

## Original article

# Neutrophils from patients with SAPHO syndrome show no signs of aberrant NADPH oxidase-dependent production of intracellular reactive oxygen species

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## Abstract

**Objective.** We aimed to investigate if aberrant intracellular production of NADPH oxidase-derived reactive oxygen species (ROS) in neutrophils is a disease mechanism in the autoinflammatory disease SAPHO syndrome, characterized by synovitis, acne, pustulosis, hyperostosis and osteitis, as has previously been suggested based on a family with SAPHO syndrome-like disease.

**Methods.** Neutrophil function was explored in a cohort of four patients with SAPHO syndrome, two of whom were sampled during both inflammatory and non-inflammatory phase. Intracellular neutrophil ROS production was determined by luminol-amplified chemiluminescence in response to phorbol myristate acetate.

**Results.** Cells from all patients produced normal amounts of ROS, both intra- and extracellularly, when compared with internal controls as well as with a large collection of healthy controls assayed in the laboratory over time (showing an extensive inter-personal variability in a normal population). Further, intracellular production of ROS increased during the inflammatory phase. Neutrophil activation markers were comparable between patients and controls.

**Conclusion.** Dysfunctional generation of intracellular ROS in neutrophils is not a generalizable feature in SAPHO syndrome. Secondly, serum amyloid A appears to be a more sensitive inflammatory marker than CRP during improvement and relapses in SAPHO syndrome.

**Key words:** NADPH oxidase, SAPHO syndrome, granulocytes, reactive oxygen species, autoinflammation, inflammation

### Rheumatology key messages

- The SAPHO syndrome is not by necessity associated with deficient neutrophil intracellular reactive oxygen species production.
- Neutrophils from patients with SAPHO syndrome are pre-primed in circulation during an inflammatory phase.
- Serum amyloid A is a more sensitive marker than CRP during SAPHO syndrome improvement and relapse.

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Submitted 10 May 2015; revised version accepted 8 March 2016

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## Introduction

SAPHO syndrome, characterized by synovitis, acne, pustulosis, hyperostosis and osteitis, was first described as a clinical entity in 1987 by Chamot *et al.* [1]. It is classified among the autoinflammatory diseases, defined by Kastner as 'abnormally increased inflammation, mediated predominantly by cells and molecules of the innate immune system, with a significant host predisposition' [2]. More specifically, SAPHO syndrome belongs to the autoinflammatory bone disorders, in which dysregulation of innate immunity causes inflammation in sterile bone [3–5]. Autoinflammatory bone disorders also include chronic recurrent multifocal osteomyelitis, chronic non-bacterial osteomyelitis and Majeed syndrome [3–8]. It remains to be clarified how these diseases differ from each other [3, 5].

The bone lesions observed in SAPHO syndrome represent a clinical spectrum from self-limiting single- or oligo-lesions to chronic lesion(s) or recurrent multifocal osteomyelitis. The histopathology of the lesions mimics infectious osteomyelitis but the lesions are typically sterile [9]. Skin lesions in SAPHO syndrome are characterized by neutrophil dermatosis, that is, severe acne, palmoplantar pustulosis and psoriasis [1, 10, 11].

Autoinflammatory diseases are by definition mediated by innate immune cells. There is growing evidence of neutrophil dysfunction in human inflammatory disorders [12–19], but their specific role in disease pathology remains largely unknown. In a study by Ferguson *et al.* [13], the function of neutrophils was investigated in a mother and daughter affected by a SAPHO syndrome-like disease. The major finding showed that the patients' cells displayed aberrant production of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived reactive oxygen species (ROS), but the biological significance of this finding was not clarified.

ROS production is a prominent neutrophil feature, contributing to, for example, antimicrobial defence. The enzyme responsible for ROS production, the NADPH oxidase, is an electron-transport system that when assembled in a membrane reduces oxygen to superoxide anion, subsequently forming other ROS. NADPH oxidase assembly can occur at the plasma membrane, resulting in release of ROS extracellularly or into a phagosome. The NADPH oxidase can also assemble at intracellular membranes, giving rise to intracellular ROS production localized in the phagolysosome as well as in non-phagosomal granules (intragranular ROS) [20].

