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Serum properdin consumption as a biomarker of C5 convertase dysregulation in C3 glomerulopathy

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Summary

Properdin (P) stabilizes the alternative pathway (AP) convertases, being the only known positive regulator of the complement system. In addition, P is a pattern recognition molecule able to initiate directly the AP on non-self surfaces. Although P deficiencies have long been known to be associated with *Neisseria* infections and P is often found deposited at sites of AP activation and tissue injury, the potential role of P in the pathogenesis of complement dysregulation-associated disorders has not been studied extensively. Serum P levels were measured in 49 patients with histological and clinical evidence of C3 glomerulopathy (C3G). Patients were divided into two groups according to the presence or absence of C3 nephritic factor (C3NeF), an autoantibody that stabilizes the AP C3 convertase. The presence of this autoantibody results in a significant reduction in circulating C3 ($P < 0{\cdot}001$) and C5 levels ($P < 0{\cdot}05$), but does not alter factor B, P and sC5b-9 levels. Interestingly, in our cohort, serum P levels were low in 17 of the 32 C3NeF-negative patients. This group exhibited significant reduction of C3 ($P < 0{\cdot}001$) and C5 ($P < 0{\cdot}001$) and increase of sC5b-9 ($P < 0{\cdot}001$) plasma levels compared to the control group. Also, P consumption was correlated significantly with C3 ($r = 0{\cdot}798$, $P = 0{\cdot}0001$), C5 ($r = 0{\cdot}806$, $P < 0{\cdot}0001$), sC5b-9 ($r = -0{\cdot}683$, $P = 0{\cdot}043$) and a higher degree of proteinuria ($r = -0{\cdot}862$, $P = 0{\cdot}013$). These results illustrate further the heterogeneity among C3G patients and suggest that P serum levels could be a reliable clinical biomarker to identify patients with underlying surface AP C5 convertase dysregulation.

Keywords: C3 glomerulonephritis, C3 glomerulopathy, C3 nephritic factor, complement, properdin

Introduction

Properdin (P) is the only known positive regulator of the complement system, acting both as a stabilizer of the alternative pathway (AP) C3 and C5 convertases and as a selective pattern recognition molecule for certain microorganisms and altered host cells (i.e. apoptotic/necrotic cells) [1]. It is a 442 amino acid-long (53 kDa) highly positive charged (pI > 9·5) glycoprotein that circulates in plasma mainly as trimers, tetramers or pentamers [2,3]. Unlike most complement proteins, which are produced mainly in the liver, P has different sites of synthesis. Neutrophils are the main producers of P, although monocytes, bone marrow progenitor cell lines and T cells also synthesize this complement protein [4]. The structural basis for the interaction of the AP C3 convertase with P has been uncovered recently. Using electron microscopy (EM), it has been shown that the N- and C-terminal ends of adjacent P monomers within the oligomers conform a curly vertex that interacts simultaneously with C3b and Bb in the AP C3 convertase [3]. Importantly, native forms of P bind with greater affinity to cell-bound C3bBb or C3bB than to cell-bound C3b [5]. In addition, and as a consequence of the oligomeric nature of P, binding to cell-bound C3b derivatives is favoured by increased avidity compared to binding to fluid phase ligands [1].

When P was first described it was considered as an initiator of the AP [6]. This original view was replaced later by the widely accepted notion that P acts as a positive regulator that amplifies the AP by extending the half-life of the

C3 and C5 convertases [7]. However, new evidence demonstrates that P can also bind to certain surfaces, and provide a platform for *de-novo* convertase assembly. Several studies supporting the latter hypothesis have reported P binding directly to various non-self surfaces such as zymosan, rabbit erythrocytes, *Neisseria gonorrhoeae* and certain *Escherichia coli* strains and to surfaces of early or late apoptotic and necrotic cells, as well as to altered-self-like live human leukaemia T cell lines and even to normal human proximal tubular epithelial cells (PTECs) [8].

The potential contribution of P to the pathogenesis of AP complement-mediated diseases has been suggested. A clinical study in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis found increased plasma levels of activated complement products (C3a, C5a, C5b-9 and Bb) but decreased P levels in patients with active disease, suggesting that P may be consumed during complement activation flares [9]. P has also been implicated in several renal diseases such as chronic and acute lupus nephritis [5] and immunoglobulin IgA nephropathy [10], where glomerular deposits of P have been detected. A recent study found decreased circulating P levels in C3 glomerulopathy (C3G) [11], but the role of P in this disease was unclear. In the context of renal disease, the main hypothesis is that only during proteinuria is urinary P available in tubular lumen. This situation promotes its interaction with heparan sulphate proteoglycan (HSPG) present on the extracellular matrix of PTECs, acting as a focal point for AP activation [12]. Recent results confirmed that deposited P via HSPG on PTECs seems to be involved in complement activation, as it can increase the deposition of C3 and C5b-9 in cellular surface *in vitro* [13]. Moreover, urinary P levels were associated with higher urinary levels of soluble C5b-9 (sC5b-9) and with worse renal function [14].

