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Original article

In linezolid underexposure, pharmacogenetics matters: The role of CYP3A5

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ABSTRACT

The exposure to linezolid is characterized by a large inter-individual variability; age, renal dysfunction and body weight explain this variability only to a limited extent and a considerable portion of it remains unexplained; therefore, we decided to investigate the role of individual genetic background focusing in particular on the risk of linezolid underexposure.

191 patients in therapy with linezolid at the standard dose of 600 mg twice daily were considered. Linezolid plasma concentration was determined at the steady state and classified as "below", "within" or "above" reference range. Genetic polymorphisms for ATP Binding Cassette Subfamily B Member 1 (ABCB1), Cytochrome P450 (CYP) enzymes CYP3A4 and CYP3A5, and Cytochrome P450 Oxidoreductase (POR) were investigated.

Age significantly correlated with drug exposure, and patients CYP3A5 expressers (GA and AA) were found at high risk to be underexposed to the drug when treated at standard dose. This association was confirmed even after correction with age. No association was found with ABCB1 polymorphism. Our data suggest that CYP3A5 polymorphisms might significantly affect linezolid disposition, putting patients at higher risk to be underexposed, while P-glycoprotein polymorphism seem not to play any role.

1. Introduction

Linezolid is an oxazolidinone antibacterial agent licensed for the treatment of pneumonia, sepsis and skin and soft tissue infections caused by multidrug-resistant Gram-positive bacteria. Nowadays, the use of linezolid in daily clinical practice has been widened to include the treatment for other difficult infections and multi-drug resistant (MDR) tuberculosis [1,2]. Linezolid selectively binds to the 30S and 50S ribosomal subunit in the translation initiation reaction and inhibits the early stage of the protein synthesis process [3].

Linezolid is licensed in the adult population at the standard dose of 600 mg twice daily orally and/or via intravenous infusion, with no specific dose modification recommendations based on patient characteristics or concomitant therapies. Recent clinical data however, showed a large inter-individual exposure to the drug with the approved regimen, with a reported twenty-fold difference in plasma linezolid trough concentrations among subjects [4,5]. Consensus reports have recently underlined the importance to maintain plasma linezolid trough concentrations in the range of 2–8 mg/L to maximize drug efficacy and limit adverse events, mainly thrombocytopenia [6]; accordingly, therapeutic drug monitoring has gained a major role as clinical strategy to evaluate the response of any individual patient [7–11].

Factors playing a key role in the observed inter-individual variability in linezolid exposure have been investigated in clinical studies. In particular, renal dysfunction was associated with a higher risk of linezolid accumulation and development of drug-related adverse events [3,6,12–15]; critically ill patients with acute renal failure were demonstrated to have a different pharmacokinetics respect to healthy patients [16]. In addition, elderly patients showed significantly higher concentration of linezolid compared with younger adults, being thus at risk of drug-related toxicity [17], suggesting a major impact of age on linezolid disposition and tolerability. The role of body weight and sex is still controversial with studies showing a significant association and studies showing none [15,17–20]. The risk of linezolid under-therapeutic exposure was also found to be higher in patients with severe thermal injuries or cystic fibrosis [9]. The role of the above mentioned covariates

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explain indeed a significant amount of the variability observed with linezolid; yet a considerable portion of the variable pharmacokinetics remains unexplained [5,19,21].

Linezolid is predominantly metabolized through oxidation of its morpholine ring to an inactive form by non-enzymatic oxidative reactions [22] therefore, cytochrome P450 enzymes seem not to play a significant role in metabolic pathway of linezolid.

However, some drug-to-drug interactions have been reported in real life [5] and a role for P-glycoprotein induction or inhibition has been suggested [23].

Recently, a potential contribution on pharmacokinetics of linezolid of ABCB1 polymorphisms, the gene encoding for the efflux pump P-glycoprotein, was proposed. In particular, it has been shown that a lower clearance of the drug has been associated with the presence of c 0.3435CT/TT genotypes [24]. However, in many cases, the reported effects of ABCB1 polymorphisms (especially c 0.3435C>T) have been inconsistent and conflicting [25].

