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Research Article

Genotype-phenotype correlation for dabigatran in patients with non-valvular atrial fibrillation (a single centre research)

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Abstract. Background: The study of the genetic factors that determine interindividual variation in drug response facilitate the dose selection in order to maximize treatment effectiveness and / or to reduce the risk of side effects. The prodrug Dabigatran etexilate's bioavailability is increased by the ABCB1 transporter – P glycoprotein (P-gp). Also, DABE is hydrolyzed by carboxylesterase CES1 in the liver to his active form dabigatran. The goal of our study was to analyze the genotype-phenotype relationship of *ABCB1* (rs1045642 and rs4148738) and *CES1* (rs8192935, rs4580160, and rs2244613) polymorphisms in patients with NVAF treated with dabigatran in clinical real-world settings. Methods: From March 2017 to June 2017, 37 consecutive Caucasian patients (70.0 years, range: 62–77, 48.6% men) with NVAF, from a single cardiology centre, who received dabigatran 150 mg twice daily, were included in the study. Genotyping for polymorphic variants of *ABCB1* and *CES1* genes was performed and the trough and peak equilibrium plasma concentrations of dabigatran were measured by liquid chromatography-tandem mass-spectrometry (LC-MS/MS). Results: In the study group dabigatran plasma levels presented important variability. No statistically significant differences were found in the trough or peak dabigatran plasma concentrations between the genotypic groups, for any studied polymorphism. Trough plasma concentrations had an inverse relationship with the body mass index ($r=-0.353$; $p<0.05$) and the body surface area ($r=-0.345$; $p<0.05$). Creatinine clearance was inversely correlated with dabigatran trough and peak plasma levels (Spearman correlation: $r = -0.215$; $p = 0.2$ and $r = -0.127$; $p = 0.4$ respectively). Conclusions: In our study, although we did not obtain a statistically significant association between *CES1* or *ABCB1* polymorphisms and dabigatran's plasma levels, we, however, observed a downward trend of its trough and peak plasma levels in variant allele carriers of SNP rs8192935 and rs4580160. The genes that encode proteins related to dabigatran's metabolism may partially explain the plasma concentration variability.

Key Words: dabigatran; ABCB1, CES1, non-valvular atrial fibrillation, P glycoprotein.

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Introduction

Non-valvular atrial fibrillation (NVAF) is the most prevalent type of AF encountered in the daily clinical practice being associated with potentially life-threatening thromboembolic complications, mainly ischemic stroke (Lip et al 2012; Dweck et al 2012; Pink et al 2013). These complications can be avoided by long-term anticoagulant administration (Kirchhof et al 2016; January er al 2019).

Dabigatran, the first non-vitamin K oral anticoagulant (NOAC) approved for preventing the cardioembolic events in patients with NVAF (Connolly et al 2009; Wallentin et al 2010), is a specific and reversible inhibitor of free and clot bound thrombin and of endogenous thrombin generation (Stangier et al 2008; Blech et al 2008).

It is orally administrated, as a prodrug - Dabigatran etexilate (DABE) and has a low bioavailability (3-7%) influenced by the ABCB1 transporter – P glycoprotein (P-gp) (Stangier et al 2008; Blech et al 2008). Aiming to increase its bioavailability, the prodrug is rapidly hydrolyzed after absorption by nonspecific esterases in two phases: by carboxylesterase CES2 in the intestine to dabigatran ethyl ester M2 and by carboxylesterase CES1 in the liver to his active form dabigatran (Stangier et al 2008; Ebner et al 2010). The peak of plasma concentration is reached at 2 hours after the intake of the capsule (Stangier et al 2008; Blech et al 2008). Distinctive from warfarin and other

NOAC – factor Xa inhibitors, CYP enzymes are not involved in dabigatran's metabolic pathway (Stangier et al 2008; Blech et al 2008).

The RE-LY (The Randomized Evaluation of Long-Term Anticoagulation Therapy) trial has demonstrated that dabigatran should be administered in two fixed daily doses and their effect does not need to be currently monitored by laboratory tests (Kirchhof et al 2016; January et al 2019; Steffel et al 2018). However, considerable variability in the plasma levels of the active metabolite of DABE was observed (European Medicines Agency. Pradaxa. Summary of Products Characteristics. Available online: n.d.) (Douxfils et al 2012). Moreover, in the exposure-response analysis from the RE-LY trial, higher trough dabigatran's plasma concentrations have been associated with a decreased risk of thromboembolism and an increased risk of bleeding (Reilly et al 2014).

