Pharmacokinetics and pharmacogenomics of β -lactam-induced neutropenia

Aim: Determine if individuals with β -lactam induced neutropenia have polymorphisms that impair function of MRP4 or OAT1/OAT3. **Methods:** Subjects with β -lactam induced neutropenia were compared to controls for the presence of *MRP4* and *OAT1/OAT3* polymorphisms, estimated plasma trough concentrations and area under the curve. **Results:** Subjects with a homozygous polymorphism at *MRP4* 3348 A to G were 5.3 times more likely to develop neutropenia ($p = 0.171$). No statistical differences were noted in pharmacokinetic parameters. Contingency analysis of children greater than 5 years of age showed neutropenia in subjects who were homozygous wild type at *MRP4* 3348 A to G was significantly associated with standard or high dosing ($p = 0.03$). **Conclusion:** *MRP4* 3348 A to G should be further studied for potential contribution to the development of β -lactam induced neutropenia.

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Keywords: β -lactam • MRP4 • neutropenia • OAT

Background

β -lactam antibiotics are the most commonly prescribed antibiotics in children. While typically safe, a reversible neutropenia may occur in up to 15% of patients receiving greater than 10 consecutive days of parenteral therapy with β -lactams antibiotics [1]. Drug-induced neutropenia is associated with an increased rate of localized infections, sepsis and even fatal complications [2,3]. A direct relationship between β -lactam antibiotic concentrations and inhibition of the progression of progenitor blood cells to neutrophils has been suggested [1,4–6]. Therefore, development of drug-induced neutropenia effectively rules out use of another β -lactam for continued therapy. It is not well understood why β -lactam antibiotics induce neutropenia; therefore, the current standard of care for out-patient antibiotic therapy with parenteral β -lactams is to monitor a complete blood count with differential every week to monitor for the complication to minimize severity [7].

Thus, a better understanding of mechanisms leading to the development of β -lactam-induced neutropenia is critical. This would allow for screening of patients who would have higher risk of developing neutropenia prior to the initiation of prolonged β -lactam antibiotic use. This would allow for closer monitoring and altered antibiotic dosing to prevent a complication which places the patient at risk for additional infections and interventions.

Efflux transporter multidrug resistance protein 4 (*MRP4*) and uptake transporters organic anion transporter 1 and 3 (*OAT1/OAT3*) have been identified on renal proximal tubule cells [8,9]. *MRP4* is also expressed on CD34⁺ stem cells and in other bone marrow cells [8]. β -lactam antibiotics are known substrates of both *MRP4* and *OAT*, and mouse models lacking these *MRP4* and *OAT3* have demonstrated increased renal concentrations and increased plasma concentrations of β -lactam antibiotics, respectively [10–13]. Additionally,

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Table 1. SNPs tested for <i>MRP4</i> and <i>OAT</i> .					
Gene	SNP	SNP ID	Location/reported effect	Reported prevalence	Ref.
<i>MRP4</i>	-1393 C>T	rs868853	5'-UTR; improved event free survival with mercaptopurine use	8.8%	[17]
<i>MRP4</i>	3348 A>G 3463 A>G	rs1751034	K1116K; increased intracellular concentrations of tenofovir	Caucasian: 16.9% African-American: 23.1% Asian: 24.2%	[18–20]
<i>MRP4</i>	912 G>T 934 A>C	rs2274407	K304N; increased thrombocytopenia with mercaptopurine use	5–17%	[17,18]
<i>MRP4</i>	4131 T>G	rs3742106	3'-UTR	40.7%	[17,19,20]
<i>MPR4</i>	2269 G>A	rs3765534	E757K; decreased activity of protein and increased leukopenia with thiopurine use	Caucasian: 1.3% African-American: 2.5% Asian: 3.3%	[15,21]
<i>MRP4</i>	95061461 G>T	rs9561778	Intron 26; increased leukopenia with cyclophosphamide use	<0.1%	[22]
<i>MRP4</i>	559 G>T	rs11568658	G187W; decreased protein activity	Caucasian: 2.5% Asian: 10.8%	[18]
<i>OAT1</i>	728 G>A	rs11568626	R50H	4.2%	[20,23]
<i>OAT3</i>	1342 G>A	rs11568486	V260I; reduced function	0.7%	[24]

there is also evidence that *MRP4* provides protection against hematopoietic toxicity secondary to thiopurine [14,15]. It is interesting to postulate that *MRP4* and *OATs* protect against hematopoietic toxicity by controlling blood and renal levels of β -lactam antibiotics.

