Phenotypical and functional characterization of neutrophils in two pyrin-associated auto-inflammatory diseases

Running head: Neutrophils in pyrin-associated autoinflammation

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Abstract

Purpose. Familial Mediterranean Fever (FMF) and Pyrin-Associated Autoinflammation with Neutrophilic Dermatositis (PAAND) are clinically distinct autoinflammatory disorders caused by mutations in the pyrin-encoding gene *MEFV*. We investigated the transcriptional, phenotypical and functional characteristics of patient neutrophils to explore their potential role in FMF and PAAND pathophysiology

Methods. RNA sequencing was performed to discover transcriptional aberrancies. The phenotypical features, degranulation properties and phagocytic capacity of neutrophils were assessed by flow cytometry. Production of reactive oxygen species (ROS), myeloperoxidase (MPO) release and chemotactic responses were investigated via chemiluminescence, ELISA and Boyden chamber assays, respectively.

Results. Neutrophils from PAAND and FMF patients showed a partially overlapping, activated gene expression profile with increased expression of *S100A8*, *S100A9*, *S100A12*, *IL-4R*, *CD48*, *F5*, *MMP9* and *NFKB*. Increased *MMP9* and *S100A8/A9* expression levels were accompanied by high plasma concentrations of the encoded proteins. Phenotypical analysis revealed that neutrophils from FMF patients exhibited an immature character with downregulation of chemoattractant receptors CXCR2, C5aR and BLTR1 and increased expression of toll-like receptor 4 (TLR4) and TLR9. PAAND neutrophils displayed an increased random, but reduced CXCL8-induced migration. A tendency for enhanced random migration was observed for FMF neutrophils. PAAND neutrophils showed a moderate but significantly enhanced phagocytic activity as opposed to neutrophils from FMF patients. Neutrophils from both patient groups showed increased MPO release and ROS production.

Conclusions. Neutrophils from patients with FMF and PAAND, carrying different mutations in the *MEFV* gene, share a pro-inflammatory phenotype yet demonstrate diverse features, underscoring the distinction between both diseases.

60 **Keywords:** autoinflammation; neutrophils; FMF, PAAND; pyrin; innate immunity

Introduction

Autoinflammatory syndromes typically present with recurrent episodes of non-infectious fever accompanied by systemic and/or local inflammation predominantly driven by dysregulation of innate immune mechanisms, in the absence of autoimmunity hallmarks such as high-titer autoantibodies or autoreactive T cells [1,2]. Multiple autoinflammatory syndromes are caused by mutations in inflammasome-related genes [3]. Inflammasomes are composed of a cytoplasmic sensor that recognizes pathogen- or danger-associated conserved structural motifs or perturbations in intracellular homeostasis, an adaptor protein and a caspase [4–6]. Activation of the sensor causes inflammasome assembly and results in consecutive activation of caspase 1, interleukin (IL-) 1β and IL-18. A major cytoplasmic sensor is pyrin, which is found primarily in neutrophils, eosinophils and monocytes/macrophages [7–9]. Human pyrin is encoded by the *MEFV* ('Mediterranean fever') gene. During homeostasis, Rho A effector protein kinases N1 and N2 (PKN1 and PKN2) mediate phosphorylation of pyrin at residues S208 and S242, thereby facilitating binding to 14-3-3 proteins, keeping pyrin inactive [10]. Pyrin inhibition is halted upon stimulation with bacterial Type III Secretion System (T3SS) effector molecules or Rho-inactivating toxins, that inactivate Rho A and PKN1/2 resulting in loss of phosphorylation and 14-3-3 protein dissociation [11–14].

Mutations in pyrin cause Familial Mediterranean Fever (FMF), the prototype monogenic autoinflammatory disorder [1,2,15]. The precise underlying mechanisms of FMF are not fully understood but increased activation of the pyrin inflammasome has been demonstrated. Clinical characteristics are recurrent febrile episodes lasting for 1 to 3 days accompanied by arthritis and/or serositis, with or without erysipelas-like rash [16]. Recently, two novel mutations in exon 2 of pyrin were linked to an autoinflammatory disorder with a distinct clinical phenotype termed 'Pyrin-Associated Autoinflammation with Neutrophilic Dermatitis' (PAAND) [17,18]. These mutations, namely S242R and E244K, disrupt a phosphorylation site or its +2 position

and binding of pyrin to 14-3-3. PAAND is a chronic recurrent neutrophilic dermatosis with long-lasting inflammatory episodes [18]. Most FMF patients carry homozygous or compound heterozygous mutations, especially in exon 10 in the *MEFV* gene, whereas PAAND is caused by a single *MEFV* mutation. FMF patients can be treated with colchicine [19]. In PAAND patients, successful treatment with anakinra (recombinant IL-1RA) was achieved in one patient [18]. Tumour necrosis factor (TNF) blockage proved more effective in another patient with limited responsiveness to IL-1 β -targeted therapy [17].

Neutrophils are the predominant cells in skin lesions from PAAND patients and the most abundant circulating leukocytes during inflammatory attacks in FMF. These innate leukocytes have important pro-inflammatory, anti-microbial effector functions including phagocytosis, production of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETs) [20,21]. The functional role as well as transcriptional and phenotypical characteristics of neutrophils in the pathology of pyrin-associated autoinflammatory syndromes, are currently unknown (for PAAND) or have been unravelled only partially (for FMF) [22–25]. In the present study, we isolated peripheral blood neutrophils during disease remission (no flare) from PAAND and FMF patients and healthy controls (HCs). The freshly isolated cells were used for comprehensive gene expression analysis by RNA sequencing (RNAseq) and for extensive phenotypical and functional characterization.

Patients & Methods

Patients

Seven PAAND patients, thirteen FMF patients and thirty healthy controls (HCs) were recruited at the University Hospital Leuven (Suppl. Table S1) after informed consent according to the declaration of Helsinki. Patients were under treatment and displayed low disease activity at the moment of sampling (Suppl. Table S1). The Ethics Committee of University Hospitals Leuven approved this study (S59874).

Cytokine, S100A8/A9 and matrix metalloproteinase (MMP)-9 measurements

Plasma concentrations of IL-1 β , IL-6, IL-10, IL-18, IL-1RA, TNF- α , interferon (IFN)- γ and CXCL8 were determined using Meso Scale Discovery multiplex technology. S100A8/A9 levels were measured by DuoSet ELISA (R&D Systems, Minneapolis, MN) and MMP-9 activity by zymography as described [26].

Neutrophil isolation

Neutrophils were isolated from fresh (within 30 min after blood donation) peripheral blood by density gradient centrifugation (10 min, 20 °C, 218 g) in Pancoll (PAN Biotech GmbH, Aidenbach, Germany) and erythrocytes eliminated by hypotonic shock (30 sec) prior to induction experiments, analysis of production of ROS and chemotaxis assays. Alternatively, neutrophils were directly isolated from whole blood using the EasySep™ Direct Human Neutrophil Isolation Kit (Stemcell Technologies, Vancouver, Canada) and used for extensive phenotyping, analysis of phagocytosis, degranulation and RNAseq. To evaluate the effect of colchicine on the phenotypical and functional properties of neutrophils, cells were stimulated for 30 min at 37°C with 150 pg/ml or 250 pg/ml of colchicine (Sigma).

RNA Sequencing

RNA was extracted from neutrophils using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNAseq, preparative techniques and gene counting were performed by the KU Leuven Genomics core as described in the supplementary M&M. Count-based differential expression analysis between HCs, FMF and PAAND was done with R-based Bioconductor package DESeq2 [29]. Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls the false discovery rate (FDR). To select significantly differentially expressed genes (DEGs), a cut-off on fold change with an absolute log₂-ratio below 0.5 was combined with a FDR value less than 0.05. Graphical visualisation of DEGs was done using the R-based packages Pheatmap and Enhancedvolcano. Gene expression levels were validated using a TaqMan gene expression assay (Applied Biosystems). Primers used are listed in Suppl. Table 2.

Flow cytometry

Cells were incubated with FcR-block (Miltenyi Biotec) and stained extracellularly in FACS buffer [phosphate buffered saline (PBS) + 2% (v/v) fetal calf serum (FCS) + 2 mM ethylenediaminetetraacetic acid (EDTA)]. Antibodies are listed in Suppl. Table S3. For intracellular staining (TLR9), cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-human CD289. Before lineage gating, all populations were first gated on live cells visualized using Zombie Aqua 516 (Biolegend) or FV620 (BD Biosciences). Forward and side scatter were used to exclude debris and doublets. After acquisition on a BD LSRFortessa X20, samples were analysed using FlowJo (LLC, V10).