In our routine clinical practice, we can encounter certain circumstances that require the individualization of treatment mostly in patients with multiple factors that interfere with the pharmacokinetics of dabigatran (drug-drug interactions, extreme weight, drug accumulation, the need of antidote administration). The field of pharmacogenomics involves the study of the genetic factors that determine interindividual variation in drug response and aims to facilitate the concept of personalized pharmacological therapy and dose selection (based on genotype) to maximize treatment effectiveness and / or to reduce the risk of side effects (Roden et al 2019). It offers clinicians the necessary tools to make informed decisions based on prognostic genetic testing. When mutations occur in genes encoding proteins as receptors, ion channels, or enzymes involved in drug metabolism, the genetic variations may alter the efficacy and safety of the therapy (Roden et al 2019; Ragia et al 2019).

Until now only one genome-wide association study has been published, also from the RE-LY trial, which established that genetic factors could have an impact on the interindividual variability in plasma levels associated with the safety and efficacy of dabigatran (Pare et al 2013).

The objective of our study was to analyze the genetic polymorphisms encoding the enzymes involved in the metabolic pathway (*ABCB1* and *CES1*), their genotype-phenotype relationship, and the possible association between the interindividual profile and the outcome of patients with NVAF treated with dabigatran in clinical real-life settings.

Methods

This analytical observational prospective monocentric study was conducted from March 2017 to June 2019 in "Niculae Stancioiu" Emergency Heart Institute and was approved by the Institute's Ethical Committee and by the Ethical Committee of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca (reg-istration number 106/08.03.2017).

We included consecutive Caucasian in- and out-patients with NVAF treated with dabigatran 150 mg twice daily, older than 18 years old, who consented to participate at the pharmacokinetic study and signed the informed agreement. The treatment and dosage were established for each patient by his attending physician based on guidelines recommendations. Patients were advised not to miss doses in the seven days before blood sampling in order to present the steady-state dabigatran levels. Those who presented valvular prosthesis, severe mitral stenosis, severe hepatic and renal failure, or needed an invasive procedure or surgical intervention that required the anticoagulant treatment interruption were excluded.

All patients were evaluated by clinical examination, ECG, and echocardiography (GE Vivid S6) in one visit during the period March 2017 – June 2017. For each individual, the CHA_2DS_2-VASc and HASBLED score were calculated to estimate thromboembolic and hemorrhagic risks. Also, the demographic and clinical parameters (age, body mass index, hepatic and renal function) and concomitant medication were registered in the database.

During the same visit, two blood samples from the antecubital vein were obtained at 12 and 2 hours respectively from the last dose intake (at 8 a.m. and 10:30 a.m.). Two vacuum plastic tubes (4ml Vacuette® tube K3 EDTA) were used for the trough (at 8 a.m.) and peak (at 10:30 a.m., after the intake of one pill) dabigatran's concentration samples. Blood was centrifuged at 2000xg for 20 min and plasma was quickly frozen and stored at −80°C until testing. Quantitative plasma concentrations of dabigatran were assessed in the Department of Toxicology of Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, using a liquid chromatography-tandem mass spectrometry method (LC-MS/MS). Patient samples, 100µL aliquots, were prepared by protein precipitation using mixed methanol-water with hydrochloric acid, containing internal standard [13C6]-dabigatran (Alsachim, Strasbourg, France). Separation of the analyte was achieved on an Acquity BEH column (C18, 1.7µm, 2.1 × 50 mm). Detection was performed by tandem-MS in positive ionization mode using a Waters TQD triple quadrupole mass spectrometer (Waters, Milford, CO, USA). The MassLynx 4.2 software (Milford, MA, USA) was used for data acquisition and processing.