We hypothesized that individuals who develop neutropenia while receiving prolonged β -lactam antibiotic therapy have SNPs that impair function of *MRP4* or *OAT* in the kidney. The resultant increased plasma concentration of the antibiotic may facilitate toxicity of progenitor blood cells and lead to a decrease in the number of neutrophils.

Methods

Study design

Individuals from age 0 to 25 years treated with prolonged (10 days or more) β -lactam therapy at Cincinnati Children's Hospital Medical Center (CCHMC; OH, USA) between January 2010 and June 2014 were identified and contacted for inclusion in this case–control study. The study took place from June 2012 to June 2014; some subjects were identified retrospectively while others were identified prospectively. Cases were defined as developing an absolute neutrophil count of less than $1500/\text{mm}^3$ during the course of their β -lactam antibiotic therapy if they were >1 year of age and less than $1000/\text{mm}^3$ if they were <1 year of age [16]. Controls were subjects who did not develop a neutrophil count less than $1500/\text{mm}^3$ (or less than $1000/\text{mm}^3$ if they were <1 year of age) with

prolonged therapy. Subjects were excluded from the study if they had history of a prior non-medication-associated neutropenic episode or a chronic medical condition known to cause periodic neutropenia that in the opinion of the investigator would interfere with the evaluation of study endpoints; they were concurrently receiving another medication that was highly associated with neutropenia, such as chemotherapy, or that interfered with renal tubular function, such as probenecid, or they had renal insufficiency (defined as $\text{GFR} < 60 \text{ ml/min/1.73 m}^2$); or had profound baseline anemia in the opinion of the investigator and would not be expected to tolerate an unnecessary blood draw.

The study was approved by the CCHMC IRB. Informed consent was obtained directly from subjects older than 18 years of age and from the parents/guardians of subjects age 0–17 years. Assent was also obtained for children age 11–17 years.

Information collected on study subjects included age, gender, race/ethnicity, primary diagnosis requiring β -lactam antibiotic therapy, type/dose/route of β -lactam antibiotic used, duration of β -lactam antibiotic use, history of chronic conditions or recurrent infections, prior treatment with β -lactam antibiotics, family history of treatment with β -lactam antibiotics, concurrent medications, baseline (at the time of initiation of antibiotic therapy), minimum and recent complete blood cell counts with differential, including absolute neutrophil count, and baseline, maximum and recent plasma creatinine and albumin levels. Past

medical and family history, as well as prior treatment with β -lactam antibiotics and current medications, was also reported directly from the subjects and/or their parents. Labs were obtained at the discretion of the attending physician, but typically were obtained weekly while receiving intravenous antibiotic therapy. The primary antibiotic for the antibiotic course was defined as the antibiotic given for the longest period of time, preferentially an intravenous antibiotic when it was given for more than 10 days but an oral antibiotic could be considered the primary antibiotic if an intravenous antibiotic was not given for more than 10 days.

Genotyping

Subjects were selectively genotyped for polymorphisms on MRP4 and OAT that had previously been described as being polymorphisms of interest. The polymorphisms tested are shown in Table 1.

Genomic DNA was extracted from blood using standard procedures (e.g., QIAGEN Blood mini kit, CA USA). All genotyping for the selected SNPs was conducted using a real-time PCR-based probe assay (TaqMan® assay, Applied Biosystems, Life Technologies, CA, USA). Forward and reverse primers and assay conditions have been described and tested [25] Known posi-

tive and negative controls for the polymorphism being tested were used when running the TaqMan assays for quality control.

