Immune complex-mediated neutrophil activation in patients with polymyalgia rheumatica

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Abstract

Objective: Neutrophils are important in host defense. However, neutrophils are also linked to inflammation and organ damage. The purpose of this study was to assess whether markers of neutrophil activation are increased in polymyalgia rheumatica.

Methods: Levels of immune complexes (IC), calprotectin, and neutrophil extracellular traps (NETs) were measured in plasma of healthy individuals (n=30) and patients with PMR (n=60), at flare and upon treatment with glucocorticoids using ELISA. Plasma-mediated neutrophil activation was assessed in presence of an FcyRIIA inhibitory antibody (IV.3).

Results: Plasma levels of calprotectin and NETs were elevated in PMR (p<0.001). Mechanistically, neutrophil activation was driven by ICs, present in plasma, able to up-regulate neutrophil activation markers CD66b and CD11b (p<0.0001) in an FcyRIIA-dependent manner (p<0.01). Of note, circulating levels of IC correlated with plasma induced CD66b and CD11b (r=0.51, p=0.004, and r=0.46, p=0.01 respectively) and decreased after glucocorticoid therapy.

In contrast to NETs, calprotectin significantly decreased after glucocorticoid therapy (p<0.001) and was higher in PMR without overlapping giant cell arteritis (GCA) compared to patients with overlapping disease (p=0.014). Interestingly, musculoskeletal involvement was associated with elevated levels of calprotectin before initiation of glucocorticoid therapy (p=0.036).
Conclusions: Neutrophil activation, including NET formation, is increased in PMR, through IC-mediated engagement of FcγRIIA. Clinically, neutrophil activation is associated with musculoskeletal involvement, with calprotectin, but not NETs, being a biomarker of treatment response in PMR patients. In all, IC-mediated neutrophil activation is a central process in PMR pathogenesis identifying potential novel therapeutic targets (FcγRIIA), as well as soluble markers for disease monitoring (calprotectin).

Key words: neutrophils, polymyalgia rheumatica, calprotectin, neutrophil extracellular traps, immune-complexes

Rheumatology key messages

- Neutrophil activation and neutrophil extracellular trap formation is increased in patients with polymyalgia rheumatica (PMR)
- Circulating immune complexes activate neutrophils through FcγRIIA in PMR patients
- Inhibition of FcγRIIA signaling could be a novel therapeutic intervention for PMR
Introduction

Polymyalgia rheumatica (PMR) is a chronic inflammatory disease of unknown etiology that primarily affects elderly women (1). Aging of the immune system plays a key role in its pathophysiology (2). PMR is often manifested with pain and stiffness in neck, shoulder and pelvic girdles and associated with elevated inflammatory markers at the onset of the disease (3). Constitutional symptoms such as fever and weight loss are some of the clinical manifestations of PMR. Recent studies have suggested a role for neutrophils in PMR, with neutrophil to lymphocyte ratio (NLR) being associated with inflammation in these patients (4). Further, serum levels of calprotectin (also known as S100A8/A9 or myeloid related protein 8/14 (MRP8/14)), a calcium-binding protein released by activated neutrophils (5), are elevated in patients with PMR and giant cell arteritis (GCA) and associated with acute phase reactants including ESR (6). However, given the challenges with measuring calprotectin in serum, i.e. spontaneous release of calprotectin upon sample processing (7), it is important to determine whether neutrophil activation occurs in PMR by assessing calprotectin levels in plasma.

When activated, neutrophils can also release neutrophil extracellular traps (NETs), a meshwork of chromatin decorated with citrullinated histones, and granule-derived enzymes, such as proteinase 3 (PR3), neutrophil elastase (NE), calprotectin, myeloperoxidase (MPO) and LL37 (8). Increased levels of NETs have been reported in several rheumatologic conditions, including rheumatoid arthritis (RA) (9), systemic lupus erythematosus (SLE) (10), systemic sclerosis (11) and vasculitis (12). Upon NET formation, several key auto-antigens, including dsDNA,
MPO, and PR3 are exposed on the NETs (13). NETs are prominent inducers of inflammation, signaling through DNA sensing TLR9 as well as the cGAS-STING pathway (10). However, the role of NET formation in PMR has not been carefully addressed.

