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Cyclosporine Metabolites' Metabolic Ratios May Be Markers of Cardiovascular Disease in Kidney Transplant Recipients Treated with Cyclosporine A-Based Immunosuppression Regimens

Ewa Hryniewiecka[1,2] · Jolanta Żegarska[2] · Dorota Żochowska[2] · Emilia Samborowska[3] · Radosław Jaźwiec[3] · Maciej Kosieradzki[4] · Sławomir Nazarewski[5] · Michał Dadlez[3,6] · Leszek Pączek[2,7]

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Abstract

Cardiovascular disease (CVD) remains one of the primary causes of death after kidney transplantation (KTX). Cyclosporine (CsA) metabolites may play a role in CVD. Metabolic ratio (MR) may be considered a measure of intra-individual differences of CsA metabolism. The study was aimed at analysis of associations of CVD with indices of CsA metabolism: MRs and dose-adjusted CsA concentrations (C/D and C/D/kg). The study was performed in the Department of Immunology, Transplant Medicine, and Internal Diseases of the Medical University of Warsaw and involved 102 KTX recipients. Whole blood concentrations of cyclosporine A, AM1, AM9, AM4N, demethylcarboxylated (dMC-CsA), dihydroxylated (DiH-CsA), trihydroxylated (TriH-CsA) cyclosporine metabolites were determined by liquid chromatography coupled with tandem mass spectrometry. Lower AM9/CsA were observed in diabetics. Patients with coronary disease and/or myocardial infarction had lower dMC-CsA/CsA and higher AM4N/CsA. Supraventricular arrhythmia (SVA) was associated with higher AM1/CsA and AM4N/CsA. Hypertriglyceridemia (hTG) was associated with lower AM9/CsA, higher C/D and C/D/kg. Decrease of AM9/CsA and AM4N and higher D/C were associated with overweight/obesity. Systolic blood pressure (BP) positively correlated with dMC-CsA/CsA and negatively with C/D/kg. Diastolic BP correlated positively with AM1/CsA, dMC-CsA/CsA, DiH-CsA/CsA and TriH-CsA/CsA. We have demonstrated the association of coronary disease/myocardial infarction, SVA, hTG, overweight/obesity and elevated arterial BP with higher MRs of AM1, AM4N, dMC-CsA, DiH-CsA and TriH-CsA, and lower MRs of AM9, which may indicate deleterious and favourable effects of individual CsA metabolites on cardiovascular system and suggest engagement of specific enzymatic pathways.

Keywords Kidney transplantation · Cyclosporine · Cyclosporine metabolites · Cardiovascular disease · Arrhythmia · Hypertriglyceridemia · Obesity · Overweight

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✉ Ewa Hryniewiecka
ewa.hryniewiecka@wum.edu.pl

[1] Department of Clinical Nursing, Medical University of Warsaw, 27 Ciolka St, Warsaw, Poland

[2] Department of Immunology, Transplant Medicine and Internal Diseases, Medical University of Warsaw, 59 Nowogrodzka St, Warsaw, Poland

[3] Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Science, 5a Pawinskiego St, Warsaw, Poland

[4] Department of General and Transplantology, Medical University of Warsaw, 59 Nowogrodzka St, Warsaw, Poland

[5] Department of General, Vascular and Transplant Surgery, Medical University of Warsaw, 1a Banacha St, Warsaw, Poland

[6] Biology Department, Institute of Genetics and Biotechnology, 5a Pawinskiego St, Warsaw, Poland

[7] Department of Bioinformatics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5a Pawinskiego St, Warsaw, Poland

Introduction

Cardiovascular disease (CVD) remains one of the primary causes of death after kidney transplantation (KTX) [1, 2]. It is estimated that cardiovascular causes (cardiac arrhythmia, cardiac arrest, heart failure, acute myocardial infarction and coronary disease) account for 28% of deaths after KTX and kidney transplant recipients have a 6.4% higher risk of deaths or major cardiovascular event compared with the general population [1, 3].

Observations and studies conducted since the introduction of cyclosporine A (CsA) to the clinical practice indicate the existence of an association between CsA therapy and cardiovascular morbidity and mortality [4, 5]. Despite many years of research, it has not been determined with certainty whether CsA metabolites play a role in the occurrence of nephrotoxic effects and cardiovascular morbidity.

