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# Prevalence of cardiovascular diseases in kidney transplant recipients and its relationship with asymmetric dimethylarginine, fibroblast growth factor-23 and multiple inflammatory markers

Paulina Wołoszyk-Chojecka[1]✉, Sylwia Małgorzewicz[2,4]✉, Andrzej Chamienia[1,2], Ewelina-Puchalska-Reglińska[3] and Alicja Dębska-Ślizień[2]

[1]Department of Pediatric and Internal Nursing, Medical University of Gdansk, Gdańsk, Poland; [2]Department of Nephrology, Transplantology and Internal Medicine, Medical University of Gdansk, Gdańsk, Poland; [3]7th Naval Hospital in Gdansk, Gdańsk, Poland; [4]Department of Clinical Nutrition, Medical University of Gdansk, Gdańsk, Poland

**Introduction and aim:** Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of endothelial nitric oxide synthase, a marker of endothelial damage and progression of atherosclerosis. Research confirms the association of ADMA with an increased risk of cardiac complications and an increased risk of death, graft loss among kidney transplant recipients (KTRs). The aim of our study was to establish the significance of ADMA and FGF-23 as biomarkers of cardiovascular risk as well as predictors of graft failure and progression of chronic transplant kidney disease in comparison to CKD subjects. In addition, an analysis of the relationship between ADMA, FGF23 and cardiovascular diseases in CKD subjects and KTRs was performed. **Material and methods:** The study group included 132 KTRs. The control group consisted of age- and sex-adjusted 40 individuals with clinically stable CKD. ADMA, FGF-23, hs-CRP and IL-6 were measured by the enzyme-linked immunoassay method (ELISA). Parameters of body mass composition such as fat mass, FTI, lean tissue mass, LTI, body water and overhydration were assessed by multi-frequency bioimpedance analysis (BIA). **Results:** Cardiovascular diseases (CVDs) were present in 31.8% of KTRs. Independent variables related to nutritional status (SGA, s-albumin), according to multivariate regression, may have an impact on the prevalence of CVD in the kidney transplant recipients' group. Our study findings suggested a correlation between ADMA and serum albumin ($r$=-0.41, $p$<0.05), oxLDL ($r$=-0.42, $p$<0.05) and overhydration (OH%, $r$=0.28, $p$<0.05). Moreover, administration of statins and/or angiotensin-converting-enzyme inhibitors was significantly related to a reduction of ADMA in KTRs. We have also identified a significant positive correlation between FGF-23 levels and inflammatory markers (hs-CRP, IL-6) and negative with overall index of renal function (eGFR-CKD EPI, eGFR-MDRD). **Conclusion:** Nutritional status, inflammation and endothelial dysfunction markers (ADMA, FGF-23) are considerably altered even in stable kidney transplant recipients.

**Abbreviations**: MBD, mineral and bone disorder; NO, nitric oxide; NOS, nitric oxide synthase; NS, not significant; NT, not tested; OH, overhydration; oxLDL, oxidized low-density lipoprotein; PTH, parathyroid hormone; ROS, reactive oxygen species; SDMA, symmetric dimethylarginine; SGA, subjective global assessment; TBW, total body water; TC, total cholesterol; TG, triacylglycerols; TNF-α, tumor necrosis factor α; VCAM, vascular cell adhesion molecule; WBC, white blood cells

## INTRODUCTION

Multiple factors, related to Chronic Kidney Disease (CKD), may contribute to endothelial dysfunction and result in the development of Cardiovascular Diseases (CVD) – the leading cause of death in kidney transplant recipients (KTR). Asymmetric dimethylarginine (ADMA), as an endogenous inhibitor of endothelial nitric oxide synthase (NOS), adversely affects the bioavailability of nitric oxide in blood vessels, which contributes to increased prevalence of cardiovascular events, as a result of enhanced proinflammatory, proaggregatory and proliferative activity in an intravascular environment. ADMA is widely regarded as a novel biomarker of the risk of endothelial cell dysfunction and progression of atherosclerosis. The turnover of arginine-methylated proteins provides monomethyl arginine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) (Post *et al.*, 2021), simultaneously both ADMA and SDMA are regarded to be uremic toxins (Oliva-Damaso *et al.*, 2019).

The enzyme, crucial for the regulation of ADMA concentration in tissues and intracellularly, is dimethylarginine dimethylaminohydrolase (DDAH), which initiates the hydrolysis of ADMA to dimethylamine (DMA) and L-citrulline (Oliva-Damaso *et al.*, 2019). DDAH is synthesized mainly in the kidney and liver, while DDAH II in tissues with endothelial NOS expression (Oliva-Damaso *et al.*, 2019). Elimination of ADMA due to urine excretion and metabolism by DDAH as well as a renal synthesis of DDAH partly explain the role of ADMA as a serological marker of increased cardiovascular burden in a patient with kidney impairment (Oliva-Damaso *et al.*, 2019). High ADMA plasma concentration may also affect renal function due to multiple mechanisms, for example, related to impaired renal blood flow, glomerular hypertension or salt accumulation (Rysz *et al.*, 2017; Oliva-Damaso *et al.*, 2019). Moreover, high dietary sodium intake increases the urinary elimination of ADMA (Oliva-Damaso *et al.*, 2019).

According to Post and others (Post *et al.*, 2021) altered protein-arginine demethylation is common in KTR.

Additionally, elevated plasma methylated form of L-arginine – ADMA levels and decreased urinary ADMA and SDMA concentrations may indicate the kidney transplant recipients with a higher risk of all-cause mortality (Post et al. 2021). Research confirms the association of ADMA with an increased risk of cardiovascular morbidity and mortality (Schlesinger et al., 2016; Oliva-Damaso et al., 2019), as well as graft loss (Frenay et al., 2015) among kidney transplant recipients.

Endothelial dysfunction (ED) is the initial phase of vascular damage and atherosclerosis. Patients with renal insufficiency may develop ED as a result of various complications, also related to altered phosphate homeostasis – hyperphosphatemia and vascular calcification (Eskandari et al., 2017). Elevated concentration of serum FGF-23 may have an impact on vascular damage (Ashikaga et al., 2010; Eskandari et al., 2017) and extraskeletal calcification process (Jean et al., 2009; Eskandari et al., 2017) as well as increased mortality and levels of inflammatory markers in CKD patients (Munoz Mendoza et al., 2012; Eskandari et al., 2017).

Fibroblast growth factor-23 (FGF-23), as a hormonal regulator of phosphaturic function (urinary phosphate excretion inductive factor) and vitamin D metabolism (1,25-dihydroxyvitamin D synthesis inhibitory factor), plays a prominent role in this process (Eskandari et al., 2017; Kawabata et al., 2019). On the other hand, Klotho Protein, as well as phosphorus, calcium, 1,25-dihydroxyvitamin D and parathyroid hormone (PTH) have an impact on the secretion and physiologic function of FGF-23 (Kawabata et al., 2019).

