

## REVIEW

# The importance of both *CYP2C19* and *CYP2B6* germline variations in cyclophosphamide pharmacokinetics and clinical outcomes

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Cyclophosphamide is an alkylating agent used in the treatment of solid and haematological malignancies and as an immunosuppressive agent. As a prodrug, it is dependent on bioactivation to the active phosphoramidate mustard metabolite to elicit its therapeutic effect. This focused review will highlight the evidence for the role of germline pharmacogenetic variation in both plasma pharmacokinetics and clinical outcomes. There is a substantial indication from 13 pharmacokinetic and 17 therapeutic outcome studies, in contexts as diverse as haematological malignancy, breast cancer, systemic lupus erythematosus and myeloablation, that pharmacogenetic variation in both *CYP2C19* and *CYP2B6* influence the bioactivation of cyclophosphamide. An additional role for pharmacogenetic variation in *ALDH1A1* has also been reported. Future studies should comprehensively assess these 3 pharmacogenes and undertake appropriate statistical analysis of gene–gene interactions to confirm these findings and may allow personalised treatment regimens.

### KEYWORDS

chemotherapy, cytochrome P450 enzymes, genetic polymorphism, genetics and pharmacogenetics, oncology

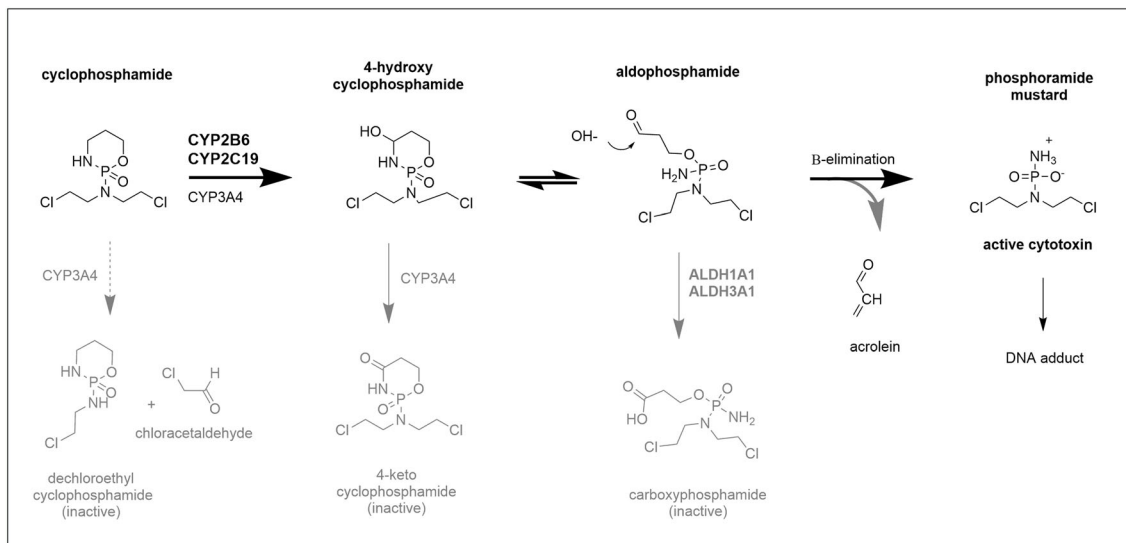
## 1 | INTRODUCTION

First synthesized over 60 years ago, cyclophosphamide (N,N-bis(2-chloroethyl)-2-oxo-1,3,2λ5-oxazaphosphinan-2-amine) remains a key chemotherapeutic agent for the treatment of a number of solid and haematological cancers inflammatory and autoimmune disorders, bone marrow transplantation (stem cell mobilization and conditioning regimens), and as prophylaxis against post-transplantation graft-vs-host disease.<sup>1–5</sup>

Cyclophosphamide is a prodrug that requires bioactivation to elicit its therapeutic effects. The complex activation and inactivation pathways for this drug, and the enzymes or chemical reactions involved, are summarised in Figure 1. Cyclophosphamide undergoes hepatic hydroxylation to form 4-hydroxycyclophosphamide (4-OHCP), which represents the major route of metabolism of the drug and accounts for 70–80% of the dose.<sup>6</sup> This route can be catalysed by any a number

of cytochrome P450 (CYP) enzymes including *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5* and *CYP2J2*.<sup>8–16</sup> *CYP2B6* and *CYP2C19* are the enzymes with the highest activity for bioactivation of cyclophosphamide.