In this study, we investigated whether patients with PMR have elevated levels of calprotectin and NETs in their plasma. We also assessed whether levels of calprotectin and NETs changed significantly before and after glucocorticoid therapy in patients with PMR. Finally, we investigated mechanisms through which neutrophil activation may occur in PMR, with an emphasis on immune complex-mediated activation through engagement of neutrophil FcγRIIA.

Materials and Methods

Patient characteristics

Patients with PMR (n=60) were recruited at time-point of active disease and were followed-up at remission at Umea University Hospital, Sweden. Demographic data, including sex, age at diagnosis, symptom duration before diagnosis, and prednisolone dose were recorded (Table 1). For all patients, laboratory data such as markers of systemic inflammation (CRP and ESR) were collected. For the PMR patients, EDTA plasma was used. At inclusion the concentration of total immunoglobulin G (IgG) levels in their plasma was also analyzed and the mean (SD) levels were found to be 11.9 (3.4) g/L (Table 1). In the general population,
the mean (SD) serum IgG levels have been reported to be 11.2 (2.5) g/L (14). Additionally, for patients with PMR, overlap with GCA was noted. EDTA plasma from healthy individuals (HC, n=30), were collected through the University of Washington, Seattle, USA. The study was approved by the appropriate local institutional review boards at University of Washington, Seattle, WA (#3100) and the Ethic Committee at Umea University (§192/96, dnr 96-138), and informed consent was obtained from all participants in accordance with the Helsinki Declaration.

**ELISAs**

Plasma levels of calprotectin (R&D Systems, Minneapolis MN, USA), and immune complexes (ICs) (MicroVue CIC-C1q EIA, Quidel, Athens OH, USA) were measured by ELISA, following the manufacturer’s instructions. The IC ELISA is based on the capacity of complement factor C1q, immobilized to the plate, to bind to circulating ICs. Quantification of circulating NETs was performed by utilizing myeloperoxidase (MPO)-DNA ELISA as described by us (10). At first, 96-well microtiter plates (Corning) were coated with anti-MPO antibody (4 μg/ml; Bio-Rad Laboratories, Hercules, CA, USA) overnight at 4°C, and then blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 hours at room temperature (RT). Then, plasma samples diluted 1:100 (MPO-DNA ELISA), were added in 1% BSA in PBS with 2 mM EDTA, and incubated overnight at 4°C. Anti-DNA-HRP from Cell Death Detection ELISA kit (clone MCA-33; Roche) was added as detection antibody for 2 hours at RT. The reaction was developed with
3,3',5,5' tetramethylbenzidine (TMB; BD Biosciences) for 20 min and stopped by the addition of 2N sulfuric acid. Known concentrations of MPO-DNA complexes (rhMPO, R&D Systems; Calf thymus DNA, Trevigon) were utilized to construct a standard curve. Absorbance was measured by a plate reader at 450 nm (Synergy 2, BioTek).

**Neutrophil isolation**

Heparinized blood from healthy individuals was layered on Polymorphpre (Axis- Shield, Dundee, UK) density gradient, according to the manufacturer’s instructions, or as described previously (15-17). Red blood cells were then lysed using RBC lysis buffer (BioLegend, San Diego, CA, USA). Neutrophils were re-suspended in serum-free RPMI-1640 medium (Life Technologies, Waltham, MA) for *in vitro* assays.

**Neutrophil activation assay**

To assess neutrophil activation, neutrophils at a concentration of 3 x 10^5 cells/well, were incubated in the presence or absence of the human FcγRIIA blocking antibody (IV.3) (5 μg/ml; Caprico Biotechnologies, Norcross, GA USA) for 30 min before addition of stimuli, such as R848 (3.5 μg/ml), or plasma samples from patients with PMR (n=8) and healthy controls (n=8) (1:50 dilution) for an additional 120 min. Approximately 90-95% of neutrophils were viable after neutrophil stimulation with plasma samples. To assess neutrophil activation, neutrophils were labeled with PE-conjugated anti-CD66b (clone G10F5, BioLegend, San Diego, CA,
USA) and APC-conjugated anti-CD11b antibodies (clone CBRM1/5, BioLegend, San Diego, CA, USA) and analyzed by flow cytometry (CytoFlex S, Beckman Coulter). Data were analyzed by FlowJo (version 10.6.2, Tree Star Inc, Ashland, OR), and results were presented as mean fluorescent intensity (MFI) of CD66b and CD11b.

