Low levels of IgM antibodies against phosphorylcholine-A increase mortality risk in patients undergoing haemodialysis

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Abstract

Background. Atherosclerosis is an inflammatory disease where oxidized low-density lipoprotein may play an important role through phosphorylcholine (PC)-exposing inflammatory phospholipids. Both atherosclerosis and its clinical consequence cardiovascular disease (CVD) are highly prevalent in patients with end-stage renal disease (ESRD). We here study the association between IgM antibodies against phosphorylcholine-A (anti-PC) and risk of death in patients undergoing haemodialysis (HD).

Methods. We performed a prospective observational study examining the relationship between anti-PC concentrations and mortality risk in a well-characterized cohort of 203 prevalent HD patients [56% men, median age 66 (interquartile range 51–74) years, vintage time 29 (15–58) months] with a mean follow-up period of 29 (14–58) months.

Results. Median anti-PC levels were lower in HD patients with systemic collagen vascular disease (18.9 versus 45.2 U/mL, \(P = 0.01\)) and in patients who died during the follow-up period (29.5 versus 53.9 U/mL; \(P = 0.0008\)). The patients with an anti-PC value below the median (42.1 U/mL) had a higher mortality rate with a crude hazard ratio (HR) of 2.13 (95% CI 1.40–3.22). These patients remained at higher risk of death (HR 1.76; 95% CI 1.13–2.74) even after adjustment for traditional risk factors (age, sex, smoking habits, CKD aetiology, CVD and diabetes), protein-energy wasting and inflammation (HR 1.70; 95% CI 1.19–2.68).

Conclusion. Low levels of natural IgM antibodies against PC are independent predictors of death among HD patients. Further studies are needed to define the clinical role of such measurements and to explore potentials for active immunization, with PC as an antigen, or passive immunization, aiming at raising levels of protective anti-PC.

Keywords: atherosclerosis; haemodialysis; inflammation; IL-6; phosphorylcholine

Introduction

The prevalence of chronic kidney disease (CKD) has reached epidemic proportions [1,2] attributable in part to the close links with cardiovascular disease (CVD) and diabetes. CKD stage 5 patients have a very poor prognosis, where CVD and infectious complications constitute the main causes of death [3,4]. Inflammation and the development of protein-energy wasting (PEW) seem to play a major role in the development of these events [5].

Atherosclerosis is generally regarded as an inflammatory condition [6], as activated immune-competent cells, producing pro-inflammatory cytokines, are abundant in lesions [7]. Phospholipase A2-modified LDL, or oxidized LDL (oxLDL), is a major factor causing the inflammation and immune reaction in the artery wall and, thus, atherosclerosis [7]. Other proposed factors include heat shock proteins (HSP) and infectious agents [8]. Oxidized LDL is abundant in atherosclerotic lesions [6], and has pro-inflammatory and immune stimulatory properties including activation of monocytes [9], endothelial cells [9,10] and T cells [11]. Studies of animal atherosclerotic models indicate that immunization with oxLDL as an antigen can ameliorate atherosclerosis development [6].

The immuno-stimulatory effects of oxLDL can be attributed to inflammatory phospholipids, such as lysophosphatidylcholine and/or platelet-activating factor (PAF)-like phospholipids, both of which contain phosphorylcholine (PC) as a major component [12,13]. PC is an interesting compound from an immunological point of view, since it is exposed not only in oxLDL, where it is a prerequisite for binding of PAF-like lipids to the PAF receptor, but also on microorganisms including bacteria, such as Streptococcus pneumonia and apoptotic cells [6]. Interestingly, PAF-receptor antagonists have anti-atherogenic properties [14].

The role of PC in oxLDL-related immune activation and inflammatory effects in atherosclerosis and CVD is therefore of considerable interest.

