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Phenoconversion of CYP2D6 by inhibitors modifies aripiprazole exposure

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1 **Abstract**

2 The efficacy of aripiprazole therapy and the risk of adverse reactions are influenced by substantial inter-
3 individual variability in aripiprazole metabolizing capacity. *In vitro* studies assigned the potential role in
4 aripiprazole metabolism to CYP2D6 and CYP3A enzymes; therefore, the association between the steady-state
5 aripiprazole plasma concentrations and patients' CYP2D6- and CYP3A-status (*CYP2D6*, *CYP3A4*, *CYP3A5*
6 genotypes and CYP3A4 expression) and/or co-medication with CYP-function modifying medications has been
7 investigated in 93 psychiatric patients on stable aripiprazole therapy. The patients' *CYP2D6* genotype had a
8 major effect on aripiprazole plasma concentrations, whereas the contribution of *CYP3A* genotypes and CYP3A4
9 expression to aripiprazole clearance was considered to be minor or negligible. The role of CYP3A4 expression in
10 aripiprazole metabolism did not predominate even in the patients with non-functional *CYP2D6* alleles.
11 Furthermore, dehydroaripiprazole exposure was also *CYP2D6* genotype dependent. Dehydroaripiprazole
12 concentrations were comparable with aripiprazole levels in patients with functional *CYP2D6* alleles, and 35% or
13 22% of aripiprazole concentrations in patients with one or two non-functional *CYP2D6* alleles, respectively. The
14 concomitant intake of CYP2D6 inhibitors, risperidone, metoprolol or propranolol was found to increase
15 aripiprazole concentrations in patients with at least one wild-type *CYP2D6**1 allele. Risperidone and 9-hydroxy-
16 risperidone inhibited both dehydrogenation and hydroxylation of aripiprazole, whereas metoprolol and
17 propranolol blocked merely the formation of the active dehydroaripiprazole metabolite, switching towards the
18 inactivation pathways. Patients' *CYP2D6* genotype and co-medication with CYP2D6 inhibitors can be
19 considered to be the major determinants of aripiprazole pharmacokinetics. Taking into account *CYP2D6*
20 genotype and co-medication with CYP2D6 inhibitors may improve outcomes of aripiprazole therapy.

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23 Keywords: aripiprazole therapy, CYP2D6 phenoconversion, risperidone, metoprolol, propranolol

24

1 **Introduction**

2 A patient's response to a drug is highly influenced by his/her drug-metabolizing capacity. Genetic
3 variations of drug-metabolizing enzymes can significantly modify the pharmacokinetics of a drug; thus, genetic
4 polymorphism is considered to account for one of the main sources of inter-individual differences in drug
5 metabolism and eventually in drug response (efficacy and/or safety) [1-3]. The non-functional or increased
6 function mutations in drug-metabolizing enzymes lead to poor or rapid metabolizer phenotypes, whereas the
7 wild-type allele is predicted to be translated to an enzyme with normal function. However, non-genetic factors,
8 such as age, sex, disease and medication, can alter the expression or the activity of drug-metabolizing enzymes;
9 therefore, homozygous wild-type genotype can be transiently switched into poor (or rapid) metabolism due to
10 phenoconversion [4-7]. Consequently, the heritable traits determine only the potential for the expression of
11 functional or non-functional enzyme, and non-genetic factors can give rise to altered phenotypes, resulting in
12 genotype-phenotype mismatch.

13 The atypical antipsychotic aripiprazole is approved by the European Medicines Agency for treatment of
14 schizophrenia as well as for treatment of moderate to severe manic episodes of bipolar I disorder [8-11].
15 Aripiprazole has unique receptor-binding properties acting as a partial agonist at dopaminergic D₂ and
16 serotonergic 5-HT_{1A} receptors, and as an antagonist at 5-HT_{2A} receptors [8, 9, 12]. It has advantages, such as
17 lower risk of extrapyramidal symptoms, hyperprolactinemia, bodyweight gain, diabetes mellitus and sedation;
18 however, the treatment discontinuation rate associated with aripiprazole inefficacy seems to be higher than that
19 of olanzapine or haloperidol [10, 13-15]. Almost complete dopamine receptor occupancy (>85%) has been
20 observed at the plasma concentration of 100-150 ng/ml, and the optimal serum concentrations that result in the
21 best improvement and no or mild side effects in patients, have been suggested to range between 150 and
22 300 ng/ml, whereas the risk of moderate to severe adverse effects increases at higher concentrations [16-19]. At
23 the same dose, considerable variability in aripiprazole concentration can occur which can be attributed to the
24 inter-individual differences in aripiprazole metabolism; therefore, therapeutic drug monitoring is recommended
25 [19, 20].

26 Aripiprazole is extensively metabolized in the liver, forming the pharmacologically active
27 dehydroaripiprazole as well as the inactive *N*-dealkyl-aripiprazole and two monohydroxy-metabolites [10, 21-
28 24]. The major metabolite dehydroaripiprazole displays similar pharmacological properties to the parent
29 compound, contributing to the antipsychotic effect of aripiprazole; therefore, the sum of the parent drug and its
30 dehydro-metabolite is often monitored [10, 17, 22, 25, 26]. CYP2D6 and CYP3A4 are primarily responsible for
31 aripiprazole metabolism, which can entail the potential for high inter-individual variability in aripiprazole
32 pharmacokinetics [17, 25, 27]. CYP2D6 is one of the most polymorphic cytochrome P450 enzymes with more