Statistics
For statistical analyses, Mann-Whitney U test, Wilcoxon signed-rank test, and Spearman's correlation were appropriately applied. Comparisons of the levels of neutrophil activation markers between cases and controls were performed using linear regression modelling adjusting for age and sex. All analyses were performed in GraphPad and SPSS software (v. 27.0 IBM Corp, USA) and considered statistically significant at p < 0.05.

Results
Demographic characteristics of the study population
The mean (SD) age of the patients with PMR at the time of diagnosis was 73 (8) years old. 73.3% of the patients were female. The mean symptom duration before the diagnosis of PMR was 2 months. The mean (SD) daily dose of prednisolone at start was 22 (7) mg. 23.3% of the patients with PMR had an overlap with GCA. The mean (SD) ESR was 60 (23) mm/hr whereas the mean CRP was 48 (37) mg/L before the initiation of glucocorticoids (Table 1). The mean age of the PMR patients were significantly higher compared to controls (Table 1). Further, the levels of
MPO-DNA (nM) and of calprotectin (ng/mL) were significantly higher in the PMR cohort. After adjusting for sex and age, only calprotectin remained significantly elevated in the PMR patients (p<0.001).

**Neutrophil activation in patients with PMR**

As depicted in Figure 1A, and consistent with prior findings in serum (6), plasma levels of calprotectin were significantly elevated in patients with PMR with active disease, and after glucocorticoid therapy as compared to healthy individuals (p<0.001). The mean concentration of calprotectin in 54 patients with active disease before glucocorticoid therapy was 3002.9 ng/ml with a standard deviation (SD) of 1812.6 ng/ml that was significantly higher (p<0.001) compared to 30 HC where it was 83.8 ng/ml with a SD of 95 ng/ml. Patients with symptoms from shoulder, thighs, and hips before initiation of glucocorticoid therapy had significantly elevated levels of calprotectin in comparison to patients having symptoms from cervical and lumbar spine, 3125.3 ng/mL vs. 1614.8 ng/mL, p=0.036.

Of note, we made the novel observation that levels of NETs, as represented by MPO-DNA complexes, were similarly elevated in PMR with active disease as well as after treatment compared to healthy individuals (p<0.001) (Figure 1B). The mean (SD) concentration of MPO-DNA complexes in 54 patients with active disease before glucocorticoid therapy was 3.2 (1.3) nM that was significantly higher than that of HC (mean (SD): 2.6 (1.5) nM, p<0.001). Interestingly, for
patients with active disease, patients with PMR without overlapping GCA had significantly elevated levels of calprotectin (mean (SD): 3243.2 (1854.7) ng/mL) compared to patients with overlapping disease (mean (SD): 1801.2 (947.3) ng/mL, p=0.01). However, levels of MPO-DNA complexes, CRP and ESR, were similar in PMR with or without overlapping GCA (data not shown).

**Mechanisms of neutrophil activation in PMR**

Given the elevated levels of neutrophil activation markers in PMR, we asked whether patients with PMR had circulating neutrophil-activating factors. To address that, neutrophils from healthy individuals were incubated with plasma from either PMR patients or healthy controls and assessed for up-regulation of degranulation markers CD66b and CD11b by flow cytometry. As compared to healthy controls, plasma from patients with PMR with active disease supported increased up-regulation of both CD66b (p<0.001, and CD11b (p<0.001) (Figures 2A and 2B, respectively).

Given the extensive neutrophil activation observed in PMR in plasma, as well as capacity of plasma to support de novo neutrophil activation, we next sought to determine what factor(s) could contribute to neutrophil activation in PMR. Prior work has demonstrated elevated levels of circulating immune complexes in patients with PMR (18,19), with immune complexes known to activate neutrophils through FcgRIIA (20). To address whether circulating immune complexes were driving neutrophil activation in PMR, neutrophils from healthy individuals were incubated with an FcgRIIA inhibitor, prior to addition of plasma from PMR patients,
and assessed for neutrophil activation by flow cytometry. Addition of the FcgRIIA inhibitor markedly reduced the capacity of plasma from PMR to induce \textit{in vitro} activation of neutrophils, as demonstrated by down regulation of CD66b and CD11b (Figures 3A and 3B respectively). In all, these results suggest that circulating immune complexes are involved in supporting neutrophil activation via FcgRIIA in PMR. Consistent with that interpretation, levels of circulating immune complexes were associated with the capacity of plasma to induce neutrophil activation \textit{in vitro} (Figures 4A and 4B) and decreased significantly after glucocorticoid therapy in patients with PMR (Figure 4C). Levels of IC correlated with IgG levels ($rs=0.332$, $p=0.03$) in patients with PMR. Similar correlations have been observed in our prior work, measuring IC levels in SLE patients, with levels of IgG correlating with IC as determined by C1q-ELISA ($r=0.19$, $p=0.03$), and our in-house flow cytometry-based IC assay ($r=0.20$, $p=0.02$) (21).