We recently reported a negative association between levels of anti-PC IgM and development of atherosclerosis in hypertensive patients [15] and that low levels of anti-PC
IgM predict development of CVD, especially stroke in men [16]. It is unknown whether anti-PC concentrations may have a prognostic role in other conditions with a high risk of CVD, such as CKD. Therefore, in this study, we aimed at investigating the predictive role of anti-PC in a high-risk population of prevalent haemodialysis (HD) patients.

Subjects and methods

Patients and experimental design

This study includes prevalent patients undergoing HD at six Swedish dialysis units in Stockholm and Uppsala. This is a post hoc analysis from a cross-sectional study with mortality follow-up that originally aimed at investigating the variability of inflammatory markers in HD patients. Because of the post hoc nature and because no study analysing anti-PC levels has been reported in the literature, no power calculation was performed. The protocol has been previously described in more detail, and patient recruitment took place between October 2003 and March 2004 [17]. From the 224 patients included in the study and further followed up for assessment of overall and cardiovascular mortality, anti-PC was determined in 203 patients (not enough plasma was available in 21 patients). The study protocols were approved by the Ethics Committee of Karolinska Institute and Uppsala University Hospital. Informed consent was obtained from all patients.

A nephrologist, who extracted data pertaining to underlying kidney disease, history of CVD, other comorbid conditions and survival data, reviewed each patient’s medical chart. The comorbidity history of each patient was determined at baseline according to the Davies comorbidity scoring [18]. Survival was determined after a mean follow-up of 41 (interquartile range (IQR) 20–48) months, with no loss of follow-up. Causes of death were registered on the basis of each patient’s medical chart.

Laboratory analyses

Blood samples were collected before the HD session after the longest interdialytic period. Plasma and serum were separated and kept frozen at −70°C if not analysed immediately. Serum concentrations of interleukin (IL)-6 were quantified by immunometric assays on an Immulite Analyser (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Plasma concentration of soluble vascular adhesion molecule (sVCAM)-1 was measured using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN, USA). High-sensitivity C-reactive protein (CRP nephelometry), white blood cell count, albumin, total cholesterol and triglyceride concentrations were analysed using certified methods at the Department of Laboratory Medicine in Karolinska University Hospital or Uppsala Academic Hospital.

Antibody determinations

Detection of low levels of IgM anti-PC antibodies was performed with an enzyme-linked immunoassay (ELISA) using a prototype of the Athera CVDefine™ kit (Athera CVDefine, Athera Biotechnologies AB, Stockholm, Sweden). The assay is based on PC covalently linked to bovine serum albumin (BSA) coated onto 96-well microtitre plates. Briefly, a 100 µL serum sample diluted 1:101 and calibrators were incubated for 30 min. After washing, the wells were incubated with 100 µL of conjugate (horseradish peroxidase-labelled anti-human IgM) washed again and incubated with 100 µL of the substrate (3, 3′, 5, 5′ tetramethylbenzidine, TMB). After incubation for 10 min, the reaction was stopped by 50 µL 0.5 M H2SO4. The optical density (OD) was read at 450 nm and antibody levels were expressed as arbitrary units (U/mL) calculated from a six-point calibrator curve containing 0, 6.25, 12.5, 25, 50 and 100 U/mL. Determinations were done in duplicates. The within coefficients of variation for the samples were <7% and the between coefficients of variation of each sample were <2%. Incubations were carried out at room temperature.

