



Different subclasses and isotypes of antibodies against phosphorylcholine in haemodialysis patients: association with mortality

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Introduction

The risk of atherosclerosis and cardiovascular disease (CVD) is increased among patients on haemodialysis (HD patients) with chronic kidney disease (CKD). The risk of death is also raised where, in addition, infections also play a role [1]. Atherosclerosis is a chronic inflammatory condition where plaques typically contain activated immune competent cells, a necrotic core of dead cells and lipids, especially oxidized low-density lipoprotein (OxLDL) taken up in macrophages, which develop into foam cells [2]. Calcification is a feature of atherosclerosis development, which increases during plaque

Summary

The risk of premature death is high among patients on haemodialysis (HD patients). We previously determined that immunoglobulin (Ig)M antibodies against phosphorylcholine (anti-PC) are negatively associated with increased risk of cardiovascular disease (CVD), atherosclerosis, some autoimmune diseases and mortality among HD patients in this cohort. Here, we also study other subclasses and isotypes of anti-PC in HD patients in relation to mortality, inflammation and gender. The study group is a cohort of 209 prevalent HD patients [median age = 66 years, interquartile range (IQR) = 51–74], vintage time = 29 months (IQR = 15–58; 56% men) with a mean follow-up period of 41 months (IQR = 20–60). Fifty-six per cent were men. We also divided patients into inflamed C-reactive protein (CRP) > 5.6 mg/ml and non-inflamed CRP. Antibody levels were determined by in-house enzyme-linked immunosorbent assay. IgG1 anti-PC below median was significantly associated with increased all-cause mortality (after adjustment for confounders: $P = 0.02$), while IgG, IgA and IgG2 anti-PC were not associated with this outcome. Among non-inflamed patients, IgM and IgG1 anti-PC were significantly associated with mortality ($P = 0.047$ and 0.02). IgG1 anti-PC was significantly associated with mortality among men ($P = 0.03$) and trending among women ($P = 0.26$). IgM (as previously reported) and IgG1 anti-PC are negatively associated with survival among HD patients and non-inflamed HD patients, but among inflamed patients there were no associations. IgG, IgA or IgG2 anti-PC were not associated with survival in these groups and subgroups. Further studies are needed to determine if raising anti-PC levels, especially IgM and IgG1 anti-PC, through immunization is beneficial.

Keywords: antibodies, haemodialysis, mortality, phosphorylcholine

development and which is also pronounced in HD patients [1,2]. In addition to being a clinical problem, studies on HD patients could thus be of interest for atherosclerosis and inflammation in general. Systemic inflammation is a feature of HD patients, and is associated with protein-energy wasting and increased risk of premature death [3]. OxLDL is proinflammatory and immune stimulatory, and could also be a cause of cell death [2]. OxLDL is increased in the circulation in uraemia [4] and we have previously reported that specifically, phosphorylcholine (PC), an important component exposed on OxLDL which we used as a measure of OxLDL, is

raised in conditions including systemic lupus erythematosus (SLE) and hypertension [5,6].

Studies on antibodies against OxLDL provide conflicting results; for example, in previous studies related to CVD they were reported to be risk markers [7,8], while we have reported that they can be protection markers [9]. LDL-oxidation is complex, difficult to standardize and can be promoted by different agents inducing somewhat different types of modified and oxidized LDL, including both enzymes and oxidizing agents as copper ions [2]. We therefore focused on defined antigens in OxLDL, in this study the lipid epitope PC, which is a damage-associated molecular pattern (DAMP), exposed on OxLDL but also on apoptotic cells. PC also avidly binds to other carriers – not only lipids, as in OxLDL, but also proteins [2]. In addition to being a DAMP, PC is a pathogen-associated molecular pattern (PAMP). It has long been known that some bacteria, including *Streptococcus pneumoniae*, expose PC and can even use PC as a way to enter cells through platelet-activating factor (PAF)-receptor. In the context of such bacteria, e.g. *S. pneumoniae*, PC is presented on carbohydrates [2]. Further, PC is abundant on other agents as nematodes and parasites, where they bind to and are exposed on proteins [10].

Five to 10% of circulating immunoglobulins (IgMs) have been reported to be IgM anti-PC [2]. In our previous studies we focused on IgM anti-PC, and reported that it is negatively associated with atherosclerosis and CVD and also in other chronic inflammatory conditions, including SLE and rheumatoid arthritis (RA) [2,11–14], findings largely confirmed and extended to other chronic inflammatory conditions by other researchers [15–21]. We previously reported that IgM anti-PC is a protection marker in HD patients [22]. Here, we study other subclasses of isotypes and subclasses in HD patients in relation to inflammation status. The implications are discussed.

Materials and methods

Patients and experimental design

The study group consisted of 209 clinically stable patients with CKD stage G5 dialyzed. We used age < 18 years, acute renal failure, signs of overt clinical infection and unwillingness to participate as exclusion criteria. The Ethics Committee of the Karolinska Institutet (EPN) approved study protocols. We obtained informed written consent from each individual and the study was conducted in adherence to the Declaration of Helsinki.

Prevalent dialysis patients. HD patients in the MIMICK1 study [23] ($n = 209$) were recruited from six different dialysis

units in Stockholm and Uppsala, Sweden. They were treated by conventional maintenance HD or haemodiafiltration. The causes of end-stage renal disease (ESRD) were chronic glomerulonephritis ($n = 37$), hypertension and renovascular disease ($n = 37$), diabetic nephropathy ($n = 35$) and others ($n = 101$).

