

Mitochondrial N-formyl methionine peptides associate with disease activity as well as contribute to neutrophil activation in patients with rheumatoid arthritis

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ABSTRACT

Objectives: Literature suggests that neutrophils of patients with rheumatoid arthritis (RA) are primed to respond to N-formyl methionine group (formylated peptides). Animal models indicate that formylated peptides contribute to joint damage via neutrophil recruitment and inflammation in joints. Non-steroidal anti-inflammatory drugs are also known to inhibit formyl peptide-induced neutrophil activation. The predominant source of formylated peptides in sterile inflammatory conditions like RA is mitochondria, organelles with prokaryotic molecular signatures. However, there is no direct evidence of mitochondrial formyl peptides (mtNFPs) in the circulation of patients with RA and their potential role in neutrophil-mediated inflammation in RA, including their clinical significance.

Methods: Levels of mtNFPs (total fMet, MT-ND6) were analyzed using ELISA in plasma and serum obtained from patients in 3 cross-sectional RA cohorts ($n = 275$), a longitudinal inception cohort ($n = 192$) followed for a median of 8 years, and age/gender-matched healthy controls (total $n = 134$). Neutrophil activation assays were done in the absence or presence of formyl peptide receptor 1 (FPR1) inhibitor cyclosporine H.

Results: Elevated levels of total fMet were observed in the circulation of patients with RA as compared to healthy controls ($p < 0.0001$) associating with disease activity and could distinguish patients with the active disease from patients with inactive disease or patients in remission. Baseline levels of total fMet correlated with current and future joint involvement, respectively and predicted the development of rheumatoid nodules (OR = 1.2, $p = 0.04$). Further, total fMet levels improved the prognostic ability of ACPA in predicting erosive disease (OR of 7.9, $p = 0.001$). Total fMet levels correlated with markers of inflammation and neutrophil activation. Circulating mtNFPs induced neutrophil activation *in vitro* through FPR1-dependent mechanisms.

Conclusions: Circulating mtNFPs could be novel biomarkers of disease monitoring and prognosis for RA and in investigating neutrophil-mediated inflammation in RA. We propose, FPR1 as a novel therapeutic target for RA.

1. Introduction

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease primarily characterized by progressive joint destruction involving complex pathophysiology [1]. Now added to this complexity are mitochondrial N-formyl peptides (mtNFPs) that we hypothesize to contribute to RA pathogenesis via neutrophil activation. Chronic inflammation orchestrated by the excessive levels of inflammatory mediators released

by resident and infiltrated cells contribute to tissue damage in RA [1]. Neutrophils are the most abundant immune cell type in the arthritic joint and play an essential role in the initiation and progression of RA [2, 3]. RA neutrophils, in general, have an activated phenotype characterized by enhanced oxidative burst, degranulation, and excessive release of neutrophil extracellular traps (NETs) [4–6]. NETs, mainly intended for microbial killing, are a potential source of citrullinated antigens, well-established principal targets of anti-citrullinated protein antibodies

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(ACPAs) in RA [7]. In a proinflammatory environment like RA, neutrophils are activated by various molecules, including immune complexes, cytokines, and damage-associated molecular patterns (DAMPs) like mitochondria [8–12]. Cell-free mitochondria, as well as mitochondrial DNA (mtDNA), have been found in the synovial fluid and circulation of RA patients suggesting mitochondrial release in the context of RA pathogenesis [13–15].

Mitochondria, owing to their microbial ancestry, possess many bacteria-like molecules, including circular mtDNA and mtNFPs. MtNFPs are known potent neutrophil chemotactic peptides which are able to induce the release of ROS from neutrophils via signaling through formyl-peptide receptor 1 (FPR1) [12,16–20]. FPR1, a member of the G-protein coupled family of receptors (GPCRs), highly expressed by myeloid cells like neutrophils, monocytes, and macrophages exhibits strong binding affinity towards NFPs, including mtNFPs [21–23]. Signaling through FPR1 leads to numerous neutrophil effector functions, including reactive oxygen species (ROS) production, degranulation, and chemotaxis, which can contribute to tissue damage upon chronic activation of neutrophils [23]. FPRs are present both on the plasma membrane and in neutrophil granules, which can be mobilized upon priming with agents like bacterial endotoxin and TNF- α , suggesting that ongoing inflammation can amplify FPR-mediated inflammatory response of neutrophils [24–27]. Indeed, neutrophils from RA patients, but not control neutrophils, exhibit higher rates of oxidative burst upon activation by standard bacterial NFP, N-formylmethionyl-leucyl-phenylalanine (fMLP) [28]. This observation suggests that neutrophils in RA patients are primed to produce ROS in response to mtNFPs. However, the role of mtNFPs, potent neutrophil activation molecules, remains to be explored in RA pathogenesis. Specifically, it is not known whether mtNFPs are elevated in RA patients, their clinical significance, or their contribution to neutrophil-mediated inflammation in RA.

To investigate this biology, we first analyzed the circulating levels of mtNFPs in several independent RA cohorts in comparison to levels in healthy individuals. Secondly, we determined whether mtNFPs in RA patients could contribute to neutrophil activation and inflammation. This aim included an *in vitro* evaluation of the inflammatory potential of mtNFPs in RA plasma using FPR1 blockade experiments. Thirdly, we sought to determine the clinical relevance of mtNFPs to RA disease activity, joint damage, and their ability to predict the development of the extra-articular disease. In brief, we made the novel and significant observation that mtNFP levels were elevated in RA patients compared to healthy individuals and associated with neutrophil-mediated inflammation and disease progression, including development of rheumatoid nodules. FPR1 may thus be a novel therapeutic target in RA.