Pharmacokinetic sampling & analysis

Subjects receiving cefazolin or ceftriaxone had albumin, creatinine and β -lactam antibiotic plasma concentration levels drawn as part of the study so that the pharmacokinetics of the β -lactam antibiotic could also be evaluated. Plasma concentrations were randomly drawn at two time points per patient, separated by a minimum of 1 h. After collection, whole blood was transported to the lab and processed within 30 min. Albumin and creatinine specimens were centrifuged at 3500 rpm for 10 min, and plasma was frozen and stored at -80°C until processing, which was performed using standard techniques by the Clinical Translational Research Center biochemistry laboratories at CCHMC. For the antibiotic concentrations, the whole blood was centrifuged at 3500 rpm for 10 min, and then 0.25 ml of plasma was pipetted into a cryovial with an equal volume (0.25 ml) of 1M, pH7.4 MOPS buffer (Teknova, CA, USA) added. The remaining plasma was pipetted into a separate cryovial. Both were flash frozen and then stored in a -80°C freezer until concentrations were determined.

Figure 1. Flow diagram of subjects included in study. 350 subjects were contacted for inclusion. 94 subjects were included in the study, of which 44 were cases and 54 were controls.

Subject variables	Cases (n = 44)	Controls (n = 54)	p-value
Demographics			
Gender, n (%) [†] :			
– Male	26 (59%)	33 (61%)	0.839
– Female	18 (41%)	21 (39%)	–
Race, n (%) [‡] :			
– White	39 (89%)	52 (96%)	0.216
– Black	3 (7%)	2 (4%)	–
– Other	2 (5%)	0	–
Age (median, IQR) [§]	4.4 (1.5–13.2)	6.5 (0.6–15.0)	0.449
Past medical history, n (%)[†]			
Cancer	2 (5%)	1 (2%)	0.586
Prior transplant	1 (2%)	0	0.449
Immune deficiency	1 (2%)	0	0.449
Hematologic disease	0	2 (4%)	0.500
GI disease	1 (2%)	4 (7%)	0.375
Cardiac disease	3 (7%)	2 (4%)	0.654
Renal disease	0	3 (6%)	0.250
Genetic/metabolic disorder	2 (5%)	7 (13%)	0.180
Neurologic disorder	10 (23%)	10 (19%)	0.624
Lung disease	11 (25%)	5 (9%)	0.053[¶]
Prematurity (<36 weeks)	4 (9%)	4 (7%)	1.000
Musculoskeletal disease	7 (16%)	10 (19%)	0.794
Recurrent infections	12 (27%)	13 (24%)	0.817
Prior prolonged course β -lactam Abx	8 (18%)	10 (19%)	1.000
Prior neutropenia with β -lactam Abx	2 (5%)	0	0.199
Sites of infection, n (%)[†]			
Bacteremia	5 (11%)	7 (13%)	1.000
CNS infection	10 (23%)	10 (19%)	0.624
Eye infection	0	3 (6%)	0.250
Sinusitis	4 (9%)	3 (6%)	0.697
Chronic otorrhea	3 (7%)	2 (4%)	0.654
Pneumonia	4 (9%)	3 (6%)	0.697
Osteomyelitis/septic arthritis/pyomyositis	19 (43%)	28 (52%)	0.422
Surgical site infection	12 (27%)	11 (20%)	0.477
Other	2 (2%)	4 (7%)	0.688
[†] Fisher's exact test. [‡] Chi squared test. [§] Rank sum test. [¶] Cases: six asthma, one cystic fibrosis, one bronchopulmonary dysplasia, three other; controls: two asthma, zero cystic fibrosis, zero bronchopulmonary dysplasia, three other. Abx: Antibiotic; GI; Gastrointestinal; IQR: Interquartile range.			

