Pentraxin 3 is elevated in haemodialysis patients and is associated with cardiovascular disease

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Abstract
Background. Pentraxins are mediators of inflammation as well as markers of the acute-phase reaction. While elevation of C-reactive protein (CRP) in patients with renal failure and its association with cardiovascular disease is well described, there are no data on pentraxin 3 (PTX3) in this population.

Methods. Plasma was obtained from 44 chronic haemodialysis (HD) patients, 35 peritoneal dialysis (PD) patients, 39 patients with chronic renal failure (CRF) not on dialysis therapy and 14 age-matched normal subjects. PTX3 production in whole blood was also investigated in samples taken before and during HD.

Results. PTX3 plasma levels were significantly higher in HD patients (5.8 ± 0.6 ng/ml) compared with the other three groups. There were no significant differences between PD patients (1.5 ± 0.4 ng/ml), CRF patients (1.5 ± 0.4 ng/ml) and normal subjects (0.76 ± 0.2 ng/ml). In dialysis patients, PTX3 levels correlated significantly with time on renal replacement therapy (RRT) and with weekly erythropoietin dose. PTX3 levels were significantly higher in patients with coronary artery disease and peripheral artery disease compared with those without. During a single HD session, PTX3 production was higher in whole blood samples taken after 3 h HD compared with samples taken before HD.

Conclusions. PTX3 levels are markedly elevated in HD patients. The increase in PTX3 production in whole blood after HD indicates that the HD procedure itself contributes to elevated PTX3 levels in HD patients. The association between PTX3 and cardiovascular morbidity suggests a possible connection of PTX3 with atherosclerosis and cardiovascular disease in HD patients.

Keywords: cardiovascular disease; chronic renal failure; C-reactive protein; haemodialysis; pentraxin

Introduction
Pentraxins are a family of acute-phase proteins that are characterized by a multimeric, usually pentameric structure. Production of pentraxins has been demonstrated in many cell types. C-reactive protein (CRP) is mainly produced by hepatocytes but also by smooth muscle and endothelial cells in atherosclerotic plaques [1,2]. The long pentraxin 3 (PTX3), is produced by many cell types, including dendritic cells, endothelial cells, vascular smooth muscle cells, fibroblasts, monocytes and others [3]. Pentraxins, and especially PTX3, are highly conserved in evolution and are considered to represent the first defence mechanisms in response to infection, the so-called innate immunity [4]. PTX3 is induced by various stimuli including lipopolysaccharide (LPS) and cytokines such as tumour necrosis factor (TNF), interleukin-1β (IL-1β) and IL-6. PTX3 levels increase dramatically during sepsis and endotoxic shock but also after myocardial infarction [5] and after surgery [6]. In the circulation, PTX3 exist as multimers, predominantly decamers with a molecular weight of 440 kDa [7]. The physiological function of PTX3 is similar to CRP and Serum amyloid P. Like CRP, PTX3 binds to apoptotic cells and to many microorganisms and serves to opsonize these structures. PTX3 binds to complement C1q, induces complement activation and thereby facilitates phagocytosis and killing of opsonized cells and microbes. By opsonizing apoptotic cells, PTX3 inhibits their recognition by dendritic cells and controls autoimmunity [4]. The role of PTX3 is underlined by experiments with PTX3-deficient or PTX3-overexpressing mouse models. PTX3-deficient mice are more susceptible to invasive pulmonary aspergillosis than control animals [8]. PTX3-overexpressing mice show an exacerbated...
inflammatory response and increased mortality following intestinal ischaemia-reperfusion injury [9].

Over recent years an association between cardiovascular events and inflammation has been reported by several groups and appears to be well established. Baseline levels of CRP in apparently healthy individuals represent an independent risk factor for cardiovascular events including myocardial infarction and peripheral artery disease (PAD) [10,11]. Moreover, the rise in CRP [12] and also PTX3 [5] after myocardial infarction or during unstable angina pectoris predicts outcome. It has been suggested that the inflammatory process may not merely be an epiphenomenon but rather a pathogenic factor in the genesis of atherosclerosis [13,14]. Cardiovascular mortality is greatly increased in haemodialysis (HD) patients [15]. Besides other explanations (hypertension, hyperlipidaemia), one reason may be the chronic inflammation observed in patients with renal failure, as indicated by elevated plasma levels of acute-phase proteins such as CRP and cytokines such as IL-6 [16,17]. Indeed, even in dialysis patients, plasma levels of CRP and IL-6 are strong predictors for mortality in subsequent years [18]. Moreover, the degree of coronary calcification and the rate of increase in calcification correlate well with CRP levels [19,20].

