Microparticles expressing myeloperoxidase and complement C3a and C5a as markers of renal involvement in antineutrophil cytoplasmic antibody – associated vasculitis

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Abstract

Objectives: To investigate expression of terminal complement components C3a and C5a on circulating myeloperoxidase (MPO)-positive microparticles (MPO^+MPs) in relation to disease activity and renal involvement in patients with ANCA-associated vasculitis (AAV).

Methods: Forty-six clinically well-characterized patients with AAV and 23 age- and sex matched healthy controls were included. The concentration of MPO^+MPs expressing C3a and C5a was analysed from citrate plasma by flow cytometry. Serum-levels of C3a and C5a were determined using commercial ELISA. The assessment of vasculitis disease activity was performed using the Birmingham Vasculitis Activity Score (BVAS). Among patients, 23 had active disease with BVAS ≥ 2 and 14 patients had active renal flares.

Results: AAV patients had significantly increased expression of C3a and C5a on MPO^+MPs compared to controls ($p<0.0001$, respectively). When the group of patients with active AAV was divided according to the presence of renal activity, the concentration of MPO^+MPs expressing C3a and C5a was significantly higher in patients with renal involvement compared to patients with non-renal disease and controls ($p<0.05$, $p<0.01$, respectively). The serum levels of C3a were significantly decreased ($p<0.01$) in the renal subgroup, while there were no changes in serum levels of C5a comparing the renal and non-renal groups. There was significant correlation between the disease activity measured by BVAS and the levels C3a and C5a expressed on MPO^+MPs.

Conclusions: Determination of C3a and C5a on MPO^+MPs might be considered as a potential novel biomarker of renal involvement in patients with AAVs and may be of importance in the pathogenetic process.

Introduction

Anti-neutrophil cytoplasm antibody (ANCA) – associated vasculitis (AAV) is a heterogeneous group of multisystem disorders characterized by pauci-immune necrotizing vasculitis, affecting small- to medium-sized blood vessels, together with neutrophil or eosinophil-enriched granulomatous inflammation. It comprises 3 distinct systemic subsets, including microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), (formerly known as Wegener's granulomatosis) and eosinophilic granulomatosis with polyangiitis (EGPA), (previously Churg-Strauss syndrome) (1).

Although AAV is generally considered as a pauci-immune disease, there is a growing body of experimental and clinical observations indicating that complement activation is critical in the development of ANCA-mediated disease (2-4). In vitro studies could demonstrate that complement activation, especially via the alternative pathway, acts as positive feedback amplification of neutrophil activation, resulting in the aggressive necrotizing inflammation in AAV (5). Additionally, the levels of complement activation fragments (Bb, C3a, C5a, and soluble C5b–9) have been shown to be elevated in the plasma and urine of patients with active disease (6). Although C3 deposits are less prominent in the glomeruli of patients with AAV than in the glomeruli of patients with immune complex diseases, C3d deposits are detected in a high percentage of patients (7,8) and have been shown to correlate with poor outcome (7,8). The crucial evidence of the involvement of complement in AAV pathogenesis is the engagement of C5a receptors (CD88) on neutrophils by C5a, suggesting that blockade of this receptor might have therapeutic efficacy in AAV patients, as shown in a recent phase 2 clinical trial (9).

C3a and C5a are ~10kDa peptides that that exhibit delicate biological effects by interactions with their widely expressed high-specific receptors C3aR, C5aR1 (CD88) and C5aR2 (C5L2 or GPR 77) (10). The C5a molecule is primarily pro-inflammatory, including leukocyte

chemotaxis, neutrophil degranulation, histamine release by basophils and mast cells and oxidative bursts on macrophages (11), but is also prothrombotic by activating platelets, endothelial cells and inducing tissue factor expression (12). In the vasculitis model, the interaction between C5a and the C5aR1 receptor creates an amplification loop that is a central contributing factor in ANCA-mediated neutrophil activation (4). The role of C3a is more subtle, as reviewed by Coulthard and Woodruff (13). In the acute setting, C3a seems to prevent mobilization of neutrophils, limits their accumulation into tissues, thereby reducing the inflammatory response at the tissue level. Yet, in certain chronic disease models, such as rheumatoid arthritis, C3a clearly demonstrates proinflammatory activity and contributes to disease progression (14). This dual role of C3a is exerted not only by interaction with C3aR, but is reported to be dependent on the presence of C5aR2, suggesting that C5aR2 may contribute to C3a signaling through interactions with C3aR (15). The role of C3a in AAV seems to be less pronounced regarding neutrophil activation (4).