The function of intragranular ROS is not fully understood, but they are thought to be involved in subcellular signalling events. A specific deficiency in intracellular ROS production has been linked to hyperinflammatory conditions in a novel form (p40<sup>phox</sup>) of chronic granulomatous disease [21]. Along the same lines, the study by Ferguson *et al.* [13] showed that intracellular ROS production was defective in neutrophils from the patients with SAPHO syndrome-like disease, which also displayed a hyperinflammatory phenotype [13].

To follow up on the study by Ferguson *et al.*, we explored neutrophil ROS production in a small cohort of

four patients with SAPHO syndrome. We found that neutrophils from these patients produced normal amounts of ROS, both intra- and extracellularly, and that the cells behaved similarly to control neutrophils when assayed *in vitro*. Our data thus demonstrate that aberrant generation of intracellular ROS in neutrophils is not a generalizable feature in SAPHO syndrome.

## Patients and methods

### Patients

Three diagnostic criteria for SAPHO syndrome, of which one is sufficient for diagnosis, were proposed by Kahn and Kahn in 1994: chronic recurrent multifocal osteomyelitis with or without skin manifestation; acute or chronic sterile arthritis associated with pustular psoriasis, palmoplantar pustulosis or severe acne; and sterile osteitis in the presence of one of the skin manifestations [22]. We included four patients diagnosed with SAPHO syndrome according to the third criterion. A summary of clinical features and investigations of included patients is given in Table 1 and detailed clinical descriptions and disease history for the patients are given in supplementary data, available at *Rheumatology* Online. When attempting to investigate whether a mechanistic feature is generalizable for all patients diagnosed with a disease (such as SAPHO syndrome), it is an advantage to include patients from different sub-cohorts, that is, of different ages, presentation and treatment approaches but all falling within the disease criteria. Therefore we found it valuable to investigate patients suffering from SAPHO syndrome who were quite different from one another, despite their constituting a relatively small cohort.

In short, patient A (age at onset, 15 years; previously healthy; male) with severe acne was brought to the hospital with pain in the right knee and fever. He was diagnosed with SAPHO and after that MRI and bone scan showed multiple lesions in knees, trochanter major, foot and clavicle compatible with osteomyelitis. He received long-term treatment with ibuprofen and was asymptomatic with normal inflammatory marker after 2.5 months of treatment.

Patient B (age at onset, 15 years; previously healthy; male) with acne fulminant was referred to the hospital with fever and pain from multiple locations. MRI and bone scan revealed multiple bone lesions compatible with osteomyelitis in sacrum, sternocostal joint, corpus sternum and vertebral bodies. The patient was diagnosed with SAPHO syndrome and treated with prednisone, MTX and NSAID. He was free of symptom with normal inflammatory markers after 6 months, but relapsed after 2 years.

Patient C (68 years old; female; with non-erosive PsA) was diagnosed with SAPHO syndrome based on palmoplantar pustulosis, osteosclerotic lesions, hyperostosis and arthritis of the sternocostal joint. Treatment with MTX (20 mg/week) was initiated and treatment outcome has yet to be evaluated.

Patient D (56 years old; female; with PsA) was diagnosed with SAPHO syndrome based on pustulosis

