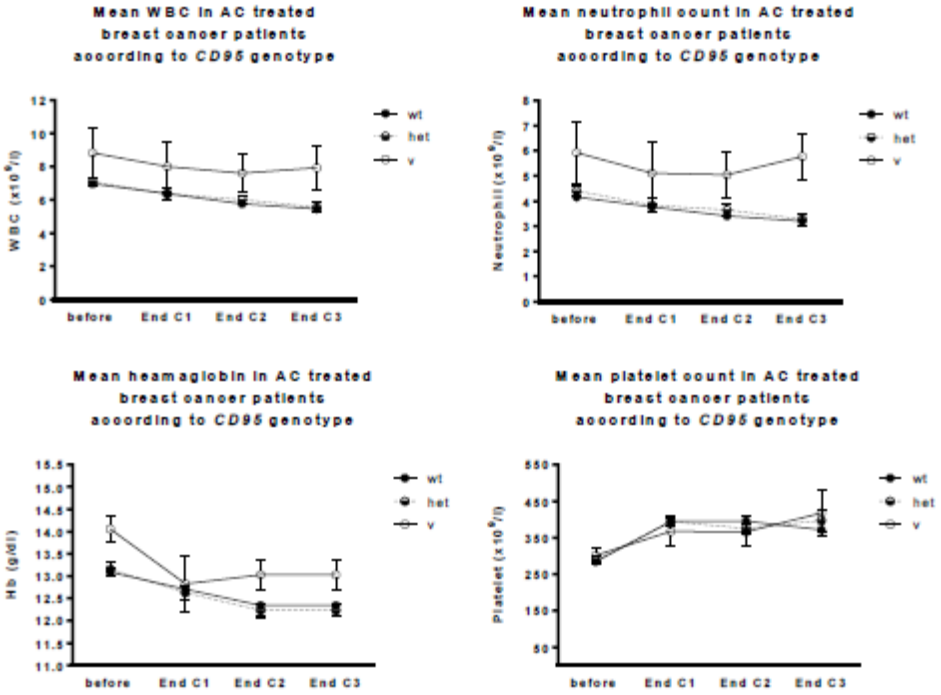
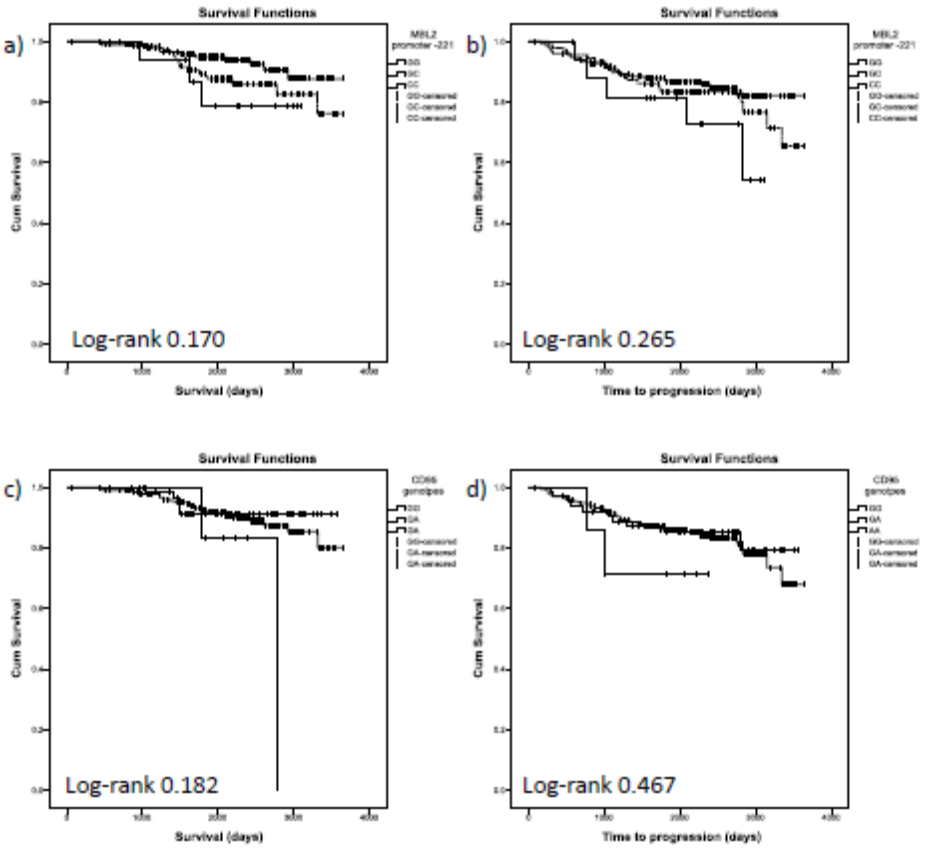