Microparticles (MPs) are membrane bound small vesicles, with diameters from 0.1-1 μm. When released from the cell membrane upon cell activation or during the apoptosis, MPs act as biological effectors in inflammation, angiogenesis, endothelial injury, and thrombosis (16). The property of carrying markers of the parental cell (MPs contain membrane, cytoplasmic and nuclear constituents characteristic of their precursor cell) enables detection of cellular origin, mainly platelets, but also red blood cells, leucocytes, or endothelial cells (17). Moreover, MPs are implicated in a variety of biological processes, such as intracellular communication and transport of surface proteins and thereby serve as markers of disease activity in variety of pathological processes (18). However, only few studies have previously elucidated the role of MPs in AAV (19-24).

Taking into consideration the putative role of complement activation in the pathogenesis of AAV, the aim of our study was to evaluate expression of C3a and C5a on MPO^{+}MPs, during active disease and remission, compared to healthy control subjects.

Patients and Methods

A group of 46 ANCA-positive patients with AAV, either GPA or MPA, were included. These patients, recruited from the Departments of Nephrology and Rheumatology at Karolinska University Hospital, were diagnosed according to the validated "EMEA algorithm" for epidemiological studies of AAV (25). The assessment of vasculitis disease activity was performed using the Birmingham Vasculitis Activity Score (BVAS, version 2003), according to the European League Against Rheumatism (EULAR) recommendations (26). Patients with a BVAS of 0 were considered to be in remission. Renal involvement was defined as pathological changes on a recent renal biopsy (seen in 14 patients with active disease, renal biopsy performed in 13 cases) and/or by the presence of significant haematuria and/or elevated creatinine values (see Table 1).

Control samples were obtained from 23 healthy age/gender matched subjects. The local ethics committee approved the study protocol (Dnr: 2008/1143-31) and informed consent for publication of study results was obtained from each subject.

Blood sampling:

Peripheral venous blood was collected into Vacutainer tubes (Becton Dickinson) containing clot activator or trisodium citrate (0.129 mol/L, pH 7.4) (1 part trisodium citrate and 9 parts blood). Serum, respectively platelet poor plasma obtained within 60 minutes of sampling by centrifugation at 2000 × g for 20 minutes at room temperature, then divided into aliquots and stored frozen at -70 °C.

Detection of microparticles using flow cytometry:

Platelet poor plasma was thawed in a water bath at 37°C for approximately 5 minutes, followed by centrifugation of samples at 2 000*g* for 20 minutes at room temperature, in order to remove any cells or debris that may interfere with the analysis. The supernatant was centrifuged again at 13 000*g* for 2 min in RT. Twenty µl of the supernatant was incubated in dark for 20 minutes with 5µl of monoclonal antibodies, anti-MPO-PE (Beckman Coulter, Brea, CA, USA) together with anti-C3a-FITC and anti-C5a-Dylite 755 (Thermo Fisher Scientific Inc., Waltham, MS, USA). After incubation, samples are fixed prior to analysis (Cellfix, BD, NJ, USA). Measurement of MPs was performed by flow cytometry on a Beckman Gallios instrument (Beckman Coulter, Brea, CA, USA). The MP gate was determined using Megamix beads (0.3–1.0µm, BioCytex, Marseille). MPO^+MPs were defined as particles < 1µm in size. Conjugate isotype-matched immunoglobulins with no reactivity against human antigens were used as negative controls. In the present study, results are shown as numbers of MPO^+MPs events measured in 30 seconds analysis. The intra- and inter-assay coefficients of variation for NMP measurement were less than 9%, respectively.

Serological markers:

ANCA were detected by standard multiplex assay (BIO-RAD, BioPlex TM 2200) according to clinical routine at the Karolinska University Hospital.

Complement factor C3 in serum was analyzed according to clinical routine using turbidimetry at the Department of Immunology at the Karolinska University Hospital. The normal range was 0.77-1.62 g/L.