1 than 100 alleles identified (<https://www.pharmvar.org/htdocs/archive/cyp2d6.htm>). In Caucasian populations,
2 several non-functional and reduced function *CYP2D6* alleles as well as the whole *CYP2D6* gene deletion
3 (*CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, *CYP2D6*10*, *CYP2D6*41*) commonly occur, leading to
4 absent or non-functional protein or decreased expression. *CYP2D6* gene duplication/multiplication of functional
5 alleles, resulting in increased expression and *CYP2D6* activity, has also been identified [2]. Genetic
6 polymorphism of *CYP2D6* is one of the main sources of inter-individual differences in metabolism of some
7 *CYP2D6* substrates, such as tricyclic antidepressants (desipramine, imipramine, nor-triptyline), fluoxetine,
8 paroxetine, risperidone, venlafaxin, metoprolol [28, 29]; however, non-genetic factors like co-administration of a
9 drug that acts as a *CYP2D6* inhibitor can also influence *CYP2D6* activity. Concomitant use of selective
10 serotonin reuptake inhibitors (duloxetine, fluoxetine, paroxetine, sertraline) has been demonstrated to decrease
11 aripiprazole metabolizing activity [30]. Therefore, both genetic variability and co-medication with *CYP2D6*
12 inhibitors are recommended to take into account in aripiprazole dosing [18, 30, 31]. *CYP3A4* activity also
13 displays high inter-individual variability, which is partly attributed to genetic factors. *CYP3A4*1B* allele is
14 assumed to increase *CYP3A4* transcription; however, the clinical significance of *CYP3A4*1B* to *CYP3A4*
15 activity is doubtful [32, 33]. *CYP3A4*22* has been demonstrated to display low hepatic *CYP3A4* expression,
16 resulting in decreased *CYP3A4* activity [34]. It has been suggested that the association between *CYP3A4*22* and
17 pharmacokinetic behaviour of *CYP3A*-substrates could be evaluated in combination with *CYP3A5* genotype
18 [35]. *CYP3A5*3* allele results in splicing defect and non-functional *CYP3A5* enzyme. Those individuals who
19 have functional *CYP3A5* enzyme are presumed to metabolize some *CYP3A*-substrates more rapidly than
20 *CYP3A5* non-expressers. Although genetic polymorphisms of *CYP3A* can influence *CYP3A* activity, non-
21 genetic factors, especially co-medication resulting in transient poor or rapid metabolism, may have much higher
22 impact on aripiprazole pharmacokinetics. The potent *CYP3A4* inducer carbamazepine has been reported to
23 substantially decrease the plasma concentrations of both aripiprazole and its dehydro-metabolite, whereas
24 *CYP3A4* inhibitors (itraconazole, fluvoxamine) seem to increase aripiprazole exposure [23, 31, 36].

25 For the estimation of patients' aripiprazole-metabolizing capacity, *CYP2D6* genotype, *CYP3A*-status
26 (*CYP3A4/5* genotype and *CYP3A4* expression) and co-medication with *CYP2D6* and *CYP3A4* inhibitors or
27 with *CYP3A4* inducers should be considered. Although CYP genotypes can be simply identified by *CYP2D6*,
28 *CYP3A4* and *CYP3A5* genotyping, the crucial task is the assessment of hepatic *CYP3A4* activity. We have
29 previously described a complex diagnostic system (CYPtest™) that estimates *CYP3A*-metabolizing capacity by
30 *CYP3A4* expression in leukocytes. *CYP3A4* mRNA levels in leukocytes were demonstrated to inform about the
31 hepatic *CYP3A4* activity [37]. The goals of the present study were to estimate the association between the
32 patients' drug-metabolizing capacity (CYP expression and CYP genotypes) and their aripiprazole therapy (dose,

1 aripiprazole plasma levels), and to analyze the influence of genetic factors and the non-genetic factor co-
2 medications on aripiprazole exposure. Taking into account patients' CYP-status and current co-medication can
3 contribute to the improvement of patients' personalized medication and can predict the risk of outlying from the
4 therapeutic concentration range.

5 **Materials and methods**

6 **Patients**

7 Ninety-three inpatients at the Department of Psychiatry and Psychotherapy, Semmelweis University
8 (Budapest, Hungary) were enrolled, and written informed consent was obtained from all participants. The study
9 was approved by the Hungarian Committee of Science and Ethics. Adult patients (≥ 18 years) on stable
10 aripiprazole dose for more than four weeks, displaying two identical concentrations at an interval of 14 days
11 were included in the study. Patients' demographic data and medication was recorded (Table 1). The aripiprazole
12 therapy was applied according to the conventional clinical protocol, initiated at low dosage (7.5 mg/day), and
13 subsequently titrated up to the target dose of 10-30 mg/day until the optimal response was achieved. Patients'
14 improvement was measured by repeated psychiatric interviews and reduction of both the total scores of PANSS
15 (positive and negative syndrome scale) and CGI (clinical global impression). Aripiprazole dosage was recorded
16 for four weeks before blood sampling for testing patients' CYP-status and for drug assay.

17 **Assaying CYP-status**

18 Patients' CYP-status was determined by *CYP2D6*, *CYP3A4* and *CYP3A5* genotyping and by analyzing
19 *CYP3A4* expression in leukocytes. Genomic DNA and leukocytes were isolated from the peripheral blood
20 samples as previously described by Temesvári et al. [37]. Hydrolysis single-nucleotide polymorphism analysis
21 for *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*6*, *CYP2D6*10*, *CYP2D6*41*, *CYP3A4*1B*, *CYP3A4*22* and *CYP3A5*3*
22 was performed using TaqMan probes (BioSearch Technologies, Novato, CA). *CYP2D6*5* (gene deletion) and
23 *CYP2D6* duplication/multiplication were analyzed by TaqMan Copy Number Assay (ThermoFisher Scientific,
24 Waltham, MA). Allele specific identification of *CYP2D6* duplication was determined as previously described
25 [38].