2. Materials and methods

2.1. Patient characteristics

Plasma samples from three independent cross-sectional RA cohorts and serum samples from one longitudinally followed inception RA cohort were analyzed in the current study. Patients with RA (Cohort 1, n = 95) defined according to the American College of Rheumatology criteria 1987, and age- and gender-matched healthy individuals were recruited to participate in research studies at the University of Washington Medical Center, Seattle. The majority of the patients in cohort 1 were female (75%), white (65%), and median (range) age at the time of diagnosis was 54 (20–78) years. Clinical disease activity index (CDAI) (information available for 70% of patients) that considered tender and swollen joints, patient global assessment, and provider global assessment was 11 (0–46) reflecting moderate disease activity. Based on CDAI scores, patients are sub-grouped as follows: patients in remission (CDAI <3), low disease activity (CDAI 3–10), moderate disease activity (CDAI 11–22), and high disease activity (CDAI ≥23). The second cross-sectional cohort of patients (Cohort 2, n = 87) with established RA as well as one RA inception cohort (Cohort 3, n = 192) followed for a

median of 8.3 years (range 4.4–19.8 years) following disease onset were recruited in Washington State. The study was approved by regional ethics boards (#3100 and #810), and informed written consent was obtained from all participants in accordance with the Helsinki Declaration. A fourth cross-sectional cohort (Cohort 4, n = 93) selected from the Studies of the Etiology of RA (SERA) cohort in Denver, Colorado (IRB# 13–2606), was included to validate key findings from Cohort 1. Subjects were randomly selected based on availability of RA disease activity measures. Overall, subjects in Cohort 4 had low disease activity with median (range) CDAI of 8 (0–50) and DAS28-CRP of median (range) 2.11 (0.97–5.26), respectively. The median age (range) of patients at the time of diagnosis is 60 (20–78) years, with the majority of patients being female (85%) and non-Hispanic white (69%). Patient cohorts 1, 2, and 3 have been reported previously [29], with additional patient characteristics, including that of cohort 4 and SLE cohort are given in Table 1.

2.2. ELISA-based methods

Plasma levels of mtNFPs and sPLA2 (human formyl methionine (fMet), and human mitochondrially encoded NADH dehydrogenase 6 (MT-ND6) and Human secreted phospholipase A2, My BioSource Inc., San Diego, CA) were determined by ELISA following manufacturer's instructions. Absorbance was measured using Synergy 2, BioTek (Winooski, VT). Standards were used as reference to calculate concentrations of measured analytes in plasma. Total fMet levels were defined as high if above the 95th percentile of healthy controls. The ability of total fMet ELISA to detect mtNFPs was validated by analyzing mitochondrial lysates as positive controls, whereas non-mitochondrial fractions collected during mitochondrial isolation were included as negative controls. The mitochondrial lysates were enriched for total fMet compared to non-mitochondrial fractions, validating the fMet assay (Supplementary Fig. 1).

2.3. Isolation of neutrophils

To isolate neutrophils from the blood of healthy subject, heparinized blood was layered on Polymorphprep (Axis-Shield, Dundee, UK) density gradient, according to the manufacturer's instructions, or as described previously [7,30]. Red blood cells were lysed with RBC lysis buffer (BioLegend). Neutrophils were re-suspended in serum-free RPMI-1640 medium (Life Technologies, Waltham, MA) for *in vitro* assays.

2.4. ROS analysis for fMet signaling

Neutrophils, plated at 3×10^5 cells/well, were incubated with a selective inhibitor of FPR1, cyclosporine H (CsH, 5 μ M) for 30 min prior to the addition of stimuli, R848 (2.5 μ g/ml), N-Formyl-Met-Leu-Phe (1 μ M), formylated MT-ND6 peptide (1 μ M) or plasma (1:100 dilution) for an additional 60 min. DHR 123 (0.5 μ M), was added during last 30 min of incubation and ROS was analyzed by flow cytometry. Published sequence of formylated MT-ND6 peptide (f-MMYALF) [31] was synthesized commercially (GenScript USA, Inc.) and was tested as endotoxin-free.

2.5. Statistics

For sample sets with a non-Gaussian distribution, non-parametric tests, Mann-Whitney U and Spearman's correlation, were used as applicable. High modified Sharp erosion score was defined as the upper quartile within the RA inception cohort [32]. All analyses were considered statistically significant at $p < 0.05$. Hierarchical clustering was performed using the R v4.0.2 pheatmap v1.0.12 (<https://www.r-pkg.org/pkg/pheatmap>).

Table 1

Demographic, clinical and treatment information of subjects.

| Cohort | RA 1 | RA 2 | RA 3 | RA4 | HC 1 | HC 2 | HC3 | SLE |
|------------------------------|--------------------------------------|----------------|------------------------------|--------------------------------|----------------|----------------|-------------------------|----------------|
| Patients (#) | 95 | 87 | 192 | 93 | 36 | 48 | 50 | 44 |
| Specimen | Plasma | Plasma | Serum | Plasma | Plasma | Serum | Plasma | Plasma |
| Age in years (median, range) | 54 (20–78) | 47 (18–64) | 43 (16–64) | 60 (20–78) | 57 (26–71) | 43 (16–64) | 59 (19–59) | 33 (19–61) |
| Gender (% female) | 75 | 100 | 100 | 85 | 85 | 100 | 84 | 91 |
| Ethnicity (white, %) | 65 | 88 | 88 | 69 (non-Hispanic White) | 100 | 88 | 80 (non-Hispanic White) | 65 |
| Seropositive (%) | 80 | 68 | 71 | 100 | N/A | N/A | 0 | N/A |
| RF (%) | 72 | 52 | 59 | 69 | N/A | N/A | 4 | N/A |
| ACPA (%) | 76 | 57 | 57 | 100 | N/A | N/A | 0 | N/A |
| Outcome measure | Disease activity | Erosion | Erosion | Disease activity | N/A | N/A | N/A | N/A |
| CDAI (median, range) | 11 (0–46) | N/A | N/A | 8 (0–50) | N/A | N/A | N/A | N/A |
| Erosive disease (%) | 50 | 54 | 10 | N/A | N/A | N/A | N/A | N/A |
| Immunosuppression (%) | 78 | 38 | 1 | N/A | N/A | N/A | N/A | N/A |
| Figures | 1A,B 2A,B,E 3A 5A-E 6A-C | 4A-C 5F-H | 2D 5I-J | 2C,2F 3B 5K | | | | 1A |
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3. Results