Detection of C3a: Serum levels of C3a were measured using MicroVue C3a Enzyme Immunoassay, Quidel Corporation, San Diego, CA, USA. This is a three-step procedure

utilizing (1) a microassay plate coated with a murinemonoclonal antibody specific for a neo-epitope on human C3a, (2) an HRP-conjugated polyclonal antibody to the C3a region of C3, and (3) a chromogenic substrate. The color intensity of the reaction mixture is proportional to the concentration of C3a present in the Standards, Controls and diluted test specimens. Results are calculated from the generated standard curve using 4-parameter analysis and expressed as ng/mL. The inter-assay CV is 5.3% and intra-assay CV is 8.3%.

Detection of C5a: Serum levels of C5a were measured using MicroVue C5a Enzyme Immunoassay, Quidel Corporation, San Diego, CA, USA. This is a direct-capture immunoassay for the measurement of C5a in human serum, plasma, and other biological or experimental samples. A three-step procedure is performed utilizing (1) a microassay plate coated with murine monoclonal antibody specific for a neo-epitope on human C5a, (2) an HRP conjugated murine monoclonal antibody to the C5a region of C5, and (3) a chromogenic substrate. The color intensity of the reaction mixture is proportional to the concentration of C5a present in the diluted test specimens, Standards, and Controls. Results are calculated from the generated standard curve using linear regression analysis and expressed as ng/mL. The inter-assay CV is 3.8% and intra-assay CV is 7.8%.

Routine laboratory analyses were carried out using standard methods at the Karolinska University Hospital, included C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and plasma creatinine levels. The Cockcroft-Gault formula was used to estimate creatinine clearance (estimated glomerular filtration rate, eGFR) (27).

Statistical analysis

Data were analysed using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). Descriptive statistics were used for presentation of patient characteristics. For

continuous variables, means and standard deviations or medians with ranges were used, whereas categorical variables were presented as percentages. For comparison of more than two groups of individuals, one-way ANOVA was used. A p-value <0,05 was regarded as statistically significant. Correlation between variables was examined using Pearson and Spearman correlation analysis, depending on data type and distribution.

RESULTS

General patient data

Detailed characteristics of the patients and controls, including age, sex, diagnosis, ANCA antibody type, disease activity score (BVAS) and renal function, are listed in Table 1. There was no difference between active and inactive patients regarding these characteristics although creatinine levels were significantly increased and eGFR levels significantly decreased in both groups of AAV-patients compared to controls, as demonstrated in Table 1.

All active patients had recent onset of disease prior to the inclusion in the study. The median time from diagnosis to inclusion and blood sampling was 5 days (range 0 - 287 days). The inactive patients had a median disease duration of 5.3 years (range 1.2 – 12 years).

Twenty-three patients had active disease defined as a BVAS greater than 0 (mean BVAS 14.0±8.1). Table 2 demonstrates clinical and laboratory variables of active, respectively inactive AAV patients. Data regarding the treatment in respective groups of patients is also shown in Table 2.

C3a and C5a positive neutrophil-microparticles

The concentration of MPO^+MPs was significantly higher in AAV-patients compared to controls, without significant differences between the subgroups of active, respectively inactive patients (Table 3, Fig 1a).

The concentration of MPO^+MPs expressing C3a was significantly higher in AAV-patients compared to controls (Table 3), while no difference was found between the active and inactive patients. However, when the group of patients with active AAV was divided according to the presence of active renal flare or non-renal disease, the concentration of MPO^+MPs expressing C3a was significantly higher in the renal group of patients compared to the non-renal group as well as inactive AAV patients and controls (Figure 1b).

As demonstrated in Table 3, the same pattern for MPO^+MPs expressing C5a was observed regarding the distribution between AAV patients and compared to controls. Figure 1c is presenting significantly increased levels of MPO^+MPs expressing C5a in patients with renal flare compared to non-renal active AAV patients.

Serum levels of C3a (Table 3) was lower in patients with active disease compared to inactive, as well as to controls ($p=0.06$, $p=0.07$ respectively) but the significance was only reached when dividing the groups of active patients into those with renal and non-renal disease (Figure 1d). This was not the case regarding serum C5a levels, neither in the whole group of patients or in the subgroup of patients with active renal flare. Accordingly, the inverse correlation between the serum levels and expression on MPO^+MPs existed only in active AAV patients and C3a levels ($p =0.01$, $r=-0.52$).

