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Original Paper

Pregnancy-Associated Plasma Protein A2 in Hemodialysis Patients: Significance for Prognosis

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Key Words
Hemodialysis • Infection • Mortality • PAPP-A • PAPP-A2 • Surface plasmon resonance

Abstract
Background: Pregnancy-associated plasma protein A (PAPP-A) is associated with adverse outcome of long-term hemodialysis patients (HD). The aim of the study was to test whether its homolog pregnancy-associated plasma protein A2 (PAPP-A2) can be detected in serum of HD patients and to define its significance. ***Methods:*** The studied group consisted of 102 long-term HD patients and 25 healthy controls. HD patients were prospectively followed up for five years (2009-2014). PAPP-A2 was measured by surface plasmon resonance biosensor, PAPP-A by time resolved amplified cryptate emission. ***Results:*** PAPP-A2, similarly as PAPP-A, was significantly increased in HD patients (median (interquartile range)) PAPP-A2: 6.2 (2.6-10.8) ng/mL, vs. 3.0 (0.7-5.9) ng/mL, p=0.006; PAPP-A: 18.9 (14.3-23.4) mIU/L, vs. 9.5 (8.4-10.5) mIU/L, p<0.001). In HD patients, PAPP-A2 correlated weakly but significantly with PAPP-A (τ=0.193, p=0.004). Unlike PAPP-A, PAPP-A2 was not significant for prognosis of HD patients when tested alone. There was a significant interaction between PAPP-A and PAPP-A2 on the mortality due to infection of HD patients (p=0.008). If PAPP-A was below median, mortality due to infection was significantly higher for patients with PAPP-A2 values above median than for patients with low PAPP-A2 levels (p=0.011). ***Conclusion:*** PAPP-A2 is increased in HD patients and interacts with PAPP-A on patients´ prognosis.

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Introduction

Pregnancy-associated plasma protein A (PAPP-A) and its homolog pregnancy-associated plasma protein A2 (PAPP-A2) belong to pappalysins, one of the family of metzincin superfamily of metalloproteinases [1]. They are responsible for proteolytic cleavage of insulin like growth factor binding proteins (IGFBPs), thus increasing insulin like growth factor (IGF) bioavailability [1, 2]. Both are expressed predominantly by placenta in pregnancy [1, 3] and required for normal growth and development [4-6].

PAPP-A (also referred to as pappalysin-1) has been intensively studied in pregnancy as well as outside pregnancy and is routinely used for screening of Down syndrome in the first trimester of pregnancy [7]. In very small amounts it is measurable also in non-pregnant women and healthy men and its increased concentration later in the life is connected with some pathological states [8, 9]. The presence of PAPP-A was shown in eroded and ruptured atherosclerotic plaques and its increase in serum in acute coronary syndrome [8]. It is a predictor of cardiovascular events among patients with cardiac chest pain and is associated with patients´ prognosis [10, 11]. Several studies documented increased PAPP-A levels in patients with chronic kidney disease [12]. Data from different patient cohorts with end-stage renal disease patients treated with long-term hemodialysis demonstrate the association of serum PAPP-A levels with adverse outcome [13-17].