HD patients (median age = 66 years; 44% women) were recruited during 2003–04 into the MIMICK1 study [23]. Each patient's medical chart was thoroughly reviewed by a nephrologist, and data were extracted on underlying kidney disease, the presence of clinically overt CVD and other co-morbid conditions such as diabetes mellitus (DM). The causes of CKD were diabetic nephropathy (25%), hypertension/renal vascular disease (20%), chronic glomerulonephritis (13%) and others (such as polycystic kidney disease), or unknown aetiologies (42%).

Collection of clinical and laboratory data

Clinical data collected at baseline visits included demographics, co-morbid conditions, causes of kidney diseases, blood pressure, body mass index and nutritional status evaluated by subjective global assessment (SGA) [24]. All blood samples were obtained in the morning after an overnight fast and kept frozen at -70°C if not analysed immediately.

Serum samples of creatinine, albumin (bromocresol purple), calcium, phosphate, intact parathyroid hormone (iPTH), ferritin, cholesterol, triglyceride (TG), haemoglobin and high-sensitivity C-reactive protein (hsCRP; by nephelometry assay; CV, 5%) were measured by routine methods at the Department of Laboratory Medicine, Karolinska University Hospital at Huddinge. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum vascular cell adhesion protein-1 (VCAM-1; R&D Systems, Minneapolis, MN, USA). Plasma concentrations of interleukin (IL)-6 [coefficient of variation (CV) = 4%], tumour necrosis factor (TNF) (CV = 2–5%) and insulin-like growth factor-1 (IGF-1, CV = 4.3%) were measured on an Immulite TM Automatic Analyser (Siemens Healthcare, Diagnostics Products Ltd, Camberley, UK), according to the manufacturer's instructions.

Antibody determination

Antibodies such as IgG, IgG1, IgG2 and IgA to PC were determined by ELISA essentially as described previously [2,12,25–27]. Pooled serum from Sigma Aldrich (St Louis, MO, USA) was used as a standard for each plate. The concentration of the antigen used in each well was 10 $\mu\text{g}/\text{ml}$. Nunc Immuno microwell plates (Thermo Labsystems, Franklin Lakes, MA, USA) were coated with PC-bovine serum albumin (BSA). Coated plates were incubated overnight at 4°C . After four washings with wash buffer, the

plates were blocked with 2% BSA-phosphate-buffered saline (PBS) for 1 h at room temperature. We followed the same washing steps then serum samples were diluted for IgG, IgG1, IgG2 and IgA (1 : 200, 1 : 100, 1 : 200 and 1 : 200, respectively) in 0.2% BSA-PBS and added at 100 μ l/well. Plates were incubated at room temperature for 2 h and washed as described above. Biotin-conjugated mouse anti-human IgG, mouse anti-human IgG1, mouse anti-human IgG2, rabbit anti-human IgA (diluted 1 : 80 000, 1 : 800, 1 : 25 000 and 1 : 15 000, respectively, in 1% BSA-PBS) was added at 100 μ l/well and incubated at room temperature for 2 h. After four washings, the plate was incubated with horseradish peroxidase conjugated streptavidin (1 : 5000, 1 : 3000, 1 : 5000 and 1 : 5000, respectively, in 0.2% BSA-PBS) (Thermo Scientific, Roskilde, Denmark) at 100 μ l/well for 20 min. The colour was developed by adding the horseradish peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (3:30, 5:50; Sigma Aldrich) at 100 μ l/well and incubating the plates for 7, 10, 15 and 10 min, respectively, at room temperature in the dark. Further reaction was stopped with stop solution 1 N H₂SO₄ at 50 μ l/well. Finally, plates were read on an ELISA Multiscan Plus spectrophotometer (Spectra Max 250; Molecular Devices, San Jose, CA, USA) at 450 and 540 nm for IgG, IgG1, IgG2, and for IgA with the Biotek 800 TS absorbance reader at 450 and 630 nm. All samples were measured in duplicate within a single assay and the CV between the duplicates was below 15% for all the antibodies.

Antibody specificity assay

Antibody specificity was tested by competition assay. The assay was performed according to previous protocol [28]. In short, diluted sera were incubated with various concentration of PC-BSA (competitor). The solution was vortexed and was tested for binding to anti-PC IgG, IgG1, IgG2 and IgA antibodies. The antigen out-competed more than 70% of binding by the respective antibodies (data not shown).

The inhibition was calculated as percentage; the formula as follows:

% inhibition

$$= \frac{(\text{OD without competitor} - \text{OD with competitor})}{\text{OD without competitor} \times 100}.$$

Statistical analyses

Continuous data were expressed as median with interquartile range (IQR) and nominal data as percentage. Statistical significance was set at the level of $P < 0.05$. Comparisons between two groups were assessed with the non-parametric Wilcoxon's test for continuous variables and χ^2 test for nominal variables. Survival during

follow-up was analysed by Kaplan–Meier. All statistical analyses were performed using STATA version 16.0 (Stata Corporation, College Station, TX, USA) and SAS version 9.4 (SAS Campus Drive, Cary, NC, USA).