Serum levels of complement factor C3 did not differ between patients and controls (1.42±0.34 vs 1.54±0.30), neither between patients with active respectively inactive patients with AAV (1.43±0.38 vs 1.42±0.30 g/L).

A correlation between the levels of C3 and C3a in serum or MPO^+MPs expressing C3a was not observed.

There were no differences found in the MPO^+MPs expressing C3a and C5a or serum levels of C3a and C5a between MPO- and PR3- ANCA positive patients.

In the group of active patients, 5 out of 23 patients were sampled before the treatment with corticosteroids was stared. The results of C3a and C5a expression on MPO^+MPs in these patients were not different as compared to the rest of the patients in the active group. There was no correlation between the prednisolone-dose and levels of C3a and C5a expressed on MPO^+MPs in the group of active patients.

In the group of inactive patients, 8 out of 23 patients were not treated with Prednisolone at the time of blood sampling. The levels of investigated parameters did not differ between patients with ongoing Prednisolone treatment and those without treatment in this group either.

There was no correlation between age of the patients, ESR and CRP at the time of blood sampling, renal dysfunction measured by creatinine or eGFR levels, and the levels of the investigated markers on MPO^+MPs or in serum observing the whole group of patients. However, a significant correlation was observed between creatinine levels and MPO^+MPs expressing C3a ($p<0.001$, $r=0.57$), whereas this correlation was not found for MPO^+MPs expressing C5a. Highly significant correlation between disease activity measured by BVAS and levels of C3a as well as C5a expressed on MPO^+MPs (Figure 2) was observed. Regarding the serum markers, only C3a was inversely correlated to BVAS ($p=0.03$, $r=-0.44$).

Representative flow cytometry plots of MPs expressing MPO together with C3a or C5a are presented on Figure 3.

Discussion

To the best of our knowledge, this is the first study to assess expression of terminal complement components C3a and C5a on MPO^+MPs in patients with AAV, stipulating the role of C3a and C5a in the pathogenesis of this disease. We have observed strong correlation between the disease activity measured by BVAS and levels of C3a as well as C5a expressed on MPO^+MPs. Moreover, the expression of C3a and C5a was significantly increased in AAV-patients with ongoing renal involvement, suggesting that these highly pro-inflammatory split products of the complement cascade might be a novel biomarker of renal flare in patients with AAV.

Previous studies have provided evidence that human neutrophil-derived MPs contain active myeloperoxidase (MPO), suggesting that they may activate endothelial cells and give rise to lesions present in vasculitis (22,23). MPO is an enzyme primarily found in granules of neutrophils but it is also expressed in monocytes, although in lower concentrations (28,29). As such, we believe that the majority of the MPO^+ MPs in the present study originates from neutrophils. Hong and colleagues (22) showed that neutrophils primed with TNFα and stimulated with ANCA-antibodies release MPs that activate endothelial cells by a reactive oxygen species (ROS)-dependent mechanism. Further, in vitro studies by Pitanga *et al.* confirmed that MPs released upon neutrophil activation are capable of causing injury to vascular endothelial cells (24). Recent studies provide evidence on tissue factor expression (TF) on NMPs, upon activation of neutrophils by ANCAs, indicating a mechanism for hypercoagulability in AAV (30,31). Although the presence of bound complement components C1q, C3b and C4b on cell-derived MPs previously were investigated in patients with other autoimmune disorders like systemic lupus erythematosus and rheumatoid arthritis (32-34), this has not been the case in patients with AAVs.

Investigation of complement activation in AAVs has been focused on C5a ever since Schreiber et al. (4) demonstrated that supernatants from ANCA-activated neutrophils can trigger the

complement system via the alternative pathway, resulting in the production of C3a and C5a. C5a could further prime neutrophils for ANCA-induced activation, and only blocking of the C5a receptor on neutrophils could block this process. Thereby, the authors could conclude that generation of C5a but not C3a upregulates the membrane expression of ANCA antigens and primes neutrophils for a subsequent ANCA-induced respiratory burst. In the complement cascade, C3 is upstream of C5 and conceivably, C3 is needed for C5a generation as opposed to a direct role in ANCA-induced neutrophil activation. Several experimental data in animal models and observations in patients with AAVs verified the role of C5a in the pathogenesis of ANCA-driven diseases, not only by amplification of neutrophil activation but also by attracting more neutrophils at the site of inflammation (2-6).

