Oral Cyclophosphamide Confounds the Relationship between *CYP2C19* and *CYP2B6* Pharmacogenetics and Cyclophosphamide-induced premature Ovarian Failure in Lupus Nephritis Patients

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Abstract Cyclophosphamide treatment of lupus nephritis is associated with ovarian toxicity. Four separate studies have previously demonstrated a lower risk of cyclophosphamide induced premature ovarian failure (POF) in carriers of the *CYP2C19**2 null function allele. This hepatic enzyme bioactivates cyclophosphamide. *CYP2B6* is also important in the activation of this prodrug however, genetic variants of *CYP2B6* have not been associated with POF risk. The aim of this study was to determine the relationship between *CYP2C19* and *CYP2B6* loss of function variants and prevalence of ovarian toxicity following treatment with monthly pulses of intravenous cyclophosphamide in lupus nephritis patients. In 28 pre-menopausal female patients, there was a 25% incidence of POF. In contrast to previous studies of a similar size we could not detect a significant relationship between the risk of POF and genetic variants of *CYP2C19*, alone or in combination with variants of *CYP2B6*. However, 71.4% of the cases with POF had been treated with additional daily oral cyclophosphamide compared with only 4.7% of controls ($P < 0.02$; OR 14.29 (95% CI, 1.4-144.5). Thus the effect of daily oral cyclophosphamide administration in addition to pulsed intravenous monthly doses may have obscured any protective effect of *CYP2C19* loss of function in this cohort.

Keywords Cyclophosphamide, Premature Ovarian Failure, Toxicity, Lupus Nephritis, *CYP2C19*, *CYP2B6*, Pharmacogenetics

1. Introduction

Lupus nephritis occurs commonly in patients with systemic lupus erythematosus and can lead to chronic kidney disease or end stage renal failure [1, 2]. Clinical trials from

the 1970s established cyclophosphamide as effective in reducing relapse and preserving renal function for patients with proliferative lupus nephritis [3-5]. Cyclophosphamide acts through its potent effects on lymphocytes [6], as it can selectively eradicate the autoimmune effectors whilst sparing the bone marrow stem cells due to the differential expression of the detoxifying enzyme ALDH [7,8]. The common toxicities of cyclophosphamide are leukopenia, haemorrhagic cystitis and gonadal toxicity, which are dose and schedule dependent [8]. Haematological toxicities only commonly occur after very high doses ($>5000 \text{ mg/m}^2 \text{ i.v. 2-4 days}$) and are relatively rare at low doses. Moreover the extensive use of supportive measures such as G-CSF can circumvent the risk of this toxicity. Bladder toxicity, particularly acute haemorrhagic cystitis, is considered to be due to the accumulation of the breakdown product acrolein in the urine [9]. Haemorrhagic cystitis rarely occurs following treatment of lupus nephritis with pulse cyclophosphamide ($1000 \text{ mg/m}^2 \text{ i.v.}$) this may be due to the routine use of prophylactic MESNA [10].

However, treatment of the autoimmune disease lupus nephritis with cyclophosphamide is associated with an increased risk of premature ovarian failure (POF) in premenopausal women [10-13].

Cyclophosphamide is a prodrug activated by liver cytochrome P450 (CYP) enzymes to form the metabolite 4-hydroxy cyclophosphamide. This metabolite then undergoes chemical conversion to the ultimate cytotoxin, phosphoramidate mustard (Figure 1). Phosphoramidate mustard cross links DNA in proliferating cells and results in depletion of lymphocytes but can also have a toxic effect on ovarian follicles. Activated cyclophosphamide causes follicular death in the ovaries by damaging the dividing granulosa cells. These granulosa cells are required for the growth and maturation of ovarian follicles.

Figure 1. Hepatic activation of the prodrug cyclophosphamide to the DNA cross linking product phosphoramidate mustard which is responsible for cytotoxicity to proliferating cells including ovarian follicle granulosa cells. Cyclophosphamide is activated by cytochrome P450 (CYP) isozymes to form the primary metabolite 4-hydroxycyclophosphamide. A number of CYP isoforms have been reported to be involved, with clinical correlations observed for CYP2C19 and CYP2B6. This metabolite is in equilibrium with its open ring tautomer (aldophosphamide). Chemical decomposition (β -elimination) then occurs to produce the DNA cross linking agent phosphoramidate mustard which is cytotoxic to proliferating cells. Metabolic routes to form inactive products are also shown (in grey).

