Endostatin, cathepsin S and cathepsin L, and their association with inflammatory markers and mortality in patients undergoing hemodialysis

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Short running title: **Endostatin, cathepsin-S and cathepsin-L in hemodialysis patients**

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Abstract

Background/aims: Although both endostatin and cathepsins S have been associated with higher mortality, data in patients with end stage renal disease (ESRD) are scarce.

Methods: Longitudinal cohort study of 207 prevalent patients undergoing hemodialysis.

Results: Cathepsins S and L were associated with soluble receptors for tumor necrosis factor (sTNFR1 and sTNFR2, rho between 0.28 and 0.43, $p<0.001$ for all). Weaker or absent associations between endostatin, cathepsins S and L were seen with other inflammatory biomarkers, i.e. CRP, interleukin 6, pentraxin 3, and TNF. In Cox and Laplace regression models adjusted for age, sex, dialysis vintage and diabetes: standard deviation increments of endostatin was associated a lower mortality (hazard ratio 0.75, 95% confidence interval (CI) 0.57-0.98), and with 6.8 months longer median survival.

Conclusions: The high levels of endostatin, cathepsins S and L, and their associations with sTNFR1 and sTNFR2 warrant further studies exploring mortality, and the angiogenic and inflammatory pathways in ESRD.

Key words: patients, TNF, Laplace regression, mortality, inflammation

Introduction

Patients with end stage renal disease (ESRD), have an estimated survival which in most cases is limited to a few years, yet their prognosis cannot be estimated successfully using established cardiovascular risk factors [1]. Biomarkers that can help identify the pathological processes underpinning the unacceptable high mortality risk in ESRD are thus warranted [2].

Proteolytic cleavage of collagen XVIII in the extra cellular matrix produce an endogenous angiogenesis inhibitor called endostatin [3]. The release of endostatin is triggered by elastase, metalloproteinases and cathepsins [4-6]. Specifically, the angiogenic activity of endostatin is believed to be regulated by cathepsins S and L [7]. Recent studies have suggested that the endostatin levels parallel decline of kidney function, and suggest that endostatin is involved in fundamental physiological processes as well as pathogenesis in the kidneys [8-12]. In addition to the effects of cathepsin S on the activity of endostatin, cathepsin S has been shown to be associated with two markers of cytokine-mediated inflammation, CRP and IL-6 [13], which also makes endostatin and cathepsins S and L interesting as potential markers of the pathophysiological inflammatory processes in different stages of CKD. Moreover, cathepsin L has been proposed as a potential kidney injury biomarker as well, as it is highly expressed in rats with renal damage [14].

Both endostatin and cathepsin S have been associated with mortality due to cancer as well as cardiovascular causes in the community [15,16]. Moreover, endostatin is being evaluated as an anti-cancer drug [17], and also cathepsins S and L are being investigated as potential drug targets by pharmaceutical companies [18,19]. Yet to date, data regarding the potential importance of endostatin, cathepsins S and L as biomarkers of pathophysiological processes and mortality in ESRD patients are limited.

Based on the associations between endostatin and cathepsins S and L with mortality as well as renal pathogenesis, we hypothesized that these biomarkers are involved in causal processes that influence the survival of hemodialysis (HD) patients. Accordingly, we aimed to explore the levels of endostatin and cathepsins S and L, and whether their concentration associates with inflammatory biomarkers and mortality in HD patients.

Methods

Patients

The current study was performed at the Karolinska University Hospital in Stockholm (including four satellite dialysis units), Danderyds Hospital, and Uppsala Academic Hospital. It is an ancillary analysis from frozen samples in a cross-sectional cohort study with prospective follow-up that originally aimed at investigating the variability of inflammatory markers over time in prevalent HD patients. Recruitment occurred from October 2003 through March 2004. Data on demographics, comorbidities [i.e. diabetes mellitus (DM) and cardiovascular disease (CVD)] were obtained from the patient records and blood samples were collected. More details about the study and its participants have been described in detail previously [20,21]. Blood samples were collected before the dialysis session. The plasma was separated within 30 min, and samples were kept frozen at-70 °C if not analyzed immediately. Sufficient plasma samples were available in 209 of the recruited patients and those were included in the present analysis.

The Ethics Committee of Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden approved the study protocol and informed consent was obtained from all patients.

