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BRIEF COMMUNICATION

# CSF/serum matrix metallopeptidase-9 ratio discriminates neuro Behçet from multiple sclerosis

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## Abstract

In neuro Behçet disease with multiple sclerosis-like features, diagnosis could be challenging. Here, we studied the cerebrospinal fluid and serum inflammatory profile of 11 neuro Behçet and 21 relapsing-remitting multiple sclerosis patients. Between the soluble factors analyzed (MMP9, TNFα, IL6, CXCL13, CXCL10, CXCL8, IFNγ, IL10, IL17, IL23, and others) we found MMP9 increased in neuro Behçet serum compared to multiple sclerosis and decreased in cerebrospinal fluid. Furthermore, neuro Behçet analysis of circulating natural killer $CD56_{DIM}$ subset suggests their potential involvement in increased MMP9 production. We believe that these findings may have a translational utility in clinical practice.

## Introduction

Today, different therapeutic approaches are available for central nervous system neuroimmune diseases; therefore, the choice of an adequate treatment depends on early diagnosis. Here, we focus our attention on those cases of neuro Behçet disease (NBD) with multiple sclerosis (MS)-similar characteristics, where a differential diagnosis is needed.[1] To date, the most widely accepted criteria for the diagnosis of Behçet disease (BD) are the International Criteria for Behçet's Disease[2] and the diagnosis of neurological involvement in BD is done mainly by clinical means.

Due to its physiological role, cerebrospinal fluid (CSF) gives information on chemokines and cytokines that in part mediate neurologic autoimmune diseases.[3–5] In NBD, CSF cytokines and chemokines (i.e., IL6, CXCL8, IL10, IL15, IFNγ, and TNFα) have been correlated with pathogenesis together with Th1 and Th17 cell polarization without identifying a unique combination of cell population/soluble factors[6–8]; all these studies indicate a complex mechanism behind the disease. Hirohata et al. in a recent work analyzed the CKs content in CSFs from NBD patients subdivided into parenchymal (nine patients) and not parenchymal (one patient) NBD, together with six headaches associated with Behçet disease and 19 controls. These authors found that parenchymal NBD differ from controls for the presence of IL6, and from viral meningitis for the lower presence of IL6, Il10, CXCL8, and TNFα.[9] Here, we studied the cytokine profile (MMP9, CXCL10, CXCL13, OPN, GM-CSF, TNFα, IFNγ, IL1α, IL1β, IL2, IL4, IL6, CXCL8, IL10, IL12p40, IL12p70, IL17, IL23, BAFF) in serum and CSF of NBD patients and relapsing-remitting MS (RRMS) patients by Milliplex multiple assays. We identified a significant difference of MMP9 CSF and serum concentrations in NBD compared to MS. Our analysis suggests that the MMP9 index, defined in this study, could be a possible biomarker in diagnostically undefined cases independently from NBD disease activity and therapy. Furthermore, we demonstrated that one key cellular source of MMP9 increased in NBD patients are circulating natural killer $CD56_{DIM}$ cells.

# Patients and Methods

## Patients

The study was approved by the local ethical committee (authorization #35/12); written informed consent was obtained from all study participants. We collected CSF and serum from 11 NBD and 21 RRMS consecutive patients undergoing routine diagnostic lumbar puncture. Patients were diagnosed with NBD according to criteria proposed by the ICBD criteria[2] and with RRMS according to McDonald criteria revised by Polman.[10] Patient and CSF characteristics are reported in Tables 1–3.

## Soluble factors

CSF and serum, stored at −80°C, were analyzed for the determination of 19 soluble factors (MMP9, CXCL10, CXCL13, OPN, GM-CSF, TNFα, IFNγ, IL1α, IL1β, IL2, IL4, IL6, CXCL8, IL10, IL12p40, IL12p70, IL17, IL23, and BAFF) by Bioplex (Biorad) using magnetic Milliplex MAP kits (Millipore).