Destruction of granulosa cells leads to a lack of estrogen and progesterone and stimulates pituitary gonadotropin production and a feedback upsurge in the recruitment of primary ovarian follicles containing ova into the pool of maturing follicles. However this increases the number of follicles containing proliferating granulosa cells available for damage by the next dose of drug and the resulting vicious cycle of hypergonadotropic hypogonadism results in accelerated depletion of ovarian reserve [14]. In older women the risk of premature ovarian failure (POF) is high due to the already diminished primary follicles.

In an attempt to balance the need to induce remission, while limiting drug toxicity intravenous cyclophosphamide is administered as monthly pulse doses. However pulse dosing still poses a considerable risk of premature ovarian failure (POF). The incidence of POF is influenced by age at initiation of therapy as well as cumulative exposure. For example In pre-menopausal women treated with less than 7 pulses of cyclophosphamide the incidence of POF is relatively low, probably due to the high ovarian reserve in this age group [13]. However, there is an incidence of 17% in this same age group treated with more than 15 pulse doses (Table 1). Although increased cumulative exposure clearly increases the risk of ovarian toxicity additional environmental or genetic risk factors may play a role since this toxicity is not observed in every woman exposed to cyclophosphamide.

Hepatic metabolism of cyclophosphamide to the 4-hydroxy cyclophosphamide by cytochrome P450 (CYP) enzymes is the rate limiting step prior to formation of the cytotoxin responsible for ovarian toxicity (Figure 1). There is extensive inter-individual variation in cyclophosphamide

metabolism and a number of studies have demonstrated the important role of CYP2C19 and CYP2B6 in formation of 4-hydroxy cyclophosphamide [15-18].

Table 1. The incidence of premature ovarian failure is reported to be influenced by age at initiation of therapy as well as cumulative exposure [13]

Number of pulses (doses) of i.v. therapy	Age at initiation of treatment		
	<25y	26-30y	>31y
<7	0%	12%	25%
>15	17%	43%	100%

An inherited loss of function of CYP2C19 activity can be accounted for by two allelic variants *CYP2C19**2 (G861A), a splice defect in exon 5; and *CYP2C19**3 (G636A), a premature stop codon in exon 4 [19].

Subjects who are homozygous for either of these variants are poor metabolisers of drugs which are metabolised by this enzyme (Table 2). The presence of the *CYP2C19**17 variant (-806 C>T, -3402 C>T) may lead to increased activity of this enzyme. Carriers of this *17 allele have been termed ultra-rapid metabolisers, however the activity is still within the normal range of wild type subjects [20].

A number of variant alleles of the *CYP2B6* gene exist. Importantly the single nucleotide polymorphisms (SNP) of *CYP2B6* can have drug-substrate specific effects on function. Notably for cyclophosphamide the *CYP2B6**5 (C1459T) variant decreases cyclophosphamide activation by up to 52% whereas the *CYP2B6**6 (G516T and A785G) variant increases cyclophosphamide activation by 140-162% [21]. Hence individuals who are homozygous for *CYP2B6**5 would be expected to be deficient activators of cyclophosphamide and subjects homozygous for *CYP2B6**6

would be expected to be rapid activators of cyclophosphamide (Table 2).

Table 2. The predicted effect of *CYP* gene variants on the hepatic metabolism of cyclophosphamide to 4-hydroxy cyclophosphamide. Wildtype activity is 100%. Subjects who are homozygous variant for both genes would be expected to have null function.

Genotype	Effect on bioactivation of cyclophosphamide relative to wild type	Activation status [reference]
<i>CYP2B6</i> *6/*6	~150%	High [21,37,38]
<i>CYP2C19</i> *17/*17	≥ 100%	Normal/High [15, 20]
<i>CYP2C19</i> *2/*2 (or *3/*3)	<50 %	Deficient [15]
<i>CYP2B6</i> *5/*5	<50%	Deficient [21]
Heterozygous combination of <i>CYP2C19</i> *2, (*3) and <i>CYP2B6</i>*5	38%	Deficient [15]

The effect of haplotype (combined *CYP2C19* and *CYP2B6* genotype) on cyclophosphamide bioactivation is complex but the presence of at least one loss of function allele (*CYP2C19**2 or *CYP2C19**3 or *CYP2B6**5) significantly decreased formation of 4-hydroxy cyclophosphamide in human liver by up to 38% and also significantly decreased activation in lupus nephritis patients [15].