Recently we have described the case of an atypical linezolid pharmacokinetic behavior potentially related to a genetically based alteration in linezolid metabolism [26], proposing a role also of cytochrome P450 CYP3A. It has been suggested that 70–90% of the interindividual variability in hepatic CYP3A activity function is attributable to genetic factors with two polymorphisms, CYP3A5*3 and CYP3A4*22, playing major roles [27] Furthermore, part of this variability may be attributed to rare variants within the CYP3A locus and/or other variants outside to it as the single nucleotide polymorphisms (SNPs) in cytochrome P450 oxidoreductase (POR) gene. POR serves as an electron donor for different CYP enzyme activities [28]; the rs1057868C>T (POR*28) is the most common polymorphism of POR and reduces its activity in vitro [29].

The aim of this study was to assess the potential contribution of individual genetic backgrounds on inter-individual differences in systemic linezolid disposition with a special focus on the risk of linezolid underexposure.

2. Materials and methods

2.1. Study population and study design

This study is based on a retrospective analysis of routine requests for therapeutic drug monitoring (TDM) and pharmacogenetic (PGx) analysis of linezolid carried out by the Unit of Clinical Pharmacology at the Luigi Sacco University Hospital (Milan, Italy) between January and December 2019, also including samples sent by other hospitals, as our center serves as reference center for TDM of linezolid in north Italy.

Eligible population included patients, who received 600 mg twice a day linezolid therapy for $\geq$ 72 h in order to ensure steady-state conditions; with a blood sample collected 12 h after the last drug intake (a time window of $\pm$ 20 min was considered acceptable), and immediately before linezolid administration the next morning to ensure that these samples can be considered as trough concentrations. If more than 1 TDM is available, the first assessment was considered in the statistical analyses. Information on date of birth, sex, and glomerular filtration (GFR estimated by the MDRD formula) at the time of the first linezolid TDM were collected.

Patients with deteriorated renal function (GFR < 30 mL/min/1.73 m^2) and patients with concentrations below the limit of quantitation (0.4 mg/L) were excluded from the analysis. In particular, in these patients, potential errors in the TDM request and/or problems with compliance to therapy cannot be excluded.

This retrospective research was conducted on data collected for clinical purposes. All data used in the study were previously anonymized, according to the requirements set by Italian Data Protection Code (leg. decree 196/2003) and by the General authorizations issued by the Data Protection Authority. Approval by Ethics Committee was deemed unnecessary because, under Italian law, such an approval is required only in the hypothesis of prospective clinical trials on medical products for clinical use (art. 6 and art. 9, leg. decree 211/2003). Informed consent for medical procedures/interventions performed for routine treatment purposes was collected for each patient by each center.

2.2. Assessment of plasma drug concentrations

Trough blood samples for the assessment of linezolid concentrations were collected into EDTA-A containing tubes and handled on ice. The plasma was separated by centrifugation at 3000 × *g* for 8 min at 4 °C and stored at − 20 °C until analysis.

Plasma concentrations were quantified using a liquid chromatography method developed and validated in our laboratory according to the European Medicines Agency Guidelines [30]. The performance of the method was tested during each analytical run using internal quality controls and blinded samples sent as part of the Instand Proficiency Testing Schemes for Antibiotic Drugs (http://www.instand-ev.de/). The method was linear from 0.4 to 48 mg/L with an inaccuracy and imprecision less than 8.2%.

Linezolid trough concentrations were classified as "below"", "within," or "above" the reference ranges of 2–8 mg/L adopted in our laboratory based on available literature [8].

2.3. Genotyping

Genomic DNA was isolated from peripheral blood cells using an automated DNA extraction system (EZ1 Advanced XL, Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were evaluated by absorbance methodology using a NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, Massachusetts).

Samples were genotyped for the most relevant SNPs in these genes: *ABCB1* c.1236C>T (rs1128503), c.2677G>T/A (rs2032582) and c.3435C>T (rs1045642); *CYP3A4*22* (rs35599367 C>T) and *CYP3A5*3* (rs776746 A>G) and *POR*28* (rs1057868 C>T). Genotypes were then grouped on the basis of functional significance described in literature [31–33]. The three *ABCB1* variants show a strong *linkage disequilibrium* and define an haplotype: the absence of any variant on both alleles was assigned the wild-type diplotype (CGC/CGC=0), while the presence of one allele with every variant was assigned the heterozygous diplotype (TTT carrier =1), and the presence of all variants on both alleles was assigned the mutant diplotype (TTT/TTT=2).