Statistical analyses

All variables were expressed as mean ± SD or as median (IQR), unless otherwise indicated. Statistical significance was set at the level of a P-value <0.05 with two-sided tests. After normality assessment, comparisons between two groups were then assessed with Student’s unpaired t-test and the Mann–Whitney test or the χ2 test, as appropriate. Because many of the variables followed a non-normal distribution, Spearman’s rank correlation (ρ) was used to determine correlations of anti-PC with other variables. In order to study whether the univariate associations observed were a reflection of the natural process of ageing, multivariate regression analyses were used including chronological age, anti-PC values and the variable of the study. Survival analyses were made with the Kaplan–Meier survival curve and the Cox proportional hazard model and using a simple dichotomization of anti-PC values according to the median value (42.1 U/mL). The univariate and multivariate Cox regression analyses are presented as hazard ratios [HR; 95% confidence intervals (CI)]. Cox adjustments were done on the basis of correction for factors that are known to be important predictors of the outcome in this disease [5]. As anti-PC has been related to both sex and smoking status in previous reports [15,16,20], these variables were also considered in multivariate adjustment. Inflammation was defined according to IL-6 values because anti-PC was related to IL-6 but not to CRP in univariate analysis. At the same time, many studies indicate that IL-6 may be the best prognosticator for mortality in CKD [21,22]. In order to evaluate the diagnostic power of anti-PC for mortality, receiver operator characteristic (ROC) curve analyses were performed. We also evaluated whether anti-PC levels add discriminatory power for mortality risk prediction to a standard risk score based on easily measurable risk factors (age, sex, albumin, IL-6 and background comorbidities) by the −2 log likelihood (−2 Log L) test in binary logistic models. This test compared different logistic regression models fitted to the same set of data. The smaller the −2 Log L value, the stronger is the agreement between the model and the observed data. The area under the curve for these models was calculated and compared. This prognostic gain analysis was then repeated in the presence and absence of inflammation (defined as median IL-6 levels), excluding IL-6 from the basic model. All statistical analyses were performed using the statistical software SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

The general characteristics of the study population are described in Table 1. Most patients were on antihypertensive medications (β-blockers, n = 100 (49%); calcium channel blockers, n = 52 (25%)); and angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blockers (ARB), n = 67 (32%)), as well as other commonly used drugs in ESRD (such as phosphate and potassium binders) and vitamin B, C and D supplementation. Sixty-five patients (32%) were on lipid-lowering medication (statins). One hundred and ninety-six patients (96%) were receiving erythropoietin-stimulating agents (ESA) at the time of evaluation.

Plasma anti-PC concentration in HD patients

Plasma levels of anti-PC followed a non-normal distribution. The median value of anti-PC was 42.1 (interquartile range 20.9–81.1) U/mL. There were no differences in...
Correlates of anti-PC

Anti-PC levels were negatively related to age (Table 1). Also, while no significant association was found with anti-PC concentration among wasted (SGA > 1) or non-wasted patients, those with/without a clinical history of diabetes, CVD or according to the different medication prescription (data not shown). Median anti-PC levels were similar among men and women (40.1 versus 45.5 U/mL, respectively; n = 113/90). With regard to other comorbidities, lower anti-PC levels were observed in those patients with clinically manifest systemic collagen vascular disease [18.9 (15.6–37.7) versus 45.2 (22.0–85.4) U/mL, n = 14/189; P = 0.01; Figure 1].

Impact of anti-PC on survival and relative risks

During the follow-up, 97 fatal events were registered. The patients who died presented lower anti-PC levels (29.5 versus 53.9 U/mL; P = 0.0008) at baseline. They were older, more often inflamed and had a higher prevalence of CVD, diabetes and PEW (Table 1). By using ROC curve analysis, anti-PC was found to have a potential diagnostic value for prediction of all-cause mortality because the area under the threshold of diagnostic indifference.

Anti-PC concentrations (per U/mL increase) had per se a significant protective impact on mortality (HR 0.986; 95% CI 0.981–0.989, P = 0.008). Thus, Kaplan–Meier curves showed that patients with concentrations below the median value of anti-PC had a worse all-cause survival (Figure 2, panel A). In a crude analysis, patients with anti-PC values below the median of distribution presented a significantly higher risk of dying (Table 2). These patients remained at higher risk when adjusting for age, sex, smoking habits, CVD and diabetes and also when further adjusting for PEW and IL-6. Different models were analysed including other inflammatory markers (CRP, S-albumin or white blood cell count) or other nutritional markers (BMI, handgrip strength, S-creatinine). However, the impact of anti-PC on mortality remained unaffected (data not shown).
Fig. 2. Kaplan–Meier curves for all-cause mortality according to median levels of anti-PC distribution (panel A) or the combined groups of IL-6 and anti-PC (panel B). In panel B, groups are created according to the median values of IL-6 (8.5 pg/mL) and anti-PC (42.1 U/mL) in the population of the study.