Degranulation and phagocytosis assay

Cells were stimulated with 50 ng/ml TNF- α (PeproTech, Rocky Hill, NJ) or 10 μ g/ml ultrapure lipopolysaccharide (LPS) from *E. coli* (Invivogen, San Diego, CA) in [PBS + 2 mM EDTA] at 37°C. After 30 min, pHrodo red *S. aureus* bioparticles (Invitrogen) were added and incubated for 30 min at 37°C. Negative controls were placed on ice without stimulation. Results were analysed by flow cytometry (*vide supra*). Degranulation was assessed based on the expression of granule-associated markers CD63, CD66b, CD11b and CD35 (Suppl. Table S3) as described [27].

Respiratory burst assay

ROS production by neutrophils was determined with a chemiluminescence-based assay as described in the supplementary M&M. Cells were stimulated with 150 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) or 50 ng/ml TNF- α in the presence of 5 mM luminol (Sigma).

Production of myeloperoxidase (MPO)

Neutrophils [2x10⁶ cells/ml in RPMI1640 complemented with 10% (v/v) FCS + 50 μ g/ml gentamycin (Gibco)] were stimulated with 200 ng/ml ultrapure LPS from *E. coli* (Invivogen, San Diego, CA). Supernatants were collected after 4 h at 37°C and analyzed for MPO using commercially available DuoSet ELISA kits (R&D Systems).

48-well Boyden chamber chemotaxis assay

The migratory responses of neutrophils towards 10 ng/ml C5a (R&D Systems), 10 ng/ml CXCL8 (72AA; PeproTech) and 10⁻⁸ M N-formylmethionyl-leucyl-phenylalanine (fMLF;

179 Sigma, Saint Louis, MO) were compared using the 48-well Boyden chamber chemotaxis assay
180 as described [28].

181

182 *Statistics*

183 A linear mixed model was used to detect statistical differences between the different study
184 groups. Correction for multiple samples per patient was done using a random intercept model.
185 Statistical analysis was performed using R.

186

Results*Neutrophils from patients with PAAND and FMF show a primed gene expression profile*

An in-depth analysis of the neutrophil transcriptome was performed by RNAseq on purified neutrophils from PAAND patients (n = 5), FMF patients (n = 5) and HCs (n = 4). Patients were under treatment with low disease activity at the moment of sampling, except for one FMF patient (Suppl. Table S1). The purity of the neutrophil transcriptome was confirmed using the cell type enrichment tool CTen. The top 50 mostly expressed genes are shown in a heatmap (Suppl. Fig. S1A).

Principal components analysis (PCA) revealed a partial overlap between neutrophils from FMF and PAAND patients, suggesting a similar transcriptome (Fig. 1A). In contrast, transcriptomes from patient and HC neutrophils were distinct, only showing a minor overlap in the PCA plot (Fig. 1A). Based on the count-based DEGs, we determined the genes that were significantly up- or downregulated after adjusting for multiple testing (adjusted p-values <0.05 were considered significant). Complete lists of DEGs are shown in Suppl. Tables S4-6. Fig. 1B shows a heatmap of the top 70 differently expressed genes between the different groups. Comparing the transcriptome of FMF patients and HCs revealed 481 DEGs, with 165 and 316 genes, respectively, being up- and downregulated in FMF. Between PAAND and HCs, 233 genes were differentially expressed, 162 genes were significantly upregulated and 71 genes were significantly downregulated in neutrophils from PAAND patients. Interestingly, 83 overlapping genes were significantly different in both PAAND and FMF when compared to HCs (Suppl. Table 7). Gene ontology showed that DEGs were involved in positive regulation of respiratory burst, phagocytosis and neutrophil regulation (Suppl. Fig. S1B). In contrast, only 58 significantly differently expressed genes in FMF compared to PAAND patients were found of which 19 were upregulated in FMF and 39 were downregulated in FMF. Fig. 1C shows the volcano plots of DEGs in neutrophils from PAAND or FMF compared to HCs (cut off adjusted

p-value: 0.05, cut off fold-change: 0.5). Genes upregulated in patient neutrophils included nuclear factor kappa-light-chain-enhancer of activated B cells (*NFKB*), factor V (*F5*), and *IL-4R*, all genes indicative of a primed phenotype. Alarmins *S100A8/9* and *S100A12* were significantly upregulated only in PAAND patients, while a non-significantly increased expression was measured in FMF neutrophils. In contrast, both *MMP9* and *CD48* were significantly upregulated in FMF whereas PAAND patients only showed a non-significantly increased expression. Importantly, increased *MMP9* and *S100A8/9* expression levels were accompanied by high levels of the encoded proteins in plasma (Table 1). Analysis of *S100A8*, *S100A9*, *S100A12*, *IL-4R*, *CD48*, *F5*, *MMP9* and *NFKB* expression levels in a second batch of RNA samples from PAAND patients (n = 7), FMF patients (n = 6) and HCs (n = 10) by single-target qPCRs revealed upregulation of all genes in both patients groups (Suppl. Fig. S2). Results from single-target qPCR analysis, but not RNAseq data, suggested upregulation of *MMP8* and *IL-1 β* expression by neutrophils from PAAND and FMF patients as compared to HCs (Suppl. Fig. 2). Collectively, these data suggest that peripheral blood neutrophils from patients with PAAND and FMF are activated cells. However, we could not identify a group of functionally relevant genes that is specifically related to FMF or PAAND.

Neutrophils from patients with PAAND and FMF display phenotypical differences and have altered maturation states

Results from gene expression studies (*vide supra*) sparked our interest to perform an extensive phenotypical and functional analysis of patient neutrophils. First, the inflammatory environment was assessed in the blood by determining the concentration of 7 cytokines, CXCL8, *S100A9/A9* and *MMP-9* (Table 1). Quantification of inflammasome-related cytokines *IL-1 β* and *IL-18* revealed enhanced *IL-18* concentrations in plasma from FMF patients as compared to individuals with PAAND or HCs (Table 1). Compared to HCs and FMF patients, PAAND patients displayed elevated plasma concentrations of *IFN- γ* and a tendency towards

increased IL-6 and TNF- α . Plasma concentrations of the neutrophil-attracting chemokine CXCL8 and anti-inflammatory IL-10 and IL-1RA did not vary significantly between the study groups. Taken together, these results support the idea that FMF and PAAND are featured by distinct plasma cytokine profiles [17]. Next, neutrophils were isolated from the blood. Neutrophils numbers were significantly higher in PAAND patients compared to HCs, whereas they were not altered in FMF (Table 1). No significant differences were found in the mean fluorescent intensity (MFI) of the neutrophil lineage-markers CD66b and CD15 (Suppl. Fig. S3A-B). The expression of the Fc γ receptor III (CD16) was moderately but significantly decreased on neutrophils of FMF patients (Suppl. Fig. S3C). The chemoattractant receptor CXCR1 was slightly but significantly upregulated on neutrophils from PAAND patients as compared to HC cells (Suppl. Fig. S4A). Neutrophils from FMF patients were featured by significantly lower levels of CXCR2, C5aR (C5a complement receptor) and BLTR1 (leukotriene B4 receptor) as compared to HCs or PAAND patients (Suppl. Fig. S4B-D). No differences in protein expression of IL-1R2 or CD11b were found on neutrophils from patients with PAAND or FMF (Suppl. Fig. S5A-B). Evaluation of the expression of TLR2, TLR4, TLR6 and TLR9 revealed significantly increased levels of TLR4 and TLR9 on neutrophils from FMF compared to PAAND patients (TLR4) or HCs (TLR4 and TLR9) (Suppl. Fig. S6). CD10 expression, which is restricted to mature cells [30], was moderately but significantly decreased only on neutrophils from FMF patients (Fig. 2A). To assess neutrophil maturation characteristics in more detail, the expression of CD16 and CD62L (L-selectin) was assessed (Fig. 2B-C, Suppl. Fig. S5C). In non-inflammatory conditions, nearly all circulating neutrophils are mature, CD16^{high}CD62L^{high} cells with 3-4 nuclear lobes. Immature neutrophils have a banded nucleus and are CD16^{dim}CD62L^{high}. Hypersegmented neutrophils are phenotypically defined as CD16^{high}CD62L^{low} cells and have more than 4 nuclear lobes. A non-significant ($p = 0.07$) increased percentage of immature neutrophils was found in FMF patients

compared to HCs (Fig. 2B, D, F). One PAAND patient showed an increased percentage of hypersegmented neutrophils (Fig. 2C, E). Since FMF patients and two PAAND patients were treated with colchicine, we evaluated the effects of colchicine on the phenotypical characteristics of HC neutrophils (Suppl. Fig. S7). In line with the observations made in FMF patients, a decreased expression of CXCR2, C5aR and BLTR1 was measured on colchicine-treated neutrophils. No differences were observed in maturation state (as evidenced by the MFI of CD10) or TLR expression, but a shedding of CD62L was measured upon colchicine treatment. Taken together, neutrophils from FMF patients, but not PAAND neutrophils, show a reduced maturation and have increased expression of TLR4 and TLR9. Alterations in expression of CXCR2, C5aR and BLTR1 on neutrophils potentially result from colchicine therapy.