Also a 2ml Vacuette® tube K3 EDTA blood sample was collected at 8 a.m. in order to determine the ABCB1 and CES1 gene variant carriers. The DNA extraction was performed in the Laboratory of the Medical Genetics Department of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca (Wizard Genomic DNA Purification Kit, Promega, Madison, USA). Genotyping for single nucleotide polymorphisms (SNPs) CES1 rs8192935, rs2244613, and ABCB1 rs1045642 was performed using custom-designed SNP TaqMan assays. Genotyping for *ABCB1* rs4148738, and *CES1* rs4580160 was performed using the predesigned SNP TaqMan assays C_1253813_10 and C_11291458_10, respectively. All genotyping assays originated from Thermo Fisher Scientific (Waltham, MA, USA).

Serum creatinine was measured from samples taken at 8 a.m. and creatinine-clearance was calculated using the Cockroft-Gault formula.

All patients were questioned by phone every 6 months to assess the compliance to dabigatran treatment and the occurrence of thromboembolic events or bleeding.

Bleeding events were classified according to the criteria of the International Society on Thrombosis and Haemostasis in: major bleeding (clinically overt bleeding and decrease in hemoglobin level of ≥2.0 g/dL or that require two units of erythrocyte mass transfusion or fatal or symptomatic intracranial, pericardial, or retroperitoneal hemorrhages); clinically relevant non-major (CRNM) bleeding (overt bleedings associated with medical

Table 1.The baseline characteristics of patients

Characteristic Median (range)		Dabigatran 150mg (*n = 37*)
Age, years		70 (62; 77)
Male, n (%)		18 (48.6%)
BMI (kg/m^2)		27.7 (26.6; 31.6)
BSA (m^2)		1.9 (1.7; 2.1)
Plasma levels (ng/ml)	at trough	80 (59; 98.3)
	at peak	130 (111.7; 180)
AF type n (%)	paroxysmal	11 (29.7%)
	persistent	7 (18.9%)
	permanent	19 (51.4%)
Anterior VKA treatment n (%)		23 (62.2%)
CHA_2DS_2-VASC score	1	5 (13.5%)
	≥ 2	32 (86.5%)
GFR CG, ml/min/1.72m^2		84.4 (69.6; 109.8)
Minor bleeding n (%)		5 (13.5%)
aPTT (seconds)	at trough	45.5 (41.2; 49.7)
	at peak	57.2 (52.6; 64)
Concomitant medication	amiodarone	4 (10.8%)
	beta-blockers	27 (73.0%)
	statins	20 (50.4%)
	antiplatelet agents	5 (13.5%)
	NSAIDs	7 (18.9%)

intervention, interruption or discontinuation of dabigatran, that did not encounter the criteria for major bleeding) and minor bleeding (non-clinically consequential overt bleedings, did not meet the criteria for major or CRNM bleeding) (Schulman et al 2005) (Hellenbart, Faulkenberg and Finks n.d.)

Statistical analysis was performed using the MedCalc Statistical Software version 18.11.3 (MedCalc Software bvba, Ostend, Belgium). Quantitative data were expressed as median and interquartile range (IQR). Qualitative data were characterized by frequency and percentage. Deviations of allelic frequencies from Hardy–Weinberg equilibrium were verified using the chi-square test. Correlations between quantitative variables were assessed using Spearman's rank correlation coefficient. The differences between groups were compared with Mann–Whitney test, the Kruskal-Wallis test, or the Chi-square test. Pearson and Spearman's coefficients were used to describe the relationships between data. A p-value < 0.05 was considered statistically significant.

Results

We registered 47 patients with 150 mg dabigatran twice-daily, from which 42 accepted to attend the study visits. Throughout the evaluation, five patients were excluded (refused the second blood sample at 10:30 a.m. after the intake of the morning pill or forgot to take the drug the evening before). Thus, the final analysis included 37 patients. All patients had been on a stable dabigatran 150 mg twice-daily dose for at least one month.

Table 2. Distribution of *ABCB1* and *CES1* genotype and allele frequencies

Gene	SNP	Genotype n (%)		MAF(%)	HWE p value
ABCB1	rs1045642	CC	11 (29.7%)	40.54	0.15
	C>T	TC	22 (59.5%)		
		TT	4 (10.8%)		
	rs4148738	GG	12 (32.4%)	37.83	0.1
	G>A	GA	22 (59.5%)		
		AA	3 (8.1%)		
CES1	rs8192935	TT	19 (51.4%)	25.67	0.21
	T>C	TC	17 (45.9%)		
		CC	1 (2.7%)		
	rs4580160	GG	21 (56.8%)	24.32	0.86
	G>A	GA	14 (37.8%)		
		AA	2 (5.4%)		
	rs2244613	AA	25 (67.5%)	16.66	-
	A>C	AC	12 (32.4%)		

The baseline characteristics of the patients are provided in Table 1. The median age was 70.0 years, range: 62–77.