Progressive loss of kidney function contributes to the increase of circulating FGF-23 levels, simultaneously, the inability of the kidney to adequately excrete urinary phosphate is contributing to hyperphosphatemia (Kawabata et al., 2019). However, excessive renal phosphorus wasting resulting in the hypophosphatemia is considered to be a possible result of the phosphaturic effect of high FGF-23 level in certain KTRs in the early time after kidney transplantation (KTx) (Ghorbani et al., 2020). Therefore, hyperphosphatemia is defined as a more characteristic condition for pre-transplant patients, whereas, hypophosphatemia frequently occurs after successful KTx and may affect approximately 40,0% of recipients within 1 month after the procedure (Kalantar-Zadeh et al., 2012; Ghorbani et al., 2020).

Mineral and bone disorder (MBD), one of the CKD frequent comorbid conditions, described primarily as abnormalities of mineral metabolism, bone morphology and turnover, leading to the increased fracture risk and potentially to vascular calcification, adversely affects morbidity and mortality of patients with CKD, particularly from cardiovascular events (Hiemstra et al., 2020).

Both, the direct impact of elevated concentration of serum FGF-23 on the renal fibrosis process and indirect suppression of 1,25-dihydroxywitamin D and promotion of proinflammatory cytokines production may exacerbate kidney function and precipitate progression of CKD (Mehta et al., 2020).

## AIM

The aim of our study was to establish the significance of ADMA and FGF-23 as biomarkers of cardiovascular risk as well as predictors of graft failure and progression of chronic transplant kidney disease in comparison to CKD subjects. In addition, an analysis of the relationship between ADMA, FGF23 and cardiovascular diseases in CKD subjects and KTRs was performed.

## MATERIALS AND METHODS

The study group consisted of 132 clinically stable KTRs (57 women; 75 men; mean age, 51.06±13.57; age range, 20–75 years). All patients were treated in the OutpatientTransplantation Unit at the Department of Nephrology, Transplantology, and Internal Medicine, Medical University of Gdansk, Poland and the majority of them were treated with triple immunosuppressive protocols, including glucocorticosteroids. This study was approved by the bioethics committee of the Medical University of Gdansk (NKBBN/291-367/2020, NKBBN/291-437/2018).

The mean time from transplantation was 39.2±61.5 (range, 1–349) months, mean estimated glomerular filtration rate (eGFR), according to the Chronic Kidney Disease – Epidemiology Collaboration (CKD-EPI) equation, was 45.2±24.9 (range, 4.0–94.4) ml/min/1.73 m² in this study population. Other data, such as presence of CVD, diabetes mellitus, and medical treatment were extracted from the medical records.

The control group consisted of 40 age- and sex-matched individuals with clinically stable CKD stage 3–5 (eGFR CKD-EPI, range 6–54 ml/min/1.73 m²). In both groups, criteria to exclude from the study are failure of such organs as lungs, liver, presence of cancer, and acute disease currently under treatment.

### Clinical characteristics

Cardiovascular diseases (CVDs) defined as hypertension and at least one additional condition – atherosclerosis, myocardial infarction, heart failure, cerebral stroke or cardiac arrhythmias, were found in 31.8% of KTRs, whereas almost 88.4% of the study population experienced hypertension. Comorbidities, such as diabetes mellitus, obesity and overweight were found in 31.0%, 20.8% and 35.4% of patients, respectively. Almost 15% of KTRs developed type 3 post-transplant diabetes. Only 7 patients received preemptive kidney transplant (5.0%), whereas 17 (12.9%) of KTRs underwent retransplantation. The majority of patients included in this study were treated with triple immunosuppressive protocols. Moreover, the most commonly prescribed immunosuppressive drugs were calcineurin inhibitors – tacrolimus (74.3%) and cyclosporine (23.8%), mycophenolate mofetil (86.6%) and glucocorticosteroids – methylprednisolone (84.8%). The remaining drugs, such as insulin, beta blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors (ACE-i) and statins were used respectively by 20.5%, 59.8%, 40.9%, 8.3% and 14.4% of KTRs.

### Control group

Most CKD patients included in the control group had a history of hypertension (84.4%) and were treated with ACE inhibitors (29.6%). Lipid disorders, particularly hypercholesterolaemia, and statin administration, were found in 43.7% and diabetes mellitus was diagnosed in 28.0% of control group subjects. Three patients had a history of coronary disease, and two of them experienced a myocardial infarction. Medical conditions that contributed to renal dysfunction in both the study and control group are presented in Table 1.

### Laboratory Assay

In all study participants plasma samples were taken after an overnight fast and stored at –80°C until analyzed.

**Table 1. Main causes of CKD in KTRs and control group.**

|  | KTRs | CG |
|---|---|---|
| Subjects | 101 | 31 |
| Chronic glomerulonephritis | 17 | 11 |
| Chronic pyelonephritis | 12 | 0 |
| Polycystic kidney disease | 18 | 2 |
| Hypertensive nephropathy | 5 | 3 |
| Diabetic Nephropathy | 15 | 2 |
| Obstructive nephropathy | 2 | 0 |
| Unknown nephropathy | 17 | 4 |
| Schoenlein-Henoch nephropathy | 2 | 0 |
| IgA nephropathy | 5 | 2 |
| Other | 8 | 7 |
| Date unknown | 31 | 9 |

The following compounds were measured in plasma or serum by an enzyme-linked immunoassay method:
- High sensitive C-reactive protein (hs-CRP, ELISA, DRG MedTek),
- Asymmetric dimethylarginine (ADMA Xpress ELISA, plasma),
- Tumor necrosis factor α (TNF-α, ELISA, Immunodiagnostik AG, serum)
- Oxidized low-density lipoprotein (oxLDL, ELISA, Immunodiagnostik AG, serum)
- Fibroblast growth factor-23 (FGF-23 Intact Human ELISA, Biomedica Poland, plasma),
- Interleukin-6 (Human IL-6 Quantikine ELISA, DRG MedTek, serum).
- Serum albumin, creatinine, blood urea nitrogen (BUN), sodium, potassium, magnesium, lipidogram, and blood morphology were assessed according to routine methods in the hospital clinical laboratory.

The following anthropometric measurements were obtained:
- Body mass (kg), arm and waist circumference (cm).
- BMI estimated according to the current body mass/height$^2$:BMI range <18.5 was considered underweight,
- 18.5–24.99 – normal weight,
- 25–30 – overweight,
- ≥30 – obesity.