Levels of mtNFPs (total fMet and MT-ND6) in plasma were measured using two different ELISAs to distinguish between total fMet levels, representing the mtDNA-encoded proteome of 13 mitochondrial proteins, as well as a peptide specific to MT-ND6, a complex I protein of the mitochondrial electron transport chain, respectively. Following the validation of fMet ELISA assay for analyzing total fMet levels (detailed in methods, [Supplementary Fig. 1](#)), analyses were extended to clinical samples.

3.1. Patients with RA have elevated levels of mtNFPs

As shown in [Fig. 1A](#), total fMet levels were elevated in all three RA cohorts compared to healthy controls ($p < 0.0001$ for all analyses). In RA cohort 4, which had overall low disease activity (median CDAI 8; media DAS28-CRP of 2.11), total fMet levels were significantly lower compared to the other three RA cohorts (median fMet 23.40 ng/mL vs. 94.69 ng/mL, 69.11 ng/mL, 71.47 ng/mL, respectively; $P < 0.0001$ for all comparisons). However, RA4 patients with active disease ($\text{DAS28-CRP} \geq 2.6$) had elevated levels of total fMet compared to healthy individuals ($p = 0.004$). Based on the evidence of mitochondrial extrusion in SLE [33,34], total fMet levels of SLE patients were included as a positive disease control for mtNFPs. As expected, SLE patients had elevated levels of total fMet compared to HC1 ([Fig. 1A](#), $p < 0.0001$). Although, patients in RA1 have elevated levels of total fMet compared to SLE (94.69 vs. 70.93 ng/mL ng/mL; $p < 0.01$), overall levels of total fMet were comparable between patients with RA and SLE, suggesting fMet levels to be elevated in conditions of inflammation. Finally, MT-ND6 levels were significantly associated with total fMet levels ($r = 0.27$, $p = 0.01$, [Supplementary Fig. 2](#)) suggesting that individual mtNFPs are also present at measurable levels in RA plasma.

3.2. MtNFPs (total fMet levels) in patients with RA associate with joint damage and disease activity

We next asked whether analysis of mtNFPs had clinical significance. Levels of total fMet correlated with markers of active disease, including swollen and tender joints ($r = 0.23$, $p = 0.03$, and $r = 0.22$, $p = 0.04$, respectively, for RA1 [Fig. 1C-D](#) and $r = 0.25$, $p = 0.02$ for RA4, [Supplementary Fig. 3](#)). For both RA cohorts 1 and 4, mtNFP levels could distinguish patients with moderate-high disease activity ($\text{CDAI} \geq 11$;

$\text{DAS28-CRP} \geq 3.2$) from patients with low disease activity and/or in remission ($\text{CDAI} \leq 10$; $\text{DAS28-CRP} < 3.2$) ([Fig. 1E](#) and F). In RA cohort 1, even patients in remission had significantly elevated levels of total fMet compared to HC1, suggesting ongoing subclinical inflammation. The strong association of fMet levels with disease activity in cohorts RA1 and RA4 is further demonstrated in clustering analysis, as shown in heat maps ([Fig. 2A](#) and B) where fMet levels were enriched in a cluster of disease activity markers, whereas they did not associate with diagnostic markers of RA, including rheumatoid factor and anti-cyclic citrullinated peptide antibody titers. Finally, levels of mtNFPs were not only associated with *current* joint involvement ([Fig. 1B](#) and C and [Supplementary Fig. 3](#)), but also with *future* joint involvement, with baseline levels of mtNFPs correlating with joint involvement several years later in the disease process ($r = 0.28$, $p = 0.003$, [Fig. 1D](#), RA3).

3.3. Levels of mtNFPs (total fMet levels) associate with disease progression in combination with ACPA

In RA cohort 2 (follow-up cohort), mtNFP levels correlated significantly with current joint erosion and joint space narrowing ($r = 0.39$, $p = 0.0003$, and $r = 0.44$, $p < 0.0001$, respectively, [Fig. 3A](#) and B). We next asked whether baseline mtNFPs levels were predictive of development of erosive disease and extra-articular manifestations. We excluded patients with radiographic evidence of erosive disease at inception (10%) from this analysis. Although total fMet levels were strongly associated with erosion score at time of blood sampling as seen in the follow-up cohort, RA2 ([Fig. 3C](#)), baseline fMet levels by themselves did not predict development of high modified Sharp erosion score ($OR = 2.3$ (0.7–7.4), $p = 0.15$). Since ACPA positivity is a strong predictor of radiographic progression of joint erosion in RA patients [35], we next explored if fMet levels improve the prognostic value of ACPA in predicting erosive disease. Patients positive for ACPA or fMet exhibited increased odds of developing erosive disease with high modified Sharp erosion score (ACPA + or fMet + OR = 7.9 (2.3–27.7), $p = 0.001$) as compared to patients positive for ACPA alone (ACPA + OR (95%, CI) = 5.7 (2.0–15.9), $p = 0.001$). The sensitivity and specificity for ACPA, fMet and 'ACPA or fMet' for development of a high modified Sharp erosion score are 32.4%, 31.6% and 31.3%, respectively, and 92.2%, 83.6% and 94.5%, respectively.