4-OHCP exists in equilibrium with its tautomer aldophosphamide in the systemic circulation.<sup>17,18</sup> It is not known how either 4-OHCP or aldophosphamide enter cells; transport is widely assumed to occur via passive diffusion; however, direct evidence of this is lacking. Once inside cells, aldophosphamide is thought to undergo spontaneous hydrolysis by nonenzymatic β-elimination, giving rise to the active alkylating compound phosphoramidate mustard and the by-product acrolein. The involvement of phosphodiesterase enzymes in this final activation step has been postulated.<sup>19</sup> Phosphoramidate mustard is a potent DNA alkylating agent that can readily form DNA interstrand cross links (ICL) via 2 reactive chloroethylamine groups. Phosphoramidate mustard reacts with the N7 of guanine on opposite



**FIGURE 1** The complex activation and inactivation pathways of cyclophosphamide. These pathways involve enzymes such as the cytochrome P450 (CYP) and aldehyde dehydrogenase (ALDH). Only the major metabolic products are shown, with the inactivation pathways shown in grey. The major route of biotransformation of cyclophosphamide is via 4-hydroxylation<sup>6</sup> whereas CYP3A4 catalysed N-dechloroethylation is a minor route contributing on average 19% of metabolic clearance<sup>7</sup>

strands of DNA and is highly selective for 5'-GNC-3' sequences.<sup>20</sup> These ICL block both DNA replication and gene transcription, resulting in cellular apoptosis. Proliferating cells are most susceptible to this genotoxicity.

A number of inactivation pathways also influence the ultimate levels of phosphoramidate mustard (reviewed in<sup>1</sup>). Cyclophosphamide can undergo N-dechloroethylation, catalysed predominantly by CYP3A4, resulting in the formation of an inactive metabolite 2-dechloroethylcyclophosphamide and equimolar amounts of the by-product chloroacetaldehyde.<sup>7</sup> This minor pathway accounts for around 19% of total metabolic clearance of cyclophosphamide. In addition, 4-OHCP can undergo secondary metabolism by CYP3A4 to form inactive 4-keto-cyclophosphamide. The intermediate product in this reaction (imino-cyclophosphamide) may also undergo glutathione-conjugation. Alcohol dehydrogenase and aldo-keto reductase are also thought to play a minor role in the elimination of aldophosphamide by reduction to alcohosphamide.<sup>1</sup> In contrast, oxidation of aldophosphamide into carboxyphosphamide, catalysed by ALDH1A1 and ALDH3A1, is an important inactivation pathway.<sup>21-23</sup> Indeed carboxyphosphamide is a major metabolite of cyclophosphamide.<sup>24</sup>

Importantly high ALDH activity is expected to protect cells, for example haematopoietic progenitor (CD34+) cells, from the cytotoxic effect of 4-OHCP.<sup>25,26</sup> This is pertinent for the use of cyclophosphamide as a conditioning agent for haematological stem cell transplantation and in the treatment of autoimmune diseases.

It is clear that there are numerous pathways that could influence interindividual variability of cyclophosphamide response. However, most pharmacogenomic studies have focussed on assessment of the genetic variants of *CYP2B6* and *CYP2C19*, the enzymes that catalyse the initial bioactivation step. The expression and activity of both *CYP2B6* and *CYP2C19* are highly polymorphic due to common single nucleotide polymorphisms (SNPs).<sup>27,28</sup> The nomenclature of the most commonly studied SNP in these 2 enzymes in the context of cyclophosphamide pharmacogenetics are summarised in Table 1. The coding region SNP in *CYP2B6* alter the amount or activity of enzyme produced, which can be substrate dependent (reviewed in<sup>28,29</sup>). For example, the 516G>T SNP (rs3745274) in recombinant systems increased enzyme activity due to homotropic co-cooperativity<sup>30</sup>; however, this SNP also causes aberrant splicing, resulting in mRNA lacking exons 4–6 and lower amounts of functional protein.<sup>31</sup> *CYP2B6*

**TABLE 1** The *CYP2C19* and *CYP2B6* polymorphisms commonly studied in cyclophosphamide pharmacogenetics

Gene	SNP	Variant (or definitive haplotype)	Allele	Effect <sup>a</sup>
<i>CYP2C19</i>	rs4244285	681G>A	*2	Null function
	rs4986893	636G>A	*3	Null function
<i>CYP2B6</i>	rs2279343	785A>G	*4	Altered function and expression
	rs3211371	1459C>T	*5	Altered function and expression
		(516G>T and 785A>G)	*6	Altered function and expression
		(516G>T, 785A>G and 1459C>T)	*7	Altered function and expression
	rs3745274	516G>T	*9	Altered function and expression
	rs4802101	-750T>C	*G	Alters an HNF transcription factor binding site (HNF1)

<sup>a</sup>In vitro studies indicate that the functional effects of *CYP2B6* variants appear to be substrate dependent.<sup>29</sup> SNP, single nucleotide polymorphism.

pharmacogenetics are complex because the common protein coding SNP can exist in various haplotype combinations. Notably, the \*6 allele (rs2279343 + rs3745274 haplotype) is most prevalent in populations with European ancestry. In contrast, the coding region SNP in *CYP2C19* result in no-function alleles, with the \*2 allele most common in people of European ancestry and the \*3 allele also prevalent in people of Asian ancestry.