26 For assaying *CYP3A4* expression, RNA was isolated from leukocytes, reverse transcribed into single-
27 stranded cDNA using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific), and real-time
28 PCR was performed using KAPA Fast Probes Mastermix (KAPA Biosystems, Cape Town, South Africa) and
29 TaqMan probes (Microsynth AG, Balgach, Switzerland). The quantities of *CYP3A4* mRNA relative to that of
30 the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined. GAPDH
31 expression is constant in all cells and independent of experimental conditions; therefore, its expression was set to

1 1 and CYP3A4 mRNA levels were normalized by GAPDH expression. Three categories of CYP3A4 expression
2 were applied to describe low, normal and high expressers. The cut-off values for CYP3A4 mRNA levels in
3 leukocytes have been previously established on the basis of the cut-off values for the hepatic CYP3A4 enzyme
4 activities (nifedipine oxidation or midazolam 1'- and 4-hydroxylation). The cut-off values for CYP3A4 (10^{-6} and
5 10^{-4}) allowed a distinction between low, normal and high expressers [37].

6 **Plasma concentrations of aripiprazole**

7 The blood samples of patients on stable aripiprazole therapy were taken between 8 and 9 am before the
8 morning dose of aripiprazole (generally 12 hr since last drug intake). The steady-state condition was confirmed
9 by reviewing the patient's therapeutic history. The blood samples were taken at the same time for CYP testing
10 and for therapeutic drug monitoring. The analytical assay was validated for routine drug monitoring. The steady-
11 state plasma concentrations of aripiprazole and dehydroaripiprazole were determined by LC-MS/MS (liquid
12 chromatography-tandem mass spectrometry). Chromatographic separation was performed using an Inertsil
13 ODS-4 (75×2.1 mm, 3 μm) column (GL Sciences Inc., Tokyo, Japan) and mobile phases of acetonitrile and
14 0.1% formic acid in gradient running mode. The samples in triplicates were analyzed using positive electrospray
15 ionization and multiple reaction monitoring (MRM) mode for quantitation of the parent compound and its active
16 metabolite (*m/z* [mass/charge] 448/285 and 448/176 for aripiprazole; *m/z* 446/285 and 446/216 for
17 dehydroaripiprazole). Calibration plots for aripiprazole and dehydroaripiprazole were linear over the range of
18 1-1000 and 1-500 ng/ml, respectively. The intraday and interday precision was less than 8%. Normalized
19 aripiprazole concentrations were calculated by dividing the concentration values by the corresponding 24-h dose
20 on a mg/kg basis.

21 **Inhibition of aripiprazole metabolism**

22 *In vitro* metabolite formation from aripiprazole was determined in human liver microsomes. Hepatic
23 microsomal fraction was isolated from three tissue donors by differential centrifugation [39]. Protein content of
24 microsomes was determined by the method of Lowry et al. [40], with bovine serum albumin as the standard. For
25 aripiprazole metabolism, the incubation mixture contained the NADPH-generating system (1 mM NADPH,
26 10 mM glucose 6-phosphate, 5 mM MgCl₂ and 2 units/ml glucose 6-phosphate-dehydrogenase), human liver
27 microsomes (1 mg protein/ml) and aripiprazole. After 60-min incubation, reactions were terminated by the
28 addition of ice-cold acetonitrile. The amounts of aripiprazole metabolites (dehydroaripiprazole, *N*-dealkyl-
29 aripiprazole, monohydroxy-aripiprazole) produced were determined by LC-MS/MS. The analytical conditions
30 were the same as described for the parent compound and dehydroaripiprazole measured in human plasma except
31 for the MRM transitions (*m/z* 231/188 and 231/152 for *N*-dealkyl-aripiprazole; *m/z* 464/285 and 464/234 for

1 monohydroxy-aripiprazole). Inhibition of metabolite formation from aripiprazole was carried out in the absence
2 and presence of risperidone, 9-hydroxy-risperidone, metoprolol or propranolol. K_i values (inhibition constants)
3 were determined by using different concentrations of aripiprazole (10, 25, 50 μM) and potential CYP2D6
4 inhibitors (1-50 μM). K_i values were calculated from Dixon plots of 1/velocity versus inhibitor concentration at
5 the three aripiprazole concentrations. The type of inhibition and the apparent K_i values were estimated from the
6 intercept of three lines of Dixon plots and expressed as the mean \pm SD of the intercepts.

7 **Data analysis**

8 Statistical significance of CYP3A4 expression, *CYP2D6* and *CYP3A5* genotypes as covariates of
9 aripiprazole plasma concentrations was analysed by principal component analysis and partial least squares (PLS)
10 modelling (SIMCA, MKS Umetrics AB, Umea, Sweden). Principal components are defined as linear
11 combination of original variables and ordered by their contribution degree to the overall data set variability. PLS
12 model is a multiple regression model that first transforms independent (input) and output variables with principal
13 component analysis to remove correlations between the variables and then calculates optimal linear model in
14 iterative procedure. Model coefficients analysis reveals original variables that provide significant information for
15 the output variable prediction. Predicted vs measured plot shows how well the model describes the data.
16 Departure from the diagonal line indicates biased model and dispersion of points around the diagonal line shows
17 how much of the total data variability can be explained by the model.

18 The data of normalized aripiprazole concentrations and the dehydroaripiprazole/parent drug ratios in the
19 patients were expressed as the median and range or mean \pm SD. Between-group-differences were calculated by
20 the use of Kruskal–Wallis analysis of variance followed by Dunn’s multiple comparisons test. For the evaluation
21 of co-medication with CYP2D6 inhibitors, the prevalence of outliers with exaggerated concentrations of
22 aripiprazole (> 300 ng/ml) was compared in patients carrying *CYP2D6*1* by the use of Fisher’s exact test. A *P*
23 value of <0.05 was considered to be statistically significant.