## Flow cytometry evaluation of MMP9 production by PBMCs

Freshly isolated PBMCs by density gradient (Biocoll) were labeled with anti-CD3 PerCP, anti-CD14 PE, anti-CD19 APC or anti-CD56 APC, anti CD8-PE-Cy7 monoclonal antibodies (eBioscience); then fixed, permeabilized (Fix&-Perm solution, Invitrogen), and intracellular stained for MMP9 detection (anti-MMP9 FITC monoclonal antibody, eBioscience). Samples were analyzed by flow cytometer Cy-Flow space (Partec-Sysmex).

**Table 1.** Clinical characteristics of NBD patients.

| | |
|---|---|
| NBD patients | *N* = 11 |
| Gender (F:M) | 6:5 |
| Age average, (min-max) | 40.4 (29–53) |
| NBD type | |
| No. of patients, (%) | |
| Parenchymal | 11 (100%) |
| Not parenchymal | 0 (0%) |
| Patients with clinical activity[1] | 2 (18.2%) |
| No. of patients, (%) | |
| Patients with brain lesions[2] | 8 (72.7%) |
| No. of patients, (%) | |
| Inflammatory lesions | 4 (36.3%) |
| Microvascular lesions | 4 (36.3%) |
| Patients under therapy | 11 (100%) |
| No. of patients, (%) | |
| Type of therapy | |
| Immunosuppressants | 8 |
| Anti-TNFα | 1 |
| Immunosup. + anti-TNFα | 2 |

[1]At least one focal neurological or neuropsychiatric manifestation within 3 months before sample collection.
[2]Presence of T2w lesions in a range of 3 months from the CSF collection.

**Table 2.** Clinical characteristics of RRMS patients.

| | |
|---|---|
| RRMS patients | *N* = 21 |
| Gender (F:M) | 14:7 |
| Age average, (min-max) | 31 (20–55) |
| Disease duration average years | 6 |
| Patients in relapse | 10 (47%) |
| No. of patients, (%) | |
| Patients with new brain lesion in the last year | 13 (65%) |
| No. of patients, (%) | |
| EDSS average, (min-max) | 1.6 (0–5.5) |
| Patients under therapy[1] | 1 (4.7%) |
| No. of patients, (%) | |

[1]Rebiff 44.

**Table 3.** CSF characteristics.

| CSF characteristics | NBD | RRMS |
|---|---|---|
| No of cells/μL | 5 (0–14)[1] | 10 (3–26)[1] |
| average (min-max) | | |
| Oligoclonal bands | 3 (33.3%) | 20 (95.2%) |
| No. of positive patients (%) | | |
| IgG Index alteration | 4 (40%) | 16 (76%) |
| No. of patients (%) | | |
| Albumin ratio alteration | 2 (18.2%) | 1 (4.7%) |
| No. of patients (%) | | |

[1]Prevalence of mononuclear cells (>95%).

## Statistical analysis

Data are expressed as mean ± SEM values. Statistical analysis was performed using Student's *t*-test, Mann–Whitney *U* test, ROC curve, where appropriate (GraphPad Prism).

# Results

## Patients

In Tables 1–3 are reported patients' and CSF characteristics. Clinical disease activity was reported in 6/11 NBD patients; 3/11 neurological (clinical and MRI) and 3/11 extraneurological. NBD, active with neurological manifestations, presented MRI new lesions in two cases and CSF alterations in all; 7/11 NBD were HLAB51-positive, 2/11 NBD had psychiatric presentations, and 3/11 stroke-like at onset. All NBD presented oral recurrent aphtosis. RRMS patients were all at early stage of disease and all but one without therapy.

