

Pharmacogenetics of adjuvant breast cancer treatment with cyclophosphamide, epirubicin and 5-fluorouracil

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Abstract

Purpose Most adjuvant breast cancer treatment regimens include the combination of an anthracycline (epirubicin or doxorubicin) and the alkylating agent cyclophosphamide. This study sought to investigate the influence of pharmacogenetics on the pharmacokinetics and metabolism of these agents.

Methods Blood samples were taken from patients treated with cyclophosphamide ($n = 51$) and epirubicin ($n = 35$), with or without 5-fluorouracil (5-FU). The pharmacokinetics and metabolism of the three drugs were investigated, together with pharmacogenetic investigations for cyclophosphamide and epirubicin. Cyclophosphamide and its metabolites and also epirubicin and epirubicinol were measured in plasma. DNA was extracted from whole blood and genotyping performed using RT-PCR.

Results Patients with at least one variant *CYP2C19**17 allele had a longer CP half-life ($p = 0.007$), as did homozygous variants for the *CYP2B6**6 allele. There was no significant effect of *GSTP1*, *CYP2B6**2, *CYP2B6**5 or *CYP2C19**2 on any pharmacokinetic parameter of CP. An *NQO2* exonic SNP was associated with a higher exposure to epirubicinol relative to epirubicin ($p = 0.011$). Other

polymorphic variants of *NQO1*, carbonyl reductase, UGT enzymes and transporters had no influence on epirubicin or its metabolite.

Conclusion Overall, pharmacogenetic factors had only a minor influence on cyclophosphamide or anthracycline-based adjuvant therapy of breast cancer.

Keywords Pharmacogenetics · Breast cancer · Cyclophosphamide · Epirubicin

Introduction

Adjuvant chemotherapy is commonly applied postsurgery with the aim of eradicating any residual disease and has been shown to improve outcomes in patients with early stage breast cancer. Although a variety of different regimens have been used, most include the combination of an anthracycline (epirubicin or doxorubicin) and the alkylating agent cyclophosphamide, often combined with 5-fluorouracil (5-FU).

Cyclophosphamide is a prodrug that requires metabolic activation to a 4-hydroxy metabolite, which subsequently breaks down to yield DNA-alkylating species [1]. The initial reaction, as well as inactivation of cyclophosphamide to keto (keto)- and dechloroethyl- (DCCP) metabolites, is catalyzed by a range of cytochrome P-450 enzymes. An additional inactivation reaction is catalyzed by aldehyde dehydrogenases to form carboxyethylphosphoramidate mustard (CX), the major metabolite seen in urine. Previous studies in patients with breast cancer [2] or with non-Hodgkins lymphoma (NHL) [3] have shown an inverse relationship between plasma concentrations of the parent drug and anti-tumor effect. Conversely, high concentrations in plasma of the inactive metabolites have been associated with relapse in the NHL patients [3].

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Epirubicin has often supplanted doxorubicin as the anthracycline component of adjuvant breast cancer regimens. The cardiotoxic effects of epirubicin are less than those associated with doxorubicin. Although not subject to the same degree of reduction by aldo-ketoreductase enzymes, epirubicin can be conjugated to form glucuronides, catalyzed by uridine glucuronosyl transferase enzymes (UGTs) [4, 5]. Epirubicin, and other anthracyclines, are also subject to transport by the ABC [6] and SLC [7, 8] families of membrane proteins, which contribute to the uptake of the drug in liver and to biliary excretion.

Genetic polymorphisms result in variations in expression and activity of CYP and UGT enzymes and of ABC and SLC transporters. Such variations can have multiple effects on the pharmacology of drugs used in cancer chemotherapy, including cyclophosphamide and epirubicin. For instance, a *UGT2B7* polymorphism has been reported to influence the outcome of adjuvant breast cancer treatment [4]. Similarly, polymorphisms in GST enzymes, which inactivate DNA-alkylating metabolites, have been shown to influence outcome in breast cancer patients treated with cyclophosphamide [9] or with anthracyclines [10]. The impact of these and other polymorphisms on the pharmacokinetics and metabolism of cyclophosphamide and epirubicin is unknown.