Given the importance of P in AP, we have measured P concentration in a selected group of patients with dense deposit disease (DDD) or C3 glomerulonephritis (C3GN), two rare diseases included in the heterogeneous group of C3G. Our results suggest that P levels in serum may be a useful biomarker to stratify C3G patients, identifying those with underlying dysregulation of the AP C5 convertase on surfaces. This initial study encourages further investigation into the functional role of P in the development, progression and treatment of diseases associated with AP dysregulation.

Materials and methods

Patients and biological samples

Forty-nine patients with biopsy-proven C3G (20 with DDD and 29 with C3GN) diagnosed between 1970 and 2015 were selected based on the availability of enough sera and plasma samples to complete all assays, as well as histopathological data (light microscopy, immunofluorescence and EM) to review the pathological diagnosis. Serum and ethylenediamine tetraacetic acid (EDTA) plasma samples were obtained under standard conditions with informed consent and with the approval of Hospital Universitario La Paz; blood was collected in plain tubes, allowed to clot at room temperature and centrifuged for 10 min at 4°C. After that, serum and plasma were collected, aliquoted and kept frozen at −80°C until use. Normal human serum (NHS) was obtained from 30 healthy volunteers.

Reagents

Commercial human C3b, FB, FD and P were obtained from Complement Technology (Tyler, TX, USA). Properdin-deficient serum, a P-depleted human serum (PDS), was generated by affinity chromatography using a monoclonal anti-P (anti-P 246.111, IgG1), a gift from Dr Mercedes Domínguez (Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid) coupled to Profinity Epoxide resin (Bio-Rad Laboratories Inc., Hercules, CA, USA), equilibrated with complement fixation buffer (4 mM Na Barbitone, 145 mM NaCl, 0·85 mM $MgCl_2$, 0·25 mM $CaCl_2$, pH 7·2).

C3, FB, C5 and sC5b-9 quantification and C3NeF detection by enzyme-linked immunosorbent assay (ELISA)

Serum C3 levels were measured by nephelometry (Siemens Healthcare, Erlangen, Germany). Serum FB levels were determined by ELISA using 100 ng/well of Protein G (GE Healthcare, Little Chalfont, UK) purified polyclonal goat IgG anti-human FB antibody (Millipore Corporation, Billerica, MA, USA) as capture antibody and a monoclonal mouse anti-human Bb (A227; Quidel, San Diego, CA, USA) as the detection antibody. C5 serum levels were determined by ELISA, as described by Delgado-Cerviño and colleagues [15], with slight modifications. Plasma sC5b-9 levels were analysed using a commercially available assay (A029; Quidel). C3NeF detection in serum samples was performed as described previously by Paixão-Cavalcante and collaborators [16].

Quantification of serum properdin levels

ELISA microtiter plates (Costar®, Corning, NY, USA) were coated with 100 ng/well of monoclonal anti-human FP1 (A233) or anti-human FP2 (A235) (both from Quidel) in carbonate-bicarbonate buffer pH 9·3 (overnight, 4°C). Plates were blocked with phosphate-buffered saline–bovine serum albumin (PBS-BSA) 1% (1 h, 37°C) and washed with PBS-Tween 0·1%. In order to measure P concentrations, a standard curve was generated with a stock solution (1 mg/ml) of commercial P (to cover a range from 13·33 μg/ml to 0·1 μg/ml). A pool of the serum from three donors was used as internal standard and P-depleted serum as negative control. All sera samples were added and

incubated at 1 : 400, 1 : 800 and 1 : 1600 dilutions in PBS-BSA 0·1% (1 h at 37°C) and run in duplicate. P detection was made using a goat anti-serum (1 : 1000; 1 h, 37°C) from Nordic Immunology (Tilburg, the Netherlands) and then with peroxidase-conjugated rabbit anti-goat Ig (Dako, Denmark A/S, Glostrup, Denmark) (1 : 1000, 1 h at 37°C) and the reactions were developed with [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid] substrate.