24 **Results**

25 **CYP-status and aripiprazole exposure**

26 Of 93 patients, 52 carried at least one non-functional (*nf*) or reduced function (*red*) *CYP2D6* allele (*nf*:
27 *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*4 \times N*, *CYP2D6*5*, *CYP2D6*6*; *red*: *CYP2D6*10*, *CYP2D6*41*), and 5
28 patients had functional *CYP2D6* duplication (*CYP2D6*1 \times N*), presumably resulting in increased CYP2D6
29 expression. Strong association was found between the patients’ *CYP2D6* genotype and the normalized
30 aripiprazole plasma concentrations (Fig. 1A). Significantly higher aripiprazole levels were observed in the
31 patients with non-functional and reduced function *CYP2D6* alleles (*CYP2D6*1/nf*, *CYP2D6*1/red* or

1 *CYP2D6**nf/nf*, *CYP2D6**nf/red*) than in those carrying homozygous wild-type genotype (*CYP2D6**1/*1) or
2 *CYP2D6* duplication (*CYP2D6**1/*1×N) (1421±384.3, 1379±466.5 or 2221±1003.7, 2191±682.7 vs
3 494.3±158.3 or 543.6±148.7 (ng/ml)/(mg dose/kg bw), $P<0.0001$). Furthermore, significant differences in
4 aripiprazole levels were found between the carriers of one and two non-functional or reduced function *CYP2D6*
5 alleles ($P<0.01$). While the normalized aripiprazole concentrations were comparable in the patients with
6 *CYP2D6**1/*1 genotype and in those with *CYP2D6* duplication (*CYP2D6**1/*1×N); however, further
7 confirmation is needed because of the relatively small number of patients with *CYP2D6**1 duplication. The
8 patients with *CYP2D6**1/*nf* genotype displayed similar aripiprazole concentrations to those with *CYP2D6**1/*red*;
9 therefore, these patients were grouped as *CYP2D6**1/*mut*. Furthermore, aripiprazole metabolizing capacity of the
10 patients carrying *CYP2D6**nf/red* was as low as those with *CYP2D6**nf/nf*, assigning them to *CYP2D6**mut/mut*
11 group. Thereafter, *CYP2D6**mut* was applied for *CYP2D6**3, *CYP2D6**4, *CYP2D6**4×N, *CYP2D6**5,
12 *CYP2D6**6, *CYP2D6**10 and *CYP2D6**41 alleles.

13 To clarify the role of CYP3A enzymes in aripiprazole metabolism, the relationship between patients'
14 CYP3A-status and aripiprazole plasma concentrations was also investigated. Patients with *CYP3A4**22 or with
15 *CYP3A4**1B allele (13/93) were predicted to display permanent low or high CYP3A4 mRNA expression;
16 however, these alleles cannot explain the high inter-individual variability in CYP3A4 activity, and non-genetic
17 factors, resulting in transiently altered metabolic capacity, are considered to substantially modify the expression
18 of functional *CYP3A4* gene. CYP3A4 expression assays revealed that most of the patients (>80%) expressed
19 CYP3A4 at normal level (Table 1). Nevertheless, no association was found between the patients' CYP3A4
20 expression and the normalized aripiprazole plasma concentrations (Fig. 1B). Furthermore, the steady-state
21 aripiprazole concentrations in the patients with functional CYP3A5 (*CYP3A5**1) were similar to those in
22 CYP3A5 non-expressers (*CYP3A5**3/*3). It may be assumed that the role of CYP3A4 in aripiprazole
23 metabolism predominates in patients with one or two non-functional *CYP2D6* alleles. However, no differences
24 in aripiprazole concentrations were observed between low and normal CYP3A4 expresser patients carrying
25 *CYP2D6**1/*mut* (1337.5±355.5 vs 1430.3±423.2 (ng/ml)/(mg dose/kg bw), $P=0.5729$). While those with
26 *CYP2D6**mut/mut* all expressed CYP3A4 at normal level; therefore, no conclusion could be drawn regarding the
27 contribution of CYP3A4 to aripiprazole clearance.

28 The plasma concentrations of dehydroaripiprazole widely varied in the patients (4.94 – 284 ng/ml);
29 however, its formation appeared to be associated merely with the patients' *CYP2D6* genotype (Fig. 2), whereas
30 the dehydroaripiprazole/aripiprazole concentration ratios were comparable in the patients expressing CYP3A4 at
31 low, normal or high level (data not shown). The patients with homozygous wild-type genotype (*CYP2D6**1/*1)
32 or with *CYP2D6* duplication (*CYP2D6**1/*1×N) displayed significantly higher dehydroaripiprazole/aripiprazole

1 ratios than those who carried one or two *CYP2D6mut* alleles (0.9 ± 0.191 or 0.967 ± 0.310 vs 0.346 ± 0.099 and
2 0.232 ± 0.085 ; $P<0.0001$). Furthermore, small but significant differences in aripiprazole dehydrogenation were
3 observed between the patients with one and with two non-functional/reduced function alleles ($P<0.001$).