\textbf{Role of glucocorticoid treatment on neutrophil activation markers}

We then investigated whether levels of neutrophil markers, blood cell-counts and acute phase reactants significantly changed after glucocorticoid treatment. As depicted in Table 2, plasma levels of calprotectin significantly decreased after treatment with glucocorticoids, consistent with prior findings in PMR (6). In contrast to calprotectin, levels of NETs were unaffected by treatment regimen. With regards to blood cell counts, there was a significant increase in lymphocytes, whereas there was a decrease in platelet count after treatment with glucocorticoids. All acute phase reactants (ESR, CRP and fibrinogen levels) significantly decreased
after treatment with oral glucocorticoids. In all, calprotectin, lymphocytes, platelet count and acute phase reactants such as ESR (Figure 4D), CRP and fibrinogen could be used as surrogate markers of disease activity and monitoring of treatment response in patients with PMR (Table 2).

**Discussion**

NET formation is a neutrophil cell death process that plays a crucial role in the pathogenesis of many autoimmune diseases (22). In the current study, we made the novel observation of elevated plasma levels of MPO-DNA complexes in patients with PMR in comparison to healthy individuals. High levels of circulating NETs in PMR could be due to exacerbated neutrophil cell death. There are reports of aberrant NET formation being related to aging, as the key enzyme for NETs, protein arginine deiminase-4 (PAD4) has been reported to spontaneously generate more NETs with aging (23,24). However, other studies demonstrated decreased capacity for NET formation in older individuals due to reduced ROS production and impaired autophagy (25), suggesting that additional factors other than aging might be responsible for the enhanced neutrophil activation. In our study, levels of NETs were not associated with age neither in the PMR cohort nor among healthy individuals (data not shown). Notably, treatment with glucocorticoids did not cause any significant change in the levels of MPO-DNA complexes. Thus, MPO-DNA complexes are not a sensitive biomarker for predicting reduction in systemic inflammation in patients with PMR.
In the subgroup analysis of patients with PMR with and without overlapping GCA, we did not find any significant difference in the plasma levels of MPO-DNA complexes either. Thus, propensity to undergo NET formation is not directly linked to disease activity or levels of inflammation, similar to what has been described in SLE (26). Further studies are needed to determine the mechanism(s) leading to peripheral MPO-DNA complexes in PMR, and long-term consequences of elevated NET formation on organ damage, including thrombosis (26).

On the other hand, calprotectin not only differed between patients with PMR and healthy controls but was also reduced upon treatment with glucocorticoids. Our findings align with prior studies that assessed serum levels of calprotectin (6) and suggest that calprotectin in the plasma could be a reliable biomarker of disease activity and/or treatment response in patients with PMR. Levels of calprotectin were similarly found elevated in patients with other rheumatic diseases such as SLE, RA, systemic sclerosis, and systemic vasculitides (10,11, 27, 28).

We also found that patients with PMR without overlapping disease with GCA had significantly higher levels of calprotectin in their circulation, as compared to patients with overlapping disease, suggesting higher levels of neutrophil recruitment, and release of calprotectin at sites of inflammation in patients with PMR without GCA. This observation could help in patient stratification and may not only reflect differences in levels of neutrophil activation, but also variability in circulating chemokines or cytokines, disease duration and burden, and absence or presence of vascular inflammation among patients with PMR and GCA.
Analysis of expression of calprotectin and NETs in tissues from synovial bursa of patients with PMR and association with disease activity and inflammatory markers might be helpful in identifying local tissue neutrophil infiltration and could be the focus of future prospective studies. For example, presence of neutrophils positive for expression of calprotectin has been identified in and around the vasa vasorum of biopsies from patients with GCA causing inflammation via activation of endothelial cells and production of reactive oxygen species (29).