Additionally, in order to validate the assay, plates were coated with 100 μg of goat IgG anti-human P purified previously with protein G and diluted in carbonate–bicarbonate buffer pH 9·3 (overnight at 4°C). The same pool of three NHS and purified P were included, and monoclonal antibodies were used to detect the captured P and measure its levels in serum samples.

Zymosan assay

Zymosan-activated human serum was prepared by the addition of zymosan A (Sigma Aldrich, St Louis, MO, USA) at a final concentration of 10 mg/ml for 60 min at 37°C. After centrifugation, the supernatant was split and stored in aliquots at −70°C until used. Assays were performed with three healthy donors' sera and PDS. For each serum assayed, two untreated control aliquots were prepared: one unheated and another incubated at 37°C for 60 min. Circulating C3, P, C5 and sC5b-9 were measured as described previously above and expressed as a percentage of a reference untreated, unheated serum. Properdin sequestered by zymosan was released by incubation with sodium dodecyl sulphate (SDS) (1% final concentration) at 95°C for 5 min followed by centrifugation at 1500 g for 5 min. Measurement of P levels was carried out by ELISA on the resulting supernatants.

Statistical analyses

Statistical calculations were performed with Prism version 5·0 (GraphPad Software, La Jolla, CA, USA). Bonferroni's multiple comparison test was used for comparisons of all the groups. Correlations among the groups were evaluated by Pearson's correlation coefficient. A P-value of $< 0{\cdot}05$ was considered statistically significant in all analyses.

Results

Properdin concentrations in C3 glomerulopathy patients

Among the 80 patients with C3G, 31 were found to be consistently normocomplementaemic and 49 were hypocomplementaemic as defined by a serum C3 level below the lower limit of the normal range (79 mg/dl). All non-hypocomplementaemic patients were excluded.

Based on the presence of C3NeF, patients were classified as C3NeF-positive ($n = 17$) and C3NeF-negative ($n = 32$).

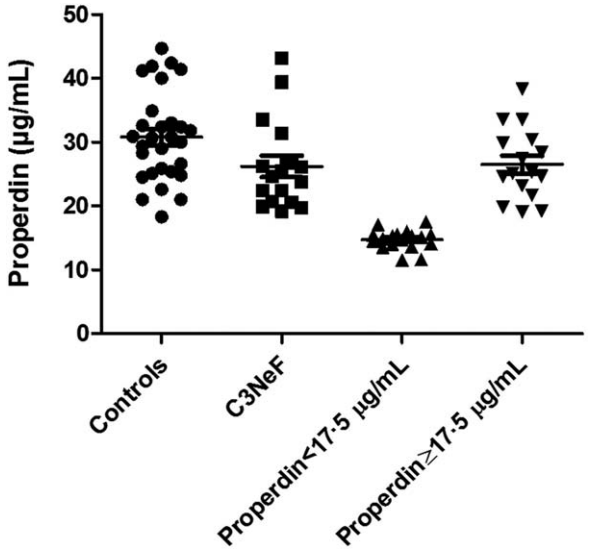

Fig. 1. Properdin levels in C3 glomerulopathy patients groups. Serum properdin of C3NeF-positive patients ($n = 17$), two C3NeF-negative groups with properdin levels equal to or above 17·5 μg/ml ($n = 15$) or below to 17·5 μg/ml ($n = 17$) was measured. Thirty healthy controls were used to calculate the normal range of properdin (17·5–45·5 μg/ml). The mean ± standard deviation of each group is indicated.

We measured the concentration of serum P in both groups of patients and a set of 30 controls. The mean P concentration in the control group was 31·4 ± 7·1 μg/ml, which is in agreement with previous reports [17,18]. To establish a P concentration normal range in serum, the lower and upper limits were calculated as the mean plus 2 standard deviations (s.d.) (17·5–45·5 μg/ml). All C3NeF-positive patients showed normal P levels; however, among the C3NeF-negative patients there were important differences in the P levels, with several of them presenting P concentrations below the calculated normal range. For analytical purposes, to clarify the P-level heterogeneity in this group, the C3NeF-negative patients were clustered further as described in Fig. 1:

- C3NeF-negative with P serum levels equal to or above 17·5 μg/ml ($n = 15$).
- C3NeF-negative with P serum levels below 17·5 μg/ml ($n = 17$).