Anthropometrics and nutrition

Body mass index (BMI) was defined as the body weight in kilograms divided by the square of patient height in meters. Self-reported appetite is a part of the subjective global assessment (SGA) questionnaire and it includes 6 different components: three subjective assessments that are performed by the patients and that concern the patient's history of weight loss, incidence of anorexia, and incidence of vomiting and three assessments that are performed by the evaluators and that are based on the subjective grading of muscle wasting, the presence of edema, and the loss of subcutaneous fat [22,23]. On the basis of these assessments, each patient received a nutritional status score: 1) normal nutritional status, 2) mild malnutrition, 3) moderate malnutrition, and 4) severe malnutrition. For the purposes of the current study, protein-energy wasting (PEW) was defined as an SGA score >1.

Laboratory analysis

Serum levels of endostatin and cathepsins S and L were analyzed using commercial sandwich enzyme linked immunosorbent assays (ELISAs; DY1098, DY952 and DY1183, R&D

Systems, Minneapolis, MN, USA), in which a monoclonal antibody specific for the peptide was coated onto microtitre plates. The soluble receptors for tumor necrosis factor-α (TNF-α), sTNFR1 and sTNFR2 were analyzed using commercially available ELISA kit (DY225 and DY726, R&D Systems, Minneapolis, MN). The assays had a total coefficient of variation (CV) of approximately 7%. High-sensitivity C-reactive protein (hsCRP) was measured by nephelometry. IL-6 was quantified in serum by an immunometric assay on an Immulite Analyzer according to the instructions of the manufacturer (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Pentraxin 3 (PTX3) and was determined by an ELISA kit (Perseus Proteomics, Tokyo, Japan), as were TNF-α levels (Immulite, DPC, Siemens, CA). Additional biochemical analyses were performed using routine methods at the Department of Clinical Chemistry at Karolinska University Hospital Huddinge.

Follow-up

Survival, censored at transplantation, was determined from the day of examination, with a median follow-up period of 31 interquartile range (IQR) 21–38 months.

Statistical analysis

Bonnet-Price 95% confidence intervals (CI) were estimated for all continuous variables, and used to test if there were any significant difference in the levels of endostatin, cathepsin S and L in individuals with high and low SGA (cut-off SGA >1), and among individuals with and without diabetes. We tested the correlations between endostatin, cathepsin S and L, as well as other inflammatory markers using the Spearman's rank correlation coefficient. The statistical software package STATA 12.1 (Stata Corporation, College Station, TX) was used.

Longitudinal analyses

We modelled endostatin, cathepsin S and L per standard deviation (SD) increment. Cox regression was used to calculate hazard ratios (HR) with 95% CI, and Laplace regression was used to calculate the difference in time (in months) until a certain percentage of the ESRD patients died during follow up [24]. We used the time until the first 25% and 50% of the patients died to identify mortality differences per SD increment of each biomarker during follow-up. The 25th survival percentile and the median survival were chosen because there were a sufficient number of observed fatalities (events) before and after these values during

the follow-up. Since different distributions and mathematical calculations are used to obtain results in Cox and Laplace regression, respectively, putting emphasis on findings significant with both methods may reduce the risk of chance findings [25,26]. The bootstrap method was used to calculate the 95% CIs in Laplace regression with an amendment for Laplace regression in Stata. The following multivariable models were used:

Model A was adjusted for age, sex, dialysis vintage and presence of diabetes mellitus. All these are well-known factors that may influence the prognosis in ESRD patients. Model B was in addition to the factors in Model A, also adjusted for CRP, albumin and PEW, to see if our estimates are independent of inflammation as well as albumin (often used as a marker of nutritional status in ESRD patients), and PEW diagnosis. Finally, we tested model B without CRP, as CRP and albumin could interfere as they are highly correlated.

RESULTS

Baseline characteristics are shown in Table 1. The mean values (SD) of endostatin and cathepsins S and L were 275.8 ± 104.9 µg/1, 13.6 ± 3.4 µg/1 and 5.3 ± 5.2 µg/1, respectively.