## Soluble factors

First, we showed that MMP9 is differently distributed in CSF and serum of NBD and RRMS: it was increased in RRMS CSF compared to NBD ($P = 0.002$) and in serum of NBD compared to RRMS ($P < 0.0001$). We observed that mean – 3SD of serum NBD MMP9 is a value that includes all the NBD and excludes all the MS (Fig. 1A). Second, to take into account the diverse protein concentrations in CSF and serum, we calculated the MMP9 index

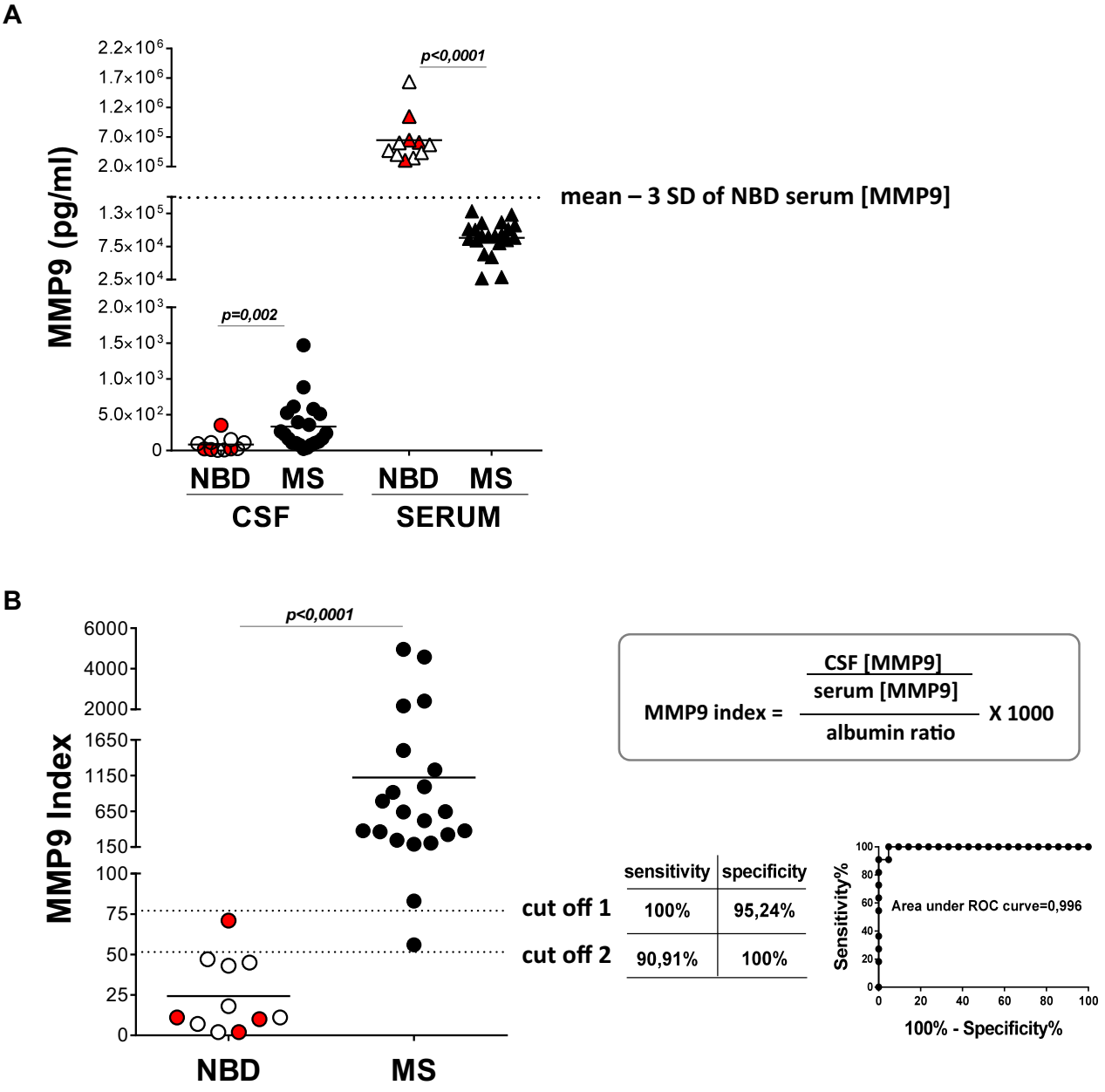


**Figure 1.** CSF and serum concentration of MMP9 in NBD and RRMS patients. (A) MMP9 concentration (pg/mL) was measured in paired CSF and serum samples of 11 NBD and 21 RRMS patients by Milliplex assay. Each dot/triangle in graph represents a single sample (in red, NBD patients with inflammatory brain lesions). Statistical significance was calculated by Mann–Whitney *t* test. Dotted line: mean value – 3 SD of serum MMP9 concentration detected in NBD samples. (B) Definition of the "MMP9 index": ratio between CSF and serum MMP9 concentration, normalized versus the albumin ratio (ratio between CSF and serum albumin concentration). The ROC curve analysis identified two possible cutoff values able to discriminate NBD and MS patients with high specificity and high sensitivity. Red dots: NBD patients with inflammatory brain lesions.

 