Although known primarily for joint inflammation, about 50% of patients with RA will also develop extra-articular manifestations [36]. Extra-articular RA is a severe condition associating with worse disease

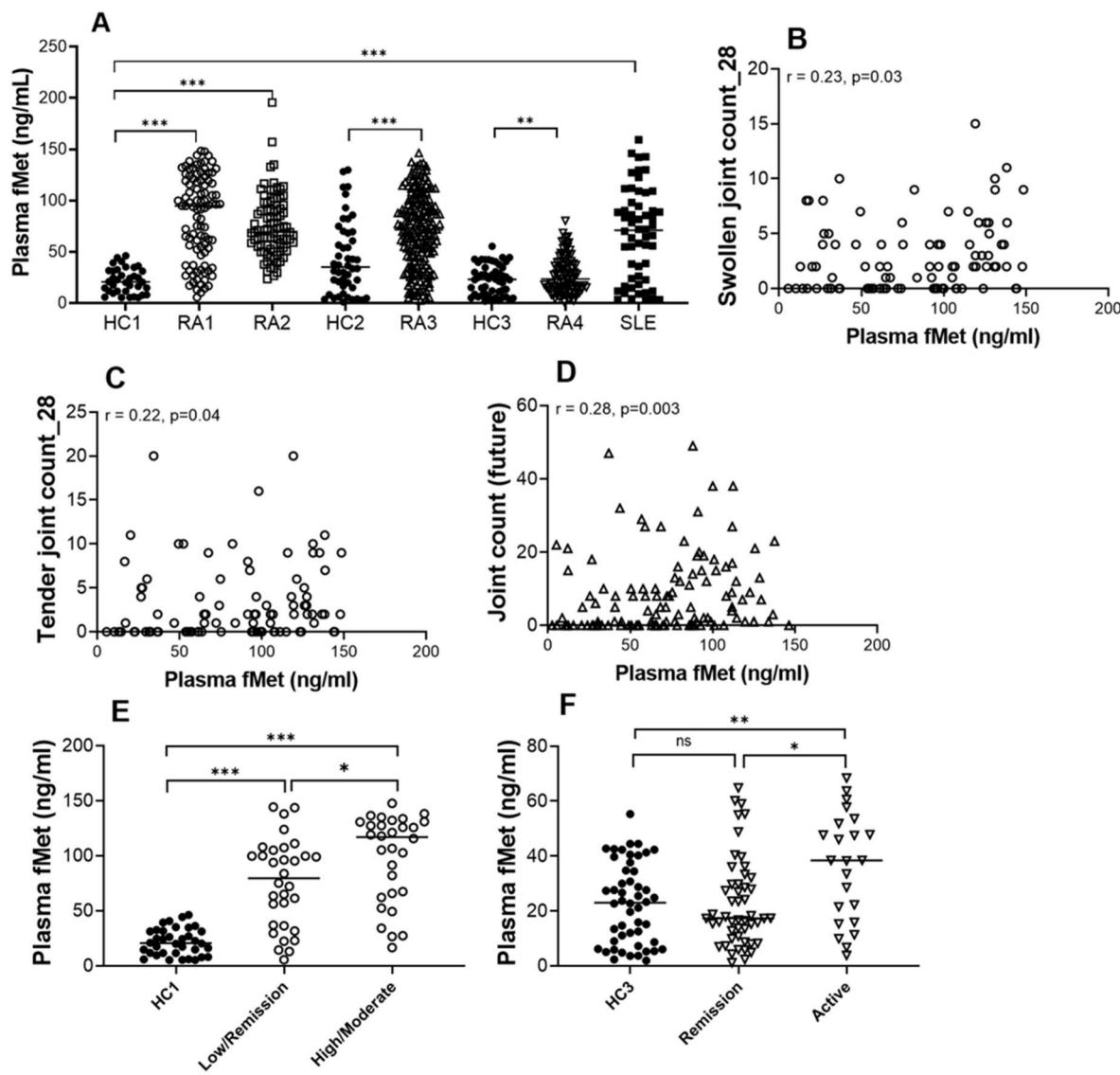


Fig. 1. Levels of mtNFPs are elevated in patients with RA and associate with disease activity. Total (fMet) and MT-ND6 specific levels of NFPs were analyzed by ELISA. A, Total fMet levels were analyzed in 4 cohorts of RA patients, a SLE cohort and 3 cohorts of healthy controls (HC). Serum samples were analyzed in cohorts RA1, RA2, RA4, SLE, HC1 and HC3. Plasma samples were analyzed in cohorts RA3 and HC2. For RA4, only fMet levels of patients with low to high DAS28-CRP (>2.6) were considered, but not levels in patients in remission (<2.6 DAS28-CRP). Correlation analysis between levels of total fMet and swollen joints (B), tender joints (C) of RA1 and future joint count (D) in the inception cohort, RA3. Healthy controls and RA patients were analyzed based on total fMet levels. Patients were further analyzed based on CDAI in RA1 (E) and DAS28-CRP in RA4 (F). Statistical analyses were done using Mann-Whitney U test, and Spearman correlation test with * P < 0.05, **p < 0.01, ***p < 0.0001, and ns, non-significant.

outcomes [36]. In an inception cohort of RA (cohort 3), 23 of 165 patients (14%) developed extra-articular nodules during follow-up, a mean of 8 years later. Further univariate analyses revealed that mtNFP levels at baseline are predictive of extra-articular (nodules) development (OR = 1.2, p = 0.04, 95% CI 1.0–1.4). The OR is for an increase of 10 ng/ml in baseline mtNFP levels.