4 To identify the major factors influencing aripiprazole steady-state concentration, the PLS model was
5 constructed with input variables, such as patients' bodyweight, aripiprazole daily dose, patients' CYP3A-status
6 (*CYP3A4* genotype, *CYP3A4* expression, *CYP3A5* genotype), *CYP2D6* genotype (Fig. 3A), and with the
7 aripiprazole plasma concentration (ng/ml) as the output variable. The number of input variables was reduced to
8 those with significant contribution regarding the distribution of centred and scaled model coefficients. The
9 patients' *CYP3A4* genotype as an independent input variable was eliminated, because *CYP3A4* expression
10 reflects the influence of *CYP3A4* genotype. While *CYP2D6*1/*1* and *CYP2D6*1/*1* \times *N* genotypes were
11 combined, because these genotypes seemed to have similar effect on aripiprazole metabolizing capacity. The
12 final PLS model inputs were aripiprazole dose, patient's bodyweight and *CYP2D6* genotype, creating the
13 following equation:

$$14 \quad cc_{\text{aripiprazole}} = 133.57 - 0.556 \cdot bw + 8.77 \cdot D + \text{constant}_{\text{CYP2D6}}$$

15 where $cc_{\text{aripiprazole}}$ is aripiprazole plasma concentration predicted from the model (ng/ml), *bw* is the patient's
16 bodyweight (kg), *D* is the daily dose of aripiprazole (mg), and the $\text{constant}_{\text{CYP2D6}}$ is -132.89 for *CYP2D6*1/*1* or
17 *CYP2D6*1/*1* \times *N* genotypes, 32.52 for *CYP2D6*1/mut*, and 210.94 for *CYP2D6mut/mut*. R^2 (0.82) and Q^2 (0.8)
18 of the PLS model indicated that the model had very good prediction power. The model also passed permutation
19 test, further indicating that the output was highly sensitive on the input values, and thus had strong predictive
20 power. The values of R^2 and Q^2 suggested that the majority (>80%) of the aripiprazole predose concentration
21 variability originated from aripiprazole dose, patient's bodyweight and *CYP2D6* genotype, whereas less than
22 20% of the variability remained unexplained by the model (Figure 3B). When removing *CYP2D6* genotype from
23 the model, only about 13% of aripiprazole concentration variability could be explained.

24 **Co-medication and aripiprazole exposure**

25 Of 93 patients, 15 patients were prescribed aripiprazole as monotherapy, whereas the majority of the
26 patients received concomitant antipsychotic medication (clozapine, haloperidol, quetiapine or risperidone) or
27 anticonvulsant drugs (clonazepam, valproic acid or lamotrigine) as adjunctive therapy. Thirty-six patients
28 received beta-adrenergic blockers (propranolol or metoprolol) and ten were on metformin therapy.
29 Co-medication with drugs known to inhibit *CYP2D6* on aripiprazole plasma concentrations was assumed to
30 modify aripiprazole clearance. In the patients with at least one *CYP2D6*1* allele, the concomitant intake of the
31 antipsychotic risperidone and/or of the beta-adrenergic blocker metoprolol or propranolol significantly influenced

1 the elimination rate of aripiprazole, whereas upon CYP2D6 inhibitor therapy, the normalized aripiprazole levels
2 were unchanged in the patients with two *CYP2D6mut* alleles (Fig. 4). The mean aripiprazole concentrations were
3 approximately 50% higher in patients with *CYP2D6*1/*1* or *CYP2D6*1/*1*×*N* genotypes ($P<0.0001$) and 20%
4 higher in the patients carrying *CYP2D6*1/mut* ($P<0.05$) as a consequence of CYP2D6 inhibitor co-medication.
5 Furthermore, co-medication of patients carrying wild-type *CYP2D6*1* allele with the CYP2D6 inhibitor
6 risperidone, metoprolol or propranolol increased the prevalence of outlier patients with exaggerated
7 concentrations of aripiprazole. Aripiprazole concentration higher than 300 ng/ml was observed more frequently
8 in the patients under CYP2D6 inhibitor therapy than in those who received no CYP2D6 inhibitor co-medication
9 (8/37 vs 2/44, $P=0.0378$). Independently from CYP2D6 inhibitor co-administration, all patients with two non-
10 functional/reduced function alleles (*CYP2D6mut/mut*) displayed exaggerated aripiprazole concentrations.

11 Inhibitory effects of risperidone and its main metabolite 9-hydroxy-risperidone as well as of propranolol
12 and metoprolol on aripiprazole metabolism were assayed *in vitro* in human liver microsomes (Table 2).
13 Risperidone and 9-hydroxy-risperidone significantly reduced the formation of dehydroaripiprazole and
14 monohydroxy-aripiprazole, and displayed competitive inhibition with the inhibitory kinetic constants (K_i) ranged
15 between 8.4 and 13.9 μM . *N*-Dealkylation of aripiprazole was also inhibited by risperidone; however, the
16 inhibitory constant was about one magnitude higher than for dehydrogenation or hydroxylation pathways. In
17 contrast to risperidone, the beta-blocker metoprolol and propranolol behaved as competitive inhibitors merely
18 towards dehydrogenation of aripiprazole, and did not inhibit hydroxylation and *N*-dealkylation pathways.
19 Substantial differences in the inhibition of dehydroaripiprazole formation were observed between metoprolol and
20 propranolol; the inhibitory constant for propranolol was 3.5-fold higher than for metoprolol, suggesting that
21 metoprolol is a more potent inhibitor of dehydrogenation than propranolol.

22 Co-medication with valproic acid was also considered as a non-genetic factor that can potentially
23 modify aripiprazole metabolism by up-regulation of CYP3A4 expression. However, in the patients on adjunctive
24 valproic acid therapy (N=11), neither aripiprazole plasma concentration nor CYP3A4 expression differed
25 significantly from those on non-valproic acid therapy (data not shown).