Gou et al. have described increased plasma levels of C3a, C5a, soluble C5b-9 and Bb in patients with active AAV compared with inactive disease (35), investigating a cohort with advanced renal involvement in AAV. Furthermore, novel findings indicate that C3d-positive glomerular staining is an independent risk factor for the development of end-stage renal disease in ANCA-associated renal vasculitis (7). Our study could not demonstrate significant differences in serum C3a and C5a levels between AAV-patients and controls. Compared to the study by Gou et al. including patients with advanced impairment of renal function due to AAV (35), we have studied a group of patients with early renal involvement and mainly preserved renal function, even in the group of patients with active AAV. The sample size in the present study may also be a contributing factor to the discrepancies, as well as eventual artefacts due to handling of the samples knowing to influence complement activation in vitro, and we can in the present setting not confirm the findings of Gou et al. However, our results of MPO^{+}MPs expressing C3a and C5a revealed clear differences and could be a more sensitive method for detection of C3a and C5a, also discriminating between renal and non-renal involvement. Serum levels of C5a remained similar even in the group of patients with active renal flare, while C3a was decreased

in these patients. The inverse relation between the levels of C3a in serum and those expressed on MPO^+MPs could be demonstrated in patients with active disease while this was not the case for C5a. Rather than existing in the soluble form in serum, we might speculate that C3a and C5a as acute pro-inflammatory split-products of the complement cascade became available on the surface of circulating MPs upon the activation of neutrophils. Thereby, these terminal complement components may be involved in the further enhancement of neutrophil activation. Previous immunohistochemical examinations of renal biopsy specimens from patients with AAVs indeed revealed Bb, C3d, and C5b-9 in glomeruli and in small arteries (36). The focal accumulation of complement activation components at sites of glomerular and vascular inflammation and necrosis in ANCA glomerulonephritis differs from the more evenly distributed immunoglobulin and complement components in immune complex mediated glomerulonephritis (36). These findings point towards complement activation at localized sites of inflammation and necrosis in ANCA-associated disease.

To conclude, our study on MPO^+ microparticles in AAV patients with varying disease phenotype and activity demonstrates that MPO^+ microparticles expressing C3a and C5a are potential novel markers of disease activity in AAV, in particular in renal disease.

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Table 1. Patients and controls general features

	Total AAV	Active AAV	AAV in remission	Healthy controls
Patient characteristics				
Subject number	46	23	23	23
Gender (M/F)	25/21	12/11	13/10	12/11
Age at sampling (mean±SD)	62.5±13.3	61.3±14.0	63.8±12.8	66±9.5
Disease characteristics				
MPA (%)	23 (50.0)	13 (56.5)	10 (43.5)	/
GPA (%)	23 (50.0)	10 (43.5)	13 (56.5)	/
MPO-ANCA positivity ever (%)	26 (54.1)ǂ	13 (54.2)	13 (54.2)	/
PR3-ANCA positivity ever (%)	22 (45.9)ǂ	11 (45.8)	11 (45.8)	/
Creatinine (mean±SD)	125.2±73.4*	145.4±94.2**	105.0±35.9*	75.5±12.4
Creatinine clearance/eGFR (mean±SD)	62.8 ±28.4**	60.7±32.9**	64.8±23.6**	85.3±16.2
BVAS (median (range) and mean±SD)	1.5 (0-31)	14.0±8.1	0	/