Knowledge about PAPP-A2 (also referred to as pappalysin-2) is rather limited and refers to pregnancy. PAPP-A2 shares approx. 46% of its amino acid residues with PAPP-A and it is synthesized as a prepro-protein [1]. Unlike PAPP-A, PAPP-A2 circulates as a noncovalent dimer [18, 19] and each subunit consists of 1558 amino acids (220kDa) [19]. Additionally, a C-terminally truncated PAPP-A2 variant was detected (less than 5% of PAPP-A2) [19]. PAPP-A2 cleaves IGFBP-5 and this cleavage is IGF-independent [1]. PAPP-A2 was able to degrade to limited extent also IGFBP-3 but not IGFBP-4 as PAPP-A [1]. IGFBP-4 is the main substrate for PAPP-A, and this cleavage is dependent on the presence of IGF [2]. IGFBP-5 can be cleaved by PAPP-A as well [20]. Similarly as PAPP-A, PAPP-A2 originates mainly from placenta and its expression is not limited either to placenta or to pregnancy [1]. Other tissues containing PAPP-A2 mRNA include pregnant uterus, fetal liver/spleen and kidney [1]. The highest PAPP-A2 mRNA expression was found in the placenta of wild-type mice but PAPP-A2 was also abundantly expressed in fetal, skeletal and reproductive tissues, mainly calvaria and prostate, and also detected in colon, lung, ovary, tibia, brain, spinal cord, testes and kidneys [5]. PAPP-A2 expression was not found in spleen, skeletal muscle, adipose tissue, thymus, uterus, lymph nodes, liver, skin, and heart [5]. Homozygous PAPP-A2 knockout mice had normal size at birth but experienced postnatal growth retardation, more pronounced in females than in males (males 10% and females 25-30% compared to wild type mice). Overall body size was decreased to higher degree than organ size [5]. On the other hand, PAPP-A knockout mice are born as proportional dwarfs [21]. PAPP-A2 deficiency altered both the size as well as the shape of bones [6]. PAPP-A2 also modulates angiogenesis in zebrafish embryos [22].

PAPP-A2 increases during pregnancy in agreement with placental synthesis. The average level at the end of pregnancy is 500-fold higher than the levels outside pregnancy [19]. This difference is much less pronounced compared to that of PAPP-A, which is more than 10,000 fold [9, 23]. However, PAPP-A2 was not detected in the cord blood, i.e. in fetal circulation [24]. Increased placental expression of PAPP-A2 [25, 26] and higher maternal levels in circulation were found in pregnancies complicated with preeclampsia and small for gestational age neonates in the first trimester as well as at term [25-28]. Moreover, PAPP-A2 was proposed as an additional marker of Down syndrome in the second trimester of pregnancy where its concentration is also increased [29]. On the other hand, PAPP-A in Down syndrome is decreased in the first trimester of pregnancy [7], and also other adverse outcomes, such as preterm delivery, intrauterine growth restriction, preeclampsia and stillbirth are also associated with lower PAPP-A levels [30]. PAPP-A2 is detectable also in non-pregnant men and women [31] but studies in different pathological states outside pregnancy are lacking.

The aim of the present study was to look whether PAPP-A2 can be measured in serum of HD patients and what is its significance. In particular, we were interested, whether PAPP-A2 is also of importance in patients´ prognosis, and if yes, whether it is a better predictor than PAPP-A; or what is the relationship between these two proteins.

Table 1. Laboratory parameters of hemodialysis patients and healthy controls

Parameter	HD Patients	Controls	HD vs. controls – p value
Number of patients (men/women)	102 (60/42)	25 (16/9)	
Age (years)	61±14	48±11	<0.001
BMI (kg/m^2)	27.0±5.3	26.2±4.2	0.4, n.s.
Hemoglobin (g/L)	111.9±10.9	143.3±8.9	<0.001
Leukocytes (x10^9/L)	7.5±2.2	6.1±1.5	0.006
Creatinine (μmol/L)	778±220	91±12	<0.001
Urea (mmol/L)	23.0±6.8	5.6±1.8	<0.001
Albumin (g/L)	40.8±3.5	47.3±3.2	<0.001
Cholesterol (mmol/L)	4.4±1.0	5.3±1.0	0.001
HDL cholesterol (mmol/L)	1.1±0.3	1.5±0.5	0.001
LDL cholesterol (mmol/L)	2.4±0.9	3.0±0.8	0.002
Triacylglycerols (mmol/L)	2.1±1.2	1.7±0.9	0.1, n.s.
CRP (mg/L)	8.5±12.4	6.1±3.0	0.7, n.s.