In the study reported here, blood samples have been taken from patients receiving treatment with the combination of epirubicin and cyclophosphamide, combined with 5-FU in some patients. The pharmacokinetics and metabolism of all three drugs have been investigated, together with genotyping for the common polymorphisms in genes known to influence the pharmacology of cyclophosphamide and epirubicin.

Methods

A total of 51 patients were recruited at the Northern Centre for Cancer Care. The protocol for the study was approved by Newcastle and North Tyneside Ethics Committee 1. The women received between 3 and 6 cycles of either 5-FU, epirubicin and cyclophosphamide (FEC) ($n = 32$), epirubicin and cyclophosphamide (EC) ($n = 3$), doxorubicin and cyclophosphamide (AC) ($n = 13$) or 5-FU, doxorubicin and cyclophosphamide FAC ($n = 3$) adjuvant chemotherapy. Twelve FEC patients had 3 cycles of FEC followed by 3 cycles of docetaxel. Forty-six patients were treated with a planned CP dose of 600 mg/m^2 and 5 (all FEC) with planned dose of 500 mg/m^2 . The dose of epirubicin was $75\text{--}100 \text{ mg/m}^2$. Dose of 5-FU was $500\text{--}600 \text{ mg/m}^2$. Each of the drugs was administered as a short infusion (3–20 min). Blood samples (10 ml) for analysis of drugs and metabolites were obtained in EDTA tubes pretreatment and

at 0.25, 0.5, 1, 2, 4, 6 and 24 h postadministration. Plasma was separated by centrifugation. Cyclophosphamide and its stable inactivated metabolites; dechloroethylcyclophosphamide (DCCP), carboxyethylphosphoramidate mustard (CX) and ketocyclophosphamide (keto) were measured by a validated LCMS method described previously [11]. Limits of quantitation were $0.5 \text{ }\mu\text{g/ml}$ for cyclophosphamide and $0.05 \text{ }\mu\text{g/ml}$ for the metabolites. Epirubicin and its major metabolite epirubicinol were quantified in plasma by a validated HPLC assay with fluorescence detection [12]. 5-FU was measured by a validated LCMS assay based on a previously published method [13]. Limits of quantitation for the epirubicin and epirubicinol were 2 and 5 ng/ml for 5-FU. Doxorubicin was not quantified in the 13 patients treated with this anthracycline.

DNA was extracted from whole blood samples using a Qiagen kit and working to the manufacturer's instructions. Genotyping for the polymorphisms described in Table 1 was performed using RT-PCR by Taqman assays-on-demand (Applied Biosystems) and one custom Taqman assay (*CYP2B6**4), again supplied by Applied Biosystems.

A non-compartmental analysis was performed using WinNonLin (version 5) for CP and its metabolites (CX, DCCP, Keto), using the linear/log trapezoidal method to calculate AUCs. A similar analysis was performed for epirubicin and epirubicinol and for 5-FU.

Data were analyzed using SPSS version 17.0. The Kolmogorov–Smirnov test was used to test for the normal distribution of parameters. A statistically significant effect of genotype on CP or metabolite (CX, DCCP, Keto) pharmacokinetics (half-life, clearance, distribution and AUC) was tested using a univariate analysis (one-way ANOVA or independent t test depending on frequency of minor allele). Other factors that may have had an influence on the pharmacokinetics and toxicity were also tested, including differences between treatment program, age and concomitant medication using independent t test and Pearson's correlation. A total of 594 statistical tests were carried out, and following Bonferroni correction for multiple testing, a threshold for statistical significance of <0.00008 was established for an Alpha of 0.05.

Results

Representative plasma concentration profiles for cyclophosphamide and metabolites, epirubicin and epirubicinol and for 5-FU are shown in Fig. 1a–c. C_{max} values for cyclophosphamide varied from 18.6 to $44.9 \text{ }\mu\text{g/ml}$, with corresponding ranges of values of 57–148 and $2.9\text{--}26.4 \text{ }\mu\text{g/ml}$ for epirubicin and 5-FU, respectively. Cyclophosphamide and epirubicin and metabolites could be quantitated throughout the 24-h sampling period. 5-FU was eliminated