Besides CRP, no other pentraxins have been investigated in patients with renal failure. We therefore examined plasma levels of PTX3 in different groups of patients with renal failure and correlated their levels with several clinical parameters and comorbid conditions.

Materials and methods

The study was approved by the Ethical Committee on Human Research of Charité University Hospital, Berlin, Germany, and performed in accordance with the Declaration of Helsinki. Non-hospitalized patients treated in an outpatient clinic were randomly chosen for comparison between groups. Plasma was obtained from 44 HD patients, 39 patients with chronic renal failure (CRF) not on dialysis therapy and 14 age-matched normal subjects. All subjects were prospectively approached and gave informed consent to the study. For HD patients, blood was drawn from a peripheral vein at the routine visit in their patient clinic were randomly chosen for comparison between groups and to avoid inter-assay variation, plasma samples from all four groups were measured in at least two dilutions until their concentrations were in the linear part of the standard curve. CRP was measured by immunoturbidimetry in the central clinical laboratory of the Virchow clinic. To facilitate comparison between groups and to avoid inter-assay variation, plasma samples from all four groups were measured on a single ELISA plate for PTX3 and IL-6.

Assay procedures

Pentraxin 3 was measured by ELISA kit from Alexis Biochemicals (Lausen, Switzerland) using a monoclonal antibody (ALX-804-464) and a biotinylated polyclonal antibody (ALX-210-365B) against PTX3 [5]. PTX3 standard was purchased from R&D systems (Wiesbaden, Germany). IL-6 was measured by high-sensitive ELISA kits (QuantiKine® HS) purchased from R&D (Wiesbaden, Germany). Samples were measured in at least two dilutions until their concentrations were in the linear part of the standard curve. CRP was measured by immunoturbidimetry in the central clinical laboratory of the Virchow clinic. To facilitate comparison between groups and to avoid inter-assay variation, plasma samples from all four groups were measured on a single ELISA plate for PTX3 and IL-6.

Statistical analysis

Plasma levels in different groups were compared using the analysis of variance (ANOVA) followed by Bonferroni/Dunn testing (Instat, Graphpad Software). Associations between different parameters were analysed by simple regression analysis followed by ANOVA. A P-value <0.05 was considered statistically significant. Results are expressed as means ± SEM for normally distributed samples and median and range for non-normally distributed samples.
Results

In preliminary experiments, several anticoagulants for measurement of PTX3 were tested. Blood was drawn from eight HD patients before HD and anticoagulated with EDTA, heparin, citrate or not anticoagulated (serum). There were large differences in PTX3 levels between anticoagulants: EDTA (2.8 ± 0.8 ng/ml), serum (0.04 ± 0.02 ng/ml), citrate (0.2 ± 0.08 ng/ml) and heparin (0.32 ± 0.16 ng/ml). We also added EDTA (1.6 mg/ml) to plasma or serum after centrifugation of the above samples. There was no increase in PTX3 levels when EDTA was added after centrifugation (data not shown). We also tested the effect of EDTA on the standard curve of the PTX3 assay in the absence of plasma/serum by diluting the recombinant PTX3 standard in EDTA solution (1.6 mg/ml). There was no effect of EDTA on the standard curve of the assay (data not shown). Therefore, in the following experiments EDTA-plasma was used for PTX3 measurements.

Clinical and demographic parameters of the four groups are given in Table 1. The groups were comparable regarding age, body mass index (BMI), and presence of comorbidities such as diabetes, CAD and pAD. Residual renal function was significantly higher in patients not yet on renal replacement therapy (RRT). HD patients received RRT significantly longer (69 ± 10 months) than PD patients (34 ± 5 months, P < 0.01).

Plasma levels of PTX3, CRP and IL-6 are shown in Figure 1. PTX3 plasma levels were significantly higher in HD patients (5.8 ± 0.6 ng/ml) compared with the other three groups. There were no significant differences between PD patients (1.5 ± 0.4 ng/ml), CRF patients (1.5 ± 0.4 ng/ml) and normal subjects (0.76 ± 0.2 ng/ml). In contrast, CRP levels were elevated in all three groups with renal failure (HD: 0.8 ± 0.2 mg/dl; PD: 1.1 ± 0.2 mg/dl; CRF: 0.9 ± 0.2 mg/dl) compared with normal subjects (0.12 ± 0.03 mg/dl), but not different between the three groups with renal failure. We observed the highest IL-6 levels in PD patients (10.8 ± 1.3 pg/ml), followed by HD patients (8.2 ± 0.8 pg/ml), CRF (4 ± 0.8 pg/ml) and normal subjects (2.5 ± 0.5 pg/ml, Figure 1). The differences were significant between both groups undergoing RRT and all other groups.