It is suggested that due to variable metabolic activity resulting from genetic polymorphisms of cytochrome P450 CYP3A4, CYP3A5 and glycoprotein P, as well as various extra-genetic factors, rate of formation of individual CsA metabolites can differ significantly between individuals [6–8]. Cyclosporine metabolites AM1, AM9 and AM4N are considered the first-generation metabolites formed during phase I metabolism and may be detected in the blood [9]. The main P450 enzyme metabolizing CsA is CYP3A4 which contributes to approximately 80% of phase I metabolism and leads to formation of AM1, AM9 and AM4N [10, 11]. Cytochrome CYP3A5 has been also shown to metabolize CsA contributing mainly to formation of AM9 [11]. Metabolic ratio (MR) has been proposed as a parameter calculated by dividing metabolite concentration by the parent drug concentration. It can be considered a measure of variability of concentrations of individual CsA metabolites [12]. The concentration to daily dose ratio or dose-adjusted concentration: C/D [ng/ml/mg/day] and dose/kg-adjusted concentration: C/D/kg [ng/ml/mg/kg/day] may be considered as a general measure of rate of cyclosporine metabolism [7, 13].

The study was aimed at analysis of associations of CVD with cyclosporine metabolites and indices of CsA metabolism: CsA metabolites' MRs and dose-adjusted CsA concentrations.

Materials and Methods

The study was performed in the outpatient clinic of the Department of Immunology, Transplant Medicine, and Internal Diseases of the Medical University of Warsaw (MUW). The study involved 102 KTX recipients from each of whom 2 ml of blood was collected during the routine outpatient visit. The study group consisted of 49 females (48%), mean age was 50.6 years (SD 12.4) and median time from KTX was 116.5 months (IQR 59.3–170.8).

The collected blood specimens were analysed by liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) using Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer (Waters, Manchester, UK) as described previously [14]. We have determined whole blood concentrations of CsA, AM1, AM9, AM4N, demethylcarboxylated cyclosporine metabolites (dMC-CsA), dihydroxylated cyclosporine metabolites (DiH-CsA) and trihydroxylated cyclosporine metabolites (TriH-CsA).

The mass spectrometer operated in the multiple-reaction monitoring–positive electrospray ionization mode. For all analytes mass spectrometer optimized settings were as follows: capillary voltage = 2.5 kV, desolvation temperature = 200 °C, cone gas flow = 150 l/h, desolvation gas flow = 800 l/h, source temperature = 150 °C. The ion transitions were m/z 1219.876 > 1202.658 and m/z 1219.876 > 1184.812 for CsA, m/z 1223.8 > 1206.5 for CsA-D4 (used as internal standard), m/z 1218.68 > 425.6 and m/z 1218.68 > 1182.85 for AM1, m/z 1218.68 > 212.4 and m/z 1218.68 > 449 for AM9, m/z 1188.64 > 661.49 and m/z 1188.64 > 1170.7 for AM4N, m/z 1251.865 > 1234.839 for DiH-CsA, m/z 1267.86 > 1250.83 for TriH-CsA, m/z 1249.88 > 1232.85 for dMC-CsA. Only first was used as quantification transition. The calibration curves range were 0.5–1000 ng/ml, 2–1500 ng/ml and 0.2–100 ng/ml for CsA, AM1 and AM4N, respectively. Concentration of AM9 was calculated using AM1 calibration curve, other CsA metabolites concentrations were quantified using CsA calibration curve.

Clinical and laboratory data were extracted from patients' medical records (chart review) and included in dedicated database. The following disease entities were included in the analyses: coronary disease and myocardial infarction, arterial hypertension (systolic and diastolic blood pressure), peripheral artery disease, stroke, supraventricular arrhythmia (SVA), ventricular arrhythmia, obesity and overweight (body mass index), hypercholesterolemia (serum total cholesterol), hypertriglyceridemia (hTG) [serum triglycerides (TG)] and total CVD. Diabetes mellitus was also included in the analyses because it is a serious problem in kidney transplant recipients and is a significant risk factor for CVD. When assessing the incidence of individual CVDs we have took into account their presence on the day of laboratory assessment, regardless of the precise time of their occurrence (before or after KTX).

