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The association between plasma proteomics and incident cardiovascular disease identifies MMP-12 as a promising cardiovascular risk marker in patients with chronic kidney disease

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HIGHLIGHTS

- Plasma MMP-12 as a potential cardiovascular risk marker in patients with chronic kidney disease (CKD) using a multiplex proteomics method.
- Higher levels of MMP-12 are associated with incident CKD in multivariable models adjusted for age, sex, kidney function, and cardiovascular risk factors.
- Additional exploration of the utility of proteomic profiling in the clinical setting of patients with CKD is needed.

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ABSTRACT

Background and aims: Previous proteomics efforts in patients with chronic kidney disease (CKD) have predominantly evaluated urinary protein levels. Therefore, our aim was to investigate the association between plasma levels of 80 cardiovascular disease-related proteins and the risk of major adverse cardiovascular events (MACE) in patients with CKD.
Methods: Individuals with CKD stages 3–5 (eGFR below 60 ml min-1 [1.73 m]-2) from three community-based cohorts (PIVUS, ULSAM, SAVA), one diabetes cohort (CARDIPP) and one cohort with peripheral artery disease patients (PADVA) with information on 80 plasma protein biomarkers, assessed with a proximity extension assay, and follow-up data on incident MACE, were used as discovery sample. To validate findings and to asses generalizability to patients with CKD in clinical practice, an outpatient CKD-cohort (Malnutrition, Inflammation and Vascular Calcification (MIVC)) was used as replication sample.
Results: In the discovery sample (total n = 1316), 249 individuals experienced MACE during 7.0 ± 2.9 years (range 0.005–12.9) of follow-up, and in the replication sample, 71 MACE events in 283 individuals over a mean ± SD change of 2.9 ± 1.2 years (range 0.1–4.0) were documented. Applying Bonferroni correction, 18 proteins were significantly associated with risk of MACE in the discovery cohort, adjusting for age and sex in order of significance, GDF-15, FGF-23, REN, FABP4, IL6, TNF-R1, AGRP, MMP-12, AM, KIM-1, TRAILR2, TNFR2, CTSL1, CSF1, PlGF, CA-125, CCL20 and PAR-1 ($p < 0.000625$ for all). Only matrix metalloproteinase 12 (MMP-12) was significantly associated with an increased risk of MACE in the replication sample (hazard ratio (HR) per SD increase, 1.36, 95% CI (1.07–1.75), $p = 0.013$).
Conclusions: Our proteomics analyses identified plasma MMP-12 as a promising cardiovascular risk marker in patients with CKD.

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1. Introduction

Chronic kidney disease (CKD) is characterized by a sustained reduction in glomerular filtration rate (GFR), and the prevalence and incidence of cardiovascular events such as stroke, myocardial infarction, and cardiovascular death in patients with CKD are substantially elevated compared to the general population [1,2]. However, the underlying mechanisms for this enhanced cardiovascular risk is incompletely understood. Recognition of mechanisms and predictive risk factors for cardiovascular disease (CVD) in renal disease is an important undertaking to elucidate the complex interplay between the kidney and the cardiovascular system. In recent years, several protein biomarker panel techniques have been proposed alongside clinical assessment predicting CKD consequences [3–6]. Urine sampling has to a greater extent been utilized in these biomarker panels, mainly due to the direct reflection of the kidney status and function of the urinary system and non-invasiveness [7–10], but the utility of plasma proteomics is less evaluated [11,12].

Our aim was to evaluate the association between 80 cardiovascular disease-related plasma protein biomarkers, assessed by a proximity extension assay, and the risk of MACE in participants with CKD stages 3–5 (but not on dialysis) in five independent research cohorts. In addition, we wanted to explore whether our findings would be viable in a more clinically related setting, therefore, we aimed at replicating our findings in an outpatient CKD-cohort.

2. Materials and methods

2.1. Discovery sample

For our discovery sample, we included 1316 participants with CKD (stages 3–5. i.e. eGFR below 60 ml min^{-1} [1.73 m]$^{-2}$) from the following cohorts:

2.1.1. Cardiovascular Risk Factors in Patients with Diabetes

A Prospective Study in Primary Care (CARDIPP; ClinicalTrials.gov: NCT01049737) is a community-based cohort launched in 2005 with the general aim to examine cardiovascular risk factors in patients aged 55–65 with type 2 diabetes. In total, 761 patients were recruited from 22 primary healthcare centers in the counties of Östergötland and Jönköping, Sweden, selected to represent different demographic areas [13]. Out of 761 consecutively registered participants, 204 participants with CKD and data on outcome and protein biomarkers were included in the present study.