The activated neutrophil phenotype observed in our patients with active disease, has been demonstrated by another group but after 12-week of steroid therapy along with augmentation in the number of circulating neutrophils and a surge in the disease activity (30). Interestingly, plasma-mediated neutrophil activation decreased in our patients with PMR upon blockade of FcγRIIA, a key neutrophil receptor for immune complexes (31). Similarly, our group recently showed plasma-mediated neutrophil activation to be abolished by blocking FcγRIIA also in systemic sclerosis (11). In the MRL/lpr lupus mouse model, administration of an immunoglobulin-binding peptide called TG19320 prevented glomerulonephritis by interfering with FcγR and IgG interaction (32). In another murine lupus model, treatment with recombinant soluble FcγRII (CD32) successfully inhibited immune complex-mediated inflammation (33). Notably, use of small chemical entities specific for the FcγRII dimer inhibited development of destructive autoimmune arthritis in FcγRIIA transgenic mice by blocking immune complex-mediated responses (34). Our results suggest that presence of circulating immune complexes may partake in neutrophil-mediated inflammation via signaling through
the FcγRIIA receptor in PMR. Thus, inhibition of FcγRIIA might be a potential attractive target for therapy in PMR. Of note, FcγRIIA is not selectively expressed on neutrophils, but also on several other important immune cells, including plasmacytoid dendritic cells, monocytes, and platelets. Further studies are warranted to determine whether ICs can similarly result in activation of those cells in PMR.

Interestingly, while there was a significant increase in the lymphocyte cell count and a drop in the platelet count, there was no significant change in the neutrophil cell count after glucocorticoid therapy in our cohort. Another study reported that neutrophils remained elevated whereas lymphoid subsets fluctuated in PMR patients during glucocorticoid therapy (35). Differences in patient populations, study designs, sample size, clinical setting, treatment duration, and follow up times may possibly explain the discordant results in our study.

Our study has few limitations. We recognize that this is a single center study susceptible to local influences. One additional limitation is the retrospective nature of our study. Another important consideration is that the source of calprotectin and MPO-DNA complexes could not be established in this study. MPO can be stored in azurophilic granules of both neutrophils and monocytes (36) and calprotectin can constitute up to 40 to 60% of the soluble cytosolic content in neutrophils, monocytes and activated macrophages (37,38).

In conclusion, our data for the first time demonstrated that levels of neutrophil activation markers and NETs are elevated in the circulation of patients with PMR implicating an important role for neutrophil recruitment and cell death in PMR. Our
data also support the use of calprotectin as a biomarker in monitoring disease activity in patients with PMR that needs to be validated by larger multicenter studies. Finally, targeting FcγRIIA by interfering with its interaction with immune complexes could be a promising pharmaceutical strategy for treating patients with PMR.

**Author Contributions:** D.M., S.R.D., C.L. contributed to the conception and design of this study. D.M., L.J., S.R.D., R.K., T.W., P.H., C.L. participated in experimental data collection and data analysis. S.R.D., L.J., contributed with patient samples and clinical data. All authors contributed to data interpretation, critically reviewed and revised the manuscript and approved the final submission.

**Funding:** This work was supported by the NIH training grant award #5T32HL007028-44 (DM) and the NIH grants 1R21EY029391 (CL) and R21AR075129 (CL).

**Declarations**

**Conflict of Interest:** Dr. Michailidou received Advisory Board fees from ChemoCentryx. Dr. Lood received research funding from Exagen Inc, Pfizer, Gilead Sciences, Horizon Therapeutics, Amytryx, Redd Pharma, and Eli Lilly. Dr. Lood is a Scientific Advisory Board member at Redd Pharma.

**Patient consent for publication:** Informed written consent was obtained from all participants in accordance with the Helsinki Declaration.
Ethics approval: The study was approved by the regional ethics board at University of Washington, Seattle, WA, (#3100) and Ethics Committee at Umea University, Sweden (§192/96, dnr 96-138).

Data availability statement: All data relevant to this study are included in the article or available upon reasonable request.