A number of studies have reported that *CYP2C19* pharmacogenetics are a risk factor in cyclophosphamide induced POF in lupus nephritis patients [22-25]. Women who were carriers of *CYP2C19* loss of function variants (*CYP2C19**2) had a significantly ($P<0.005$) decreased risk (RR= 0.1) of POF [22]. This was confirmed in a later study [23] (OR=0.14; $P=0.009$). Moreover, in patients treated with a cumulative dose of cyclophosphamide greater than 23.75g there was 11 fold higher odds of ovarian toxicity in *CYP2C19* wild type compared with *CYP2C19**1/*2 or *2/*2 genotype [25]. The protective effect of loss of function variants of *CYP2C19* is assumed to be due to decreased formation of the cytotoxic agent. Interestingly, when assessed, these studies have not found any association with *CYP2B6* variants, despite the important role of this enzyme

in the bioactivation of cyclophosphamide [22-23]. To date no studies have analysed the relationship of the combined *CYP2C19-CYP2B6* haplotype on the risk of POF in pre-menopausal lupus patients treated with cyclophosphamide (Figure 2).

Figure 2. The possible role of deficient activation of cyclophosphamide as a protective factor against treatment induced ovarian toxicity (POF, premature ovarian failure).

The aim of this study was to determine the relationship between loss of function variants of *CYP2C19* and *CYP2B6* and prevalence of ovarian toxicity following treatment with monthly doses of i.v. cyclophosphamide in New Zealand lupus nephritis patients.

2. Materials and Methods

Ethics approval for the clinical study was obtained from the New Zealand Health and Disability Northern X Regional Ethics Committee (NTX /06/09/112). The study (NCT00441220) was registered on <http://www.clinicaltrials.gov/>. Patients who had received pulse i.v. cyclophosphamide for treatment of biopsy proven

Table 3. The association between CYP genotype and the development of premature ovarian failure. ^aG861A or G636A SNP are the loss of function alleles in *CYP2C19**2 and *3 respectively. ^bC1459T is the loss of function SNP in the *CYP2B6* *5 and *7 allele. *P* values for differences between distribution of extensive metabolisers and loss of function metabolisers in POF or no toxicity group were determined by Fishers exact test. *P* values <0.05 are considered statistically significant. The odds ratio (OR) with 95% confidence intervals (CI) are shown.

Genotype	Case Ovarian failure n=7	Control Not affected n=21	
<i>CYP2C19</i> extensive metabolisers (n=16)	3	13	<i>P</i>=0.42 OR 0.46 95% CI 0.08-2.62
<i>CYP2C19</i> *17*17	0	2	
<i>CYP2C19</i> *17*1	0	2	
<i>CYP2C19</i> *1*1	3	9	
<i>CYP2C19</i> loss of function carriers (n= 12)	4	8	
<i>CYP2C19</i> *1*2 ^a	4	3	
<i>CYP2C19</i> *1*3 ^a	0	3	
<i>CYP2C19</i> *17*2 ^a	0	2	
<i>CYP2B6</i> extensive metabolisers (n= 25)	6	19	<i>P</i>=1.00 OR 0.63 95% CI 0.05-8.26
<i>CYP2B6</i> *6*6	0	1	
<i>CYP2B6</i> *4*6	0	0	
<i>CYP2B6</i> *6*1	3	12	
<i>CYP2B6</i> *9*1	0	2	
<i>CYP2B6</i> *1*1	3	4	
<i>CYP2B6</i> loss of function carriers (n=3)	1	2	
<i>CYP2B6</i> *1*5 ^b	0	1	
<i>CYP2B6</i> *1*7 ^b	1	1	
Normal activators (n=13) <i>CYP2C19</i> *17*17, *1*17, *1*1 and <i>CYP2B6</i> 6*6, *4*6, *1*6*, 1*9, *1*1	2	11	
Deficient activators (n=15) <i>CYP2C19</i> *1*2, *1*3, *17*2 and/or <i>CYP2B6</i> *1*5, *1*7	5	10	

proliferative lupus nephritis (WHO class III/IV) at any time up to 10 years prior to the recruitment date were enrolled into the study. Following written informed consent a blood sample (8.5 mL) was collected into PAXgene™ blood tubes (Qiagen, Hilden, Germany) for *CYP2C19* and *CYP2B6* genotyping.

