Complement inhibitor C4b-binding protein in primary Sjögren's syndrome and its association with other disease markers.

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AFZ performed experiments and evaluations of the results and wrote paper
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The abbreviations used are:

AECC: American European Consensus Criteria;
C4BP: C4b-binding protein;
CRP: C-reactive protein;
hsCRP high sensitive C-reactive protein
ELISA: Enzyme-linked immunosorbent assay;
NHS: Normal human serum;
pSS: Primary Sjögren's Syndrome;
RCA: Regulators of complement activity;
anti-SSA/Ro: anti –Sjögren's Syndrome A/Ro-autoantibodies;
anti-SSB/La: anti -Sjögren's Syndrome B/La-autoantibodies;
VAS: Visual Analogue Scale

Abstract
A subgroup of patients suffering from primary Sjögren's syndrome (pSS) display unexplained low levels of complement components C3 and/or C4, which is associated with increased risk of non-Hodgkin's lymphoma. C4b-binding protein (C4BP) is a major fluid-phase complement inhibitor, which can influence C4 and C3 levels. Therefore we analyzed C4BP levels in the sera of patients with pSS to better understand the disturbances in complement in pSS. Associations with other disease markers were also investigated to define a possible role of C4BP as marker of high risk disease course. Plasma levels of C4BP were analysed in pSS patients (n=86) and in controls (n=68) by ELISA. C4BP levels from 49 patients were correlated to disease activity markers and autoantibody profiles. We found that total C4BP plasma levels were significantly higher in pSS patients compared with controls. C4BP levels correlated to the acute phase response, to levels of C4 and C3 as well as to the CD4+/CD8+ T-cell ratio. C4BP levels were inversely related to IgG levels, extent of autoantibody production and global disease activity. C3dg levels, a marker of complement activation, displayed a negative correlation to C4 levels but interestingly not to C4BP levels. In conclusion**,** C4BP levels are increased in patients suffering from pSS proportional to their acute phase response. However, in the most active cases, with the most widespread autoantibody production, C4BP levels were decreased in parallel with levels of C3 and C4 and CD4+ T cells, suggesting that disturbed complement regulation may contribute to pathogenicity in pSS.

Introduction
PSS is the second most common autoimmune disease after rheumatoid arthritis, with a prevalence of around 0.4% when defined strictly according to the American European Consensus Criteria (AECC) [1] . Uncomfortable symptoms (dry mouth and eyes, gastrointestinal and urogenital discomfort), reduced health related quality of life, disabling fatigue and increased risk for and death due to non-Hodgkin's lymphoma make pSS a difficult condition [2-5] . Autoantibodies reported in pSS are mainly directed against the 52 kDa SSA/Ro, 60 kDa SSA/Ro, SSB/La, components of ribonucleoprotein complex, presented to the immune system during apoptosis, viral infections or by other mechanism. The antibody response to Ro and La and its diversification is regulated by MHC class II haplotypes HLA DR3 (DRB1*0301) and DR15 (DRB1*1501) being independent risk factors for Ro and La seropositivity in pSS [6,7] .
While the autoantibodies are important for diagnosing and classifying the disease, disturbances in the function of the complement system, low levels of C3 and C4, have been repeatedly identified as markers of unfavourable outcome, such as lymphoma, severe disease manifestations and premature death [3,4,8,9]. It is unknown if low complement levels are caused by consumption or by genetic influence. Next to true consumption of complement also poor inhibition/regulation of complement activation could be responsible for the low levels of C4 and or C3. The fluid-phase complement inhibitor which interacts with both C4 and C3 is C4b-binding protein (C4BP). C4BP has a central role in regulating the classical and lectin complement pathways [10] . To inhibit complement, C4BP acts as a cofactor for factor I-dependent degradation of C4b and C3b [11,12] and accelerates decay of C3-convertases [12,13] . C4BP is a large multimeric glycoprotein (570 kDa) and circulates in plasma in three isoforms composed of different combinations of α–chains and β–chain [14] with the predominant isoform (α7β1) and two minor isoforms (α7β0) and (α6β1) [15-17]. The term total-C4BP will be used to describe all different isoforms and the term β–chain positive C4BP to describe only those isoforms that do contain a β–chain. Complement-regulatory activity depends on the C4BP α-chain whereas the C4BP β-chain binds to protein S, which gives the complex the capacity to bind to negative phospholipids such as those present on dead cells [18].

