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CONSENSUS ARTICLE

Genetic risk factors for clozapine-induced neutropenia and agranulocytosis in a Dutch psychiatric population

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Prescription of clozapine is complicated by the occurrence of clozapine-induced reduction of neutrophils. The aim of this study was to identify genetic risk factors in a population of 310 Dutch patients treated with clozapine, including 38 patients developing neutropenia and 31 patients developing agranulocytosis. *NQO2 1541AA* (NRH quinone oxidoreductase 2; protects cells against oxidative metabolites) was present at a higher frequency in agranulocytosis patients compared with control (23% versus 7%, $P = 0.03$), as was *ABCB1* (ABC-transporter-B1; drug efflux transporter) *3435TT* (32% versus 20%, $P = 0.05$). In patients developing neutropenia, *ABCB1 3435TT* and homozygosity for $GSTT1^{null}$ (glutathione-S-transferase; conjugates reactive clozapine metabolites into glutathione) were more frequent compared with control (34% versus 20%, $P = 0.05$ and 31% versus 14%, $P = 0.03$), whereas $GSTM1^{null}$ was less frequent in these patients (31% versus 52%, $P = 0.03$). To investigate whether combinations of the identified genetic risk factors have a higher predictive value, should be confirmed in a larger case–control study.

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INTRODUCTION

Clozapine is an atypical antipsychotic drug and is used with high efficacy in the treatment of refractory schizophrenia, defined as an insufficient treatment result after two consecutive antipsychotic trials.[1,2] However, the use of clozapine is limited due to the risk of developing agranulocytosis, a potentially lethal drop in number of neutrophils, which occurs in about 0.4–0.8% of patients.[3] Because clozapine-induced agranulocytosis (CIA) has a fatality rate of 2.2–4.2%, even while patients are monitored,[3] its prescription is subject to strict regulations and requires regular control of white blood cells. The risk for CIA is highest within the first 18 weeks, although cases have been described in which CIA occurred years later.[4,5] Physicians prescribing clozapine are confronted with several dilemmas. Should patients who oppose blood drawing be started on clozapine? Should patients with a decrease in neutrophils be continued on clozapine? How to proceed when a patient is prescribed clozapine and is having a fever? A better assessment of the individual risk could aid in decision making in clozapine treatment regimen and blood monitoring protocols. As twin studies have indicated a genetic component, the individual risk might be predicted by genetic polymorphisms.[6,7] Several association studies have been performed in order to identify the genetic factors determining the susceptibility for CIA and neutropenia. Roge *et al.*[8] suggested that the mechanism behind CIA and clozapine-induced neutropenia may be different.

The first associations between genetic markers and CIA were found for alleles of human leukocyte antigens (*HLAs*), implicating a role for the immune system in the pathogenesis of agranulocytosis.[9] However, the initial studies on the association of CIA with *HLA* genes often concerned small numbers (~100 patients in total), extended *HLA* haplotypes and depended on ethnicity (reviewed in Chowdhury *et al.*[10]). The generally complex associations with *HLA* genes were suggested to originate from linkage disequilibrium with other polymorphisms located in nearby genes.[11] Two candidate genes, located close to genes of the major histocompatibility complex are *TNF-α* (tumor necrosis factor alpha) and *Hsp70-2* (heat shock protein 70-2). Clozapine treatment is accompanied by an increase in TNF-α.[12,13] Moreover, *TNF* alleles are in linkage disequilibrium with *HLA* haplotypes and one study showed an association between *TNF* microsatellites and CIA.[14,15] Corzo *et al.*[11,16] observed a linkage disequilibrium between the *Hsp70-2* 8.5 kb/9 kb *PstI* polymorphic site and CIA-associated *HLA* haplotypes. Consequently, an association between this polymorphic site and CIA was observed.[11]

CIA is generally considered to be mediated by the oxidative metabolism of clozapine in neutrophils by the combination of NAD(P)H-oxidase and myeloperoxidase (MPO).[17,18,] The reactive nitrenium ions and/or radicals formed have been shown to cause increased levels of oxidative stress and induction of apoptotic pathways in neutrophils of patients with CIA.[19] Both MPO and the NAD(P)H-oxidase subunit CYBA (cytochrome b-245 light chain) are genetically determined. The *CYBA 640A>G* mutation is associated with lower NAD(P)H-oxidase activity, and the *-463G>A* mutation in the promotor site of *MPO* leads to lower MPO expression. A lower frequency would be expected in CIA patients, because these mutations will result in lower clozapine bioactivation. However, previous studies did not show statistically significant differences, possibly due to a small sample size (Table 5).[20,21]

