Original Research

Serum protease activity in chronic kidney disease patients: The GANI_MED renal cohort

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Impact statement

- Renal and cardiac diseases are very common and often occur concomitantly, resulting in increased morbidity and mortality. Understanding of molecular mechanisms linking both diseases is limited, available fragmentary data point to a role of the renin–angiotensin system (RAS) and, in particular, Ras-related peptidases.
- Here, a comprehensive analysis of serum peptidase activities in patients with different stages of chronic kidney disease (CKD) is presented, with special emphasis given to RAS peptidases
- The serum activities of the peptidases angiotensin I-converting enzyme 2 and dipeptidyl peptidase 4 were identified as closely associated with kidney function, specifically with the estimated glomerular filtration rate. The findings are discussed in the context of available data suggesting protective roles for both enzymes in reno-cardiac diseases.
- The data add to our understanding of pathomechanisms underlying development and progression of CKD and indicate that both enzymes might represent potential pharmacological targets for the preservation of renal function.

Abstract

Serum or plasma proteases have been associated with various diseases including cancer, inflammation, or reno-cardiovascular diseases. We aimed to investigate whether the enzymatic activities of serum proteases are associated with the estimated glomerular filtration rate (eGFR) in patients with different stages of chronic kidney disease (CKD). Our study population comprised 268 participants of the ''Greifswald Approach to Individualized Medicine'' (GANI_MED) cohort. Enzymatic activity of aminopeptidase A, aminopeptidase B, alanyl (membrane) aminopeptidase, insulin-regulated aminopeptidase, puromycin-sensitive aminopeptidase, leucine aminopeptidase 3, prolyl-endopeptidase (PEP), dipeptidyl peptidase 4 (DPP4), angiotensin I-converting enzyme, and angiotensin I-converting enzyme 2 (ACE2) proteases was measured in serum. Linear regression of the respective protease was performed on kidney function adjusted for age and sex. Kidney function was modeled either by the continuous Modification of Diet in Renal Disease (MDRD)-based eGFR or dichotomized by eGFR < 15 mL/min/1.73 m^2 or <45 mL/min/1.73 m^2, respectively. Results with a false discovery rate below 0.05 were deemed statistically significant. Among the 10 proteases investigated, only the activities of ACE2 and DPP4 were correlated with eGFR. Patients with lowest eGFR exhibited highest DPP4 and ACE2 activities. DPP4 and PEP were correlated with age, but all other serum protease activities showed no associations with age or sex. Our data indicate that ACE2 and DPP4 enzymatic activity are associated with the eGFR in patients with CKD. This finding distinguishes ACE2 and DPP4 from other serum peptidases analyzed and clearly indicates that further analyses are warranted to identify the precise role of these serum ectopeptidases in the pathogenesis of CKD and to fully elucidate underlying molecular mechanisms.

Keywords: Protease activity, angiotensin-converting enzyme 2, dipeptidyl-peptidase IV, renin–angiotensin system, chronic kidney disease, estimated glomerular filtration rate

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Introduction

In the last decade, membrane-bound proteases/peptidases (ectopeptidases) have gained considerable research interest due to their contribution in the formation, competitive metabolism, and degradation of a broad variety of peptide hormones that function as key regulators for intercellular

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communication. Pathophysiological conditions and, in particular, cardio-renal diseases greatly affect the release (shedding) of ectopeptidases into extracellular compartments including blood plasma. Members of the *A Disintegrin And Metalloprotease* family are prototype ''sheddases'' implicated in this process. Soluble peptidases/proteases shed into the plasma may represent a possible source of biomarkers. Thus, in this study a comprehensive analysis of serum ectopeptidases activities in patients with chronic kidney disease (CKD) at different disease stages is performed. Special emphasis is given to peptidases/proteases of the renin–angiotensin system (RAS), as dysregulation of the activity of both the classical and alternative axes of the RAS has been associated with cardiac and renal diseases. Both, cardiac and renal diseases, are very common and often occur concomitantly, resulting in increased morbidity and mortality.[1] To date, 5–10% of the world population is affected by CKD.[2] In the next decades, CKD prevalence will further increase due to rising prevalence of major risk factors for CKD including diabetes, hypertension, and cardiovascular disorders and the increasingly older population.[3,4] CKD is defined by structural pathology (or kidney transplant), a decline in estimated glomerular filtration rate (eGFR) ($<60\,mL/min/1.73\,m^2$) over three-month time with clinical implications or albuminuria.[2]