Of the P-gp inhibitors only amiodarone was prescribed as a concomitant medication in 10.8% of patients. None of the patients was treated with verapamil or quinidine. Proton pump inhibitors or dietary supplements were sporadically used with more than four weeks before blood sampling. Median aPTT values were 57.2 (52.6; 64) at 2 hours and 45.5 (41.2; 49.7) at 12 hours from the dabigatran intake.

The mean follow-up time was 17.74 ± 3.01 months (limits: 12-24). Patients underwent treatment for 16.82 ± 14.06 months (1-60 months) until the blood sampling. All through this period no thromboembolic, major or clinically relevant non-major hemorrhagic events occurred. Only minor bleedings appeared in 13.5% of patients.

In Table 2 is presented the distribution of the genotypes and allele frequencies. The heterozygous genotypes of the *ABCB1* gene polymorphisms predominated. For SNP rs1045642 11 patients (29.7%) presented CC genotype, whereas 22 (59.5%) the CT genotype and four patients (10.8%) the TT genotype. Similarly, 12 patients (32.4%) presented the GG genotype for SNP rs 4148738, 22 (59.5%) the GA genotype, and three patients (8.1%) carried the AA genotype.

Regarding *CES1* polymorphisms, most of the patients were homozygous for the major allele in all subgroups: 51.4% presented TT genotype for the SNP rs8192935, while 17 patients (45.9%) carried TC genotype and only one patient presented the CC genotype (2.7%); 56.8% had the GG genotype of rs4580160, 14 patients presented GA genotype and only two patients carried the AA type (5.4%); finally, for rs2244613 homozygous AA predominated (66.6%), but none of the patients carried the CC genotype. For all other polymorphisms, the genotypes distribution was in agreement with the Hardy–Weinberg equilibrium.

No statistically significant differences were found in the trough or peak dabigatran plasma concentrations between the genotypic groups, for any studied polymorphism. A downward trend of

the dabigatran plasma levels at *CES1* rs8192935 and rs4580160 minor allele carriers was observedbut without statistical significance. The peak plasma levels were lower in the *ABCB1* minor allele carriers for both SNP, with no significance and the trough levels were similar between genotypic subgroups.

Both trough plasma concentration (mean 80 (59; 98.3) ng/mL) and peak plasma concentration (mean 130 (111.7; 180) ng/mL), determined by LC-MS/MS, presented important variability. They were higher in women and in patients treated with amiodarone, but without statistical significance. Patients with heart failure had lower values of plasma concentrations. Creatinine clearance was inversely correlated with dabigatran trough and peak plasma levels (Spearman correlation: $r = -0.215$; $p = 0.2$ and $r = -0.127$; $p = 0.4$ respectively).

An inverse moderate correlation between dabigatran residual plasma concentrations and the BMI (Spearman correlation: $r = -0.353$; $p < 0.05$), and BSA ($r = -0.345$; $p < 0.05$) was noticed. A weak correlation was also observed between trough levels and aPTT values at 12 hours ($r = 0.308$; $p = 0.07$).

Discussions

The remarkable high variability of dabigatran's plasma levels was a fiercely debated topic in the last year's literature. It was mentioned from the beginning in the product characteristics (European Medicines Agency. Pradaxa. Summary of Products Characteristics. Available online: n.d.) and was considered insignificant in patients' treatment (Stangier et al 2008, Douxfils et al 2015; Hawes et al 2013; Chan et al 2015). So far the therapeutic limits for dabigatran's plasma concentrations have not been established. It is considered that residual values lower than 30-50 ng/dl increase the risk of thromboembolic events (Testa et al 2016) and the results derived from RE-LY trial showed that trough plasma levels correlate with hemorrhagic and stroke risk in patients with AF (Chan et al 2015).