TABLE 1 Clinical features and investigations of included patients

| Parameter   | Patient A  | Patient B   | Patient C  | Patient D   |
|---|--|---|--|---|
| Gender  | Male   | Male  | Female   | Female  |
| Age at onset, years   | 15   | 15  | Not known  | 45  |
| Presenting symptom  | Fever; right knee pain   | Fever; hip, wrist and thorax pain   | Thorax, back and shoulder pain                                 | Synovitis of sternocostal joint; hip and wrist pain |
| Skin findings   | Cystic acne  | Acne fulminans  | Pustulosis palmoplantaris                                      | Pustulosis palmoplantaris                           |
| Location of bone lesions on bone scan <sup>a</sup> and/or MRI | Both knees, right trochanter major, left foot and left medial clavicle | Left sacrum, right sternocostal joint, corpus sternum, Th7, Th8 and Th9   | L4, L5 and bilateral sternocostal joints                       | Bilateral sternocostal and sacroileac joints        |
| CRP at admission, mg/l  | 160  | 84  | 12   | <5  |
| ESR, mm/h   | 95   | 115   | 34   | <20   |
| Blood culture   | Negative × 2   | Negative × 2  | Negative   | Negative  |
| Biopsy histology  | Normal tissue  | NP  | Normal   | NP  |
| Biopsy culture  | No signs of malignancy, inflammation or infection                      | NP  | NP   | NP  |
| Biopsy 16S rRNA   | <i>Staphylococcus hominis</i><br><i>Streptococcus milliaris</i>        | NP  | NP   | NP  |
| Duration first episode(onset to asymptomatic)                 | 2.5 months   | 6 months  | Still symptomatic, recently started antiinflammatory treatment | 3 months  |
| Treatment, duration <sup>b</sup>                              | Isoxicillin IV (9 d)<br>NSAID (1 y)<br>Amoxicillin (6 w)               | Cefotaxime IV (13 d)<br>NSAID (1 y)<br>Prednisone (3 w)<br>Isoxicillin (6 w)<br>Omeprazol (3 w)<br>MTX (1 y)<br>3 years | MTX  | Infliximab (15 months)                              |
| Follow-up time  | 2 years  | 3 years   | 1 month  | 2.5 years   |
| Number of episodes  | 1  | 2   | First episode ongoing  | 1   |

<sup>a</sup>Tc-99m HDP bone scintigraphy. <sup>b</sup>Duration given in d, w, mo or y. y: years; mo: month; w: weeks; d: days. NP: not performed.

palmoplantar and hyperostosis of sternocostal joints. Treatment with infliximab resulted in remission and is continued on a monthly basis.

#### Complete blood cell count, acute phase reactants and cytokine measurements

Complete blood cell count and white blood cell differential were determined using an ADVIA cell counter (ADVIA, Siemens Healthcare, Erlangen, Germany). Acute phase proteins (CRP; serum amyloid A, SAA) were measured in serum by ELISA. Inflammatory cytokines were measured in plasma by Bio-Plex Pro Human Cytokine Group I 10-Plex Assay and Bio-Plex Manager Software (Bio-Rad Laboratories, Hercules, CA, USA).

#### Blood sampling of patients and definition of inflammatory/non-inflammatory patient status

Patients A and B were followed with CRP and SAA levels in serum over time as part of routine care (Fig. 1). At two time-points, corresponding to an inflammatory phase (defined by increased CRP and SAA, Table 2; samples A1 and B1) and a non-inflammatory phase (defined by normalized CRP and SAA, Table 2; samples A2 and B2), heparinized venous blood and plasma was collected (see below) for analysis of neutrophil function and cytokine levels, respectively (Fig. 1). The sample from patient C was collected during an inflammatory phase (slightly elevated CRP) while patient D was under treatment with an anti-TNF $\alpha$  inhibitor (infliximab) and was non-inflammatory at time of sampling. Samples from adult controls were analysed in parallel.