Experimental and clinical data indicate that the administration of inhibitors of the angiotensin-converting enzyme (ACEi) or angiotensin II type 1 receptor (AT1R) blockers can limit the progression of kidney injury (albuminuria, damage of podocytes, decrease of eGFR) due to chronic volume overload.[5,6] Angiotensin II (AngII) promotes the production of reactive oxygen species and renal fibrosis via direct (activation of NADPH oxidase) and indirect (increased aldosterone plasma concentrations) mechanisms.[7] Accordingly, aldosterone receptor antagonists have been also shown to diminish histological signs of kidney injury, creatinine serum concentrations, and proteinuria.[8] In the SOLVD study, enalapril reduced proteinuria in diabetic patients.[9] Captopril stabilized the GFR in postinfarct patients with heart failure.[10] However, the CONSENSUS study showed a 10–15% increase in serum creatinine in patients treated with enalapril[11] and a slight decrease in the GFR was observed for valsartan in the VALHEFT study. Furthermore, candesartan was not able to reduce proteinuria in the CHARM-Added study.[12] In summary, available studies do not provide a simple answer to the question what the precise role of the RAS and, in particular, of its different axes, in the development and progression of CKD actually is. A better understanding might be hampered by the extensive heterogeneity among patients with CKD with respect to underlying pathomechanisms and concomitant diseases. However, any disease-, gender-, or age-dependent dysregulation of typical RAS proteases might be partly compensated by modulating the expression/activity of other ectoproteases. In support of this view, it was shown previously that an age-dependent decrease in ACE activity in the pericardial fluid of elder women is compensated by increased activities of membrane alanyl-aminopeptidase (APN, CD13) and dipeptidyl-peptidase IV (DPPIV, DPP4, CD26).[13] Advanced age and ACEi treatment could increase the pathophysiological relevance of non-RAS proteases.[14,15] In this study, we determined enzymatic activities of the RAS-related ectopeptidases ACE, ACE2, aminopeptidase A (APA), aminopeptidase B (APB), APN, and insulin-regulated aminopeptidase (IRAP) as well as of DPP4, puromycin-sensitive aminopeptidase (PSA), leucine aminopeptidase 3 (LAP), and prolyl-endopeptidase (PEP) in serum samples from patients with different stages of CKD (eGFR groups 1–5). Protease characteristics are summarized in Table 1.

Materials and methods

Study design and patients

Patients were selected from the ''Greifswald Approach to Individualized Medicine'' (GANI_MED) project. GANI_MED aims at implementing individualized diagnostic and therapeutic measures in patient cohorts with common cardiovascular, cerebrovascular, or metabolic conditions, with pulmonary disease, sepsis, and in patients with adverse medication effects. All GANI_MED patients were recruited in the university hospital or from special dialysis treatment centers. Patients are subjected to highly standardized anamnestic, somatometric, basic laboratory diagnostic analyses, which also include determination of eGFR. For cohort profile and detailed study procedures, please refer to Grabe *et al.*[21] GANI_MED was approved by the ethics committee of the Medical Faculty of the University of Greifswald. All participants provided informed written consent.

In the present study, data from 244 GANI_MED patients enrolled between July 2011 and December 2013 in the ''renal and renovascular disease cohort'' were analyzed. None of these patients underwent a renal transplantation before inclusion of the study. All patients consented to blood sampling and subsequent measurements of serum protease activities and plasma creatinine concentrations were performed. The 244 patients were assigned to the different CKD stages (1–5) according to their eGFR: group 1 (CKD $1+2$; $eGFR \geq 60\,mL/min/1.73\,m^2$), group 2 (CKD 3A; $eGFR > 45–60\,mL/min/1.73\,m^2$), group 3 (CKD 3B; $eGFR > 30–45\,ml/min/1.73\,m^2$), group 4 (CKD 4; $eGFR > 16–30\,mL/min/1.73\,m^2$), group 5 (CKD 5; $eGFR \leq 15\,mL/min/1.73\,m^2$). As the number of patients in the CKD stages 1–3 was naturally low in the renal cohort patients, 24 age- and sex-matched patients from the GANI_MED ''cardiovascular cohort'' were selected to fill up these stages, so that each group comprised more than 25 patients.