Neutrophils from patients with PAAND and FMF display altered pro-inflammatory activities

Upon activation, neutrophils may execute multiple pro-inflammatory activities including the release of ROS and phagocytosis. FMF neutrophils, in particular those from patients with homozygous mutations, displayed significantly enhanced ROS production under basal conditions as well as after stimulation with TNF- α compared to neutrophils from HCs (Fig. 3A-B). Neutrophils from PAAND also displayed enhanced basal and TNF- α -induced ROS production as compared to HCs (Fig. 3A-B). Colchicine treatment had no major impact on the basal ROS production of HC neutrophils (Suppl. Fig. S8A). Numbers of phagocytosing neutrophils were not significantly different between the study group, although a tendency towards an increased percentage of cells having beads engulfed was observed for PAAND patients (Fig. 3C). The number of engulfed beads (represented by the MFI) was significantly lower for FMF neutrophils compared to HCs whereas PAAND neutrophils showed a moderately but significantly enhanced phagocytic activity (Fig. 3D). Colchicine did not affect

the phagocytic capacity of HC neutrophils (Suppl. Fig. S8B-C). Examination of cell culture supernatants 4 h after sub-cultivation under basal conditions or in the presence of LPS revealed a tendency towards an increased release of MPO – an enzyme stored in azurophilic granules that converts H₂O₂ to hypochlorous acid – by neutrophils from some PAAND and FMF patients (Fig. 3E-F). Our results indicate that neutrophils from PAAND and FMF patients show a tendency of elevated pro-inflammatory activity.

Neutrophils from patients with PAAND and FMF show a primed phenotype

Neutrophil priming occurs when quiescent neutrophils are (continuously) exposed to inflammatory agents or mechanical stress, and results in increased responsiveness to subsequent stimulation. The activated gene expression profile, increased ROS production and the observed tendency towards increased concentrations of the granule-associated protease MMP-9 and cytosolic alarmin S100A8/A9 in plasma from FMF and PAAND patients already suggest enhanced neutrophil activation (Table 1). Interestingly, an increased percentage of small and large granular neutrophils was found in most PAAND patients as compared to FMF patients or HCs (Fig. 4A-B). Degranulation of neutrophils upon *in vitro* stimulation with LPS or TNF- α was studied (Fig. 4 and Suppl. Fig. S9) based on surface expression of CD35 (secretory vesicles), CD66b (specific granules), CD11b (specific granules, gelatinase granules, secretory vesicles) and CD63 (azurophilic granules) [31]. No significant differences in expression of CD35 were detected (Fig. 4C and Suppl. Fig. S9). PAAND neutrophils displayed significantly higher levels of CD66b and CD63 upon stimulation with TNF- α , but not after LPS stimulation or in medium conditions, as compared to HCs (Fig. 4D/F). Additionally, neutrophils from PAAND patients showed enhanced CD11b expression in medium conditions but no effects were observed upon LPS or TNF- α stimulation (Fig. 4E & Suppl. Fig S9).

In conclusion, neutrophils from PAAND patients show a tendency of increased degranulation that is reflected by the increased plasma levels of S100A8/9 and MMP-9.

Random migration and chemotactic responses of neutrophils from PAAND and FMF patients

The migratory behavior of PAAND, FMF and control neutrophils was evaluated *in vitro*. As compared to HCs, neutrophils from PAAND patients displayed enhanced spontaneous migration and a tendency towards increased random migration was observed for FMF (Fig. 5A). Migration induced by the classical neutrophil-chemoattractants CXCL8, C5a and fMLF was examined by calculating the chemotactic indices (CI) [28]. Comparison between CI from PAAND patients and HCs showed impaired CXCL8-dependent chemotaxis and a tendency towards reduced C5a-induced migration (Fig. 5B-C). Neutrophils from FMF patients showed a tendency towards decreased CXCL8- and C5a-mediated migration as compared to HCs. Differences were less clear for fMLF-induced migration (Fig. 5D). However, in absolute cell numbers, neutrophils from FMF patients showed a tendency towards enhanced fMLF-induced chemotaxis (Suppl. Fig. S10). Colchicine treatment did not affect migration of HC neutrophils (Suppl. Fig. S11). Taken together, neutrophils from both PAAND and FMF patients show a (tendency towards) increased spontaneous migration *versus* reduced chemoattractant-directed migration.

Discussion

PAAND and FMF are caused by disease-specific mutations in the *MEFV* gene, resulting in an excessive pyrin-dependent maturation of IL-1 β and IL-18. In contrast to FMF, patients with PAAND, have severe recurrent neutrophilic dermatosis and prolonged fever attacks [17,18]. Distinct mechanisms of pyrin activation may contribute to the disparate clinical features in FMF and PAAND patients. Indeed, whereas mutations causative for PAAND presumably result in

loss of phosphorylation and spontaneous activation of pyrin, no scientific consensus exists regarding the precise underlying mechanism of pyrin activation in FMF.

FMF and PAAND patients typically present with neutrophilia and a massive influx of neutrophils into affected tissues [2,18]. Neutrophils express high pyrin levels and were identified as important IL-1 β source in several models of inflammation and infection [32–38]. However, only limited transcriptional, functional and phenotypical data are available on neutrophils from PAAND and FMF patients. Published results revealed that neutrophils from FMF patients display an activated phenotype at the mRNA level, even during disease remission [22]. Consistently, FMF neutrophils displayed enhanced spontaneous CD62L shedding during the first two hours of *ex vivo* subcultivation [25]. In addition, the formation of NETs is proposed to play a major role during inflammatory attacks in FMF [24].

These clinical and experimental findings support the notion that neutrophils may play an important pathological role in pyrin-associated autoinflammation, sparking our interest to study neutrophils from FMF and PAAND patients. The present study is the first to provide a detailed overview of transcriptional, phenotypical and functional characteristics of neutrophils derived from the patients' peripheral blood. The study population included seven related PAAND patients with a S242R mutation, thirteen FMF patients with M680I or M694V mutations, and thirty age- and sex-matched HCs. All included individuals were adults and patients had low disease activity when samples were collected.

In-depth analysis of the neutrophil transcriptome by RNAseq and single-target qPCR revealed that neutrophils from FMF and PAAND patients have an activated gene expression profile, as indicated by enhanced expression of *S100A8*, *S100A9* and *S100A12*, *IL-1 β* , *IL-4R*, *CD48*, *F5*, *MMP8*, *MMP9* and *NF κ B*, among others. As expected, profound differences were observed between neutrophils from HCs and the two patient groups, while a partial overlap was found between the transcriptomes of FMF and PAAND neutrophils with genes indicating an activated

state of the neutrophils. We did not identify a group of functionally relevant genes that is specifically related to FMF or PAAND.

Analysis of the neutrophil maturation state uncovered an increased number of immature neutrophils – a hallmark of systemic inflammation – in FMF. In contrast, one PAAND patient showed an increased percentage of hypersegmented neutrophils, which are prone to activation and associated with inflammation. Compared to HCs, neutrophils from FMF patients showed a reduced phagocytic activity, increased ROS release and a tendency towards increased MPO production. Enhanced spontaneous MPO production by FMF neutrophils was recently described in another patient cohort five hours after subcultivation [25]. PAAND neutrophils showed a significantly increased phagocytic response, displayed an enhanced granularity and enhanced ROS production. These data suggest that neutrophils from patients with pyrin-associated autoinflammation display a primed phenotype, even during the remission phase.