References


## Table 1. Baseline demographic characteristics of patients with PMR and HC

<table>
<thead>
<tr>
<th>Descriptive data</th>
<th>Patients, N=54</th>
<th>HC, N=30</th>
<th>p-value, Mann-Whitney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women, n (%)</td>
<td>44 (73.3)</td>
<td>21 (70)</td>
<td>0.667&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age at inclusion, mean (SD) years</td>
<td>73 (8)</td>
<td>40 (14)</td>
<td>&lt;0.001</td>
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<tr>
<td>Symptom duration before diagnosis, mean (SD) months</td>
<td>2 (2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>First follow up, mean (SD) months</td>
<td>3.7 (2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MPO-DNA, mean (SD) nM</td>
<td>3.2 (1.25)</td>
<td>2.6 (1.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calprotectin, mean (SD) ng/mL</td>
<td>3002.9 (1812.6)</td>
<td>83.8 (95.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucocorticoid dose, mean (SD) mg</td>
<td>22 (7)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ESR, mean (SD) mm/hour</td>
<td>60 (23)</td>
<td>not measured</td>
<td></td>
</tr>
<tr>
<td>CRP, mean (SD) mg/L</td>
<td>48 (37)</td>
<td>not measured</td>
<td></td>
</tr>
<tr>
<td>IgG, mean (SD) g/L</td>
<td>11.9 (3.4)</td>
<td>not measured</td>
<td></td>
</tr>
<tr>
<td>GCA, n (%)</td>
<td>14 (23.3)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

SD, standard deviation, ESR, erythrocyte sedimentation rate, CRP, C-reactive protein, NA, not applicable

<sup>a</sup> calculated using Pearson Chi-square
Table 2. Mean concentration of neutrophil markers, blood cell counts, and acute phase reactants in PMR.

<table>
<thead>
<tr>
<th>*Biomarker</th>
<th>Before glucocorticoids</th>
<th>After glucocorticoids</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Wilcoxon signed rank test</td>
</tr>
<tr>
<td></td>
<td>N=54</td>
<td>N=54</td>
<td></td>
</tr>
<tr>
<td>MPO-DNA, nM</td>
<td>3.2 (1.25)</td>
<td>3.1 (1.13)</td>
<td>0.38</td>
</tr>
<tr>
<td>Calprotectin, ng/mL</td>
<td>3002.9 (1812.6)</td>
<td>1065.4 (767.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils (x 10^3/μl)</td>
<td>5.8 (1.4) a</td>
<td>5.9 (1.6)</td>
<td>0.63</td>
</tr>
<tr>
<td>Monocytes (x 10^3/μl)</td>
<td>0.80 (0.3) e</td>
<td>1.3 (1.5)</td>
<td>0.57</td>
</tr>
<tr>
<td>Lymphocytes (x 10^3/μl)</td>
<td>1.58 (0.4) e</td>
<td>2.0 (0.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Basophils (x 10^3/μl)</td>
<td>0.03 (0.02) e</td>
<td>0.4 (1.0)</td>
<td>0.19</td>
</tr>
<tr>
<td>Eosinophils (x 10^3/μl)</td>
<td>0.2 (0.9) e</td>
<td>0.3 (0.8)</td>
<td>0.30</td>
</tr>
<tr>
<td>Platelets (x 10^9/L)</td>
<td>357.2 (92.7) b</td>
<td>267.0 (61.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>4.9 (1.4) b</td>
<td>2.6 (1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESR, mm/hour</td>
<td>59.8 (23.3)</td>
<td>11.9 (6.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>48.6 (37.9) c</td>
<td>11.1 (3.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a) N=23 cases, b) n=35 cases, c) n=50 cases, d) n=51 cases, e) n=22 cases