It has been suggested that synthesis of these two subunits is regulated differentially [19] . C4BP is synthesized by hepatocytes and activated monocytes [20,21] and its production is upregulated by inflammatory cytokines (IFN-γ, IL-1, IL-6, and TNF-α) [21,22] . In addition, its expression levels are also influenced by genetic variations in the Regulators of Complement Activation genes (RCA cluster) on chromosome 1q32 [23] . C4BP interacts with several ligands relevant for autoimmunity such as apoptotic and necrotic cells, DNA and C-reactive protein (CRP) [18,24-27] . Although several questions are still open, one publication suggested that C4BP would stimulate B cells via CD40 [28] , which could be relevant for autoimmune diseases like pSS.
The present pilot study was undertaken to analyse plasma levels of C4BP in pSS patients and controls and to relate C4BP levels in the patients to C4 and C3 levels and other markers of disease activity.

Patients and Methods:

***Patients and controls*:** A total of 86 consecutive unselected patients with pSS according to the AECC [1] were included for comparison of plasma protein levels with healthy controls. Sera from 68 unrelated healthy persons from the same area in southern Sweden were used as controls. For 49 of the patients simultaneously collected detailed information was available regarding both laboratory and clinical parameters of disease activity. Blood samples were drawn simultaneously for analysis of C4BP, C3, C4 C3dg, disease activity markers and autoantibody status. Several factors were analyzed directly on fresh material, other analyses were performed on frozen samples. For the remaining 37 patients only the levels of C4BP were determined.

Determination of total C4BP and β−chain positive C4BP levels

Total C4BP and β-chain positive C4BP plasma levels were analysed in pSS patients and controls using ELISA. For the detection of both total C4BP and β−chain positive C4BP, microtiter plates (Maxisorp Nunc, Roskilde, Denmark) were coated with 100 µl of rabbit polyclonal antibodies against human C4BP (PK 9008, prepared in house) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C.The wells were blocked with 100 µl of quenching solution (washing buffer supplemented with 3% fish gelatine, Norland Products Cranbury, USA) for 1 h at room temperature. After quenching, the plates were Washed four times with washing buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$ and 0.1% Tween 20; pH 8.0). The same washing procedure was used between each step of the assay. Appropriately diluted test samples, standards and controls were added to the plates and incubated for 1h at 37°C. Samples were diluted in quenching solution and serial dilutions of a pool of normal human serum (NHS), prepared from six healthy blood donors, was used as a standard. This NHS was always included in each assay as an internal control.
After another washing, the MK-104 (for detection of C4BP α−chain for total C4BP) or MK-2B (for detection of C4BP β chain for β-chain positive C4BP) diluted in quenching solution were added and plates were incubated for 1h at 37°C. Both monoclonal antibodies and the PK9008 were kind gift of prof. Dahlbäck (Lund University, Lund, Sweden). Goat anti-mouse antibody, conjugated with horseradish peroxidase (Dako, Glostrup, Denmark), diluted in quenching solution was added after another washing step and incubated for 1h at 37°C. After the last washes the plates were developed using 1,2-phenylenediamine dihydrochloride as substrate (OPD) (DakoCytomation, Glostrup, Denmark) following the instructions of the supplier. Concentrations of C4BP were calculated relative to a standard of pooled NHS of which we had previously determined the C4BP concentrations to be 148.4 µg/mL of total C4BP and 127.0 µg/mL of β−chain positive C4BP respectively. The intra-assay coefficient of variation

for the ELISA was 5.7 % (MV= 105.2 μg/mL) for total C4BP and 4.9% (MV=95.0 μg/mL) for β-chain positive C4BP. Inter-assay coefficient of variation was 3.3% (MV=105.2 μg/mL) for total C4BP and 4.1% (MV=95.0 μg/mL) for β-chain positive C4BP. Patients and controls were analysed on the same day. The person performing the assays was blinded to the clinical characteristics of the patients.