Laboratory testing

Blood sampling. Eight milliliters of blood was drawn from a cubital vein using an 18-gauge needle (Becton Dickinson Diagnostic Systems, USA) and a SST II Advance, vacutainer® (Becton Dickinson Diagnostic Systems, USA) tube for serum samples. Blood samples rested 30 min to undergo coagulation which was followed by centrifugation at $2800 \times g$ for 10 min. The supernatant was filled into 1.5 mL sample tubes (Eppendorf, Germany) and stored at

Table 1 Characteristics of proteases analyzed

Protease				Substrate			Inhibitor	
Name	Gene ID	CD number	EC number	Name	Concentration (mM)	Source	Name (concentration (M))	Ref.
Leucine aminopeptidase 3 (LAP)	LAP3		3.4.11.1	H-Leu-pNA-HCl	4.375	Sigma	–	
Alanyl (membrane) aminopeptidase (APN)	ANPEP	CD13	3.4.11.2	H-Ala-pNA-HCl	2.925	Bachem	RB3014 [1×10^{-7}]	Chen *et al.*[62]
Leucyl/cystinyl aminopeptidase (insulin-regulated aminopeptidase, IRAP)	LNPEP		3.4.11.3	H-Leu-pNA-HCl	4.375	Sigma	RB3014 [10^{-7}]	Aroor *et al.*[16]
Arginyl aminopeptidase (aminopeptidase B) (APB)	RNPEP		3.4.11.6	H-Arg-pNA-2HCl	2.5	Bachem	–	
Glutamyl aminopeptidase (aminopeptidase A) (APA)	ENPEP	CD249	3.4.11.7	H-Glu-pNA	5.0	Bachem	–	
Puromycin-sensitive aminopeptidase (PSA)	NPEPPS		3.4.11.14	H-Ala-pNA-HCl	2.925	Bachem	RB3014 [1×10^{-7}]	Riera *et al.*[17]
Dipeptidyl-peptidase 4 (DPPIV, DPP4)	DPP4	CD26	3.4.14.5	H-Gly-Pro-pNA-HCl	1.65	Bachem	Lys-Z-NO-thiazolidid [1×10^{-4}]	Ansorge *et al.*[63], Schön *et al.*[64]
Angiotensin I-converting enzyme (ACE)	ACE	CD143	3.4.15.1	ACE color-Kit		MAST-DIAGNOSTICA-GmbH	–	
Angiotensin I-converting enzyme 2 (ACE2)	ACE2		3.4.17.23	ACE color-Kit		MAST-DIAGNOSTICA-GmbH	Lisinopril [0.3×10^{-6}]	Lehmann *et al.*[65]
Prolyl endopeptidase (PEP)	PREP		3.4.21.26	H-Gly-Pro-pNA-HCl	1.65	Bachem	Lys-Z-NO-thiazolidid [1×10^{-4}]	Ansorge *et al.*[63], Schön *et al.*[64]

−80°C until analysis. Determination of protease activities and plasma creatinine was performed from separate collection tubes that were, however, obtained during one blood draw.

Determination of protease activity. Enzymatic activity of ACE, ACE2, APA, APB, APN, LAP, PEP, PSA, IRAP, and DPP4 was determined from serum samples after one freeze–thaw cycle using the substrates and inhibitors specified in Table 1.

The assays for determination of APA, APB, APN, LAP, PEP, PSA, IRAP, and DPP4 enzymatic activity were carried out in 50 mM Hepes buffer containing 200 mM NaCl, 10 μM $ZnCl_2$, and 1% DMSO pH 6.8. Enzyme reactions were performed in flat-bottom 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). The total reaction volume amounting to 100 μL consisted of 65 μL buffer, 10 μL serum and 25 μL substrate. The reaction was started by addition of the substrate. When appropriate, inhibitors were used to increase the specificity of the assays, e.g. RB3014 was applied to discriminate between APN (sensitive) and PSA (insensitive) Ala-pNA-hydrolyzing activity as well as between IRAP (sensitive) and LAP (insensitive) Leu-pNA-hydrolyzing activity. Lys-Z-NO-thiazolidid was used to distinguish DPP4 (sensitive) from PEP (insensitive). When an inhibitor was applied, a preincubation with the inhibitor was performed for 30 min at 37°C before addition of the substrate. The optical density at the starting point was used as blank sample. After incubation at 37°C for 1 h in the dark, the optical density was measured at 405 nm with 620 nm reference wavelength by a microtiter plate reader infinite M200 (Tecan, Crailsheim, Germany). The enzymatic activity of ACE and ACE2 was determined using the ACE color-Kit (MAST-DIAGNOSTICA-GmbH, Reinfeld, Germany) according to the manufacturer's instructions adapted to 96-well plates, with the lisinopril-insensitive fraction being considered as ACE2 activity.