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Next to genetic polymorphisms at the level of clozapine-bioactivating enzymes, also polymorphisms of protective enzymes involved in inactivation of reactive clozapine metabolites potentially determine susceptibility to CIA. Quinone oxidoreductases *NQO1* and *NQO2*, which protect cells against oxidative metabolites, such as quinones and quinoneimines, are known to be strongly genetically determined.[22,23] Their role in inactivation of reactive clozapine metabolites has not yet been demonstrated experimentally. A small association study on the role of *NQO2* polymorphisms in CIA showed a higher frequency of the *NQO2 1541A* allele for CIA patients compared with control patients (Table 5).[24] The nucleotide substitution *1541G>A* in the first intron is believed to affect a binding site for the myeloid zinc finger protein MZF1,[24] which has a general role in the regulation of hematopoietic gene expression.[25] This suggests that the resulting lower expression of NQO2 might increase the risk for CIA. The role of polymorphism *NQO1 C609T* in CIA, which results in very low expression levels of NQO1,[26] has not yet been studied.

Several glutathione S-transferases (GSTs) have been shown to catalyze the conjugation of reactive clozapine metabolites into glutathione.[27] In humans, marked interindividual differences exist in the expression of class Alpha (GSTA), Mu (GSTM), Pi (GSTP) and Theta (GSTT) GSTs,[28] due to genetic polymorphisms. In case of GSTM1 and GSTT1, part of the population completely lack the enzyme as a consequence of genetic deletions ('null genotypes'). Several studies have shown association of these GST-null genotypes with increased sensitivity to antitumor compounds[29] and an increased susceptibility to adverse drug reactions.[30] The presence of GSTA1 C-69*T*, one of the four linked base substitutions present in the GSTA1*B variant, results in a low hepatic expression in homozygous patients.[31] Dependent of the substrate, the GSTP1 A313G mutation can result in altered catalytic efficiency, due to changes in the active site[29] and was shown to be associated with a better response to platinum-based chemotherapy.[32,33] Generally speaking, reduction in the ability to detoxify electrophilic reactive metabolites by GSTs may result in a higher risk for toxicity due to a higher exposure to the reactive metabolites.

Clozapine serum levels are influenced by polymorphisms in the P-glycoprotein (P-gp) transporter gene *ABCB1* (*MDR1*).[34,35] However, no direct relationship has been observed between clozapine serum levels and leukocyte counts.[36] Interestingly, one case report described a CIA patient with a normal clozapine serum level, but an abnormally high clozapine level in neutrophils.[37] Furthermore, one case report describes twin brothers who developed CIA and were heterozygous for both *ABCB1 C3435T* and *G2677T/A*.[6] These data suggest a link between *ABCB1* polymorphisms and the risk for CIA, possibly by affecting clozapine levels in neutrophils, although further research is warranted.

The aim of this study was to investigate the above mentioned genetic risk factors for clozapine-induced neutropenia and agranulocytosis in a large Dutch psychiatric population prescribed with clozapine. Thirteen candidate gene polymorphisms were selected for investigation. The choice of polymorphism was based on reported association with CIA in other populations (*CYBA A640G, HLA-DQB1 G6672C, Hsp70-2 G1276A, MPO G-463A (rs2333227)* and *NQO2 G1541A*), putative role in CIA based on similarity with reported CIA-associated polymorphisms (*NQO1 C609T (rs1800566)* and *TNF G-308A(rs1800629)*) and putative role in clozapine transport (*ABCB1 G2677T/A (rs2032582)* and *ABCB1 C3435T (rs1045642)*) and elimination (*GSTA1 C-69T*, *GSTP1 A313G*, and *GSTT1* and *GSTM1* gene deletion).

MATERIALS AND METHODS

Patients and setting

This retrospective study was conducted in the Dutch Psychiatric Hospital GGz Centraal location Meerkanten and in the Mental Health Services Rivierduinen. According to national guidelines. Measurement of the number of leukocytes was performed just before the start of clozapine treatment, once a week in the first 18 weeks and once a month until the end of treatment.[38] Patient selection occurred based on the criteria for CIA and neutropenia as described by the Food and Drug Administration.[38,39] CIA patients were selected based on at least one neutrophil count $\leqslant 500\ \mu l^{-1}$. Neutropenia patients were selected on at least one neutrophil count between 500 μl and $1500\ \mu l^{-1}$ or two neutrophil counts $< 2000\ \mu l^{-1}$ during clozapine treatment. Patients were excluded when a neutrophil count $< 2000\ \mu l^{-1}$ was recorded before the start of clozapine treatment.