Clinical disease activity:
Due to lack of a consensus instrument for disease activity in pSS evaluation was performed using a Visual Analogue Scale (VAS) for physician's global assessment with 0 as "no activity" and 10 as "maximal disease activity" as it was used as a gold standard in two recent investigations [29,30] . The VAS evaluation was performed before the C4BP levels were determined.

Analysis of serological disease activity markers and autoantibody profiles
All analyses were done at the Immunology Department in the Lund University Hospital, Lund, Sweden. Antinuclear antibody titres were determined by immunofluorescence while SSA/Ro and SSB/La antibodies were detected qualitatively by immunoblotting. IgG was analysed by nephelometry and IL-6 by chemiluminescence. High sensitive CRP (hsCRP) and C3, C4 were measured by turbidometry. Blood samples for C3dg analysis were collected using EDTA-tubes according to instructions of the laboratory of Clinical Chemistry. Plasma was prepared as fast as possible and stored at - 80°C. C3dg levels were determined using non-pre-treated EDTA-plasma using double rocket immunoelectrophoresis technique with antibodies specific for C3d and C3c [31]. CD4 and CD8 lymphocyte markers were measured using flow cytometry.

Statistical analysis
Differences between the patients and controls and between subgroups of pSS patients were evaluated by unpaired Student's T-test. Correlation between total C4BP and β-chain positive C4BP levels and correlations between C4BP levels and other disease characteristics were done by Pearson correlation coefficient analysis. In variables not fulfilling the assumptions for Pearson correlation we used Spearman rank correlation instead. P values less than 0.05 were considered statistically significant. The study was approved by the regional ethics committee.

Results

C4BP levels are increased in plasma of pSS patients
The plasma levels of fluid-phase complement inhibitor C4BP were analyzed in 86 unselected patients suffering from pSS and in 68 healthy controls. Baseline characteristics of patients and controls are reported in Table 1. Mean C4BP levels were significantly higher in the pSS patients than in the controls, $p<0.0001$ for total C4BP and $p=0.0032$ for β-chain positive C4BP (Fig.1A, B). None of the pSS patients was deficient in C4BP but we observed a broader range of C4BP levels in the patients in comparison to the controls.

Levels of total C4BP are correlated to levels of β-chain positive C4BP but not at the highest concentrations of total C4BP.
We analyzed to what extent the levels of total C4BP are correlated to the levels of β-chain positive C4BP. For this purpose we plotted β-chain positive C4BP versus total C4BP levels of each individual patient or control. In healthy individuals the total C4BP and β-chain positive C4BP were very well correlated ($r^2=0.79$) (Fig. 2A). For the pSS patients a similar strong correlation was observed ($r^2=0.87$; Fig. 2B). Interestingly, for those pSS patients with total

C4BP levels exceeding 400 µg/mL there does not seem to be a similar linear increase in β-chain positive C4BP (r^2 =0.047) as can be observed below 400 µg/mL.
Next we analyzed if the C4BP levels in the pSS patients were related to their clinical characteristics. From the 86 pSS patients we had detailed simultaneously collected clinical and laboratory data available for 49 patients. There were no major differences in clinical characteristics between these 49 patients and the total 86 pSS patient group (Table 1).

Plasma C4BP levels correlate positively to plasma C3 and C4 levels in pSS patients.
Plasma levels of C4BP were positively correlated to the circulating levels of C3 and C4 (Table 2) indicating that indeed lower levels of C4BP are associated with lower levels of C3 and C4.
Next we analyzed to what extent the levels of C3 and C4 were associated with complement consumption by measuring complement activation breakdown product C3dg. No significant correlation was found between C3 and C3dg but levels of C4 showed a strong negative correlation with the levels of the breakdown product C3dg (r^2 = -0.441; p=0.002). However, no correlation was observed between C3dg and either total C4BP or β-chain positive C4BP (r^2 = 0.061; p=0.683 and r^2 = - 0.21; p=0.888 respectively).
The three patients with very low levels of C4 (<0.12 g/l) had very high levels of C3dg (>15 mg/l), which indicated that low C4 levels in pSS may not mainly be due to genetically determined low production but also due to increased consumption. The C4BP levels in these patients were relatively low possibly indicating a lack of regulation. Since this study was cross sectional it was not possible to conclude if the low C4BP levels preceded the consumption of C4 and C3, or if C4BP was consumed together with C4 and C3 due to complement activation.