Data are expressed as mean±SD (standard deviation). Abbreviations: BMI, body mass index; CRP, C-reactive protein; HD, hemodialysis; HDL, high density lipoprotein; LDL, low density lipoprotein; n.s., not significant.

Subjects and Methods

Study subjects

The studied group consisted of 102 long term hemodialysis patients (60 men and 42 women, mean age 61±14 years) from 2 dialysis centres in the Czech Republic and 25 healthy controls (16 men and 9 women, mean age 48±11 years) for comparison of baseline biochemical parameters of interest. All patients were in stable clinical status when entering the study. Primary renal diagnosis of studied hemodialysis patients were as following: diabetic nephropathy (26.5%), hypertensive nephropathy (4.9%), interstitial nephritis (21.6%), glomerulonephritis (18.6%), polycystic kidney disease (14.7%) and multifactorial or unknown (13.7%). The majority of the patients was dialyzed 3 times a week for 4 hours using polysulphone or modified cellulosic membranes, and their dialysis treatment lasted for median 25.5 months (range 1 – 247 months). Basic laboratory characteristics of hemodialysis patients and controls are shown in Table 1. More than one third of the patients (41.2%) were diabetics, more than half of them (57.8%) had dyslipidemia and the majority (94.1%) had hypertension. The case history of the patients included cardiovascular disease in 34.3% (acute myocardial infarction in 14.7%), cerebrovascular diseases in 3.9% and peripheral vascular disease in 12.7%. 16% of the patients were smokers. The patients were treated with standard medication which was changed over time as required (at baseline antihypertensives in 38.2%, diuretics in 50%, hypolipidemics in 48.5%, aspirin or other antiplatelet drugs in 17.6%, iron, erythropoietin, phosphate binders and vitamin D and its analogs). This studied group is part of the group presented in Kalousová et al. focused on PAPP-A polymorphisms in hemodialysis patients [32].

Hemodialysis patients were prospectively followed up for five years between April 2009 and April 2014. Causes of death and eventual date of transplantation were recorded. Causes of death were finely classified based on patients´ history by two independent physicians as cardiovascular, infection, tumor or other. Half of the patients died during the follow up. In 20 patients (i.e. 19.6%) the causes of death were cardiovascular events while 17 patients (i.e. 16.7%) died due to infection. In 4 patients (3.9%) the cause of death was advanced tumor and in 10 patients (9.8%) the cause was other or unknown. 28 patient were transplanted and 4 of them died (2 due to cardiovascular events and 2 due to infection).

This study was performed in adherence to the principles of the Helsinki Declaration and approved by the Institutional Ethical Committee. All participants gave informed consent prior to entering the study.

Laboratory methods

Blood samples of hemodialysis patients were obtained after the long dialysis interval (2 days) via puncture of the arteriovenous fistula. Blood was collected before starting the dialysis session and prior to heparin administration. In controls, blood was collected after overnight fasting via puncture of the cubital vein. In both studied groups, blood collection occurred simultaneously with blood collection for routine laboratory examinations. Blood was collected into tubes without anticoagulant, centrifuged for 10 min at 3000 rpm (rotations per minute) and serum was stored at –80 °C.

PAPP-A2 concentration in serum was measured by surface plasmon resonance (SPR) biosensor following the protocol recently described by Bocková et al [31]. The used biosensor combines a single surface referencing approach [33] and an antibody sandwich assay with functionalized gold nanoparticles for signal enhancement [34]. Prior to determination of PAPP-A2 concentration in clinical samples, the calibration curve for the SPR biosensor was established from the sensor responses to known concentrations of PAPP-A2 spiked into blood plasma. Then, the sensor response obtained for a clinical sample was converted to a PAPP-A2 concentration using this calibration curve. The cross-reactivity to PAPP-A was evaluated and was less than 2%. Results are expressed in ng/mL.