Control patients were selected for prescription of clozapine for at least 1 year, no record of either neutrophil counts $< 2000\ \mu l^{-1}$ or leukocyte counts $< 4000\ \mu l^{-1}$. In all groups patients aged under 18 were excluded, as were patients for whom DNA samples could not be obtained. For patients of GGz Centraal, genotyping of *CYP2D6* and *CYP2C19* polymorphisms was performed routinely upon admission to the clinic and anonymized DNA samples were stored at −70 °C for research purposes and therefore for this study. For patients of Mental Health Services Rivierduinen, DNA samples are not routinely collected, therefore DNA was obtained after selection of the patients.

The condition of at least 1 year of clozapine use was justified by the fact that 80% of the total incidence of CIA occurs in the first 18 weeks of use,[38,40] which is why in Europe patients are weekly monitored for blood cell counts during these first months. Less frequent cases of agranulocytosis were reported to occur during the first year,[41,42] after which only incidental cases of CIA have been reported after up to 19 years of clozapine use.[5]

The Medical Research Ethics Committee in Amsterdam assessed the project and stated that it was not subject to further investigation under the Medical Research (Human Subjects) Act. The study has been approved by the research board (Innova) of Psychiatric Hospital GGz Centraal. Patients were not subjected to any procedures besides routine clinical practice and were informed on the use of data for research purposes. Data were excluded from research upon objection.

Genotyping methods

Thirteen polymorphisms were selected for analysis: *ABCB1 G2677T/A (rs2032582), ABCB1 C3435T (rs1045642), CYBA A640G, HLA-DQB1 G6672C, Hsp70-2 G1276A, MPO G-463A (rs2333227), NQO1 C609T* (also known as *NQO1*2, rs1800566), NQO2 G1541A, TNF G-308A (rs1800629), GSTA1 C-69T, GSTP1 A313G, and GSTT1 and GSTM1* gene deletion. The *Hsp70-2 G1276A* corresponds to the polymorphic *PstI* site (8.5 and 9.0 kb allele) investigated in previous studies.[43] Primer sequences, PCR extension and elongation conditions, restriction enzymes and length of obtained DNA fragments are shown in Table 1.

With exception of *HLA-DQB1* and the GSTs, determinations were performed using allele-specific digestion of PCR products. Primer sequences and PCR extension and elongation conditions are shown in Table 1. *HLA-DQB1* determination was performed using allele-specific reverse primers in combination with a common forward primer. For all polymorphisms except the GSTs, PCR reaction mixes contained 2–4 ng μl^{-1} DNA, 0.4 μM of each primer, 0.2 mM dNTPs, 0.04–0.1 U μl^{-1} Thermoprime Plus DNA polymerase (Abgene House, Epsom, UK) and 1.0x ReddyMix PCR buffer. For *NQO2 G1541A*, a semi-nested PCR was performed using primers HL1541F+HL1541R on the PCR product obtained with primers HL1541F+HL1541R1. PCR products were digested and analyzed by gel electrophoresis. For *MPO G-463A* and *ABCB1 G2677T/A*, gel electrophoresis was performed after addition of SDS (1% final concentration) to the digestion products.

The presence of at least one *GSTM1* and/or *GSTT1* allele was determined as described by Arand *et al*.[44] Briefly, 10 ng of DNA