C4BP levels are correlated to the acute phase response as well as to the CD4+/CD8+ T-cell ratio
Since C4BP was reported previously to be an acute phase protein we analyzed if the levels of C4BP would be related to the plasma levels of other acute phase markers such as CRP and IL-6. Indeed we found a strong positive correlation between the levels of C4BP in the pSS samples and the levels of IL-6 as well as CRP using a high sensitive CRP assay (hsCRP) (Table 2).
Similarly to low complement levels also a low CD4+/CD8+ T-cell ratio confers an increased risk for the development of lymphoma in pSS patients [4] . We observed that C4BP levels were positively correlated to the CD4+/CD8+ T-cell ratio. Thus lower levels of C4BP correlated with the high risk profile of hypocomplementemia and CD4+ T-cell lymphocytopenia.

C4BP levels are inversely correlated to total IgG levels, disease activity, autoantibody production and non-exocrine disease manifestations.
In the pSS patients (n=49), total plasma IgG levels were inversely correlated to the level of β-chain positive C4BP (Table 2). When analysing subgroups of pSS patients separately, depending on their autoantibody status, we observed that individuals having autoantibodies to the La (SSB) antigen displayed significantly lower levels of both total-C4BP and β-chain positive C4BP compared to those lacking an anti-La response (Table 3). Also in the four patients positive for cryoglobulins we observed a trend for lower levels of β-chain positive C4BP than those without cryoglobulinemia (Table 3). Likewise patients with non-exocrine disease manifestations presented significantly lower β-chain positive C4BP levels than those who had a disease course restricted to glandular signs and symptoms (Table 3).
Currently no validated disease activity index is available for pSS and therefore, we have used the physician's global assessment on a VAS (0-10) as the best available measure to describe the overall disease activity of the patients. Using this physician's global assessment we

observed that C4BP levels correlated negatively with the overall disease activity, indicating that patients with most severe disease had lower levels of C4BP (Table 2).

Discussion:

Patients suffering from pSS display among other phenomena also an altered complement profile, which is an essential characteristic of the high risk type 1 subtype of the disease [4,8,9]. This study was undertaken to investigate how the endogenous fluid-phase complement inhibitor C4BP is related to the clinical and laboratory parameters of these patients and if impaired complement regulation would explain the low C3 and C4 levels in high risk patients.

Mean C4BP levels were significantly higher in pSS patients than in controls, interpreted as an expression of inflammatory activity also mirrored by increased IL-6 and CRP levels. Increased plasma levels of C4BP have been reported before for other inflammatory diseases and C4BP has been reported to be an acute phase protein [32] . Its production is also reported in *in-vitro* assays to be upregulated following stimulation with IL-6. Here we report a direct relation *in vivo* between IL-6 levels and increased levels of C4BP. Interestingly, the gradual increase in C4BP levels that was correlated to IL-6 and CRP levels was not observed in the most severe cases of pSS, that is, the patients with the broadest range of autoantibody production against both Ro and La and highest immunoglobulin levels and in those judged as having the highest global disease activity and signs of non-exocrine disease.