Genomic DNA was extracted from whole blood using a PAXgene™ blood DNA kit according to manufacturer's instructions (Qiagen, Hilden, Germany). *CYP2C19* genotype of individuals was determined by PCR-RFLP analysis of the two major null function allelic variants (*CYP2C19**2 and *CYP2C19**3) and the possible gain of function variant (*CYP2C19**17) using previously published methods [19,26]. *CYP2B6* genotype was determined by PCR-RFLP analysis of the G516T, A785G and C1459T SNPs, this allows determination of the *4 (A785G), *5 (C1459T), *6 (G516T, A785G), *7 (G516T, A785G and C1459T) and *9 (G516T)

alleles as described previously [27].

Premature ovarian failure was defined as sustained amenorrhea occurring before 45 years of age. Clinical assessment of evidence of POF was assessed from the patient medical records independent of the genotyping data. Determination of genotype was undertaken without access to the clinical data.

An unpaired Student's *t* test was used to determine statistical difference between groups except for data sets with non-normal distribution, when a Mann Whitney U test was used. Fisher's exact test was used to analyse categorical (genotype) data. The results are reported as two sided *P* values or odds ratios (OR) with 95% CI. Statistical tests were adjusted for the effects of multiple testing by Bonferroni correction. Values of *P*<0.05 were considered to be statistically significant. All data were analysed using GraphPad Prism version 5.02.

3. Results

Thirty one pre-menopausal female patients who had received pulse i.v. cyclophosphamide for treatment of lupus nephritis over the previous 10 years were recruited into the study. Two patients had received gonadotropin releasing hormone antagonists, such as goserelin acetate to prevent accelerated recruitment of ovarian follicles and were excluded from the analysis. In addition one patient had evidence of primary infertility prior to cyclophosphamide treatment and was also excluded from the analysis.

Of the remaining 28 female patients (age 28.6 ± 7.2 years when treatment commenced), seven women (25%) experienced cyclophosphamide induced premature ovarian failure (POF). The 28 patients were then stratified as *CYP2C19* extensive metabolisers (*1 or *17) or *CYP2C19* loss of function carriers (*2 or *3). There were 16 subjects who were either wild type or *17 variant and 12 subjects who were heterozygous carriers of the loss of function alleles (Table 3). No patients were homozygous variant for any *CYP2C19* allele. There was no increase in the prevalence of *CYP2C19* extensive metabolisers in the POF group compared with the not affected group ($P = 0.42$, OR 0.46, 95%CI 0.1-2.6). This is in contrast to the significant relationship observed in previous studies of a similar sample size [22-25].

Only one of the 7 patients with POF (14.3%) was a *CYP2B6**5 (C1459T) loss of function carrier compared with 9.5% of those not affected. No patient was homozygous for *CYP2B6**5. There was no relationship between *CYP2B6*

genotype and risk of cyclophosphamide induced POF ($P = 1.00$, OR 0.63, 95%CI 0.05-8.3). This is in agreement with previous studies [22-23].

The combined *CYP2C19*-*CYP2B6* haplotype of each individual patient was then determined and the subjects were stratified into normal/high activators (i.e. no loss of function alleles at either gene) or deficient activators (i.e. at least one loss of function allele at either gene; Figure 2). There was no increased prevalence of normal/high activators in the POF group compared to those not affected ($P = 0.39$; OR = 0.37, 95% CI 0.1-2.3).

The 28 patients in this study received 9.9 ± 5.2 (median = 9) i.v. pulses of cyclophosphamide with a mean cumulative i.v. dose of 9.1 ± 12.4 g (median 7.9 g). The patients with POF had fewer pulses of cyclophosphamide compared with those who did not have POF, but this was not significant (Table 4). The subjects with POF also had a lower cumulative dose (6.9 ± 1.0 g versus 12.5 ± 2.4 g) but due to the wide variance in the case data this was not significant ($P = 0.148$).

Following careful examination of the patients medical records we noted that five of the women (71.4%) with POF had been administered daily oral cyclophosphamide at some stage in addition to i.v. therapy. This was significantly higher ($P < 0.05$) than the number of women (4.7%) in the not affected group who had received daily oral cyclophosphamide (Table 4). Despite this additional treatment there was no significant difference in the total i.v. plus p.o. dose received between the cases and the controls.

Table 4. The confounding effects of dosing regimen on risk of cyclophosphamide induced ovarian failure. Statistical analysis was by ^aStudent's T-test (unpaired, 2 tailed), ^bnon-parametric Mann Whitney U test (due to the significant difference in the variation between the groups). ^cFisher's exact test. The odds ratio (OR) with 95% confidence intervals (CI) are shown. P values < 0.05 are considered statistically significant.