3.4. MtNFPs (total fMet levels) associate with neutrophil activation markers and inflammation in patients with RA

Considering the ability of mtNFPs to induce neutrophil activation, we assessed the association of mtNFP levels with neutrophil activation markers. In all three RA cohorts we found circulating mtNFP levels to be associated significantly with neutrophil activation markers including levels of S100A8/9 or calprotectin, NETs and peroxidase (fMet vs. S100A8/9 and peroxidase, for RA1: r = 0.43, r = 0.43, p < 0.0001 for all analyses, Fig. 4A and Supplementary Fig. 4A; fMet vs. S100A8/9 and

NETs for RA2: r = 0.47, r = 0.57, p < 0.0001 for all analyses, Fig. 4B and Supplementary Fig. 4B; fMet vs. NETs and S100A8/9 for RA3: r = 0.25, p < 0.0005, r = 0.25, p < 0.0002, Supplementary Fig. 4C and D). There were also significant correlations between mtNFP levels and clinical markers of systemic inflammation, ESR and CRP (fMet vs. CRP and ESR for RA1: r = 0.60, p < 0.0001, r = 0.37, p < 0.004, Fig. 4C and D; fMet vs. CRP for RA2 and RA4: r = 0.74, r = 0.83, p < 0.0001 for all analyses, Fig. 4E and F). Further analysis revealed that total fMet levels in RA1 cluster with levels of neutrophil activation markers (peroxidase, and S100A8/9) and a clinical marker of inflammation, CRP (Supplementary Fig. 4E).

3.5. Circulating mtNFPs (total fMet) induce ROS production from neutrophils in an FPR1-dependent manner

MtNFPs signal through FPRs to induce neutrophil effector functions, including ROS generation. We thus asked whether NFPs, present in RA

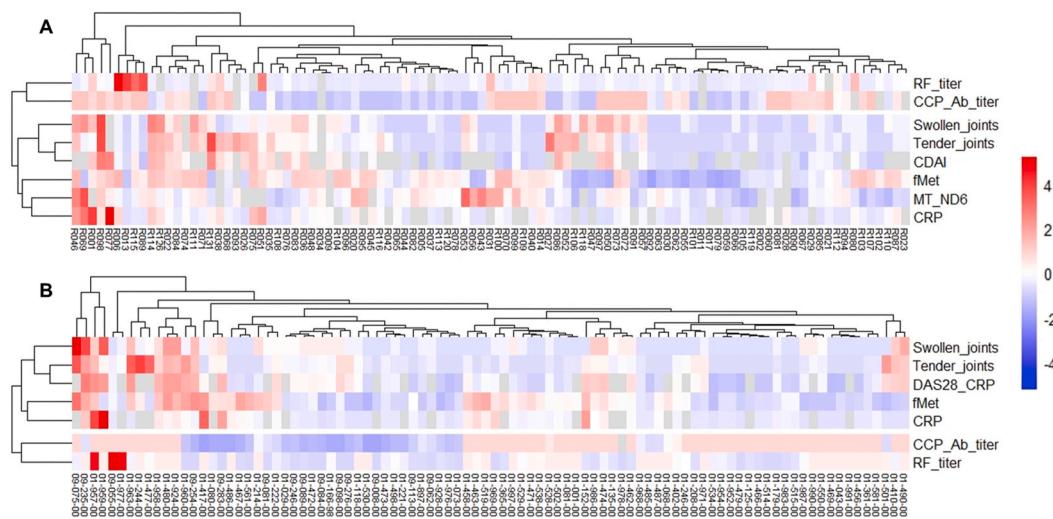


Fig. 2. Levels of mtNFPs (total fMet) in RA patients cluster with markers of disease activity and inflammation. Shown in A and B are heat maps showing hierarchical clustering of various disease activity, diagnostic and inflammatory markers with levels of mtNFPs in cohorts RA1 (A) and RA4 (B). Rows and columns represent markers and patients, respectively. Hierarchical clustering was performed using the R v4.0.2 pheatmap v1.0.12.

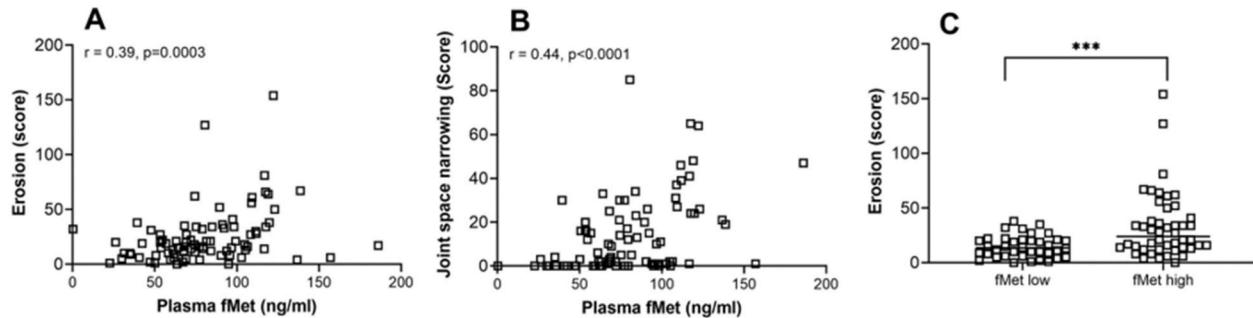


Fig. 3. MtNFPs (total fMet) circulating in RA plasma associate with joint damage. Correlation analysis between levels of total fMet and erosion score (A) joint space narrowing (B) and (C) comparison of erosion scores between patients stratified based on fMet levels as fMet low or fMet high (fMet levels above 95th percentile of HC are considered as fMet high) for follow-up cohort, RA2 white square □. Statistical analyses were done using Mann-Whitney U test, and Spearman correlation test with *** $p < 0.001$.