Demographic details and basic clinical information are listed in Table 1. In our cohort, the mean age of the C3NeF-positive patients group was lower than that of the C3NeF-negative group. Patients were mainly female, except in the C3NeF-negative group with normal P, where 60% were male. Hypertension was reported more often in C3NeF-negative patients when compared to the C3NeF-positive group. All groups presented typically with altered serum levels of creatinine and albumin. However, although all groups presented with proteinuria, the C3NeF-negative

Table 1. Demographic and clinical findings in C3 glomerulopathy patients.

		C3NeF (n = 17)	Properdin < 17·5 μg/ml (n = 17)	Properdin ≥ 17·5 μg/ml (n = 15)
Age (years)	Mean ± s.d. (range)	18 ± 12 (6–55)	34 ± 23 (6–79)	41 ± 24 (5–77)
Sex	Male	5	7	9
	Female	12	10	6
Hypertension		5/17 (29·4%)	7/17 (41·2%)	8/15 (53·3)
s-Cr (0·5–1·0 mg/dl)	Mean ± s.d.	1·0 ± 1·1	1·5 ± 0·9	1·6 ± 1·0
s-Alb (3·4–5·0 g/dl)	Mean ± s.d.	2·9 ± 1·0	2·9 ± 0·6	3·3 ± 0·8
Proteinuria (g/24 h)	Mean ± s.d.	2·4 ± 3·0	8·0 ± 4·9	2·2 ± 2·0
Subtype C3G	C3GN	3/17 (17·6%)	14/17 (82·4%)	12/15 (80·0%)
	DDD	14/17 (82·4%)	3/17 (17·6%)	3/15 (20·0%)

All data referred to years; s-Cr, s-Alb and proteinuria are represented as mean ± standard deviation (s.d.). C3NeF = C3 nephritic factor; s-Cr = serum creatinine; s-Alb = serum albumin; C3G = C3 glomerulopathy; C3GN = C3 glomerulonephritis; DDD = dense deposit disease.

group with low P levels had a higher degree of proteinuria (mean ± s.d., 8·0 ± 4·9 g/24 h) compared with the other two groups (C3NeF-positive, 2·4 ± 3·0 g/24 h; C3NeF-negative with normal P, 2·2 ± 2·0 g/24 h). The proportion of patients diagnosed wth DDD was noticeably greater in the C3NeF-positive group (82·4%), while C3GN is more frequent in the C3NeF-negative patients.

Complementary complement assays in C3 glomerulopathy patients

AP activation: circulating C3 and FB levels. Circulating C3 was reduced severely in the three patient groups compared with controls [median 104·0 mg/dl (range, 80·2–175·0)] (Fig. 2a). C3NeF-positive patients exhibited the lowest C3