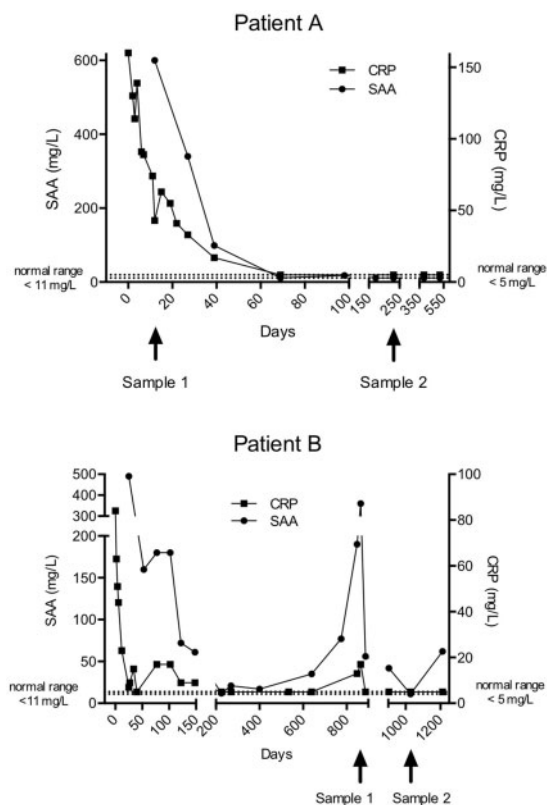
The inflammatory status of patients A and B was confirmed by complete blood cell counts and white blood cell differentials (Table 2), showing that the absolute numbers of neutrophils, monocytes and thrombocytes were increased during the inflammatory phase as compared with the non-inflammatory phase, although the absolute numbers of neutrophils and monocytes were within the normal range (Table 2). For patients C and D, the blood cell counts were within the normal range (Table 2).

The inflammatory status of patients A and B at the first sampling was supported by plasma cytokine levels; patient A showed high amounts of IL-6 (but no other cytokines) and patient B displayed an increase in IL-6, IL-12, IFN- $\gamma$  and GM-CSF (supplementary Fig. S1, available at *Rheumatology* Online). Both patients were within normal range of cytokines at the second sampling (non-inflammatory phase). Samples from patients C and D were not analysed for plasma cytokines.

#### Control samples

Each patient sample was processed and analysed in parallel with a control sample. These samples were collected from healthy adult blood donors from the Sahlgrenska University Hospital Blood Centre, Gothenburg. We did not use age-matched controls for the adolescent patients A and B, as such controls could not be obtained for parallel analysis of cell function. In addition, the patient data were compared with a compiled set of control data from healthy adult blood donors collected over time in our

Fig. 1 SAA and CRP values over time in patients A and B



Serum content of SAA (left axis) and CRP (right axis) for patient A (top) and patient B (bottom) are plotted over time. Arrows indicate the time points at which samples were collected for neutrophil analysis. Samples (1) were collected during an inflammatory phase while samples (2) were collected during a non-inflammatory phase. SAA: serum amyloid A.

laboratory, comparing cytokine content in plasma ( $n = 11-20$ ) and neutrophil production of ROS ( $n = 29$ ).

#### Neutrophil isolation

Peripheral blood neutrophils were isolated according to Böyum *et al.* [23]. After isolation, neutrophils were resuspended in Krebs-Ringer glucose (KRG) buffer supplemented with  $\text{Ca}^{2+}$  (KRG-Ca; 1 mM), and stored on ice.

#### ROS production measured by luminol/isoluminol-enhanced chemiluminescence

NADPH oxidase-derived ROS production was measured using luminol/isoluminol-enhanced chemiluminescence (CL) [24]. Extracellular ROS was measured in the presence of isoluminol ( $56 \mu\text{M}$ ; Sigma-Aldrich, St Louis, MO, USA; membrane-impermeable) and horseradish peroxidase (4 U/ml) while intracellular ROS was measured in the presence of luminol ( $56 \mu\text{M}$ ; Sigma-Aldrich; membrane-permeable), and the extracellular ROS scavengers superoxide dismutase (50 U/ml; Worthington Biochemical Corp., Lakewood, NJ,