Results

The role of IgG, IgG1, IgG2 and IgA anti-PC in clinical outcome

We divided patients according to medians of IgG, IgG1, IgG2 and IgA anti-PC and compared to lower *versus* higher, and determined the association between clinical outcome, biochemical and medications. Data are shown for IgG in Table 1, for IgG1 in Table 2, for IgG2 in Table 3 and for IgA in Table 4.

We performed Kaplan–Meier survival estimates for all inflamed and non-inflamed HD patients. The two groups, inflamed and non-inflamed, were divided according to CRP level. We performed Kaplan–Meier survival estimates for male and female patients in separate analyses.

The IgG median levels for all inflamed and non-inflamed antibodies were not associated with outcome. Also, no significant association was seen for gender-based analysis (Supporting information, Fig. S1). We decided to go further and study the levels of these antibodies, with subclasses IgG1 and IgG2.

We found that individuals with IgG1 anti-PC lower than median had a worse outcome ($P = 0.02$) and among subgroups of non-inflamed patients ($P = 0.004$) and in males ($P = 0.03$), while there was no significant difference among inflamed patients and in women (data for IgG1 in Fig. 1).

In contrast to anti-PC IgG1, anti-PC IgG2 was not significantly associated with mortality as outcome when we compared patients with low levels (below median) with those with high levels (data for IgG2 in Fig. 2). Interestingly, the graphs show that the trend was that the lower the levels of anti-PC, the higher was survival (not significant).

IgA anti-PC patients were divided according to median levels, and we found no statistically significant differences in clinical, biochemical and medication treatments. When we compared all-cause mortality by Kaplan–Meier survival estimates, we found that in all patients and patients with and without inflammation were not statistically significant (Supporting information, Fig. S2).

We have previously published that IgM anti-PC is a protection marker for mortality in this population [22]. Further subgroup analyses herein indicate that this association is present among non-inflamed and men ($P = 0.04$, $P = 0.002$), but among inflamed patients and women this association did not reach statistical significance (data not shown).

Table 1. Baseline characteristics according to lower and higher levels of IgG anti-PC in HD patients

	Lower	Higher	
Demography and clinical characteristics			
Age (years)	66.0 (53.0–74.0)	67.0 (48.0–74.0)	0.67
Males, <i>n</i> (%)	49 (47.1%)	66 (63.5%)	0.018
Diabetes mellitus, <i>n</i> (%)	29 (27.9%)	22 (21.2%)	0.26
Cardiovascular disease, <i>n</i> (%)	61 (58.7%)	71 (68.3%)	0.15
Nutritional status			
Malnutrition (SGA > 1), <i>n</i> (%)	46 (45.1%)	49 (48.0%)	0.67
Body mass index (kg/m ²)	23.9 (21.0–27.1)	23.9 (21.2–28.2)	0.64
Handgrip strength (%)	59.3 (51.0–74.1)	59.3 (44.4–74.1)	0.89
Biochemicals			
Haemoglobin (g/l)	115.0 (108.0–121.5)	116.0 (106.5–126.5)	0.63
Albumin (g/l)	35.0 (33.0–38.0)	34.0 (32.0–37.0)	0.22
hsCRP (mg/l)	5.4 (2.1–15.0)	5.8 (2.6–18.0)	0.56
Triglyceride (mmol/l)	1.6 (1.1–2.3)	1.6 (1.1–2.4)	0.62
Total cholesterol (mmol/l)	4.4 (3.7–5.2)	4.2 (3.6–4.7)	0.32
Ferritin (µg/l)	430.0 (255.0–649.0)	441.0 (266.0–659.0)	0.91
Fibrinogen (g/l)	3.9 (3.2–4.5)	4.1 (3.2–4.8)	0.20
IL-10 (pg/ml)	1.3 (0.9–2.2)	1.3 (0.9–2.6)	0.88
IL-6 (pg/ml)	7.4 (4.5–14.7)	7.8 (5.5–16.0)	0.22
TNF-α, pg/ml	13.5 (10.7–16.3)	13.4 (11.5–17.3)	0.59
Leucocyte count (10 ⁹ /l)	7.1 (5.9–8.8)	7.9 (6.2–9.2)	0.26
T3, nmol/ml	0.9 (0.7–1.0)	0.9 (0.7–1.1)	0.50
T4, nmol/ml	68.2 (55.3–84.9)	70.8 (51.5–88.8)	0.87
TSH, mIU/ml	1.5 (1.0–2.6)	1.5 (0.8–2.5)	0.86
Pro-BNP, ng/ml	10 824.5 (3009.0–27 054.0)	10 122.0 (4105.0–25 304.0)	0.79
Medications			
β-blockers, <i>n</i> (%)	50 (48.1%)	53 (51.0%)	0.68
ACEi/ARB, <i>n</i> (%)	30 (28.8%)	39 (37.9%)	0.17
Statins, <i>n</i> (%)	35 (33.7%)	33 (31.7%)	0.77

Data are presented as median (interquartile range) for continuous measures and *n* (%) for categorical measures. HD = haemodialysis; PC = phosphorylcholine; IL = interleukin; TNF = tumour necrosis factor; hsCRP = high-sensitive C-reactive protein; T3 = triiodothyronine; T4 = thyroxine; TSH = thyroid-stimulating hormone; pro-BNP = pro-B-type natriuretic peptide; ACEi = angiotensin converting enzyme inhibitors; ARB = angiotensin receptor blocker.