(ratio between CSF and serum MMP9 concentration normalized vs. the albumin ratio) (Fig. 1B) and we utilized the ROC curve analysis to calculate the best cutoff value where several are possible. In this case, the ROC curve identified two cutoff indexes (index 51.5 and 77) able to discriminate NBD and MS patients with high specificity and high sensitivity (Fig. 1B). Third, in Figure 2A is reported, the quantity of BAFF, equal in the two sample, IL6, increased in NBD CSF ($P = 0.02$), and TNFα increased in CSF ($P < 0.0001$) and serum ($P = 0.0005$) compared to RRMS. Our data on recall chemokine profiles revealed that in NBD CSF CXCL8 is increased ($P = 0.01$) and CXCL13 decreased ($P = 0.02$) compared to RRMS; CXCL10 is present at the same level in both samples (Fig. 2B). Although MMP9 may be modulated by cytokine and chemokine and vice versa, we did not found any significant correlations between MMP9 level and the other cytokine and chemokines investigated. Moreover, we did not find any correlations between MMP9 production and the clinical and laboratory data (not shown). Last, our data show that serum level of MMP9 might discriminate between NBD and RRMS; to gain insight into this finding, we investigated if the two samples differ also in intracellular MMP9 production, looking specifically to blood circulating mononuclear cells (PBMCs). In Fig. 2C, we show that MMP9 production in PBMCs was significantly increased in NBD compared to RRMS ($P = 0.0006$). This difference was in part due to circulating natural killer cells (NKs), $CD56_{DIM}$ subset (Fig. 2D upper dot plot) and in this subset, the MMP9 producing CD8+ population did not differ from the CD8-, in respect to the whole PBMCs (Fig. 2D lower dot plot).