Regulation of the transcription of *CYP2B6* and *CYP2C19* is controlled by a number of transcription factors which include: constitutive androstane receptor (CAR), pregnane X receptor (PXR), glucocorticoid receptor (GR), estrogen receptor- $\alpha$  and GATA-4.<sup>27,28,32-34</sup> *CYP2B6* is a highly inducible enzyme<sup>28</sup> and can undergo autoinduction by cyclophosphamide.<sup>35</sup> Whilst *CYP2C19* is also inducible<sup>27</sup> the effect of cyclophosphamide on the regulation of this gene is not known.

## 2 | PLASMA PHARMACOKINETICS

There is substantial variation in the plasma pharmacokinetics of cyclophosphamide and total clearance ranges from 1.0–12.6 L/h.<sup>6,36-38</sup> The major route of clearance is nonrenal and 4-hydroxylation is the predominant route of hepatic metabolism of the drug. Cyclophosphamide can induce its own metabolism within 24 hours following continuous infusion or after repeated administration over several days.<sup>38</sup>

In adult cancer patients, *CYP2C19* genotype, but not *CYP2B6*, is significantly associated with cyclophosphamide elimination rate ( $K_e$ ), but this was only observed in patients receiving  $\leq 1000$  mg/m<sup>2</sup> dose.<sup>39</sup> Population-pharmacokinetic modelling of data from adult patients ( $n = 124$ ) receiving 4 day cyclophosphamide regimens, found a trend between *CYP2C19*\*2 status and decreased induced clearance (via 4-hydroxylation) with an effect size of 14%.<sup>40</sup> This regimen included thiotepa, a *CYP2B6* inhibitor, which will have influenced the relative contribution of *CYP2B6* in cyclophosphamide clearance in these patients. However, inclusion of *CYP2C19* and *CYP2B6* covariates did not influence the POP-PK model of clearance (induced) in data from 21 patients undergoing myeloablation with high dose cyclophosphamide. It is not clear which of the *CYP2B6* SNPs were assessed in this study.<sup>41</sup>

Paediatric use of cyclophosphamide for malignancies such as non-Hodgkin's lymphoma, acute lymphoblastic leukaemia, neuroblastoma and rhabdomyosarcoma suggests that plasma half-life is shorter in children than adults.<sup>37</sup> This is assumed to be due to higher metabolic clearance in children.<sup>6</sup> A study in 51 children (median age 5.5 years) did not find a correlation between cyclophosphamide clearance and *CYP2B6* genotype.<sup>14</sup> This study did not assess *CYP2C19*. In contrast, in children (mean age  $11.2 \pm 4$  years) with non-Hodgkins lymphoma ( $n = 49$ ) an association was observed between the *CYP2B6*\*6 allele and lower clearance of cyclophosphamide both at dose 1 and at dose 5 (after autoinduction).<sup>42</sup> No association was found in this study with *CYP2C19*\*2. It is of note that hepatic *CYP2B6* enzyme appears to reach adult activity levels as early as 1 year postnatal age.<sup>43</sup> In contrast, maturation of hepatic *CYP2C19* enzyme activity does not occur until around 10 years of age.<sup>44</sup> Hence, assessment of *CYP2C19*

genotype in very young children is unlikely to uncover any associations with cyclophosphamide clearance.

The plasma pharmacokinetics of 4-OHCP are also highly variable, and there is a strong correlation between cyclophosphamide and 4-OHCP plasma AUC.<sup>45</sup> The *bioactivation ratio*, the ratio of 4-OHCP to the parent drug, is often used as a descriptor of this relationship. Using this bioactivation ratio approach, an association with *CYP2C19*, but not *CYP2B6* genotype was demonstrated in 68 breast cancer patients.<sup>46</sup> In contrast, a study in a cohort of 103 cancer patients found no significant association between the bioactivation ratio and either *CYP2C19* or variants of *CYP2B6*.<sup>47</sup> Assessment of the bioactivation ratio in a paediatric population ( $n = 51$ ) also found no association with *CYP2B6* genotype; however, *CYP2C19* variants were not assessed.<sup>14</sup> Most notably, assessment of 4-OHCP plasma pharmacokinetics at first dose in a large study ( $n = 567$ ) of non-Hodgkin's lymphoma patients demonstrated strong ( $P < 0.0001$ ) independent associations of both *CYP2B6* and *CYP2C19*\*2 genotype with 4-OHCP AUC.<sup>48</sup>