β -lactam antibiotic plasma concentrations were determined using validated high performance liquid chromatography assays performed, according to recently described methods, by the clinical pharmacology laboratory at CCHMC according to recently described methods [26,27]. In brief, unbound concentrations of cefazolin and ceftriaxone were quantified using an Agilent 1100 Series (CA USA), modular 3D HPLC system equipped with Diode Array Detector and temperature controlled Autosampler and Column compartments. Analytical grade cefazolin (200 mg free acid, cat. 1097603, lot L1K284) and ceftriaxone (350 mg monosodium salt, cat. 1098184, lot H0J296) (US Pharmacopeia, MD, USA) were used to prepare (external) assay standards at concentrations of 1, 5, 10, 25, 50, 100 and 250 $\mu\text{g/ml}$ and assay control standards at 7.5 $\mu\text{g/ml}$ (low), 40 $\mu\text{g/ml}$ (medium) and 150 $\mu\text{g/ml}$ (high) concentrations. During analyses, prepared standards and patient samples were maintained in the Autosampler at 4°C (with light excluded) and resolved on a 250 x 3.00 mm SYNERGI Hydro-RP column (4 μm x 80 Å; Phenomenex, CA, USA) which was maintained at 20°C. Analyte was eluted from the column by a 4–60% gradient of acetonitrile added against 20 mM aqueous (pH 7.4) solution of KH_2PO_4 (resultant gradient: 96–40%). Cefazolin and ceftriaxone were optimally detected by background-corrected UV absorption of a 4 nm bandpass at 240 nm and 275 nm respectively using a 40 nm bandpass centered at 360 nm as the reference signal. Under these conditions, the assay lower limit of detection and lower limit of qualification were calculated to be 1.2 and 3.9 $\mu\text{g/ml}$ respectively for cefazolin and 3.1 and 10.2 $\mu\text{g/ml}$ for ceftriaxone. The method was validated using criteria

established for guidance of industrial bioanalytical methods [28]. Analytical standard matrix performance and suitability were validated by comparison of standard measurements from spiked, individual donor's plasma to spiked, pooled donor plasma. To validate intraday sample integrity, all of the cefazolin and ceftriaxone control standards (low, medium, high) were assayed five-times a day with 12-h spacing between the first and last measurement of the day. To evaluate if storage at -20°C affected assay reproducibility and/or the integrity of samples/standards, the above described validation assay was repeated on 3 separate days. A summary of the intraday and interday variation is presented in [Supplementary Table 1](#). For the final quantitation of unbound plasma cefazolin and ceftriaxone each study sample was assayed in quadruplicate. Blank and calibrator measurements were repeated between each replicate set of subjects sample measurements.

Pharmacokinetic modeling

Pharmacokinetic modeling with Bayesian estimation using MW/Pharm (MW/Pharm, Version 3.82, Mediware, Groningen, The Netherlands) was performed to predict individual pharmacokinetic parameter estimates, clearance, volume of distribution and area under the curve ($\text{AUC}_{24\text{h}}$). The ceftriaxone model used was based on a model published by Stoeckel *et al.* [29], while the cefazolin model used was based on a model published by Scheld *et al.* [30]. To convert to the models to pediatrics, they were parameterized using the allometric option with a fixed coefficient of 0.75 for clearance [31,32]. Standard dosing for ceftriaxone and cefazolin were defined as 50 mg/kg/day and 75 mg/kg/day, respectively. High dosing for ceftriax-

Table 3. Antibiotic use and laboratory evaluation of cases and controls.

Subject variables	Cases (n = 44)	Controls (n = 54)	p-value
Antibiotic class, n (%)[†]			
Penicillin	13 (30%)	14 (26%)	0.895
Cephalosporin	27 (61%)	34 (63%)	–
Carbapenem	4 (9%)	6 (11%)	–
Total therapy days, median (IQR) [‡]	28 (23–44)	33 (22–56.5)	0.289
Laboratory evaluation, mean (SD)			
Lowest white blood cells (K/mcl) [‡]	3.4 (2.6, 5.3)	6.5 (4.9, 8.4)	<0.001*
Lowest absolute neutrophil count [‡]	940 (618, 1205)	2210 (1755, 3492)	<0.001*
Lowest hematocrit (%) [§]	29.1 ($\pm$ 4.8)	29.5 ($\pm$ 5.1)	0.366
Lowest platelets (K/mcl) [§]	214 ($\pm$ 94)	283 ($\pm$ 109)	<0.001*
[*] p < 0.05 [†] Chi-squared test. [‡] Rank sum test. [§] T test. IQR: Interquartile range; SD: Standard deviation.

Figure 2. Distribution of genotypes comparing cases (n = 44) and controls (n = 54). MRP4 is multidrug resistance protein 4. OAT is organic anion transporter. The bar graphs are normalized to 100% and the numbers represent the number of cases and/or controls that were homozygous for the wild type (homozygous WT), heterozygous or homozygous for the polymorphism (homozygous SNP). WT: Wild type.

one and cefazolin were defined as 75–100 mg/kg/day and 100–150 mg/kg/day, respectively.