Statistical analysis

All calculations were performed using IBM SPSS software (IBM Corp., Armonk, NY, USA). Shapiro–Wilk and Bartlett's tests were used for identification of normally distributed variables for continuous and categorical variables respectively. Variables with normal distribution are presented as mean and standard deviation (SD) and were analysed using Pearson correlation coefficient, Student's *t* test; not-normally distributed variables are presented as median and interquartile range (IQR) and were analysed using Spearman correlation coefficient and *U* Mann–Whitney test. Multivariable analyses were performed by logistic regression and included independent variables: patients' age and gender, eGFR, time from KTX, steroid therapy, precursors of mycoohenolic acid (pMPA) therapy and CsA concentration or daily dose. The differences were considered statistically significant when $p < 0.05$.

Results

Demographic, clinical and laboratory data of the study participants are summarized in Table 1. In addition to anti-rejection drugs kidney transplant recipients took many other medications. Due to the number of medicines taken and the small group size, it was not possible to analyse the relationship between individual drug groups and the parameters of cyclosporine and its metabolites. However, analysed patients did not take many agents listed in the summary of product characteristics as entering the most relevant and best documented interactions, i.e. barbiturates, carbamazepine, oxcarbamazepine, phenytoin, naficillin, intravenous sulfadimidine, probucol, orlistat, ticlopidine, sulfinpyrazone, terbinafine, bosentan, rifampicin, octreotide, metoclopramide, high doses of methylprednisolone, imatinib, colchicine, nicardipine, nefazodone. The most numerous groups of drugs taken by the patients were

Table 1 Clinical and laboratory characteristics of the study group

Parameter	Number (%)	Mean (SD)/median (IQR)
Prednisone	77 (75.5)	–
Mycophenolic acid precursors	67 (65.7)	–
Cyclosporine dose (mg/day)	–	150.0 (125.0–200.0)
Cyclosporine dose (mg/kg/day)	–	2.0 (1.6–2.7)
Cyclosporine concentration	–	87.3 (66.6–114.1)
Cyclosporine dose-adjusted concentration (ng/ml/mg)	–	41.8 (31.3–63.7)
Cyclosporine dose-adjusted concentration per kg (ng/ml/mg/kg)	–	0.56 (0.47–0.77)
Diabetes mellitus	30 (29.4)	–
Coronary disease + myocardial infarction	12 (11.8)	–
Arterial hypertension	97 (95.1)	–
Systolic blood pressure (mmHg)	–	131.0 (12.2)
Diastolic blood pressure (mmHg)	–	80.4 (7.9)
Peripheral artery disease	22 (21.6)	–
Stroke	4 (3.9)	–
Supraventricular arrhythmia	7 (6.9)	–
Ventricular arrhythmia	4 (3.9)	–
Total cardiovascular disease	39 (38.2)	–
Obesity/overweight	15 (14.7)	–
BMI (kg/m^2)	–	26.0 (4.9)
Graft insufficiency (eGFR < 30 ml/min/1.73 m^2)	–	–
eGFR (ml/min/1.73 m^2)	–	49.2 (18.4)
Alanine transaminase (ALT) (IU/ml)		18.0 (13.0–28.9)
Aspartate transaminase (AST) (IU/ml)		18.0 (15.0–27.0)
Total bilirubine (mg/dl)		0.59 (0.47–0.83)
Hypercholesterolemia	70 (68.6)	–
Total cholesterol (mg/dl)	–	201.0 (174.5–230.0)
Hypertriglyceridemia	53 (52.0)	–
Triglycerides (mg/dl)	–	124.0 (107.0–170.0)

BMI body mass index, *eGFR* estimated glomerular filtration rate, *IQR* interquartile range, *SD* standard deviation

medicines used in the treatment of hypertension, coronary heart disease, dyslipidaemia and hyperglycaemia: loop diuretics (41.2%), thiazide diuretics (7.8%), spironolactone (4.9%), angiotensin convertase inhibitors (21.6%), angiotensin receptor blockers (10.8%), alpha-adrenergic blockers (19.6%), beta-adrenergic blockers (70.6%), calcium channel blockers (41.2%), imidazoline receptor agonists (16.7%), insulin (9.8%), oral antidiabetic drugs (11.8%), HMG-CoA reductase inhibitors (52.9%).