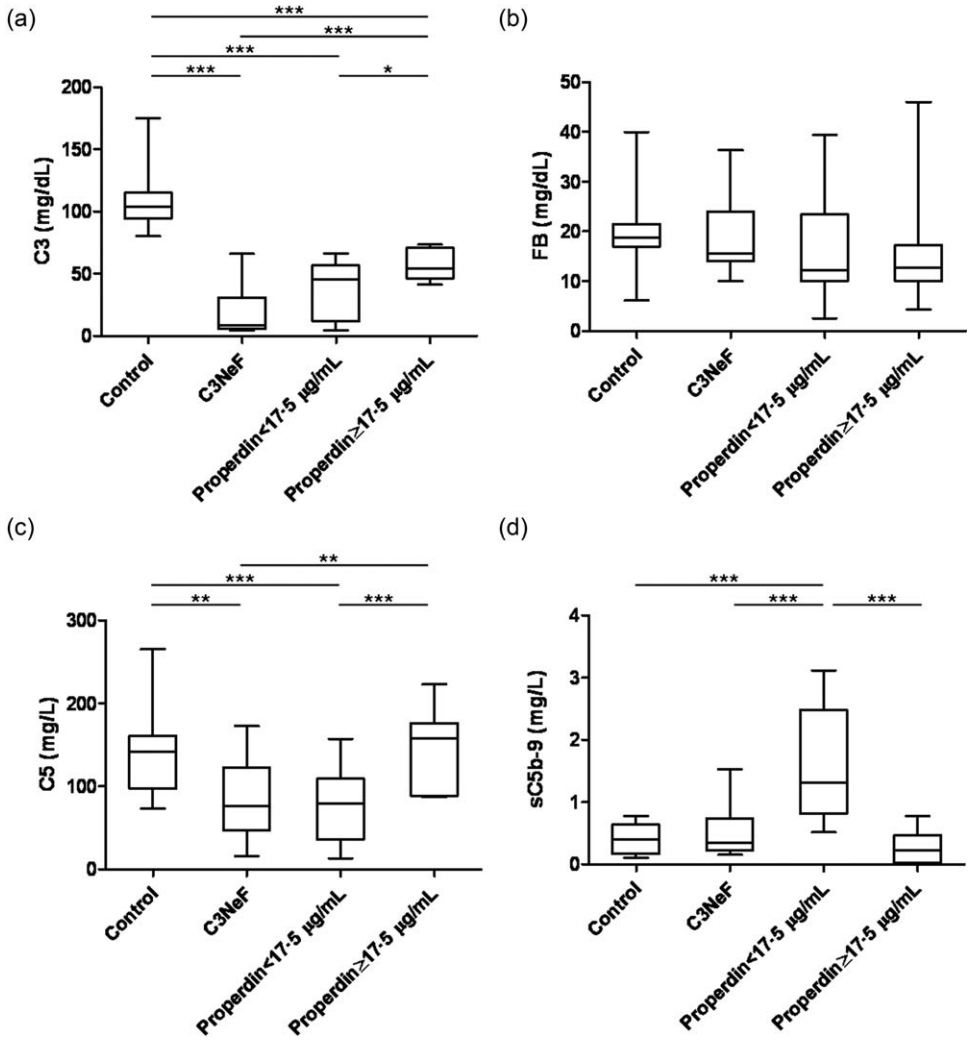

Fig. 2. Complement profile in the C3 glomerulopathy cohort. (a) Circulating C3 levels, (b) FB levels and (c) C5 levels and (d) sC5b-9 levels. For all box-and-whisker plots, the first and the third quartiles are represented by the bottom and top ends of the box, the median is represented by the inner horizontal line and the whiskers represent the interquartile range. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

levels [8·7 mg/dl (4·3–66·0, P 0·001], followed by C3NeF-negative patients with P serum levels below the normal range [45·7 mg/dl (4·8–66·4), $P < 0·001$]) and C3NeF-negative patients with P serum levels within the normal range [54·4 mg/dl (41·4–73·4), $P < 0·001$]. Serum C3 levels differed significantly ($P < 0.05$) between the two C3NeF-negative groups of patients, being significantly lower in those patients with low P levels.

Serum FB levels were within the normal range in all groups of patients, although there was a slight decrease of FB levels among the two C3NeF-negative group of patients compared to the other groups (Fig. 2b).

Terminal pathway activation: circulating C5 and sC5b-9 levels. The assembly and activation of the C5 convertase, which cleaves C5 to C5a and C5b, trigger activation of the terminal pathway (TP). In our cohorts, C5 concentration was reduced severely in C3NeF-positive patients [75·9 mg/l (16·0–172·6)] and in those C3NeF-negative patients with low P levels [79·2 mg/l (13·0–157·0)] compared with controls [141·3 mg/l (73·2–265·0), $P < 0·01$ for both] (Fig. 2c). There were no significant differences between controls and C3NeF-negative patients with normal P levels.

Plasma levels of sC5b-9 have been used as a biomarker of terminal complement activation on cell surfaces in complement-mediated diseases [19]. Notably, we found that circulating sC5b-9 was significantly higher in patients with low P serum levels [1·3 mg/l (0·5–3·1)] when compared to the other C3G patient groups [C3NeF-positive: 0·35 mg/l (0·2–1·5), $P < 0·001$ and C3NeF-negative patients with normal P levels: 0·23 mg/l (0–0·8), $P < 0·001$], which present similar sC5b-9 levels to the control group [0·4 mg/l (0·1–0·8); $P < 0·001$] (Fig. 2d).

Association between circulating properdin levels, complement components and clinicopathological parameters of patients with C3 glomerulopathy

The analysis between P levels and complement components showed a significant correlation with only C3 and C5 ($r = 0·798$, $P = 0·0001$; $r = 0·806$, $P < 0·0001$, respectively) in patients with low P. Accordingly, plasma sC5b-9 levels correlated marginally inversely with P consumption ($r = -0·683$, $P = 0·043$). In addition, the higher degree of proteinuria observed in patients with low P levels correlated with a severe serum P reduction ($r = -0·862$, $P = 0·013$). In the other two patient groups (C3NeF-positive and C3NeF-negative with normal P), P levels neither correlated with other complement components nor with clinical parameters (Table 2).

Table 2. Correlations among serum properdin levels, complement components and renal clinical parameters.