PAPP-A concentration in serum was measured by TRACE (Time Resolved Amplified Cryptate Emission) using standard kits and Kryptor analyser (Brahms GmbH, Thermo Fisher Scientific, Henningsdorf, Germany). Results are expressed in mIU/L. One IU/L is equivalent to 4500 ng/mL (Maternal Control Data sheet, Randox Laboratories Ltd., UK) [35].

Routine laboratory parameters were measured with standard methods using automated analyzers.

Statistical analysis

Statistical analysis was performed using SPSS v.16 statistics software (SPSS Inc., Chicago, IL, USA, www.spss.com).

Data are expressed as mean ± SD (standard deviation), except for PAPP-A2 and PAPP-A which are provided as medians and interquartile ranges (IQR). Comparison between HD patients and controls and between different subgroups was done non-parametrically, using Mann-Whitney U test. Receiver operating characteristics (ROC) and area under the curve (AUC) were used for assessment of discrimination between HD patients and controls. Kendall's tau correlation coefficient was used for description of the relationship between parameters.

Overall mortality, cardiovascular mortality and mortality due to infection were tested. Hazard ratios (relative risks of mortality, HR) per quartile and appropriate 95% confidence intervals (CI) were calculated using the Cox proportional hazard model with adjustment for age and sex. Transplantation was considered as time-dependent covariate and stratification for the dialysis center was done. PAPP-A and PAPP-A2 were evaluated as continuous variables as percentiles of the concentration in each patient in relationship to the whole patients´ population in order to avoid the influence of extreme results (logarithmic transformation was not possible as due to several zero results of PAPP-A2). When interaction between PAPP-A and PAPP-A2 was studied, PAPP-A was evaluated as percentiles and PAPP-A2 as values below and above median. Final results of HR and 95% CI are expressed per quartile. Kaplan-Meier analysis was used to construct real survival curves for PAPP-A2 and PAPP-A below and above median levels. The medians for PAPP-A and PAPP-A2 calculated for the whole group of patients are used all over the study. For overall mortality, time to death or till the end of the study was considered. For specific causes of mortality, additionally other causes of death were regarded as censoring. Curves were compared with log-rank test. All results were considered statistically significant at $p<0.05$.

Results

PAPP-A2, similarly as PAPP-A, was significantly increased in HD patients (PAPP-A: HD vs. controls: 18.9 (14.3-23.4) mIU/L, vs. 9.5 (8.4-10.5) mIU/L, $p<0.001$ (Fig. 1A), PAPP-A2: HD vs. controls: 6.2 (2.6-10.8) ng/mL, vs. 3.0 (0.7-5.9) ng/mL, $p=0.006$ (Fig. 1B)). Based on receiver operating characteristics, PAPP-A provided better discrimination between HD patients and controls (PAPP-A: AUC 0.961, $p<0.001$; PAPP-A2: AUC 0.678, $p=0.006$). There was no difference either in PAPP-A2 or PAPP-A between men and women, either in HD patients or in controls. The difference between HD and controls was still evident when a