In patients on RRT (HD and PD patients combined) there was a significant correlation between PTX3 and weekly erythropoietin-dose and time on RRT (Table 2). CRP levels were significantly associated only with erythropoietin dose, IL-6 levels only with age (Table 2).

Table 1. Clinical and demographic characteristics of the subjects studied

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
<th>PD</th>
<th>CRF</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>44</td>
<td>35</td>
<td>39</td>
<td>14</td>
</tr>
<tr>
<td>Age (year)</td>
<td>59.8 ± 2.2</td>
<td>55.6 ± 2.3</td>
<td>60.4 ± 2.2</td>
<td>60.3 ± 1.1</td>
</tr>
<tr>
<td>BMI</td>
<td>25 ± 0.9</td>
<td>24.8 ± 0.7</td>
<td>25.5 ± 0.7</td>
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</tr>
<tr>
<td>Residual GFR (ml/min)</td>
<td>1.0 ± 0.2</td>
<td>4.3 ± 0.7</td>
<td>27 ± 3.3**</td>
<td>n.avail.</td>
</tr>
<tr>
<td>Months on RRT</td>
<td>69 ± 10**</td>
<td>34 ± 5</td>
<td>n.appl.</td>
<td>n.appl.</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>25</td>
<td>42</td>
<td>28</td>
<td>n.avail.</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>29</td>
<td>25</td>
<td>18</td>
<td>n.avail.</td>
</tr>
<tr>
<td>CAD (%)</td>
<td>43</td>
<td>57</td>
<td>69</td>
<td>n.avail.</td>
</tr>
<tr>
<td>pAD (%)</td>
<td>34</td>
<td>34</td>
<td>23</td>
<td>n.avail.</td>
</tr>
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</table>

** P < 0.01 vs PD-patients; ## P < 0.01 vs HD and PD patients.

n.appl., not applicable; n.avail., not available. CRF, chronic renal failure; BMI, body mass index; RRT, renal replacement therapy; CAD, coronary artery disease; PAD, peripheral artery disease.

Fig. 1. Box plots for plasma levels of PTX3, CRP and IL-6 in HD and PD patients, those with CRF and normal subjects. The horizontal line represents the median, the diamond indicates the mean. *P < 0.05 and **P < 0.01 vs all other groups.
When all four groups of subjects were combined, there was no correlation either between PTX3 and CRP or between PTX3 and IL-6. The correlation between CRP and IL-6 was highly significant ($R = 0.41, P < 0.001$).

To investigate whether the HD treatment induces a rise in PTX3 plasma levels, blood was taken from 22 HD patients before and after as well as 24 h after HD. For convenience to sample blood at the 24 h time point, hospitalized patients were selected for these experiments. As shown in Figure 2, there were no significant differences in plasma levels of PTX3, CRP and IL-6 between the investigated time points.

To further elucidate the PTX3 stimulatory role of the HD procedure, we sampled whole blood before and at 60 and 180 min during HD and measured PTX3 production in these samples after incubation. In preliminary experiments, we found that the production of PTX3 in whole blood is very slow and increased only after 48 h, with a plateau reached after 72 and 96 h. Blood samples were therefore incubated for 96 h. Spontaneous production of PTX3 in pre-HD blood samples was significantly higher than in blood from normal subjects (Figure 3). LPS-induced PTX3 production was similar in HD-patients compared with normal individuals. Spontaneous and LPS-induced production of PTX3 was significantly higher in blood sampled at 180 min of HD compared with pre-HD blood samples (Figure 3).

To investigate possible associations of PTX3, CRP and IL-6 with atherosclerosis, we analysed all patients with renal failure for the presence of CAD and pAD. Levels of PTX3, CRP and IL-6 were higher in patients with CAD or pAD compared with patients without these diseases (Figure 4). The difference between the presence or absence of the disease was highest for PTX3, and significant for both PTX3 and IL-6. The difference in CRP levels did not reach significance (Figure 4).

### Discussion

The present study is the first description of PTX3 levels in patients with renal failure. PTX3 plasma levels were significantly higher in HD patients compared with the other three groups. This difference is probably due to induction of PTX3 by the HD session. Moreover, PTX3 levels were significantly associated with clinical parameters such as time on RRT, weekly erythropoietin dose and atherosclerosis.

