Complement Activation and Plasma Levels of C4b-Binding Protein in Critical Limb Ischemia Patients

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Abbreviations: APC, activated protein C; $C4BP_{tot}$, total C4b-binding protein; $C4BP_{\beta}$, β-chain of C4BP; CLI, critical limb ischemia; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; LDL, low-density lipoprotein; TCC, terminal complement complex; TNF-α, tumor necrosis factor-α

ABSTRACT

Objective: Critical limb ischemia (CLI) is a peripheral arterial disease manifested by drastically diminished blood flow to the legs, pain at rest, non-healing wounds and gangrene caused by atherosclerosis. Significant tissue necrosis is associated with late stage CLI and the patients have a poor prognosis. Necrotic and apoptotic cells activate complement and bind complement inhibitor C4b-binding protein (C4BP). The major isoform of C4BP is composed of seven identical α-chains and one β-chain, here termed $C4BP_{\beta}$, whereas upon inflammation a normally less abundant isoform is upregulated that is exclusively composed of α-chains. Measuring the α-chains of C4BP includes both isoforms and is termed total C4BP ($C4BP_{tot}$).
The hypothesis of this study was that levels of complement activation and C4BP are predictive for the severity of the disease and that their measurement might be of clinical advantage.

Methods: This was a prospective, single-center study of 259 consecutive patients with CLI admitted to a secondary referral center for vascular diseases. Interventions included evaluation of soluble terminal complement complexes (C5b-9), $C4BP_{tot}$ and $C4BP_{\beta}$, lipid levels, the inflammatory mediators tumor necrosis factor-α, interleukin-6, 8-iso-protaglandin $F_{2\alpha}$, high-sensitivity C-reactive protein, neopterin, plasma homocysteine and plasma endothelin-1 in plasma as well as resistance to activated protein C and ankle blood pressure. All data were compared to an age-matched population based control group of 219 currently healthy individuals.

Results: The data are presented as mean±SEM/median. CLI patients showed systemic complement activation (1.17±0.06/1.13 AU/ml versus 0.69±0.07/0.59 AU/ml in healthy controls, $P < .0001$), which was even higher in patients with gangrene (1.33±0.11/1.28 AU/ml versus 1.1±0.08/1.0 AU/ml, $P = .0264$), who also showed increased C4BP levels (421±28.6/386 μg/mL versus 341±10.8/318 μg/mL for $C4BP_{tot}$, $P = .0248$; 374±25.4/332 μg/mL versus 305±9.5/285 μg/mL for $C4BP_{\beta}$, $P = .0581$). C4BP plasma levels were significantly elevated in CLI patients in comparison to healthy controls (351±8.1/322 μg/mL versus 297±8.0/288 μg/mL for $C4BP_{tot}$, $P =$

.0001; 314±7.0/287 μg/mL versus 265±7.0/263 μg/mL for $C4BP_{\beta}$, $P = .0004$) and correlated to levels of interleukin-6 ($P_{tot/\beta} = .0048/.0019$), high-sensitivity C-reactive protein ($P < .0001$), leukocyte ($P_{tot/\beta} = .0086/.0043$) and platelet count ($P = .0001$), LDL/HDL ratio ($P_{tot} = .0151$) and HDL ($P_{tot/\beta} = .0047/.0177$), but not to tumor necrosis factor-α.
Conclusions: Increased complement activation and C4BP plasma levels are related to the degree of tissue necrosis and disease severity of critical limb ischemia. This knowledge in combination with the found correlations to other biomarkers is useful for understanding the pathophysiology of the disease.

INTRODUCTION

Critical limb ischemia (CLI) is characterized by chronic ischemic rest pain, ulcers, or gangrene attributable to arterial occlusive disease.[1] The incidence is approximately 500 to 1000 per million a year in western countries, with the highest rates among older subjects, smokers and diabetics.[1,2] The one-year mortality in CLI is around 20 %[3] and the rate of primary amputation ranges from 10 % to 40 %. Due to the negative impact on quality of life and the poor prognosis, both in terms of limb salvage and survival, CLI is a critical public health issue.[2,4]
The complement system is an essential part of the innate immune system in front line of defense against bacterial infections. It bridges innate and adaptive immunity and is also important for the removal of immune complexes and dying cells.[5] Complement is tightly controlled by several soluble or membrane bound complement inhibitors, but erroneous or excessive activation contributes significantly to the pathology of many inflammatory diseases. C4b-binding protein (C4BP) is the major soluble inhibitor of the classical and lectin pathways of complement and circulates in complex with the vitamin K-dependent protein S, a cofactor to the coagulation inhibitor activated protein C (APC), thereby linking complement with the coagulation system.[6] C4BP is a large plasma glycoprotein of 500 kDa and mainly produced in the liver. The major isoform of C4BP is composed of seven identical α-chains and one β-chain to which protein S is bound (here termed $C4BP_{\beta}$).[7] The subunits are covalently linked by disulfide bonds at the C terminus.[8] C4BP is an acute phase reactant, because its plasma level increases during inflammation and after trauma.[9] However, upon trauma there is mainly upregulation of the normally less abundant isoform of C4BP that is composed exclusively of α-chains.[10] By measuring the α-chains, all forms of C4BP are detected and hence they are termed total C4BP ($C4BP_{tot}$). Accordingly, the expression of α- and β-chains has been shown to be differentially regulated by cytokines.[11] Recent investigations revealed that C4BP regulates complement also on apoptotic and necrotic cells.[12,13]