The relationship between plasma pharmacokinetics and these pharmacogenes has also been studied in patients with (systemic lupus erythematosus, SLE) and vasculitis. A pharmacokinetic study in 23 patients with these autoimmune diseases demonstrated that carriers of *CYP2B6* 516G>T had a significantly lower cyclophosphamide elimination rate ( $K_e$ ), however, this study did not assess *CYP2C19* genotype.<sup>49</sup> These autoimmune diseases can result in glomerulonephritis (i.e. lupus nephritis). A preliminary assessment in a small group of lupus nephritis patients ( $n = 16$ ) suggested that the combination genotype based on these 2 pharmacogenes may relate to the bioactivation ratio.<sup>15</sup> A further study assessed cyclophosphamide and 4-OHCP pharmacokinetics in 18 lupus nephritis patients did not find an association with *CYP2C19* genotype<sup>50</sup> but did not determine *CYP2B6* genotype. However, a large study (189 SLE patients) found strong correlations between both *CYP2C19* and *CYP2B6* variants and 4-OHCP plasma concentrations.<sup>51</sup> Multivariate linear regression indicated that *CYP2C19*\*2 genotype accounted for 23.6% of variation in plasma 4-OHCP. No correlations were observed for the coding region variants of *CYP2B6*. However, an SNP in the promoter region of the *CYP2B6* gene, (-750T>C;\*G, rs4802101), was found to be important. A combined genotype determined across the 2 gene loci (*CYP2C19* and *CYP2B6*) strongly associated with plasma 4-OHCP concentrations and accounted for 47.9% of the individual variability. It is of note that the 5' promoter region variants, -750T>C (\*G) and -2320T>C, were also found to have significant correlations with the bioactivation ratio in a cohort of 103 patients with lymphoma and breast cancer.<sup>47</sup> Since *CYP2B6* is a highly inducible gene, these promoter region variants may influence the known ability of cyclophosphamide to autoinduce its own metabolism.<sup>1,35,52</sup>

Only a small number of studies have assessed the relationship between pharmacogenetics and the inactivation pathways. Formation of the inactive metabolites (keto-cyclophosphamide, carboxy-cyclophosphamide or dechloroethyl-cyclophosphamide) did not associate with *CYP2B6* variants or *CYP2C19*\*2 in 49 children with non-Hodgkins lymphoma.<sup>42</sup> Assessment of these same inactivated metabolites in 51 breast cancer patients also did not find any

statistical associations with *CYP2B6* or *CYP2C19* after Bonferroni correction for multiple testing.<sup>53</sup> Formation of 4-OHCP was not determined in these studies. In contrast, a gene-dose-dependent association of *ALDH3A1* with increased bioactivation ratio has been reported. Notably, this effect is in the opposite direction to the association between *CYP2C19* genotype and bioactivation ratio in the same patients.<sup>46</sup>

Thus, whilst there are substantial inconsistencies in the design and power of each individual study, there does appear to be substantial evidence of an association between both *CYP2C19* and *CYP2B6* genetic variation and interindividual differences in the bioactivation of cyclophosphamide to 4-OHCP. These studies are summarised in Table 2. However, the time-to-maturation of *CYP2C19* (for paediatric studies), use of *CYP2B6* inhibitors in concomitant chemotherapy regimens as well as the autoinduction effect of high dose/continuous dosing schedules, which are likely to influence *CYP2B6* expression, may be confounding factors in some of these studies. The possible additional influence of *ALDH3A1* on the plasma concentrations of 4-OHCP has not been well characterised.