## Discussion

In this report, we investigated the inflammatory profile of CSF and serum in a group of Italian patients affected by NBD and RRMS. A significant difference of MMP9 level distribution was found: MMP9 in RRMS was higher in CSF and lower in serum compared to NBD. Moreover, the cutoff value of the concentration in serum identify all NBD samples with 100% specificity. In order to take into account the differences between intrathecal and peripheral protein production, we normalized the MMP9 levels with albumin ones, by calculation of "MMP9 Index."[11] MMP9 has been widely investigated in MS[12–14]; recently Song et al. found that during experimental autoimmune encephalomyelitis (EAE, animal model of MS) MMP9 has a pivotal role at the parenchymal border of CNS, increasing the migration of leukocytes. Furthermore, in EAE MMP9 activity was found regulated by cytokines produced by infiltrating leukocytes and was determinant for the induction of astrocytes chemotactic activity.[15] In MS patients, MMP9 activation and lesion development are linked.[16] We are aware of the differences between metallopeptidase production and activity; nonetheless, we interpret our data of increased MMP9 content in MS CSF compared to NBD CSF as reinforcing the hypothesis that MMP9 has a more specific role in CNS cell invasion during MS lesions formation, than in NBD. Our findings in part differ from previous data, where MMP9 measured by ELISA was found only slightly increased in CSF of MS patients compared to NBD.[17] Of note, in our sample, mean value of NBD CSF MMP9 content was 10 times lower than the quantity reported previously. In our opinion, this may be due to the different methodology, Milliplex versus ELISA or to patients' clinical phase at CSF collection, or both. These considerations may explain also the conflicting reports of BAFF levels in NBD and MS.[18,19] Moreover, in NBD, compared to MS, recall of B lymphocytes (CXCL13) decreased while the recall of neutrophils (CXCL8) increased. Based on our observations, we hypothesize the occurrence of a distinctive inflammatory environment in CSF of MS and NDB. In NBD CSF, TNFα and IL6 levels were higher in respect to MS ones; overall, the measured levels of CXCL8, IL6, and TNFα confirm the inflammatory response, previously described in NBD and in Behçet disease.[20,21] We know that neutrophils are important in Behçet disease inflammation [22] and the presence of CXCL8 in CSF suggests their involvement in the neuronal form too. In the NBD periphery, where MMP9 is increased compared to MS, we investigated the possible MMP9 source between circulating mononuclear cells. A low percentage of T cells, B cells, and monocytes produce MMP9 (from 0.5% to 0.07%), whereas NK cells were MMP9-positive for 1.9%. We identified NKs $CD56_{DIM}$ subset as the key producers of MMP9, in agreement with previous findings that indicate NK $CD56_{DIM}$ involved in inflammation of several autoimmune diseases, including Behçet.[23–25] In Behçet, this NK subset was found depleted in blood during disease activity, probably because trafficking into disease active tissues and decreased during treatment with azathioprine.[26] In our sample, NBD patients were all under therapy at the time they developed the neurological form and we confirmed the effect of azathioprine on NK depletion in three NDB patients under therapy compared to the other NBD cases (data not shown). Finally, many different chemokines and cytokines are responsible for MMP9 regulation,[27] nevertheless, we did not find correlations between any of the investigated soluble factors and MMP9 production.

In conclusion, by comparing NBD and MS, we confirmed the distinctive role of TNFα and IL6 in NBD inflammatory events and suggested the differential central and peripheral involvement of MMP9. It will be important in the future to validate MMP9 differences in

 