plasma, could promote neutrophil activation, such as ROS generation *in vitro*. We optimized the assay with fMLP, a prototype bacterial NFP, and formylated MT-ND6 peptide that are known to induce ROS generation in neutrophils. Cyclosporine H (CsH), an FPR1 inhibitor, could completely block the ROS generation induced by fMLP and formylated MT-ND6 whereas non-FPR1 triggered ROS generation (e.g. TLR7/8 agonist) could not be inhibited, thus confirming the specificity of the inhibitor (Supplementary Fig. 5). Compared to HC plasma, plasma from RA patients caused significant induction of ROS release from healthy control neutrophils ($p = 0.02$, Fig. 5A). However, this ROS induction could be due to many inflammatory mediators present in plasma, including proinflammatory cytokines. To determine whether RA plasma-induced ROS generation was mtNFP-dependent, RA plasma that were high ROS inducers (above 85th percentile of ROS induced by HC plasma) were analyzed for ROS induction by neutrophils in presence or absence of CsH. Although CsH treatment did not reduce ROS generation to baseline ($p = 0.001$, Fig. 5B) as for prototype NFPs, it did inhibit a significant proportion, ranging from 15% to 53% (Fig. 5C), of RA plasma-induced ROS formation by neutrophils, suggesting that mtNFPs in plasma are contributing to neutrophil activation. To further investigate the relevance of these circulating mtNFPs to disease activity, we compared neutrophil ROS-induction potential of plasma from patients with active disease and patients in remission with normal CRP (<3 mg/L) in FPR1-dependent manner i.e. by stimulating neutrophils with patient plasma in the presence or absence of CsH. Consistent with the

association of mtNFPs with disease activity (Fig. 1E), plasma from patients with active disease induced significantly elevated levels of ROS from neutrophils compared to plasma from patients in remission (Fig. 5D). Further, neutrophil ROS-induction by plasma from patients with active disease but not by plasma from patients in remission could be significantly attenuated with FPR1-inhibition suggesting that mtNFPs primarily contribute to disease-relevant inflammation in active disease.

4. Discussion

This study presents evidence of an association between mtNFP-mediated neutrophil activation in RA patients and inflammation, disease activity, and joint damage, supporting their potential role in amplifying a central disease-promoting process of RA pathogenesis via chronic neutrophil activation. Overall, we propose mtNFPs as novel clinical biomarkers for measure of disease activity and disease severity and mtNFP-mediated signaling as a potential therapeutic target of RA.

Several cell types, including neutrophils, activated platelets, mast cells, and damaged cells, are known sources of extracellular mitochondria and their derived products in sterile inflammatory pathologies like RA. Consistently Boudreau et al. [15] have demonstrated that synovial fluid of RA patients, but not osteoarthritis patients, have elevated levels of platelet-derived extracellular mitochondria. MtNFPs are intramitochondrial. Potential mechanisms of mtNFPs release from mitochondria include the digestion of mitochondrial membrane by enzymes