### 3 | THERAPEUTIC OUTCOMES

Several articles have also studied associations with the *CYP2B6* and *CYP2C19* pharmacogenes and therapeutic outcomes. A recent study of chronic lymphocytic leukaemia patients ( $n = 44$ ), assessed *CYP2B6*\*6 genotype and found no correlation with disease response.<sup>55</sup> In contrast, in multiple myeloma patients ( $n = 26$ ) treated with high-dose cyclophosphamide for myeloablation, an association was observed between progression free survival and *CYP2B6* (785A>G).<sup>56</sup> However, *CYP2C19* was not assessed in these patients. A larger study ( $n = 119$ ) of patients with chronic lymphocytic leukaemia found that *CYP2B6*\*6 carriers had inferior treatment response compared with noncarriers of this haplotype (odds ratio [OR] 0.27,  $P = 0.004$ ).<sup>57</sup> Unfortunately, *CYP2C19* was again not assessed in these studies. In lymphoma patients ( $n = 93$ ) treated with high-dose cyclophosphamide prior to haematological stem cell transplantation, associations between disease relapse, overall survival and *CYP2B6* (1459C>T and the \*7 haplotype), but not *CYP2C19*\*2, have been reported.<sup>58</sup> However, in a study of acute myelogenous and lymphocytic leukaemia patients ( $n = 359$ ) receiving high-dose cyclophosphamide for myeloablation prior to haematological stem cell transplantation, patients homozygous for *CYP2C19*\*2 had significantly worse progression free and overall survival compared with individuals who were heterozygote carriers or wild type at this allele.<sup>59</sup> This study also assessed *CYP2B6* coding region variants and classified patients by assumed phenotypes (poor metabolisers: *CYP2B6*\*6 homozygous individuals; ultrarapid metabolisers: *CYP2B6*\*4 heterozygote carriers; extensive metabolisers: individuals who were any other genotype). Better progression free survival was observed in *poor metabolisers* compared with *ultrarapid metabolisers*. Unfortunately, multivariate analysis of genetic variants at the 2 loci was not undertaken in this study. Most recently, significant associations between treatment response and

both *CYP2C19*\*2 and *CYP2B6* (785A>G) genotype were observed in a large study of lymphoma patients ( $n = 567$ ). Patients homozygous for *CYP2C19*\*2 had poor response (OR 0.26, 95% confidence interval [CI] 0.05–0.58). Binary logistic models indicated that patients with both *CYP2C19*\*2 and *CYP2B6* 785G alleles had the worse treatment response.<sup>48</sup>

A study of *CYP2B6* haplotypes in 38 breast cancer patients found a significant gene-dose relationship and time to relapse,<sup>60</sup> while no association was observed with outcomes and *CYP2B6* haplotypes in a larger cohort of breast cancer patients ( $n = 350$ ).<sup>61</sup> However, although this study did not assess *CYP2C19*, disease-free survival was associated ( $P = 0.02$ , unadjusted) with *CYP3A4*\*1B. Importantly, a comprehensive study of both *CYP2C19* and *CYP2B6* in breast cancer patients ( $n = 230$ ) found associations with overall survival and these pharmacogenes.<sup>62</sup> *CYP2B6* 516G>T and A785A>G homozygous individuals were more likely to have poor overall survival ( $P = 0.04$  and  $0.036$ , respectively). *CYP2C19*\*2 homozygous individuals had an overall survival hazards ratio of 6.2 (95% CI 0.79–487;  $P = 0.083$ ) compared with wildtype individuals. Unfortunately, multivariate analysis of the epistatic effect of both *CYP2C19* and *CYP2B6* was not undertaken. Indeed, a separate study using both multivariate and multifactor dimensionality reduction analysis found strong gene-gene interactions for *CYP2C19* and *CYP2B6* with treatment response in 111 breast cancer patients.<sup>63</sup>

Of note, a recent global screening (Illumina SNP array) approach confirmed the importance of *CYP2C19*\*2 with disease outcome in 250 Indian breast cancer patients.<sup>64</sup> This *CYP2C19* SNP (rs 4244285), along with an SNP in *ALDH1A1* (rs6151031), was identified as important ( $p < 5 \times 10^{-8}$ ). Also a significant gene-dose effect of *CYP2C19*\*2 with risk of poor response was observed. Of particular note, this genomic approach, which assessed 700 000 SNPs, did not find any association with *CYP2B6*. However, *CYP2B6* may be difficult to accurately assess using this type of genomic analysis due to the presence of the adjacent pseudogene (*CYP2B7P1*), fusion alleles and copy number variation.<sup>54</sup>

The relationship between cyclophosphamide pharmacogenetics and outcomes in autoimmune disease has not been as widely studied. Assessment of lupus nephritis patients ( $n = 62$ ) found that homozygosity for *CYP2C19*\*2 and *CYP2B6* (1459C>T) was associated with increased risk of developing end stage renal disease after treatment with cyclophosphamide.<sup>65</sup> In contrast, in another study of lupus nephritis patients ( $n = 36$ ), there was no association between *CYP2C19*\*2 or *CYP2B6* (1459C>T) and disease remission.<sup>66</sup> However, this study did not assess other *CYP2B6* haplotypes that contribute to bioactivation. An additional study of lupus nephritis patients ( $n = 70$ ) also found no association with *CYP2C19* or *CYP2B6* and remission from disease. Instead, a polymorphism in *GSTP1* (105 Ile > Val) was identified as possibly important ( $P = 0.047$ ).<sup>67</sup> Most recently, a combined genetic marker (*CYP2C19*\*2 and *CYP2B6*-750C) significantly associated with worse disease remission and longer response time in a gene-dose dependent manner in SLE patients ( $n = 189$ ).<sup>51</sup> A further study ( $n = 136$ ) of lupus nephritis patients also found that *CYP2C19*\*2 genotype associated with poor response.<sup>50</sup> Additional epistatic effects