2.1.2. Study of Atherosclerosis in Västmanland (SAVa)

enrolled 2315 patients between November 2005 and May 2011 with various manifestations of cardiovascular disease (CVD). The cohort is comprised of three study populations, Västmanland Myocardial Infarction Study (VaMIS, n = 1008; ClinicalTrials.gov: NCT01452178), Peripheral Arterial Disease in Västmanland (PADVa; ClinicalTrials.gov: NCT01452165) and a control group, SAVa-control [14]. The present study uses data and samples from PADVa and SAVa-control.

2.1.2.1. PADVa study cohort. Participants referred to the Vascular Ultrasound Laboratory of Västmanland County Hospital, Västerås, Sweden, were recruited if they satisfied one of the three consecutive inclusion criteria: (i) claudication symptoms with an ankle–brachial pressure index ≤0.90 in the ipsilateral lower extremity (ii); claudication symptoms with evidence of occlusive arterial disease in the ipsilateral extremity on ultrasonography examination or (iii) internal carotid artery occlusion or stenosis. Out of 614 patients fulfilling the inclusion criteria, 452 (73.6%) constituted the final study population [14]. The current study includes 187 individuals with CKD in SAVa-control with data on outcome and protein biomarkers.

2.1.2.2. SAVa study cohort. Control participants (total of 855 individuals) were randomly chosen from the Swedish National Population Register and matched to the VaMIS patients on age, sex, and municipality. The current study includes 229 individuals with CKD in SAVa-control with data on outcome and protein biomarkers (for more information, please see https://savastudy.se/controls/).

2.1.3. The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS)

The study started in 2001 with the intention to longitudinally evaluate measures on endothelial function and arterial compliance in a sample of individuals, aged 70 years and residing in Uppsala community, Sweden [15]. Initially, 1016 out of 2025 (50.2%) invited individuals were enrolled, composing the final study population. Follow-up biomedical assessments have been performed at 5-year intervals (for more information, please see www.medsci.uu.se/pivus/). Four hundred fifteen participants with CKD and data on outcome and protein biomarkers were included in the current study.

2.1.4. The Uppsala Longitudinal Study of Adult Men (ULSAM)

The study started in 1970 and of 2841 invited middle aged male (born between 1920 and 1924) residents of Uppsala county, Sweden, 2322 (81.7%) participated [16]. A continuous screening examination program has been performed repeatedly since the beginning of the study (for more details, please see www.pubcare.uu.se/ulsam/). The present study includes 281 participants with CKD and data on outcome and protein biomarkers at the fourth investigation period, when the participants were aged 77 years.

2.2. Replication sample

The Malnutrition, Inflammation and Vascular Calcification (MIVC) cohort aimed at studying risk factors in kidney disease was initiated between 2010 and 2013 at the Dante Pazzanese Institute of Cardiology, São Paolo, Brazil [17] was used as the replication sample. The study enrolled 300 successive outpatients with CKD stages 3–5 prior to commencement of dialysis. In the current investigation, 283 participants with available outcome data and protein biomarkers were included in the analyses.

2.3. Multiplex proximity extension assay

We used a proximity extension assay (PEA), the Olink Proseek® Multiplex Cardiovascular I 96x96 kit (http://www.olink.com/), simultaneously measuring the concentration of 92 cardiovascular candidate proteins. The mentioned proteomics approach has previously been applied to investigate the relationship between novel protein biomarkers and cardiovascular pathologies [18–20]. In short, the assay is performed in a 96-well microplate with 92 pairs of oligonucleotide-labeled antibodies and four internal controls. When the oligonucleotide-labeled antibodies bind to their specific target proteins, the oligonucleotide functions as a distinctive reporter sequence, subsequently amplified and quantified with a Fluidigm Biomark™ HD real-time polymerase chain reaction (PCR) platform. The lower limit of detection was defined as 3 standard deviations above background noise. PCR values above the detection threshold were log_2-transformed and corrected for technical variation based on negative and interplate controls and transformed to a mean of zero and standard deviation (SD) of 1. Log_2-transformed values portrays the relative protein abundance but are not easily transformed to absolute concentrations. Validation of the assay was implemented at different laboratories with a mean coefficient of validation (CV) intra-assay and inter-assay variations observed to be 8% and 15%, respectively [18,19]. A quality control (QC) was made in which proteins with > 15% missing values were excluded and missing values for biomarkers between 0 and 15% missingness were imputed by the lower limit of detection (LOD) threshold divided by two

(Supplementary Table 1). After carrying out the QC, 12 proteins were excluded, leaving 80 out of 92 proteins included in the analysis.