Nutritional status was estimated by 7-SGA (7-point subjective global assessment):
- 6–7 – good nutritional status (not at risk for malnutrition),
- 3–5 – mild malnutrition (at risk for malnutrition),
- 1–2 – severe malnutrition

Parameters of body mass composition were assessed by the multi-frequency bioimpedance analysis (BIA) method with a BCM (Body Composition Monitor, Fresenius SA, Bad Homburg, Germany). Evaluated body mass composition parameters included:
- lean tissue mass (LTM) and lean tissue index (LTI),
- fat tissue mass (FAT) and fat tissue index (FTI),
- body water (TBW, total body water; ICW, intracellular water; ECW, extracellular water; E/I ratio; OH, overhydration).

The biochemical and anthropometric characteristics of the study and control group are presented in Table 2.

## Statistical Analysis

Statistical analysis was performed using Statistica 13.3 version for Windows. All data are presented as mean ± S.D. Comparisons between the groups were examined by Student's $t$-test (for parametric data) and U Mann-Whitney Rank Sum Test (for non-parametric data).

Spearman's correlation was used for a nonparametric measure of statistical dependence between 2 variables. Independent associations among variables were assessed with stepwise multiple regression analysis. For all performed analyses $p < 0.05$ was considered as statistically significant.

**Table 2. The biochemical and anthropometric characteristics of the study and control group.**

|  | KTRs | Control group |
|---|---|---|
| Sex (F/M) | 57/75 | 14/26 |
| Age (years) | 51.06±13.57 | 52.76±19.67 |
| BMI | 25.87±4.09 | 27.07±5.65 |
| BMI ≤ 25 (%) | 56.1 | 65.7 |
| Creatinine (mg/dL) | 2.00±1.60 | 2.83±1.92 |
| eGFR – CKD EPI (ml/min/1.73 m$^2$) | 47.00±23.70 | 29.97±14.41 |
| hs-CRP (mg/L) | 4.88±4.15 | 5.55±3.71 |
| WBC (10$^9$/L) | 8.70±3.19 | 7.02±3.86 |
| Haemoglobin (g/dL) | 11.67±2.56 | 11.46±2.37 |
| BUN (mg/dL) | 41.85±26.00 | 43.95±23.00 |
| Proteinuria (g/24h) | 0.44±0.53 | NT |
| Uric Acid (mg/dL) | 7.10±1.81 | NT |
| Glucose (mg/dL) | 114.58±42.40 | 113.5±14.65 |
| Albumin (g/L) | 35.40±6.21 | 34.75±5.95 |
| TC (mg/dL) | 210.65±45.29 | 184.00±47.48 |
| TG (mg/dL) | 171.40±80.24 | 148.31±77.02 |
| LDL (mg/dL) | 136.27±41.47 | 108.71±40.08 |
| HDL (mg/dL) | 46.73±10.48 | 48.00±19.67 |
| Sodium (mmol/L) | 139.36±3.23 | NT |
| Potassium (mmol/L) | 4.37±0.49 | 4.63±0.51 |
| Magnesium (mg/dL) | 1.81±0.30 | NT |
| Phosphate (mg/dL) | 3.50±1.40 | 3.79±0.71 |
| Calcium (mg/dL) | 9.33±0.99 | 9.35±0.66 |

BMI, Body Mass Index; BUN, Blood Urea Nitrogen; CKD-EPI, Chronic Kidney Disease, Epidemiology Collaboration equation; hs-CRP, high sensitivity C-reactive protein; eGFR, estimated Glomerular Filtration Rate; HDL, High-density lipoprotein; TC, Total cholesterol; LDL, Low-density lipoprotein; NT, not tested; TG, Triacylglycerols; WBC, white blood cells

**Table 3. Patient characteristics according to nutritional status (SGA + s-albumin) in KTX group**

| Parameters | Total Sample Median (% or IQR or SD) | Not at Risk Median (IQR) | At Risk for Malnutrition Median (IQR) |
|---|---|---|---|
| Subjects | 128 | 92 (71.9%) | 36 (28.1%) |
| **Sex** | | | |
| Male | 72 (56.3%) | 51 (55.4%) | 21 (58.3%) |
| Female | 56 (43.7%) | 41 (44.6%) | 15 (41.7%) |
| SGA | 3–7 | 6–7 | ≤5 |
| BMI | 25.87±4.12 | 26.26±4.24 | 24.89±3.68 |
| FAT (%) | 34.20±8.98 | 34.32±9.16 | 33.85±8.57 |
| FTI | 12.40±4.55 | 12.61±4.70 | 11.76±4.07 |
| LTM (%) | 50.55±12.04 | 50.81±12.00 | 49.77±12.38 |
| LTI | 12.68±2.50 | **13.01±2.53*** | **11.70±2.20*** |
| TBW (%) | 48.92±6.34 | 48.67±6.18 | 49.64±6.81 |
| ECW (L) | 17.71±3.62 | 17.84±3.68 | 17.32±3.45 |
| ICW (L) | 18.56±4.05 | **19.13±4.13*** | **16.87±3.31*** |
| E/I (L) | 0.97±0.14 | **0.94±0.14*** | **1.04±0.13*** |
| OH (%) | 11.83±9.38 | **10.41±9.87*** | **15.88±6.41*** |
| Arm circumference (cm) | 30.43±3.70 | **31.34±3.58*** | **28.02±2.88*** |
| Serum albumin level (g/L) | 35.40±6.21 | 36.37±6.88 | 33.00±3.28 |
| Age (years) | 51.00±13.65 | **48.47±13.18*** | **57.44±12.83*** |
| Time after TX (months) | 39.17±61.48 | 39.75±62.50 | 37.62±59.56 |
| ADMA (µM/L) | 0.96±0.31 | 0.95±0.32 | 0.97±0.30 |
| FGF-23 (pg/ml) | 570.14±840.86 | 515.33±800.50 | 688.06±923.62 |
| hs-CRP (mg/L) | 4.85±4.10 | 4.67±4.13 | 5.26±4.04 |
| Diabetes | 41 (31.0%) | 24 (26.1%) | 17 (42.2%) |
| CVD | 42 (31.8%) | 25 (27.2%) | 17 (47.2%) |

*p<0.05; ADMA, Asymmetric dimethylarginine; FGF-23, Fibroblast growth factor-23; IL-6, Interleukine-6; SGA, Subjective Global Assessment; BMI, Body Mass Index; CRP, C-reactive protein; hs-CRP, high sensitivity C-reactive protein; FTI, fat tissue index; LTM, Lean tissue mass; LTI, lean tissue index; TBW, Total body water; ECW, Extracellular water; ICW, Intracellular water; E/I, extracellular water/intracellular water ratio; OH, overhydration

## RESULTS

### Anthropometry

Body mass index correlated positively with body fat ($r$=0.54, $p$<0.05), arm ($r$=0.79, $p$<0.05) and waist ($r$=0.84, $p$<0.05) circumferences respectively. The mean percentage of body fat was 34.20±8.98% (range, 4.3–55.6%). Fat tissue index (FTI) was 12.40±4.55 kg/m², and lean tissue index (LTI) was 12.68±2.50 kg/m². Patients with excessive body mass were identified by BMI; 35.4% of KTRs were classified as overweight and 20.8% were obese.