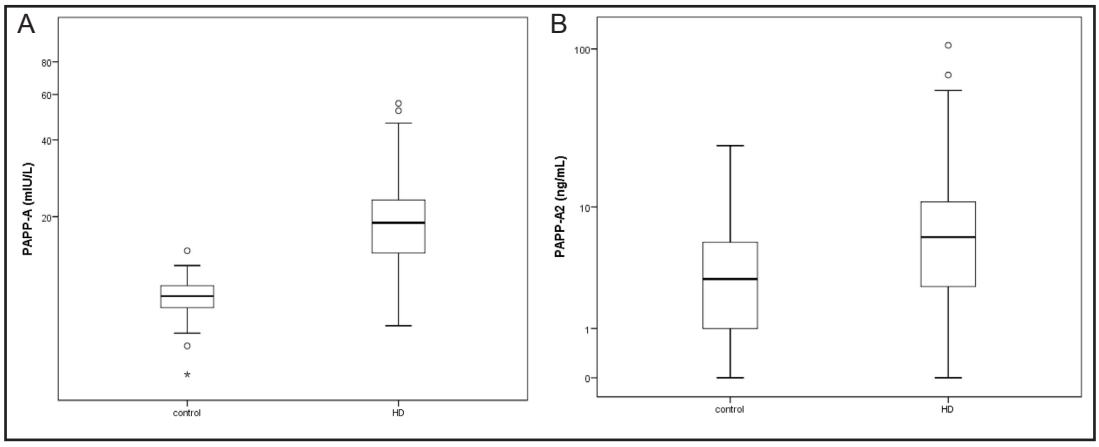

Fig. 1. PAPP-A and PAPP-A2 in HD patients and healthy controls. A PAPP-A: HD vs. controls (median, interquartile range): 18.9 (14.3-23.4) mIU/L, vs. 9.5 (8.4-10.5) mIU/L, p<0.001. B PAPP-A2: HD vs. controls (median, interquartile range): 6.2 (2.6-10.8) ng/mL, vs. 3.0 (0.7-5.9) ng/mL, p=0.006. Abbreviations: HD, hemodialysis; PAPP-A, pregnancy-associated plasma protein A, PAPP-A2, pregnancy-associated plasma protein A2.

subgroup of controls with more comparable age was selected (10 oldest controls, mean age 57 years, 4 men and 6 women) and compared with HD patients: PAPP-A (10 controls) 8.7 (8.5-10.5) mIU/L, p<0.001 vs. HD patients, PAPP-A2 (10 controls) 2.5 (0.3-5.8), p=0.042 vs. HD patients.

In HD patients, PAPP-A2 correlated weakly but significantly with PAPP-A (τ=0.193, p=0.004). In about 60% cases high PAPP-A (i.e. above median) is connected with high PAPP-A2 and low PAPP-A (below median) with low PAPP-A2. There was also a significant correlation of PAPP-A2 with age (τ=0.171, p=0.01), with albumin (τ= -0.186, p=0.006) and with CRP (τ= -0.203, p=0.004) and a correlation of PAPP-A with albumin (τ= -0.240, p<0.001). None of these correlations was seen in controls. We did not find any difference either in PAPP-A2 or in PAPP-A levels between diabetics and non-diabetics, between patients with and without cardiovascular, peripheral vascular or cerebrovascular diseases in the personal history and there was no relationship to hypertension. There was no difference among various primary renal diagnoses either.

PAPP-A2 alone was not significant for survival of HD patients. Results of Cox regression analysis are provided in Table 2. Survival curves for PAPP-A2 below and above median in Kaplan Meier analysis did not show any difference for overall mortality (p=0.547, n.s. – not significant), cardiovascular mortality (p=0.585, n.s.) and slight but non-significant difference for mortality due to infection (p=0.069, n.s.). Cox regression confirmed the significance of PAPP-A for the prognosis of HD patients (Table 2). Kaplan-Meier analysis revealed borderline non-significance of PAPP-A for overall mortality (p=0.085, n.s.), significance for cardiovascular mortality (p=0.002), but non-significance for mortality due to infection (p=0.844, n.s.). Evaluation of mortality due to tumours is difficult as just 4 patients died due to tumours. However, all these patients had PAPP-A below median. An interaction between PAPP-A and PAPP-A2 on mortality of HD patients was observed. It was of borderline non-significance for overall mortality (p=0.088, n.s.) and significant for mortality due to infection (p=0.008). For HRs and 95% CIs for PAPP-A when PAPP-A2 is below or above median see Table 2. In Kaplan Meier analysis when PAPP-A and PAPP-A2 below and above median were evaluated, for combination of high PAPP-A2 (above median) and low PAPP-A (below median), there is highest increase of hazard function, while for both low PAPP-A and low PAPP-A2 (below median) the increase of hazard function is the lowest (p=0.090, n.s.). Additionally, from Kaplan-Meier analysis it can be taken that if PAPP-A is below median, mortality due to infection significantly differs for PAPP-A2 values below and above median being worse for