Due to the fact that CLI is an inflammatory disease and often associated with significant tissue necrosis, we hypothesized that complement is activated and that levels of C4BP are increased in CLI patients and correlate with the degree of tissue damage and with other biomarkers of the disease. Therefore, we have measured level of complement activation as well as C4BP plasma levels in CLI patients and correlated these values with a number of inflammatory markers.

METHODS

Participants. The Vascular Centre at Malmö University Hospital is the referral center for all patients with CLI in southern Sweden. During a 14-month period, 316 consecutive patients were referred to the centre with a confirmed diagnosis of CLI, and 259 consented to participate in the

present study. The study protocol conformed to the Declaration of Helsinki, and all participants gave written informed consent. The Lund University Regional Ethical Review Board approved the study.

The diagnosis of CLI was made in accordance with TransAtlantic Inter-Society Consensus scientific criteria[1,4] of ulceration, gangrene, or rest pain caused by peripheral arterial disease proven by ankle pressure (<50 to 70mm Hg), reduced toe pressure (<30 to 50mm Hg) or reduced transcutaneous oxygen tension (TC_{PO2}). Diagnosis was confirmed by an experienced vascular surgery consultant and by toe pressure measurements in those patients where the arteries in the affected leg were noncompressible and the ankle pressure was >50 to 70mm Hg.[1]

A population based control group of currently healthy individuals including 219 subjects of both sexes and without symptomatic cardiovascular disease or peripheral arterial atherosclerosis was also analyzed. These patients were a part of a follow-up program for the Preventive Medicine Project in Malmö.[14]

Patient variables. Gangrene was defined as visible tissue loss on clinical examination. Body mass index was calculated as weight in kilograms/height in m2. Serum levels of triglycerides and total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol were determined by a DAX 48 automatic analyzer. Plasma homocysteine, a risk factor for cardiovascular diseases, was analyzed by a high-performance liquid chromatographic assay and resistance to APC with a predilution of sample plasma in factor V-deficient plasma. All methods were previously described in detail.[15]

Blood sampling. EDTA-plasma was immediately prepared after taking the blood samples, kept cold until stored in a -70°C freezer within one hour.

Determination of terminal complement complex (TCC) formation. Deposition of TCCs on the solid phase was based on reaction with mAb aE11 specific for a neoepitope exposed when C9 is incorporated in the complex[16] followed by biotin-conjugated mAb 9C4-RQ-B directed against human C6[17] and HRP-conjugated streptavidin (Dako) with 1,2-phenylenediamine dihydrochloride (OPD, Dako) as substrate using absorbance values at 490 nm to represent the relative amount of bound TCCs in the wells.

ELISA for determination of C4BP concentrations. The plates were coated with 5 μg/mL rabbit antibody PK9008 (generated in house) overnight at 4°C in 75 mM sodium carbonate. After quenching for 1 h with 50 mM Tris-HCl, 0.15 M NaCl, 0.1 % Tween, pH 7.5 (washing buffer) supplemented with 3 % fish gelatin, the plates were washed four times and incubated for 1 h with the plasma. After another four washes 5 μg/mL of MK104 (for detection of $C4BP_{tot}$) and MK2B (for detection of $C4BP_{\beta}$) were added and incubated for 1 h. Goat anti-mouse antibody, conjugated with horseradish peroxidase (Dako), was added after another four washes and incubated for 1 h. After the last four washes, the plates were developed using OPD as substrate, and the absorbance at 490 nm was measured spectrophotometrically. The antibodies were a kind gift of prof. B. Dahlbäck (Lund University).