Sample size & statistical analysis

Sample size calculations were based on the assumptions; a 10% prevalence of neutropenia; there would be three genotypes with 0, 1 and 2 risk alleles, and; our cases and controls would be matched 1–1.5. As such, the estimated sample sizes to reach 80% power and $\alpha = 0.005$ (Bonferroni correction for 10 comparisons) by allele frequency association test (degrees of freedom = 1) for a risk allele frequency (q) of 0.2 and an odds ratio (OR) of 4 for the presence of neutropenia would be 32 cases and 48 controls. For a q of 0.1 and an OR of 4, we would need 48 cases and 72 controls.

Descriptive statistics were used to summarize the main demographic features of the enrolled cases and controls. The comparability of the two groups were assessed by Fisher's exact test or Chi-squared test for

categorical features and T test or Rank-Sum test for quantitative features, depending upon whether the data was parametric or nonparametric in SigmaPlot (Systat Software Inc., CA, USA). The heterozygous and homozygous polymorphism for each polymorphism was also analyzed separately in a nonadditive model to generate an odds ratio in SigmaPlot. Finally, we used inferential statistics to test our hypothesis by logistic regression analysis to generate an odds ratio in SAS (SAS Institute Inc., NC, USA). The model includes the subject's genotype (coded as 0, 1 and 2 for additive model) as our independent variable and the development of neutropenia as our dependent variable.

Results

Participants

Review of hospital records between January 2010 and June 2014 identified 350 eligible subjects. Of these, 104 consented to participate in the study. Six subjects were

excluded leaving 98 subjects in the analysis; 44 were cases and 54 were controls (Figure 1). As the controls were not perfectly matched to the cases in a 1.5:1 ratio, the analysis was performed in an unmatched fashion.

Demographics

There was no significant difference in gender, race or age between the cases and controls (Table 2). There also was no significant difference in past medical history or sites of infection for which the antibiotic was given for cases and controls (Table 2). The most common underlying conditions were neurologic disorders (23% cases and 19% controls, $p = 0.624$) and lung disease (25% cases and 9% controls, $p = 0.053$). The primary driver for the difference in lung disease between cases and controls was an underlying condition of asthma (six cases and two controls). A similar percentage of subjects reported recurrent infections (27% cases and 24% controls, $p = 0.817$). The most common sites of infection were bone and joint or muscle infection (43% cases and 52% controls, $p = 0.422$), surgical site infection (27% cases and 20% controls, $p = 0.477$) and CNS infection (23% cases and 19% controls, $p = 0.624$).

Antibiotic use & laboratory evaluation

Cases and controls also had similar receipt of antibiotics (Table 3). The primary antibiotic class given to the subjects was penicillin for 30% of cases and 26% of controls, a cephalosporin was given to 61% of cases and 63% of controls and a carbapenem was given to 9% of cases and 11% of controls ($p = 0.289$). Cases received antibiotics for a median of 28 (23, 44) days while controls received antibiotics for a median of 33 (22, 56.5) days ($p = 0.289$).

Cases and controls differed in their laboratory evaluations (Table 3). As defined, cases were significantly more likely to have lower white blood cell counts (3.4 K/ μ l vs 6.5 K/ μ l, $p < 0.001$) and lower absolute neutrophil counts (940 vs 2210, $p < 0.001$) than controls. In addition, cases were found to have statistically significant lower platelet counts than controls (214 K/ μ l vs 283 K/ μ l, $p < 0.001$), although the difference is not likely clinically significant.

Genotyping

The distribution of genotypes is shown in Figure 2. There was no significant difference in minor allele frequency between cases and controls (Supplementary Table 2). Logistic regression analysis using the subject's genotype (coded as 0, 1 and 2 for additive model) as our independent variable and the development of neutropenia as our dependent variable found no statistical significance (Table 4). Each SNP was also analyzed in a nonadditive model by calculating an odds ratio for the heterozygous and homozygous polymorphism individually and the results are shown in Table 5. In this analysis, subjects with a homozygous polymorphism at MRP4 3348 A>G (rs1751034) were 5.3-times more likely to develop neutropenia (0.6–49.2, $p = 0.171$), but heterozygotes were protected (OR: 0.4 [0.2–1.1], $p = 0.100$). Because we had not controlled for ontogeny, a contingency analysis of children >5 years of age was performed for this polymorphism. It found that neutropenia in those subjects who were homozygous wild type was significantly associated with whether they received standard or high dosing ($p = 0.03$) (Figure 3). However, there was no such association for those subjects who were heterozygous or homozygous

Table 4. Logistic regression analysis for additive model (coded as 0, 1 and 2).