Except for the RE-LY trial, few individual studies assessed the dabigatran's plasma levels variability, endeavoring to identify patients at increased risk of adverse events. Very few have used LC-MS/MS, the gold standard in determining these concentrations and all have obtained values with very large variations for both free and total dabigatran (Testa et al 2016; Antovic et al 2013; Skeppholm et al 2014). In our study, all patients presented considerable variations of the trough and peak concentrations, with 8-13 times differences between the minimum and maximum values. Median residual and peak values were similar to those reported in previous studies (Testa et al 2016; Antovic et al 2013; Skeppholm et al 2014).

The NOAC pharmacogenomics data already published are sparse and heterogeneous. The first and unique genome-wide association study (GWAS), using a subset of patients from the RE-LY trial and developed to identify potentially clinically significant genetic determinants of dabigatran blood concentration variability, was published in 2013 (Pare et al 2013). Three genotypes that influenced the plasma levels of dabigatran were identified (two involving the esterase CES1 gene and one the *ABCB1* transporter gene). The only SNP associated with the clinical outcome was *CES1* rs2244613 found in 32.8% of the patients. It was correlated with a 15% decrease in dabigatran's trough plasma concentrations and with a lower risk of bleeding, but not with thromboembolic events (Pare et al 2013).

Subsequently, these *CES1* polymorphisms were investigated in six individual studies that provided inhomogeneous results. The first, by Chin et al, in patients from New Zeeland, demonstrated that the estimates of renal function, but not the studied polymorphisms, can explain 32–47 % of the variability in dabigatran trough plasma concentrations (2014). In the second individual study, with 92 patients with NVAF, Dimatteo et al. revealed a significant association of the SNP *CES1* rs8192935 with dabigatran trough plasma levels (Dimatteo et al 2016), but not with peak levels as it was highlighted in GWAS study (Pare et al 2013). The in vitro study in 104 normal human liver samples, by Shi at al., analyzed the relationship between genetic polymorphisms and DABE activation, the CES1 enzyme's expression, and activity. Neither SNP *CES1* rs2244613, nor rs8192935 correlated, but another polymorphism, *CES1* G143E proved his role in DABE and his intermediate metabolites activation (Shi et al 2016). In the pharmacogenetic study of Gouin-Thibault et al. dabigatran and rivaroxaban were simultaneous investigated in healthy Caucasian males and SNP *CES1* rs2244613 carriers presented lower dabigatran's peak levels ($p=0.43$) (Gouin-Thibault et al 2017), while in the Italian study this SNP was associated with a 2-3% reduction in trough plasma concentrations (Dimatteo et al 2016). Meanwhile, it was published the case of a Chinese patient with a second cardio-embolic stroke and a large thrombus in the left atrial appendage during the anticoagulant treatment with dabigatran. The genetic test showed that this patient was a heterozygous carrier of SNP *CES1* rs8192935 and rs4580160 and homozygous carrier for *CES1* rs2244613, rs4122238 (Gu et al 2018). The most recent individual study, performed in an orthopedic department in Russia did not reveal any significant effect of SNP *CES1* rs2244613 on dabigatran's plasma peak and trough levels (Sychev et al 2018).

In our study, although we did not obtain a statistically significant association between *CES1* polymorphisms and dabigatran's plasma levels, we, however, observed a downward trend of its trough and peak plasma levels with allele carriers of both SNP rs8192935 and rs4580160. Unfortunately, the small number of patients did not allow us to reach a statistical significance.

Also for *ABCB1* gene variations few and conflicting data were published up to the present. In the GWAS study ABCB1 rs4148738 was modestly associated with an increase of dabigatran peak concentration, without influencing the adverse events (Pare et al 2013) and in the individual study of Dimatteo et al (2016) it determined a decrease of trough levels without statistical significance. The SNP ABCB1 rs1045642 was associated with higher peak, but not with trough dabigatran's plasma concentrations in the „crossover" study (Gouin-Thibault et al 2017) and in patients with knee arthroplasty (Sychev et al 2018). In the present research, the peak plasma levels were lower in the ABCB1 minor allele carriers for both SNP without statistical significance; the trough levels were similar between genotypic subgroups.