Mean WBC in AC treated breast cancer patients according to CD95 genotype
WBC (x10⁹/l)
wt
het
v
before
End C1
End C2
End C3
Mean neutrophil count in AC treated breast cancer patients according to CD95 genotype
Neutrophil (x10⁹/l)
Mean haemaglobin in AC treated breast cancer patients according to CD95 genotype
Hb (g/dl)
Mean platelet count in AC treated breast cancer patients according to CD95 genotype
Platelet (x10⁹/l)

Figure 3


a)
Survival Functions
Cum Survival
Survival (days)
Log-rank 0.170
b)
Survival Functions
Cum Survival
Time to progression (days)
Log-rank 0.265
c)
Survival Functions
Cum Survival
Survival (days)
Log-rank 0.182
d)
Survival Functions
Cum Survival
Time to progression (days)
Log-rank 0.467

**Pharmacogenetic association of MBL2 and CD95 polymorphisms with grade 3 infection following adjuvant therapy for breast cancer with doxorubicin and cyclophosphamide.**

David Jamieson, Nicola Sunter, Sara Muro, Lucie Pouché, Nicola Cresti, Johanne Lee, Julieann Sludden, Melanie J Griffin, James M. Allan, Mark W. Verrill, Alan V. Boddy

Supplementary material

Figure S1

a) *MBL2* gene structure illustrating positions of SNPs.

b) Linkage disequilibrium of *MBL2* SNPs in a cohort of 363 breast cancer patients showing D' values on the bottom left side of the matrix and Fisher's exact 2 sided p values on the top right half.

c) Impact of MBL2 SNPS on expression of mean MBL in plasma of 101 of the patients. The solid lines illustrate the mean values associated with individual SNPS and the dashed lines with haplotypes constructed from precedents in the literature. Predicted MBL level phenotypes are assigned to individual genotypes and haplotypes according to the literature as follows:

For the promoter SNP rs11003125 the minor allele is associated with greater expression.

For the promoter SNP rs7096206 the minor allele is associated with lower expression.

For all three of the exon 1 SNPs the minor allele is associated with lower activity.

For the promoter haplotype individuals with at least one minor allele for the rs11003125 SNP and wild type for the rs7096206 SNP were assigned to the high phenotype group. Individuals with at least one minor allele for the rs7096206 SNP and wild type for rs11003125 the SNP were assigned to the low phenotype group. All others were assigned to the intermediate group.

For the exon haplotype any minor allele for the exon 1 SNPS was assigned the allelic designation O and a major allele for all three SNPs assigned the designation A. Individuals

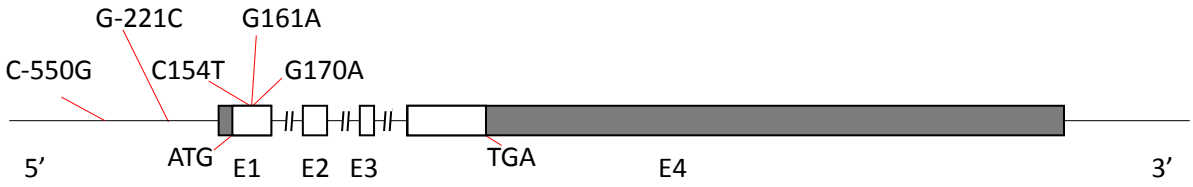
homozygous for the A allele were assigned to a high phenotype group, individuals with one minor allele were assigned to the intermediate group and individuals with at least two minor alleles assigned to a low phenotype group.