In our study group, KTRs with altered body composition – excessive body mass (BMI ≥25) with the simultaneous decline of lean tissue index < 12 (LTI, 11.56±1.37 kg/m²) and high fat tissue index >14 (FTI, 16.59±3.89 kg/m²) – presented a significant and approximately two-fold increase in the level of hs-CRP (6.51±4.68 mg/L *vs.* 3.14±3.14 mg/L), than patients with normal range of above-mentioned parameters (BMI<25, LTI>12, FTI<14). The nutritional status and anthropometric characteristics of kidney transplant recipients are included in Table 3.

### Cardiovascular risk factors

Cardiovascular diseases (CVDs) were present in 31.8% of KTRs, and the prevalence of CVD in relation to BMI is presented in Table 4. Inflammatory and endothelial dysfunction markers in the study and control group did not differ significantly. Only the FGF 23level was significantly higher in KTRs ($p$<0.05).

The negative correlation between ADMA and serum albumin ($r$=–0.41, $p$<0.05) and positive with overhy-

**Table 4. The prevalence of CVD depending on the BMI**

| BMI | The Percentage of Patients Diagnosed with CVD |
|---|---|
| <18.5 | 0 (0.0%) |
| 18.5–24.99 | 13 (30.95%) |
| 25–30 | 18 (42.85)% |
| ≥30 | 11 (26.20%) |

BMI, body mass index; CVD, cardiovascular diseases

**Table 5. Inflammatory and endothelial dysfunction markers in the study and control group**

| | KTRs | Control group |
|---|---|---|
| hs-CRP (mg/L) | 4.88±4.15 | 5.55±3.71 |
| IL-6 (pg/ml) | 10.58±16.48 | 14.95±18.55 |
| ADMA (µM/L) | 0.96±0.31 | 0.90±0.68 |
| FGF-23 (pg/ml) | **573.29±847.12*** | **258.68±360.87*** |
| TNF-α | 6.88±5.37 | NT |
| oxLDL (U/L) | 187.20±280.52 | NT |

*p<0.05; ADMA asymmetric dimethylarginine; FGF-23 fibroblast growth factor-23; IL-6 interleukine-6; TNF-α tumor necrosis factor α; CRP C-reactive protein; hs-CRP high sensitivity C-reactive protein; oxLDL oxidatively modified low-density lipoprotein; NT-not tested

dration (OH%, $r$=0.28, $p$<0.05) was noticed. Moreover, administration of statins and/or angiotensin-converting-enzyme inhibitors were significantly related to lower ADMA levels in KTRs (no statins 1.0±0.31 *vs.* statins 0.79±0.24; no ACEI 0.99±0.3 *vs.* ACEI 0.75±0.3 µM/L, $p$<0.05).

We have also identified a significant positive correlation between FGF-23 levels and inflammatory markers (hs-CRP, IL-6) and negative with an overall index of re-

**Table 6. Inflammatory markers and other parameters depending on FGF-23 levels in the KTRs (*p*<0.05)**

|  | FGF-23 ≤ 100pg/ml | FGF-23 > 100pg/ml |
|---|---|---|
| FGF-23 (pg/ml) | 71.77±20.35 | 945.40±963.35 |
| hs-CRP (mg/L) | 3.10±3.12 | 6.10±4.22 |
| IL-6 (pg/ml) | 6.41±7.37 | 13.95±20.60 |
| eGFR – CKD EPI (ml/min/1.73 m2) | 60.60±18.70 | 31.10±21.1 |
| BUN (mg/dL) | 26.37±14.31 | 55.14±27.42 |
| Proteinuria (g/24h) | 0.21±0.33 | 0.60±0.63 |
| Time after TX (months) | 69.60±63.64 | 28.15±59.80 |

BUN, Blood Urea Nitrogen; eGFR, estimated glomerular filtration rate; FGF-23, fibroblast growth factor-23; hs-CRP, high sensitivity C-reactive protein; IL-6, interleukine-6

**Table 7. The Spearman correlation between different variables (*p*<0.05).**

|  | ADMA | FGF-23 | hs-CRP | IL-6 | FTI | LTI |
|---|---|---|---|---|---|---|
| IL-6 | R=0.088 (NS) | R=0.388 | R=0.533 | – | R=0.329 | R=−0.326 |
| eGFR-MDRD | R=−0.008 (NS) | R=−0.661 | R=−0.420 | R=−0.324 | R=−0.254 | R=0.080 (NS) |
| Albumin | R=−0.410 | R=−0.350 (NS) | R=−0.133 (NS) | R=−0.177 (NS) | R=0.358 | R=0.120 (NS) |
| Proteinuria | R=0.354 | R=0.597 | R=0.207 (NS) | R=0.261 (NS) | R=−0.207 (NS) | R=0.178 (NS) |

ADMA, asymmetric dimethylarginine; eGFR-MDRD, estimated Glomerular Filtration Rate – The Modification of Diet in Renal Disease; FGF-23, fibroblast growth factor-23; FTI, fat tissue index; hs-CRP, high sensitivity C-reactive protein; IL-6, Interleukine-6; LTI, lean tissue index; NS, not significant

**Table 8. The prevalence of CVD depending on the nutritional status**

| Regression Model | B | Standard Error | Beta | *p*-Value |
|---|---|---|---|---|
| Constant | 3.5 | 1.0 |  | 0.002 |
| SGA | −0.20 | 0.17 | −0.40 | 0.03 |
| s-Albumin | −0.05 | 0.21 | −0.58 | 0.01 |
| Proteinuria | 0.05 | 0.21 | 0.04 | 0.82 |
| eGFR (CKD-EPI) | 0.00 | 0.20 | 0.04 | 0.84 |

CVD, cardiovascular disease; eGFR (CKD-EPI), estimated glomerular filtration rate Chronic Kidney Disease Epidemiology Collaboration; s-Alb, serum albumin; SGA, Subjective Global Assessment

nal function (eGFR-CKD EPI). Detailed information on inflammatory and endothelial dysfunction parameters in study groups is included in Table 5 and Table 6. The correlations between studied parameters are presented in Table 7 and Figs 1–3.