All samples were analyzed in duplicate. Reanalysis was indicated when values differed by >5%. The enzymatic activities are given in nkat/L and were calculated as follows: $A = (\Delta E\ Vt)/(t\ \varepsilon\ d\ Vs)/L$.

Creatinine measurement and eGFR. Plasma creatinine concentrations in Gani_Med were measured at the Institute of Clinical Chemistry and Laboratory Medicine in the University Medicine Greifswald (Greifswald, Germany). Gani_Med was started in 2011, when creatinine measurements were performed with a modified kinetic Jaffé method on the Siemens Dimension Vista (Siemens Healthcare Diagnostics, Eschborn, Germany). In November 2012 a new, enzymatic method for creatinine measurement on the Siemens Dimension Vista was introduced in our lab. All subsequent Gani_Med samples were measured with this method. As method changes may lead to uncomparable values, a method comparison was performed. For this comparison, 327 plasma samples (0–700 μmol/L creatinine) were measured with each method. The comparison of the respective pairs revealed that the enzymatic method measures lower creatinine concentrations (difference based on the median: −13.4%) than the modified kinetic Jaffé method. Therefore, it was considered necessary to convert the creatinine concentrations measured with the Jaffé method to the newer enzymatic method. Passing–Bablok regression ($r = 0.993$) yielded the coefficients for the following formula: Creatinine enzymatic μmol/L = Creatinine Jaffé μmol/L *0.915 – 3.61. This formula was applied to convert the 142 measurements obtained by the Jaffé method to the enzymatic method and all analyses are based on the converted data. The eGFR was calculated using the four-variable MDRD equation.[22] The MDRD formula was chosen, as about 90% of subjects included in the present study have an eGFR < 60 mL/min/1.73 m^2, values at which the MDRD formula has a high accuracy.[23]

As mentioned above, for eGFR groups 1–3 only very few samples were available in the CKD cohort. By design, samples from the cardiovascular cohort were selected randomly and matched by age and sex. In detail, two, 10, and 12 samples were added to eGFR groups 1, 2, and 3, respectively. The sample sizes per group after successful measurement of protease activities are presented in Table 2.

Statistical analyses

For all analyses, a linear regression of the respective protease activity on kidney function adjusted for age, in years, and sex was calculated. Kidney function was modeled either using the continuous eGFR values (eGFRcrea_MDRD), the groups, or dichotomized by eGFR < 15 mL/min/1.73 m^2 (egfr_group15) or <45 mL/min/1.73 m^2 (egfr_group45), respectively.

Table 2 Characterization of eGFR groups

eGFR group (eGFR range (mL/min/1.73 m^2))	CKD stage	N samples	N men (%)	Mean age (SD)	N medication (%)
1 (≥60)	1 + 2	27	19 (70.4)	60.6 (13.2)	21 (84.0)
2 (45 – <60)	3A	29	23 (79.3)	68.5 (10.4)	21 (72.4)
3 (30 – <45)	3B	31	19 (61.3)	70.8 (11.6)	16 (51.6)
4 (15 – <30)	4	52	30 (57.7)	72.5 (10.5)	30 (61.2)
5 (<15)	5	129	75 (58.1)	67.8 (14.0)	67 (54.0)
Total		268	166 (61.9)	68.4 (13.0)	155 (60.1)

ATC code C9: agents acting on the renin–angiotensin system; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; SD: standard deviation.

The dichotomizations and the calculations based on groups were performed as sensitivity analyses and to address potential non-linear relationships between the eGFR values and the protease activities.

The results were adjusted for multiple testing by applying the false discovery rate (FDR)[24] and claimed significant having an FDR < 0.05.

All analyses were carried out in R v3.1.3 (www.r-project.org).

Results

A total of 268 patients were included in the study. The classification of the patients in the eGFR groups is given in Table 2. Multivariable regression analysis revealed a significant association of patients' eGFR with the serum enzymatic activities of ACE2 and DPP4. These associations remained significant after adjusting for age and sex (Table 3). For the other proteases analyzed, no association of their enzymatic activity with eGFR was detected (Table 3).