Table 1. PCR primers and conditions and digestion patterns

		Primer sequences (5′–3′)	*PCR cycling conditions*	*Restriction enzyme*	*DNA fragment length (bp)*
ABCB1 G2677T/A rs2032582	Forward	TGCAATAGCAGGAGTTGT[a]	5′95 °C, (30″95 °C, 30″58 °C, 30″72 °C) × 41, 5′72 °C	BseYI	G: 275+76T/A: 351
	Reverse	AAAGTGGGGAGGAAGGAAGA[a]			
ABCB1 C3435T rs1045642	Forward	TGTTTTCAGCTGCTTGATGG[b]	5′95 °C, (30″95 °C, 30″60 °C, 30″72 °C) × 41, 5′72 °C	BfuCI	T: 197
	Reverse	AAGGCATGTATGTTGGCCTC[b]			C:160+37
CYBA A640G	Forward	AGCAGTGGACGCCCATCGAGCCCAA[c]	5′95 °C, (30″95 °C, 30″67 °C, 30″72 °C) × 41, 5′72 °C	DraIII	G: 258A: 229+29
	Reverse	CGCTGCGTTTATTGCAGGTGGGTGC[c]			
HLA-DQB1 G6672C	Forward	TTGGAGGCTTCGTGCTGG	5′95 °C, (30″95 °C, 30″60 °C, 2′72 °C) × 31, 5′72 °C	NA	*WT primer* G: 1123 C: no product
	Reverse WT	TGGGCCTGCCATCTCCCCC			*MUT primer* G: no product C: 1123
	Reverse MUT	TGGGCCTGCCATCTCCCCG			
Hsp70 G1276A	Forward	TCCGAAGGACTGAGCTCTTG[d]	5′95 °C, (30″95 °C, 1′60 °C, 5′72 °C) × 41, 10′72 °C	PstI	A: 2076
	Reverse	CAGCAAAGTCCTTGAGTCCC[d]			G: 1137+939
MPO G-463A Rs 2333227	Forward	CGGTATAGGCACACAATGGTGAG[e]	5′95 °C, (30″95 °C, 30″56 °C, 30″72 °C) × 41, 3′72 °C	AciI	A: 355+61
	Reverse	GCAATGGTTCAAGCGATTCTTC[e]			G: 171+123+61
NQO1 C609T Rs 1800566	Forward	AAGCCCAGACCAACTTCT[f]	5′95 °C, (30″95 °C, 30″56 °C, 30″72 °C) × 41, 3′72 °C	HinfI	C: 172T: 131+41
	Reverse	ATTTGAATTCGGGCGTCTGCTG[f]			
NQO2 G1541A	Forward	CGGGCTGCTTAGGTTGGCAC[g]	5′95 °C, (30″95 °C, 30″59 °C, 30″72 °C) × 41, 5′72 °C	BstEII	G: 204+20T: 224
	Reverse R1 Reverse R	CAGTCCGGGAACTGTCCTTC[g] CCTTCCAAGTCCCCCGGTCAC[g]			
TNF G-308A Rs 1800629	Forward	AGGCAATAGGTTTTGAGGGCCAT[h]	5′95 °C, (30″95 °C, 1′60 °C, 1′72 °C) × 41, 5′72 °C	NcoI	G: 85+22A: 107
	Reverse	TCCTCCCTGCTCCGATTCCG[h]			
GSTA1 C-69T	Forward reverse Sequence primer	AGTAGGTGGCCCCTTGGC TGTCACCGTCCTGGCTCGAC (biotin) GGCTTTTCCCTAACTTGAC			
GSTT1 del	Forward	TTCCTTACTGGTCCTCACATCTC	15′95 °C, (30″95 °C, 30″55′ °C, 30″72 °C) × 35, 10′72 °C		480 bp
	Reverse	TCACCGGATCATGGCCAGCA			
GSTM1 del	Forward	GAACTCCCTGAAAAGCTAAAGC	15′95 °C, (30″95 °C, 30″55′ °C, 30″72 °C) × 35, 10′72 °C		215 bp
	Reverse	GTTGGGCTCAAATATACGGTGG			
Albumin	Forward	GCCCTCTGCTAACAAGTCCTAC	15′95 °C, (30″95 °C, 30″55′ °C, 30″72 °C) × 35, 10′72 °C		350 bp
	Reverse	GCCCTAAAAAGAAAATCGCCAATC			

Allelic discrimination assays were adaptations from assays described by: [a]Hüebner *et al.*[45] [b]Cascorbi *et al.*[64] [c]Inoue *et al.*[65] [d]Milner *et al.*[66] [e]London *et al.*[67] [f]Zhang *et al.*[68] [g]Ostrousky *et al.*[24] [h]Wilson *et al.*[69]

was taken to amplify representative sequences of the genes of *GSTT1*, *GSTM1* and albumin (as reference gene). Hotstart PCR mastermix was used from Qiagen (Venlo, The Netherlands). The PCR products of the *GSTT1* and *GSTM1* were detected by agarose gel electrophoresis. The rs1695 genotype of *GSTP1-1* (mutation *A313G*; Ile105Val) was determined by allele-specific PCR using a predesigned Taqman assay (C___3237198_20) from Life Technologies (Nieuwerkerk a/d IJssel, The Netherlands) and analyzed on the 7500 real time PCR system (Life Technologies). The presence of *GSTA1 C-69T* was determined by pyrosequencing on a Pyrosequencer 96MA (Qiagen). Sequence to analyze was C/TCTTCTTTCA and dispensation order was GTCGTCTCA.