2.4. Inclusion criteria, outcome definition and number of eligible participants

To establish the CKD stages, we then estimated GFR in accordance with the Chronic Kidney Disease Epidemiology (CKD-EPI) Creatinine Equation formula in all cohorts [21]. All Individuals without available fasting frozen plasma or serum samples, eGFR above 60 ml min^{-1} $[1.73\ m]^{-2}$ were excluded. MACE was defined as a new episode of fatal or non-fatal myocardial infarction (I21 in ICD-10; www.who.int/classifications/icd/en/) or fatal/non-fatal stroke (I60–I63), whichever occurred first, and was obtained from hospital and death register linkage.

2.5. Statistical analysis

In our primary analyses, age and sex-adjusted Cox proportional hazard regression model was used to investigate the associations between each protein and time-to-MACE as an outcome. As the protein assay does not provide standard concentration units, protein values were transformed to a mean of zero and SD of 1. Participants were at risk until the occurrence of MACE, death or until the last day of follow-up.

In our pre-defined analyses plan, we used the patients with CKD from the above mentioned five cohorts as a discovery cohort in which proteins associated with MACE at a Bonferroni corrected *p*-value of 0.000625 were considered statistically significant. As a second step, we wanted to investigate the possible significance of the associations in a clinical setting using the CKD outpatient MIVC-cohort. A nominal p-value ($p < 0.05$) was considered statistically significant in the replication. As an additional step we also performed additional multivariable Cox-models adjusting for established cardiovascular risk factors (age, sex, systolic blood pressure, LDL-cholesterol, diabetes, smoking status, eGFR) for proteins that were consistently associated with MACE in both cohorts. All analyses were carried out in STATA 15 (Stata corp, College Station, TX).

3. Results

3.1. Baseline characteristics

The baseline characteristics of all participants are shown in Table 1. The discovery cohort combined participants with CKD stages 3–5 in CARDIPP (n = 204; 23 events over 7.7 ± 1.9 years), PADVa (n = 187; 43 events over 4.9 ± 1.8 years), SAVa-control (n = 229; 27 events over 4.9 ± 1.7 years), PIVUS (n = 415; 84 events over 8.6 ± 2.7 years), and ULSAM (n = 281; 85 events over 7.7 ± 3.5 years). The discovery cohort included a total of 1316 individuals out of which 249 experienced MACE during 7.0 ± 2.9 years (range 0.005–12.9). The MIVC cohort documented 71 MACE events in 283 individuals over a mean ± SD change of 2.9 ± 1.2 years (range 0.1–4.0).

In the discovery cohort, 18 proteins were associated with a possible risk of MACE at the pre-defined Bonferroni significance threshold corresponding to $p < 0.000625$ after adjusting for age and sex. In order of significance, these included, growth/differentiation factor 15 (GDF-15), fibroblast growth factor 23 (FGF23), renin (REN), fatty acid-binding protein, adipocyte (FABP4), interleukin-6 (IL6), tumor necrosis factor receptor 1 (TNF-R1), agouti-related protein (AGRP), matrix metalloproteinase-12 (MMP-12), adrenomedullin (AM), kidney injury molecule 1 (KIM-1), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2), tumor necrosis factor receptor 2 (TNF-R2), cathepsin L1 (CTSL-1), macrophage colony-stimulating factor 1 (CSF-1), placenta growth factor (PIGF), ovarian cancer-related tumor marker CA 125 (CA-125), C–C motif chemokine 20 (CCL20), and proteinase-activated receptor 1 (PAR-1) (Table 2). The association between all 80 proteins and MACE are presented in Supplementary Table 2. Of the 18 proteins that were significantly associated with MACE in the discovery phase, only MMP-12 was significantly and consistently associated with MACE in the replication sample (Table 2). The associations between MMP-12 and MACE were similar when adjusting for established cardiovascular risk factors (Discovery; HR 1.24, 95% CI 1.08–1.43, $p = 0.003$ Replication; HR 1.33, 95% CI 1.03–1.72, $p = 0.027$).