Discussion

We report here that IgG1 anti-PC are negatively associated with mortality among CKD patients undergoing haemodialysis. Having low levels (below median) was associated with significantly higher mortality in the entire study group. Further, this association was only significant among men, not women. If divided into non-inflamed and inflamed patients (inflamed defined as CRP < 5.6), a low levels of IgG1 was found to be significantly protective marker only in case of Non-Inflamed patients and was not significant in case of inflamed patients.

However, in contrast, anti-PC IgG2 was not associated with mortality in the entire study group and in subgroups such as inflamed *versus* non-inflamed and women or men. Of note, one important cause of early death in this patient group is infections which, together with CVD, are the main cause of premature death [1].

Total IgG or IgA anti-PC were not significantly associated with mortality in the entire group, nor in the subgroups mentioned.

We have previously reported that IgM anti-PC was associated significantly with mortality as a protection marker in this patient cohort [22], and one rationale with the present study was to investigate the properties of other subclasses and isotypes of anti-PC. We also note that low IgM anti-PC is significantly associated with mortality among non-inflamed patients, but only showing a trend among inflamed patients. If women and men were studied separately, IgM anti-PC was a significant protection marker only among men, not women, where the association with protection in relation to mortality did not reach significance.

In previous studies, we were not able to detect IgG3 and IgG4 anti-PC at any significant levels and this was thus not studied here [26].

Subclasses and isotypes of anti-PC other than IgM have not been studied among patients with low kidney function in CKD patients, and knowledge in the general population or other disease groups is scarce. We have previously demonstrated differences between IgG1 and

Table 2. Baseline characteristics according to lower and higher levels of IgG1 anti-PC in HD patients

	Lower	Higher	
Demography and clinical characteristics			
Age (years)	69.0 (54.5–75.0)	62.0 (47.0–74.0)	0.050
Males, <i>n</i> (%)	54 (51.9%)	62 (59.0%)	0.30
Diabetes mellitus, <i>n</i> (%)	30 (28.8%)	21 (20.0%)	0.14
Cardiovascular disease, <i>n</i> (%)	70 (67.3%)	63 (60.0%)	0.27
Nutritional status			
Malnutrition (SGA > 1), <i>n</i> (%)	47 (46.1%)	49 (47.6%)	0.83
Body mass index (kg/m ²)	23.8 (21.1–26.9)	24.0 (21.1–28.1)	0.46
Handgrip strength (%)	56.3 (44.7–74.1)	61.2 (51.0–73.5)	0.14
Biochemicals			
Haemoglobin (g/l)	115.5 (107.0–125.5)	115.0 (107.0–123.0)	0.79
Albumin (g/l)	34.0 (32.0–37.0)	35.0 (33.0–38.0)	0.34
hsCRP (mg/l)	5.3 (2.4–16.9)	5.8 (2.4–17.0)	0.89
Triglyceride (mmol/l)	1.6 (1.1–2.2)	1.6 (1.2–2.3)	0.61
Total cholesterol (mmol/l)	4.4 (3.7–5.2)	4.3 (3.6–4.9)	0.56
Ferritin (µg/l)	407.0 (255.0–621.0)	460.0 (270.0–701.0)	0.45
Fibrinogen (g/l)	4.1 (3.2–4.7)	3.9 (3.2–4.8)	0.88
IL-10 (pg/ml)	1.2 (0.9–2.1)	1.4 (0.9–2.6)	0.75
IL-6 (pg/ml)	8.5 (5.0–14.7)	7.3 (4.8–15.2)	0.66
TNF-α, pg/ml	13.6 (10.8–16.3)	13.4 (11.1–17.3)	0.63
Leucocyte count (10 ⁹ /l)	7.1 (5.9–8.8)	8.1 (6.2–9.5)	0.071
T3, nmol/ml	0.8 (0.7–1.0)	0.9 (0.7–1.1)	0.024
T4, nmol/ml	66.9 (48.9–82.4)	71.4 (56.0–87.5)	0.24
TSH, mIU/ml	1.6 (1.1–2.5)	1.4 (0.8–2.5)	0.13
Pro-BNP, ng/ml	9696.0 (2820.5–30 097.0)	10 856.0 (3668.0–22 467.0)	0.68
Medications			
β-blockers, <i>n</i> (%)	51 (49.0%)	53 (50.5%)	0.84
ACEi/ARB, <i>n</i> (%)	29 (28.2%)	40 (38.1%)	0.13
Statins, <i>n</i> (%)	36 (34.6%)	32 (30.5%)	0.52

Data are presented as median (interquartile range) for continuous measures and *n* (%) for categorical measures. HD = haemodialysis; PC = phosphorylcholine; IL = interleukin; TNF = tumour necrosis factor; hsCRP = high-sensitive C-reactive protein; T3 = triiodothyronine; T4 = thyroxine; TSH = thyroid-stimulating hormone; pro-BNP = pro-B-type natriuretic peptide; ACEi = angiotensin converting enzyme inhibitors; ARB = angiotensin receptor blocker.

IgG2 anti-PC where the former were protection markers for atherosclerosis progress, in contrast to the latter, findings thus in line with the present study [26]. Anti-PC can differ in affinity for PC, which can also be presented as p-nitrophenyl phosphorylcholine (NPPC) [26]. We determined that human anti-PC could be divided into two populations, group I (IgM and IgG1) and group II (IgG2) [26]. Group I anti-PC recognizes both forms of PC, while group II antibodies only recognize NPCC, and here the phenyl-ring attached to PC is of importance for the antigenicity. IgG2 anti-PC is known to be directed against capsulated bacteria and has bactericidal properties, recognizing carbohydrate antigens [26,29,30].