Gene	SNP	SNP ID	OR (95% CI)	p-value
MRP4	-1393 C>T	rs868853	0.666 (0.285–1.514)	0.3340
MRP4	3348 A>G	rs1751034	1.078 (0.542–2.183)	0.8305
	3463 A>G	–	–	–
MRP4	912 G>T	rs2274407	1.439 (0.488–4.575)	0.5168
	934 A>C	–	–	–
MRP4	4131 T>G	rs3742106	1.026 (0.590–1.792)	0.9269
MPR4	2269 G>A	rs3765534	0.396 (0.018–4.270)	0.4561
MRP4	95061461 G>T	rs9561778	0.808 (0.094–6.958)	0.8344
MRP4	559 G>T	rs11568658	0.977 (0.526–1.830)	0.9395
OAT1	728 G>A	rs11568626	0.396 (0.018–4.270)	0.4561
OAT3	1342 G>A	rs11568486	<0.001 (...–4.722) [†]	0.9856

[†]Computational issue as only one genotype was nonwild type.
OR: Odds ratio.

Table 5. Odds ratio for developing neutropenia in cases versus controls for various SNP of *MRP4* and *OAT*.

Polymorphism	Cases (n = 44)	Controls (n = 54)	OR (95% CI)	p-value
MRP4				
rs868853 (-1393 C>T):				
– Homozygous WT [†]	31 (71%)	43 (79%)	–	–
– Heterozygous	12 (27%)	10 (19%)	1.7 (0.6–4.3)	0.430
– Homozygous SNP	1 (2%)	1 (2%)	1.2 (0.1–20.3)	1.000
rs1751034 (3348 A>G):				
– Homozygous WT	32 (73%)	34 (63%)	–	–
– Heterozygous	8 (18%)	19 (35%)	0.4 (0.2–1.1)	0.100
– Homozygous SNP	4 (9%)	1 (2%)	5.3 (0.6–49.2)	0.171
rs2274407 (912 G>T):				
– Homozygous WT	38 (86%)	44 (81%)	–	–
– Heterozygous	6 (14%)	10 (19%)	0.7 (0.2–2.1)	0.707
– Homozygous SNP	0	0	–	–
rs3742106 (4131 T>G):				
– Homozygous WT	19 (43%)	23 (42%)	–	–
– Heterozygous	18 (41%)	22 (41%)	1.0 (0.5–2.3)	0.850
– Homozygous SNP	7 (16%)	9 (17%)	0.9 (0.3–2.8)	0.862
rs3765534 (2269 G>A):				
– Homozygous WT	42 (95%)	53 (98%)	–	–
– Heterozygous	2 (5%)	1 (2%)	2.5 (0.2–28.8)	0.586
– Homozygous SNP	0	0	–	–
rs9561778 (95061461 G>T):				
– Homozygous WT	28 (63%)	34 (63%)	–	–
– Heterozygous	14 (32%)	19 (35%)	0.8 (0.4–2.0)	0.892
– Homozygous SNP	2 (5%)	1 (2%)	2.5 (0.2–28.8)	0.586
rs11568658 (559 G>T):				
– Homozygous WT	42 (95%)	52 (96%)	–	–
– Heterozygous	2 (5%)	2 (4%)	1.2 (0.2–9.2)	1.000
– Homozygous SNP	0	0	–	–
OAT1				
rs11568626 (728 G>A):				
– Homozygous WT	43 (98%)	52 (96%)	–	–
– Heterozygous	1 (2%)	2 (4%)	0.6 (0.1–6.9)	1.000
– Homozygous SNP	0	0	–	–
OAT3				
rs11568486 (1342 G>A):				
– Homozygous WT	44 (100%)	53 (98%)	–	–
– Heterozygous	0	1 (2%)	–	–
– Homozygous SNP	0	0	–	–
[†] Wild type. OR: Odds ratio; WT: Wild type.				

for the polymorphism.