Table 2a. Patient information

	Population (N)	*Male/female*	*Age at lowest ANC*	*Clozapine dose*	*Plasma level*	*Lifetime duration clozapine treatment (months)*
Agranulocytose	31	20 (65%)/11 (35%)	46.2 ± 14.8	331 ± 170	354 ± 344	57.9 ± 62.5
Neutropenia	38	21 (55%)/17 (45%)	44.2 ± 16.9	240 ± 139	240 ± 146	40.0 ± 46
Control	241	165 (68%)/76 (32%)	43.9 ± 19.1	345 ± 187	253 ± 105	67.8 ± 49
Total	310	206 (66%)/104 (34%)	44.2 ± 17.0	332 ± 184	264 ± 157	63.8 ± 50.4

Table 2b. Co-medication

	Number	*Valproic acid use*		*Carbomazepine use*		*Olanzapine use*	
		Number	*Average dose (mg)*	*Number*	*Average dose (mg)*	*Number*	*Average dose (mg)*
Agranulocytosis	31	4 (13%)	1438 ± 597	1 (3%)	600	0	NA
Neutropenia	38	3 (8%)	900 ± 361	3 (8%)	433 ± 153	1 (3%)	15
Control	241	24 (10%)	1283 ± 457	2 (1%)	400 ± 0	5 (2%)	12 ± 12
Total	310	31 (10%)	1266 ± 471	6 (2%)	450 ± 122	6 (2%)	13 ± 7

Abbreviation: NA, not applicable.

For *ABCB1 G2677T/A*, no distinction could be made between *2677T* and *2677A;* for simplicity the allele was classified as the more common *2677T* in this manuscript.[45] Assays were validated by sequencing of one homozygous wild type, one homozygous mutant and one heterozygous sample (BaseClear, Leiden, The Netherlands). For *HLA-DQB1*, no homozygous *6672CC* sample was available for sequencing.

Statistics

We calculated the necessary sample size (25 cases) based on a desired increased allele frequency in cases by 2.5-fold in case of a frequency of 0.2 and a 1.6-fold increase of a frequency of 0.5, assuming a 1:10 sample ratio of cases versus controls and a significance level of $\alpha = 0.05$ and 80% power ($\beta = 0.2$). Statistical analyses were performed using SPSS for Windows (SPSS version 15.0, Chicago, IL, USA). Cases were grouped according to neutrophil counts (see above). Subgroups were based on genotype; for all investigated polymorphisms differences in frequency of the three possible genotypes (homozygous for one or the other allele or heterozygous for both alleles) were analyzed, comparing frequencies in neutropenia and CIA patients with control patients and with each other. Statistical analyses for frequencies of genotypes were performed using Fisher exact probability test, showing two-tailed probability values. The strength of the associations between the polymorphism with neutropenia and CIA were expressed as odds ratios (ORs) with a 95% confidence interval (CI).

RESULTS

A total of 310 patients treated with clozapine were included in this study; 31 patients exhibited CIA, 38 patients had neutropenia and 241 patients served as controls. Additional patient and clozapine use information and co-medication are presented in Tables 2a and 2b.

When comparing CIA patients with controls, as presented in Table 3, of the polymorphisms previously reported to be associated with CIA (*CYBA 640, HLA-DQB1 G6672C, Hsp70-2 G1267A, NQO2 1541G and MPO G-463A)* only *NQO2 1541G* had significantly different allele and genotype frequencies. Absolute numbers of different genotypes in the three different groups (CIA, neutropenia and controls) are presented in Supplementary 1. The *NQO2 1541AA* genotype, which is expected to result in low NQO2 expression, was present at a higher frequency in the case population (23%) than in the control population (7%; $P = 0.004$; Table 3) and the *NQO2 1541GG* wild-type genotype was present in 53% of the control patients compared with only 29% in CIA patients. The odds ratio for developing CIA was 5.5 times higher for the homozygous mutant *1541AA* genotype (Table 4).

A genetic test based on detection of *NQO2 1541AA* would have a sensitivity of 23% and a specificity of 97%, with a negative predictive value of 90%. For the other polymorphisms which were not yet studied in relation to CIA (*ABCB1 G2677T*, *ABCB1 C3435T*, *NQO1 C609T*, *TNFα G-308A, GSTA1 -69T, GSTP1 313G, GSTT1*^{null}, and *GSTM1*^{null}), the *ABCB1 3435TT* genotype was more frequent in CIA patients (32 versus 20% in controls; $P = 0.05$; Table 3) and the wild-type *ABCB1 3435CC* genotype was more frequent in controls (31% versus 16% in CIA patients). Homozygous mutant (*3435TT*) patients had a threefold higher chance on developing CIA (Table 3). In contrast, we observed a trend towards less CIA patients having the *ABCB1 2677 TT* genotype (10%) compared with controls (20%, $P = 0.07$). On the basis of these data, testing for *ABCB1 3435TT* would have a sensitivity of 32% and a specificity of 80%, with a negative predictive value of 90%.