26 Discussion

27 Due to high inter-individual variability of aripiprazole metabolizing enzymes, therapeutic drug
28 monitoring for aripiprazole is recommended to reduce poor response or development of adverse effects [18-20].
29 CYP2D6 and CYP3A4 have been reported to be involved in aripiprazole metabolism, and the association
30 between *CYP2D6* genotype and steady-state concentrations of aripiprazole has been demonstrated by several
31 authors, whereas the impact of *CYP3A* polymorphisms (e.g. *CYP3A5* and *CYP3A4* genotypes) on aripiprazole

1 pharmacokinetics has not been clearly proved [31, 41, 42]. Although *CYP3A5*3*, the most frequent allele in
2 Caucasians, is associated with loss of CYP3A5 expression and a consequence of no enzyme activity, and
3 *CYP3A4*1B* and *CYP3A4*22* lead to altered expression of CYP3A4, the substantial inter-individual variability
4 in CYP3A activity cannot be explained exclusively by genetic polymorphisms. CYP3A5 is expressed in the liver
5 at much lower concentration than CYP3A4 (approximately 1% and 30% of the total hepatic CYP content,
6 respectively) [2]; however, the functional CYP3A5 enzyme can display much higher affinity towards certain
7 CYP3A substrates, such as tacrolimus, than CYP3A4, and CYP3A5 expresser patients carrying *CYP3A5*1* can
8 metabolize tacrolimus more rapidly than those with *CYP3A5*3/*3* genotype [43]. Current studies have reported
9 that *CYP3A5* and *CYP3A4* polymorphisms hardly had an impact on aripiprazole pharmacokinetics [44, 45];
10 however, drawing conclusion from *CYP3A* genotypes on the potential role of CYP3A enzymes in aripiprazole
11 metabolism is considered to be the limitations of these studies. CYP3A4 activity seems to be influenced by non-
12 genetic factors rather than by genetic polymorphisms. The non-genetic factors, such as age, hormonal status or
13 transcriptional induction by CYP3A-inducers, such as carbamazepine or valproic acid, can mask the effect of
14 genetic factors on CYP3A4 expression [2, 46]; therefore, for the categorization of the patients regarding
15 CYP3A4 expression, more useful information can be obtained from CYP3A4 mRNA levels than from
16 *CYP3A4*-genotyping. CYP3A-metabolizing capacity determined by CYP3A4 expression in leukocytes was
17 demonstrated to inform about the hepatic activity towards CYP3A substrates, such as ciclosporin, tacrolimus or
18 clonazepam [43, 47].

19 Our findings confirmed the results previously reported in the literature that *CYP2D6* genotype has a
20 major impact on aripiprazole plasma concentrations normalized by the dose [42, 44, 45, 48]; however, some
21 overlap in the aripiprazole metabolizing activity between *CYP2D6* genotypes was observed. The aripiprazole
22 metabolizing capacity of *CYP2D6nf/red* patients was as reduced as that of the individuals carrying two non-
23 functional *CYP2D6* alleles (*CYP2D6nf/nf*), despite the fact that intermediate and poor metabolizer phenotypes
24 were assigned to *CYP2D6nf/red* and *CYP2D6nf/nf*, respectively [49]. The aripiprazole plasma concentrations in
25 *CYP2D6*1/nf* genotype group were close to those subjects carrying *CYP2D6*1/red*, but significantly different
26 from those with two functional alleles (*CYP2D6*1/*1*), although these individuals with *CYP2D6*1/nf*,
27 *CYP2D6*1/red* or *CYP2D6*1/*1* were listed in the same normal CYP2D6 metabolizer phenotype. Furthermore,
28 the patients with *CYP2D6*1/*1* × *N* genotype predicted to be ultra-rapid metabolizers displayed aripiprazole
29 concentrations similar to the normal metabolizer patients with *CYP2D6*1/*1*. Our results confirm the findings of
30 Hendset et al. [48] predicting similarly reduced aripiprazole metabolizing activity of *CYP2D6red* and *CYP2D6nf*
31 alleles. The present study was the first that attempted to investigate the association between aripiprazole plasma
32 concentrations and the patients' CYP3A-status (CYP3A4 expression together with *CYP3A5* genotype). CYP3A

1 activity estimated from the patients' CYP3A-status did not appear to significantly influence aripiprazole
2 clearance. Principal component analysis of the patients' drug-metabolizing capacity parameters as well as of
3 demographic and aripiprazole dosing data also identified *CYP2D6* genotype as the main factor in aripiprazole
4 elimination, whereas the contribution of CYP3A activity to aripiprazole pharmacokinetics may be minor or
5 negligible. Following repeated administration of aripiprazole, dehydroaripiprazole exposure was also found to be
6 *CYP2D6* genotype dependent. Dehydroaripiprazole plasma concentrations were comparable with aripiprazole
7 levels in the patients carrying *CYP2D6**1/*1 or *CYP2D6**1/*1×N genotype, whereas the patients with one or
8 two non-functional *CYP2D6* alleles displayed dehydroaripiprazole concentrations at 35% or 22% of aripiprazole
9 levels, respectively. This means that in patients with non-functional *CYP2D6* alleles, the higher plasma
10 concentrations of aripiprazole were associated with a decrease in dehydroaripiprazole concentrations.