We observed a strong correlation between levels of total C4BP and β-chain positive C4BP. However, the proportional increase in β-chain positive C4BP was not observed for total C4BP levels higher than 400 μg/mL. This phenomenon was reported before for SLE patients and is suggested to prevent depletion of free protein S levels [19] . The C4BP β-chain has a very high affinity for protein-S and the instant binding of protein-S to the β-chain largely inactivates the function of protein-S as an anticoagulant [10], which can have major consequences. Decrease in free protein-S level is strongly related to occurrence of thrombosis [19] and the body appears to have developed sophisticated regulation mechanism to prevent decrease of free protein-S levels as a consequence of increased production of C4BP. The observation that β-chain positive C4BP correlates better with autoantibody status and extraglandular disease could be the result of the lack of correlation between total C4BP and β-chain positive C4BP above 400 μg/mL.

A recent study on pSS patients revealed an abnormal distribution of genetic subtypes within the RCA region, also including the genes for C4BP, which contributed to Ro and La diversification when in epistasis with HLA DR3 [23]. Therefore the positive correlation that we observed between C4BP and C4 and C3 could result from the fact that genetic subtypes associated with low C4BP production result in impaired complement regulation which, on the background of defined HLA subtypes, can increase the risk for B cell activation, autoantibody production and immune complex formation. On the other hand it is also possible that complement activation results in depletion of circulating C4 levels paralleled by depletion of C4BP, which would also explain the positive correlation between C4 and C4BP levels. The partial correlation between C4BP and C3 and C4 is likely to be the result of both genetic influences and complement mediated depletion that influence plasma levels of these proteins independently. The current data clearly indicate that C4, C3 and C4BP levels are influenced together in pSS patients, but the cause and effect relation remains to be established.

We found that there was a strong negative correlation between C4 and C3dg and no correlation between C4BP and C3dg, which indicated that it was more likely that low C4 and lower C4BP were the result of complement activation rather than that C4 levels were low because of low levels of C4BP, but formally this would have to be shown in a longitudinal study.

C4BP correlated better with C3 than with C4. The importance of lowered C3 in primary SS is controversial in contrast to the accepted negative prognostic role for low C4. Partly this

controversy is due to different cut-off values and several studies show that low C3 is an as good marker of lymphoma and death risk as low C4 [4,9]. Our ongoing study on genetic abnormalities within complement and complement regulator genes in a larger SS population will hopefully contribute to clarifying some of the unexplained findings in this small study.
C4BP levels may be useful as a biochemical marker of disease activity as they inversely correlated with physician's global assessment of disease activity. An ongoing study will allow a comparison of peripheral complement levels with genetic variants within complement factors and complement regulators. The results of these studies will contribute to the further clarification of the yet widely unknown pathogenetic mechanisms in pSS.
Overall conclusions: C4BP levels are increased in patients suffering from pSS proportional to their acute phase response. Only in the more severe cases with intensive ongoing autoantibody production and systemic extraglandular disease manifestations, C4BP levels are decreased in parallel with C3, C4 and CD4+ T-cell counts.

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Table 1 Baseline characteristics of patients and controls

	All patients (n=86)	**Patients included in correlation study (n=49)**	**Controls (n=68)**
Age: years, median (range)	61 (22-85)	59 (30-85)	59 (25-72)
Disease duration: years, median (range)	10 (0-22)	12 (1-22)	
Sex: female/male	78/8 (91%/9%)	43/6 (88%/12%)	55/13 (81%/19%)
Salivary gland biopsy: Autoimmune sialadenitis / unspecific inflammation or normal / not done or invalid biopsy	70/8/8 (81%/9%/9%)	38/7/4 (78%/14%/8%)	
SSA positivity	56 (65 %)	35 (71%)	
SSB positivity	35 (41 %)	32 (65%)	
ANA positivity	72 (84%)	43 (88%)	
C3 mean/std (g/l)		1.15 +/-0.29	
C4 mean/std (g/l)		0.25 +/-0.08	

Table 2: Correlation analysis between C4BP and clinical and laboratory disease markers