In contrast to CRP, levels of PTX3 are higher in HD compared with PD patients. One reason might be that HD-patients were on RRT longer (69 ± 10 months) compared with PD patients (34 ± 5 months). We also observed a highly significant correlation between time of RRT and PTX3 levels, i.e. the longer the time of RRT, the higher the PTX3 levels. Thus, the longer time of RRT in HD patients may account for the higher PTX3 levels. However, we adjusted for time on RRT and still, PTX3 levels were significantly higher in HD-patients (adjusted levels: 5.5 ± 0.52, confidence intervals 4.46–6.5 ng/ml) compared with PD patients (2.0 ± 0.62, confidence intervals 0.79–3.3 ng/ml). It is also unlikely that residual renal function accounts for higher PTX3 levels in HD patients since the difference in residual renal function was small between HD and PD patients compared with patients with CRF but PTX3 levels were similar in PD and CRF patients. It is possible that PD removes more PTX3 than HD. We did not investigate PTX3 concentrations in peritoneal dialysate effluates. However, PTX3 is a large molecule of 40 kDa [3]. Gel electrophoresis demonstrates that PTX3 are assembled to form multimers predominantly
of 440 kDa apparent molecular mass, corresponding to decamers; moreover, gel filtration reveals even higher forms corresponding to an apparent molecular mass of 900 kDa [7] which makes removal by peritoneal dialysis highly unlikely. In our view, the most likely explanation is that the HD procedure induces more PTX3 than PD. Our data support the hypothesis that HD induces PTX3 because PTX production from whole blood leaving the dialyzer was higher than from blood sampled before HD (Figure 3). Certainly, because of the relatively low number of subjects per group, the differences in PTX3 between the groups should be interpreted with caution. Inter- and intra-individual variation in PTX3 levels might yield different results when investigating larger populations.

In healthy subjects, PTX3 levels were reported to average 0.99 ± 0.51 ng/ml [22], another study reported 1.34 ± 0.9 ng/ml PTX3 in 100 healthy individuals [5]. Thus, the levels in control subjects observed in the present study (0.76 ± 0.26 ng/ml) are well comparable to previous studies. In an earlier study using the same PTX3 assay, we reported PTX3 levels to average 0.54 ± 0.3 ng/ml in six living kidney donors before nephrectomy that increased to 38 ± 3 ng/ml 3 days after nephrectomy [6]. We therefore believe that the PTX3 assay used is valid and gives reliable plasma levels when EDTA is used for anticoagulation.

The observation that PTX3 could only be measured in EDTA-anticoagulated plasma but not in plasma obtained with other anticoagulants or serum suggests that a cation, presumably calcium, is required for detection of PTX3. We excluded an effect of EDTA on the ELISA test and confirmed that EDTA had to be present before centrifugation of plasma. Adding EDTA after centrifugation had no effect on PTX3 levels. It therefore seems unlikely that the ELISA detects only multimeric forms of PTX3 and calcium is required to form multimers. It appears more likely that in the presence of calcium PTX3 binds to cells and is removed together with cells by centrifugation. Although binding of PTX3 to complement C1q is calcium independent, binding to other ligands such as the extracellular matrix component TNF-stimulated gene 6 (TSG-6) appear to be calcium dependent [4]. However, in general, binding characteristics of PTX3 are far less thoroughly investigated compared with CRP [23].

We observed an association between PTX3 levels and the presence of CAD and PAD. Although CRP levels were also higher in patients with than those without these atherosclerotic diseases, in contrast to PTX3, the difference in CRP levels was not significant. It is therefore tempting to speculate that the extensive discussion about the role of CRP in the pathogenesis of atherosclerosis [13] extends to other pentraxins as well, in particular PTX3. The hypothesis that the association between CRP and atherosclerosis is not only an association, but that CRP might directly promote atherosclerosis, has recently been reinforced by the observation that inhibition of CRP may reduce the extent of myocardial infarction in rats [23].

One reason to examine PTX3 is its production by many cell types including monocytes and cells of the vascular wall [3]. On a simple weight basis, circulating PTX3 levels are ~10-times lower than those of CRP, but the PTX3 monomer is twice the molecular mass than CRP [7]. Thus, the lower PTX3 levels do not exclude an important biological effect in comparison with CRP. In addition, the local production of PTX3 within the tissue and vascular wall may enable PTX3 to exert complement-inducing and pro-atherosclerotic effects above and beyond its circulating fraction. When investigating the effect of targeting CRP, one should be aware of the presence of other pentraxins. Unlike CRP, the association between PTX3 and cardiovascular diseases has not been investigated in a large population. Our data suggest that at least in subpopulations such as HD patients with high
levels, PTX3 may exert important effects in addition to CRP.

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Conflict of interest statement. None declared.

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