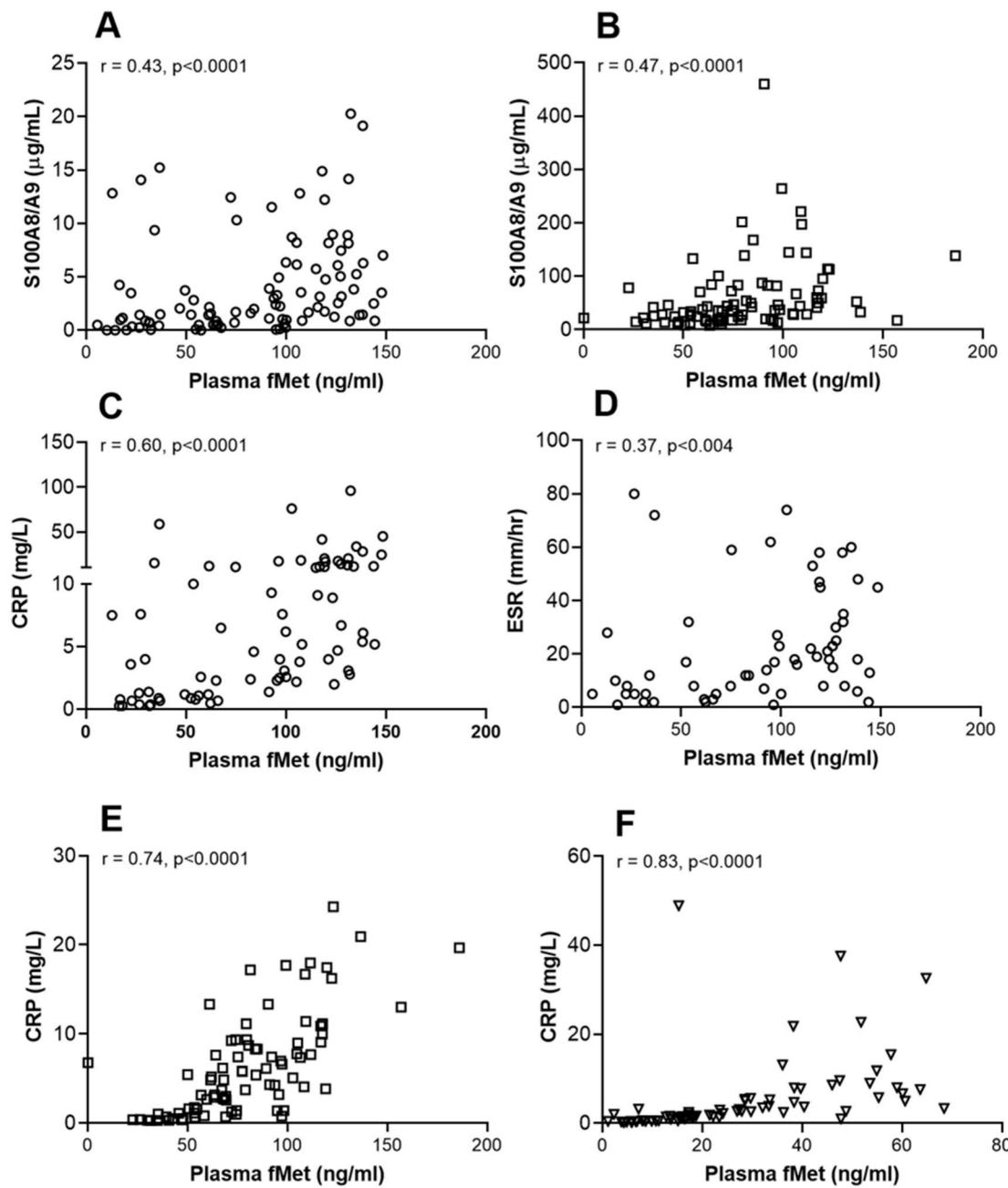


Fig. 4. Levels of mtNFPs (total fMet) in RA patients associate with neutrophil activation markers and clinical measures inflammation. Total fMet and S100A8/A9 levels were analyzed by ELISA. Shown in figures A, C and D, figures B and E and figure F are data from cohorts: RA1 (white circle \circ), RA2 (white square \square), and RA4 (white down-pointing triangle ∇), respectively. Statistical analyses were done using Spearman correlation test.

like secreted phospholipase A2 and/or complement-mediated lysis [15, 37]. Incidentally, secreted phospholipases A2 are observed at increased concentrations in the circulation of inflammatory conditions, including RA [38]. Consistent with the literature, we also found elevated levels of SPLA2 in the plasma of our RA patient cohort compared to controls (Supplementary Fig. 6), demonstrating the presence of a mechanism that can potentially contribute to elevated fMet levels in RA. However, further studies are needed to determine the pathway(s) essential in promoting mitochondrial disruption in RA.

Once released into the extracellular space, mtNFPs can be sensed by high-affinity FPR1 predominantly expressed on phagocytic leukocytes, including monocytes, macrophages with a high expression in neutrophils [39]. Activation of FPR1 by mtNFPs, akin to bacterial NFPs, elicit signaling cascades that culminate in diverse neutrophil effector

responses, including oxidative burst [18,23]. While not in the RA context, mtNFPs released during sterile injury were demonstrated to cause neutrophil migration and degranulation and elicit neutrophil-mediated organ injury [18]. However, the role of mtNFPs in neutrophil activation contributing to RA pathogenesis is unknown. We have previously reported on elevated levels of neutrophil activation markers in RA patients able to predict erosive disease and joint space narrowing [29]. Our current data demonstrate that plasma from RA patients had increased ability to induce ROS from neutrophils of healthy blood donors. FPR1 blockade experiments suggested that mtNFPs circulating in RA plasma contribute significantly to this immune activation of neutrophils. As further evidence, we found that RA patients have elevated levels of mtNFPs associating with neutrophil activation markers like calprotectin (S100A8/A9), peroxidase, and NETs, all of

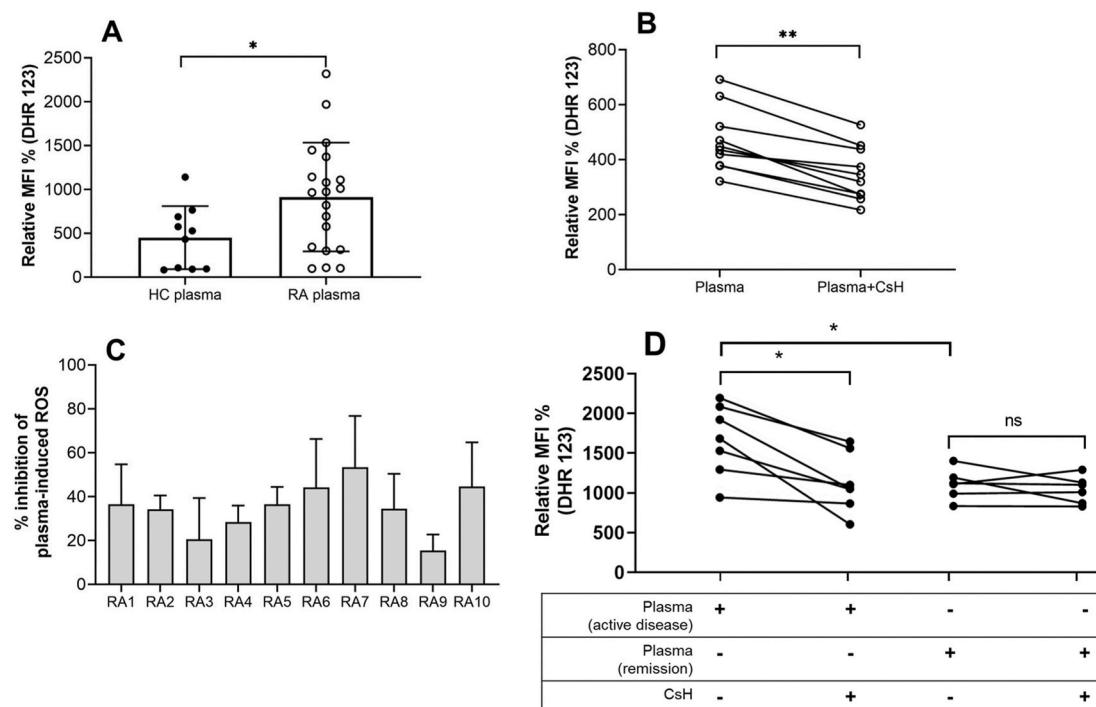


Fig. 5. MtNFPs (total fMet) circulating in RA plasma can induce ROS release from neutrophils in FPR1-dependent manner. (A) Neutrophil ROS induced by RA plasma compared to HC plasma. (B) Inhibition of RA plasma-induced neutrophil ROS by CsH. (C) Percent inhibition of RA plasma-induced neutrophil ROS. (D) Comparison of ROS-induction potential of plasma from patients with active disease and patients in remission from neutrophils with and without pretreatment with FPR1 inhibitor, CsH. Healthy neutrophils plated at 3×10^5 cells/well were incubated with a selective inhibitor of FPR1, CsH ($5 \mu\text{M}$) for 30 min prior to the addition of plasma (1:100 dilution) for an additional 60 min. DHR 123 ($0.5 \mu\text{M}$), was added during last 30 min of incubation and ROS was analyzed by flow cytometry. Relative MFI % was calculated as ROS induced by stimuli divided by media control $\times 100$; % inhibition was calculated as ROS induced by plasma only – ROS induced by plasma pretreated with CsH condition divided by ROS induced by plasma only $\times 100$. Statistical analyses were done using Mann-Whitney U test and Wilcoxon test for paired analyses with * <0.05 , and ** $p < 0.01$. Samples belong to RA1, white circle ○.