Categorical analysis of eGFR groups confirmed the significant associations of ACE2 and DPP4 serum activities with the eGFR group (overall P values DPP4: $P = 0.000487$; ACE2: $P = 0.0043$; Figure 1). As shown in Figure 1(a), of all eGFR groups, the groups 1 and 2 showed the lowest ACE2 activities. Thus, a dichotomous analysis was performed to compare ACE2 protease activity in patients with eGFR $< 45\,mL/min/1.73\,m^2$ to that in patients with eGFR $\geq 45\,mL/min/1.73\,m^2$. The result show a significantly higher ACE2 activity in patients with eGFR $\geq 45\,mL/min/1.73\,m^2$ ($P < 0.00016$) (Supplementary Table S1). For DPP4, the eGFR group 5 exhibited the highest serum activity levels of all groups (Figure 1(b)). Dichotomous analysis of DPP4 activity in patients with eGFR $< 15\,mL/min/1.73\,m^2$ versus $\geq 15\,mL/min/1.73\,m^2$ confirmed significantly higher DPP4 serum activities in patients with eGFR $< 15\,mL/min/1.73\,m^2$ compared to patients with higher eGFR ($p = 3.73 \times 10^{-5}$) (Supplementary Table S2). Of note, ACE, ENPEP, and NPEPPS passed the significance threshold in the analyses in patients with eGFR < 45 and $< 15\,mL/min/1.73\,m^2$, respectively, but in no other analysis.

Table 3 Associations between protease activity and eGFR.

Protease	Beta	SE	p	FDR	n
ACE2	−0.135	0.040	8.00E-04	**0.008**	268
DPP4	−0.744	0.252	0.003	**0.017**	268
ACE	−0.846	0.378	0.026	0.065	268
PREP	−0.231	0.102	0.024	0.065	268
ENPEP	−0.148	0.083	0.075	0.150	268
LNPEP	−0.164	0.170	0.334	0.477	268
NPEPPS	0.173	0.179	0.334	0.477	268
LAP3	0.365	0.485	0.452	0.503	268
RNPEP	0.350	0.460	0.447	0.503	268
ANPEP	0.504	0.770	0.513	0.513	268

ACE. angiotensin I-converting enzyme; ACE2: angiotensin I-converting enzyme 2; ANPEP: memnrane alanyl-aminopeptidase (APN); DPP4: dipeptidyl peptidase 4; ENPEP: glutamyl-aminopeptidase (APA); LAP3: leucine aminopeptidase 3; LNPEP: leucyl/cystinyl aminopeptidase; NPEPPS: aminopeptidase puromycine sensitive (PSA); PREP: prolyl endopeptidase (PEP); RNPEP: arginyl aminopeptidase (APB); eGFR: estimated glomerular filtration rate; FDR: false discovery rate (statistically significant values are shown in bold); SE: standard error.
Results from linear regression models adjusted for age in years and sex. Results were adjusted for multiple testing by applying the FDR.

DPP4 and PEP were inversely correlated with age (Table 4), whereas none of the others proteases studied here showed significant age- or sex-dependent changes in serum activity (Supplementary Tables S3 and S4). Among the 268 patients included in the study, 80 had atrial fibrillation (AF) in their medical history. In agreement with the established fact that AF prevalence increases with age, in our study AF was positively correlated with age ($r = 0.27$, $p = 1.02 \times 10^{-5}$). AF was not correlated to protease activity (Supplementary Table S4).

ACE activity was influenced by medication (ATC code C9; agents acting on the RAS). However, the significant association between eGFR and DPP4 or ACE2 activity, respectively, remained after adjusting for medication intake.

Discussion

We performed a comprehensive analysis of serum protease activity to assess their potential association with CKD. Of the 10 protease activities studied, that of ACE2 and DPP4 were found to be associated with eGFR.

ACE2 is fairly well characterized as part of the alternative ACE2/Ang-(1–7)/Mas axis of the RAS. The kidney is among the tissues with highest ACE2 expression levels,[25,26] and in polarized kidney cells ACE2 appears to be preferentially expressed on the apical surface.[27] ACE2 is proteolytically cleaved from the apical surface to release a soluble form.[27,28] In human plasma, ACE2 enzymatic activity is rather low and, dependent on the assay applied and the clinical setting, might even be too low to be measured.[29] Nevertheless, plasma or serum enzymatic activity of ACE2 exerts significant protective effects due to its ability to convert AngII, the major effector peptide of the classical ACE/AngII/AT1R RAS axis, to Ang-(1–7). Such protective activity of Ang-(1–7) has been observed in animal models of Xu *et al.*[30] and patients with CKD.[31] Accordingly, loss of ACE2 was shown to exacerbate experimental renal ischemia–reperfusion injury[32] and pharmacological inhibition of ACE2 aggravated albuminuria in (5/6) nephrectomy mice; the latter effect could be prevented by the AT1R antagonist, losartan.[33]