Table 1 Genetic variants investigated

Gene	*Designation	rs Number	Taqman assay details	HWE (<i>p</i>)	Genotype (cDNA CDS)	Amino acid change	Reported phenotype of minor allele	References
GSTP1	*2	1695	C_3237198_20	0.998	313A>G	Ile105Val	Substrate-specific modulated activity	Zimniak et al. [32]
CYP2B6	*2	8192709	C_2818162_20	0.886	64C>T	Arg22Cys	↑ Activity	Lamba et al. [33]
	*5	3211371	C_30634242_40	0.294	1459C>T	Arg487Cys	LoF	Lamba et al. [33]
	*6	2279343	Forward primer, 5'-TGG AGAAGCACCCGTGA AACC-3' Reverse primer, 5'-TGGAGCAGGTA GGTGTCGAT-3' Probe CCCCCA(A VIC/G FAM)GGACCTC	0.524	785A>G	Lys262Arg	↓↑ Activity	↑ Xie (cyclo) et al. [34], ↓ Rotger et al. [35]
CYP2C19	*2	3745274	C_7817765_60	0.393	516G>T	Gln172His	Poor survival	Bray et al. [24]
	*17	4244285	C_25986767_70	0.082	681G>A	Splice Variant	LoF	Satyanarayana et al. [36]
NQO1		12248560	C_469857_10	0.692	-806C>T	5'UTR	↑ Expression	Sim et al. [37]
		1800566	C_2091255_30	0.522	559C>T	Pro187Ser	Degraded protein	Siegel et al. [38]
NQO2		1143684	C_8774861_1	0.473	139T>C	Leu47Phe	↓ Activity	Jamieson et al. [28, 26]
SLC22A16		714368	C_2256675_10	0.201	146A>G	His49Arg	↑ Doxorubicin AUC	Lal et al. [29]
		6907567	C_22272571_10	0.201	312T>C	Synonymous	↑ Doxorubicin AUC	
CBR1		723685	C_2953081_10	0.599	755T>C	Val252Ala	?	
		12210538	C_22271871_10	0.21	1226T>C	Met409Thr	?	
CBR3		9024	C_2440206_1	0.909	+133G>A	3'UTR	↓ Clearance of doxorubicin	Lal et al. [7]
		1056892	C_9483603_10	0.692	730G>A	V244 M	↑ Cardiotoxicity	Blanco et al. [39]
ABCB1		8133052	C_2561992_20	0.303	11G>A	C4Y	↓ Expression	Fan et al. [40]
		1128503	C_7586662_10	0.434	1236C>T	Synonymous	?	Hoffmeyer et al. [42]
		2032582	C_11711720C_30 & C_11711720D_40	0.985	2677G>T/A	Ala893Ser/Thr	?	
ABCC1		1045642	C_7586657_20	0.923	3435C>T	Synonymous	↓ Expression	
		4148350	C_25614362_10	0.713	1988 + 219G>T	Intronic	↑ Cardiotoxicity	Visser et al. [31]
UGT2B7		7439366	C_32449742_20	0.357	802C>T	His268Tyr	Good survival	Parmar et al. [4]