Analysis of inflammatory mediators. In order to determine the inflammatory immune status of the patients, the following acute phase reactants and inflammatory mediators were analyzed: the proinflammatory cytokines plasma tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were measured by ELISA using commercially available test kits (Pharmingen). Plasma neopterin, another indicator for a proinflammatory immune status, was determined by ELISA and the acute phase protein serum high-sensitivity C-reactive protein (hs-CRP) by rate turbidimetry. We measured the vasoconstricting peptide plasma endothelin-1 (ET-1), a marker for arterial hypertension, by radioimmunoassay and the prostaglandin-related inflammatory mediator plasma

8-iso-protaglandin $F_{2\alpha}$ by enzyme immunoassay. Detection limits as well as intra- and inter-assay coefficients were previously described.[15]

Statistics. All variables were recorded in a database, and statistical analyses and calculations were performed using JMP 7 (SAS Institute) and SPSS. Statistical significance was assessed using univariate analysis, whereby Spearman's rank correlation was performed for continuous variables and Mann-Whitney U-test for binary variables. All tests were non-parametric and two-tailed, and $P < .05$ was considered significant. In order to further specify the univariate results, a multiple regression analysis was used. Log-transformations of the outcome variables (C4BP or TCC) have been used in order to decrease the influence of outliers and in order to obtain regression residuals that are more symmetrically distributed. All variables with $p<0.30$ in the univariate analysis were entered in a multiple regression model. One variable was then omitted at a time, starting with the variable with the highest p-value and stopped when all remaining variables had $p < .10$. Using this method, variables that are highly significant at the 5% level and that reveal the strongest direct correlations to the outcome variables could have been determined. The multiple regression p-values are separately listed for the corresponding variables. If not otherwise stated, the results are presented as mean±standard error of mean/median (25^{th}-75^{th} percentile).

RESULTS

CLI patients show systemic complement activation and elevated C4BP plasma levels

Complement activation, defined by the presence of pathological levels of circulating TCCs (> 0.9 AU/ml), could be measured in the patient cohort with the highest levels in those with gangrene (Fig 1, A). The controls showed no complement activation. C4BP plasma levels were significantly higher in CLI patients compared to age-matched healthy controls (Fig 1, B). Further dividing the patient group, the ones suffering from gangrene showed significantly higher $C4BP_{tot}$ levels than those without (Fig 1, C). In accordance with that, significantly elevated levels of C4BP were found in patients who were considered for amputation (Table II). Neither TCC nor C4BP levels were significantly increased in patients with leg ulcers in comparison to patients without ulcers.

Gender and age correlation of C4BP

In the healthy control population, the concentration of C4BP was significantly higher in women than in men (310 μg/mL versus 278 μg/mL, $P = .002$ for $C4BP_{tot}$; 274 μg/mL versus 253 μg/mL, $P = .01$ for $C4BP_{\beta}$). However, no such difference could be demonstrated in CLI patients. Neither in patients nor in healthy controls, C4BP plasma levels correlated with age. The participant's demographic data are summarized in Table I.

Correlation between C4BP and plasma levels of inflammatory mediators

In patients, both $C4BP_{tot}$ and $C4BP_{\beta}$ were significantly correlated with IL-6 (Fig 2, A). However, no significant correlation was found with TNF-α (Fig 2, B), although levels of this proinflammatory cytokine were increased in patients [controls: 1.23±0.13/0.77 (0.55-1.22) pg/mL; patients: 2.21±0.22/1.55 (0.99-2.53) pg/mL]. The strongest correlation in patients was found between C4BP and hs-CRP levels (Fig 2, C; multiple regression p-value: $P < .0001$). As shown before, hs-CRP was significantly correlated with both proinflammatory cytokines, IL-6 and TNF-α.[15] Furthermore, the level of circulating TCCs was significantly correlated with IL-6

(P = .0431, r = .2425) and hs-CRP (P = .0045, r = .3483; multiple regression p-value: P = .027). Both forms of C4BP correlated significantly with leukocyte count (Fig 2, D), but not with 8-iso-protaglandin $F_{2\alpha}$ and neopterin.

Correlation between C4BP plasma levels and lipid metabolism

In the patients, positive correlations were observed between C4BP and triglyceride levels (Fig 3, A; multiple regression p-value: P < .001) as well as for C4BP and LDL/HDL ratio (Fig 3, B). In consistence with that C4BP correlated negatively with HDL (Fig 3, C). No correlation was determined with cholesterol levels. In the controls, C4BP correlated significantly with triglyceride levels (P_{tot} = .0002, r_{tot} = .2628; P_{β} = .0041, r_{β} = .2049) and cholesterol (P_{tot} = .0140, r_{tot} = .1758), but not with LDL/HDL ratio or HDL.