When comparing neutropenia patients with controls, again the risk of having neutropenia was about threefold higher in patients with the *ABCB1 3435TT* genotype; the *ABCB1 3435TT* genotype was more frequent in neutropenia patients (34% versus 20% in controls) and the wild-type *ABCB1 3435CC* genotype was more frequent in controls (31% versus 18% in neutropenia patients; $P = 0.05$; Table 3). The *GSTP1 313GG* appears to be more frequent in neutropenia patients (25% versus 10% in controls), with an odds ratio of 2.5, this is not statistically significant due to the small sample size. *GSTT1* deficiency was more frequently observed in neutropenia patients (31% versus 14% in control patients; $P = 0.03$; Table 3), with an odds ratio of 2.64 (Table 4). In contrast, 33% of neutropenia patients lacked the gene of GSTM1, compared to 53% in the control group. This difference does not appear to be statistically significant (OR = 0.45).

DISCUSSION

Agranulocytosis is a very serious, life-threatening adverse effect of clozapine occurring in 0.4–0.8% of the patients. Although the

Table 3. Allele and genotype frequencies for CIA, neutropenia and control patients

	Allele frequency			*Homozygote frequency*[a]			P-*value*[a]	
	Control	*Neutropenia*	*CIA*	*Control*	*Neutropenia*	*CIA*	*Neutropenia*	*CIA*
ABCB1 2677T	0.45	0.46	0.31	0.20	0.24	0.10	0.82	0.07
ABCB1 3435T	0.44	0.58	0.58	0.20	0.34	0.32	**0.05**	**0.05**
CYBA 640A	0.51	0.42	0.53	0.26	0.16	0.29	0.14	1.00
HLA-DQB1 6672G	0.96	0.96	0.95	0.93	0.93	0.90	1.00	0.70
Hsp70-2 1267G	0.44	0.47	0.52	0.18	0.21	0.29	0.61	0.29
NQO1 609T	0.19	0.26	0.11	0.03	0.03	0.00	1.00	0.60
NQO2 1541A	0.27	0.20	0.47	0.07	0.03	0.23	0.32	**0.004**[b]
MPO -463A	0.23	0.33	0.23	0.07	0.11	0.16	0.26	0.37
TNF -308A	0.14	0.16	0.19	0.02	0.03	0.00	0.52	1.00
GSTA1 -69T	0.41	0.33	0.39	0.16	0.11	0.16	0.31	1.00
GSTP1 313G	0.37	0.44	0.40	0.10	0.25	0.10	0.10	0.73
GSTT1^{null}	0.14	0.31	0.13	0.14	0.31	0.13	**0.03**	1.00
GSTM1^{null}	0.53	0.33	0.48	0.53	0.33	0.48	**0.05**	0.71

Abbreviation: CIA, clozapine-induced agranulocytosis. [a]Regarding homozygous presence of indicated allele. [b]Significant difference between neutropenia and CIA patients ($P = 0.01$).

Table 4. Odds ratios and 95% confidence intervals for CIA, neutropenia and control patients

	Control versus neutropenia		*Control versus CIA*		*Control versus neutropenia+CIA*	
	OR[a]	*95% CI*	*OR*[a]	*95% CI*	*OR*[a]	*95% CI*
ABCB1 2677T	1.12	0.44–2.85	0.30	0.08–1.08	0.66	0.31–1.43
ABCB1 3435T	**2.90**	1.08–7.79	**3.13**	1.01–9.70	**2.99**	1.36–6.57
CYBA 640A	0.44	0.16–1.26	1.14	0.40–3.27	0.70	0.33–1.51
HLA-DQB1 6672G	1.03	0.19–5.66	0.71	0.16–3.20	0.84	0.23–3.06
Hsp70-2 1267G	1.32	0.48–3.60	1.86	0.67–5.18	1.56	0.73–3.35
NQO1 609T	1.87	0.94–3.72	0.55	0.23–1.32	1.13	0.65–1.97
NQO2 1541A	0.30	0.04–2.33	**5.53**	1.83–16.68	1.72	0.69–4.31
MPO –463A[b]	1.85	0.93–3.69	1.24	0.58–2.62	1.54	0.90–2.64
TNF –308A[b]	0.87	0.41–1.85	0.56	0.26–1.22	0.71	0.24–1.77
GSTA1 –69T	0.53	0.16–1.68	0.88	0.29–2.66	0.66	0.29–1.57
GSTP1 313G	2.48	0.95–6.50	1.19	0.30–4.76	1.95	0.85–4.51
GSTT1^{null}	**2.64**	1.19–5.86	0.89	0.29–2.70	1.73	0.88–3.41
GSTM1^{null}	**0.45**	0.22–0.95	0.85	0.40–1.79	0.61	0.35–1.06