Analysis of associations between dose-adjusted and dose/kg-adjusted CsA concentrations has revealed negative correlations of AM9/CsA with C/D ($r=-0.32$, $p=0.01$) and of dMC-CsA/CsA ($r=-0.5$, $p=0.03$).

We have observed lower AM9/CsA in patients with diabetes mellitus (DM) (61.5 [IQR 49.2–71.4] vs 74.8 [IQR 60.1–90.1], $p=0.03$) and it was confirmed in multivariable analysis ($\beta=-0.04$, OR 0.96, 97.5% CI 0.93–0.99, $p=0.01$) (Fig. 1a).

Patients with coronary disease (CD) and/or myocardial infarction (MI) had lower dMC-CsA/CsA ratios (0.53 [IQR 0.43–0.6] vs 0.84 [IQR 0.62–1.14], $p=0.02$). In multivariate analysis CD/MI was associated with higher AM4N/CsA ($\beta=0.83$, OR 2.3, 97.5% CI 1.1–5.3, $p=0.03$).

SVA was associated with higher AM1/CsA (471.6 [IQR 460.8–483.7] vs 389.1 [IQR 317.0–461.6], $p=0.046$) and AM4N/CsA (3.9 [IQR 2.5–4.3] vs 2.8 [IQR 2.1–3.6],