Correlations between C4BP plasma levels and other patient variables

Patients with pathologic levels of TCCs showed significantly increased platelet count (P = .0132, r = .2949). Furthermore, the platelet count correlated significantly with both forms of C4BP (P_{tot} = .0001, r_{tot} = .2714; P_{β} = .0001, r_{β} = .2693; multiple regression p-value: P = .015). Interestingly, the APC-resistant patients (n=24) showed significantly increased levels of TCCs [1.79±0.23/1.75 (1.41-2.35) AU/ml versus 1.09±0.08/1.0 (0.62-1.37) AU/ml, multiple regression p-value: P = .007], but no change in C4BP levels.

Only in patients, $C4BP_{tot}$ significantly correlated with BMI (P_{tot} = .0361, r_{tot} = .1525) and with patient weight (P_{tot} = .0410, r_{tot} = .1477).

C4BP was not correlated with ET-1, homocysteine, hemoglobin, creatinine or ankle pressure in the patients.

C4BP plasma levels and pharmacological therapy

The C4BP plasma levels were significantly increased in patients treated with antibiotics (Table II). Since the patients were treated with different kinds of antibiotics, the elevated C4BP level might be due to a general influence of these drugs on liver metabolism or it might be due to infections and following acute phase reactions. Both $C4BP_{tot}$ and $C4BP_{\beta}$ levels were significantly lower in patients treated with the anticoagulant warfarin or inhibitors of the renin-angiotensin system in comparison to patients without such treatments (Table II). The correlation of C4BP with the inhibitors of the renin-angiotensin system was not significant anymore after the multiple regression analysis and therefore it most likely involves an indirect effect. The following treatments did not affect the concentration of C4BP in plasma: anti-platelet drugs, β-blockers, diuretics, calcium channel blockers or lipid lowering drugs.

Influence of other/previous diseases and lifestyle on C4BP plasma levels

No significant differences in C4BP plasma level could be detected between patients with or without diabetes, previous stroke, angina pectoris or acute myocardial infarction. Furthermore, neither in patients nor in controls smoking influenced C4BP and C4BP was not correlated with one-year survival.

Summarizing the data, a multiple regression analysis revealed, that the C4BP levels are most strongly and directly correlated to hs-CRP and triglyceride levels as well as to platelet count and warfarin-treatment. The most important determinants correlated to TCC levels are hs-CRP levels and APC resistance. All correlations found with the univariate regression method are equally

statistically significant, but they might reflect indirect influences of the parameters via a third variable.

DISCUSSION

In this study, we demonstrated that patients suffering from CLI showed on-going complement activation, as determined by presence of pathological levels of circulating TCCs that are the final product of the complement cascade. Furthermore, the plasma level of the complement inhibitor C4BP was upregulated in these patients compared to healthy controls. Both levels of plasma TCCs and $C4BP_{tot}$ were highest in patients with gangrene.

CLI is an inflammatory disease, which is caused by reduced blood flow to the external limbs; this might lead to significant tissue necrosis especially when gangrene arises. In this context, complement can either be activated by CRP or directly by necrotic and apoptotic cells. According to the multiple regression analysis CRP was correlated strongest with C4BP and TCC, which indicates that it involves a direct relationship, whereas the correlation to gangrene might be indirect. However, the complement inhibitor C4BP appears to be upregulated by the organism when there is significant tissue necrosis. It binds damaged cells and prevents excessive inflammation evoked by these cells due to the fact that they also bind large amounts of C1q, the initiator of the classical complement pathway.[12,18] Therefore it should be beneficial to increase C4BP synthesis in states with pronounced tissue destruction as this still allows complement activation to the C3-level but prevents further inflammatory events such as the generation of anaphylatoxins. It is unclear, however, which mediators are responsible for this upregulation. Since there was no significant correlation between levels of C4BP and TCCs, it seems that the activation of complement itself does not initiate the upregulation of C4BP. Hence, the increase in C4BP might arise as result of general acute phase reaction.

The measurement of systemic levels of TCC and C4BP does not allow conclusions about local effects in the skin or muscles and it does not differentiate between increased consumption and decreased expression of the proteins, but it is most likely that both TCC and C4BP levels are much higher in necrotic tissue, since complement activation is known to take place in such areas and since C4BP is known to bind to dying cells. Plasma levels provide information about the balance between newly synthesized and consumed protein. It would also be interesting to measure the local concentrations of C4BP and TCC in specific organs of CLI patients. We plan to investigate this issue in the future.