	Plasma levels (mean/std) range		**C4BP total**	**β-chain positive C4BP**
C3 (g/l)	1.13 (0.29)	r^2	0.489	0.573
	0.38-1.75	p	<0.001	<0.001
C4 (g/l)	0.25 (0.08)	r^2	0.288	0.319
	0.09-0.44	p	0.045	0.025
High sensitive CRP (mg/l)	4.34 (4.27)	Rho	0.332	0.313
	0.29-22.90	p	0.020	0.028
IgG (g/l)	16.51 (6.41)	r^2	-0.272	-0.296
	6.40-33.30	p	0.058	0.039
Il 6 (ng/l)	10.71 (12.20)	Rho	0.384	0.312
	2.79-75	p	0.008	0.032
Ratio CD4+/CD8+ T-cells	2.29 (2.13)	Rho	0.163	0.347
	0.15-14.00	p	0.269	0.016
Physician's global disease activity scoring (VAS 0-10)	3.35 (1.92)	r^2	-0.14	-0.288
	0-7	p	0.33	0.044

Table3: Comparison of subgroups of SS patients with regard to C4BP levels

	C4BP total μg/mL	**β-chain positive C4BP μg/mL**
SSA+SSB positives (n=17)	204.94 (60.15) * [A]	141.34 (26.12) ** [A]
SSA positives, but SSB negative (n=18)	266.45 (105.58)	184.85 (44.57)
SSA+SSB negatives (n=14)	241.04 (68.39)	164.85 (41.49)
Cryoglobulin positives (n=4)	213.79 (60.03)	144.26 (32.88)
Cryoglobulin negatives (n=40)	243.61 (90.35)	169.11 (43.37)
Non-exocrine disease (n=35)	228.87 (84.10)	154.74 (38.48)* [B]
Isolated exocrine disease (n=14)	260.29 (83.72)	187.79 (41.79)

* $p < 0.05$ ** $p < 0.01$

A (patients with anti SSB autoantibodies vs all patients without anti SSB autoantibodies)

B (patients with non-exocrine disease manifestations vs patients with pure glandular disease)

Figure legends

Figure 1
C4BP plasma levels are increased in the pSS patients as compared to age and sex matched controls.
Levels of total C4BP and β-chain positive C4BP of healthy controls (n=68) and pSS patients (n=86) by ELISA. Mean concentration in controls for total C4BP was 205.5 +/- 49.2 μg/mL and for β-chain positive C4BP 160.0+/- 30.5 μg/mL. Mean concentration in pSS for total C4BP was 266.9 +/- 95.8 μg/mL and 181.6 +/- 52.8 μg/mL for β-chain positive C4BP.

Figure 2
Total C4BP levels are correlated to β-chain positive C4BP levels in pSS patients and controls.
The levels of β-chain positive C4BP were plotted against the levels of total C4BP for controls (A) and pSS patients (B). β-chain positive C4BP is very well correlated with the levels of total C4BP in controls (r^2=0.79) and pSS patients (r^2=0.87).

Figure 1.