The Spearman's rank correlation (rho) between endostatin and cathepsin S was 0.21, p=0.003; endostatin and cathepsin L (rho=0.29, p<0.001); cathepsin S and L (rho=0.63, p<0.001). There were no significant differences in median levels of endostatin, cathepsins S or cathepsin L in patients with and without PEW (SGA >1) based on their Bonnet-Price CIs (p>0.09 for all). No significant difference in median levels of endostatin, cathepsins S or cathepsin L in patients with and without diabetes was noted (p>0.12 for all) (data not shown in tables).

The Spearman's rank correlations between endostatin, cathepsin S and cathepsin L with inflammatory markers at baseline were calculated (CRP, interleukin 6 [IL-6], pentaxin 3 [PTX3], sTNFR1, sTNFR2 and TNF-α). Only cathepsin L was associated with CRP (rho=0.18, p=0.01). None of the markers were associated with IL-6 (p>0.2 for all). Weak associations with PTX3 (endostatin rho=-0.12, p=0.11), (cathepsin S rho=0.13, p=0.071), (cathepsin L rho=0.16, p=0.03) were noted. Cathepsins S and L were both highly associated with sTNFR1 and sTNFR2 (rho between 0.28 and 0.43, p<0.001 for all). Endostatin was

associated with sTNFR1 (rho=0.25, p<0.001), but less so with sTNFR2 (rho=0.13, p=0.06). Only cathepsin S was associated with TNF (rho=0.18, p=0.011) (data not shown in tables).

The Spearman's rank correlations between endostatin, cathepsin S and cathepsin L, and adiponectin, ghrelin and leptin were also calculated in secondary analyses. Only one weakly significant association was detected, between endostatin and ghrelin (rho=0.16, p=0.02) (data not shown in tables).

Table 2 shows the association between SD increments in the serum levels of endostatin, cathepsins S and cathepsin L, and mortality in Cox regression models. High levels of endostatin were associated with slightly lower mortality in model A, but the association was attenuated and no longer significant in model B. Neither cathepsins S nor cathepsin L was associated with mortality in the Cox regression models.

Table 3 shows the association between SD increments in the serum levels of endostatin, cathepsins S and L, respectively, and survival time in Laplace regression models. In model A, a SD higher endostatin level was associated with about 6 months longer time until the first 25% and 50% of the patients died (p<0.01). The results for endostatin were attenuated and only significant for the first 50% that died (4.7 months later, p=0.023) in model B. In agreement with the Cox regression models, neither SD increments in cathepsins S or L were significantly associated with any difference in survival time until the first 25% and 50 % of the HD patients had died. Although the cathepsins were non-significant, all risk estimates for all three markers portrayed longer survival time in individuals with high levels.

Finally, the results in model B remained essentially unaltered without adjustments for CRP (data not shown in tables).

Discussion

In a cohort of prevalent HD-patients, high levels of endostatin were associated with a lower relative mortality risk and with several months longer median survival in Laplace regression models. The results were attenuated after adjustments for CRP, albumin and PEW. The biomarkers that regulate the anti-angiogenic activity of endostatin; cathepsins S and cathepsin L were not significantly associated with mortality risk, but higher levels had consistent risk estimates toward a lower mortality risk and longer survival in Laplace regression models. Endostatin, cathepsins S and L were associated with various inflammatory markers to

different degrees, making them interesting targets to explore in studies exploring the inflammatory milieu in HD-patients.

In contrast to the findings in the present study in HD patients, where endostatin was associated with lower mortality risk, higher levels of endostatin has been shown to be associated higher mortality risk in the community [15]. A higher mortality risk with higher levels of cathepsin S has also been seen in community based studies [16]. Yet the risk estimates with higher levels of cathepsins S and L in the present study pointed, although non-significant, towards a lower mortality, which could be a "spill-over" effect from endostatin, considering the high correlation between endostatin and the cathepsins in the present study.

Elevated endostatin levels have been suggested as a biomarker of a rapid turnover of the extracellular matrix in patients with malignant diseases, such as renal carcinoma [27], and in patients with hypertensive renal disease [28]. Yet, initiated extra cellular matrix remodeling has been shown to be present already in early CKD stages [10,27,29], and may thus progress further during HD. The effects of endostatin have been suggested to be kidney protective in diabetic rats with diabetes [8]. Our findings of a lower overall mortality risk, suggest that protective effects of endostatin itself or that rapid extracellular matrix turnover is associated with favourable pathophysiological processes in HD-patients. Another explanation to the lower risk in patients with higher levels of endostatin is that the anti-angiogenic properties may exert protective effects [30].