	C3NeF		Properdin <17·5 μg/ml		Properdin ≥17·5 μg/ml	
	r	*P*	*r*	*P*	*r*	*P*
C3 (mg/dl)	−0·209	0·420	0·798	0·0001	0·123	0·661
FB (mg/dl)	0·158	0·574	−0·455	0·118	0·379	0·163
C5 (mg/l)	−0·210	0·434	0·806	<0·0001	0·154	0·583
sC5b-9 (mg/l)	0·194	0·617	−0·683	0·043	−0·495	0·258
s-Cr (mg/dl)	0·016	0·956	0·527	0·064	0·098	0·774
s-Alb (g/dl)	−0·247	0·438	0·261	0·439	0·561	0·092
Proteinuria (g/24 h)	−0·289	0·388	−0·862	0·013	0·155	0·649

$P < 0·05$ was considered significant. C3NeF = C3 nephritic factor; s-Cr = serum creatinine; s-Alb = serum albumin.

Table 3. Complement component variation in presence of zymosan.

		NHS 1	NHS 2	NHS 3	PDS
% C3	Non-treated	100·0%	100·0%	100·0%	100·0%
	37°C	100·0%	100·0%	97·8%	100·0%
	Z 10 mg/ml	82·8%	92·6%	83·0%	99·1%
% C5	Non-treated	100·0%	100·0%	100·0%	100·0%
	37°C	92·6%	92·6%	100·0%	100·0%
	Z 10 mg/ml	10·2%	10·2%	6·4%	55·7%
% Properdin	Non-treated	100·0%	100·0%	100·0%	0·0%
	37°C	100·0%	96·4%	92·5%	0·0%
	Z 10 mg/ml	37·0%	38·3%	44·9%	0·0%
% sC5b-9	Non-treated	0·0%	0·0%	0·0%	0·0%
	37°C	23·0%	37·9%	24·5%	0·0%
	Z 10 mg/ml	93·4%	95·7%	94·2%	18·7%

Three different normal human serum (NHS) were used. Values were expressed as a percentage of a reference untreated, unheated serum. PDS = properdin-depleted serum; Z = zymosan.

Serum properdin consumption was due to sequestration by activated surfaces

In *in-vitro* conditions using zymosan as activator surface, 60% of properdin was consumed in healthy donors' serum (Table 3). The serum–zymosan mixture was subsequently centrifuged, serum-containing supernatant was extracted and the pellet including the zymosan was then treated with SDS 1% to detach complement components from the zymosan particles. Under these conditions, P was released fully, thus recovering normal P serum levels in the supernatant (Fig. 3a). In the presence of zymosan, C3 levels from NHS were reduced by between 10 and 20% compared with untreated and 37°C incubated samples, and a marked C5 consumption was observed in relation with increased sC5b-9 levels (Table 3). However, in the absence of P (PDS), C5 was only reduced by approximately 45% and sC5b-9 levels were increased to 18·7% (Table 3). Additionally, when PDS was assayed with zymosan-P coated incubated previously with NHS, C5 was markedly consumed (around 90%) and this reduction correlated with an increase of the same extent in sC5b-9 levels (Fig. 3b).

Discussion

Properdin ensures optimal rates of AP activation by its ability to bind and stabilize the inherently labile C3 and C5