Abbreviations: CI, confidence interval; CIA, clozapine-induced agranulocytosis; OR, odds ratio. [a]Regarding homozygous presence of indicated allele versus homozygous presence of other allele. [b]Regarding homo- or heterozygous presence of indicated allele. Bold values indicate significant result $P > 0.05$.

exact mechanism remains to be established, formation of reactive metabolites such as nitrenium ions are considered to have a role in development of CIA.[46,47] In addition, CIA is thought to be immune mediated.[48] Therefore, variability in activity of the enzymes involved in bioactivation and inactivation as well as enzymes involved in immune reactions might be important factors determining interindividual susceptibility for these adverse drug reactions. In our study we tested whether polymorphisms in genes encoding such enzymes may serve as genetic predictors for CIA or neutropenia.

In the present study, a higher frequency of the *NQO2 1541A* allele was found for CIA patients, compared with control patients. Herewith, we confirm the findings of an Israeli study, with 98 clozapine users and 18 CIA patients (Table 5, ref. 24). In contrast with Ostrousky's study, where all CIA patients were heterozygous for *NQO2 1541G>A*, also patients that were homozygous wild type or mutant were represented in the patients with CIA. Still, the allele frequencies in control and CIA patients are comparable, as are the numbers of 1541A carriers in either group (47 and 51% in controls and 71 and 100% in CIA patients, this study versus Ostrousky *et al*.[24]). The *NQO2 G1541A* mutation results in disruption of the MZF1 binding site.[24] MZF1 is specifically expressed in myeloid cells and essential for granulopoiesis.[25] *NQO2* mRNA expression was found to be lower in neutrophils of CIA patients than in control patients.[24] Functional studies on the detoxification of clozapine metabolites by NQO2, however, are warranted to support the results.

Previously, the importance of NQO1 in neutrophiles was demonstrated using NQO1$^{-/-}$ mice, which were shown to be resistant to mitomycin-C-induced neutropenia.[49] In humans, mutation *NQO1 C609T* is associated with reduced NQO1 activity. An association study in benzene-exposed adult Chinese workers showed *NQO1 C609T* is associated with greater risk of neutropenia, indicative for a protective role of NQO1 in human neutrophils.[50] However, in the present study no association was found between *NQO1 C609T* and CIA, indicating that the enzyme function *per se* is not indicative of the development of CIA. The different substrate selectivities of NQO1 and NQO2 might explain their different contribution in protection against benzene and clozapine neutropenia.

A role for P-gp, a cell membrane-bound efflux pump encoded for by the ABCB1 gene, in clozapine transport across blood cell membranes has not yet been established. In the current study, the homozygous presence of *ABCB1 3435TT* seems to be a risk factor

Table 5. Results from other studies control versus CIA

	Allele frequency		*Homozygote frequency*[a]					
	Control	*CIA*	*Control*	*CIA*	P-*value*[a]	*OR*[b]	*95% CI*	*Cases/controls*
CYBA 640A[19]	0.40	0.41	0.19	0.31	0.09	2.26	0.96–5.35	78/75
HLA-DQB1 6672G[58]	0.99	0.89	0.98	0.78	< 0.001	0.06	0.01–0.26	79/125
Hsp70-2 1267G[15]	0.59	0.78	0.35	0.56	0.01	2.40	0.9–6.14	32/43
NQO2 1541A[23]	0.28	0.50	0.05	0.00	< 0.001	ND	ND	18/80
MPO -463A[b,19]	0.20	0.22	0.03	0.10	0.11	1.12	0.59–2.14	81/78
MPO -463A[b,20]	0.19	0.23	0.01	0.06	0.21	0.90	0.38–2.14	31/77

Abbreviations: CI, confidence interval; CIA, clozapine-induced agranulocytosis; ND, not determined; OR, odds ratio. [a]Regarding homozygous presence of indicated allele. [b]Regarding homo- or heterozygous presence of indicated allele.

for clozapine-induced decrease of neutrophils. The low frequency of the *ABCB1 3435CC* genotype in CIA patients suggests a protective effect of *ABCB1 3435CC* against lethal neutrophil diseases. Henning *et al.*[51] showed that inhibitors of P-gp did not alter intracellular accumulation of clozapine in HL-60 cells.[51] However, the level of expression and activity of P-gp does depend on the type of blood cell.[52] Also literature on *ABCB1 G2677A/T*, resulting in the amino-acid substitution A893S/T, does not provide a definite conclusion on the effect of this polymorphism on expression or function of the transporter *in vivo*. Depending on patient groups or cell type, the *ABCB1 2677G* allele was associated with increased, decreased or similar transcription and/or expression levels.[53–57] Both the *ABCB1 2677GG* genotype and the *ABCB1 3435CC* were associated with reduced serum levels of clozapine, *ABCB1 3435CC* showing the most prominent effects.[34,35] Clozapine concentrations in neutrophils could well be increased for patients homozygous for *ABCB1 2677GG* or *3435CC*, despite lower serum levels. A case report describing a normal clozapine serum level, but abnormally high clozapine levels in neutrophils in a CIA patient does indicate a role for clozapine transporters in CIA.[37] In literature no direct relationship has been observed between clozapine serum levels and leukocyte counts, most likely because cellular uptake, bioactivation and inactivation are important factors.