4. Discussion

4.1. Principal findings

In the current study, we used a proximity extension assay with 80 cardiovascular disease-related proteins to establish biomarker signatures associated with risk of major adverse cardiovascular events in individuals with chronic kidney disease that participated in 5 different research cohorts. In the discovery sample, 18 proteins were significantly associated with MACE applying a pre-defined Bonferroni significance threshold. As a second step, we used an independent CKD outpatient-cohort to explore if results were generalizable to patients with CKD in clinical practice. Of the 18 proteins identified in the discovery phase, only plasma MMP- 12 was associated with outcome in the replication sample.

4.2. Comparison with the literature

Little is known regarding the role of matrix metalloproteinases (MMPs) in plasma and the subsequent association with cardiovascular events, particularly regarding MMP-12 [22]. One study reported the up-regulation of both MMP-2, and MMP-9 in diabetic CKD with subsequent arterial stiffening, endothelial dysfunction and reduced angiogenesis [23]. Besides MMP-2 and -9, MMP-1 and -3 has also been more extensively studied in relation to the kidney and succeeding cardiovascular events [24]. As for MMP-12, a recent meta-analysis presented a possible role for the metalloproteinase in ischemic stroke [25], yet another study suggested involvement of MMP-12 in the pathway of MMP-mediated macrophage invasion with tissue damage and atherosclerotic rupture as a consequence [26]. Also, genome-wide association studies (GWASs) supports a causative role for MMP-12 in strokes [27]. We are not aware of any previous studies on the association of MMP-12

Table 1
Baseline data.

Variable	Discovery sample	Replication sample
Events/total N	249/1316	71/283
Follow-up, years	7.0 ± 2.9	2.9 ± 1.2
% women	64	63
Age, years	71 ± 6.4	60 ± 10
BMI, kg/m^2	28 ± 4.4	29 ± 5.8
eGFR, ml min^{-1} $[1.73\ m]^{-2}$	50 ± 7.7	16 ± 8.0
Diabetes, %	27	49
Systolic blood pressure	148 ± 22	154 ± 28
Diastolic blood pressure	79 ± 11	81 ± 15
Total cholesterol, mmol/l	5.2 ± 1.1	4.8 ± 1.4
LDL-cholesterol, mmol/l	3.2 ± 1.0	2.7 ± 1.1
HDL-cholesterol, mmol/l	1.4 ± 0.4	1.2 ± 0.4
Current smoker, %	10	57
Antihypertensive medication, %	56	98

Continous variables are given as mean ± SD.
Discovery cohort: CARDIPP, Cardiovascular Risk Factors in Patients with Diabetes: a Prospective Study in Primary Care; PADVa, Peripheral Arterial Disease in Västmanland; SAVa-control, The Study of Atherosclerosis in Västmanland; PIVUS, The Prospective Investigation of the Vasculature in Uppsala Seniors; ULSAM, The Uppsala Longitudinal Study of Adult Men.
Replication cohort: MIVC, The Malnutrition, Inflammation and Vascular Calcification.

Table 2
Associations of circulating protein markers and MACE in discovery and replication cohorts.

Discovery cohort (CARPIDD, PADVa, SAVa, PIVUS, ULSAM)	Adjusted for age and sex	
Protein	HR (95% CI)	*p*-value
Growth/differentiation factor 15 (GDF-15)	1.75 (1.52–2.02)	1.00e-14
Fibroblast growth factor 23 (FGF23)	1.61 (1.41–1.83)	6.30e-13
Renin (REN)	1.42 (1.24–1.63)	2.74e-07
Fatty acid-binding protein, adipocyte (FABP4)	1.43 (1.24–1.65)	6.45e-07
Interleukin-6 (IL6)	1.30 (1.17–1.45)	6.52e-07
Tumor necrosis factor receptor 1 (TNF-R1)	1.51 (1.28–1.78)	8.78e-07
Agouti-related protein (AGRP)	1.44 (1.25–1.67)	9.68e-07
Matrix metalloproteinase-12 (MMP-12)	1.42 (1.23–1.63)	1.11e-06
Adrenomedullin (AM)	1.61 (1.33–1.96)	1.19e-06
Kidney injury molecule 1 (KIM-1)	1.42 (1.23–1.65)	3.91e-06
TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2)	1.29 (1.14–1.46)	0.0000494
Tumor necrosis factor receptor 2 (TNF-R2)	1.31 (1.15–1.50)	0.0000648
Cathepsin L1 (CTSL1)	1.38 (1.17–1.63)	0.0001129
Macrophage colony-stimulating factor 1 (CSF-1)	1.45 (1.19–1.76)	0.0002105
Placenta growth factor (PIGF)	1.27 (1.12–1.45)	0.0002291
Ovarian cancer-related tumor marker CA 125 (CA-125)	1.40 (1.17–1.68)	0.0002914
C–C motif chemokine 20 (CCL-20)	1.25 (1.10–1.41)	0.0004281
Proteinase-activated receptor 1 (PAR-1)	1.27 (1.11–1.46)	0.0005305
Replication cohort (MIVC)	Adjusted for age and sex	
Protein	HR (95% CI)	p-value
Matrix metalloproteinase-12 (MMP-12)	1.36 (1.07–1.75)	0.013