**TABLE 2** Summary of studies assessing the relationship between CYP2B6 and CYP2C19 genetic polymorphisms and cyclophosphamide bioactivation plasma pharmacokinetics

Disease	Study size (n)	Population ancestry <sup>a</sup>	Variant allele assessed <sup>b</sup>		Multivariate analysis	Significant association		
			CYP2B6 SNP (haplotypes assessed)	CYP2C19		CYP2B6	CYP2C19	
Cancer	14	51		516G>T, 785A>G, 1459C>T (*4, *5, *6)	ND	-	ND	
	39			64C>T, 516G>T, 777C>A, 785A>G, 1459C>T (*2, *3, *4, *5, *6, *7)	*2	-	*2	
	42	49		516G>T, 785A>G, 1459C>T (*5, *6)	*2, *17	*5, *6	-	
	40	124		64C>T, 516G>T, 785A>G, 1459C>T	*2	-	-	
	45	29		516G>T (*9)	*2	*9	-	
	46	68	Pakistan	516G>T, 785A>G, 1459C>T (*4, *5, *6)	*2	*4, *5, *6	-	
	47	103		-2320T>C, -750T>C, 136A>G, 296G>A, 419G>A, 415A>G, 516G>T, 785A>G, 1172T>A, 1459C>T, 15582C>T, 18492T>C (*G, *H, *4, *5, *6, *7, *8, *9, *11, *12, *14, *15)	*2, *3	*H	-	
	48	567	Chinese	64C>T, 516G>T, 785A>G, 1459C>T (*2, *4, *6, *9, *29, *30)	*2, *3	*2, *4, *6, *9, *29, *30	*2	
	Autoimmune disease	41c	21		64C>T, 516G>T, 777C>A, 785A>G (*2, *3, *6)	*2	-	-
		15	16	Polynesian	516G>T, 785A>G, 1459C>T (*4, *5, *6, *7, *9)	*2, *3	*5	*2, *3
49		23	African American	516G>T, 785A>G, 1459C>T (*4, *5, *9)	ND	*9	ND	
50		18	Indian	ND	*2	ND	-	
51		189	Chinese	-2320T>C, -750T>C, 516G>T, 785A>G, 1459C>T, 15582C>T	*2, *3	*G, *H, 15582C>T	*2	

<sup>a</sup>European ancestry was major ethnicity, unless indicated.

<sup>b</sup>Alleles are as described in Table 1 with additional alleles (SNP or haplotypes) as follows: CYP2B6\*H (-750T>C and -2320T>C) = rs4802101 and rs7254579; \*2 (64C>T) = rs8192709; \*3 (777C>T) = rs45482602; \*8 (415A>G) = rs12721655; \*11 (136A>G) = rs35303484; \*12 (296G>A) = rs36060847; \*14 (419G>A) = rs35793040; \*15 (1172T>A) = rs35979566; \*29 and \*30 are CYP2B6-CYP2B7 hybrids (crossover in intron 4)<sup>5,4</sup>;

CYP2B6 15582C>T = rs4803419, 18492T>C = rs2279345; CYP2C19\*17 (-806C>T) = rs12248560.

<sup>c</sup>This study did not fully disclose the CYP2B6 and CYP2C19 SNP assessed. ND, not determined; SNP, single nucleotide polymorphism.

**TABLE 3** Summary of studies assessing the relationship between CYP2B6 and CYP2C19 genetic polymorphisms and therapeutic outcomes

Disease	Study size (n)	Population ancestry <sup>a</sup>	Variant allele assessed <sup>b</sup>		Multivariate analysis	Significant association	
			CYP2B6 SNP (haplotypes assessed)	CYP2C19		CYP2B6	CYP2C19
Cancer	48	Chinese	64C>T, 516G>T, 785A>G, 1459C>T (*2, *4, *6, *9, *29, *30)	*2, *3	Yes	*4	*2
	55		516G>T, 785A>G (*6)	ND	No	-	ND
	56		516G>T, 785A>G, 1459C>T (*4, *5, *6, *9)	ND	No	*4	ND
	57		516G>T, 785A>G (*6)	ND	No	*6	ND
	58		516G>T, 785A>G, 1459C>T (*5, *6, *7)	*2	Yes	*5, *7	-
	59 <sup>c</sup>		516G>T, 785A>G, 1459C>T (*4, *5, *6, *7)	*2, *3	No	*6	*2, *3
	60 <sup>c</sup>		516G>T, 785A>G, 1459C>T (*4, *5, *6, *9)	ND	No	*5, *6, *9	ND
	61		516G>T, 785A>G, 1459C>T (*4, *5, *6, *7, *9)	ND	Yes	-	ND
	62		64C>T, 415A>G, 516G>T, 777C>A, 785A>G, 1459C>T (*2, *3, *4, *5, *8, *9)	*2	No	*2, *4, *8, *9	*2
	63		516G>T (*9)	*2	Yes	-	*2
	64		GWAS (700 000 SNP)	-	-	-	-
	Autoimmune disease		50	Indian	ND	*2	Yes
51 <sup>d</sup>		Chinese	-750T>C, -2320T>C, 785A>G, 1459C>T, 516G>T, 15582C>T	*2, *3	Combined genotype	CYP2B6*H - and -CYP2C19*2	