A significant increase in C4BP was observed in patients who were considered for amputation. Due to atherosclerosis, insufficient oxygen supply often leads to tissue necrosis in the limbs. If there is no chance for tissue rescue, amputation is often the last possibility to protect the healthy limb parts. Since binding of C4BP to dying cells is important to prevent inflammation, it is reasonable that we found increased C4BP levels in these patients. In summary, TCC and C4BP plasma levels are related to the degree of tissue necrosis in CLI and their determination can give information about the disease extent.

C4BP is an acute phase protein and increases in concentration upon infection and inflammation.[19] Acute phase proteins are upregulated by IL-6, IL-1 and TNF-α, and these three cytokines have been suggested to increase synthesis of C4BP *in vitro*. We found that both $C4BP_{tot}$ and $C4BP_{\beta}$ are significantly correlated with IL-6 *in vivo*, which is consistent with earlier *in vitro* findings.[9] However, no significant correlation was found between C4BP and TNF-α, although levels of this proinflammatory cytokine were increased in the patients. This demonstrates that TNF-α is not a main inducer of C4BP synthesis *in vivo*, at least in CLI. These

results are in agreement with studies of C4BP secretion from HepG2 cells, where it was observed that IL-6 upregulates C4BP secretion more significantly than IL-1 and TNF-α.[9,20] Furthermore, TNF-α did not upregulate C4BP in Hep3B and counteracted effects of IL-6.[11] A relation between complement activation and IL-6 has been documented before. On the one hand, C5a induces an increase in IL-6[21] but IL-6 also plays a role in upregulation of the C5a receptor.[22]

The strongest correlation from the multiple regression analysis was obtained between C4BP levels and hs-CRP a major acute phase protein. A highly significant correlation between total C4BP (not distinguishing C4BP forms) and CRP with $r = .345$ has been previously reported,[19] while we found a correlation coefficient of $r = .3642$ for $C4BP_{tot}$. Another study reported an r-value of .567.[23] It is remarkable that levels of C4BP and CRP are so closely correlated. CRP binds C1 of the classical pathway, which leads to activation of complement. We found increased amounts of TCCs in patients with elevated CRP. This is in agreement with data showing that CRP frequently colocalizes with TCC in the intima of early atherosclerotic lesions of coronary arteries.[24] A certain level of complement activation is usually beneficial, as targets opsonized with CRP and following complement factors will be taken up by phagocytes. However, too much activation could lead to overt inflammation and tissue damage. Therefore, in order to control the level of complement activation, CRP interacts also with complement inhibitors C4BP[25] and factor H.[26] Strikingly, even though C4BP was only correlated with IL-6 and not with TNF-α, hs-CRP was, as expected, highly correlated with both proinflammatory cytokines. This indicates a specific interaction between the C4BP and IL-6 regulating pathways.

The positive correlations between both forms of C4BP and leukocyte count in patients are in agreement with an earlier study, showing that C4BP correlated with erythrocyte sedimentation rate ($r = .506$),[19] another marker of inflammation. Notably, C4BP was also correlated with platelet count in the patient group. This implies that there are functional correlations between C4BP and some aspects of platelet physiology, since activated platelets initiate complement cascades[27] and bind C4BP-protein S complex to exposed phosphatidylserine.[13] The significant correlation between platelet count and levels of TCCs was expected, since it has been shown before that C5b-9 enhances thrombin-induced platelet secretion.[28] Furthermore it is feasible that elevated levels of C4BP provide increased protection of platelets to counteract complement attack, finally leading to increased platelet count. APC-resistant patients showed higher levels of circulating TCCs and the multiple regression analysis revealed that it involves a direct correlation. Since complement activates platelets and platelets can activate complement, it is difficult to determine whether hypercoagulation leads to more complement activation or vice versa, but membrane assembly of C5b-9 markedly accelerates the rate of platelet-catalyzed plasma clotting,[29] suggesting a direct link between C5b-9-mediated prothrombinase activation and procoagulant activity accompanying immunologic damage to the platelet.

In agreement with previous findings,[19] we found correlations between C4BP and blood lipids both in patients and healthy controls. C4BP associates with chylomicrons, which perhaps would otherwise cause complement activation.[30] One study revealed significantly increased C4BP levels in patients suffering from aortic atherosclerosis and it has been concluded that C4BP serum level can serve as an independent indicator of aortic atherosclerosis.[31] Possibly, the determination of C4BP plasma levels in CLI patients could also be used as a predictor of the degree of atherosclerosis. Moreover, C4BP might be associated with lipid metabolism in other ways. However, one has to be careful in judging serum lipid levels in our study, since they may be falsely low in patients with CLI.[32]