### Multivariate regression

Multivariate regression model was established to predict the impact of the independent variables, related to nutritional status (SGA, s-albumin), on the prevalence of CVD in the kidney transplant recipients' group (the adjusted R2 of the model was 0.36; *p*=0.002). Analysis showed that nutritional status assessed by SGA and s-albumin may have a significant impact on the prevalence of CVD in the kidney transplant recipients' group.
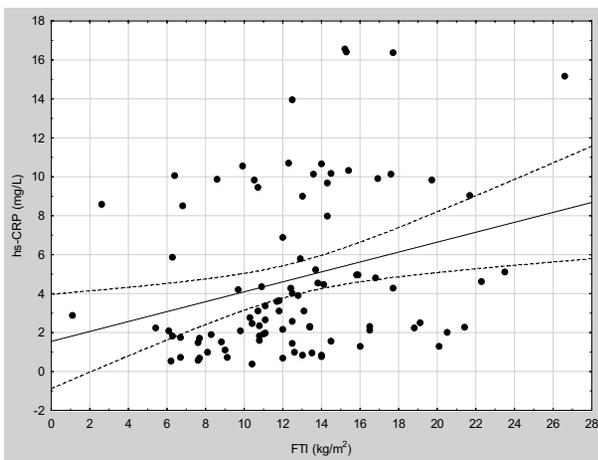
Detailed information is provided in Table 8.
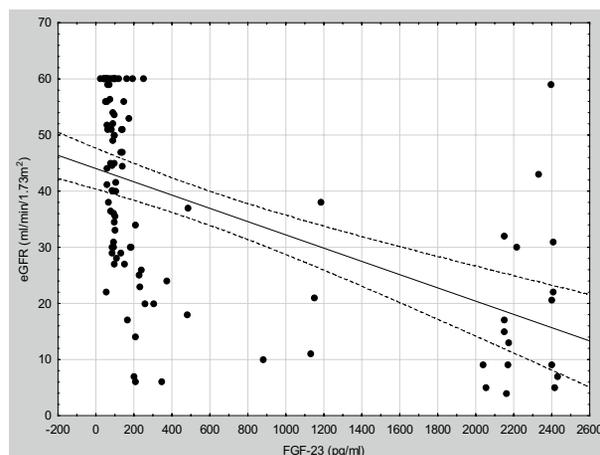


**Figure. 1. The correlation between FGF-23 and eGFR in KTR group.**
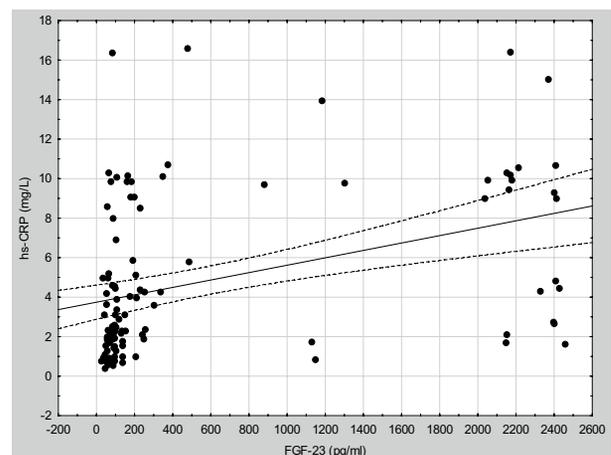


**Figure. 2. The correlation between FGF-23 and hs-CRP in KTR group.**

**Figure. 3. The correlation between FTI and hs-CRP in KTR group.**

## DISCUSSION

Our study focused on investigating the correlation between ADMA and FGF-23 level and the prevalence of CVD among patients after kidney transplantation. Results show a high prevalence (31.8%) of CVD in KTRs. ADMA and FGF 23 were associated with laboratory signs of inflammation, nutritional status and graft function. The highest percentages of patients with CVD diagnosis were overweight. The presented results confirm that CVD is a major and complex health problem among KTRs.

## ADMA

The discovery of asymmetric dimethylarginine may enable a more complete understanding of nitric oxide's importance and role in cardiovascular protection. The normal range of ADMA in a general population of healthy subjects is 0.22–0.69 μmol/L (Deneva-Koycheva et al., 2011; Vilcea et al., 2020). Elevated plasma levels of ADMA may contribute to the promotion of atherosclerosis processes, cardiovascular events and their further consequences – higher mortality rate and diminished quality of life (Mehta et al., 2020). The connection between asymmetric dimethylarginine (ADMA) and endothelial dysfunction adversely affects the intima-media thickness of the carotid arteries (Wang et al., 2018), moreover, it may also determine the risk of coronary heart disease, stroke and peripheral arterial disease (Vilcea et al., 2020). ADMA, as an independently functioning cardiovascular risk factor, simultaneously involved in the increased progression of chronic renal disease, due to direct inhibition of the endothelial nitric oxide synthesis (eNOS) and promotion of inflammation and oxidative stress, which contributes to cardiovascular and renal pathology (Vilcea et al., 2020). Moreover, diminished renal excretion of ADMA as a result of possible hypertension or reduced renal blood perfusion after heart failure may have an additional impact on ADMA's elevated concentration in plasma (Liu et al., 2018). Therefore, precise monitoring of ADMA level and evaluation of renal function, especially in the hypertensive patient group, may have an important role in the prevention of cardiovascular events (Vilcea et al., 2020).

According to clinical studies, approximately less than 20% increase in ADMA plasmatic level is generally noted in certain cardiovascular diseases (e.g. coronary artery

disease, hypertension, heart failure), in contrast to severe CKD patients, who may reach even 4 to 10 fold higher level of ADMA concentration in blood (Liu et al., 2018). Therefore, adverse effects of ADMA excess – involving abnormalities of the endothelial cell function, angiogenesis process, vascular injury repair, NO and ROS altered generation, in conjunction with the severity of CKD, profoundly affect cardiovascular morbidity and mortality (Liu et al., 2018). According to Zoccali's et al. (2001) prospective evaluation of 225 hemodialysis patients, an increase in plasma concentration of asymmetrical dimethylarginine was the second (after age) strong risk factor of overall mortality and cardiovascular mortality in the ESRD population. Moreover, according to Fliser and others study (Fliser et al., 2005), the elevated ADMA level may contribute to significantly faster (52.8 mo vs. 71.6 mo) nephropathy progression. In our study group of KTRs, plasma ADMA concentration correlated negatively with the eGFR ratio, emphasizing its importance in the process of renal impairment. According to Kielstein and others (Kielstein et al., 2004), elevated levels of ADMA cause decreased renal perfusion. Possible mechanisms of diminished renal plasma flow might be related to proteinuria, which leads to the damage of renal tubules cells, subsequently, DDAH enzyme insufficient renal expression, caused by tubular dysfunction, results in the excess of ADMA concentration in the blood and tissues (Kielstein et al., 2007; Raptis et al., 2013). As a consequence, renal perfusion continually decreases, with its negative effects on ADMA cumulative concentration, kidney impairment progression and hypertension. In our study group statistical analysis confirmed a significant positive correlation between ADMA concentration and proteinuria ($r=+0.35$, $p<0.05$); additionally, we also identified a negative correlation with serum albumin ($r=-0.42$, $p<0.05$).