<sup>a</sup>European ancestry was major ethnicity, unless indicated.

<sup>b</sup>Alleles are as described in Table 1 with the additional alleles (SNP or haplotypes) as follows: CYP2B6\*H (-750T>C and -2320T>C) = rs4802101 and rs7254579; \*2 (64C>T) = rs8192709; \*3 (777C>T) = rs45482602; \*8 (415A>G) = rs12721655; \*11 (136A>G) = rs35303484; \*12 (296G>A) = rs36060847; \*14 (419G>A) = rs35773040; \*15 (1172T>A) = rs35979566; \*29 and \*30 are CYP2B6-CYP2B7 hybrids (crossover in intron 4)<sup>54</sup>; 15582C>T = rs4803419; CYP2C19\*17 (-806C>T) = rs12248560.

<sup>c</sup>These studies stratified patients by a putative metaboliser status based on various combinations of CYP2B6 alleles assessed.

<sup>d</sup>This study stratified patients by a putative metaboliser status based on combinations of both CYP2B6 and CYP2C19 alleles assessed. GWAS, genome-wide association study; ND, not determined; SNP, single nucleotide polymorphism.

of *CYP3A4* and *GSTP1* were suggested. However, logistic regression analysis indicated that *CYP2C19\*2* genotype independently associated with response (Coeff 0.99 ± SE 0.34, OR 2.69 (95% CI 1.36–5.32),  $P = 0.0043$ ). Unfortunately, *CYP2B6* variants were not assessed in this study.

In a study of patients with renal disease due to vasculitis ( $n = 93$ ), no association between response and *CYP2C19\*2* or *CYP2B6* (1459C>T) genotype was observed.<sup>68</sup> Other important *CYP2B6* variants and haplotypes were not assessed. This study did find an association between disease outcome and Fcγ receptor gene (*FCGR*). Further to this, an Illumina array based analysis of 491 617 SNPs in lupus nephritis patients ( $n = 109$ ) also found that treatment response at 6 months associated with the *FCGR2B* locus.<sup>69</sup> Hence, renal remission outcomes in these autoimmune diseases may be more strongly influenced by the pathology of the disease (autoimmune reaction to IgG-complexes) rather than the drug treatment. However, this genome array-based approach, as noted earlier in this review, may not report on *CYP2B6* SNP due to the complexity of this genomic region. The studies reporting associations of *CYP2B6* and/or *CYP2C19* genetic polymorphisms with therapeutic outcomes are summarised in Table 3.

Despite the inconsistent assessment of both *CYP2B6* and *CYP2C19* in many of the studies reviewed *supra vide* there does appear to be substantial evidence for the role of both *CYP2B6* and *CYP2C19* pharmacogenes with cyclophosphamide response. It is important to note that these associations were observed in many different contexts despite the use of different dosages, dosing regimens, drug combinations, as well as differences in the intrinsic resistance in different neoplastic and autoimmune diseases. However, the different statistical methods and gene analysis methods employed across these studies may hide or enhance associations.

It is clear that the majority of the therapeutic outcome or pharmacokinetic studies have focussed on *CYP2B6* and/or *CYP2C19* as candidate genes. However, some studies have also reported on selected SNP in other candidate genes such as *CYP2C9*, *GST* or *ABCB1*.<sup>39–42,46,48–51,55,56,61–65,67,68</sup> Whilst not the focus of this review, it is important to note that the role of pharmacogenetics in the risk of excessive toxicity (due to high levels of bioactivation of this cytotoxic drug) have also been assessed. Associations between *CYP2C19* and/or *CYP2B6*, as well as other pharmacogenes such as *ALDH*, *GSTP1* and *CYP3A4*, and the severity of neutropenia or premature ovarian failure have been reported.<sup>40,65,67,70–75</sup>

## 4 | CONCLUSION

It appears from pharmacokinetic studies that both *CYP2C19* and *CYP2B6* influence the bioactivation of cyclophosphamide to 4-OHCP. Inherited variation in both of these genes also appear to influence therapeutic outcomes. However, many studies do not comprehensively investigate both of these pharmacogenes and this has led to inconsistencies in the quality of the data. Approximately 25% of the reported studies have not assessed *CYP2C19* loss of function variants and in the light of the recent large studies,<sup>48,51,62</sup> which have

demonstrated significant associations with the *CYP2C19\*2* null function variant, future studies should include assessment of this locus. Consideration of individuals who are compound heterozygous for null function alleles (*\*2/\*3*) should also be incorporated in these studies, particularly in non-European populations. This appears to have been overlooked<sup>48,51</sup> despite the high prevalence of the *\*3* allele in these populations.