The individual causes of death are summarized in Table 3. The main cause of death was cardiovascular complications, followed by infections. Thus, we attempted to study the impact of anti-PC levels in these specific mortality risks. In both cases, we could observe a significant effect, i.e. patients with anti-PC values less than or equal to median had 3.2 times higher risk of dying from infections (HR 3.2; 95% CI 1.1–8.9, \( P = 0.01 \)) and two times higher risk of dying from cardiovascular events (HR 1.98; 95% CI 1.02–3.84, \( P = 0.03 \)). Due to a reduced sample size in each case, no further adjustments were performed.

Cross-classification between IL-6 and anti-PC

We have recently postulated that in a persistently inflamed uraemic environment, elevated cytokine levels may alter the natural action and prognostication of circulating molecules [23–25]. Therefore, we studied the prognostic use of anti-PC in the presence or absence of concurrent elevated cytokine levels. Different groups with high and low concentration values were established according to the median value of anti-PC and IL-6 and cross-classified (Table 4). The proportion of patients dying during the follow-up rose across declining groups of anti-PC for any group of IL-6 levels. Inflamed groups had a worse survival (Figure 2, panel B), and the group of patients with both inflammation and low anti-PC exhibited the highest probability of dying. However, the risk was not significantly different from that of the inflamed counterpart with high anti-PC levels. Nonetheless, non-inflamed patients with low anti-PC levels showed a mortality risk 3.8 times higher than non-inflamed patients with high anti-PC levels. To confirm this observation, we used ROC curve analysis. In the absence of inflammation, the area under the curve (71%) was significantly greater \( (P < 0.01) \) than the threshold of diagnostic indifference suggesting that anti-PC could be useful as a diagnostic tool. This was not the case in the presence of inflammation, in which case the area under the curve was smaller (46%) and non-significant \( (P = 0.8) \). Adding anti-PC values to a standard risk score based on age, sex, albumin, IL-6 and background comorbidities versus all-cause mortality resulted in a 4% prognostic gain (Table 5). In the subset of patients without inflammation (IL-6 < median value), adding anti-PC determinations improved the prognosis of the model by 9% (Figure 3, panel A), whereas in inflamed patients, only 1% was gained (Figure 3, panel B).
Table 4. Crude all-cause mortality risk according to median values of anti-PC and IL-6 cross-combined

<table>
<thead>
<tr>
<th></th>
<th>Total deaths, n (%)</th>
<th>Crude</th>
<th>P-value</th>
<th>Model 1</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Low IL-6, high anti-PC</td>
<td>56</td>
<td>7 (13%)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Low IL-6, low anti-PC</td>
<td>46</td>
<td>22 (48%)</td>
<td>3.92 (1.74–8.08)</td>
<td>0.0009</td>
<td>3.81 (1.69–8.59)</td>
</tr>
<tr>
<td>High IL-6, high anti-PC</td>
<td>45</td>
<td>27 (60%)</td>
<td>5.60 (2.34–12.35)</td>
<td>&lt;0.0001</td>
<td>3.68 (1.64–8.23)</td>
</tr>
<tr>
<td>High IL-6, low anti-PC</td>
<td>56</td>
<td>41 (73%)</td>
<td>8.09 (3.74–17.19)</td>
<td>&lt;0.0001</td>
<td>4.38 (2.00–9.58)</td>
</tr>
</tbody>
</table>

Hazard ratios, 95% confidence interval and the level of significance are indicated. Groups are created according to the median value of IL-6 (8.5 pg/mL) and anti-PC (42.1 U/mL) in the population of the study; model 1 considers adjustment for age, sex, the prevalence of cardiovascular disease, diabetes and the presence of protein-energy wasting (SGA > 1).