In line with this are interesting observations on periodontitis. In this disease condition the risk of CVD is raised, which has been attributed to a combination of traditional and non-traditional risk factors [31]. IgG2 but not IgG1 anti-PC is also raised, which is line with other findings, as IgG2 anti-PC is mainly against bacterial carbohydrate-carried PC [32,33].

It is not known how IgM anti-PC is influenced by vaccination with *S. pneumoniae* among humans, but it is interesting to note that IgM antibodies to OxLDL are not induced, and thus IgM anti-PC probably are not [34]. IgM anti-PC was showed a trend towards an association with protection among inflamed patients, which may indicate that the effects of bacterial infections are not so strong, otherwise IgM anti-PC would have been higher among inflamed individuals and not associated with protection.

In general, T cell-dependent protein antigens induce mainly IgG1, whereas T cell-independent carbohydrate antigens induce mainly IgG2 antibodies. IgG1 is more effective than IgG2 in binding Fc-gamma-receptors, which could favour phagocytosis of dead cells in atherosclerosis and in complement activation; what this could mean in the context of the present study is unclear. There could also be other differences in effector functions [35].

Based on these findings, including from the present study, we hypothesize that anti-PC IgG2 are mainly related

Table 3. Baseline characteristics according to lower and higher levels of IgG2 anti-PC in HD patients

	Lower	Higher	
Demography and clinical characteristics			
Age (years)	66.0 (51.0–74.0)	65.5 (48.5–72.5)	0.34
Males, <i>n</i> (%)	49 (53%)	55 (60%)	0.37
Diabetes mellitus, <i>n</i> (%)	23 (25%)	22 (24%)	0.86
Cardiovascular disease, <i>n</i> (%)	52 (57%)	66 (72%)	0.031
Nutritional status			
Malnutrition (SGA > 1), <i>n</i> (%)	43 (48%)	39 (43%)	0.46
Body mass index (kg/m ²)	23.6 (20.4–26.3)	24.3 (21.4–28.4)	0.11
Handgrip strength (%)	56.3 (46.9–74.1)	59.3 (44.4–71.4)	0.60
Biochemicals			
Haemoglobin (g/l)	117.0 (110.5–124.0)	115.0 (106.0–123.0)	0.28
Albumin (g/l)	35.0 (33.0–37.0)	35.0 (32.5–38.0)	0.70
hsCRP (mg/l)	5.1 (2.4–13.0)	7.2 (2.9–21.0)	0.14
Triglyceride (mmol/l)	1.6 (1.1–2.2)	1.6 (1.2–2.3)	0.78
Total cholesterol (mmol/l)	4.3 (3.6–5.0)	4.2 (3.6–4.8)	0.83
Ferritin (µg/l)	472.0 (289.0–627.0)	380.5 (227.0–620.5)	0.24
Fibrinogen (g/l)	3.9 (3.2–4.5)	4.2 (3.0–4.8)	0.36
IL-10 (pg/ml)	1.2 (0.9–2.0)	1.3 (0.9–2.6)	0.58
IL-6 (pg/ml)	7.4 (4.5–11.5)	7.8 (5.0–17.5)	0.18
TNF-α, pg/ml	13.6 (10.6–16.6)	13.4 (11.5–16.8)	0.51
Leucocyte count (10 ⁹ /l)	7.1 (5.8–8.8)	8.0 (6.6–9.5)	0.050
T3, nmol/ml	0.9 (0.7–1.0)	0.9 (0.6–1.1)	0.58
T4, nmol/ml	70.8 (55.3–88.2)	66.9 (51.5–83.7)	0.37
TSH, mIU/ml	1.5 (1.1–2.5)	1.5 (0.8–2.5)	0.51
Pro-BNP, ng/ml	10 440.0 (3112.0–27 367.0)	10 856.0 (4105.0–25 304.0)	0.95
Medications			
β-blockers, <i>n</i> (%)	40 (43%)	52 (57%)	0.077
ACEi/ARB, <i>n</i> (%)	26 (29%)	35 (38%)	0.17
Statins, <i>n</i> (%)	29 (32%)	29 (32%)	1.00

Data are presented as median (interquartile range) for continuous measures and *n* (%) for categorical measures. HD = haemodialysis; PC = phosphorylcholine; IL = interleukin; TNF = tumour necrosis factor; hsCRP = high-sensitive C-reactive protein; T3 = triiodothyronine; T4 = thyroxine; TSH = thyroid-stimulating hormone; pro-BNP = pro-B-type natriuretic peptide; ACEi = angiotensin converting enzyme inhibitors; ARB = angiotensin receptor blocker.

to bacterial infections where the antigen is mainly carbohydrate-related. IgM and IgG1 are mainly associated with CVD and atherosclerosis, where PC is an important antigen on inflammatory phospholipids in OxLDL, causing immune activation [36]. The lack of association with outcome for anti-PC IgG2 could reflect raised levels already at baseline due to ongoing infections. It is also possible that IgG2 anti-PC are less effective in protection due to differences in some effector functions, such as clearance of dead cells.