Elevated inflammatory markers (CRP, IL-6), together with ADMA high plasma levels in subjects with advanced renal failure, according to numerous studies (Zoccali et al., 2001; Zoccali et al., 2002; Aucella et al., 2009; Tripepi et al., 2011), remain in clinically confirmed correlation and play an important role as a predictive factor for atherosclerotic changes, cardiovascular events and overall mortality.

Based on the results obtained from the current study, the correlation between ADMA and serum albumin ($r=-0.41$, $p<0.05$) and overhydration (OH%, $r=0.28$, $p<0.05$) are strongly suggested. Also, regression analysis shows an association between the nutritional status (albumin) and CVD which may suggest that low albumin as an acute phase protein and indicator of malnutrition is associated with a higher risk of endothelium damage.

According to Frenay and others (Frenay et al., 2015), certain kidney transplant recipient subpopulation classified with ADMA concentration ≥0.66 μmol/l was characterized by excessive albuminuria and also elevated levels of FGF-23, PTH and serum phosphorus concentration.

According to our findings, ADMA elevated plasma concentration in the subgroup of patients with the presence of CVDs were non-statistically significant, however, we have observed an upward trend in comparison to KTRs without CVDs and in the control group, nonetheless, comparison to healthy subjects is still recommended. The suppressive effect of hyperglycemia on DDAH enzyme activity, due to diabetes mellitus and/or insulin resistance, may partly explain a presumptive mechanism of ADMA excessive accumulation in these conditions, however, a comprehensive pharmacotherapy approach to

the treatment of diabetes mellitus (with metformin and thiazolidinediones usage) might have a positive impact on ADMA regulation (Napora *et al.*, 2006).

Numerous studies have suggested the positive therapeutic impact of statin administration on circulating asymmetric dimethylarginine reduction. According to Serban's and others (Serban's *et al.*, 2015) meta-analysis study, the hydrophilic statin administration (rosuvastatin, pravastatin and fluvastatin), but not hydrophobic statins, significantly decreased plasma ADMA concentration. In the present study, the administration of statins and/or angiotensin-converting-enzyme inhibitors was significantly related to a lower level of ADMA in KTRs.

## FGF-23

Fibroblast growth factor 23 (FGF-23), as a regulative component of renal phosphate reabsorption and 1,25(OH)2D synthesis, is a crucial part of mineral and bone homeostasis (Chu *et al.*, 2021). Laboratory evaluation of FGF-23 concentration in humans, allows the detection of two available types of FGF-23 – an intact FGF-23 form (iFGF-23) and C-terminal fibroblast growth factor 23 form (cFGF-23), additionally, the cFGF-23 ELISA assay might be able to provide more precise results (Chu *et al.*, 2021), since, according to recent findings (Sharma *et al.*, 2020; Chu *et al.*, 2021), inflammation and iron status might interfere with the effects of iFGF-23 correlations. Notwithstanding those reservations, in our study, we evaluated the plasma concentration of intact fibroblast growth factor 23 form in KTRs and control subjects.

Current literature strongly suggest that an increased circulating level of FGF-23 is independently associated with cardiovascular and all-cause mortality after kidney transplantation (Wolf *et al.*, 2011; Baia *et al.*, 2013), overall graft loss in kidney transplant recipients (Wolf *et al.*, 2011; Chu *et al.*, 2021) and progression of chronic kidney disease (CKD) (Wolf *et al.*, 2011). Moreover, recent research emphasizes plasma FGF-23 importance in the process of inflammation, erythropoiesis, functional iron deficiency and anaemia (David *et al.*, 2016; David *et al.*, 2017; Edmonston *et. al.*, 2020; Mace *et al.*, 2020). Our findings suggest the link between considerably diminished renal function evaluated by reduced glomerular filtration rate and the higher fibroblast growth factor 23 concentration in KTRs.

Additionally, a significant correlation for FGF-23, obtained in our study group, has been associated with hs-CRP ($r=0.46$, $p<0.05$), IL-6 ($r=0.4$, $p<0.05$), hemoglobin ($r=-0.55$, $p<0.05$) and ferritin ($r=0.56$, $p<0.05$).

Research results submitted by Malyszko *et al.* (2014), which concern the mechanisms of endothelial cell injury in kidney transplant recipients, have also emphasized the importance of disturbances in the FGF23-Klotho system for future cardiac outcomes (Liabeuf *et al.*, 2018). According to Yilmaz and others (Yilmaz *et al.*, 2013), after a successful kidney transplant procedure, the apparent improvement in endothelial function actually begins to occur simultaneously with a gradual decline in FGF-23 levels (Liabeuf *et al.*, 2018). In addition, numerous observational studies have revealed independent correlations of excessive FGF-23 plasma levels with other parameters, such as eGFR (Baia *et al.*, 2013), serum phosphate (Baia *et al.*, 2013; Malyszko *et al.*, 2014), hemoglobin (Baia *et al.*, 2013), donor type (living or deceased) (Baia *et al.*, 2013), IL-6 (Malyszko *et al.*, 2014), vascular cell adhesion molecule (VCAM) (Malyszko *et al.*, 2014), time after transplantation (Malyszko *et al.*, 2014), mean corpuscular volume (Malyszko *et al.*, 2014) and more. Our study findings clearly suggest a statistically significant positive correlation between FGF-23 levels and inflammatory markers (hs-CRP, IL-6) and negative with graft function (eGFR-CKD EPI, eGFR-MDRD).

## LIMITATIONS AND OUTLOOK

Our findings suggest that the inflammatory and endothelial dysfunction markers, as well as nutritional status, are considerably altered in kidney transplant recipients. The recent interest in novel endothelial dysfunction markers creates an opportunity for early prediction of future cardiac outcomes and/or renal disease progression. Accurate diagnosis and early treatment are crucial for improving patient outcomes. Further research is needed to entirely understand the link between investigated biomarkers and their association with life- threatening complications of the cardiovascular system. The limitation of our study is the relatively small group of patients, but despite its limitation, the results of the present study are valuable because they indicate a problem of occurrence of untraditional risk factors of CVD also in patients after TX, such as inflammation and nutritional status. Further studies in KTRs (e.g., multicenter) are needed to confirm our results.