Plasma cytokine levels were determined to provide more insights into the original environment of isolated neutrophils. We confirmed the differential cytokine profiles in both patient groups [17]. In line with the more profound features of systemic inflammation in FMF, increased concentrations of IL-18 were detected in plasma from FMF patients. Interestingly, recent experimental evidence suggests that neutrophils from FMF patients are a potentially important source of IL-18 [25]. PAAND patients showed increased plasma concentrations of IFN- γ and a tendency towards enhanced IL-6. A tendency towards increased MMP-9 activity was observed in plasma from FMF and PAAND patients. Moreover, levels of S100A8/A9 were at least ten times higher in plasma from FMF and PAAND patients as compared to HCs. These observations are in line with the increased expression of the corresponding genes.

In addition to neutrophil activation, also neutrophil migration requires tight regulation. We found that neutrophils from PAAND patients show increased spontaneous migration. FMF neutrophils also displayed a tendency towards enhanced random migration. The increased

spontaneous migration was recently reported to be significantly increased in another cohort of FMF patients [39]. When we analyzed the chemoattractant-directed migration, a significantly reduced migration in response to CXCL8 for neutrophils from PAAND patients was found if chemotactic indices were assessed. Neutrophils from FMF patients also displayed a tendency towards impaired chemoattractant-induced migration. The reduced chemoattractant-directed migratory responses can be explained partially by the observed increased random migration of patient neutrophils. Moreover, the moderately but significantly reduced protein levels of the major chemoattractant receptors CXCR2, BLTR1 and C5aR on neutrophils from FMF patients may also contribute to the reduced chemotactic response, although the expression levels of chemoattractant receptors on cells do not necessarily recapitulate the functional response induced upon ligand stimulation [40–43]. Worth mentioning, the differences in chemoattractant receptor levels were only found at the protein level and suggest an altered receptor turn-over rate rather than transcriptional differences.

We acknowledge that our sample size is small due to the rarity of PAAND and the practical limitations that come with neutrophil experiments (including the need for freshly isolated cells). Individual PAAND patients were included multiple times at different time points. We corrected for the presence of replicates of individual patients by using a random intercept statistical model. Additionally, FMF patients had distinct mutation states and patients were at differential treatment strategies (Suppl. Table S1). The use of colchicine in particular may interfere with downstream analyses. This inhibitor of microtubule polymerization accumulates in neutrophils and may interfere with cellular processes including migration and adhesion, phagocytosis and production of cytokines and superoxide [44–48]. In our hands, colchicine-treatment induced a reduction of CXCR2 and C5aR expression levels on HC neutrophils and promoted TNF- α -induced ROS production, but did not affect basal ROS production, phagocytosis and chemotaxis.

411 To conclude, our results indicate that neutrophils from patients with PAAND and FMF have an
412 activated gene expression profile and are characterized by unique primed phenotypes with
413 enhanced pro-inflammatory properties (Suppl. Table S8 summarizes our main conclusion).
414 Comparison between neutrophils from the two pyrin-associated autoinflammatory syndromes
415 favors the idea that PAAND and FMF are distinct diseases.

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Authorship contributions

All authors contributed to the study conception and design. Erika Van Nieuwenhove, Albrecht Betrains, Lien De Somer, Steven Vanderschueren, Ellen De Langhe and Carine Wouters were responsible for diagnosis and recruitment of patients and/or collection of clinical data. Bert Malengier-Devlies, Mieke Metzemaekers, Mieke Gouwy, Maaïke Cockx, Lotte Vanbrabant and Noëmie Pörtner performed experiments and analyzed data. Bert Malengier-Devlies and Mieke Metzemaekers performed statistical analysis under supervision of Jorgen Vercauteren. Paul Proost, Patrick Matthys and Carine Wouters jointly supervised the study. The first draft of the manuscript was written by Bert Malengier-Devlies and Mieke Metzemaekers and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosure of conflicts of interest

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Table 1. Plasma cytokine levels

	HCs	PAAND	FMF
Age in years	28 (18-64)	49 (19-76)	34.5 (20-62)
Gender (M/F)	11/19	4/3	6/7
IL-1β (pg/ml)	0,01 (0.01-0.01) (n = 12)	0,01 (0.01-4.10) (n = 6)	0,01 (0.01 -0.42) (n = 8)
IL-6 (pg/ml)	0,47 (0.08-0.96) (n = 12)	2,64 (0.43-15.11) (n = 6)	0,43 (0.04-46.4) (n = 8)
IL-10 (pg/ml)	0,18 (0.11-0.57) (n = 12)	0,27 (0.07-5.01) (n = 6)	0,29 (0.03-1.92) (n = 8)
IL-18 (pg/ml)	440,9 (310.1-1684.0) (n = 12)	939,1 (567.9-1571.0) \$ (n = 6)	4330,0 (1262.0-20076.0) *, \$ (n = 8)
IL-1RA (pg/ml)	150,4 (72.64-316.0) (n = 12)	209,1 (143.5-13910.0) (n = 6)	187,0 (93.1-597.7) (n = 8)
IFN-γ (pg/ml)	7,13 (4.20-15.96) (n = 12)	21,80 (4.13-336.30) *, \$ (n = 6)	6,3 (1.0-16.2) \$ (n = 8)
TNF-α (pg/ml)	1,63 (0.22-7.31) (n = 12)	2,68 (1.17-22.70) (n = 6)	1,7 (0.5-5.1) (n = 8)
CXCL8 (pg/ml)	2,34 (1.05-2.71) (n = 12)	2,24 (1.14-12.32) (n = 6)	2,1 (1.1-10.5) (n = 8)
S100A8/A9 (ng/ml)	249,4 (69.4-597.0) (n = 12)	3287 (741.8-5710) (n = 6)	2495 (1102-97687) (n = 8)
MMP-9 (pmol/ml)	1,95 (0.48-5.07) (n = 12)	3,34 (0.44-30.20) (n = 6)	2,83 (1.29-19.68) (n = 8)
Neutrophils (cells/ml)	1,65*10 ⁶ (0.77-2.24)	2,375*10 ⁶ (1.06-3.34) ***, \$	1,475*10 ⁶ (0.85-3.64) \$

Overview of patients and controls (HCs). Age, neutrophil numbers (determined after immuno-magnetic purification as cells per ml of blood), plasma cytokine and S100A8/A9 concentrations and MMP-9 activities are represented as median (range). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * p<0.05, *** p<0.001; for statistical differences between patients and HCs, \$ p<0.05; for statistical differences between patient groups

Figure Legends**Fig. 1. Distinct gene expression profile in neutrophils from PAAND or FMF patients**

RNA-sequencing was performed on neutrophils from 5 PAAND patients, 5 FMF patients and 4 HCs. Neutrophils were isolated by immunomagnetic negative selection. (A) Principle component analysis (PCA) using the most variable genes (B) Heatmap of DEGs. Colors indicate expression profiles (as Z-scores:red high, blue low expression). (C) Vulcano plot of DEGs with adjusted p-values. Plot shows statistical significance versus magnitude of gene expression change (fold-change). A cut-off of 0.05 and 0.5 was (dotted lines) chosen for the adjusted p-values and fold-change, respectively.