Patients with other dyplotypes can't be categorized in any of these groups and so were excluded from statistical analysis.

All genotypes were determined by Real-Time PCR using LightSNiP (TIB-MolBiol, Berlin, Germany) or TaqMan Assays® (Thermo Fisher Scientific, Waltham, Massachusetts) on LightCycler 480 (Roche, Basel, Switzerland), according to the manufacturer's instructions. Data analysis was performed as described in the LightCycler 480 Instrument operator's manual.

Genotyping performance was estimated through use, in each analysis, of known-genotype internal quality controls.

2.4. Statistical analysis

Analysis of the data was done using the management software linked to the Information System Laboratory (DNLab –NoemaLife, Dedalus Italia S.p.A) that collects, processes and archives the results.

Normal distribution of the continuous variables was confirmed by the Kolmogorov–Smirnov test. Not-normally distributed variables were expressed as median (interquartile range IQR) and normally distributed variables as mean $\pm$ standard deviation (SD).

Continuous variables were analyzed between genders by Student *t*-test or Mann-Whitney non parametric test. Comparisons among three groups were made with parametric analysis of variance (ANOVA) or non-parametric (Kruskal–Wallis) tests, according to the significance of

the Levene's test for equality of variances. Correlation analyses were also used to evaluate factors potentially associated with plasma drug concentrations.

Analyses were also performed considering as the dependent variable the drug concentrations categorized as "below"", "within," or "above" the reference ranges of 2–8 mg/L for linezolid trough concentrations.

Genotype frequencies for the various SNPs were assessed for deviation from Hardy–Weinberg equilibrium using the $\chi 2$ test.

Pearson chi-squared analyses were performed to compare characteristics expressed as frequencies, according to the number of observations. This was applied to different genotypes and categorized drug concentrations.

Multinominal logistic regression was employed to analyze the risk to have concentrations below or above the range, respect to be within the range according to genotype. An odds ratio (OR) and a 95% confidence interval were calculated to evaluate the strength of any association.

The statistical significance level was defined as a p-value of less than 0.05. All statistical calculations were performed using the Stata v 16.1 software (StataCorp, USA).

3. Results

The study included 196 patients, 78 women and 118 men. Six patients (2 women and 4 men) were excluded a priori from the analysis because the concentration of linezolid was below the limit of quantification (0.4 mg/L).

All the 190 patients included in the analysis were Caucasian and no patient was in therapy with rifampin or other CYP3A5 inducer. Characteristic of the patients are reported in Table 1.

All patients were between the ages of 18 and 98 years (median age 70 years) with no differences between sex (67 and 68 years for females and males, respectively, $p = 0.627$). Median serum creatinine and GFR were 0.97 mg/dL (IQR: 0.7–1.26 mg/dL) and 73.4 mL/min/1.73 m^2 (IQR: 50.3–88.6 mL/min/1.73 m^2), respectively.

A wide distribution of plasma linezolid trough concentrations was observed (from 0.66 to 43.35 mg/L; inter-individual CV=77%). Mean linezolid concentration was 10.5 ± 8.1 mg/L with no statistically significant differences between sex (males: 10.8 ± 7.9, females: 10.2 ± 8.2 mg/L, $p = 0.641$).

Table 1
Patient characteristics and genotype frequencies of SNPs.

Total, n	190
Males, n (%)	114 (60)
Females, n (%)	76 (40)
Age, years, median (IQR)	70 (59–79)
Creatinine values, mg/dL, median (IQR)	0.94 (0.7–1.26)
GFR, mL/min/1.73 m^2, median (IQR)	73.4 (50.3–88.6)
ABCB1 c.3435 C>T rs1045642 (%)	CC (29,5)
	CT (44,7)
	TT (25,8)
ABCB1 c.2677 G>T/A rs2032582 (%)	GG (32,1)
	GT (44,2)
	GA (2,6)
	TA (2,1)
	AA (0)
	TT (18,9)
ABCB1 c.1236 C>T rs1128503 (%)	CC (31,1)
	CT (48,4)
	TT (20,5)
CYP3A4*22 C>T rs35599367 (%)	CC (*1/*1) (85,3)
	CT (*1/*22) (14,2)
	TT (*22/*22) (0,5)
CYP3A5*3 A>G rs776746 (%)	AA (*1/*1) (1,1)
	AG (*1/*3) (13,2)
	GG (*3/*3) (85,8)
POR*28 C>T rs1057868 (%)	CC (*1*1) (55,8)
	CT (*1/*28) (38,4)
	TT (*28/*28) (5,8)

IQR:interquartile range; GFR: glomerular filtration rate

72 patients out of 190 (37.9%) had linezolid trough concentrations within the therapeutic range; 52.4% and 9.4% had, respectively, linezolid trough concentrations above and below the therapeutic range (Fig. 1).