which are reported to be increased in RA patients [29]. Considering the clinical implications, mtNFP levels were associated with CRP and ESR, inflammatory markers commonly incorporated in disease activity indexes. In the current analysis both ACPA and RF, current diagnostic markers of RA, did not associate with disease activity index. In contrast, levels of mtNFPs significantly associated with disease activity and could distinguish patients based on disease activity as demonstrated in two independent RA cohorts. Additional analysis also revealed that mtNFPs do not cluster with RF and ACPA suggesting mtNFPs to be independent indicators of disease activity. Of note, levels of mtNFPs were elevated in some patients in clinical remission (CDAI ≤ 2.8), suggesting mtNFP could be a sensitive marker of ongoing subclinical disease activity. This is consistent with prior work from our group, and others, demonstrating a neutrophil activation signature in RA patients in clinical remission [29, 40]. Hence, when analyzed, circulating biomarkers like mtNFPs and neutrophil-related markers in combination with conventional blood markers may be useful in clinical practice to detect low-grade inflammatory activity of RA patients in clinical remission.

One of the key findings from the current investigation relates to the capacity of mtNFPs to associate with disease progression, e.g., development of future joint damage as well as rheumatoid nodules and mtNFPs, in combination with ACPAs, showed an improved ability to predict the development of the erosive disease than ACPA alone, which is one of the strongest predictors of radiographic progression of joint erosion in RA [35]. Interestingly, NETs were shown to make similar predictions, strengthening our proposition of mtNFP-driven neutrophil activation in RA [29]. The mechanistic details of mtNFP-driven extra-articular development in RA remains to be investigated. Given the study design, causality cannot be implied. However, consistent with our findings, prior work in a mouse model of septic arthritis clearly demonstrate that bacterial formylated peptides are sufficient in

mediating *S. aureus*-induced arthritis [41]. In that study, mice immunized with the wild-type strain of *S. aureus*, but not with an isogenic mutant strain lacking the ability to produce formylated peptides, developed arthritis, and severe joint destruction accompanied by the increased infiltration of neutrophils. Future studies involving arthritic animal models deficient in FPR1 are needed to characterize the mechanism of mtNFP-mediated joint injury in RA.

The study limitation includes analyses and experiments considering mtNFP-mediated activation of neutrophils alone. Although FPR1 is highly expressed on neutrophils, other immune cells implicated in RA pathogenesis, including platelets, monocytes, and macrophages, also express FPR1 suggesting the potential activation of those cells by circulating mtNFPs *in vivo*, warranting future biomarker and RA animal model studies analyzing these additional cell types. Further limitation includes the effect of different treatment regimens on the levels of mtNFPs considering that release mechanisms of mtNFPs are associated with inflammation. Hence, the inclusion of pre-clinical and treatment-naïve patients with RA in future studies should determine treatment effects on the levels of mtNFPs and validate the prognostic capacity of mtNFPs for disease activity. Although our longitudinal cohort allowed us to conduct predictive analyses for the joint destruction, sampling at the inception alone limited further analysis to assess the change in mtNFPs levels with disease progression and activity and substantiate the potential association of mtNFPs with joint destruction. Another potential limitation could be the alternate sources of formylated peptides other than mitochondria in the circulation of RA patients, which include bacteria, the prokaryotic ancestors of mitochondria. Given the variable evidence of increased intestinal permeability (i.e., leaky gut) in RA patients [42–44], whereby gut bacteria can gain access to the bloodstream, we cannot rule out the possibility that some of the measured NFPs in our study are potentially from bacterial sources and thus are not exclusively

of mitochondrial origin. The role of the gut-joint axis in RA pathogenesis majorly encompasses the molecular mimicry between antigens of gut microbiota and host proteins that can be enabled by increased intestinal permeability. This suggests a hypothesis where bacterial NFPs in the circulation of RA patients could contribute to chronic neutrophil activation as a novel mechanism through which leaky gut and its associated risk factors such as pathological inflammation across intestinal barrier and gut dysbiosis can contribute to RA pathogenesis; this will need to be investigated in future studies.

5. Conclusion

For the first time, we have demonstrated that mtNFPs are elevated in the circulation of patients with RA and promote neutrophil activation through formyl peptide receptor 1 (FPR1). Our data demonstrate the clinical value of mtNFPs in monitoring disease activity and in predicting disease severity, including extra-articular disease in RA patients, although these observations remain to be validated in larger patient cohorts. Several non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit fMLP-induced neutrophil activation [45–47]. Thus, it is likely that FPR1 antagonistic property of NSAIDs might contribute to, at least, some of their anti-inflammatory effects. Further, genetic deletion or pharmacological inhibition of FPR1 demonstrated significant anti-inflammatory effects in various *in vitro* and *in vivo* studies [48–50]. Hence, FPRs in the context of sterile injury and conditions of unresolved chronic inflammation like RA represent important therapeutic targets for ameliorating neutrophil-dominant inflammation.

Author contributions

BD and CL conceived the study. BD and CL designed experiments, analyzed data, and interpreted results. BD and AAB performed experiments. JLN, MKD, KDD, and MLF provided materials and clinical cohorts. BD wrote the manuscript and CL critically reviewed the manuscript. All authors reviewed and approved the manuscript.

Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2021.102630>.

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