Fig. 2. Phenotypical analysis of neutrophils from patients with pyrin-associated autoinflammation

Neutrophils from PAAND patients, FMF patients and HCs were isolated by immunomagnetic negative selection and analysed by flow cytometry and cytopsin. (A) Box whisker plot indicate expression levels on neutrophils [mean fluorescence intensity (MFI)] of CD10. (B-C) Based on CD16 and CD62L expression, neutrophils were divided into immature ($CD16^{dim}CD62L^{high}$) (B) and hypersegmented neutrophils ($CD16^{high}CD62L^{low}$) (C) and their relative number was shown. (D-F) Representative neutrophil cytopsin images from a HC (D), a PAAND patient (E) and an FMF patient (F). Hypersegmented neutrophils and immature neutrophils were indicated by an asterisk (*) or a hashtag (#), respectively. Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$

Fig. 3. Altered ROS production and phagocytic activity of neutrophils from patients with pyrin-associated autoinflammation

(A-B) Neutrophils from PAAND patients, FMF patients and HCs were exposed to (A) RPMI1640 medium alone or (B) 50 ng/ml TNF- α in the presence of 5 mM luminol and kinetic measurements of luminol oxidation were performed during 3 h at 37°C. ROS production was assessed by calculating fold changes of luminol oxidation, *i.e.* the differences between the lowest value and the maximal value during the incubation period. (C-D) Neutrophils were incubated with pHrodo red *S. aureus* bioparticlesTM during 30 min at 37°C and bead uptake was evaluated by flow cytometry. Results are presented as (C) percentage of cells that engulfed beads and (D) the amount of engulfed beads/cell (represented by the MFI). (E-F) Neutrophils were cultured in the absence (E) or presence of 200 ng/ml ultrapure LPS (F). Supernatants were collected after 4h of incubation at 37°C and MPO concentrations were determined by ELISA. Each dot in box whisker plots represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. Altered granularity and degranulation capacity of neutrophils from patients with pyrin-associated autoinflammation

(A-B) Forward scatter (FSC) and side scatter (SSC) were used to evaluate the neutrophil size and granularity. (A-B) Percentage of small and large granular neutrophils in HCs, patients with PAAND or FMF. (C-F) Neutrophils were cultured for 1 h with 50 ng/ml TNF- α at 37°C. Relative expression levels (MFI) of CD35 (secretory vesicles), CD66b (specific vesicles),

CD11b (gelatinase vesicles) and CD63 (azurophilic vesicles) were plotted to assess degranulation of neutrophils. Each dot represents an individual donor ($n \geq 5$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Migratory behavior of neutrophils from patients with pyrin-associated autoinflammation

Random and chemoattractant-induced migratory responses of freshly isolated neutrophils from PAAND patients, FMF patients and HCs were investigated *in vitro* using the 48-well Boyden chamber chemotaxis assay. Cells were seeded in the top well of the chamber and exposed in the lower well to (A) buffer alone, (B) 10 ng/ml CXCL8, (C) 10 ng/ml C5a or (D) 10 nM fMLF and migration was allowed during 45 min at 37°C. Results are represented as number of migrated cells in 10 high power microscopic fields or as chemotactic indices (*i.e.* total number of neutrophils that migrated in response to chemoattractant divided by the number of cells that migrated in response to buffer alone) for random and chemoattractant-induced migration, respectively. Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$.

647 **Fig. 1**

Fig. 2

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Phenotypical and functional characterization of neutrophils in two pyrin-associated auto-inflammatory diseases

Running head: Neutrophils in pyrin-associated autoinflammation

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Supplementary Material for Review

Supplementary Material & Methods*Respiratory burst assay*

Cells were suspended in Roswell Park Memorial Institute medium 1640 (RPMI1640, Gibco, Paisley, Scotland) (without phenol red) at a final concentration of 1.5×10^6 cells per ml, seeded in a white 96-well plate (PerkinElmer, Waltham, MA) and stimulated in duplicate. Kinetic measurements of luminol oxidation were performed during 3 h at 37°C using a Clariostar Monochromator Microplate Reader (BMG Labtech, Ortenberg, Germany). The results were subtracted by values obtained with PMA stimulation in the absence of luminol and converted to fold changes of luminol oxidation *i.e.* (maximum – minimum)/minimum. The maximum and minimum values refer to the maximum and minimum luminescence values during the 3 h incubation period.

RNA sequencing, library preparation and quality check

Sequence libraries were prepared with the Lexogen QuantSeq 3' mRNA-Seq Library prep kit according to the manufacturer's protocol. Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies) with the DNA 1000 kit (Agilent Technologies) according to the manufacturer's recommendations. Libraries were diluted to a final cDNA concentration of 2 nM and subsequently sequenced on an Illumina HiSeq4000 platform according to the manufacturer's recommendations. Single-end reads of 50 bp length were produced with a minimum of 1M reads per sample. Quality control of raw reads was performed with FastQC v0.11.7. (1). Adapters were filtered with ea-utils fastq-mcf v1.05. (<https://github.com/ExpressionAnalysis/ea-utils>). Splice-aware alignment was performed with Hisat2 (<http://daehwankimlab.github.io/hisat2/>) against the human reference genome hg19 using the default parameters. Reads mapping to multiple loci in the reference

genome were discarded. Resulting BAM alignment files were handled with Samtools v1.5. (2). Quantification of reads per gene was performed with HT-seq Count v2.7.14.

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Supplementary Figures & Tables

Suppl. Fig. S1. Top 50 expressed genes identified from the transcriptome of neutrophils

B

GO biological process complete	Number of genes	Adjusted p value
B cell receptor signaling pathway	132	8,25E-04
regulation of immune system process	1734	1,12E-03
humoral immune response	386	1,41E-03
phagocytosis, recognition	106	2,09E-03
defense response to protozoan	25	2,10E-03
positive regulation of respiratory burst	9	4,58E-03
phagocytosis, engulfment	131	5,51E-03
regulation of immune effector process	476	5,64E-03
neutrophil degranulation	482	6,00E-03
glomerular filtration	13	7,69E-03
complement activation, classical pathway	161	9,74E-03
defense response to Gram-positive bacterium	99	1,06E-02
humoral immune response mediated by circulating immunoglobulin	167	1,11E-02
positive regulation of B cell activation	170	1,19E-02
negative regulation of immune response	345	1,21E-02
innate immune response in mucosa	22	2,08E-02
hormone-mediated signaling pathway	134	3,28E-02
opsonization	73	3,62E-02
cellular response to steroid hormone stimulus	144	4,09E-02
regulation of signal transduction	3164	4,12E-02
translational initiation	144	4,13E-02
negative regulation of macromolecule metabolic process	2928	4,14E-02
retina homeostasis	79	4,33E-02
negative regulation of response to external stimulus	565	4,84E-02

RNA-sequencing was performed on neutrophils from 5 PAAND patients, 5 FMF patients and 4 HCs. Neutrophils were isolated by immunomagnetic negative selection. Heatmap showing

the top 50 highly expressed genes. Colours indicate normalized gene expression (scale represents number of counts x10 000)

Suppl. Fig. S2. Analysis of gene expression by qPCR

Neutrophils from PAAND patients, FMF patients and HCs were isolated by immunomagnetic negative selection and analysed by flow cytometry. RNA was extracted and relative expression levels were analyzed by qPCR. (A-J) Box whisker plot show the expression levels of *S100A8*

(A), *S100A9* (B), *S100A12* (C), *MMP8* (D), *MMP9* (E), *IL1B* (F), *F5* (G), *CD48* (H), *NFKB* (I) and *IL4R* (J), normalized to expression of hypoxanthine guanine phosphoribosyltransferase (*HPRT*) for each individual patient. Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supp. Fig. S3. Phenotypical analysis of neutrophils from patients with PAAND and FMF.

Neutrophils from PAAND patients, FMF patients and HCs were isolated by immunomagnetic negative selection and analysed by flow cytometry. (A-C) Box whisker plots indicate expression levels on neutrophils [mean fluorescence intensity (MFI)] of CD66b (A), CD15 (B) and CD16 (C), for individual patients. Representative histograms were plotted showing the

expression levels (MFI) on purified neutrophils of HC (red), PAAND patients (blue) and FMF patients (yellow). Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$.

Supp. Fig. S4. Phenotypical analysis of neutrophils from patients with PAAND and FMF.

Neutrophils from PAAND patients, FMF patients and HCs were isolated by immunomagnetic negative selection and analysed by flow cytometry. (A-D) Box whisker plots indicate expression levels on neutrophils [mean fluorescence intensity (MFI)] of CXCR1 (A), CXCR2

(B), C5aR (C) and BLTR1 (D), for individual patients. Representative histograms were plotted showing the expression levels (MFI) on purified neutrophils of HC (red), PAAND patients (blue) and FMF patients (yellow). Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Suppl. Fig. S5. Phenotypical analysis of neutrophils from patients with PAAND and FMF.

Neutrophils from PAAND patients, FMF patients and HCs were isolated by immunomagnetic negative selection and analysed by flow cytometry. Expression levels (MFI) of IL-1R2 (A), CD11b (B) and CD62L (C) were measured on neutrophils and are shown for the individual patients. Each dot represents an individual donor (n ≥ 6). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. *p<0.05.

Suppl. Fig. S6. Phenotypical analysis of neutrophils from patients with PAAND and FMF.