Linezolid trough concentrations resulted positively correlated with patients' age as shown in Fig. 2($r = 0.2118$, $p = 0.0034$).

Conversely, no association was found between linezolid trough concentrations and GFR ($r = -0.053$ $p = 0.6601$).

The distribution of genotypes for ABCB1, CYP3A4, CYP3A5 and POR polymorphisms are shown in Table 1, all polymorphisms being in Hardy-Weinberg equilibrium.

No association was found between the c.3435C>T ABCB1 polymorphism and linezolid exposure, either when considering linezolid trough concentrations as a continuous variable stratified according to patients' genotype (linezolid trough concentrations resulted as follows: CC 11.4 ± 9.1 mg/L; CT 10.2 ± 8.0 mg/L; TT: 9.8 ± 6.8 mg/L; $p = 0.561$), or when clustering data as below, within or above the therapeutic ranges (CC+CT: below range 9.9% vs within range: 38.3% vs above range: 51.8% TT; 8.2%; vs 36.7%; vs 55.1% respectively). These results were confirmed also taking in consideration the haplotype of ABCB1 (Table 2).

A significant relationship was instead found in the frequency distribution of linezolid trough concentrations when looking at the rs776746 polymorphism of the CYP3A5 gene. Indeed, as shown in Table 2, the large majority of patients CYP3A5 GG were within or above linezolid range. (under range: GG 6.8% vs AA+AG 25.9%, $p = 0.002$).

The multinomial logistic regression analysis confirmed that CYP3A5 expressers (GA and AA) were at higher risk to be underexposed to the drug [OR 9.09 (95% CI: 2.32–32.25) $p = 0.001$] even after correction with the age [OR 5.88 (95% CI: 1.47–23.08) $p = 0.013$) (Table 3).

Furthermore, POR genotype played a role in the exposure to the drug [OR 8.28 (95% CI: 1.36–50.28) $p = 0.022$].

4. Discussion

Our study confirms a wide inter-individual distribution of linezolid plasma trough concentrations in patients treated with the conventional drug dose of 600 mg twice daily, a regimen that in the large majority of centers worldwide is still used. Linezolid trough plasma concentration although less precise than the area under the concentration-versus-time curve (AUC) over 24 h, is linearly correlated to it [19,34] such that it provides a practical alternative assessment of daily drug exposure and represent a step forward compared with the fixed-dose approaches to identify patients at risk of inefficacy or toxicity [8]. It should be emphasized that the exposure (or the trough concentration) must be evaluated in the context of the minimum inhibitory concentration (MIC) of the pathogen to evaluate accurately the probability of efficacy, even if in some clinical conditions linezolid is given empirically, and MIC values are not available [8].

In our population, collected in real life, we found that the risk of drug overexposure is much greater than the risk of drug underexposure.

Patients' age was the variable strongly associated with trough linezolid concentration. This is not an unexpected finding: consistent literature is available documenting that elderly patients are at higher risk of linezolid accumulation and development of drug-related hematologic toxicity [35]. Accordingly, we have recently proposed to consider these patients as a special population deserving the use of reduced linezolid dose [17]. By analyzing a large cohort of data with more than 3200 results of linezolid TDM, a highly significant and progressive increment in the linezolid trough concentrations was found moving from patients aged < 50 years to those aged > 90 years, with an overall increment of 30% per decade of age. Nearly 30%, 50%, and 65% of patients aged < 65 years, 65–80 years, and > 80 years, respectively, had supra-therapeutic linezolid trough concentrations at the first therapeutic drug monitoring assessment [17].