Fig. 3. Areas under the curve showing the prognostic gain of adding anti-PC levels to a logistic regression analysis including age, sex, albumin and background CVD, in the absence (panel A) or presence (panel B) of systemic inflammation.

Table 5. Percentage gain in the predictive power for all-cause mortality attributable to adding anti-PC in a basic regression model of easily measurable risk markers

<table>
<thead>
<tr>
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<th>(−2 Log L)</th>
<th>AUC</th>
<th>% gain</th>
</tr>
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<tbody>
<tr>
<td>All patients (n = 203)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard model</td>
<td>309.8</td>
<td>0.80</td>
<td>4</td>
</tr>
<tr>
<td>Standard model + anti-PC</td>
<td>281.0</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Non-inflamed patients (n = 102)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard model*</td>
<td>101.7</td>
<td>0.84</td>
<td>9</td>
</tr>
<tr>
<td>Standard model* + anti-PC</td>
<td>93.3</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Inflamed patients (n = 101)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard model*</td>
<td>179.9</td>
<td>0.77</td>
<td>1</td>
</tr>
<tr>
<td>Standard model* + Anti-PC</td>
<td>159.7</td>
<td>0.78</td>
<td></td>
</tr>
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</table>

The standard model included age (median), sex, albumin (median), IL-6 (median) and background cardiovascular events (presence/absence). Anti-PC groups were defined according to the median value, and inflammation was defined according to the median IL-6. *In this model, IL-6 was not included.

Discussion

We here report that low levels of IgM antibodies to PC (anti-PC) predict increased risk of death in HD patients. Furthermore, the prognostic value of low levels of anti-PC was independent of both traditional and novel risk markers, such as inflammation and wasting, suggesting that low anti-PC concentrations reflect a mortality risk that operates outside these pathophysiological pathways.

Although the presence of natural anti-PC antibodies has been documented for a long time and the role of PC in immune reactions has been discussed [26], relatively little has been reported about their role in disease development in humans. In mice, it has been shown that anti-PC antibodies from the T15 lineage protect against severe meningococcal infections [27]. Even though there may be an overlap between CVD- and infection-related deaths in our study group, an interesting possibility that deserves further study is that also in humans, low anti-PC may predict increased risk of infections as anti-PC could help neutralizing the major antigen PC from microorganisms. In previous reports with younger age groups, we reported that women have higher anti-PC levels than men [15,20]. Whether this is related to the later onset of CVD in women, or due to biological differences between men and women in relation to anti-PC, is currently unknown. In contrast, the present study shows no difference in anti-PC among male and female HD patients. This may be related to the generally late onset of CKD manifestation and the more advanced form of...
Anti-PC and mortality risk in HD patients

Anti-PC levels have been reported to be decreased in patients with systemic lupus erythematosus, another persistently inflamed patient group where the risk of CVD is exceedingly high [29]. Because anti-PC extracted from human Ig inhibited the effects of inflammatory phospholipids, low anti-PC may predispose not only to CVD but also to systemic inflammatory diseases like systemic lupus erythematosus by decreasing the individual’s anti-inflammatory capacity [29]. It is therefore interesting to note that in the present study, individuals with rheumatic conditions, including vasculitis, had lower levels of anti-PC as compared to CKD patients with other aetiologies. However, low anti-PC was a risk marker for early death, also when these patients were excluded (data not shown). The causes of low anti-PC levels are not clear but could depend on a uraemia-related insufficient immune response in CKD [5], as well as on sequestration of PC-containing compounds, including oxLDL, into atherosclerotic lesions [30]. Other possibilities may in principle include anti-PC-containing immune complexes, but irrespective of the cause, low anti-PC levels could predispose to CVD. Finally, as genetic causes of low anti-PC levels cannot be excluded, the heritability of anti-PC is presently under investigation in our laboratory.