LoF loss of function, AUC area under the plasma concentration–time curve

Source Pharmgkb <https://www.pharmgkb.org/> and <http://www.cypalleles.ki.se/>

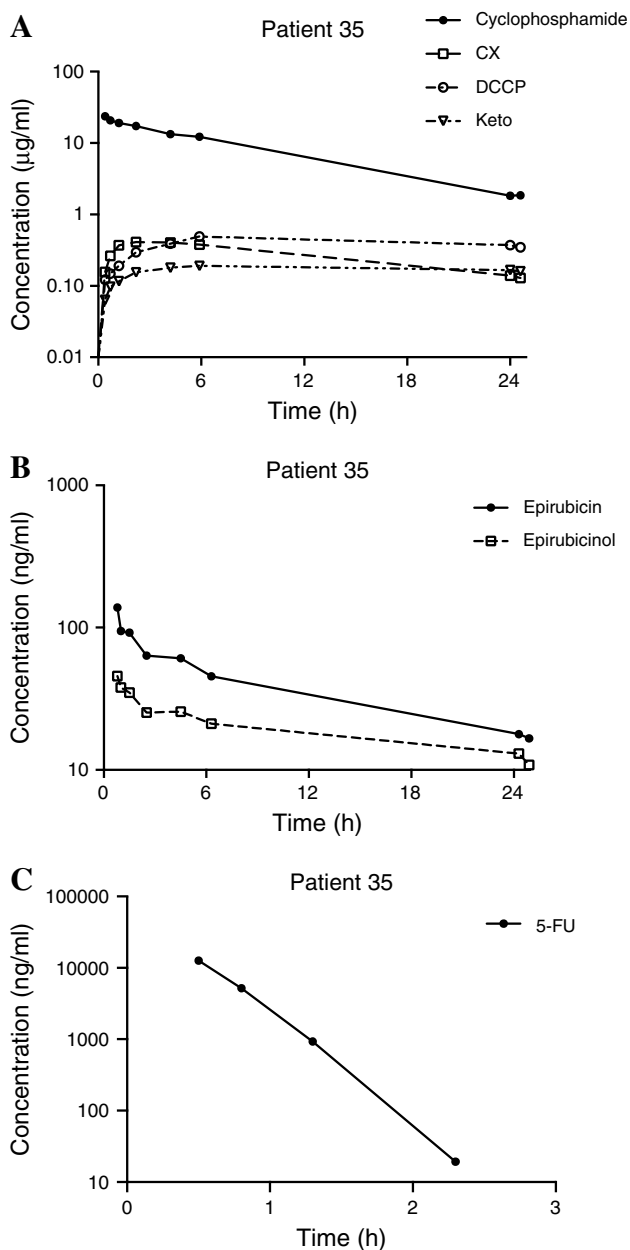


Fig. 1 Plots of log concentration against time for cyclophosphamide and its metabolites (a), for epirubicin and epirubicinol (b) and for 5-fluorouracil (c) in a representative patient

very rapidly and was not detectable beyond 4 h after administration. Pharmacokinetic parameters for each of the drugs were normally distributed and are given in Table 2, together with AUC values for the metabolites. With the exception of epirubicin clearance, which is higher than that reported previously (181 ± 46 l/h cf 61.7 (33.3 – 107.9) l/h [14]), all of the parameters are in keeping with previously published data [15, 16].

In relation to cyclophosphamide exposure, genotypes of *CYP2B6**2, *CYP2B6**4, *CYP2B6**5, *CYP2B6**9,

Table 2 Pharmacokinetics of cyclophosphamide, epirubicin and 5-fluorouracil in FEC treated breast cancer patients

Drug/metabolite	Half-life (h)	AUC _{0–24}	Cl (l/h)	V(l)
Cyclophosphamide	6.06 ± 1.20	228 ± 36	4.43 ± 0.78	37.9 ± 6.2
CX	11.3 ± 4.3	11.3 ± 3.9		
DCCP	29.3 ± 20.1	8.0 ± 3.6		
Keto	37.7 ± 18.9	9.3 ± 2.5		
Epirubicin	12.8 ± 3	779 ± 165	181 ± 46	$3,294 \pm 940$
Epirubicinol	21.6 ± 7.6	339 ± 125		
Ratio		0.44 ± 0.13		
5-Fluorouracil	$10.8 \pm 2.5^*$	$6,126 \pm 2,732$	174 ± 87	45.3 ± 26.1

Data are mean \pm SD

AUC in (ng/ml.h)

Cyclophosphamide AUC normalized to dose of 600 mg/m^2

For 5-FU, AUC normalized to dose of 500 mg/m^2 and * half-life in minutes

For epirubicin and epirubicinol, AUC normalized to dose of 100 mg/m^2

CYP2C19, *CYP2C19**2, *CYP2C19**17 and *GSTP1* were determined for all 51 patients. All SNPs were in Hardy–Weinberg equilibrium, and the frequencies were consistent with those of a European population. Linkage disequilibrium was investigated using a Fisher’s exact test and a 3 by 3 contingency table. Strong linkage disequilibrium was seen between the *CYP2B6* SNPs 758A>G and 516G>T ($p < 0.001$), and this haplotype describes the *CYP2B6**6 genotype. This linkage disequilibrium is consistent with previously reported data [17]. Since only one patient had the *4 allele (785A>G), patients who were homozygous for the *CYP2B6**6 alleles were compared as a group to all other patients. None of the other SNPs were in linkage disequilibrium.