In our study both $C4BP_{tot}$ and $C4BP_{\beta}$ levels were significantly lower in CLI patients treated with the anticoagulant warfarin in comparison to patients without such treatment. A diminution in C4BP plasma concentration was already found in patients with inherited protein S deficiency;[33] and the concentration of protein S itself is known to likewise decrease during warfarin therapy.[34] The molecular mechanisms for the correlation between decreased protein S and decreased C4BP levels during warfarin treatment still have to be elucidated, but our finding implies that the level of protein S influences the synthesis of $C4BP_{tot}$ and $C4BP_{\beta}$ by hepatocytes.
In conclusion, CLI patients showed elevated plasma levels of circulating TCCs and of the complement inhibitor C4BP. Both parameters were particularly high in patients with gangrene, most likely due to significant tissue necrosis. Thus, levels of C4BP and C5b-9 are related to the degree of tissue necrosis and reflect disease severity in CLI. Moreover, the found correlations with other biomarkers help to understand the pathophysiology of the disease and can facilitate the research for new diagnostic tools and clinical assays. The results of this study suggest that a treatment of CLI patients with emerging complement inhibitors might be beneficial to limit inflammation and improve the clinical outcome.

ACKNOWLEDGMENTS
The authors thank Barbro Palmqvist for technical assistance and Jonas Björk from the Department of Biostatistics (Competence Centre for Clinical Research) for his assistance with the statistical analyses.

SOURCES OF FUNDING
The authors acknowledge the financial support of Swedish Foundation for Strategic Research, Swedish Research Council, research grant from the University Hospital in Malmö and Foundations of Kock, Österlund, King Gustav V's 80th anniversary, Ernhold Lundström, Hulda Almroth and US Immunodeficiency Network. MM is a recipient of postdoctoral stipend from the Anna-Greta Crafoord foundation.

DISCLOSURES
The authors have no conflicting financial interests.

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LEGENDS

Figure 1. ***Systemic complement activation and elevated C4BP plasma levels in critical limb (CLI) ischemia patients.*** Panel A shows complement activation, determined by presence of circulating terminal complement complexes (TCCs), in CLI patients [1.17±0.06/1.13 (0.73-1.45) AU/ml; n = 70] in comparison to healthy controls [0.69±0.07/0.59 (0.37-0.88) AU/ml; n = 47] and in patients with [1.33±0.11/1.28 (1.07-1.70) AU/ml; n = 24] and without gangrene [1.1±0.08/1.0 (0.72-1.37) AU/ml; n = 48]. Panel B illustrates increased plasma levels of $C4BP_{tot}$ and $C4BP_{\beta}$ in CLI patients [351±8.1/322 (255-412) μg/mL for $C4BP_{tot}$; 314±7.0/287 (222-366) μg/mL for $C4BP_{\beta}$; n = 194] compared with healthy controls [297±8.0/288 (247-329) μg/mL for $C4BP_{tot}$; 265±7.0/263 (227-294) μg/mL for $C4BP_{\beta}$; n = 196). Panel C demonstrates $C4BP_{tot}$ and $C4BP_{\beta}$ plasma levels in CLI patients with [421±28.6/386 (296-469) μg/mL for $C4BP_{tot}$; 374±25.4/332 (255-458) μg/mL for $C4BP_{\beta}$; n = 24] and without gangrene [341±10.8/318 (244-403) μg/mL for $C4BP_{tot}$; 305±9.5/285 (220-363) μg/mL for $C4BP_{\beta}$; n = 170]. * denotes significant *P* value, evaluated using Mann-Whitney U-test, the horizontal line for each data set represents the median.

Figure 2. ***Correlation between C4BP and plasma levels of inflammatory mediators.*** Panel A illustrates the correlation of IL-6, panel B of TNF-α and panel C of hs-CRP plasma levels with $C4BP_{tot}$ and $C4BP_{\beta}$ plasma levels in CLI patients. Panel D demonstrates the correlation between leukocyte count and $C4BP_{tot}$ as well as $C4BP_{\beta}$ plasma levels. * denotes significant *P* value, *r* = Spearman's rank correlation coefficient.

Figure 3. ***Correlation between C4BP and lipid plasma levels.*** Panel A illustrates the correlation of triglyceride levels, panel B the correlation with LDL/HDL ratio and panel C the correlation of HDL levels with $C4BP_{tot}$ and $C4BP_{\beta}$ plasma levels in CLI patients. * denotes significant *P* value, *r* = Spearman's rank correlation coefficient.