The chief observation of the present study is the independent prognostic value of low anti-PC levels with regard to mortality in HD patients. Consideration of traditional risk factors and baseline comorbidities only slightly reduced the observed hazard ratio. Moreover, the prognostic value of low anti-PC seemed unaffected by further adjustments for markers of PEW or inflammation, being able to add 4% prognostic gain to a standard risk score of common risk markers. Our study shows as well that if causes of death were studied separately, low anti-PC levels increased the risk of cardiovascular deaths and fatal infectious complications, the main causes of death in this population. However, as our study is relatively small, these findings need to be interpreted with caution. At the same time, the boundaries between CVD- and non-CVD-related deaths may be overlapping in the setting of uraemia and the exact cause of death can often not be determined. In any case, our observations are compatible with the reported effects of anti-PC on preventing lethal infections in mice [26,27] or in predicting atherosclerotic complications [15] or future cardiovascular events [16] in non-renal populations. Clearly, studies with larger patient groups are needed to establish the exact role of anti-PC IgM in HD patients in relation to CVD and other causes.

Although the value of inflammatory markers in predicting ESRD mortality risk is clear [5], we tend to underestimate the elevated mortality risk also evident in ESRD patients in whom inflammation is not clinically evident. A notable observation in our study is that in non-inflamed HD patients, low anti-PC levels predicted a multivariate adjusted 3.8-fold increased risk of dying, a risk comparable to that of inflamed patients with high anti-PC. This observation reinforces the concept that anti-PC may operate through pathways independent of inflammation. In patients in whom inflammation was not apparent, adding anti-PC determinations to a basic score of established risk markers added a prognostic gain of 9%, a modest but possibly clinically relevant improvement in mortality prognostication. Although we cannot derive from our analysis the mechanisms behind this implication on CKD prognosis, we can extrapolate from recent studies in mice indicating that both active immunization with PC [31] and passive immunization with anti-PC [32] abrogate atherosclerosis. Thus, immunization with S. pneumoniae leads to both less atherosclerosis and increased anti-PC levels [33]. Also, mouse IgM anti-PC antibodies reduced oxLDL uptake via scavenger receptors in macrophages [34], a key process in the development of atherosclerosis, as scavenger receptor-deficient mice develop less atherosclerosis than controls [35].

Some limitations of our study deserve attention: clearly, our study should be recognized as a hypothesis-generating study that warrants further confirmation. This is a cross-sectional clinical patient material where comorbidities or death certificates are extracted from medical charts. At the same time, the prevalent nature of our cohort may represent a selection of patients who have survived CVD or survived despite the presence of factors potentially contributing to increased mortality risk. Finally, even though the overall population was relatively large, stratifying patients into those with and without inflammation resulted in smaller subgroups and reduced study power. Thus, our subgroup analyses regarding inflammation would need further confirmation in a large-scale study.

In conclusion, we here report that low anti-PC levels independently predict death risk among prevalent HD patients. Because this risk prediction was independent of both traditional risk factors and risk markers associated with inflammation and wasting, our study suggests that anti-PC concentrations may reflect a mortality risk that operates outside these pathophysiological pathways. These findings may encourage the development of methods to raise anti-PC levels in patients at risk as occurs in CKD, particularly in late stages of the disease. One option could be to accomplish this through active immunization, with PC as an antigen. However, such a procedure may involve a risk for unwarranted effects and furthermore it may be difficult to monitor the effects of such treatment. Another interesting possibility that deserves further study is passive immunization, e.g.
to administer anti-PC as monoclonals or polyclonals, thus raising levels of protective anti-PC.

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Conflict of interest statement. BL is affiliated with Baxter Healthcare Inc. JF is named as co-inventor on pending patents relating to anti-PC and CVD.

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