The mutual influence of the classical AngII/ACE/AT1R and alternative Ang-(1–7)/ACE2/Mas axis of the RAS is also emphasized by the interesting data of Reich *et al.*,[34] who found that ACE2 mRNA levels were reduced whereas ACE mRNA levels were increased in glomerular and proximal tubular cells of diabetic patients. Another study revealed no differences in renal expression of ACE2 between patients with diverse primary and secondary renal diseases, including patients with diabetic nephropathy.[35] Podocytes were shown to preferentially express the components of an alternative RAS axis, neprilysin, aminopeptidase A, ACE2, and renin, which leads to the primarily formation of Ang-(1–7) and simultaneous degradation of AngII.[36]

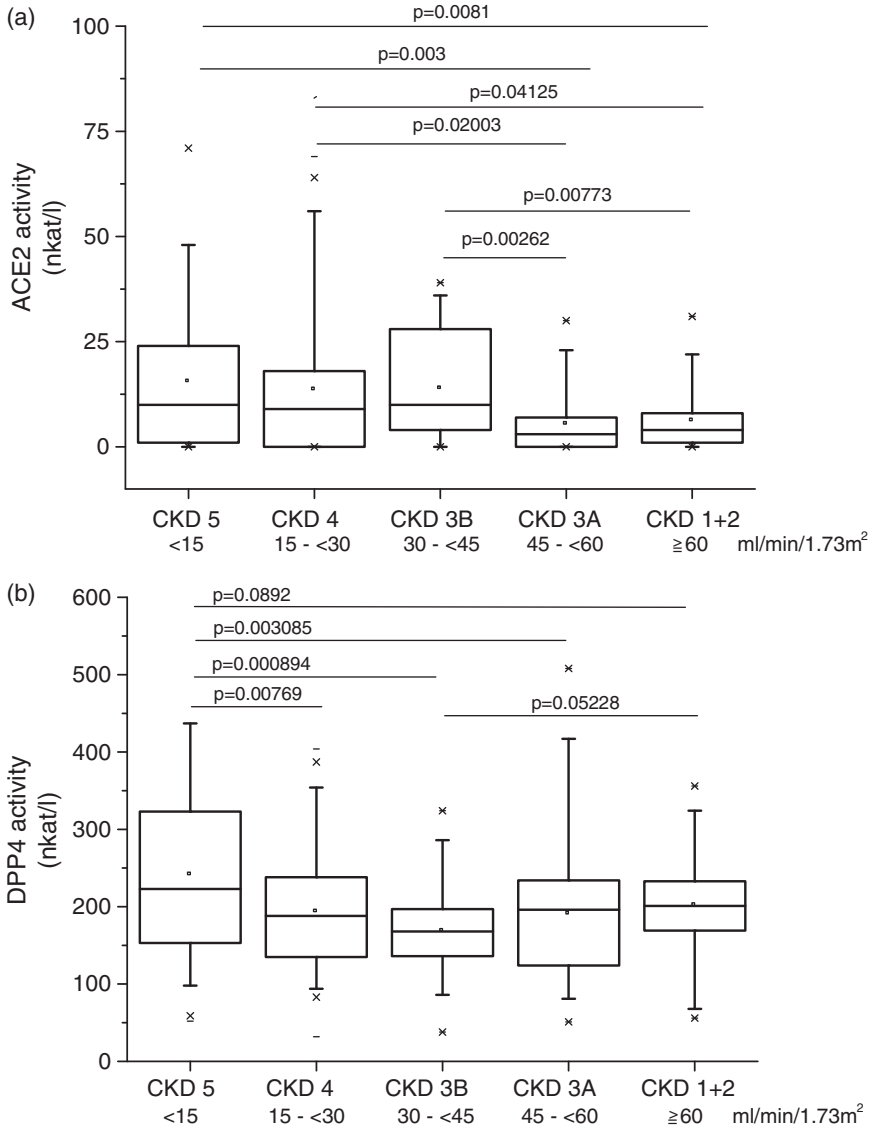

Figure 1 Serum ACE2 and DPP4 activities according to eGFR groups. (a) ACE2 serum activity was inversely correlated with eGFR. Patients with well-preserved eGFR (eGFR groups 1 and 2; >45 mL/min/1.73 m^2) had lower ACE2 activities than groups 3–5 ($P < 0.00016$). (b) DPP4 activity according to eGFR groups. Significantly higher DPP4 serum activities were observed in patients with eGFR < 15 mL/min/1.73 m^2 compared to serum activity of patients with eGFR ≥ 15 mL/min/1.73 m^2 ($p = 3.73 \times 10^{-5}$).
ACE2: angiotensin I-converting enzyme 2; DPP4: dipeptidyl-peptidase 4; eGFR: estimated glomerular filtration rate