Neutrophils from PAAND patients, FMF patients and HCs were isolated by immunomagnetic negative selection and analysed by flow cytometry. (A-D) Box whisker plots indicate expression levels on neutrophils [mean fluorescence intensity (MFI)] of TLR2 (A), TLR4 (B), TLR6 (C) and TLR9 (D), for individual patients. Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$, ** $p < 0.01$.

Suppl. Fig. S7. Effects of colchicine treatment on the phenotypical characteristics of neutrophils.

HC neutrophils were isolated by immunomagnetic negative selection, stimulated with vehicle or colchicine (150 pg/ml or 250 pg/ml) and analysed by flow cytometry. Expression levels

[mean fluorescence intensity (MFI)] of CD66b (A), CD15 (B), CD16 (C), CD10 (D), CXCR1 (E), CXCR2 (F), C5aR (G), BLTR1 (H), TLR2 (I), TLR4 (J), TLR6 (K), TLR9 (L) and CD62L (M) were measured on neutrophils and are shown for neutrophils from two healthy controls.

Suppl. Fig. S8. Effects of colchicine treatment on ROS production and phagocytosis

HC neutrophils were isolated by centrifugation in a density gradient (for ROS production assays) or immunomagnetic negative selection (for phagocytosis experiments) and incubated for 30 min in the presence of vehicle or colchicine (150 pg/ml or 250 pg/ml). (A) Cells were exposed to RPMI1640 medium supplemented with 5 mM luminol and kinetic measurements of luminol oxidation were performed during 3 h at 37°C. ROS production was assessed by calculating fold changes of luminol oxidation, *i.e.* the differences between the value at the start of the incubation and the maximal value. (B-C) Neutrophils were incubated with pHrodo red *S. aureus* bioparticlesTM during 30 min at 37°C and bead uptake was evaluated by flow cytometry. Results are presented as (B) percentage of neutrophils that engulfed beads and (C) the amount of engulfed beads/cell (represented by the MFI).

Suppl. Fig. S9. Activation state of neutrophils from PAAND and FMF patients upon *in vitro* stimulation

(A-H) Neutrophils from PAAND patients, FMF patients and HCs were cultured for 1 h without (A, C, E, G) or with 10 μ g/ml ultrapure LPS (B, D, F, H) at 37°C. Relative expression levels

(MFI) of CD35 (secretory vesicles) (A-B), CD66b (specific vesicles) (C-D), CD11b (gelatinase vesicles) (E-F) and CD63 (azurophilic vesicles) (G-H) were plotted to assess degranulation of neutrophils. Each dot represents an individual donor ($n \geq 5$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient. * $p < 0.05$.

Suppl. Fig. S10. Altered migration behaviour of neutrophils from patients with pyrin-associated autoinflammation.

Chemotactic responses of neutrophils from PAAND patients, FMF patients and HCs were investigated *in vitro* using the 48-well Boyden chamber chemotaxis assay. Cells were added to the upper well of the chamber and exposed in the bottom well to (A) 10 ng/ml CXCL8, (B) 10 ng/ml C5a or (C) 10 nM fMLF and migration was allowed during 45 min at 37°C. Results are represented as absolute number of migrated cells microscopically counted in 10 high power fields. Each dot represents an individual donor ($n \geq 6$). Symbols with the same shape in the PAAND patient group represent replicate measurements of the same patient assessed at different time points. Symbols in the FMF group represent patients with a homozygous (●), a heterozygous (■) or a compound heterozygous (▲) mutation, respectively (Suppl. Table S1). Results were statistically analysed using a linear mixed model. A random intercept model was used to correct for multiple samples per patient.

Suppl. Fig. S11. Effects of colchicine treatment on migration

HC neutrophils were isolated by centrifugation in a density gradient and incubated for 30 min in the presence of vehicle or colchicine (150 pg/ml or 250 pg/ml). (A-B) Random and chemoattractant-induced migratory responses of neutrophils were investigated *in vitro* using the 48-well Boyden chamber chemotaxis assay. Cells were loaded in the upper part of the chamber and (A) buffer alone or (B) 10 ng/ml CXCL8 (72 amino acid form) were added below the chemotaxis membrane. Migration was allowed during 45 min at 37°C and migrated cells were counted by microscopy. Results are represented as number of migrated cells in ten high power fields.

Supplementary Table S1. Patient characteristics**Supplementary Table S2. Overview of primers used for qPCR****Supplementary Table S3. Overview of antibodies used for flow cytometry****Supplementary Table S4: Differentially expressed genes in PAAND neutrophils versus HC neutrophils**

Significantly differentially expressed genes as determined by RNA-sequencing in purified neutrophils from 5 PAAND patients and 4 age-matched HCs. The logFC represents the logarithm of the fold change (FC) comparing the HC neutrophil gene expression to the PAAND neutrophil gene expression. Genes with an expression logFC > |0.5| and false discovery rate (FDR) <0.05 were considered significant. FC.

Supplementary Table S5: Differentially expressed genes in FMF neutrophils versus HC neutrophils

Significantly differentially expressed genes as determined by RNA-sequencing in purified neutrophils from 5 FMF patients and 4 age-matched HCs. The logFC represents the logarithm of the FC comparing the HC neutrophil gene expression to the FMF neutrophil gene expression. Genes with an expression logFC > |0.5| and FDR <0.05 were considered significant. FC. Fold change; FDR. False discovery rate.

Supplementary Table S6: Differentially expressed genes in FMF neutrophils versus PAAND neutrophils

Significantly differentially expressed genes as determined by RNA-sequencing in purified neutrophils from 5 FMF patients and 5 PAAND patients. The logFC represents the logarithm of the FC comparing the PAAND neutrophil gene expression to the FMF neutrophil gene

expression. Genes with an expression $\log FC > |0.5|$ and $FDR < 0.05$ were considered significant.

FC. Fold change; FDR. False discovery rate.

Supplementary Table S7: Overlapping differentially expressed genes in FMF and PAAND neutrophils versus HC neutrophils

Overlapping significantly differentially expressed genes as determined by RNA-sequencing in purified neutrophils from 5 FMF patients and 5 PAAND patients compared to 4 age-matched HCs.

Supplementary Table S8. Summary

Summary of the observed results

Supplementary Table S1. Patient characteristics

Patient	Disease	Age in years ^a	Gender (M/F)	Ethnicity	Mutation statue	Gene	Mutation	Treatment	Number of times sampled	Disease state at the moment of blood collection	Symbol used in figures
FMF1	FMF	29	M	Armenian	Homozygous	<i>MEFV</i>	M680I	Colchicine	2	remission	●
FMF2	FMF	20	F	Turkish	Homozygous	<i>MEFV</i>	M694V	Colchicine	1	remission	●
FMF3	FMF	48	M	Turkish	Heterozygous	<i>MEFV</i>	M680I	Colchicine, NSAIDs	2	remission (once), flare (once)	■
FMF4	FMF	31	F	N.D.	Homozygous	<i>MEFV</i>	M694V	Colchicine, NSAIDs	1	remission	●
FMF5	FMF	35	F	N.D.	Homozygous	<i>MEFV</i>	M694V	Colchicine	1	remission	●
FMF6	FMF	33	F	Turkish	Compound heterozygous	<i>MEFV</i>	V726A/E167D - F479L	Colchicine	1	remission	▲
FMF7	FMF	20	F	Turkish	Homozygous	<i>MEFV</i>	M694V	Colchicine, canakinumab	1	remission	●
FMF8	FMF	46-48	M	Turkish	Heterozygous	<i>MEFV</i>	M694V	Colchicine, canakinumab	2	remission	■
FMF9	FMF	62	M	Turkey	Homozygous	<i>MEFV</i>	M694V	Colchicine, canakinumab	1	remission	●
FMF10	FMF	40	M	Turkey	Homozygous	<i>MEFV</i>	M694V	Colchicine, canakinumab	1	remission	●
FMF11	FMF	49	F	Turkey	Homozygous	<i>MEFV</i>	M694V	Colchicine	1	remission	●
FMF12	FMF	34	M	Armenia	Compound heterozygous	<i>MEFV</i>	V726A/M680I	Colchicine	1	remission	▲
FMF13	FMF	23	F	Armenia	Compound heterozygous	<i>MEFV</i>	A761H/M694V	Colchicine, canakinumab	1	remission	▲
PAAND1	PAAND	53-54	M	Caucasian	Heterozygous	<i>MEFV</i>	S242R	Colchicine, anakinra, methylprednisolone , metoprolol	3	remission	△
PAAND2	PAAND	19-20	F	Caucasian	Heterozygous	<i>MEFV</i>	S242R	Etanercept, methotrexate	4	remission	▲
PAAND3	PAAND	46-48	F	Caucasian	Heterozygous	<i>MEFV</i>	S242R	No treatment	3	remission	◇
PAAND4	PAAND	52-53	M	Caucasian	Heterozygous	<i>MEFV</i>	S242R	Adalimumab, methylprednisolone, tramadol, acetaminophen, fentanyl, NSAIDs, metoprolol	4	remission	□
PAAND5	PAAND	24-25	M	Caucasian	Heterozygous	<i>MEFV</i>	S242R	Mycophenolate mofetil, tacrolimus, amlodipine, acetaminophen	4	remission	○
PAAND6	PAAND	75-76	M	Caucasian	Heterozygous	<i>MEFV</i>	S242R	Colchicine, methylprednisolone	3	remission	■
PAAND7	PAAND	50	F	Caucasian	Heterozygous	<i>MEFV</i>	S242R	NSAIDs, sulfalazine	1	remission	●