**TABLE 2** Laboratory variables in blood samples from SAPHO syndrome patients during an inflammatory (A1, B1 and C) or a symptom-free (A2, B2 and D) phase

| Patient         | CRP,<br>mg/l | SAA,<br>mg/l | Hb,<br>g/l | Platelets,<br>× 10 <sup>9</sup> /l | WBC,<br>× 10 <sup>9</sup> /l | ANC,<br>× 10 <sup>9</sup> /l | ALC,<br>× 10 <sup>9</sup> /l | AMC,<br>× 10 <sup>9</sup> /l | AEC,<br>× 10 <sup>9</sup> /l | ABC,<br>× 10 <sup>9</sup> /l |
|-----------------|--------------|--------------|------------|------------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| A1              | 55           | >600         | 122        | 476                                | 6.4                          | 4.3                          | 1.6                          | 0.35                         | 0.18                         | 0.03                         |
| A2              | <5           | <11          | 145        | 268                                | 3.6                          | 1.9                          | 1.3                          | 0.22                         | 0.15                         | 0.02                         |
| B1              | 17           | 360          | 137        | 383                                | 10.7                         | 7.1                          | 2.8                          | 0.70                         | 0.06                         | 0.03                         |
| B2              | <5           | <11          | 141        | 275                                | 5.7                          | 2.3                          | 3.0                          | 0.41                         | 0.04                         | 0.02                         |
| C               | 9            | —            | 110        | 349                                | 9.7                          | —                            | —                            | —                            | —                            | —                            |
| D               | <5           | 120          | 115        | 314                                | 7.8                          | —                            | —                            | —                            | —                            | —                            |
| Normal<br>range | <5           | <11          | 130–160    | 150–350                            | 4.5–15                       | 1.5–8.5                      | 2–9.5                        | 0.1–1                        | 0.04–0.4                     | 0–0.1                        |

ABC: absolute basophil count; AEC: absolute eosinophil count; ALC: absolute lymphocyte count; AMC: absolute monocyte count; ANC: absolute neutrophil count; Hb: haemoglobin; SAA: serum amyloid A; WBC: white blood cell count.

USA) and catalase (2000 U/ml; Worthington) [25]. Neutrophils ( $5 \times 10^5$  cells/ml) and reagents were incubated in KRG-Ca for 5 min at 37 °C before stimulation with the protein kinase C activator phorbol myristate acetate (PMA;  $5 \times 10^{-8}$  M; Sigma-Aldrich). The CL activity was measured continuously in a six-channel Biolumat LB 9505 (Berthold Technologies, Zug, Switzerland) or Mithras LB 940 CL (Berthold Technologies) for 20 min and peak CL values were used for comparison between samples.

#### Hydrogen peroxide production measured with *p*-hydroxyphenylacetic acid

Hydrogen peroxide production was measured as oxidation of *p*-hydroxyphenylacetic acid (PHPA) [24]. Neutrophils ( $7.5 \times 10^5$ ) were incubated in KRG-Ca with PHPA (3.3 mM; Sigma-Aldrich) and horseradish peroxidase (4 U/ml, Roche, Mannheim, Germany) at 37 °C and stimulated with PMA ( $5 \times 10^{-8}$  M) after which PHPA oxidation was measured over time in a fluorometer (Perkin Elmer, Waltham, MA, USA) (excitation/emission wavelengths 317/400 nm). The PHPA molecule is membrane-impermeant, thus extracellular hydrogen peroxide was exclusively measured. The total amount of hydrogen peroxide (intracellular plus extracellular) was measured in the presence of azide (1 mM), which inhibits intracellular consumption of ROS [24] and allows for intracellular hydrogen peroxide to leak out of the cell and be measured extracellularly. Intracellular hydrogen peroxide production was calculated as total minus extracellular hydrogen peroxide production. The results show the accumulated PHPA fluorescence after 15 min stimulation.

#### Myeloperoxidase quantification assay

Neutrophils ( $10^6$  cells/ml) were lysed with Triton X-100 (0.1%; Merck, Darmstadt, Germany) for 10 min at 4 °C after which the samples were centrifuged for 15 s at 16 100g. Myeloperoxidase (MPO) content in the cell homogenates was measured by ELISA (Novakemi).