Table I. Demographic data of the participants [mean±SEM/median (25^{th}-75^{th} percentile); n]

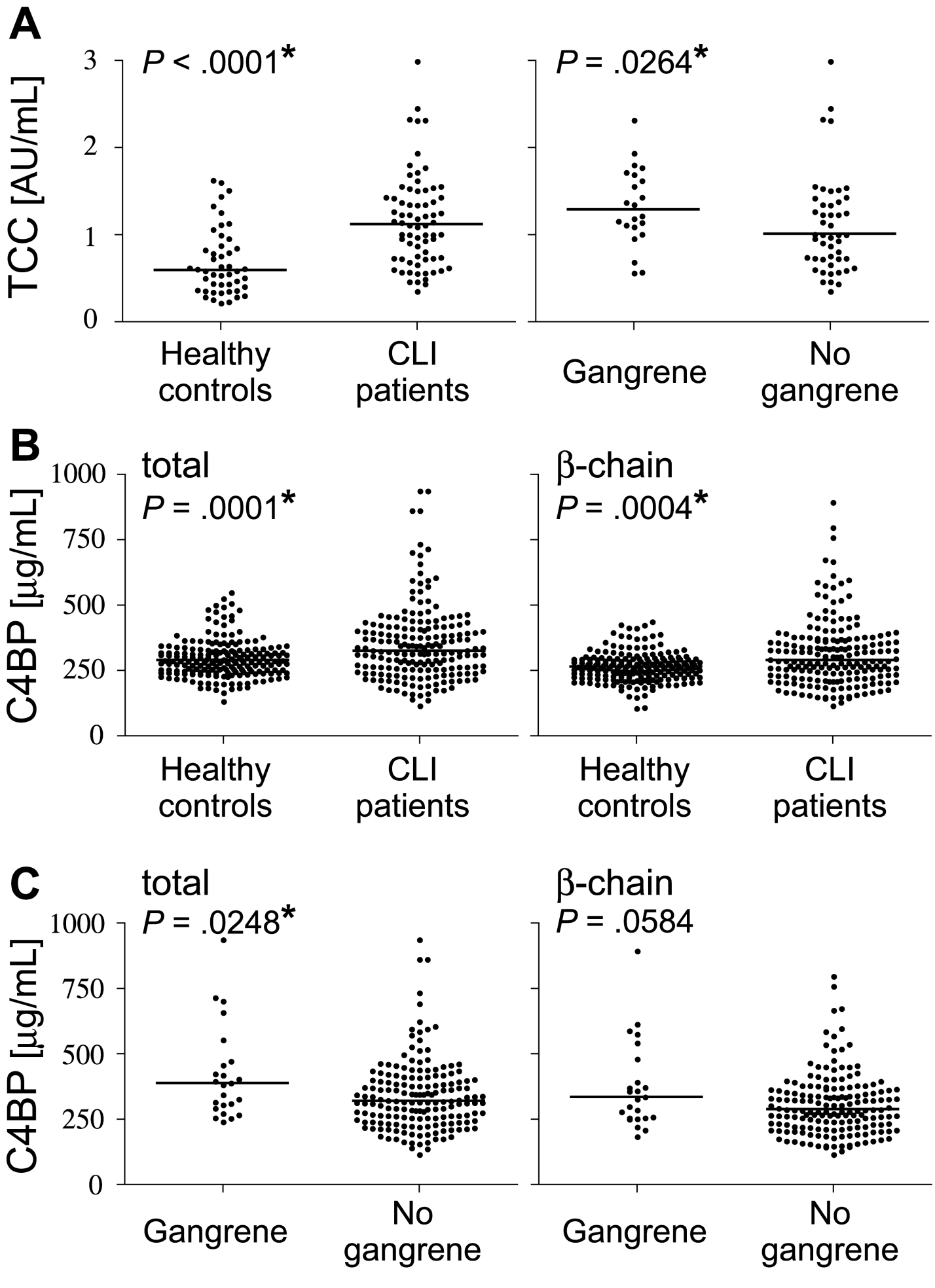
	CLI Patients	Controls
Age (years)	75.1±0.64/76 (68-82)	67.9±0.11/67 (67-68)
Female/Male	121/138	117/101

Table II. C4BP plasma concentrations in different subgroups of patients with critical limb ischemia.

	$C4BP_{tot}$ [μg/mL]	***P***	**$C4BP_{\beta}$** [μg/mL]	***P***
Amputation (n=24)	418±28.6/377 (285-508)	.0430*	383±25.3/340 (258-499)	.0240*
No amputation (n=170)	341±10.8/317 (247-403)		304±9.5/284 (221-361)	
Antibiotic-treated (n=47)	378±20.7/371 (267-458)	.0411*	338±18.3/335 (241-414)	.0496*
Not antibiotic-treated (n=147)	342±11.7/316 (250-394)		305±10.4/284 (221-354)	
Warfarin-treated (n=24)	258±28.2/241 (213-311)	< .0001* (<.0001)[#]	218±24.8/209 (178-250)	< .0001*
Not warfarin-treated (n=170)	364±10.6/338 (265-423)		327±9.3/308 (246-375)	
ACE inhibitor-treated (n=67)	319±17.2/289 (230-399)	.0219* (.084)[#]	285±15.2/263 (205-358)	.0210*
Not ACE inhibitor-treated (n=127)	368±12.5/339 (268-419)		329±11.1/303 (246-372)	

Significance of differences was determined using Mann-Whitney U-test, displayed are medians (25^{th}-75^{th} percentile), * denotes significant *P* value, [#] denotes multiple regression p-value.