There was a significant effect of *CYP2C19**17 on both CP and CX half-life. Having at least one variant allele was associated with a longer CP half-life (6.72 ± 1.14 h vs. 5.76 ± 1.12 h, $p = 0.007$, Fig. 2a) and a longer CX half-life (13.8 ± 6.0 h vs. 10.1 ± 2.4 h, $p = 0.030$, Fig. 2b). The magnitude of the effect was small, and there was no statistical effect of *CYP2C19**17 on any of the other pharmacokinetic parameters. Although 4 patients were taking the *CYP2C19* inhibitor omeprazole, the effect of *CYP2C19**17 was retained when these patients were omitted from the analysis. There was similarly a significant effect of *CYP2B6**6 on CP half-life, however, not on CX half-life. Homozygote *CYP2B6**6 patients showed a longer half-life compared to all others (7.12 ± 1.43 h vs. 5.92 ± 1.11 h, $p = 0.020$, Fig. 2c). There were no significant effects of the *CYP2B6**6 allele on any of the other pharmacokinetic parameters. There was also no significant effect of *GSTP1*, *CYP2B6**2, *CYP2B6**5, *CYP2C19**2 or *NQO1/2* on any pharmacokinetic parameter (Supplementary Fig. 1).

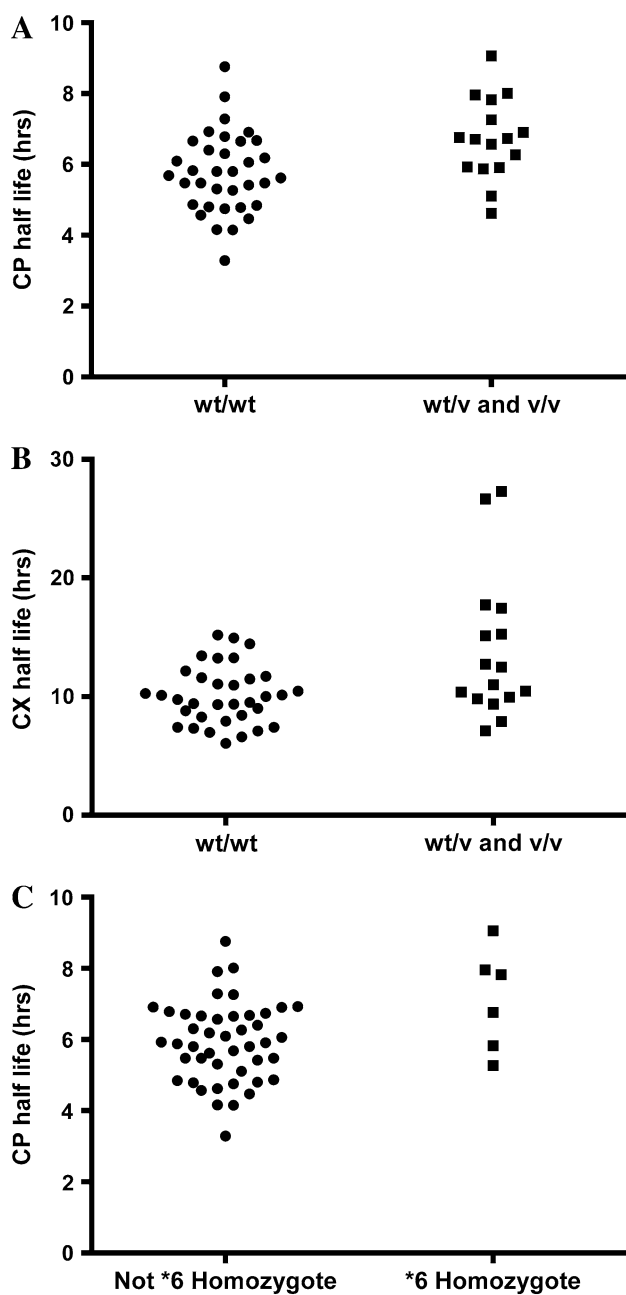


Fig. 2 Influence of pharmacogenetics on CP pharmacokinetics. Effect of *CYP2C19**17 on CP half-life (a) and on CX half-life (b) and of *CYP2B6**6 on CP half-life(c). Although these differences had *p* values less than 0.05, none were significant after applying the Bonferroni correction