11 The potential role of CYP2D6 in aripiprazole clearance, clearly demonstrated by the strong association
12 between the patients' *CYP2D6* genotype and the plasma aripiprazole concentrations or the dehydro-
13 metabolite/aripiprazole ratios, also means that aripiprazole pharmacokinetics can be sensitive to potential
14 CYP2D6 inhibitors. Multi-drug therapy is often applied for patients with psychiatric disorders; thus,
15 combination of antipsychotics and/or of antipsychotics with antidepressants or with drugs for the treatment of
16 comorbid medical conditions can increase the risk of pharmacokinetic drug interactions. Selective serotonin
17 reuptake inhibitors are often prescribed with antipsychotics to treat depressive periods; however, many of them
18 (such as paroxetine, fluoxetine, sertraline or duloxetine) are strong CYP2D6 inhibitors resulting in transient poor
19 metabolism of CYP2D6 substrates due to phenoconversion [30]. Aripiprazole clearance was significantly
20 decreased by co-administration of the CYP2D6 inhibitor paroxetine or fluoxetine [31, 50]. In the present study,
21 none of these selective serotonin reuptake inhibitors was applied because of an increased risk of akathisia. Co-
22 medication with risperidone, metoprolol and propranolol was nevertheless assumed to modify aripiprazole
23 metabolism, because these drugs are CYP2D6 substrates and have the capability to inhibit CYP2D6 activity [51-
24 54]. We have observed that concomitant use of risperidone, metoprolol or propranolol was associated with an
25 increase in aripiprazole concentration. Risperidone was demonstrated to be an inhibitor of aripiprazole
26 dehydrogenation and hydroxylation pathways, displaying inhibitory potencies (K_i) similar to that was reported
27 for the CYP2D6-selective bufuralol 1'-hydroxylation ($6.9 \pm 4.1 \mu\text{M}$) [51]. Eap et al. [55] found risperidone to be a
28 weak *in vivo* inhibitor of dextromethorphan *O*-demethylation catalysed by CYP2D6; however, the patients
29 involved in the study received risperidone dose lower than usual. The inhibitory potency of the main risperidone
30 metabolite, 9-hydroxy-risperidone, towards dehydrogenation and hydroxylation of aripiprazole was found to be
31 similar to that of risperidone. This means that the hydroxylation of risperidone did not abolish the inhibition of
32 aripiprazole metabolism. Furthermore, 9-hydroxy-risperidone is a pharmacologically active metabolite and has

1 been marketed as paliperidone [56]; therefore, co-medication with paliperidone may be expected to increase
2 aripiprazole plasma concentrations as well. The presence of metoprolol or propranolol also decreased
3 aripiprazole metabolism; however, metoprolol was found to be more potent inhibitor than propranolol. Using
4 various CYP2D6-selective substrates, such as bufuralol or dextromethorphan, *in vitro* inhibitory potencies (IC₅₀)
5 of metoprolol and propranolol towards CYP2D6 have been reported to be within the same order of magnitude
6 (varied between 2.5 and 9.8 µM) [52-54]. Although metoprolol was concluded to be a clinically not relevant
7 inhibitor of venlafaxin metabolism [57], Kirschbaum et al. [18] have found a significant increase in aripiprazole
8 blood concentration in patients treated with metoprolol which is in line with our results. From *in vitro* inhibition
9 parameters, Turpeinen et al. [54] estimated relatively high *in vivo* CYP2D6 inhibition percentage for propranolol
10 (32%). In the current study, these beta-adrenergic receptor blockers inhibited merely the formation of the active
11 dehydroaripiprazole metabolite, and did not influence hydroxylation and *N*-dealkylation pathways. Since
12 *N*-dealkyl-aripiprazole and hydroxy-aripiprazole are inactive metabolites, the inactivation pathways may become
13 the principal route of aripiprazole metabolism upon co-medication with metoprolol or propranolol, resulting in
14 potential modification of aripiprazole efficacy. Aripiprazole has been reported to have a significantly higher risk
15 of akathisia compared to other second generation antipsychotics [58], and beta-adrenergic receptor blockers, such
16 as propranolol or metoprolol, are frequently used in the clinical management of antipsychotics induced akathisia
17 [59]. The fact that co-administration of aripiprazole and beta-blockers is common, underlines the clinical
18 significance of our findings. Co-medication with risperidone, metoprolol or propranolol was found to entail
19 higher prevalence of exaggerated aripiprazole concentrations which may increase the risk of moderate and
20 severe side effects developing more frequently above 300 ng/ml [18].

21 One of the limitations of the present study is that it investigated the pharmacokinetic interactions of
22 relatively weak CYP2D6 inhibitors with aripiprazole; however, not only the simple co-administration of
23 aripiprazole with risperidone or with beta-blockers, but the multiple combinations of these drugs are also
24 frequent which might substantially reduce CYP2D6 function. Due to the fact that this was a naturalistic study,
25 the number of patients treated with CYP2D6 inhibitors was low; however, the relatively narrow range of
26 aripiprazole blood concentrations (primarily in *CYP2D6**1/*1 group) indicated that these three drugs have
27 similar inhibitory potential on CYP2D6 and on aripiprazole metabolism *in vivo*. For acceptable statistical
28 analysis, we pooled the inhibition data which is another limitation of the present study and further investigation
29 is required to confirm our findings. Although the genetic variations of transporter components responsible for the
30 absorption, distribution and elimination of many drugs (e.g. ABCB1) were not investigated, the present study
31 attempted to clarify the functional role of CYP2D6 and CYP3As as well as to identify some potentially
32 interacting drugs in aripiprazole clearance. Our findings demonstrated that patients' *CYP2D6* genotype primarily

1 influenced steady-state aripiprazole plasma concentration and dehydroaripiprazole formation, whereas
2 contribution of CYP3A4 to *in vivo* aripiprazole clearance was considered to be minor or negligible. Additionally,
3 co-administration of CYP2D6 inhibitors, such as risperidone, metoprolol or propranolol, was found to modify
4 aripiprazole exposure in patients carrying wild-type *CYP2D6*1* allele. Our results suggest that patients' *CYP2D6*
5 genotype and co-medication with CYP2D6 inhibitors can be considered to be the major determinants of
6 aripiprazole pharmacokinetics. However, further studies should be performed to clearly demonstrate whether
7 taking into account patients' *CYP2D6* genotype and co-medication with CYP2D6 inhibitors can improve
8 outcomes of aripiprazole therapy.