The endostatin levels were about five-fold higher in the present study as compared with elderly individuals in the general population [28], which may partly be explained by the fact that endostatin has been shown to be associated with many risk factors that are highly prevalent in HD patients: gluco-metabolic disturbance [31,32], elevated blood lipids [33], and long term hypertension [28]. The high levels may in HD patients may additionally be explained by the fact that urinary excretion is the major elimination route of endostatin [34]. The high endostatin levels probably reflect a combination of increased extracellular matrix turnover and lack of renal clearance.

An explanation to the findings towards the null despite high levels of cathepsins S and L, could be the heterogeneity of the HD patients or that the aforementioned risk factors and associated CKD disease progression have been going on for too long periods of time, for the observed levels of the cathepsins to show variability in their prognostic value for mortality.

Parts of the findings of the present study may be explained by the filtration of these biomarkers. Cathepsin S, cathepsin L and endostatin are all derived from larger propeptides but the final peptides have molecular weights in the 20-30 kDa range (cathepsin B 24 kDa, cathepsin L 25 kDa and endostatin 20 kDa). The sizes indicate that these peptides should be freely filtered through the glomeruli as they have similar molecular weights as cystatin C, beta-trace protein and beta2-microglobulin. In patients with reduced ESRD and reduced GFR the plasma concentrations of these peptides will increase due to the reduced glomerular filtration. The diagnosis of ESRD is predominantly based on creatinine based eGFR in this study. Creatinine is a small molecule compared to these peptides. It has been shown that there an increased filtration of small molecules like creatinine during pregnancy combined with a decreased filtration of medium-sized (10-30 kDa) molecules [35]. It seems possible that such qualitative changes in glomerular filtration may also occur in some patients with reduced kidney function. The increased use of cystatin C in combination with creatinine may provide information on the occurence of qualitative changes in glomerular filtration in patients with CKD. Both cathepsins and endostatin will interact with other molecules in vivo such as collagen, fibronectin, histones, proteoglycans and glycoproteins with glycosaminoglycans. When bound to other molecules the size of the complexes will increase thus reducing the glomerular filtration. Previous studies have shown that conventional hemodialysis with low-flux membranes effectively eliminates small molecules such as urea and creatinine but do not reduce the levels of proteins in the 20-30 kDa range. Post-dilution haemodiafiltration and pre-dilution haemofiltration reduced cystatin C (13.3 kDa) and beta2-microglobulin (11.8 kDa) but was less efficient for removing beta-trace protein (23-29 kDa) [36]. Cathepsin S, cathepsin L and endostatin are in the same molecular range as beta-trace protein which means that these dialysis methods will not reduce the plasma levels.

There were also no salient associations between endostatin or the cathepsins with adipokines, and no significant difference in the levels of these markers in individuals with and without PEW, factors that may be of greater importance for the health of HD-patients than these biomarkers.

In contrast to a large study of elderly community dwelling men [13], cathepsin S was not associated with CRP and IL-6 in the present study. Yet, cathepsin L was associated with IL-6. More importantly, both cathepsins were associated with both sTNFR1 and sTNFR2, which

may be of clinical importance since the role of these biomarkers have been shown to be of importance for different stages of CKD and mortality [37-40]. Yet, the association between endostatin as well as cathepsins S and L levels with mortality and inflammatory biomarkers may be only associative, and do not clearly demonstrate a role of these molecules in the pathogenesis of diseases associated with CKD and dialysis. In addition, all these molecules may be involved in CKD progression as well as endothelial dysfunction. In fact, inhibition of angiogenesis may play a pivotal role in the capillary rarefaction, and endothelial cell injury that is present in ESRD patients.

Studies investigating cathepsins S and L in earlier stages of CKD and diabetes nephropathy, as well as studies confirming our findings in the present study in HD-patients are warranted.

Strengths and limitations

Strengths of our investigation include the prospective longitudinal study design, the detailed characterization of study participants, and relatively large sample of HD-patients with no loss to follow-up. The high correlation between cathepsin S and L and between these cathepsins and endostatin adds validity to our findings and support the robustness of our data. Limitations include the observational design, including prevalent patients with varying disease duration and low statistical power in some of our analyses. It should also be acknowledged that as it has been reported that mortality is far more common than dialysis at all stages of CKD [41], suggesting that the present cohort of prevalent HD-patients might constitute a selected group of survivors not representative of CKD patients.