There could be different, non-mutually exclusive explanations of why low IgG1 and IgM anti-PC are associated with a worse outcome in CKD (and in other inflammatory conditions). One is that these antibodies are consumed, and thus decreased in the circulation for that reason. They could, for example, bind to atherosclerotic lesions.

Another possibility that we have not studied is that immune complexes containing anti-PC could form and, if present, could contribute to lower levels of circulating

anti-PC. In addition, they could play other roles, and immune complexes containing one modified form of LDL, malondialdehyde (MDA)-modified, is associated with atherosclerosis [37].

Recently, mouse IgM anti-PC were reported to be regulated by programmed cell death ligand 2 (PD-L2), which could represent a mechanism of regulation of anti-PC in addition to antigen exposure [38].

There could thus be different mechanisms causing low levels of these antibodies, but it could still be argued that irrespective of the cause of low levels, IgM and IgG1 antibodies are still predicting mortality and if they are also causatively involved, raising them could be beneficial.

Studies in experimental mouse models support the notion that some anti-PC could cause protection against atherosclerosis and CVD, as immunization with PC ameliorates atherosclerosis development in a mouse model [39].

Of note, in humans, we could not determine such a dominating clone. Instead, human anti-PC showed signs

Table 4. Baseline characteristics according to lower and higher levels of IgA anti-PC in HD patients

	Lower	Higher	
Demography and clinical characteristics			
Age (years)	66.5 (57.0–74.0)	64.5 (47.0–74.0)	0.20
Males, <i>n</i> (%)	48 (47.1%)	66 (64.7%)	0.011
Diabetes mellitus, <i>n</i> (%)	24 (23.5%)	24 (23.5%)	1.00
Cardiovascular disease, <i>n</i> (%)	64 (62.7%)	64 (62.7%)	1.00
Nutritional status			
Malnutrition (SGA > 1), <i>n</i> (%)	45 (45.0%)	48 (48.0%)	0.67
Body mass index (kg/m ²)	23.7 (20.4–27.1)	23.9 (21.6–27.5)	0.42
Handgrip strength (%)	59.3 (51.0–74.1)	59.2 (46.9–74.1)	0.71
Biochemicals			
Haemoglobin (g/l)	116.0 (107.0–125.0)	115.0 (107.0–123.0)	0.62
Albumin (g/l)	35.0 (33.0–38.0)	35.0 (32.0–37.0)	0.62
hsCRP (mg/l)	5.5 (2.8–13.0)	6.3 (2.4–19.0)	0.70
Triglyceride (mmol/l)	1.7 (1.1–2.5)	1.5 (1.1–2.0)	0.21
Total cholesterol (mmol/l)	4.5 (3.8–5.2)	4.2 (3.5–4.8)	0.037
Ferritin (µg/l)	405.0 (255.0–654.0)	441.0 (248.0–659.0)	0.69
Fibrinogen (g/l)	4.1 (3.2–4.5)	3.8 (3.3–5.1)	0.37
IL-10 (pg/ml)	1.4 (0.9–2.2)	1.2 (0.9–2.6)	0.51
IL-6 (pg/ml)	7.4 (4.7–12.6)	7.8 (5.1–17.5)	0.24
TNF-α, pg/ml	13.0 (10.5–16.9)	13.6 (11.3–17.2)	0.47
Leucocyte count (10 ⁹ /l)	7.1 (6.1–8.7)	8.0 (6.1–9.4)	0.14
T3, nmol/ml	0.9 (0.7–1.1)	0.9 (0.7–1.1)	0.79
T4, nmol/ml	69.5 (59.2–83.7)	68.2 (51.5–87.5)	0.81
TSH, mIU/ml	1.5 (0.9–2.5)	1.5 (1.0–2.5)	0.71
Pro-BNP, ng/ml	9085.0 (3589.0–27 367.0)	11 135.0 (3368.0–23 934.0)	0.99
Medications			
β-blockers, <i>n</i> (%)	50 (49.0%)	53 (52.0%)	0.67
ACEi/ARB, <i>n</i> (%)	30 (29.4%)	39 (38.6%)	0.17
Statins, <i>n</i> (%)	28 (27.5%)	38 (37.3%)	0.13

Data are presented as median (interquartile range) for continuous measures and *n* (%) for categorical measures. HD = haemodialysis; PC = phosphorylcholine; IL = interleukin; TNF = tumour necrosis factor; hsCRP = high-sensitive C-reactive protein; T3 = triiodothyronine; T4 = thyroxine; TSH = thyroid-stimulating hormone; pro-BNP = pro-B-type natriuretic peptide; ACEi = angiotensin converting enzyme inhibitors; ARB = angiotensin receptor blocker.

of Ig-switch and somatic mutation and in addition, anti-PC are T cell-dependent in humans [28,40].

There are different potential cellular and molecular mechanisms by which anti-PC IgG1 and IgM could be protective in CKD, but also in other chronic inflammatory conditions, especially CVD.

Atherosclerosis is characterized by the presence of OxLDL in lesions, accumulation of dead cells in a necrotic core, and inflammation, where activated immune competent cells in lesions are present. In all these three key features of atherosclerosis (which is a major cause of CVD among CKD patients), human anti-PC could be protective, according to published research.