Table 2. PAPP-A2 and PAPP-A as mortality predictors for overall mortality, cardiovascular mortality and mortality due to infection in HD patients and their interaction

HR (95% CI), p	Overall mortality	Cardiovascular mortality	Mortality due to infection
PAPP-A2	0.993 (0.779-1.264), p=0.952, n.s.	1.146 (0.768-1.709), p=0.505, n.s.	1.106 (0.719-1.702), p=0.647, n.s.
PAPP-A	1.674 (1.189-2.357), p=0.003	2.647 (1.377-5.089), p=0.004	2.389 (1.080-5.283), p=0.032
Interaction between PAPP-A and PAPP-A2	p=0.088, n.s.	p=0.619, n.s.	p=0.008
PAPP-A, if PAPP-A2 below median	1.603 (1.133-2.268), p=0.008	2.744 (1.385-5.436), p=0.004	2.079 (0.910-4.750), p=0.083, n.s.
PAPP-A, if PAPP-A2 above median	1.060 (0.704-1.594), p=0.781, n.s.	1.944 (0.851-4.444), p=0.115, n.s.	0.700 (0.401-1.222), p=0.210

HRs and 95% confidence intervals are given per quartile and were obtained using Cox analysis with adjustment for age and sex. Transplantation was considered as time-dependent covariate and stratification for the dialysis center was done. Abbreviations: CI, confidence interval; HD, hemodialysis; HR, hazard ratio; n.s., not significant; PAPP-A, pregnancy-associated plasma protein A; PAPP-A2, pregnancy-associated plasma protein A2.