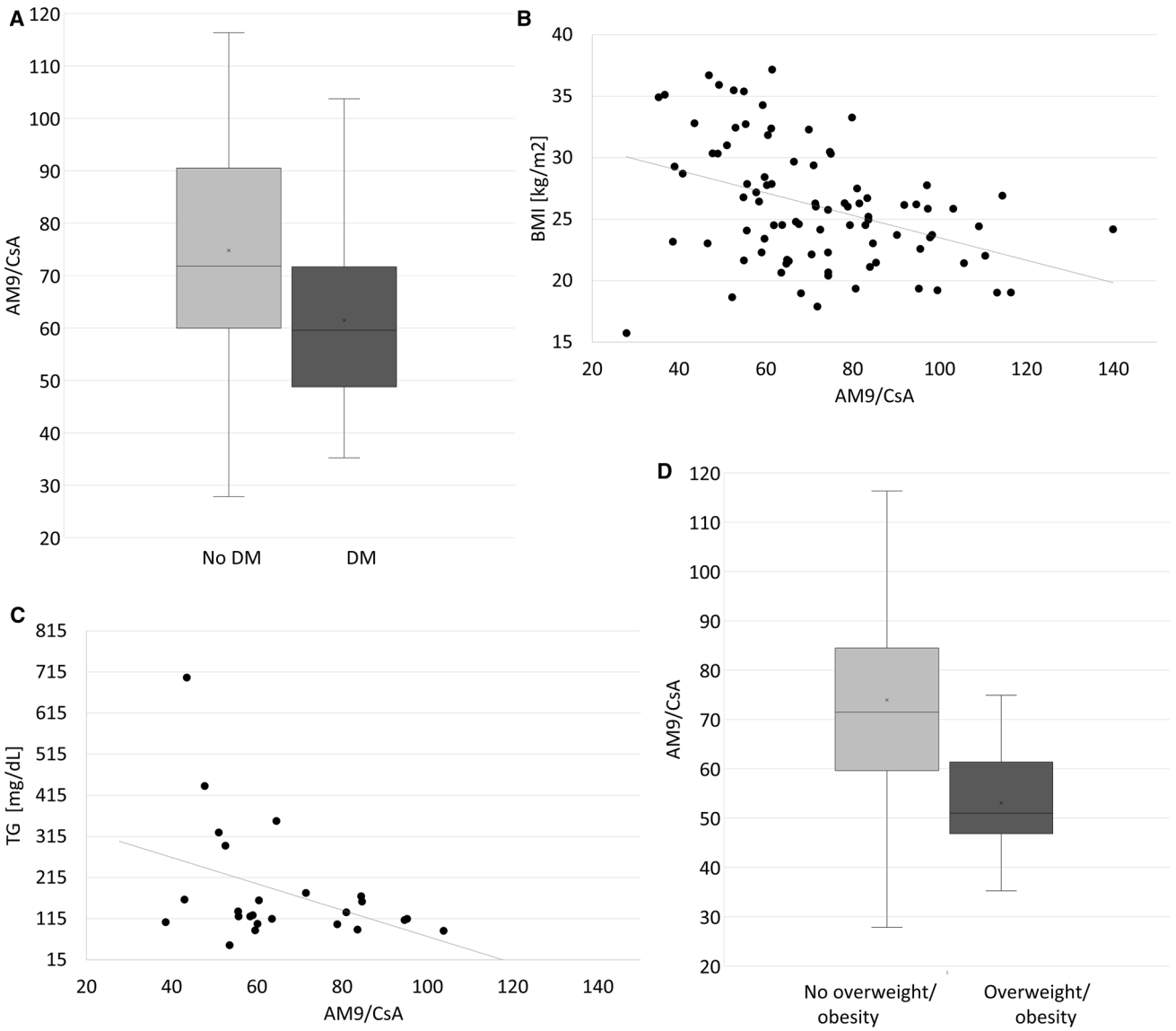

Fig. 1 Associations of AM9/CsA with clinical and laboratory parameters. **a** Lower AM9/CsA levels in patients with diabetes mellitus; **b** negative correlation of AM9/CsA with body mass index; **c** negative correlation of AM9/CsA with blood triglyceride levels; **d** lower AM9/CsA in patients with overweight/obesity. *BMI* body mass index, *DM* diabetes mellitus, *TG* triglycerides

$p=0.03$) which was confirmed in multivariate analysis ($\beta=1.24$, OR 3.5, 97.5% CI 1.6–10.4, $p=0.007$).

hTG was associated with lower AM9/CsA (61.3 [IQR 52.2–74.9] vs 74.3 [IQR 61.8–94.6], $p=0.008$) which was confirmed in multivariate analysis ($\beta=-0.02$, OR 0.98, 97.5% CI 0.95–0.99, $p=0.04$). There was also a negative correlation of TG levels with AM9/CsA ($\beta=-5.6$, $p=0.0003$) (Fig. 1c). hTG was associated with lower metabolism rate expressed by higher dose-adjusted CsA concentrations: C/D (53.7 ng/ml/mg/day [IQR 38.9–67.2] vs 32.6 ng/ml/mg/day [IQR 29.0–41.2], $p=0.000001$) and C/D/kg (0.65 ng/ml/mg/kg/day [IQR 0.54–0.84] vs 0.49 ng/ml/mg/kg/day [IQR 0.45–0.59], $p=0.008$). It was confirmed in multivariate analysis for C/D ($\beta=0.11$, OR 1.12, 97.5% CI 1.05–1.21, $p=0.002$) and C/D/kg ($\beta=3.9$, OR 51, 97.5% CI 2.5–2609, $p=0.03$).

Decrease of AM9/CsA was associated with overweight and obesity (51.0 [IQR 46.8–61.4] vs 71.4 [IQR 59.6–84.4], $p=0.001$) (Fig. 1d) with similar trend for AM4N/CsA (2.32 [IQR 1.95–2.8] vs 2.83 [IQR 2.23–3.7], $p=0.054$), and it was confirmed in multivariate analysis ($\beta=-0.06$, OR 0.04, 97.5% CI 0.89–0.98, $p=0.004$ and $\beta=-0.85$, OR 0.43, 97.5% CI 0.18–0.86, $p=0.03$, respectively). There was also negative correlation of AM9/CsA with BMI ($r=-0,39$, $p=0.00001$) confirmed in multivariate analysis ($\beta=-.07$, $p=0.003$) (Fig. 1b), which revealed also a positive correlation of dMC-CsA/CsA with BMI ($\beta=4.9$, $p=0.003$). In patients with obesity/overweight we have observed higher dose-adjusted CsA concentrations D/C (63.7 ng/ml/mg/day [IQR 48.9–73.5] vs 40.0 ng/ml/mg/day [IQR 30.7–55.9], $p=0.02$) and it was confirmed in multivariate analysis ($\beta=0.06$, OR 1.07, 97.5% CI 1.01–1.13, $p=0.02$).

Using multivariable analysis we have found positive correlation of systolic blood pressure (SBP) with dMC-CsA/CsA ($\beta=9.8$, $p=0.04$), and negative correlation with dose/kg-adjusted CsA concentration C/D/kg ($\beta=-21.3$, $p=0.03$). Diastolic blood pressure (DBP) correlated positively with AM1/CsA ($r=0.22$, $p=0.03$ and $\beta=0.02$, $p=0.03$), dMC–CsA/CsA ($\beta=11.3$, $p=0.0005$), DiH-CsA/CsA ($r=0.34$, $p=0.001$ and $\beta=0.12$, $p=0.0006$) and TriH-CsA/CsA ($\beta=2.2$, $p=0.002$).