No association was found between linezolid trough concentrations

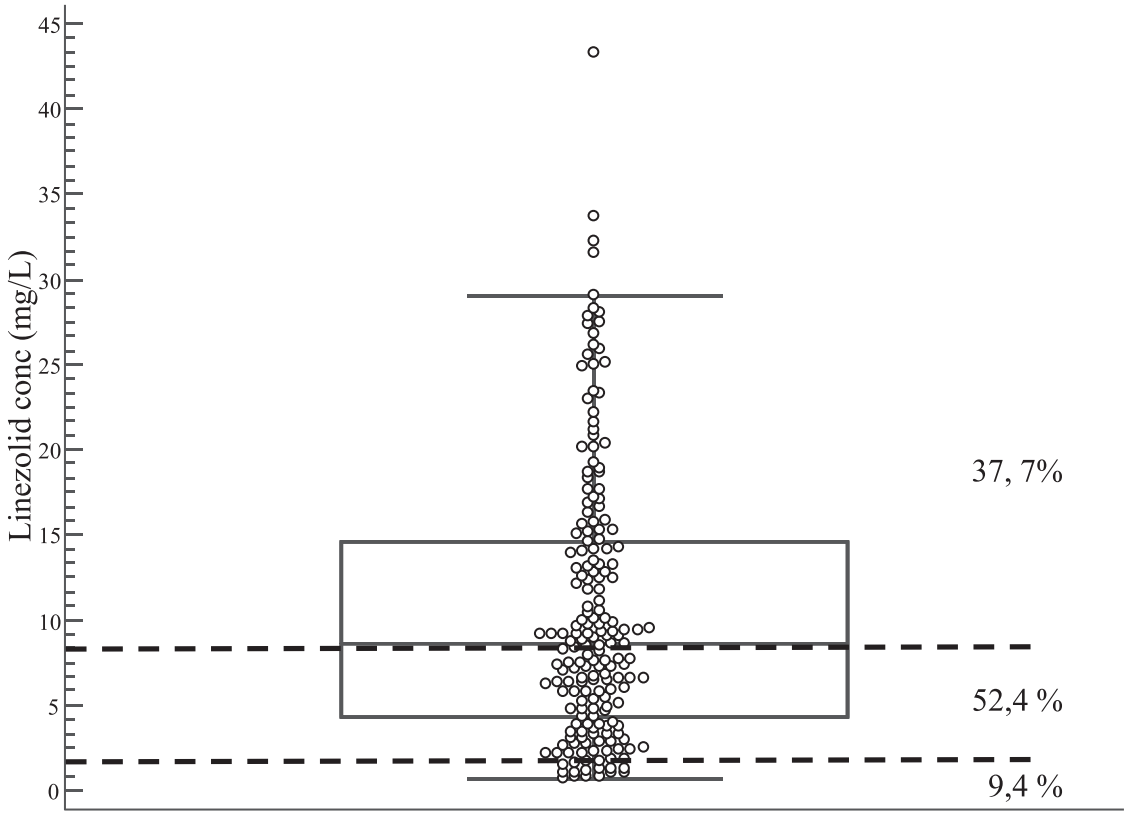

Fig. 1. Box-and Whisker plot of linezolid plasma trough concentrations. Dashed lines represent the concentration therapeutic window. Percentages of samples below, within or above the therapeutic concentrations are also reported.