## CONCLUSIONS

Nutritional status, inflammation and endothelial dysfunction markers (ADMA, FGF-23) are considerably altered even in stable kidney transplant recipients. hs CRP, Il-6 and ADMA were comparable to CKD patients, and FGF 23 was higher in KTRs. The upward trend of elevated ADMA plasma concentration in KTRs with the presence of CVDs as well as the link between hs-CRP and the higher blood level of fibroblast growth factor 23 – were observed in our study. The presented study indicated a problem of occurrence of untraditional risk factors of CVD in patients after kidney transplantation, such as inflammation and deterioration of the nutritional status.

## REFERENCES

Ashikaga E, Honda H, Suzuki H, Hosaka N, Hirai Y, Sanada D, Nakamura M, Nagai H, Matsumoto K, Kato N, Mukai M, Watanabe M, Takahashi K, Shishido K, Akizawa T (2010) Impact of fibroblast growth factor 23 on lipids and atherosclerosis in hemodialysis patients. *Ther Apher Dial* **14**: 315–322. https://doi.org/10.1111/j.1744-9987.2009.00796.x

Aucella F, Maas R, Vigilante M, Tripepi G, Schwedhelm E, Margaglione M, Gesualdo L, Boeger R, Zoccali C (2009) Methylarginines and mortality in patients with end-stage renal disease: a prospective cohort study. *Atherosclerosis* **207**: 541–545

Baia LC, Humalda JK, Vervloet MG, Navis G, Bakker SJ, de Borst MH; NIGRAM Consortium (2013) Fibroblast growth factor 23 and cardiovascular mortality after kidney transplantation. *Clin J Am Soc Nephrol* **8**: 1968–1978. https://doi.org/10.2215/CJN.01880213

Chu C, Elitok S, Zeng S, Xiong Y, Hocher CF, Hasan AA, Krämer BK, Hocher B (2021) C-terminal and intact FGF23 in kidney transplant recipients and their associations with overall graft survival. *BMC Nephrol* **22**: 125. https://doi.org/10.1186/s12882-021-02329-7

David V, Francis C, Babitt JL (2017) Ironing out the cross talk between FGF23 and inflammation. *Am J Physiol Ren Physiol* **312**: F1–F8. https://doi.org/10.1152/ajprenal.00359.2016

David V, Martin A, Isakova T, Spaulding C, Qi L, Ramirez V, Zumbrennen-Bullough KB, Sun CC, Lin HY, Babitt JL, Wolf M (2016) Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. *Kidney Int* **89**: 135–146. https://doi.org/10.1038/ki.2015.290

Deneva-Koycheva TI, Vladimirova-Kitova LG, Angelova EA, Tsvetkova TZ (2011) Plasma asymmetric dimethylarginine levels in healthy

people. *Folia Med* **53**: 28–33. https://doi.org/10.2478/v10153-010-0024-z

Edmonston D, Wolf M (2020) FGF23 at the crossroads of phosphate, iron economy and erythropoiesis. *Nat Rev Nephrol* **16**: 7–19. https://doi.org/10.1038/s41581-019-0189-5

Eskandari Naji H, Ghorbanihaghjo A, Argani H, Raeisi S, Safa J, Alirezaei AH, Rashtchizadeh N (2017) Serum sTWEAK and FGF-23 levels in hemodialysis and renal transplant patients. *Int J Organ Transplant Med* **8**: 110–116

Fliser D, Kronenberg F, Kielstein JT, Morath C, Bode-Böger SM, Haller H, Ritz E (2005) Asymmetric dimethylarginine and progression of chronic kidney disease: the mild to moderate kidney disease study. *J Am Soc Nephrol* **16**: 2456–2461. https://doi.org/10.1681/ASN.2005020179

Frenay AR, van den Berg E, de Borst MH, Beckmann B, Tsikas D, Feelisch M, Navis G, Bakker SJ, van Goor H (2015) Plasma ADMA associates with all-cause mortality in renal transplant recipients. *Amino Acids* **47**: 1941–1949. https://doi.org/10.1007/s00726-015-2023-0

Ghorbani M, Ossareh S (2020) Early postkidney transplantation hypophosphatemia. *J Res Med Sci* **25**: 36. https://doi.org/10.4103/jrms.JRMS_452_19

Hiemstra TF, Smith JC, Lim K, Xu D, Kulkarni S, Bradley JA, Paapstel K, Schoenmakers I, Bradley JR, Tomlinson L, McEniery CM, Wilkinson IB (2020) Effect of kidney donation on bone mineral metabolism. *PLoS One* **15**: e0235082. https://doi.org/10.1371/journal.pone.0235082

Jean G, Terrat JC, Vanel T, Hurot JM, Lorriaux C, Mayor B, Chazot C (2009) High levels of serum fibroblast growth factor (FGF)-23 are associated with increased mortality in long haemodialysis patients. *Nephrol Dial Transplant* **24**: 2792–2796. https://doi.org/10.1093/ndt/gfp191

Kalantar-Zadeh K, Molnar MZ, Kovesdy CP, Mucsi I, Bunnapradist S (2012) Management of mineral and bone disorder after kidney transplantation. *Curr Opin Nephrol Hypertens* **21**: 389–403. https://doi.org/10.1097/MNH.0b013e3283546ee0

Kawabata C, Komaba H, Ishida H, Nakagawa Y, Hamano N, Koizumi M, Kanai G, Wada T, Nakamura M, Fukagawa M (2019) Changes in fibroblast growth factor 23 and soluble klotho levels after hemodialysis initiation. *Kidney Med* **2**: 59–67. https://doi.org/10.1016/j.xkme.2019.09.007

Kielstein JT, Fliser D (2007) Lowering asymmetric dimethylarginine – a new mechanism mediating the renoprotective effects of renin-angiotensin system inhibitors in proteinuric patients? *Blood Purif* **25**: 324–326. https://doi.org/10.1159/000107409

Kielstein JT, Impraim B, Simmel S, Bode-Böger SM, Tsikas D, Frölich JC, Hoeper MM, Haller H, Fliser D (2004) Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans. *Circulation* **109**: 172–177. https://doi.org/10.1161/01.CIR.0000105764.22626.B1

Liabeuf S, Cheddani L, Massy ZA (2018) Uremic toxins and clinical outcomes: the impact of kidney transplantation. *Toxins* **10**: 229. https://doi.org/10.3390/toxins10060229