Considering the numerous associations between AM9/CsA MR and CV diseases, this metabolite was used to test new parameter: AM9 to total metabolites' concentration ratio (AM9/M_{total}). In general, the results obtained did not differ significantly from those found in respect to AM9/CsA MR. Univariate and multivariable analysis showed lower values of AM9/M_{total} MR in diabetes mellitus (11.8 [IQR 9.9–14.1] vs 13.7 [IQR 11.9–16.2], $p=0.01$ and $\beta=-0.23$, $p=0.01$); obesity and overweight (10.4 [IQR 9.0–13.4] vs 13.6 [IQR 11.6–16.1], $p=0.001$ and $\beta=-0.3$, $p=0.003$), hTG (12.4 [IQR 10.4–13.9] vs 14.6 [IQR 11.9–17.3] and $\beta=-018$, $p=0.008$) and negative correlation with BMI ($r=-0.48$, $p=0.000002$ and $\beta=-0.37$, $p=0.00009$). In addition, we have observed higher AM9/M_{total} MR in patients with CD + MD ($\beta=0.28$, $p=0.006$) in multivariable analysis.

There were no statistically significant differences or correlations for arterial hypertension, peripheral artery disease, stroke, ventricular arrhythmia, hypercholesterolemia and CVD in general. Detailed presentation of analysed relationships can be found in Table 2.

Discussion

The first interesting finding of this study was the general lack of associations of dose-adjusted (C/D) and dose/kg-adjusted (C/D/kg) CsA concentrations with MRs of individual cyclosporine metabolites. If we assume that C/D and C/D/kg ratios are a general measure of the rate of CsA metabolism, then their higher values should occur in so called "poor metabolizers". Those subjects should have lower MR values. However, the inverse relationship was demonstrated only for AM9/CsA and C/D, and dMC-CsA/CsA. This probably means that the greater metabolic clearance of the drug does not fully reflect the extent of the formation of individual metabolites. Thus, C/D and C/D/kg ratios cannot be used as a surrogate for direct measurements of individual CsA metabolite concentrations.

It is well established that CsA therapy is associated with CVD including high incidence of arterial hypertension [15, 16]. Histopathological changes affecting cardiac muscle both in atria and ventricles, such as degenerative changes, myocardial fibrosis and myofibrils' disorganization, were also described [17–19]. Searching for the causes of these complications has led to the identification of certain mechanism that link CsA to adverse effects on the cardiovascular system [5]. In this context the effect of CsA metabolites was also suspected, however, small number of studies were carried out to clarify this issue and they have concentrated mainly on histopathological issues or unfavourable influence of parent drug CsA [18, 20]. The majority of studies analysing mechanism of nephrotoxicity and vasoconstriction pointed at cyclosporine, AM4N, AM1 and AM9 as having the highest nephrotoxic potency [21]. While tubular and mesangial cells viability and proliferation are inhibited mainly by cyclosporine, AM9, AM1A, AM1c, AM1c9 AM4N, AM1 and AM19 [22, 23].

Our analyses revealed significant associations of higher AM4N/CsA MR with coronary disease/myocardial infarction and SVA. This may suggest both the deleterious effect of AM4N on coronary arteries and the promotion of atherosclerotic processes, as well cardiomyocyte damage also at the level of atria. Interestingly, the occurrence of obesity/overweight and higher BMI was accompanied by lower