In our patient cohort, direct serum ACE2 activity (without removal of any endogenous inhibitor prior to activity measurement) was inversely associated with eGFR. Lowest ACE2 activities were found in patient groups exhibiting an eGFR ≥ 45 mL/min/1.73 m^2. It has been shown previously that plasma ACE2 levels are low in healthy subjects, but substantially increase under pathologic conditions including cardiac disease or diabetes.[37–39] Higher serum ACE2 activity in patients with lower eGFR as observed here could represent a similar compensatory and protective mechanism aimed at counteracting disease-associated induction of the classical AngII/ACE/AT1R RAS axis. Elevated AngII levels have been associated previously with various cardiovascular or cardio-renal risk factors including diabetes, hypertension, obesity, and hyperlipidemia.

In a recent study, however, Roberts *et al.*[40] report lower ACE2 plasma activities in patients on hemodialysis when compared to CKD patients who do not require hemodialysis (stage 3–4; eGFR ≥ 15 – <60 mL/min/1.73 m^2). This is particularly true for the ACE2 activity after inhibitor removal (indirect activity; about 2.5-fold decrease), whereas the direct ACE2 activity was not significantly affected (1.25-fold lower). The latter finding might partly explain the discrepant results. It should be emphasized, however, that the ACE2 activities measured in our study are in the range of those previously reported.[39,40] Notably, the direct ACE2 enzymatic activities of healthy controls (8.3 nkat/L in Wang *et al.*[39]) were closest to that of patients with well-preserved eGFR (≥60 mL/min/1.73 m^2) in our study (6.25 nkat/L). A recent study observed lower circulating ACE2 activity in CKD

Table 4 Associations between protease activity and age.

Protease	Beta	SE	p	FDR	n
PREP	–0.790	0.191	4.706E-05	**4.706E-04**	268
DPP4	–1.368	0.474	0.004	**0.021**	268
ENPEP	–0.318	0.155	0.041	0.136	268
LAP3	–1.829	0.899	0.043	0.107	268
ANPEP	–2.571	1.425	0.072	0.145	268
NPEPPS	–0.467	0.332	0.161	0.268	268
RNPEP	–0.980	0.852	0.251	0.359	268
LNPEP	–0.358	0.315	0.256	0.320	268
ACE	–0.666	0.706	0.346	0.385	268
ACE2	–0.021	0.075	0.779	0779	268

ACE. angiotensin I-converting enzyme; ACE2: angiotensin I-converting enzyme 2; ANPEP: memnrane alanyl-aminopeptidase (APN); DPP4: dipeptidyl peptidase 4; ENPEP: glutamyl-aminopeptidase (APA); LAP3: leucine aminopeptidase 3; LNPEP: leucyl/cystinyl aminopeptidase; NPEPPS: aminopeptidase puromycine sensitive (PSA); PREP: prolyl endopeptidase (PEP); RNPEP: arginyl aminopeptidase (APB); FDR: false discovery rate (statistically significant values are shown in bold); SE: standard error.
Results were adjusted for multiple testing by applying the FDR.

patients without concomitant cardiovascular disease (stages 3–5 and 5/dialysis) when compared to controls.[41] Urinary ACE2 levels were found to be increased in diabetic patients and in patients with CKD stage 4 (compared to CKD1-3).[42] Urinary ACE2 seems to reflect glomerular damage as it was associated with albuminuria and urinary liver-type fatty acid binding protein levels.