Martin *et al.* Fig. 1



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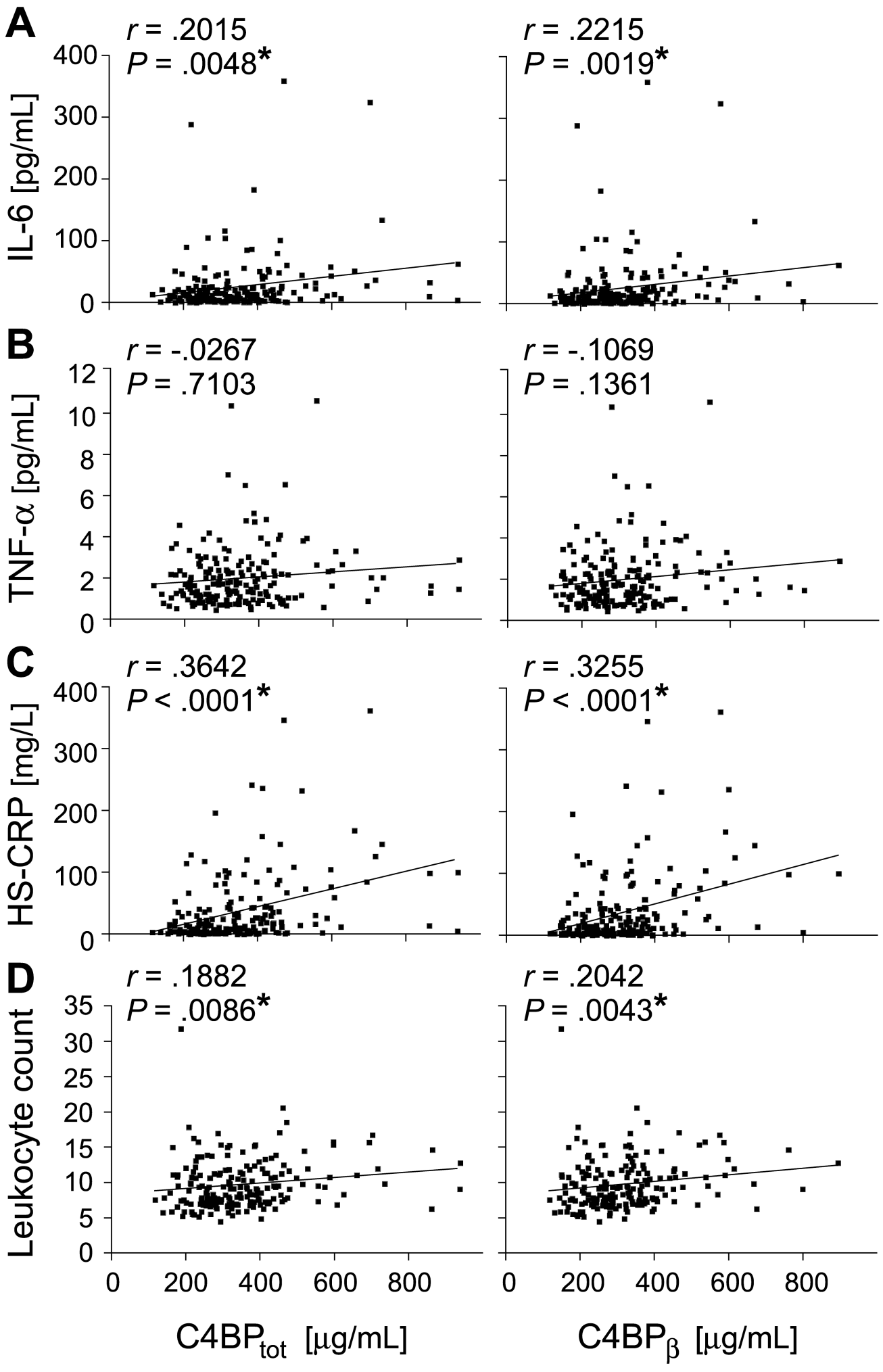

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