#### Neutrophil activation assays

Upregulation of CD35 (complement receptor 1) and CD11b ( $\alpha$ -chain of CR3) on the cell surface as well as

shedding of CD62L (L-selectin) were used as neutrophil activation markers. Peripheral blood was left untreated at room temperature or incubated for 20 min at 37 °C with or without TNF $\alpha$  (10 ng/ml; a neutrophil secretagogue; Sigma-Aldrich). Leucocytes were fixated and erythrocytes lysed with FACS lysing solution (BD) for 15 min at 4 °C. Leucocytes were then stained with phycoerythrin-conjugated anti-CD35 (BD Biosciences, Franklin Lakes, NJ, USA), anti-CD11b (BD) or anti-CD62L (BD) antibodies for 1 h on ice. The cells were washed in PBS and a minimum of 10 000 neutrophils (gated on basis of forward and side scatter) was analysed by flow cytometry (BD Accuri C6 and FlowJo software).

#### Evaluation of apoptosis

Neutrophil cell death was monitored essentially as described earlier [26]. Cells ( $5 \times 10^6$  cells/ml) were suspended in RPMI 1640 medium with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria) and incubated at 37 °C in 5% CO<sub>2</sub> in the absence or presence of anti-CD95 monoclonal antibody (Fas ligand; pro-apoptotic stimulus; Nordic Biosite, Täby, Sweden) or lipopolysaccharide (LPS; anti-apoptotic stimulus; Sigma-Aldrich). After 20 h incubation the cells were stained with Annexin V-FLUOS (for apoptosis; Roche) and 7-aminoactinomycin D (for necrosis; BD). A minimum of 10 000 neutrophils were analysed by flow cytometry.

#### Ethics statement

The children were enrolled and received care at the Pediatric Department, NU-Hospital Group in Trollhättan and Uddevalla, in consultation with pediatric rheumatologists at the Queen Silvia Children's Hospital, Gothenburg. The adult patients were enrolled at the Department of Rheumatology, Sahlgrenska University Hospital, Gothenburg. Informed consent from patients and parents was obtained in accordance with the Declaration of Helsinki. The Regional Ethical Review Board, Gothenburg, Sweden, approved the study.

## Results

### Neutrophils from patients with SAPHO syndrome have normal extra- and intracellular ROS production capacity

Neutrophil NADPH oxidase activity was investigated using the isoluminol/luminol CL system that can be used to distinguish between extra- and intracellular ROS production. Neutrophils from patients with SAPHO syndrome responded to PMA stimulation with ROS production both extracellularly (Fig. 2A) and intracellularly (Fig. 2B). All samples showed ROS production that was within normal range (samples C and D, data not shown) as compared with CL activity in internal controls as well as compiled data from healthy donors (Fig. 2A and B; samples C and D, data not shown). Notably, both patients A and B produced more both extra- and intracellular ROS when they were in an inflammatory state (A1 and B1) compared with a later time point when the inflammation had resolved (A2 and B2; Fig. 2A and B).

Intracellular ROS production was also investigated by the PHPA method. In line with the CL results, neutrophils from patients with SAPHO syndrome produced similar amounts of both extra- and intracellular hydrogen peroxide in response to PMA, as compared with control cells (Fig. 2C; samples C and D, data not shown). Taken together, neutrophil ROS production was normal in our cohort of patients with SAPHO syndrome, both during an inflammatory flare and after resolution of inflammation.