Pharmacokinetic modeling

Comparison of antibiotic exposure defined by the estimated plasma trough concentration at steady state and average 24 h AUC_{24h} between cases and controls with plasma antibiotic concentrations obtained for ceftriaxone and cefazolin are shown in [Supplementary Table 3](#). We also evaluated antibiotic exposure as defined by AUC_{24h} to genotype, which is shown in [Table 6](#). While the total numbers were low, no significant difference could be seen in either comparison.

Discussion

Antibiotics remain a mainstay of therapy in pediatric patient, being prescribed in one of five pediatric office visits and up to 40% of hospitalized children [33,34]. β -lactam antibiotics approximate about 60% of the total antibiotic use each year in USA [35]. However, the common side effect of neutropenia can be limiting

when trying to treat children for serious infections with long courses of antibiotic. The incidence of neutropenia is estimated to range from 5% to more than 15% of patients treated with high doses of long-term β -lactam antibiotics, and more than 90% of cases occur with more than 10 days of therapy [15]. Bone marrow aspirates of subjects with β -lactam-induced neutropenia revealed a lack of well-differentiated myeloid elements with numerous granulocyte precursors still present, suggesting inhibition during the proliferation stage [1]. Also, *in vitro* the addition of β -lactam antibiotics to either a promyelocytic cell line or bone marrow cultures collected from hematologically normal human donors showed a dose-dependent inhibition of granulopoiesis [1,4,6]. The median concentrations of penicillins and cephalosporins to cause 50% inhibition of colony formation ranged from 105–550 $\mu\text{g/ml}$ and 7–35 $\mu\text{g/ml}$, respectively, which is within the expected range of normally delivered parenteral doses [1]. In fact, all but five of our 21 subjects with cephalosporin

Figure 3. Contingency analysis of children >5 years of age for *MRP4* 3348 A>G. Cases versus controls are compared using standard versus high dosing of β -lactam therapy. Standard dose for ceftriaxone was 50 mg/kg/day. High dose for ceftriaxone was 75–100 mg/kg/day. Standard dose for cefazolin was 75 mg/kg/day. High dose for cefazolin was 100–150 mg/kg/day. WT: Wild type.

Table 6. Pharmacogenomic analysis of 24 h area under the curve.					
Gene	SNP	Homozygous SNP (mean ± SD)	Heterozygous (mean ± SD)	Homozygous WT (mean ± SD)	p-value†
MRP4	-1393 C>T	NA	2282.57 (798.84)	1816.91 (659.21)	0.315
MRP4	3348 A>G	1872.38 (0)	1991.20 (942.75)	1850.48 (617.47)	0.639
	3463 A>G				
MRP4	912 G>T	NA	1601.75 (550.89)	1958.01 (717.41)	0.392
	934 A>C				
MRP4	4131 T>G	1980.85 (690.22)	1874.24 (865.15)	1818.62 (488.49)	0.923
MPR4	2269 G>A	NA	1526.3 (0)	1905.73 (715.18)	0.623
MRP4	95061461	NA	1997.23 (627.10)	1776.29 (753.55)	0.506
	G>T				
MRP4	559 G>T	NA	2331.14 (71.06)	1837.38 (722.88)	0.372
OAT1	728 G>A	NA	NA	1886.76 (701.96)	NA
OAT3	1342 G>A	NA	NA	1886.76 (701.96)	NA
†ANOVA. NA: Not applicable; SD: Standard deviation; WT: Wild type.					

plasma concentrations obtained had approximated trough concentrations greater than that range (Table 4).

This study sought to better identify the molecular mechanisms behind the development of neutropenia to provide clinicians with a potential screening test that could identify those subjects more at risk. Although the study included a moderate sample size, a statistically significant effect could not be found. However, a trend to development of neutropenia was detected in patients who had homozygous polymorphisms at rs1751034 on MRP4. It is possible that with a larger sample size, significant differences may be found.