The combined haplotype built on the exonic haplotype by assigning the groups promotor haplotype assignations described above only to individuals who were homozygous wild type for all three of the exonic alleles. AO and OO individuals remained intermediate and low respectively, irrespective of promoter haplotype.

Figure S2

Blood counts in breast cancer patients during doxorubicin and cyclophosphamide chemotherapy according to *MBL2* -221 genotype.

Figure S1




| | | rs11003125 C-550G | rs7096206 G-221C | rs5030737 C154T | rs1800450 G161A | rs1800451 G170A |
|---|---|---|---|---|---|---|
| rs11003125 | C-550G | 1 | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| rs7096206 | G-221C | **0.998** | 1 | **0.002** | **<0.001** | 0.064 |
| rs5030737 | C154T | **0.932** | 0.82 | 1 | 0.16 | 0.312 |
| rs1800450 | G161A | **0.939** | **0.894** | 0.556 | 1 | 0.841 |
| rs1800451 | G170A | **0.995** | 0.711 | 0.869 | 0.114 | 1 |

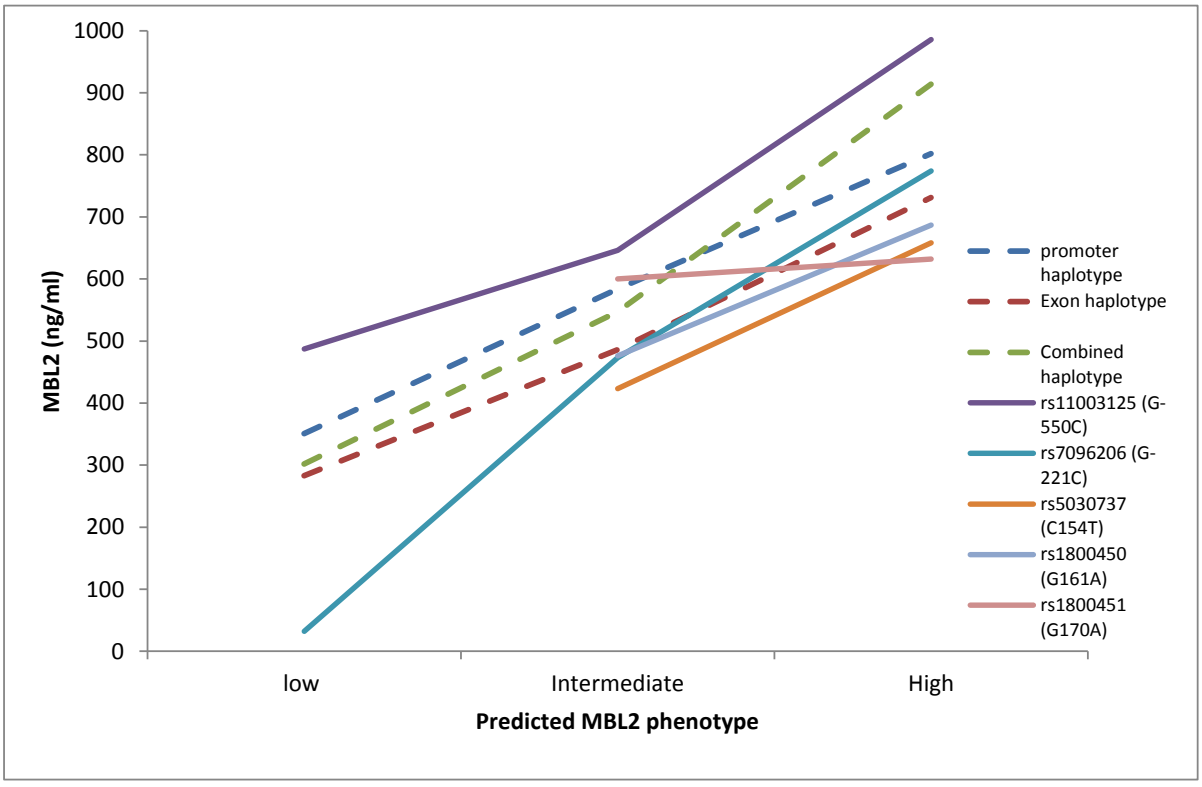
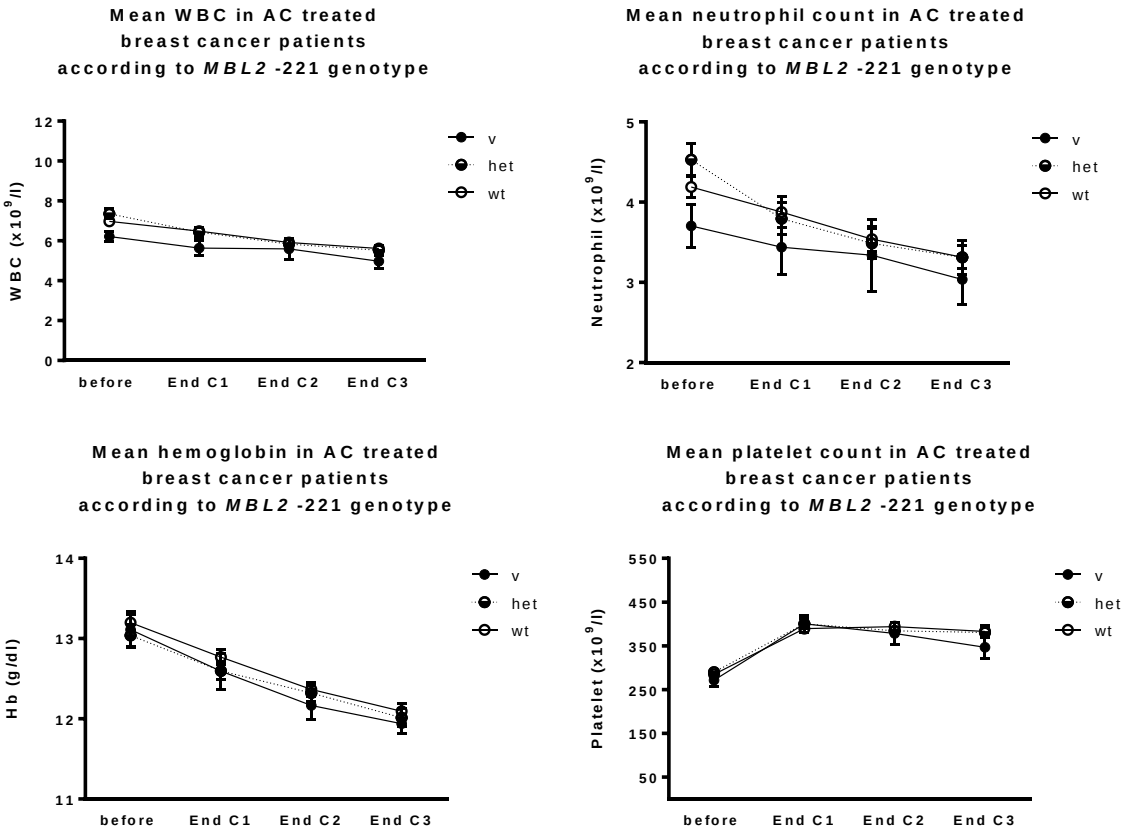


Figure S2


Mean WBC in AC treated breast cancer patients according to MBL2 -221 genotype
WBC (x10^9/l)
12
10
8
6
4
2
0
before
End C1
End C2
End C3
v
het
wt
Mean neutrophil count in AC treated breast cancer patients according to MBL2 -221 genotype
Neutrophil (x10^9/l)
5
4
3
2
before
End C1
End C2
End C3
v
het
wt
Mean hemoglobin in AC treated breast cancer patients according to MBL2 -221 genotype
Hb (g/dl)
14
13
12
11
before
End C1
End C2
End C3
v
het
wt
Mean platelet count in AC treated breast cancer patients according to MBL2 -221 genotype
Platelet (x10^9/l)
550
450
350
250
150
50
before
End C1
End C2
End C3
v
het
wt