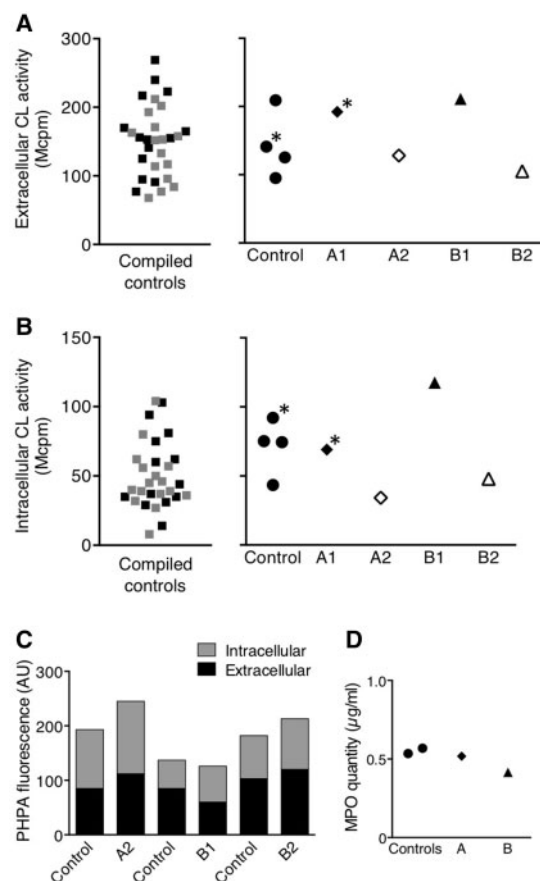
Determination of intracellular ROS using luminol-amplified CL is MPO-dependent [24]. We therefore quantified MPO in neutrophil lysates to ensure that altered MPO levels did not influence the CL results. We found similar amounts of MPO in controls and patients (Fig. 2D and data not shown).

### Neutrophils from patients with SAPHO syndrome can be primed and show normal receptor upregulation and shedding

In circulation, neutrophils are in a quiescent state. After transmigration from the vessels into the (inflamed) tissue, neutrophils become primed, that is, increasingly responsive to inflammatory stimuli. This is in part achieved by mobilization of intracellular granules to the cell surface, leading to increased receptor exposure (CD35 and CD11b) and shedding of cell-to-cell interaction molecules (CD62L) involved in transendothelial migration [27]. Primed neutrophils are normally not found in circulation, but can be present under certain infectious or aseptic inflammatory conditions [14, 17, 28–30].

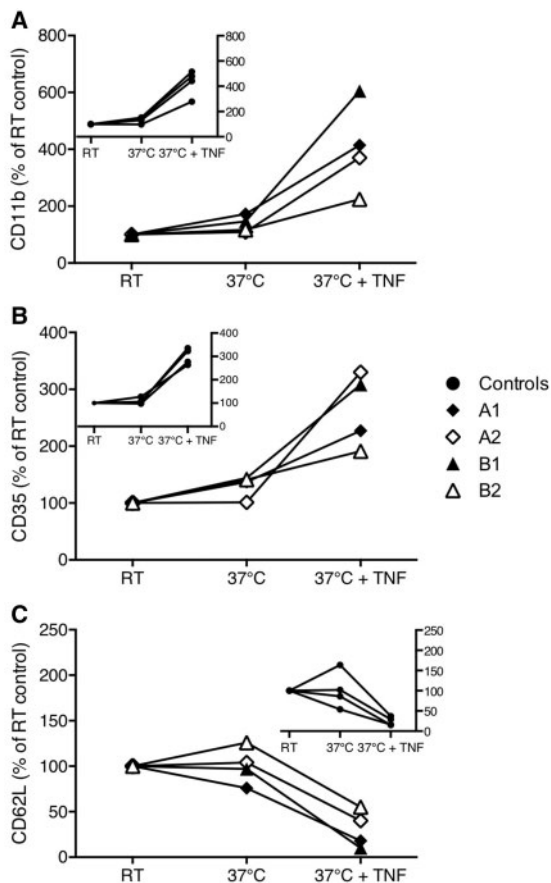
Receptor exposure was measured on neutrophils in blood to investigate whether cells from patients with SAPHO syndrome had a primed phenotype. The cell surface markers CD35, CD11b and CD62L were measured in neutrophils from blood kept at room temperature or at 37°C without or with the priming agent TNF $\alpha$ . There were no major differences in basal levels of CD11b, CD35 or CD62L on any of the patient neutrophils as compared with control neutrophils (data not shown).