ǂ Two patients were double positive for PR3-ANCA and MPO-ANCA

BVAS ≥ 1 is defined as active disease; * $p<0.01$; ** $p<0.001$

Table 2. Clinical manifestations and treatment of patients with AAV

	Active AAV n=23	Inactive AAV n=23	*p* value
CRP	14.8 ± 16.9	4.6±5.3	<0.001
ESR	37.5 ± 28.8	19.9±14.4	<0.01
MPO-ANCA (median (range))	n=13; 81 (0-800)	n=9; 7.9 (0-150)	ns
PR3-ANCA (median (range))	n=10; 40.9 (0-800)	n=6; 1.5 (0-12)	ns
Organ involvement; ongoing / previous (%)			
General (arthralgia/arthritis)	4 (17.4%)	0 / 5 (21.7%)	
Skin	7 (30.4%)	0 / 3 (13.0%)	
ENT (ear, nose, throat)	6 (26.1%)	0 / 12 (52.2%)	
Gastrointestinal tract	2 (8.6%)	0 / 0 (0%)	
Lung	8 (34.8%)	0 / 10 (43.5%)	
Renal	13 (56.5%)	0 / 19 (82.6%)	
Peripheral nerves	1 (4.3%)	0 / 2 (8.6%)	
Treatment			
Prednisolone mg/day (range)	30.0 (0-75.0)	5 (0-20)	
Methotrexate n (%)	3 (13%)	4 (17.4%)	
Azathioprine n, (%)	1 (4.3%)	6 (26.1%)	
Mycophenolate mofetil n (%)	5 (21.7%)	1 (4.3%)	
Cyclophosphamide n (%)	5 (21.7%)	0 (0%)	
No immunosupressive treatment n (%)	9 (38.7%)	7 (30.1%)	

Abbreviations: CRP- C-reactive protein, ESR - Erythrocyte Sedimentation Rate

Table 3. Serum levels of C3a and C5a and the concentration of MPO^+microparticles; C3a positive and C5a positive MPO^+microparticles in plasma of patients with AAV as well as controls.

	Total AAV n=46	Active AAV n=23	Inactive AAV n=23	Controls n=23
MPO^+MPs	190.8 ± 28.4	225.7 ± 50.1	154.4 ± 24.1	46.6 ± 23.2
$C3a^+MPO^+$MPs (mean±SD)	110.8 ± 49.7**	120.2 ± 51.3***‡	101.4 ± 47.3*	62.2 ± 32.1
C3a serum (ng/mL; mean±SD)	1887 ± 744.1	1738 ± 658.8	2036 ± 807.6	2066 ± 837.9
$C5a^+MPO^+$MPs (mean±SD)	83.7 ± 71.8**	92.7.4 ± 57.6**‡	74.7 ± 44.7**	29.9 ± 15.0
C5a serum (ng/mL; mean±SD)	22.6 ± 10.8	23.3 ± 10.5	21.9 ± 11.3	20.7 ± 7.8

Patients vs controls: *p<0.05; **p<0.01; ***p<0.001

Figure 1. Concentration of MPO$^+$ microparticles in patients with active, respectively inactive AAV-patiens and controls (a). C3a- and C5a positive MPO$^+$ microparticles in patients with AAV (active, with renal flare and inactive) and controls (b and c). Levels of C3a in serum in patients with AAV (active, with renal flare and inactive) and controls (d).