Table 2 Relationships' between CVDs and cyclosporine metabolites parameters

		CsA (ng/ml)	AM1/CsA MR	AM9/CsA MR	AM9/T-CsA-MR	AM4N/CsA MR	dMC-CsA/CsA MR	DiH-CsA/CsA MR	TriH-CsA/CsA MR	C/D [ng/ml/mg]	C/D/kg [ng/ml/mg/kg]
Diabetes mellitus	U	0	0	↓	↓	0	0	0	0	0	0
	M	–	–	↓	↓	–	0	–	–	–	–
Coronary disease + myocardial infarction	U	0	0	0	0	0	↓	0	0	0	0
	M	–	0	–	↑	↑	0	0	0	–	–
Arterial hypertension	U	0	0	0	0	0	0	0	0	0	0
	M	–	0	0	0	0	–	–	–	–	–
Systolic blood pressure (mmHg)	U	0	0	0	0	0	0	0	0	0	0
	M	–	–	–	0	–	↑	–	–	–	↓
Diastolic blood pressure (mmHg)	U	0	↑	0	0	0	↑	↑	↑	0	0
	M	–	↑	–	0	–	↑	↑	↑	–	0
Peripheral artery disease	U	–	0	0	0	0	0	0	0	0	0
	M	–	–	–	0	–	–	0	–	–	–
Stroke	U	0	0	0	0	0	0	0	0	0	0
	M	–	–	–	0	–	–	–	–	–	–
Supraventricular arrhythmia	U	0	↑	0	0	↑	0	0	0	0	0
	M	–	0	0	0	↑	–	0	–	–	–
Ventricular arrhythmia	U	0	0	0	0	0	0	0	0	0	0
	M	0	–	–	0	–	–	0	–	–	–
Total cardiovascular disease	U	0	0	0	0	0	0	0	0	0	0
	M	–	–	–	0	–	–	0	–	–	–
Obesity/overweight	U	0	0	↓	↓	↓	↑	0	0	↑	0
	M	–	–	↓	↓	↓	–	–	–	↑	–
BMI (kg/m^2)	U	0	0	↓	↓	↓	0	0	0	↑	0
	M	0	0	↓	↓	↓	↑	–	0	↑	↓
Hypercholesterolemia	U	↑	0	0	0	0	0	0	0	0	0
	M	–	–	–	0	–	–	–	–	–	–
Total cholesterol (IU/l)	U	↑	0	0	0	↑	0	0	0	0	0
	M	↑	–	–	0	0	–	–	–	↓	0
Hypertriglyceridemia	U	↑	0	↓	↓	0	0	0	0	↑	↑
	M	–	–	↓	↓	–	0	–	–	↑	↑
Triglycerides (IU/l)	U	0	0	0	0	0	0	0	0	0	0
	M	0	0	↓	0	↓	–	0	0	–	–

BMI, body mass index; dMC-CsA, demethylcarboxylated cyclosporine metabolites; DiH-CsA, dihydroxylated cyclosporine metabolites; TriH-CsA, trihydroxylated cyclosporine metabolites; T-CsA-M, total cyclosporine metabolites; U, univariate tests; M, multivariable tests; MR, metabolic ratio; 0, no relationship; –, analytical model building not possible; ↑, increase of analysed cyclosporine metabolite parameter; ↓, decrease of analysed cyclosporine metabolite parameter

MRs of AM4N/CsA and AM9/CsA. In contrast, in obese/overweight KTX recipients higher dMC-CsA/CsA and dose-adjusted CsA concentrations were observed. Differences in the direction of relationships between MRs of individual metabolites and dose-adjusted CsA concentrations emphasize the variability of the proportion of studied metabolites with respect to general rate of CsA metabolic clearance. The cross-sectional evaluation carried out in this study can not answer weather there is real causal relationship between cardiovascular disorders and the generation of individual metabolites.

Particularly noteworthy is the observation that higher MR AM9/CsA is associated with absence of diabetes mellitus, hTG, overweight/obesity and lower BMI. Akhlaghi et al. have described lower MR of AM1, AM9, AM1c, AM1c9 and AM19 in patients with diabetes mellitus suggesting that it may be the consequence of reduced hepatic biotransformation in diabetic patients [24]. However, it can be also hypothesized that shifting the balance in the formation of individual CsA metabolites towards AM9 may contribute to the reduction of synthesis of other more harmful CsA metabolites. Thus, the increased formation of AM9 would indicate a favourable cyclosporine metabolic profile in an individual KTX recipient. It may be also important that in the formation of AM9 CYP3A5 isoenzyme is involved, while other cyclosporine metabolites are mainly produced by CYP3A4 [11]. The constellation of activity of these two isoenzymes in a given patient may translate into a final metabolic profile of cyclosporine. In the study group we have performed the analysis of genetic polymorphisms of CYP3A4 and CYP3A5 associated with their enzymatic activity, but the incidence of individual genotypes was too low to allow the analysis of their relationships with CVD.