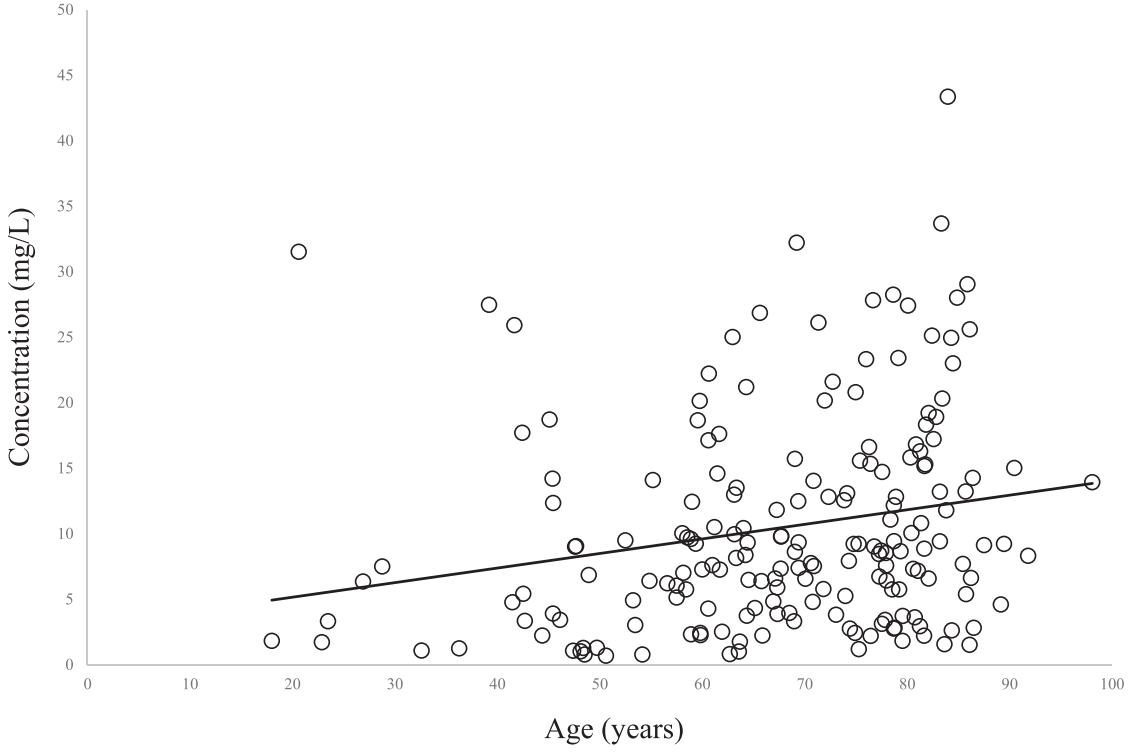

Fig. 2. Correlation between linezolid concentration and patient age. Solid line represent the linear trend.

Table 2
Genotype frequencies of SNPs tested for potential association with linezolid overexposure and underexposure.

Variable	Genotype	Therapeutic range			
		Above range GF %	Within range GF %	Below range GF %	P-value
CYP3A4*22 rs35599367C>T	CC	54.3	36.4	9.3	
	CT+TT	42.9	46.4	10.7	0.53
CYP3A5*3 rs776746 A>G	AA+AG	55.6	18.5	25.9	**0.002**
	GG	52.1	41.1	6.8	
POR*28 rs1057868C>T	CC+CT	53.1	38.6	8.4	
	TT	45.5	27.3	27.3	0.11
ABCB1 rs1045642 c.3435 C>T	CC+CT	51.8	38.3	9.9	0.90
	TT	55.1	36.7	8.2	
ABCB1 c.1236C>T+c.2677G>T/A+c.3435C>T	CGC/CGC + TTT carriers	53.7	38.1	8.2	
	TTT/TTT	51.7	37.9	10.3	0.93

GF: Genotype frequency.

Table 3
Multinomial logistic regression (without or with age variable) for potential association with linezolid underexposure ($C_{min} < 2$ mg/L).

Variable	Genotype	Unadjusted for age OR (95% CI)	p	Adjusted for age OR (95% CI)	p
CYP3A4*22 rs35599367C>T	CC CT+TT	0.90 (0.23–3.60)	0.89	0.95 (0.22–4.02)	0.95
CYP3A5*3 rs776746 A>G	AA+AG GG	9.09 (2.32–32.25)	**0.001**	5.88 (2.29–23.08)	**0.013**
POR*28 rs1057868C>T	CC+CT TT	4.6 (0.84–25.05)	0.078	8.28 (1.36–50.28)	**0.022**
ABCB1 rs1045642 c.3435C>T	CC+CT TT	0.85 (0.25–2.94)	0.80	0.86 (0.24–3.05)	0.82
ABCB1 c.1236C>T+c.2677G>T/A+ c.3435C>T	CGC/CGC + TTT carriers TTT/TTT	1.26 (0.30–5.30)	0.75	1.28 (0.29–5.62)	0.74

OR: odds ratio, CI: confidence interval, p: p-value.

and renal function, apparently challenging available literature in the field [4,10,14,15]. It must be stressed, however, that we deliberately decided to exclude from the present study patients with severe renal impairment (that is GFR <30 mL/min/1.73 m^2) as we wanted to focus on factors explaining linezolid underexposure, which is a clinically relevant, yet unexplored issue.