First, uptake of OxLDL by macrophage is inhibited by human IgM anti-PC [41]. Macrophages take up OxLDL and develop into foam cells filled with lipids and stay in plaques; many of the dead cells in the plaques were foam cells [2]. While uptake of LDL occurs through the LDL-receptor, OxLDL is taken up through different

scavenger receptors which, in contrast to the LDL-receptor, are not down-regulated when exposed to increasing amounts of the antigen. Foam cell formation may be a defence system initially, by protecting from the proinflammatory OxLDL, but later this appears not to be the case, as foam cells become an important part of the necrotic core and are inert, remaining in the plaques. In line with this notion, genetically modified mice with a defective scavenger receptor function develop less atherosclerosis [42].

Secondly, human IgM anti-PC promotes clearance of dead cells by phagocytes both in mouse [43] and human experimental systems [28]. This could be of importance in atherosclerosis and also in other chronic inflammatory conditions, such as autoimmunity [44,45]. Having low levels of IgM anti-PC may thus contribute to increased atherosclerosis through this mechanism. Another mechanism by which IgM anti-PC could decrease the burden of dead cells is through inhibition

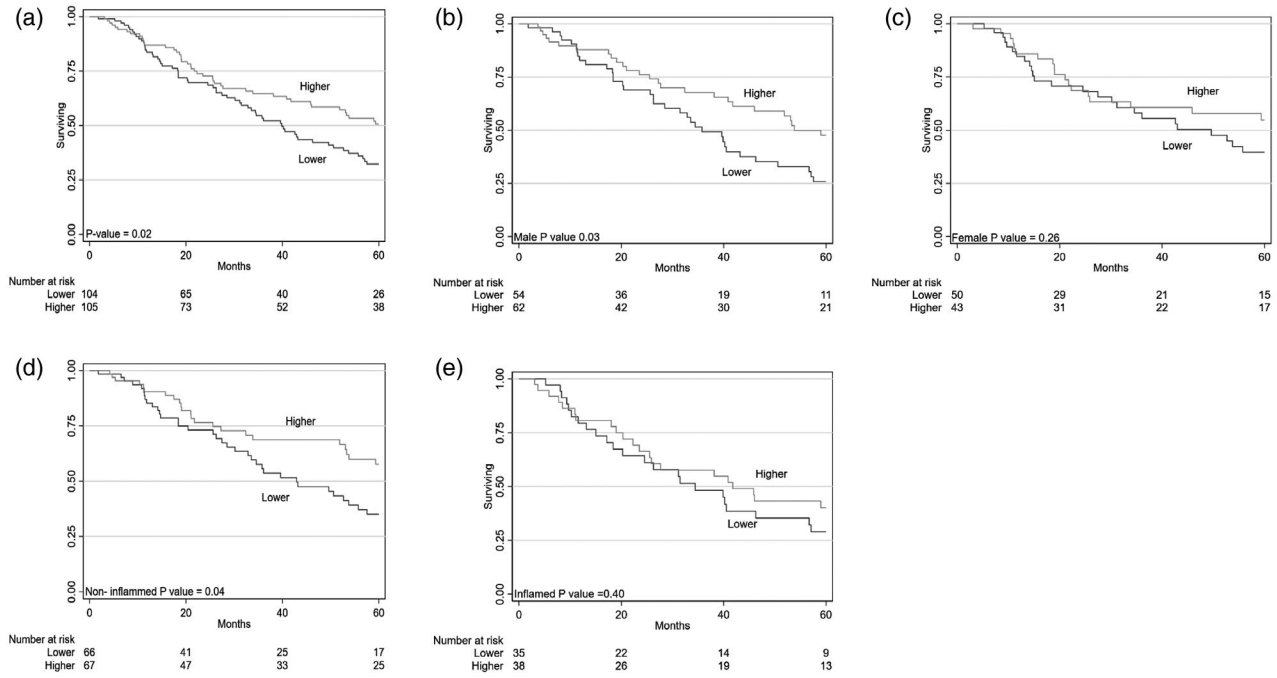


Fig. 1. Patients were divided into five groups: 1A, all patients; 1B, males; 1C, females; 1D, non-inflamed patients; and 1E, inflamed patients. Antibody levels were divided according to median into lower *versus* higher levels of immunoglobulin (Ig)G1 anti-phosphorylcholine (PC) for surviving *versus* months. All patients: $P = 0.02$, males, $P = 0.03$; females, $p = 0.26$; non-inflamed, $P = 0.04$; inflamed, $P = 0.40$.