9

10 **Conflict of Interest**

11 The authors declare that they have no conflicts of interest.

12

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- 8

1 Table 1. Patients' demographic and clinical characteristics

		N	%
Patients		93	
Sex, male/female		41/52	44.1/55.9
Diagnosis			
	Schizophrenia	85	91.4
	Bipolar disorder	8	8.6
Age (year)*		31 (18; 65)	
Bodyweight (kg)*		80 (47; 145)	
Aripiprazole daily dose (mg)*		15 (5; 30)	
Serum levels (ng/ml)			
	Aripiprazole*	193 (6.2; 819)	
	Dehydroaripiprazole*	86.8 (4.9; 284)	
CYP genotype			
<i>CYP2D6</i>	<i>CYP2D6*1/*1</i>	36	38.7
	<i>CYP2D6*1/nf**</i>	26	28.0
	<i>CYP2D6*1/red***</i>	14	15.0
	<i>CYP2D6nf/nf**</i>	7	7.5
	<i>CYP2D6nf/red***</i>	5	5.4
	<i>CYP2D6*1/*1xN</i>	5	5.4
<i>CYP3A4</i>	<i>CYP3A4*1/*1</i>	80	86
	<i>CYP3A4*1/*1B</i>	4	4.3
	<i>CYP3A4*1/*22</i>	9	9.7
<i>CYP3A5</i>	<i>CYP3A5*1/*3</i>	8	8.6
	<i>CYP3A5*3/*3</i>	85	91.4
CYP expression			
<i>CYP3A4</i>	low expressers	14	15.0
	normal expressers	77	82.8
	high expressers	2	2.2
Co-medication			
	no	15	16.1
	clozapine	30	32.2
	haloperidol	8	8.6
	quetiapine	12	12.9
	risperidone	16	17.2
	clonazepam	20	21.5
	valproic acid	11	11.8
	lamotrigine	3	3.2
	propranolol	21	22.6
	metoprolol	15	16.9
	metformin	10	10.8

2 * median (min; max); ** *CYP2D6nf*: *3, *4, *5, *6 and *4xN; *** *CYP2D6red*: *10 and *41

1 Table 2. K_i values (μM) for *in vitro* inhibition of aripiprazole metabolism by risperidone,
 2 9-hydroxy-risperidone, propranolol and metoprolol*

	Risperidone	9-Hydroxy-risperidone	Propranolol	Metoprolol
Dehydroaripiprazole	8.4±2.64	9.3±2.97	48.2±9.16	13.4±2.80
Hydroxy-aripiprazole	9.1±1.70	13.9±0.26	-	-
<i>N</i> -dealkyl aripiprazole	57.0±7.11	-	-	-

3 **In vitro* inhibition of aripiprazole metabolism was carried out at three different
 4 concentrations of aripiprazole (10, 25, 50 μM) and eight concentration points of risperidone,
 5 9-hydroxy-risperidone, propranolol or metoprolol (1-50 μM) using hepatic microsomes of
 6 three tissue donors.

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Figure legends

Fig. 1: The influence of the patients' *CYP2D6* genotypes (A) and CYP3A-status (*CYP3A5* genotype and *CYP3A4* expression) (B) on aripiprazole plasma concentrations.

nf: non-functional *CYP2D6* variations (*3, *4, *5, *6 and *4×N); *red*: reduced function variations (*10 and *4I); ×N: allele duplication; Δ: *CYP3A5**1 carrier; *: $P < 0.01$; **: $P < 0.0001$

Fig. 2: The influence of the patients' *CYP2D6* genotypes on dehydroaripiprazole/aripiprazole concentration ratios.

mut: non-functional and reduced function *CYP2D6* alleles (*3, *4, *5, *6, *4×N, *10 and *4I); ×N: allele duplication; *: $P < 0.001$; **: $P < 0.0001$

Fig. 3: PLS model for aripiprazole plasma concentration. (A) Principal component analysis with input variables of aripiprazole daily dose/patients' bodyweight, patients' CYP3A-status (*CYP3A4* expression, *CYP3A5* genotype), *CYP2D6* genotype; (B) Aripiprazole plasma concentrations predicted by the PLS model.

To simplify the data for the PLS modelling, some of the *CYP2D6* genotype groups were combined and three *CYP2D6* genotype groups were created, *CYP2D6**1/*1 (for *CYP2D6**1/*1 and *CYP2D6**1/*1×N), *CYP2D6**1/*mut* (for *CYP2D6**1/*nf* and *CYP2D6**1/*red*) and *CYP2D6**mut*/*mut* (for *CYP2D6**nf*/*nf* and *CYP2D6**nf*/*red*).

Fig. 4: The effect of co-medication with CYP2D6 inhibitors (risperidone, metoprolol or propranolol) on patients' aripiprazole plasma concentrations. Boxes with the line inside represent the range and median, whereas the whiskers are for minimum and maximum values. The number of patients (N) in each group is also indicated. In *CYP2D6**1/*1 and *1/*1×N group, co-medication with CYP2D6 inhibitor risperidone, metoprolol and propranolol was for 7, 7 and 8; in *CYP2D6**1/*mut* group, 7, 7 and 10; in *CYP2D6**mut*/*mut* group, 2, 1 and 3, respectively.

mut: loss-of-function *CYP2D6* allele; ×N: allele duplication; *: $P < 0.05$; **: $P < 0.0001$