The activity of those enzymes is influenced by many other factors, including liver function, concomitant medications, co-morbidities, certain foods and plant preparations. In the study group liver function could not affect the obtained results because in all patients liver parameters were within normal limits (Table 1). Our patients received numerous medications, mainly antihypertensive and cholesterol lowering drugs. Multivariable analyses included coadministration of steroids and MPA precursors only. Our patients did not take many agents listed in the summary of product characteristics as entering the most relevant and best documented interactions, i.e. barbiturates, carbamazepine, oxcarbamazepine, phenytoin, nafcillin, intravenous sulfadimidine, probucol, orlistat, ticlopidine, sulfinpyrazone, terbinafine, bosentan, rifampicin, octreotide, metoclopramide, high doses of methylprednisolone, imatinib, colchicine, nicardipine, nefazodone. Analyses regarding the impact of allopurinol, ursodeoxycholic acid and oral contraceptives could not be performed due to small number of cases.

Negative correlation of AM9/CsA with higher triglycerides blood levels may be also the consequence of altered CsA metabolism in patients with dyslipidaemia. It was found that hypercholesterolemia inhibits CsA metabolism and leads to higher C/D AUC [25]. We have not observed relationship between CsA metabolites and hypercholesterolemia or total cholesterol blood levels. However, in our study group hTG was associated with higher dose-adjusted and dose/kg-adjusted CsA concentrations. It may suggest that similarly to cholesterol triglycerides could inhibit cyclosporine metabolism.

Similarly to AM4N/CsA MR, increased AM1/CsA ratios were observed in patients with SVA and higher DBP values suggesting unfavourable effect of this metabolite on cardiovascular system. Higher dMC-CsA/CsA MR was found in overweight/obese patients and in relationship with higher SPB and DBP. The only parameter that correlated with DiH-CsA/CsA and TriH-CsA/CsA MR was DBP. This relationship was independent of kidney graft function even though the effect of DiH-CsA and TriH-CsA on eGFR values in KTX patients was observed by others [26]. It has confirmed our previous preliminary results [14]. This may suggest a similar mechanism that contributes to rise of blood pressure and worsening of renal function in the presence of elevated DiH-CsA and TriH-CsA. We cannot exclude that those adverse effects are associated with endothelin-mediated vascular dysfunction observed during 21-day cyclosporine treatment in rats [27]. The importance of those and maybe other metabolites in inducing a rise in arterial pressure indicated the existence of a negative correlation between dose/kg-adjusted CsA concentration and SBP.

It is the first study revealing associations between CVD and individual CsA metabolites and their MRs. We have pointed to specific cyclosporine metabolites, such as AM1, AM9, AM4N, dMC-CsA, DiH-CsA and TriH-CsA, which may play significant role in the development of cardiovascular complications after KTX. The observed relationships may also suggest the contribution of P450 isoenzymes activity to the development of CVD. Due to frequency of occurrence of individual gene polymorphisms determining enzymatic activity of P450, the study group turned to be too small to test this hypothesis and it is the main weakness of the study. Available research data seem to be too weak to recommend monitoring of cardiovascular complications with analysed cyclosporine parameters in routine clinical setting. In addition, due to possibility of the influence of other drugs on the CsA metabolism, further studies in larger patients' groups using standardized co-administered treatment regimens are required. It seems that CsA metabolites' profile in the study group was not affected by graft function because multivariate analyses have included eGFR as one of the independent variables.

In conclusion, we have demonstrated the association of coronary disease/myocardial infarction, SVA, hTG, overweight/obesity and elevated arterial blood pressure with higher MRs of AM1, AM4N, dMC-CsA, DiH-CsA and TriH-CsA, and lower MRs of AM9, which may indicate deleterious and favourable effects of individual cyclosporine metabolites on cardiovascular system and suggest engagement of specific enzymatic pathways.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval The study protocol was approved by the MUW Ethical Committee. All procedures performed were in accordance with the ethical standards of the MUW Ethical Committee and with the 1964 Helsinki declaration and its later amendments.

Research Involving Human and Animal Participants This article does not contain any studies with animal performed by any of the authors.

Informed Consent Before the study procedures all patients have given their written informed consent.

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