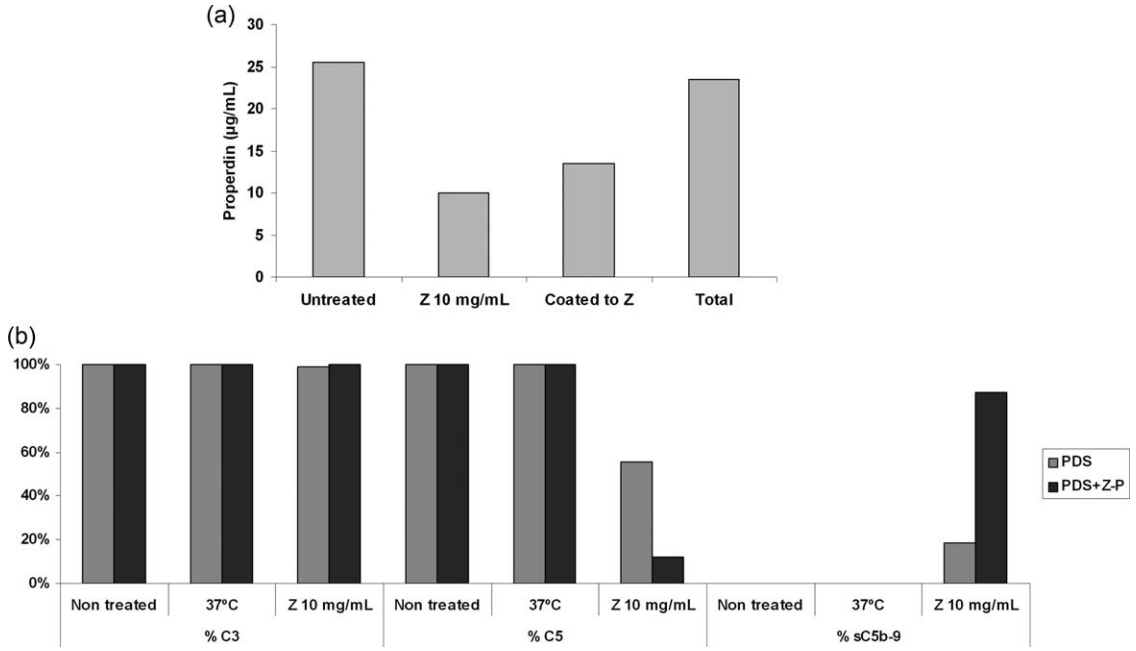

Fig. 3. Properdin is critical for C5 convertase activation on activator surfaces. (a) Serum properdin was reduced after treatment with zymosan (Z) by sequestration on Z surface. The remaining properdin quantity was released from Z particles after treatment with sodium dodecyl sulphate (SDS) 1% at 95°C for 5 min. (b) Using Z-properdin-covered particles (Z-P), C5 convertase activity was increased, as reflected by C5 consumption and elevation of sC5b-9 levels when a properdin-depleted serum (PDS) was used and in comparison with the same PDS without Z-P. In serum samples treated at 37°C, the values of C3, C5 and sC5b-9 were not changed compared to untreated serum. Values are expressed as a percentage of a reference untreated, unheated serum.

convertases complexes (C3bBb and C3bBbC3b). Given that P participates only in AP activation, the use of this serum parameter as a biomarker of AP dysregulation may provide a more detailed and mechanistic understanding of the complement contribution in those diseases associated with a defective AP control. Therefore, we considered it important to determine the P serum concentration in patients with such pathologies.

In the current study, we searched for correlations between the P serum levels and other clinical parameters in a group of C3G patients, a complement-mediated renal pathology that encompasses the very rare kidney diseases DDD and C3GN [11,20].

The most common anti-complement autoantibodies reported in patients with C3G is C3NeF. This autoantibody has been detected in approximately 50% of C3GN cases and up to 80% of DDD patients [21]. In our cohort, the presence of C3NeF was higher in DDD than C3GN patients (82·4% in DDD *versus* 17·6% in C3GN) (Table 1). The C3NeF stabilizing effect of the C3bBb convertase results in a higher and prolonged activity, C3 and C5 consumption and activation of the TP [21]. Our results were in agreement with this stabilizing effect (Fig. 2a,c). Within the C3NeF-positive group, FB (Fig. 2b) and P levels were similar to those observed in the control group (Fig. 1). These preserved FB and P levels could be explained by a lower ratio of C3 convertase formation. Interestingly, sC5b-9 levels in the C3NeF-positive group were similar to the controls (Fig. 2d). This result is in agreement with the observation from other authors in DDD patients, where sC5b-9 levels were normal in a situation of C5 consumption [11].

Serum P levels below the normal range have been detected in a group of C3NeF-negative patients, most of them diagnosed with C3GN (Table 1). This group showed a significant C3 reduction (Fig. 2a). Also, C5 convertase activity was increased in these patients, as evidenced by a marked C5 consumption (Fig. 2c). Although the C3NeF-positive group exhibited reduced C5 levels, C3NeF-negative patients with low P levels showed a significant increase in sC5b-9 levels (Fig. 2d). By correlation analysis, it was demonstrated that P consumption was associated significantly with a reduction of circulating C3 and C5 ($r = 0{\cdot}798$, $P = 0{\cdot}0001$; $r = 0{\cdot}806$, $P < 0{\cdot}0001$, respectively) and with an elevation of sC5b-9 levels ($r = -0{\cdot}683$, $P = 0{\cdot}043$) (Table 2). These results indicate that the low level of P in the C3NeF-negative group is due probably to marked C5 convertase activation.

The main difference between the two groups with low C5 levels, C3NeF-positive and C3NeF-negative with low P, is the location where TP activation takes place. Nowadays, it is accepted that C3NeF-positive patients exhibit a profound AP dysregulation in the fluid phase. In contrast, our data suggest that in the C3G subset with low P, significant complement activation takes place on cell surfaces.