Comprehensive assessment of the multiple SNP variants in *CYP2B6* is also variable across the 28 studies reviewed. *CYP2B6* is a more challenging genomic region than *CYP2C19*. *CYP2B6* is a highly polymorphic locus and the correct assignment of allele (*\*star number*) is important, since there appear to be substantial substrate-dependent differences in the *in vitro* activity of each variant and haplotype combination (reviewed in<sup>29</sup>). The 516G>T variant is in strong linkage disequilibrium (>0.8) with the 785A>G variant, hence the *\*6* haplotype is common. However, it is important to note that both of these variants can also be found in isolation (*\*4* or *\*9* allele). Most studies in this review have not directly assessed the chromosome location (maternal or paternal) of the SNP variant (i.e. diplotype). Thus, for example, individuals who are carriers of both 516G>T and 785A>G variants could be either heterozygous for the *\*6* allele (diplotype: GA/TG), or compound heterozygous (*\*4/\*9*) with a diplotype of GG/TA. Statistical methods for imputation of the most likely haplotype (e.g. PHASE algorithm<sup>76</sup>) could be incorporated in future studies; however, this approach may still incorrectly assign a proportion of the diplotype calls.

*CYP2B6* is located adjacent to a highly homologous pseudogene (*CYP2B7P1*) and duplication and deletion fusion alleles between *CYP2B6-2B7P1* (*\*29* and *\*30*) also influence cyclophosphamide bioactivation.<sup>48</sup> The complexity of this genomic region and the presence of the *CYP2B6* SNP (e.g. 785A>G) in the pseudogene can result in poor data quality when using next generation sequencing or genome-wide SNP arrays. This may affect the routine reporting of this region in this genomic methods and could explain why despite the substantial signal from candidate gene studies (Table 2 and 3) the 2 genome-wide association studies reviewed<sup>64,69</sup> did not detect the *CYP2B6* locus as important for treatment outcomes. Long read amplification of this region is required to accurately call haplotype in heterozygous carriers of common coding region SNP of *CYP2B6*. Newer techniques, such as high-resolution melt analysis, multiplex-ligation-probe amplification and nanopore sequencing,<sup>77–79</sup> will probably be required to appropriately genotype patients for *CYP2B6* variants and fully assess the role of this pharmacogene in cyclophosphamide outcomes.

Finally, autoinduction of cyclophosphamide bioactivation, following high dose or continuous dosing regimens, is likely to have an important influence on the pharmacogenetic relationships with cyclophosphamide bioactivation. Notably, some studies<sup>46,50</sup> have found associations with SNPs in the promoter region of *CYP2B6*. These SNPs are thought to alter the binding of HNF1 and HNF4 transcription factors to the *CYP2B6* promoter<sup>80</sup> and could influence an individual's susceptibility to autoinduction. Assessment of the relationships between *CYP2B6* promoter variants and cyclophosphamide bioactivation

pharmacokinetics at the initial, as well as subsequent doses, could clarify the importance of these regulatory SNPs.

Future studies to confirm the role of the *CYP2B6* and *CYP2C19* pharmacogenes should include investigation of epistatic gene–gene interactions using appropriate statistical analysis such as multifactor dimensionality reduction or reporting individual patients combined genotype at the 2 gene loci. This multivariate approach has already shown some utility in population sizes between 189 and 567 patients.<sup>50,51</sup> Remarkably, considering the important role in the inactivation of 4-OHCP, few studies have included the assessment of *ALDH1A1* on clinical response to cyclophosphamide therapy. This detoxification pathway is likely to influence the amount of circulating 4-OHCP-aldophosphamide available for uptake into cells and thereby influence the number of DNA ICL formed within cells, and thus patient outcomes. This candidate gene should be included in future studies of cyclophosphamide pharmacogenetics. Using either a comprehensive candidate gene approach or by suitable genome-wide assessment, understanding the inherited factors that influence response to this important drug may allow appropriate treatment stratification based on an individual patient's combined genotype at these loci.

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## COMPETING INTERESTS

The authors have no conflicts of interest to declare.

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