	Case Ovarian failure n=7	Control Not affected n=21	
Number of cyclophosphamide pulses	8.6 ± 0.92	9.86 ± 1.14	$P=0.54^a$
Cumulative i.v. dose (g)	6.9 ± 1.0	12.5 ± 2.4	$P= 0.148^b$
Number (%) of subjects who received additional daily oral cyclophosphamide	5 (71.4%)	1 (4.7%)	$P=0.015^c$ OR 14.29 95% CI 1.4-144.5
Total dose (i.v. + p.o.) received (g)	17.5 ± 5.9	12.8 ± 2.4	$P=0.38^a$

4. Discussion

POF is a significant side-effect in patients with lupus nephritis. A number of strategies have been used to induce remission. Studies comparing oral versus intravenous therapy have not shown benefit [28,29], low dose versus high dose i.v. cyclophosphamide have shown either, no benefit [30], or improvement [31,32]. More recently comparison of mycophenolate mofetil with cyclophosphamide treatment resulted in a lower incidence of POF [33-34].

Here we explore the hypothesis that genotyping may also be used to determine patients at risk of POF from cyclophosphamide. We confirmed the lack of relationship between *CYP2B6* genotype and cyclophosphamide induced ovarian toxicity that had been observed in previous studies [22-25]. Indeed an additional study in <45 year old female breast cancer patients receiving combination chemotherapy schedules which included cyclophosphamide also demonstrated no relationship between *CYP2B6* genotype and POF [36].

However, in contrast to the previous studies in lupus patients which demonstrated a significant relationship between *CYP2C19* genotype and cyclophosphamide induced POF we did not find any protective effect of *CYP2C19* loss of function.

Since both enzymes have been shown to be important in the activation of cyclophosphamide we also analysed the relationship between combined *CYP2C19-CYP2B6* loss of function haplotype and the risk of POF. This also did not identify a protective effect of deficient activation of cyclophosphamide in therapy induced POF.

The 28 patients in this study received a median of nine i.v. pulses of cyclophosphamide and a median cumulative i.v. dose of 7.9 g. Based on previous data [13] the minimum incidence of POF in this age group following this dosage should be at least 12%. The incidence of POF in the current study was 25%, which is higher than predicted based on age and i.v. dose (Table 1). However, this incidence of POF is lower than the previously reported rates of between 48-59.7% in the studies which have demonstrated a protective effect for *CYP2C19* loss of function [22,23,25].

Cumulative dose is known to influence the risk of POF and the previously reported protective effect of *CYP2C19* was observed in patients who received >15 pulse doses. Notably, Ngamjanyaporn *et al* 2011 [25] found that *CYP2C19*1*1* patients who received a high cumulative dose (>23.75 g) were 11-fold more likely to have toxicity than patients who had low cumulative dose (<23.75 g) and this risk was lower (2.3 fold) if patients were carriers of *CYP2C19*2*. The low cumulative i.v. dose (median 11 g) in the current study may have limited any protective effect of deficient activation status on risk of POF.

To control disease symptoms many patients require additional oral cyclophosphamide treatment. There were significantly more women who had been treated with daily oral cyclophosphamide in the POF group than in the women

who did not have this toxicity. Although there was no difference in the total cyclophosphamide dose received (i.v. plus p.o.) between the groups, continuous exposure to cyclophosphamide following daily oral administration is known to cause more ovarian toxicity than monthly pulse i.v. cyclophosphamide [12].

No assessment of the risk of other toxicities relative to genotype was undertaken. This is because the risk of leukopenia and haemorrhagic cystitis are proactively managed clinically by administration of G-CSF and MESNA and hence the incidence of these events will be confounded by these preventative measures. In addition haematuria is one of the renal symptoms of lupus and hence it is difficult to accurately assess symptoms of haemorrhagic cystitis from retrospective case records of these patients.

5. Conclusion

In conclusion, there is increasing evidence from the literature that *CYP2C19* genotype can influence the risk of cyclophosphamide premature ovarian failure in patients with lupus. However, we did not find any relationship between the incidence of POF and *CYP2C19* or *CYP2B6* genotype. This was probably due to the confounding effect of additional daily oral cyclophosphamide treatment in our cohort. Further studies to investigate *CYP2C19* status as a risk factor are however still warranted but these studies should include in the design and analysis other important therapeutic factors which can influence this toxicity such as: cumulative cyclophosphamide i.v. dose, age at initiation of therapy and importantly whether a patient has received any additional daily oral cyclophosphamide.

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