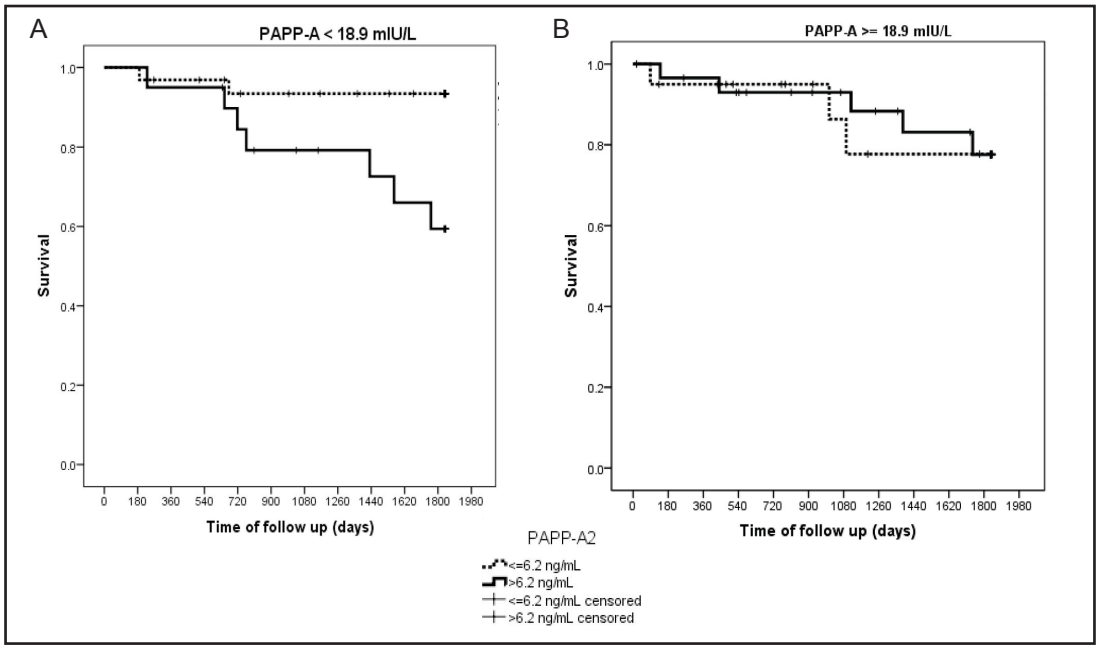

Fig. 2. Kaplan-Meier analysis demonstrating mortality due to infection of HD patients. A for PAPP-A below median (<18.9 mIU/L, 52 patients) and PAPP-A2 values below and above median (6.2 ng/mL, 32 and 20 patients), p=0.011 (log-rank test). B for PAPP-A above median (≥18.9 mIU/L, 50 patients) and PAPP-A2 values below and above median (6.2 ng/mL, 20 and 30 patients), p=0.893 (log-rank test). Survival until the end of the study as well as other causes of death than due to infection were regarded as censoring. Abbreviations: HD, hemodialysis; PAPP-A, pregnancy-associated plasma protein A; PAPP-A2, pregnancy-associated plasma protein A2.

higher PAPP-A2 levels (p=0.011) (Fig. 2A). Such result was not observed in the group with PAPP-A above median (Fig. 2B). Cumulative hazard function for mortality due to infection for PAPP-A when PAPP-A2 is below and above median is depicted in Fig. 3.

Taken together, PAPP-A2 is increased in HD patients compared to healthy controls. Unlike PAPP-A, PAPP-A2 is not significant for the prognosis of HD patients when tested alone.

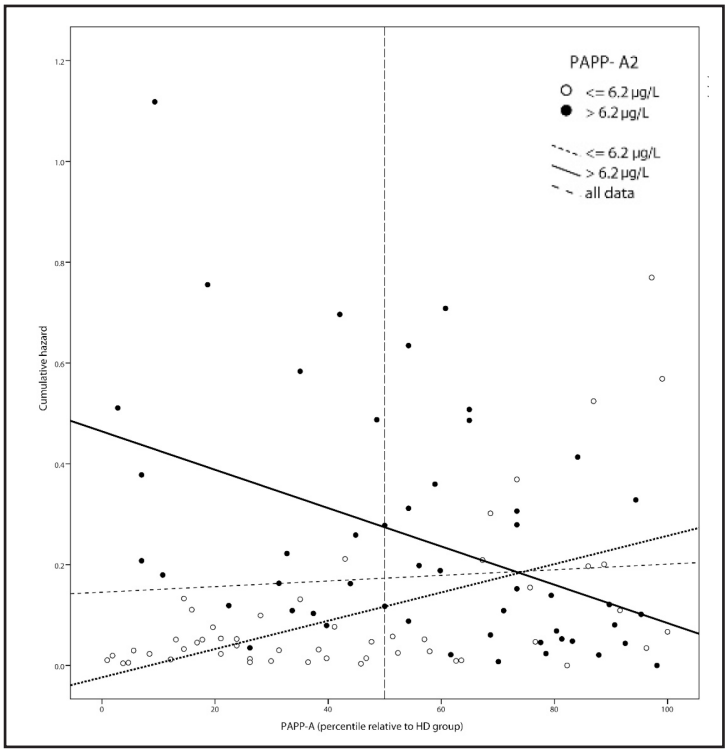

Fig. 3. Cumulative hazard function for mortality due to infection of HD patients for PAPP-A when PAPP-A2 is below and above median (6.2 ng/mL). HRs were obtained using Cox analysis with adjustment for age and sex. p=0.032 for PAPP-A when evaluated alone. p=0.011 for PAPP-A2 below and above median when PAPP-A is approaching to zero. p=0.008 interaction between PAPP-A and PAPP-A2. Abbreviations: HD, hemodialysis; PAPP-A, pregnancy-associated plasma protein A; PAPP-A2, pregnancy-associated plasma protein A2.