Conclusions

Our findings indicate that the prognostic use for endostatin, cathepsins S and L in prevalent HD patients are limited. Yet, the high levels of these markers, and the high correlations with sTNFR1 and sTNFR2 warrants further studies exploring the angiogenic and inflammatory pathways using these markers in studies of CKD progression, as well as their association with specific morbidities.

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Disclosures

The study was investigator-initiated and -driven. The authors report no conflicts of interests in connection with this study.

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Table 1: Baseline characteristics.

	n (%) or median (95% Bonnet-Price confidence interval)
Number of subjects	209
Men	118 (56 %)
Age (years)	66 (63-69)
Dialysis vintage (months)	29 (24-33)
Endostatin (ng/mL)	254.3 (237.2-271.4)
Cathepsin S (ng/mL)	13.4 (12.7-14.0)
Cathepsin L (ng/mL)	4.64 (4.37-4.91)
sTNFR1 (ng/mL)	17.7 (17.0-18.3)
sTNFR2 (ng/mL)	24.5 (23.7-25.2)
Body mass index (kg/m^2)	23.9 (23.3-24.6)
C-reactive protein (mg/L)	6.7 (5.2-8.1)
Interleukin 6 (pg/mL)	8.4 (7.5-9.3)
Pentraxin 3 (ng/mL)	10.2 (9.1-11.3)
TNF (pg/mL)	14.1 (13.5-14.7)
Adiponectin (µg/mL)	22.2 (19.8-24.6)
Ghrelin (pg/mL)	367 (344-390)
Leptin (ng/mL)	16.1 (12.6-19.5)
Plasma Albumin (g/L)	35 (34-36)
Subjective global assessment >1	93 (45 %)
Ischemic heart disease	65 (31%)
Chronic renal failure of uncertain origin	23 (11%)
Polycystic kidneys	19 (9%)
Renal vascular disease (all types)	31 (15%)
Glomerulonephritis	24 (11%)
Polyonephritis (all types)	14 (7%)
Cystic kidney disease	7 (3%)
IgA nephropathy	6 (3%)
Kidney tumour	4 (2%)
Diabetes	49 (23 %)

Table 2 Multivariable Cox regression models showing the association between endostatin, cathepsin S and cathepsin L, respectively, with mortality

Biomarker	Model	Hazard Ratio	95% confidence intervals	p-value
Endostatin	A	0.75	0.57-0.98	0.040
	B	0.85	0.64-1.12	0.24
Cathepsin S	A	0.90	0.69-1.17	0.43
	B	0.84	0.64-1.09	0.20
Cathepsin L	A	0.83	0.54-1.29	0.40
	B	0.79	0.53-1.21	0.29

Model A was adjusted for age, sex, number of days on dialysis before baseline and presence of diabetes mellitus. Model B was in addition to the factors in Model A, also adjusted for CRP, albumin and SGA.

Table 3 Multivariable Laplace regression models showing the difference in survival in months that it takes for the first 25% and 50% of the ESRD patients to die, per standard deviation increment in endostatin, cathepsin S and cathepsin L, respectively

		Endostatin			Cathepsin S			Cathepsin L		
Percentile	**Model**	**Months**	**95% CI**	**p-value**	**Months**	**95% CI**	**p-value**	**Months**	**95% CI**	**p-value**
25%	A	6.04	2.07, 10.03	0.003	2.24	-4.11, 8.90	0.51	4.57	-2.52, 11.65	0.21
	B	4.68	0.65, 8.71	0.023	3.42	-3.01, 9.86	0.30	3.30	-2.07, 8.67	0.23
50%	A	6.80	2.59, 11.02	0.002	2.58	-3.55, 8.71	0.41	4.36	-3.42, 12.14	0.27
	B	2.08	-2.07, 6.23	0.33	2.19	-1.24, 5.63	0.21	3.53	-0.14, 7.22	0.059

Model A was adjusted for age, sex, number of days on dialysis before baseline and presence of diabetes mellitus. Model B was in addition to the factors in Model A, also adjusted for CRP, albumin and SGA.