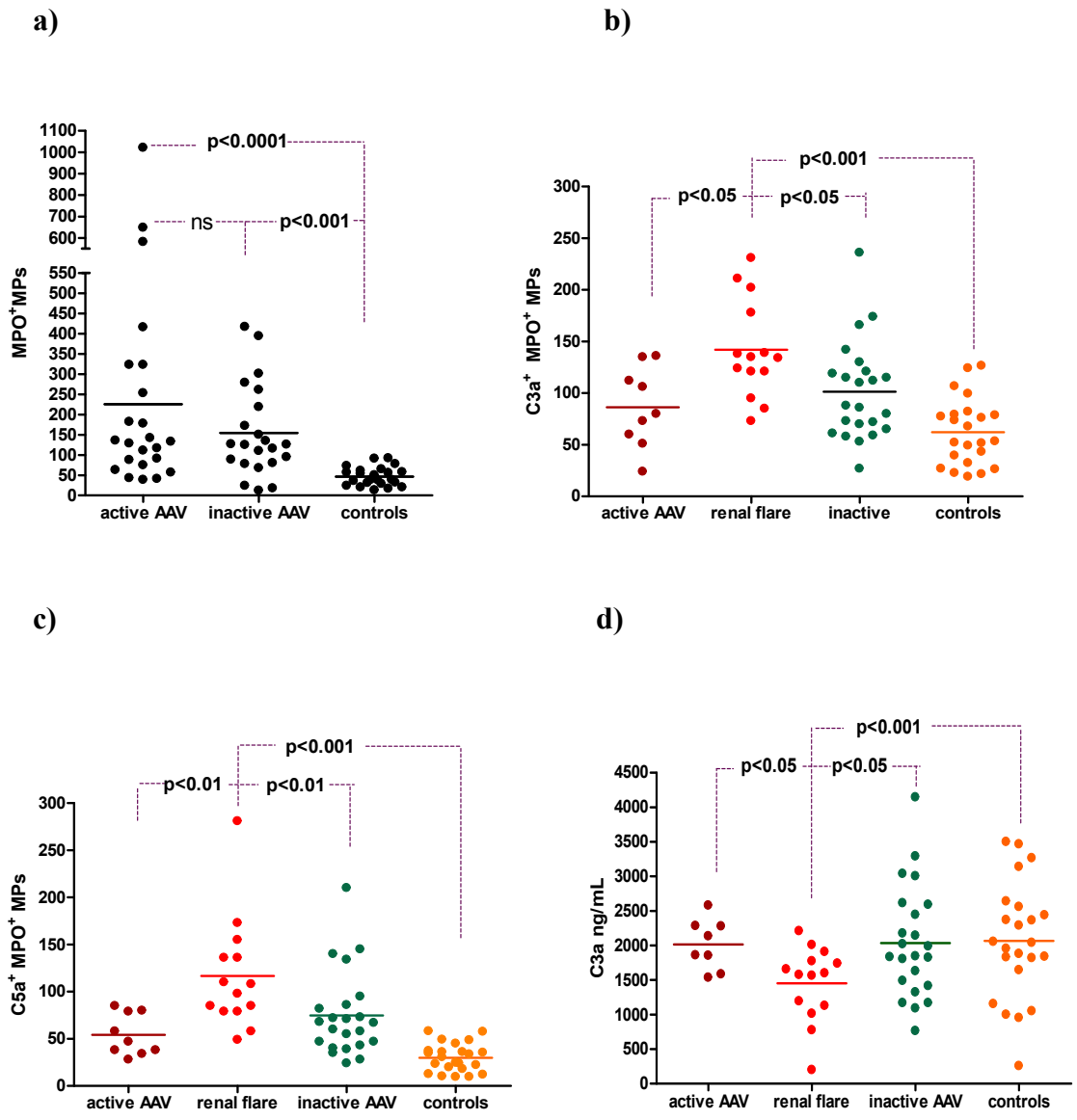

Figure 2. Correlation between disease activity measured by BVAS and levels of C3a and C5a expressed on MPO-positive microparticles.

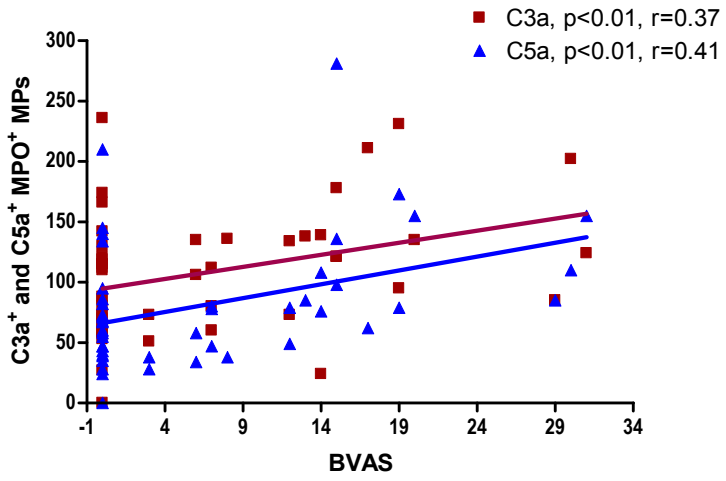

Figure 3. Representative Flow Cytometry plots of MPs expressing MPO together with C3a or C5a. Microparticles are measured by flow cytometry and defined as particles < 1µm in size. Moreover, the MPs were phenotyped based on MPO and C3a and C5a expression. A) MPs in the MP-gate in a patient and control sample. B) MPs in a patient sample co-expressing MPO together with C3a and/or C5a. C) MPs in a control sample co-expressing MPO together with C3a and/or C5a.