In any case, data in literature suggest that patients' age and renal function explain only partially the wide interindividual variability in linezolid exposure [19]. Most importantly, aging and renal insufficiency are well known players associated with an important risk of linezolid overexposure whereas factors explaining linezolid underexposure (observed in nearly 10% of our patients) are presently ill defined. To address this issue, we decided here to investigate the potential involvement of pharmacogenetic factors.

A recent study did not find a significant impact of ABCB1 polymorphisms on the pharmacokinetics of linezolid in terms of area under the concentration-time curve of linezolid or trough levels. The study was however on a limited number of patients [24]. The potential chance due to the limited number of patients was in our case avoided. Our data do not support an involvement of ABCB1 polymorphisms both considering the polymorphism rs1045642, and the haplotype containing the mutated alleles that has been demonstrated to produce major structural modifications responsible of changes in the conformation of the binding sites and a subsequent decrease in P-gp activity [36].

This result seems to confirm the data of Gandelman et al. that suggested that linezolid itself is not a P-glycoprotein substrate [37]. The drug to drug interaction that have been reported, principally with rifampicin, that has been gained increasing attention, could be ascribed not to P-glycoprotein inhibition or induction, but to the contribution of CYP3A on linezolid clearance, as hypothesized by Bolhuis [38].

For these reasons, we decided to explore also the role of the most important functional CYP3A polymorphisms on the linezolid disposition and in the POR gene, involved in the regulation of CYP3A4/5 enzymes.

Patient CYP3A5 expressers (*1/*1 and *1/*3) were found to be more at risk to be underdosed respect to CYP3A5 non expressers (*3/*3). The presence of the CYP3A5*3 allele results in a truncated protein, leading to a decreased functional CYP3A5 enzyme and lower metabolic rate [39]. The CYP3A5*3 homozygosity is the most frequent condition in Caucasian population, where 95% of the individuals are non expressers. Normally, drugs that are metabolized by CYP3A5, like tacrolimus, are expected to be found at higher concentrations in CYP3A5 non expressors, but this was not demonstrated for linezolid. A possible explanation can be the one found by Gandelman [37]. In this study, he found that, when a powerful inducer like rifampin is co-administered with linezolid, the metabolism of linezolid was increased by 1.3- to 1.6-fold in human hepatocytes and the area under the plasma concentration–time curve over the dosing interval and maximum concentration values for linezolid were reduced approximately by 32% and 21%, respectively. Therefore, his hypothesis was that the large increase in expression level of a CYP enzyme that typically has a small contribution to linezolid clearance could cause a small decrease in linezolid exposure.

In our case no inducer of CYP3A5 was present, but the presence of a functional CYP3A5 enzyme (patient genotypes *1/*1 or *1/*3), would result in an increase in CYP3A expression levels that can yield in a small increase in linezolid metabolism with decreased linezolid trough levels, confirming the results found by Gandelman.

The enzyme P450 oxidoreductase (POR) enables the activity of CYP450 enzymes by transferring electrons from nicotinamide adenine dinucleotide phosphate-oxidase to microsomal CYP enzymes. In particular the POR*28 (rs1057868) SNP seems to exert isoform-specific effect on CYP activity [39]. Data from literature, suggest that this relationship is manifest with CYP3A5: in transplant CYP3A5 expresser patients in therapy with tacrolimus, T allele carriers had lower predose concentrations and reached target levels later [40,41].

We found, after correction with age, that patient with POR*28 were at higher risk to have lower concentrations. Unfortunately, due to the lower number of patients with CYP3A5*1, we could not verify the impact of POR*28 in these patients.

A possible limitation of this "real life" retrospective study is the lack of additional patient information; therefore, we cannot exclude other possible covariates associated with linezolid exposure (i.e. drug-drug interactions beside rifampicin, poor compliance to therapies, body weight and/or body mass index, etc). In addition, patients with impaired renal function were excluded from the analysis. The limited number of CYP3A5 expressers could influence the results; therefore, other studies could confirm the data we obtained.

5. Conclusions

In conclusion, our study indicated that CYP3A5 polymorphisms might significantly affect linezolid disposition, putting patients at higher risk to be underexposed, while P-glycoprotein polymorphism does not play any role.

Declaration of conflicting interests

The authors declare no conflict of interest.

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