The *NQO2* rs1143684 variant was associated with a higher ratio of epirubicinol/epirubicin AUC (Fig. 3). The mean and standard deviation of the ratio for carriers and minor allele homozygotes were 0.73 ± 0.26 compared with a value of 0.50 ± 0.11 for wild-type homozygotes ($p = 0.011$, *t* test). Although a wide range of genotypes linked to the pharmacology of epirubicin was explored, no

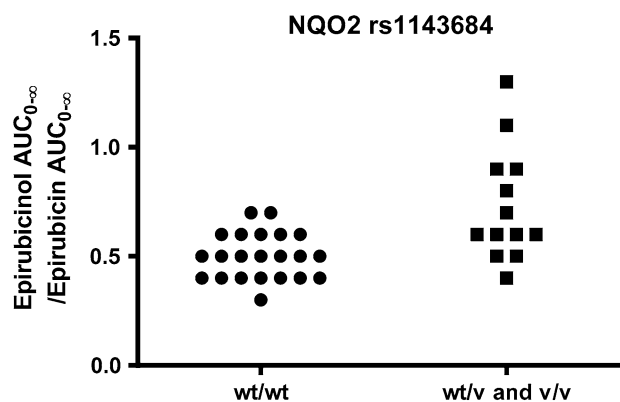


Fig. 3 Greater exposure to epirubicinol relative to epirubicin associated with the presence of at least one *NQO2* rs1143684 SNP ($p = 0.011$, *t* test)

other influence of genotype on pharmacokinetics was identified. Plots of epirubicin CI for the genetic variants most frequently observed or reported to influence epirubicin pharmacokinetics are given in Supplementary Fig. 2.

None of the pharmacogenetic effects on cyclophosphamide or epirubicin pharmacokinetics retained statistical significance after Bonferroni correction for multiple testing.

There were statistically significant differences in epirubicin clearance (264 ± 47 vs. 173 ± 39 l/h, $p < 0.0001$) and volume of distribution ($4,732 \pm 1,361$ vs. $3,159 \pm 795$ l, $p = 0.004$) between patients who had received EC ($n = 3$) compared with those who had received FEC ($n = 32$), resulting in a lower AUC in the EC group. However, this may have been confounded by age, with the EC patients tending to be older (64.3 ± 4.9 vs. 55.3 ± 9.4 years), although this difference was not significant ($p = 0.194$).

In four patients who received concomitant treatment with omeprazole, the half-life of cyclophosphamide was longer than in the other 47 patients (7.33 ± 0.64 h vs. 5.95 ± 1.18 h, $p = 0.027$). There was no impact on pharmacokinetics of any other concomitant medication.

Although the primary aim of this study was to analyze the effects of genotype on pharmacokinetics, toxicity was also considered. Due to the short follow-up time for this study, this was measured by investigating patients who experienced a dose delay or a dose reduction, inability to complete planned course, or who were suffering from a grade 3 infection. None of the SNPs had a significant effect on indicators of, or surrogates for, toxicity.

Discussion

The adjuvant treatment of early breast cancer now commonly includes an anthracycline, cyclophosphamide and

possibly the addition of other agents such as 5-FU. In recent years, doxorubicin has been replaced by epirubicin as the preferred anthracycline, largely on the basis of a lower risk of cardiotoxicity. Although a regimen of EC or FEC is reasonably well tolerated, some patients experience toxicity that limits dose intensity, and some patients suffer recurrent disease. The hypothesis that pharmacogenetic variability, mediated by an effect on pharmacokinetics or metabolism of these adjuvant agents, influences response to treatment is explored in this study. Although the effect of dihydropyrimidine dehydrogenase on the pharmacology of 5-FU is profound, genetic variants in the *DPYD* gene are rare in a Caucasian population and the clinical impact of *DPYD* pharmacogenetics is unlikely to be seen in this small population [18, 19]. Although CPIC guidelines indicate that dose reduction may be appropriate for individuals heterozygous for *DPYD* variants, the frequency of these is cited to be only 3–5 %. Therefore, the primary focus of the study reported here is on cyclophosphamide ($n = 51$) and epirubicin ($n = 35$). These are relatively small numbers for a pharmacogenetic study, but the aim was to study the direct influence of pharmacogenetics on the metabolism of these two drugs rather than on outcome.