However, when evaluated simultaneously with PAPP-A, there is an interaction between these two parameters.

Discussion

In this study we describe for the first time increased levels of PAPP-A2 in long-term hemodialysis patients. PAPP-A2 correlates weakly but significantly with PAPP-A. Our study demonstrated that PAPP-A2 modulates the effect of PAPP-A, on mortality of HD patients, and confirmed the significance of PAPP-A on overall mortality, cardiovascular mortality and mortality due to infection.

PAPP-A is a mortality predictor in HD patients as demonstrated in different patients´ cohorts [13-17]. In the study which included 1098 diabetic HD patients, PAPP-A was associated with sudden death, infectious complications and stroke [16]. PAPP-A provides better information about patients risk compared to other pregnancy protein placental growth factor (PlGF) and to IGFBP-4 and IGF-1 which are influenced by its action [15]. Based on the presented study, PAPP-A is superior to PAPP-A2. If PAPP-A2 was tested alone, no significance for patients´ survival was seen. However, if PAPP-A2 was evaluated simultaneously with PAPP-A, there is an interaction between these two parameters. This was observed in case of overall mortality (borderline non-significance) and significantly in case of mortality due to infection. If PAPP-A is low, i.e. beneficial, PAPP-A2 selects a subgroup with high PAPP-A2 levels which has significantly worse prognosis than the subgroup with low PAPP-A2.

Although the mechanism is not clear, we should consider high homology of these two proteins (almost 50%) and also the difference in cleavage of IGFBPs by them [1, 2, 20]. Unlike PAPP-A, PAPP-A2 does not bind to cells [36, 37]. Even if evaluating states outside pregnancy, we should take a lesson from pregnancy where despite some similarities between these two proteins, i.e. production by placenta and increase during pregnancy, there are also some differences. First, PAPP-A increase is much more pronounced compared to the increase of PAPP-A2 [9, 19, 23]. Second, in case of Down syndrome or pregnancy pathologies there is early decrease of PAPP-A [7, 30] while PAPP-A2 increases [25-29]. Third, PAPP-A2 knockout

mice had normal size at birth but there is postnatal growth retardation [5], while PAPP-A knockout mice are born as proportional dwarfs [21].

In the present study, similarly as in pregnancy, the increase of PAPP-A2 was less pronounced compared to the increase of PAPP-A. Despite the high homology between PAPP-A2 and PAPP-A, PAPP-A2 correlates with PAPP-A only weakly suggesting different regulation of both proteins. PAPP-A2 unlike PAPP-A correlated with C-reactive protein and probably has closer relationship to inflammation than PAPP-A. On the other hand, both PAPP-A and PAPP-A2 play a role in bone metabolisms [5,6,38-40]; and it remains a question whether they could be involved also in vascular calcifications which are common in HD patients. Additional question is whether they could act in different manner or time period based on their variable influence on growth prenatally and postnatally. Moreover, there may be also different effect of acute and chronic increase: chronic PAPP-A (transgene mice) increase was related to accelerated progression of atherosclerotic plaques [41] but was linked to decreased neointimal formation after vascular injury, i.e. insufficient acute increase [42]. Unfortunately, to our knowledge, no data regarding PAPP-A2 dealing with this problem are available to date.

We have to admit also some study limitations. Mainly, healthy controls which were used for comparison with HD patients were significantly younger than patients. However, the difference both in PAPP-A and PAPP-A2 serum concentration between HD patients and controls was still present when a subgroup of controls with more comparable age was selected and compared with HD patients.

Conclusion

PAPP-A2 is increased in HD patients and interacts with PAPP-A on patients´ prognosis. Further studies are required to clarify the pathophysiological mechanisms of PAPP-A2 action in patients with renal failure before therapeutic measures can be established. Estimation of relevant cut-offs in a larger patients´ population would also be essential for future clinical usage of this new biomarker.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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