^aSome patients were sampled more than once. In that case an age range is indicated for the sampling period.

NSAID, non-steroidal anti-inflammatory drug

Supplementary Table S2. Overview of primers used for qPCR

Gene	Primer	Company
<i>S100A8</i>	Hs.PT.58.19654111.gs	Integrated DNA Technologies
<i>S100A9</i>	Hs.PT.58.20989743	Integrated DNA Technologies
<i>S100A12</i>	Hs.PT.58.39942209	Integrated DNA Technologies
<i>IL1B</i>	Hs.PT.58.1518186	Integrated DNA Technologies
<i>NFKB</i>	Hs.PT.58.20344216	Integrated DNA Technologies
<i>F5</i>	Hs.PT.58.2237261	Integrated DNA Technologies
<i>IL4R</i>	Hs.PT.58.14553110	Integrated DNA Technologies
<i>CD48</i>	Hs.PT.58.3602385	Integrated DNA Technologies
<i>MMP8</i>	Hs.PT.58.38449046	Integrated DNA Technologies
<i>MMP9</i>	Hs.PT.58.22814824.g	Integrated DNA Technologies
<i>GAPDH</i>	Hs.PT.39a.22214836	Integrated DNA Technologies
<i>HPRT</i>	Hs.PT.39a.22214821(1)	Integrated DNA Technologies

Supplementary Table 3. Overview of antibodies used for flow cytometry.

Antigen	Clone	Company	Dilution	Host species	Colour	Cat. number
BLTR-1	203/14F11	BD	1/200	Mouse	BV510	744913
CD10	HI10a	BD	1/100	Mouse	BV786	564960
CD11b	ICRF44	BD	1/66	Mouse	BV510	563088
CD11b	ICRF44	Biologend	1/100	Mouse	PE-Cy5	301308
CD121a	Polyclonal	R&D	1/500	Goat	PE	FAB269P
CD121b	REA744	Miltenyi Biotec	1/66	Recombinant human	FITC	130-111-274
CD14	61D3	Invitrogen	1/160	Mouse	PE-Cy5	15-0149-42
CD15	HI98	BD	1/100	Mouse	BUV395	563872
CD15	W6D3	BD	1/200	Mouse	BV786	741013
CD16	3G8	BD	1/200	Mouse	AlexaFluor700	557920
CD16	3G8	BD	1/200	Mouse	BUV395	563784
CD181	5A12	BD	1/20	Mouse	PE	555940
CD182	6C6	BD	1/10	Mouse	FITC	551126
CD282	11G7	BD	1/100	Mouse	BV421	565350
CD284	HTA125	Invitrogen	1/20	Mouse	AlexaFlur488	53-9917-42
CD286	TLR6.127	Biologend	1/20	Mouse	PE	334708
CD289	eB72-1665	Invitrogen	1/20	Rat	APC	17-9099-82
CD35	E11	Biologend	1/100	Mouse	FITC	333404
CD62L	DREG-56	Invitrogen	1/100	Mouse	APC	17-0627-42
CD63	H5C6	Biologend	1/100	Mouse	PERCPCy5.5	353020
CD66b	G10F5	BD	1/100	Mouse	BV421	562940
CD66b	G10F5	Biologend	1/14	Mouse	PerCP5.5	305108
CD88	S5/1	Biologend	1/40	Mouse	PerCPCy5.5	344312

Supplementary Table S4.

Gene Symbol	logFC	FDR
SRPK1	-1.3	2.0e-09
PAPSS1	-2.7	5.5e-09
ARG1	-2.7	5.1e-08
IGHA2	5.1	1.6 e-07
IL4R	-1.8	3.4e-07
MS4A7	2.8	1.0e-06
ANXA3	-2.0	1.4 e-06
GYG1	-2.1	3.9 e-06
IGHG1	5.7	5.9 e-06
LMNB1	-1.5	5.9 e-06
MLLT4	-2.8	5.9 e-06
GADD45A	-1.5	7.3e-06
F5	-1.8	9.0e-06
C9orf72	-1.1	1.3 e-05
MACF1	-1.4	1.6e-05
MAPK14	-1.1	1.8e-05
CST7	-2.5	2.0e-05
MLTK	-1.9	2.7e-05
PAG1	-1.3	2.8e-05
CTA-384D8.34	1.8	3.8 e-05
PEBP1	2.8	4.5e-05
CHST2	-1.8	8.7e-05
TLR5	-2.2	0.000108
FCER1G	-1.0	0.0001186
ASPH	-1.3	0.000177
PGS1	-1.3	0.00032
METTL7A	2.5	0.0005761
IGHA1	4.7	0.00074
IGLC7	6.0	0.000714
PROK2	-1.4	0.000714
RNF10	-1.2	0.000714
TMED8	-1.2	0.000714
DEFA3	-3.9	0.000725
TECPR2	-0.7	0.00080287
GPCPD1	-1.1	0.000887
PPFIBP2	2.4	0.0010
ORM1	-2.4	0.0010
MSL3	-1.2	0.0012
NAGK	1.7	0.0012
ALPL	-1.6	0.00154
EIF1AY	-8.1	0.0015
KCNJ15	-1.0	0.0015
MPEG1	1.8	0.0016
TSPO	-1.6	0.00183
CD44	-1.5	0.00238
EMILIN2	-1.5	0.0025
KCNQ10T1	-2.4	0.0027
ARFGAP3	-0.9	0.00333
CHCHD10	2.8	0.00386
IGHG3	6.0	0.0040

Gene Symbol	logFC	FDR
SH3GLB1	-0.8	0.0041
FGD2	2.8	0.0042
SERPINB1	-1.0	0.0042
ATF6	-0.8	0.0043
ATP5G1	3.0	0.0043
CASP5	-1.6	0.0043
ZNF438	-0.8	0.005076
ACSL1	-0.8	0.0055
SNX3	-1.3	0.0055
MZB1	5.5	0.006069
GALM	2.0	0.0061
HRH2	-0.9	0.0061
ST3GAL4	-1.2	0.0061
SYNE1	-1.2	0.0061
IGHG4	8.1	0.00637
HPSE	-1.5	0.0071
ATP11B	-0.8	0.00734
DHRS13	-1.2	0.00753
UPP1	-1.6	0.00753
HSPH1	1.9	0.00765
SAMSN1	-1.3	0.00765
FMNL2	2.2	0.008053
WDFY3	-0.8	0.00818
TMEM260	-1.0	0.00836
CDKN1C	1.7	0.0087
C19orf59	-2.5	0.0087
OSBPL1A	-2.0	0.0087
POR	-1.2	0.0087
STAT5B	-0.8	0.0087
GPR97	-1.0	0.009005
RP11-290F20.1	-2.0	0.00941
USB1	-0.9	0.00941
CEACAM6	-2.9	0.0097
TBC1D15	-0.7	0.0098
AL928768.3	5.1	0.011
AC074289.1	1.0	0.011
BMX	-1.2	0.013
CECR1	2.2	0.013
ZDHHC2	-1.7	0.013
IGLC2	5.4	0.013
HP	-3.9	0.013
ATP2B4	-1.0	0.014
FAM129A	-0.8	0.014
PABPC4	1.5	0.014
PLP2	-0.9	0.014
ZNF254	-1.9	0.014
FAM118A	2.6	0.014
FLOT2	-0.9	0.014
CDK5RAP2	-1.4	0.015
CR1	-1.1	0.015

Supplementary Table S5.