*Calculated for matched pairs
Figure 1. Levels of calprotectin and NETs in patients with PMR. Plasma levels of (A) calprotectin and (B) MPO-DNA complexes were measured by ELISA in healthy controls (HC, n=30), and patients with polymyalgia rheumatica (PMR) with active disease (n=54) and after treatment with glucocorticoid therapy (n=54). Statistical analyses were performed using Mann-Whitney U test and Wilcoxon signed rank test, respectively, with ***p < 0.001. Each circle represents an individual sample, with the bar representing the median of the group.
Figure 2. Neutrophil activation induced by plasma from patients with PMR. Neutrophils from a healthy individual were incubated with plasma from healthy controls (HC, n=20) and patients with polymyalgia rheumatica (PMR, n=45) and analyzed for the expression of the neutrophil cell surface degranulation markers (A) CD66b and (B) CD11b by flow cytometry. Results are presented as the mean fluorescent intensity (MFI). Statistical analyses were done using Mann-Whitney U test with ***p<0.001. Each circle represents an individual sample, with the bar representing the median of the group.
Figure 3. FcγRIIA-mediated neutrophil activation induced by plasma from patients with PMR. Plasma from healthy controls (HC, n=8), and patients with polymyalgia rheumatica (PMR, n=8) were incubated in vitro with neutrophils isolated from a healthy object in the presence or absence of the FcγRIIA-blocking antibody (Clone IV.3) for 120 min and assessed for its capacity to up-regulate the neutrophil activation surface markers (A) CD66b and (B) CD11b. Results are shown as the mean fluorescent intensity (MFI). Statistical analyses were done using Mann-Whitney U test as well as Wilcoxon signed-rank test with **p < 0.01, and ***p<0.001.
Figure 4. Association of circulating immune complexes with plasma-mediated neutrophil activation and treatment in patients with PMR. Plasma-mediated neutrophil activation as assessed by (A) CD66b and (B) CD11b was correlated with plasma levels of immune complexes (IC) from patients with PMR (n=30). Expression of neutrophil activation markers is presented as mean fluorescent intensity (MFI). IC levels (C) were measured by ELISA in HC (n=20) and patients with PMR with active disease (n=38), and after treatment (n=38). (D) Analysis of ESR levels before and after treatment in PMR patients. Statistical analysis was done using Spearman’s correlation test, Mann-Whitney U test, and Wilcoxon signed rank test, with ***p < 0.001. Each circle represents an individual sample, with the bar representing the median of the group.
Figure 1. Levels of calprotectin and NETs in patients with PMR. Plasma levels of (A) calprotectin and (B) MPO-DNA complexes were measured by ELISA in healthy controls (HC, n=30), and patients with polymyalgia rheumatica (PMR) with active disease (n=54) and after treatment with glucocorticoid therapy (n=54). Statistical analyses were performed using Mann-Whitney U test and Wilcoxon signed rank test, respectively, with ***p < 0.001. Each circle represents an individual sample, with the bar representing the median of the group.
Figure 2. Neutrophil activation induced by plasma from patients with PMR. Neutrophils from a healthy individual were incubated with plasma from healthy controls (HC, n=20) and patients with polymyalgia rheumatica (PMR, n=45) and analyzed for the expression of the neutrophil cell surface degranulation markers (A) CD66b and (B) CD11b by flow cytometry. Results are presented as the mean fluorescent intensity (MFI). Statistical analyses were done using Mann-Whitney U test with ***p<0.001. Each circle represents an individual sample, with the bar representing the median of the group.
Figure 3. FcγRIIA-mediated neutrophil activation induced by plasma from patients with PMR. Plasma from healthy controls (HC, n=8), and patients with polymyalgia rheumatica (PMR, n=8) were incubated in vitro with neutrophils isolated from a healthy object in the presence or absence of the FcγRIIA-blocking antibody (Clone IV.3) for 120 min and assessed for its capacity to up-regulate the neutrophil activation surface makers (A) CD66b and (B) CD11b. Results are shown as the mean fluorescent intensity (MFI). Statistical analyses were done using Mann-Whitney U test as well as Wilcoxon signed-rank test with **p < 0.01, and ***p<0.001.
Figure 4. Association of circulating immune complexes with plasma-mediated neutrophil activation and treatment in patients with PMR. Plasma-mediated neutrophil activation as assessed by (A) CD66b and (B) CD11b was correlated with plasma levels of immune complexes (IC) from patients with PMR (n=30). Expression of neutrophil activation markers is presented as mean fluorescent intensity (MFI). IC levels (C) were measured by ELISA in HC (n=20) and patients with PMR with active disease (n=38), and after treatment (n=38). (D) Analysis of ESR levels before and after treatment in PMR patients. Statistical analysis was done using Spearman's correlation test, Mann-Whitney U test, and Wilcoxon signed rank test, with ***p < 0.001. Each circle represents an individual sample, with the bar representing the median of the group.