The extent to which dabigatran plasma levels influence the clinical evolution of patients with AF is highly dependent on demographic factors (Chan et al 2015). Individual clinical studies showed that old age, low body weight, female sex, and renal dysfunction were most commonly associated with high residual plasma concentrations (Šinigoj et al 2015). Also, high CHA_2DS_2-VASc and HAS-BLED scores in combination with low creatinine clearance and APTT ratio of 1.20 were predictive

factors for increased dabigatran plasma concentrations (Owada et al 2015). Similar to the data published by Stangier et al., in our cohort, the values measured in women were higher than those measured in men (Stangier et al 2010). A possible explanation would be that in women *CES1* is more expressed, thus the exposure to dabigatran and its activity are higher (Shi et al 2016; Shi et al 2016). We also obtained negative correlations between dabigatran's plasma levels and BMI as reported by Chan et al (2015).

It is well known that renal function is the major determinant of plasma concentrations (Stangier et al 2010; Blech et al 2008) and patients with chronic renal failure need plasma concentrations monitoring and dose adjustment (Olesen et al 2012; Nishimura 2018). Compared to patients from the RE-LY trial (Connolly et al 2009), in our study, no patients had severe renal impairment. Renal function was only slightly impaired as most patients had creatinine clearance above 70 ml/ min/1.72 m^2.

Another factor that may influence the plasma levels of dabigatran is represented by the drug-drug interaction with P-gp inhibitors (amiodarone, dronedarone, quinidine, verapamil, clarithromycin, ketoconazole) (Heidbuchel et al 2015). In our studied group, of the P-gp inhibitors, only amiodarone was prescribed as concomitant medication. In these patients, both trough and peak plasma concentrations were higher without reaching statistical significance. Being a mild to moderate P-gp inhibitor, amiodarone moderately influences dabigatran's plasma levels (European Medicines Agency. Pradaxa. Summary of Products Characteristics. Available online: n.d.) and the guidelines do not recommend reducing the dose in patients who do not have moderate or severe renal failure (Kirchhof et al 2016; Heidbuchel et al 2015).

We also observed, in the present study, that patients with heart failure had lower values of plasma concentrations. In their paper, Testa et al emphasize the importance of specific laboratory measurements and of dose adjustment in certain patients with very low or high anticoagulant levels at steady state (2018). They claim that the NOAC's plasma levels variability increases considerably in phase IV (postmarketing) studies compared with the healthy subjects or uncomplicated patients in phase II trials (Testa et al 2018). However, a subgroup analysis of patients with symptomatic HF's outcomes from the RE-LY trial, made by Ferreira et al showed that there was no relevant relationship between dabigatran's efficacy or safety and the presence of HF (Ferreira et al 2013). Moreover, few real-world observational studies have evaluated the NOAC's effects in NVAF patients with or without HF. They found that dabigatran was associated with a lower risk of bleeding in NVAF patients with HF compared to warfarin (Amin et al 2019).

The present study has several limitations - the most important, the small number of studied patients. Anticoagulation in AF is a lifelong treatment and dabigatran is an expensive drug, which is not reimbursed by the national health insurance. This is the reason why only a small number of patients afford to buy it and it is also the explanation for the relatively homogeneous features of the study group. Second, adherence to treatment could not be optimally evaluated, becoming a bias. Also, there were a limited number of SNP studied and for each a single determination. Regarding the pharmacokinetic data from the recent literature, all studies highlight the same tendency: the *CES1* gene polymorphisms decrease dabigatran plasma concentrations. Even if all these results are inhomogeneous, the importance of this association lies in the fact that the dabigatran trough plasma levels along with other factors linearly correlate with the patient's clinical outcome. Very few or none of our real-life patients carried the minor alleles for *CES1* polymorphisms which might explain the lack of clinical events. Concerning *ABCB1* polymorphisms, the clear effect on dabigatran pharmacokinetics and clinical importance has not been yet defined.

Conclusions

The genes that encode proteins related to dabigatran's metabolism may explain part of the dabigatran's plasma concentrations variability. The plasma levels of dabigatran are influenced by demographical parameters. Much larger studies are needed to confirm the relationship between genetic polymorphisms and dabigatran's plasma levels and their impact on the clinical consequences in patients with NVAF.

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