ABCB1 G2677T and *C3435T* are reported to be genetically linked,[58] as was the case in our study (data not shown). However, although we find comparable allele frequencies in control patients, the *3435T* allele was more frequent in CIA, whereas the *2677T* frequency was lower. In a previous study, mutation ABCB1 C3435T, but not G2677T, was found to be related to neutropenia induced by the antitumor agent amrubicin in lung cancer patients.[59] These results and our results suggest that the mutation C3435T might have a more prominent effect on ABCB1 activity than G2677T.

For *CYBA 640* and *MPO-463* our results do not notably differ from those obtained in another study (Table 5).[20] However, for *Hsp70-2 1267* we could not reproduce the results obtained by Corzo *et al.*[16] (Table 5): although we did find an OR that approximates the reported one, our findings do not reach statistical significance. The small overall sample size of the former study and the limited number of cases in both studies are likely the cause of this discrepancy.

GSTM1, GSTA1 and GSTP1 were shown to be involved in glutathione conjugation of the nitrenium ion of clozapine, GSTP1 having the highest activity.[27] In the present study, GSTP1 polymorphism was not associated with either CIA or neutropenia, which can be explained by the fact that the mutation only has a minor effect on inactivation of the reactive nitrenium ion of clozapine.[60] Although GSTT1 appeared to be inactive in inactivation of this reactive metabolite,[27] an overrepresentation of *GSTT1*^{null} genotype in patients with clozapine-induced neutropenia was found in this study. Deficiency of GSTM1 was less frequent in patient with neutropenia, therefore seems a protective factor. Deficiency of GSTM1 was found previously to cause resistance against acetaminophen-induced hepatotoxicity in mice, by disrupting a cellular pathway involved in cytotoxicity.[61] Whether a similar mechanism might be applicable in neutrophiles remains to be established.

HLA-DQB1 6672C has been associated with CIA previously,[62] and even the pharmacogenetic test PGxPredict:CLOZAPINE (PGxHealth, New Haven, USA, discontinued in March 2011) was based on this polymorphism. Furthermore, in a recent GWAS study on 98 CIA cases, an amino-acid substitution in HLA-DQB1 that is in linkage disequilibrium with the *6672C* SNP was found to be associated with CIA.[63] The results of the present study did not confirm these associations, (Table 5) although the overall frequencies of both cases and controls combined were comparable (9%[15] versus 8% in our study), the previously reported frequency of the marker in cases versus control (22 versus 2%, Table 5))[15] differs notably from our findings (10 versus 7%).

It is conceivable that, for example, an aberrant transport of clozapine, resulting in increased intracellular levels, poses a much greater risk for CIA if combined with an aberrant reaction of neutrophils to stress. However, we could not find such combinations in our study since our sample size does not allow for finding combined genetic predictors. Of note, with a prevalence of 0.4–0.8% for CIA and 3% for neutropenia, it is challenging to include more cases. Further limitations of this study are the variations in dose, co-medication and treatment duration. Which are related to the retrospective structure of the study. Dose variations were previously shown not to influence the occurrence of CIA.[36] Treatment duration was used as a selection criterion for control patients. Co-medication was limited (Table 2b) and did not vary between groups.

In conclusion, this study describes 31 patients who developed clozapine-induced agranulocytosis and 38 patients who developed neutropenia in a group of 310 clozapine users. The most significant association with CIA was found with mutation NQO2 G1541A, making it one of the candidate markers for the prediction of CIA, with in this study a specificity of 97% and a sensitivity of 23%. Furthermore, an association was found between CIA and a P-gp transporter polymorphism (ABCB1 3435T) for the first time. So far the predictive value of individual genetic polymorphisms is not strong enough to predict CIA with high accuracy. Therefore most likely combinations of risk factors, such as increased cellular uptake, high bioactivation, low bioinactivation, and immunological factors are required to result in CIA. Much larger association studies, allowing the study of combinations of genetic markers and risk factors, will be required to identify the high-risk combinations for clozapine-induced agranulocytosis and neutropenia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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