Gene Symbol	logFC	FDR
CTA-384D8.34	2.7	2.7e-09
IFIT3	2.8	2.8e-07
DHRS9	1.5	9.1e-07
BATF2	2.2	2.9e-06
GIMAP2	2.2	4.3e-06
RPS27A	0.9	4.3e-06
GBP3	2.7	5.0e-06
TSC22D3	1.3	9.2e-06
IFIT2	2.4	1.2e-05
OASL	3.0	1.7e-05
F5	-1.7	1.8e-05
SEC11C	3.4	2.2e-05
GBP7	5.7	2.3e-05
LY6E	5.7	2.5e-05
IRF7	1.8	3.7e-05
LAP3	2.5	4.4e-05
PDZD8	-1.3	4.4e-05
VIM	-1.2	5.4e-05
DHX58	2.9	5.7e-05
APOL6	1.7	6.4e-05
ETV7	2.9	6.4e-05
STAT2	1.9	6.4e-05
MOV10	2.6	6.6e-05
PARP14	1.9	7.0e-05
GIMAP8	2.2	7.7e-05
RPH3A	-4.6	8.1e-05
GBP4	2.4	8.5e-05
PPM1K	3.1	0.00010
DTX3L	0.9	0.00010
TIMM10	4.1	0.00012
CD48	-1.1	0.00013
GBP1	1.9	0.00015
C5orf56	1.8	0.00017
MMP9	-2.0	0.00017
METTL7A	2.6	0.00019
PARP12	2.4	0.00019
TREX1	2.7	0.00020
APOL1	1.3	0.00023
GBP1P1	2.0	0.00024
IFIH1	2.0	0.00024
IFIT5	2.1	0.00024
IGHA2	5.6	0.00024
NQO2	-1.4	0.00024
RPL37A	1.2	0.00026
FRMD3	2.6	0.00030
RPL5	1.6	0.00034
SAMSN1	-1.8	0.00034
TRIM22	1.8	0.00038
CTB-61M7.2	-1.4	0.00050
CCL5	4.0	0.00057

Gene Symbol	logFC	FDR
RPL10A	2.0	0.00057
AQP3	5.2	0.00057
FGD2	3.8	0.00058
DDX58	1.4	0.00058
GPR97	-1.2	0.00058
IGHG1	3.4	0.00058
CDA	-1.2	0.00060
UBE2L6	1.4	0.00062
TRIB2	3.8	0.00062
FBXW2	-1.1	0.00065
MARCKS	-0.9	0.00070
ODF3B	1.5	0.00070
RNF213	1.3	0.00070
RPS12	1.2	0.00070
RPS4X	1.8	0.00070
HLA-DRB1	2.7	0.00071
SERPING1	2.0	0.00072
RTP4	4.5	0.00073
PEBP1	2.5	0.00078
STAT1	1.3	0.00078
DSE	-1.4	0.00079
SAMHD1	1.3	0.00079
RPS19	1.1	0.00081
IFI6	2.7	0.00082
CHMP5	1.1	0.00087
GBE1	-1.8	0.00087
RARRES3	1.6	0.00087
TAP1	1.0	0.00087
OAS1	4.2	0.00087
S100A10	2.3	0.00087
LDHB	2.6	0.00089
MT2A	3.1	0.00091
OXSRI	-0.9	0.00096
IFI44L	4.3	0.0011
OAS2	4.9	0.0011
TAP2	1.0	0.0011
MEGF9	-0.8	0.0011
OR52K3P	1.4	0.0012
SAMD9L	1.6	0.0012
DDX60	3.1	0.0013
PDIA4	2.8	0.0013
ZDHHC2	-2.0	0.0013
ISG15	3.7	0.0013
ACPP	-1.2	0.0013
SP140	1.5	0.0014
NUB1	0.9	0.0014
PPP2R5A	-0.8	0.0014
FBL	2.3	0.0015
GNG2	-0.9	0.0015

Supplementary Table S6.

Gene Symbol	logFC	FDR
G0S2	-1.3	3.0e-06
PSMB8	1.1	4.0e-06
LL22NC03-2H8.5	3.2	2.6e-05
GZMA	4.2	0.00018
EMR2	1.0	0.00021
HSPA6	0.8	0.00031
MACF1	1.2	0.00040
CLEC2B	1.3	0.00043
LACTB	1.1	0.00051
GZMB	3.1	0.00065
SNHG6	1.2	0.0011
MT-ND1	-1.1	0.0012
DOCK4	1.6	0.0035
MT-ND2	-1.4	0.0035
GNLY	3.1	0.0046
IRF1	0.7	0.0053
HCAR3	1.0	0.0058
ARL4C	2.3	0.0077
KLRB1	4.8	0.0086
C9orf72	0.7	0.0092
SLK	0.6	0.015
DHRS9	1.2	0.016
HIP1	-1.4	0.016
MAML2	1.0	0.016
TXNIP	0.8	0.016
MT-CYB	-1.3	0.018
MT-ND5	-1.3	0.018
MT-ND4L	-1.5	0.021
RPS27A	0.7	0.021
GIMAP2	2.0	0.023
CD247	2.7	0.024
GIMAP4	1.3	0.024
NASP	1.0	0.024
CCL5	3.8	0.024
SAMD3	3.6	0.024
PAPSS1	1.9	0.025
RPS20	0.9	0.027
VCL	-1.0	0.028
MTND2P28	-2.2	0.030
ZFAS1	0.6	0.032
TSC22D3	1.0	0.032
GBP3	2.1	0.032
DIAPH2	-1.1	0.033
ERO1L	-0.9	0.034
ZYG11B	-0.8	0.034
C5orf56	1.4	0.034
MT-ND4	-1.2	0.035
PPFIBP2	-1.7	0.036
NXPE3	-1.2	0.040
TBX21	3.0	0.040

Gene Symbol	logFC	FDR
TRAF3IP3	-1.0	0.042
ZEB2	0.7	0.042
CNPY3	-0.6	0.045
SETD8	-1.1	0.046
FCGR2C	1.2	0.048
PGLYRP1	-1.2	0.048
HNRNPU-AS1	1.5	0.048
VNN2	0.7	0.051

Supplementary Table S7.

Gene Symbol
CTA-384D8.34
F5
SEC11C
GBP7
PDZD8
METTL7A
IGHA2
NQO2
SAMSN1
FGD2
GPR97
IGHG1
MARCKS
RPS4X
PEBP1
NAMPTL
OR52K3P
PDIA4
ZDHHC2
PPP2R5A
ITM2C
RGL4
RPL4
CAMP
OSBPL1A
HMGB2
AL928768.3
IFRD1
IGJ
SRGAP2
ARHGAP24
RNASE3
RPS8
MZB1
SOS2
IGHG3
BMX
ARG1
SRPK1
ZNF281
LAMTOR5
IRAK3
SYNE1
IGHA1
SSR4
RABGAP1L
CHURC1
PTTG1
LPIN2
EIF1AY

Gene Symbol
GADD45A
IGLC7
TEP1
BASP1
SNX3
POR
ZBTB16
BTN3A2
GALM
CST7
KIF1B
PADI2
CLSTN1
CALR
CD44
ALPL
CD177
IRF4
ORM1
PLP2
PABPC4
ACSL1
ANKRD36BP2
DEFA3
NFIL3
IGHG4
IL4R
RPS28
EMBP1
CHCHD10
MS4A7
PYGL
CLSTN1

Supplementary Table S8.

Feature	PAAND	FMF
Number of neutrophils	↑	=
Maturation	=	Immature
MFI chemoattractant receptors (CXCR2, C5aR, BLTR1)	=	↓
MFI TLR4/TLR9	=	↑
ROS production	↑	↑
Phagocytosis	↑	↓
MPO release	↑	↑ (n.s.)
Granularity neutrophils	↑	=
Degranulation	↑	=?
Random migration	↑	↑ (n.s.)
Gene expression	Activated gene expression profile	Activated gene expression profile

Abbreviations: n.s.; not significant; ↑, ↓ and = indicate enhanced, decreased or unaltered parameters compared to healthy controls