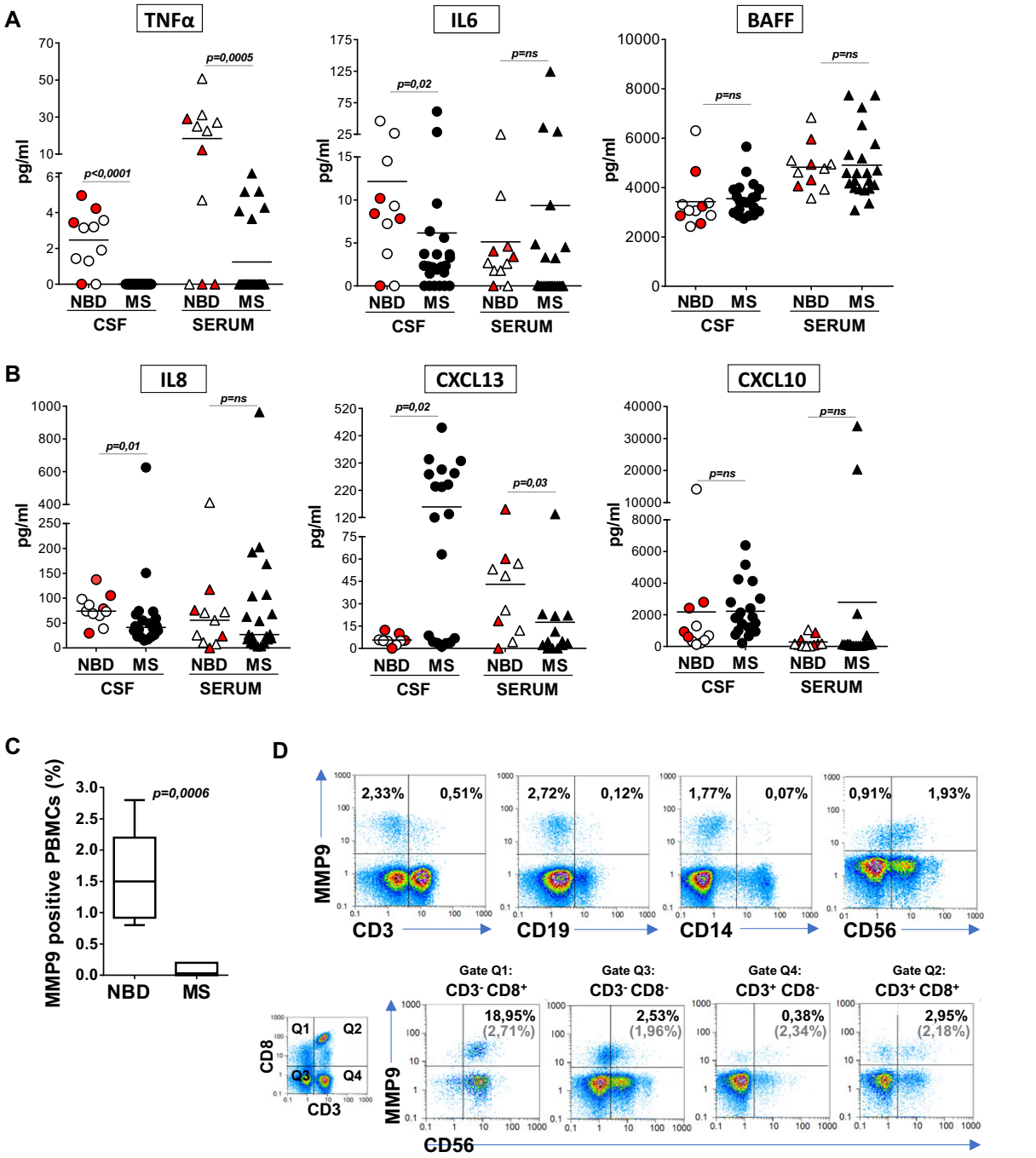


**Figure 2.** Characterization of cytokines/chemokines profile of NBD patients compared to RRMS patients and phenotypic analysis of cells producing MMP9 in peripheral blood. (A–B) Measurement of cytokines (TNFα, IL6, BAFF) and chemokines (CXCL8, CXCL10, CXCL13) in paired samples of CSF and serum of 11 NBD and 21 RRMS patients (Milliplex assay). Each dot/triangle in graph represents a single sample, (in red, NBD patients with inflammatory brain lesions); statistical significance was calculated by Mann–Whitney *t* test. (C) Percentage of cells producing MMP9 in peripheral blood, evaluated in 11 NBD and 14 RRMS patients by MMP9 intracellular staining and flow cytometry analysis. The graph shows the median (with range) of the percentage of cells positive for MMP9 staining in PBMCs; statistical significance was calculated by Mann–Whitney *t* test. (D) Phenotype of PBMCs producing MMP9 in NBD. Upper panel: percentage of T cells (CD3+), B cells (CD19+), monocytes (CD14+), and NK cells (CD56+) expressing MMP9 at the intracellular level (gate on total PBMCs). Lower panel: MMP9 intracellular production by NK cell subsets. Plots from one NBD patient representative of 9 out of 11 examined samples. Percentages reported on the plots refer to the specific gate indicated over the plot and, given in brackets, to total PBMCs.

 

independent cohorts of patients, in order to properly define it a disease biomarker.

## Author Contribution

AA contributed to the acquisition and analysis of data and drafting figures; EB, TB, MMDE, GE, LE, ES, AMR, and AB contributed to the acquisition and analysis of data; CB contributed to the conception and design of the study and drafting manuscript.

## Conflict of Interest

None declared.

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