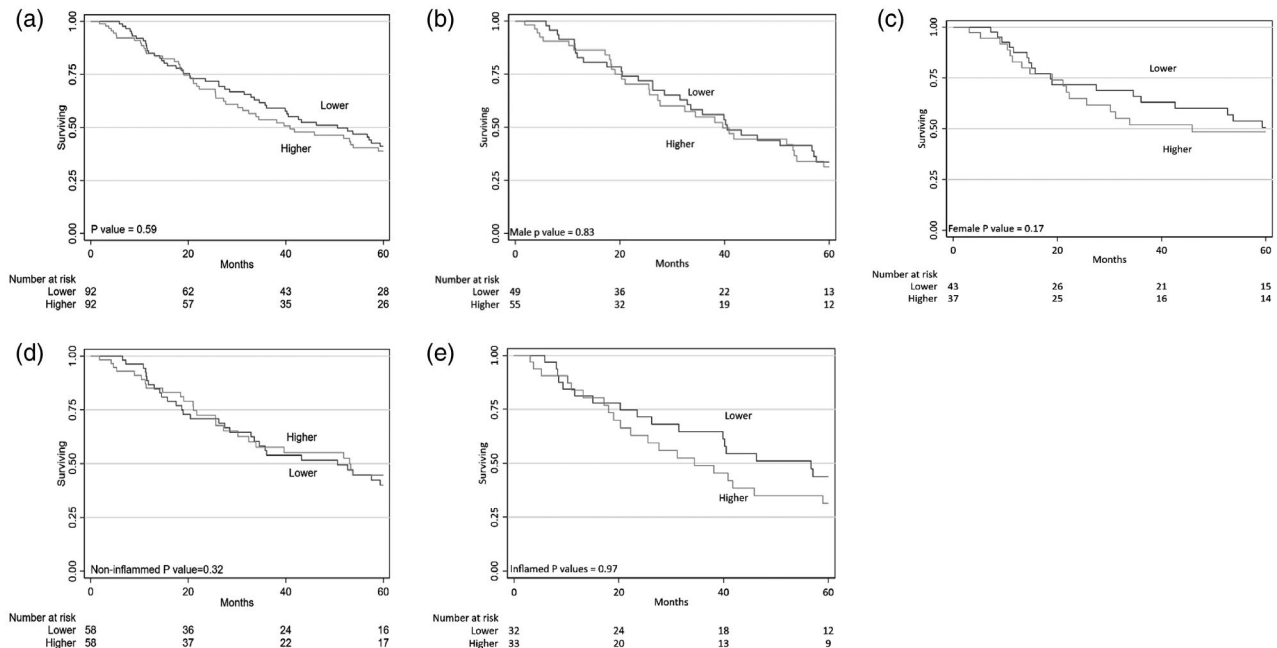


Fig. 2. Patients were divided into five groups: 2A, all patients; 2B, males; 2C, females; 2D, non-inflamed patients; and 2E, inflamed patients. Antibody levels were divided according to median into lower *versus* higher levels of immunoglobulin (Ig)G2 anti-phosphorylcholine (PC) for surviving *versus* months. All patients: $P = 0.59$; males, $P = 0.83$; females, $P = 0.17$; non-inflamed, $P = 0.32$; inflamed, $P = 0.97$.

of lysophosphatidylcholine-induced cell death, where lysophosphatidylcholine is a major phospholipid in plaques [26].

Also, in relation to inflammation, both IgG and IgM anti-PC could play a role, being anti-inflammatory. One mechanism is a direct inhibition by IgG anti-PC of the

proinflammatory effects of PC-exposing lipids [14]. Another is promotion by IgM anti-PC of regulatory T cells, which have immune modulatory properties which could be beneficial not only in atherosclerosis and autoimmunity [46], but also in chronic renal disease [47].

An additional feature of atherosclerosis is calcification, which is often advanced among CKD patients. We recently reported that IgM anti-PC is a protection marker for vascular ageing where calcification was one included feature [48]. However, it is not known if anti-PC could play a role in different stages of calcification. Our previous finding, that IgM antibodies act against oxidized cardiolipin but not against cardiolipin, illustrates that not all IgMs have protective properties in this context [49].

Several limitations should be considered when these results are interpreted. First, our results cannot prove causality due to the observational study design; the true association between anti-PC and mortality might be underestimated when analysing anti-PC at baseline. Secondly, the sample size is relatively small in survival analysis and results are based upon univariate Kaplan–Meier analysis. Our data therefore need confirmation in larger HD cohorts. Conversely, strengths of this study include adjustments for clinically relevant confounders (e.g. risk factors represented by the Davies co-morbidity score, lipid-lowering medications and nutritional status and inflammation, which are factors influencing survival in HD patients) and the relatively long observational period with no patients being lost to follow-up.

We recently proposed the hypothesis that low exposure to PC-exposing microorganisms as nematodes, parasites and some bacteria could be one underlying cause of low levels of anti-PC (especially IgM and IgG1). This notion was based on our studies on Kitavans, from Papua New Guinea who, at the time of the investigation, lived a traditional life as hunters, gatherers and horticulturalists. Here, chronic inflammatory conditions as CVD and rheumatic disease were extremely uncommon, if known at all. These individuals' lifestyle was beneficial to most traditional risk factors, but also had much higher levels of anti-PC than Swedish controls [25,27].

Taken together, IgM and IgG1 but not IgG2 or IgA anti-PC are protection markers for mortality among CKD patients. Whether such immunity could also play an underlying mechanistic role in disease development deserves further study. One possibility could be immunization with PC, and if so our results could favour that the methodology used should induce an antibody response with a dominance of IgG1 and IgM anti-PC.

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Disclosures

J. F. is named as inventor on patents related to anti-PC.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Patients were divided in 5 groups: 2A All patients, 2B for Non-inflamed patients, 2C for Inflamed patients, 2D for Female and 2E for Males. Antibody levels were divided according to median into Lower *versus* Higher levels of IgG anti-PC for Surviving *versus* Months.

Fig. S2. Patients were divided in 5 groups: 2A All patients, 2B for Non-inflamed patients, 2C for Inflamed patients, 2D for Female and 2E for Males. Antibody levels were divided according to median into Lower *versus* Higher levels of IgG anti-PC for Surviving *versus* Months.