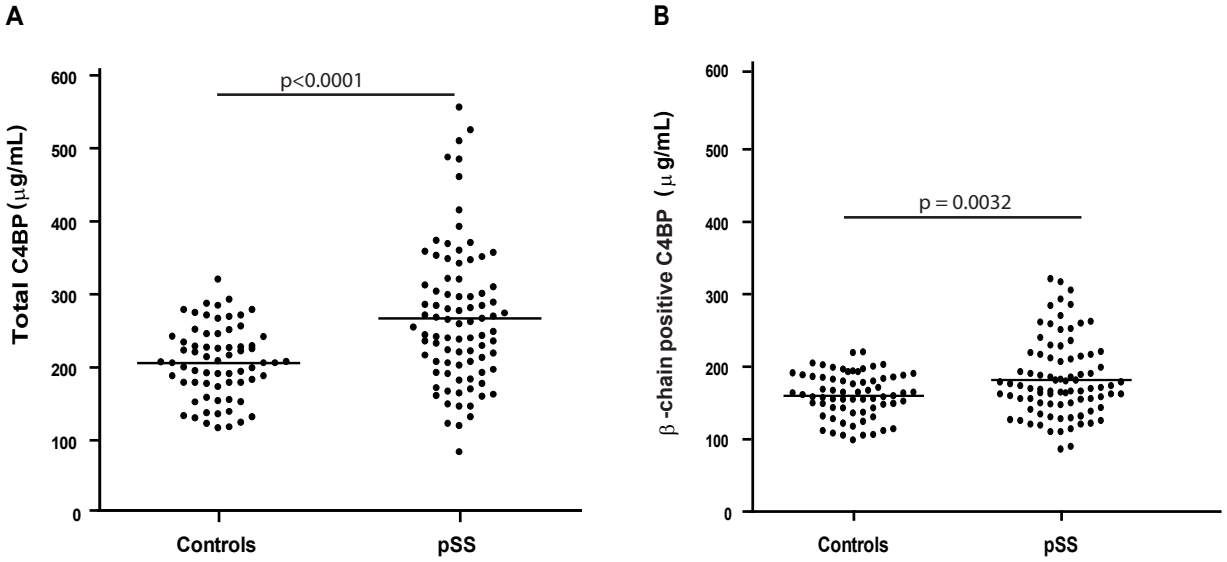
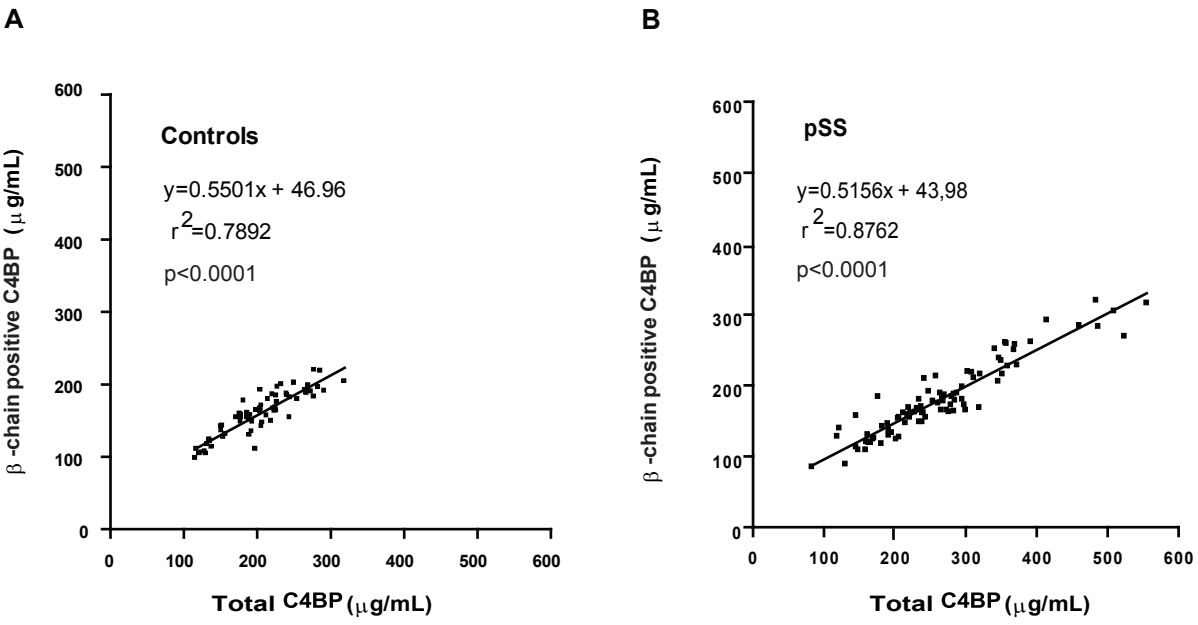

A
Total C4BP (μg/mL)
600
500
400
300
200
100
0
p<0.0001
Controls
pSS
B
β -chain positive C4BP (μg/mL)
600
500
400
300
200
100
0
p = 0.0032
Controls
pSS

Figure 2.


A
Controls
y=0.5501x + 46.96
r2=0.7892
p<0.0001
β -chain positive C4BP (μg/mL)
Total C4BP (μg/mL)
0
100
200
300
400
500
600
B
pSS
y=0.5156x + 43,98
r2=0.8762
p<0.0001
β -chain positive C4BP (μg/mL)
Total C4BP (μg/mL)