The only effect of pharmacogenetic variation on cyclophosphamide pharmacokinetics was the impact of *CYP2C19* and *CYP2B6* genotype on half-life. Individuals with a variant *CYP2C19**17 allele or homozygous for *CYP2B6**6 had a longer half-life than patients lacking these variants. These effects were fairly modest and were not linked to a significant consistent effect of genotype on clearance, as would be expected by the direct inverse relationship between these two parameters. Previous studies have reported a similarly modest effect of *CYP2C19* genotype on cyclophosphamide pharmacokinetics [17, 20]. The *CYP2C19**17 variant is usually associated with an ultrafast metabolizer phenotype [21], which seems inconsistent with the longer half-life of cyclophosphamide observed. However, the impact of genotype on metabolism is substrate dependent and may be confounded by differences between normal volunteers and cancer patients [22]. The modest interaction with omeprazole is also consistent with a significant role for *CYP2C19* in the metabolism of cyclophosphamide. Interestingly, a similar impact of *CYP2B6**9 variants (a constituent of the *6 genotype) on cyclophosphamide elimination rate constant, consistent with the effect seen on half-life here due to *CYP2B6**6, was reported previously in patients with nephritis [23]. The clinical significance of these modest differences in pharmacokinetics is unclear; however, variants linked to *CYP2B6**6 have previously been associated with worse overall survival [24], which is consistent with the slower rate of activation of cyclophosphamide.

A number of polymorphisms in genes believed to be associated with the metabolism (*CBR1*, *CBR3*, *NQO1* and *NQO2*), conjugation (*UGT2B7*) and transport (*ABCB1*, *ABCC1* and *SLC22A16*) of anthracyclines were investigated in this study. Individuals carrying at least one minor allele for the *NQO2* SNP rs1143684 had a higher exposure to epirubicinol when corrected for epirubicin exposure. *NQO2* is a homolog of the quinone reductase, *NQO1*, which has previously been shown to be associated with response to anthracycline-containing regimens [25, 26]. However, while *NQO1* can use both NADH and NADPH to catalyze the obligate two-electron reduction in quinones to yield a hydroquinone product, *NQO2* can use neither efficiently [27, 28], and it remains to be demonstrated that *NQO2* retains a functional enzymatic activity. We have previously reported that the same *NQO2* polymorphism is associated with a worse outcome following adjuvant AC therapy for hormone receptor negative breast cancer and that stable expression of the *NQO2* rs1143684 mutant confers resistance to doxorubicin in MCF7 cells, while wild-type *NQO2* sensitizes their isogenic partner cells [26]. The previous clinical and in vitro observations are consistent with the apparent increased accumulation of epirubicinol reported in this study. The mechanism behind this relationship remains to be elucidated, but given the lack of endogenous *NQO2* activity and the lack of activity of the homologous *NQO1* with anthracyclines as a substrate, it is unlikely to involve a direct reduction in the quinone group of epirubicin.

Although SNPs in *SLC22A16* [29], *CBR1* and *CBR3* [7], or expression of *ABCB1* [30] have been associated with the pharmacology of epirubicin and other anthracyclines, no significant effect of known, functionally significant SNPs was observed. A SNP in the *UGT2B7* gene, associated with the glucuronidation of epirubicin [5], has previously been reported to have a profound influence on survival outcome [4]. However, this SNP was also found to have no impact on epirubicin pharmacokinetics or metabolism. The rs4148350 T allele in the *ABCC1* gene has been associated with a greater risk of cardiotoxicity in children receiving anthracycline therapy [31] and febrile neutropenia in breast cancer patients treated with FEC [8]. However, only the SNP in *NQO2* had any effect on epirubicin pharmacokinetics (Fig. 3).

This study sought to investigate the effect of variants in key genes thought to influence the pharmacology of cyclophosphamide and epirubicin. Detailed data on pharmacokinetics and metabolism, in relation to pharmacogenetic variants, were generated in a relatively small population. Although larger studies would be required to discern more subtle effects, the data presented here indicate only a modest impact of pharmacogenetics on FEC therapy for breast cancer.

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Conflict of interest All authors declare that they have no conflict of interest.

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