HR and 95% CI are given for an age and sex adjusted model.
CI, confidence interval; HR, hazard ratio. CARDIPP, Cardiovascular Risk Factors in Patients with Diabetes: a Prospective Study in Primary Care; PADVa, Peripheral Arterial Disease in Västmanland; SAVa-control, The Study of Atherosclerosis in Västmanland; PIVUS, The Prospective Investigation of the Vasculature in Uppsala Seniors; ULSAM, The Uppsala Longitudinal Study of Adult Men. MIVC, The Malnutrition, Inflammation and Vascular Calcification. Bonferroni corrected p-value < 0.000625 was considered statistically significant in primary analyses, and a nominal p-value < 0.05 was considered statistically significant in secondary analyses (for details, see Materials and Methods section).

and MACE in patients with CKD.

4.3. Potential mechanisms

Potential mechanisms in renal pathology may be infiltration of macrophages and succeeding expression of MMP-12 in the glomerular compartment with renal function decline as a consequence [28,29], together with constitutive up-regulation of MMP-12 from macrophages in the cardiovascular system, increasing the risk of cardiovascular events [26]. However, considering we were not able to determine an association in our observational analyses; possible underlying mechanisms for the association between MMP-12 and MACE in the present study are hypothetical. The causal pathways in this process is beyond the scope of this observational study and needs further exploration.

4.4. Clinical implications

The interplay between the different MMPs is multifaceted and much of previous data originate from animal studies, which, compared to studies in humans, are quite divergent with reference to MMP-expression patterns [26,28,30]. Substantial evidence suggests individual MMPs as targets for therapeutical intervention in renal and cardiovascular disease [24,28,31,32]. However, considering the complexity of MMP expression and function, inhibiting single pathologically related MMPs at specific time points, without affecting advantageous MMPs is an intricate undertaking. The utility of MMP-12 measurements for risk prediction purposes in patients with CKD needs to be addressed in future larger studies.

4.5. Strengths and limitations

The major strength of our study is the longitudinal design and the novel proteomics approach measuring 80 CVD protein biomarkers in a discovery approach. Our study also has limitations. First, even though we used the same definition of CKD, kidney disease severity in the discovery sample and replication sample was quite different. Second, the use of a PEA technique does not allow for absolute quantification of the proteins, which limits a clinically viable assessment. Third, the time between plasma sampling and protein analysis may have impinged upon protein levels, but pre-analytical biases should have been kept to a minimum seeing that sample collection was done in a consistent fashion and samples stored properly.

4.6. Conclusion

Our proteomics approach identified plasma MMP-12 as a risk marker in patients with CKD, which could be due to its effects on macrophages that merits additional investigation. Furthermore, our data encourage additional exploration of the utility of proteomic profiling in the clinical setting of patients with CKD.

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CRediT authorship contribution statement

Tobias Feldreich: Investigation, Methodology, Visualization, Validation, Writing - review & editing, Writing - original draft, Formal analysis. **Christoph Nowak:** Validation, Writing - review & editing. **Axel C. Carlsson:** Validation, Writing - review & editing. **Carl-Johan Östgren:** Validation, Writing - review & editing. **Fredrik H. Nyström:** Validation, Writing - review & editing. **Johan Sundström:** Validation, Writing - review & editing. **Juan-Jesus Carrero-Roig:** Data curation, Validation, Writing - review & editing. **Jerzy Leppert:** Data curation, Validation, Writing - review & editing. **Pär Hedberg:** Data curation, Validation, Writing - review & editing. **Vilmantas Giedraitis:** Validation, Writing - review & editing. **Lars Lind:** Validation, Writing - review & editing. **Antonio Cordeiro:** Data curation, Validation, Writing - review & editing. **Johan Ärnlöv:** Conceptualization, Funding acquisition, Data curation, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2020.06.013.

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