Liu X, Xu X, Shang R, Chen Y (2018) Asymmetric dimethylarginine (ADMA) as an important risk factor for the increased cardiovascular diseases and heart failure in chronickidney disease. *Nitric Oxide* **78**: 113–120. https://doi.org/10.1016/j.niox.2018.06.004

Mace ML, Olgaard K, Lewin E (2020) New aspects of the kidney in the regulation of fibroblast growth factor 23 (FGF23) and mineral homeostasis. *Int J Mol Sci* **21**: 8810. https://doi.org/10.3390/ijms21228810

Malyszko J, Koc-Zorawska E, Matuszkiewicz-Rowinska J, Malyszko J (2014) FGF23 and Klotho in relation to markers of endothelial dysfunction in kidney transplant recipients. *Transplant Proc* **46**: 2647–2650. https://doi.org/10.1016/j.transproceed.2014.09.015

Mehta R, Cai X, Lee J, Xie D, Wang X, Scialla J, Anderson AH, Taliercio J, Dobre M, Chen J, Fischer M, Leonard M, Lash J, Hsu CY, de Boer IH, Feldman HI, Wolf M, Isakova T (2020) CRIC Study investigators. serial fibroblast growth factor 23 measurements and risk of requirement for kidney replacement therapy: the CRIC (Chronic Renal Insufficiency Cohort) study. *Am J Kidney Dis* **75**: 908–918. https://doi.org/10.1053/j.ajkd.2019.09.009

Munoz Mendoza J, Isakova T, Ricardo AC, Xie H, Navaneethan SD, Anderson AH, Bazzano LA, Xie D, Kretzler M, Nessel L, Hamm LL, Negrea L, Leonard MB, Raj D, Wolf M (2012) Chronic renal insufficiency cohort. fibroblast growth factor 23 and inflammation in CKD. *Clin J Am Soc Nephrol* **7**: 1155–1162. https://doi.org/10.2215/CJN.13281211

Napora M, Zdrojewski Z (2006) Role of asymmetric dimethylarginine (ADMA) in cardiovascular diseases. *Nefrol Dializoter Pol* **10**: 95–100

Oliva-Damaso E, Oliva-Damaso N, Rodriguez-Esparragon F, Payan J, Baamonde-Laborda E, Gonzalez-Cabrera F, Santana-Estupiñan R, Rodriguez-Perez JC (2019) Asymmetric (ADMA) and symmetric (SDMA) dimethylarginines in chronic kidney disease: a clinical approach. *Int J Mol Sci* **20**: 3668. https://doi.org/10.3390/ijms20153668

Post A, Bollenbach A, Bakker SJL, Tsikas D (2021) Whole-body arginine dimethylation is associated with all-cause mortality in adult renal transplant recipients. *Amino Acids* **53**: 541–554. https://doi.org/10.1007/s00726-021-02965-1

Raptis V, Kapoulas S, Grekas D (2013) Role of asymmetrical dimethylarginine in the progression of renal disease. *Nephrology* **18**: 11–21 https://doi.org/10.1111/j.1440-1797.2012.01659.x

Rysz J, Gluba-Brzózka A, Franczyk B, Jablonowski Z, Cialkowska-Rysz A (2017) Novel biomarkers in the diagnosis of chronic kidney disease and the prediction of its outcome. *Int J Mol Sci* **18**: 1702. https://doi.org/10.3390/ijms18081702

Schlesinger S, Sonntag SR, Lieb W, Maas R (2016) Asymmetric and symmetric dimethylarginine as risk markers for total mortality and cardiovascular outcomes: a systematic review and meta-analysis of prospective studies. *PLoS One* **11**: e0165811. https://doi.org/10.1371/journal.pone.0165811

Serban C, Sahebkar A, Ursoniu S, Mikhailidis DP, Rizzo M, Lip GY, Kees Hovingh G, Kastelein JJ, Kalinowski L, Rysz J, Banach M (2015) A systematic review and meta-analysis of the effect of statins on plasma asymmetric dimethylarginine concentrations. *Sci Rep* **5**: 9902. https://doi.org/10.1038/srep09902

Sharma S, Katz R, Bullen AL, Chaves PHM, de Leeuw PW, Kroon AA, Houben AJHM, Shlipak MG, Ix JH (2020) Intact and C-terminal FGF23 assays-do kidney function, inflammation, and low iron influence relationships with outcomes? *J Clin Endocrinol Metab* **105**: 4875–4885. https://doi.org/10.1210/clinem/dgaa665

Tripepi G, Mattace Raso F, Sijbrands E, Seck MS, Maas R, Boger R, Witteman J, Rapisarda F, Malatino L, Mallamaci F, Zoccali C (2011) Inflammation and asymmetric dimethylarginine for predicting death and cardiovascular events in ESRD patients. *Clin J Am Soc Nephrol* **7**: 1714–1721

Vilcea A, Darabantiu D, Puschita M (2020) The importance of a new cardiovascular risk factor – asymmetric dimethylarginine. *Maedica* **15**: 373–375. https://doi.org/10.26574/maedica.2020.15.3.373

Wang F, Xiong R, Feng S, Lu X, Li H, Wang S (2018) Association of circulating levels of ADMA with carotid intima-media thickness in patients with CKD: a systematic review and meta-analysis. *Kidney Blood Press Res* **43**: 25–33. https://doi.org/10.1159/000486743

Wolf M, Molnar MZ, Amaral AP, Czira ME, Rudas A, Ujszaszi A, Kiss I, Rosivall L, Kosa J, Lakatos P, Kovesdy CP, Mucsi I (2011) Elevated fibroblast growth factor 23 is a risk factor for kidney transplant loss and mortality. *J Am Soc Nephrol* **22**: 956–966. https://doi.org/10.1681/ASN.2010080894

Yilmaz MI, Sonmez A, Saglam M, Yaman H, Kilic S, Turker T, Unal HU, Gok M, Cetinkaya H, Eyileten T, Oguz Y, Caglar K, Vural A, Mallamaci F, Zoccali C (2013) Longitudinal analysis of vascular function and biomarkers of metabolic bone disorders before and after renal transplantation. *Am J Nephrol* **37**: 126–134. https://doi.org/10.1159/000346711

Zoccali C, Benedetto FA, Maas R, Mallamaci F, Tripepi G, Salvatore Malatino L, Böger R (2002) Asymmetric dimethylarginine, C-reactive protein, and carotid intima-media thickness in end-stage renal disease. *J Am Soc Nephrol* **13**: 490–496. https://doi.org/10.1681/ASN.V132490

Zoccali C, Bode-Böger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, Cataliotti A, Bellanuova I, Fermo I, Frölich J, Böger R (2001) Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet* **358**: 2113–2117. https://doi.org/10.1016/s0140-6736(01)07217-8