Dipeptidyl peptidase 4 (DPP4, CD26, EC3.4.14.5) is a 110 kDa type II membrane protein. In the plasma membrane it occurs as a 220–240 kDa homodimeric peptidase. The enzyme is ubiquitously expressed with highest expression levels in kidney, spleen, and lung.[43] Furthermore, it has been established as a T-cell activation marker[44,45] and implicated in T-cell function.[46,47] Various diseases, in particular cancer, are associated with altered expression/activity of DPP4.[15,48] Bone marrow-derived cells but not the kidney have been identified as a source of soluble DPP4.[43] Serum DPP4 levels are subject to change; altered activities have been associated with various cancers[49–51] and activity increased after oral glucose load in healthy subjects.[52] The postproline cleaving substrate specificity makes DPP4 relatively unique among other proteases.[53,54] It preferentially removes X-Pro, but also X-Ala dipeptides from the N-terminus of substrates. GLP-1 is among the established DPP4 substrates and, therefore, DPP4 inhibitors (gliptins) emerged as antidiabetic compounds with increasing clinical use.[55] DPP4 has been implicated in the preservation of renal function in streptozotocin-induced diabetic rats[56] and in the non-obese diabetic mouse.[17] In the latter, insulin improved renal function as well as circulating and renal ACE2 activity.[17] However, inhibition of DPP4 appears to be nephroprotective by mechanisms independent of changes in blood glucose, too.[57] This view is supported by a recent study demonstrating in two different animal models of renal injury (db/db mice and adriamycin-induced nephropathy) that the DPP4 inhibitor, DA-1229, improved proteinuria, renal fibrosis, and urinary nephrin loss without affecting glycemic control.[58] Similarily, gemigliptin suppressed albuminuria and reduced apoptosis of podocytes independent of its glucose-lowering effects.[59] Furthermore, inhibition of DPP4 by vildagliptin was shown to restore urinary flow, GFR, and proteinuria in a rat model of heart failure-induced renal dysfunction.[60] Recent data provide mechanistic insight into the role of DPP4 by demonstrating that linagliptin prevents TGF-β1/Smad2/3-signaling in a mannose-6-phosphate receptor-dependent manner in a model of diabetic nephropathy.[61] Furthermore, the DPP4 inhibitor, MK0626, normalized the AngII-dependent decrease in the expression of megalin, an endocytic receptor of the proximal tubule.[16] This emphasizes the important contribution of an inappropriately activated RAS (due to, e.g. hypertension, AF, obesity, hyperlipidemia, or hyperglycemia) to CKD.[16,58] DPP4 inhibitors have been shown to reduce fibrotic and inflammatory markers with NF-κB, TGF-β1/Smad, AngII, and mitogen-activated kinase Erk1/2 being possible mediators identified.[16,18,61]

In this study we observed a negative association between eGFR and serum DPP4 activity. Highest activities (240 ± 113 nkat/L; mean ± SD) were found in patients with eGFR < 15 mL/min/1.73 m^2. This activity range is about 50% lower than of previously published reference values.[19] Of note, this previous work did not consider the contribution of other members of the DPP4 family to the Gly-Pro-pNA-hydrolyzing activity that was used for measuring serum/plasma activities in the assay. In our study, DPP4 activity was defined as the fraction of Gly-Pro-pNA-hydrolyzing activity that could be inhibited by Lys-Z-nitro-thiazolidid, thereby excluding the non-inhibited fraction that rather represents PEP activity. Whereas Durinx *et al.*[19] observed a decline of serum DPP4 activity with growing age and higher activities in men than in women, these effects could be observed here only for age. Again, the apparent discrepancy might be due to the application of a specific DPP4 inhibitor in our study. An age-dependent increase in Gly-Pro-pNA-hydrolyzing activity has been observed previously in the pericardial fluid of elder female patients (> 80 years) with coronary artery disease, whereas elder male patients showed a tendency toward decreased DPP4 activities.[13] In another study, where DPP4 activity was higher in patients with appropriate implantable cardioverter/defibrillator (ICD) intervention than in patients without ICD intervention, DPP4 activity was negatively correlated with age.[20] This finding suggests that the specific response to diverse pathophysiological conditions may depend on age and sex. Although ACE, ENPEP, and NPEPPS passed the significance threshold in one of the dichotomous analyses, further studies are needed to test whether these enzymatic activities are truly associated with the eGFR, since both associations were close to the significance level only in one specific dichotomous analysis and not in the other tests, and thus giving inconclusive results.

In summary, our data indicate that ACE2 and DPP4 enzymatic activities but not the enzymatic activities of several other serum peptidases are associated with the eGFR in patients with CKD. It remains to be established if and to what extent altered protease activities may serve as useful

markers for monitoring development and progression of CKD.

However, our findings clearly show that further analyses are warranted to identify the precise role of serum ectopeptidases in the pathogenesis of CKD and to fully elucidate underlying molecular mechanisms.

Authors' contributions: All authors participated in the design of the study, interpretation of data, and review of the manuscript. AH and AT performed statistical analyses. AH, CW, UL performed the laboratory experiments. BF, KE, NE, RR, SA, SBF, and SS contributed data/materials/analysis tools. CW and UL wrote the manuscript. AH and RR checked and polished the manuscript. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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