We elected to study MRP4 as a likely candidate gene based on prior research. MRP4 is an efflux transporter present on both on the apical membrane of renal proximal tubule cells as well as the surface of CD34⁺ stem cells and other bone marrow cells [8]. It has been shown that various β-lactam antibiotics are substrates of MRP4 using tandem mass spectrometry and *in vitro* studies of membrane vesicles expressing human MRP4 [10,11]. MRP4 also was demonstrated to be involved in the luminal efflux of ceftizoxime and cefazolin in the renal tubule of a mouse model; MRP4 knockout mice demonstrated higher renal concentrations of the cephalosporin antibiotics [11]. Additionally, MRP4 knockout mice have also demonstrated bone marrow aplasia and enhanced hematopoietic toxicity with exposure to other studied drugs, such as thiopurine, suggesting that MRP4 may provide progenitor cell specific protection in its role as a surface cell transporter [14,15]. Importantly, MRP-4 expression has also been demonstrated to be related to hematopoietic stem cell differentiation *in vitro* [36].

Secondly, the distribution in the population and the impact of this polymorphism on other drugs could explain what we saw in our patient population. The polymorphism, a nucleotide change from A to G at nucleotide position 3348, is present in approximately 17% of Caucasians, 20% of Mexican-Americans, 23% of African-Americans and 24% of Asians [18]. This is similar to the rate of neutropenia seen with prolonged β-lactam use of up to 15% [1]. In addition, homozygous polymorphisms also have been associated with a 35% increased intracellular concentration of tenofovir diphosphate [20]. In our study, the administration of high dose β-lactam therapy remained a risk factor for developing neutropenia based on the contingency analysis, but plasma concentrations alone did not appear to explain the entire contribution to toxicity.

Limitations to the study included a convenience-based recruitment strategy. Specifically, subjects were recruited prospectively from patients treated with β-lactam antibiotics by the Infectious Disease Division at CCHMC between October 2012 and June 2014, and retrospectively from patients treated from January 2010 to October 2012 who had previously consented to have remnant blood samples stored in a biorepository. Another limitation was the use of specimens from the biorepository, as receiving the correct specimens was critical. One subject included in the above analysis was later identified to have had the wrong DNA specimen supplied for genotyping. However, the lack of identifying information on the sample precluded the ability to exclude the subject from analysis. In addition, the epidemiologic and pharmacologic data collected were

a combination of retrospective and prospective data, so the accuracy of data collected is dependent upon correct charting in the electronic medical record. We did not include differential expression of MRP4 or OAT based on age in our analysis although this has been suggested in rat models, which is another limitation to the study [37]. Lastly, to maximize the ability of a study to identify genotypic influence, it is important to have a large cohort with well-defined phenotypes and homogeneous exposures. We estimated sample size based on an odds ratio of 4, which was likely too high. Thus, the sample size and use of multiple types of β -lactam antibiotics are also limitations to this study.

Conclusion & future perspective

In conclusion, β -lactam-induced neutropenia continues to be a common problem with prolonged use of these antibiotics. No specific polymorphisms were identified in this study as a risk factor for the development of neutropenia. However, the results from this study suggest that a polymorphism in *MRP4*, specifically at 3348 A>G (rs175034), should be further investigated. Future studies will need to be performed in a larger cohort of subjects to better define this possible association and further elucidate the molecular mechanisms.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/pgs-2015-0008

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Disclaimer

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- β -lactam antibiotics are associated with the development of neutropenia.
- *MRP4* and *OAT* are β -lactam transporters that could be associated with the development of neutropenia

Methods

- This case-control study examined the relationship between SNPs in *MRP4* and *OAT* and the development of β -lactam induced neutropenia.

Results

- Subjects with a homozygous polymorphism at *MRP4* 3348 A to G were 5.3 times more likely to develop neutropenia ($p = 0.171$).
- Contingency analysis of children greater than 5 years of age showed neutropenia in subjects who were homozygous wild type at *MRP4* 3348 A to G was significantly associated with standard or high dosing ($p = 0.03$).

Conclusion

- No specific polymorphisms were identified in this study as a risk factor for the development of neutropenia.
- However, the results from this study suggest that a polymorphism in *MRP4*, specifically at 3348 A to G, should be further studied

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