Previous results in the double factor H (FH)/P knock-out (KO) mice revealed a much higher increase in C5 levels than in C3 levels compared with their single FH KO littermates [22], which suggest that C5 consumption in these double KO mice occurs mainly on surfaces and is P-dependent, while C3 consumption is a fluid phase P-independent event.

Recent investigation suggests that complement activation can also occur through a P-directed pathway (PDP), which is initiated on specific targets by P and uses the same components as the AP [2,23–25]. We hypothesize that serum P consumption in our cohort of patients could be due to P sequestration in the altered renal tubule of C3G patients; in this scenario, P acts as an initiator and amplifies complement activation at local sites by the PDP. Another hypothesis suggests that P could also be sequestered through C3 and C5 convertases that were being generated at a high level in the altered renal tubule. In any case, the P effect is linked to complement activation on activating surfaces. In agreement with these hypotheses, strong P staining has been observed on the luminal surfaces of the tubules in kidney biopsies from patients with proteinuric renal disease, but not in non-proteinuric controls [26]. However, studies investigating the correlation between tubular P deposits and P serum levels in disease conditions are lacking, and we could not address this fundamental question in our cohort due to the unavailability of fresh renal biopsies.

With the zymosan assay model, we showed here that P sequestration by zymosan surfaces mimics the reduction in P serum levels observed in C3G patients. P was also critical to increased C5 convertase activity on surfaces, which is in agreement with previous results [22]. According to this, when using PDS as a negative control, C5 convertase activation was lower than in the presence of P. However, when the same serum was tested with zymosan-P coated particles incubated previously with NHS, C5 consumption and sC5b-9 production is equivalent to that of NHS (Fig. 3b). In conclusion, these results demonstrate that P is deposited on zymosan surfaces during complement activation and that the observed effects of this assay on C5 and C5b-9 levels mirror the complement levels observed in C3G patients undergoing pathological complement activation, and suggest that P is an important determinant in TP triggering on activator surfaces.

Zaferani and colleagues identified that tubular heparan sulphate proteoglycan (HSPG) served as a docking platform for P in proteinuric renal disease [26]. P participation in PDP on the cell surface of PTECs enhances C3b and C5b-9 deposition, leads to tubulointerstitial damage and worsens renal function [12,13,26]. In connection with these evidences, serum P consumption in our patients can be explained by the deposition of P on altered renal tubules. In addition, P consumption in our cohort was associated with a higher degree of proteinuria (Table 2), suggesting a role for P and PDP-associated C5 consumption in proteinuria-mediated renal damage.

The first anti-complement therapy available is eculizumab (Soliris®; Alexion Pharmaceuticals, Cheshire, CT, USA), a fully humanized monoclonal antibody that binds with high affinity to C5 and prevents the generation of C5b-9. Eculizumab was first approved for the treatment of paroxysmal nocturnal haemoglobinuria, and more recently for atypical haemolytic uraemic syndrome treatment [27].

The experience in using eculizumab for C3G treatment is limited to a few case reports and the results from a 1-year, open-label study of eculizumab therapy in six subjects. Heterogeneous responses were reported indicating that eculizumab may be an adequate treatment in only a subgroup of patients with C3G [28]. Therefore, one of the major challenges in treating C3G patients with the anti-complement therapy may be how to identify those with primarily C5 convertase dysregulation.

Levels of soluble C5b-9 may be informative of a high degree of C5 convertase dysregulation on surfaces [28]. In our study, we show a correlation between high sC5b-9 levels and P consumption, supporting that P is also a useful biomarker of C5 convertase activation on cell surfaces. Moreover, P levels are a useful clinical parameter to predict renal damage, as is reflected by the association of P consumption with the high degree of proteinuria. Therefore, serum P measurement is a potential deciding parameter for therapeutic assessment in C3NeF-negative patients, although further studies are required to validate its significance and impact in clinical practice.

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Disclosure

The authors declare no financial conflicts of interest. S. R. de C. has received honoraria from Alexion Pharmaceuticals for giving lectures and participating in advisory boards. None of these activities has had any influence on the results or interpretation in this paper.

Author contributions

Concept of the study: F. C.; design of the experiments: F. C., M. B. G -M., M. L -T.; experimental work: F. C., S. G.; collection and characterization of clinical samples: F. C., M. L.-T., P. N.; manuscript writing: F. C., M. L.-T., P. N., S. G., A. T., S. R. de C.

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