**Fig. 2** ROS production in neutrophils from patients with SAPHO syndrome and controls



Extracellular (A) and intracellular (B) ROS production stimulated with PMA (50 nM) was measured with isoluminol/luminol-enhanced CL. Results show the peak activity. To the left is data collected from neutrophils isolated from buffy coats (grey) or peripheral blood (black) from healthy donors. To the right, results are shown for patients with SAPHO syndrome and healthy controls measured in parallel. Note that patient A1 and its control were measured in a plate reader but the values have been converted to match CL activity as measured for the other samples (asterisks). (C) ROS production measured by PHPA oxidation after 15 min stimulation with PMA (50 nM). Extracellular and total H<sub>2</sub>O<sub>2</sub> productions were measured in the absence or presence of azide (1 mM). Intracellular H<sub>2</sub>O<sub>2</sub> was calculated as the difference between the total and extracellular production. Results show the fluorescence of PHPA in arbitrary units. (D) The MPO quantity in neutrophil cell lysates was measured by ELISA. CL: chemiluminescence.

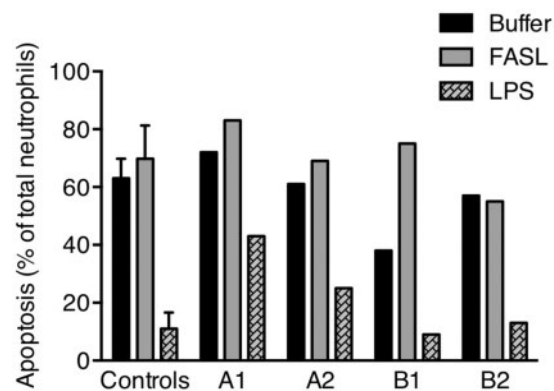
Incubation of the cells from patients A and B at 37°C in the absence of TNF did not substantially affect the levels of CD11b and CD35, but after TNF stimulation these granule markers had significantly increased on the cell surface (Fig. 3A and B), in line with the results on control cells

**Fig. 3** Surface molecule exposure

The surface molecules CD11b, CD35 and CD62L were measured in neutrophils from peripheral blood incubated at room temperature (RT) or at 37 °C without or with TNF $\alpha$  (10 ng/ml) for 20 min. The surface expression of the markers was measured with fluorophore-conjugated antibodies against the respective protein in non-permeabilized cells and the fluorescence was measured in a flow cytometer. Results show the mean fluorescence intensity as a percentage of the value in RT cells.

(insets). Correspondingly, the cleavage of CD62L was primarily seen after TNF treatment of the neutrophils, and to similar extents as for the controls (Fig. 3C). Taken together, these data show that neutrophils from patients with SAPHO syndrome show no alterations in responsiveness to priming agents, and have a normal degranulation function.

Nevertheless, neutrophils in the inflammatory sample from patient A (A1) showed slightly increased CD11b after incubation at 37 °C and a corresponding cleavage of CD62L (Fig. 3). Also patient C showed upregulation of neutrophil CD11b as compared with control cells (data not shown) in the 37 °C sample, in line with the inflammatory status of this patient (Table 2). These data suggest that an inflammatory episode of SAPHO syndrome can be associated with the neutrophils taking on a pre-primed phenotype.

**Fig. 4** Neutrophil apoptosis as measured by Annexin V expression

Isolated neutrophils were incubated for 20 h *in vitro* in the absence (buffer; spontaneous apoptosis) or presence of FASL (pro-apoptotic stimulus) or LPS (anti-apoptotic stimulus). The cells were then stained with Annexin V and 7AAD, and analysed by flow cytometry to investigate the level of apoptosis (Annexin V positive, 7AAD negative). The graph shows the percentage of apoptotic neutrophils in the different samples. The controls (n = 4) are shown as means and SD.

Neutrophils from patient C responded well to TNF $\alpha$  priming (data not shown), that is, the priming mechanisms were intact in these cells as for patients A and B. Neutrophils from patient D, however, did not respond to TNF $\alpha$  priming *in vitro* (data not shown). Since this patient was on anti-TNF $\alpha$  treatment at the time of sampling, and the assay was done in whole blood and not with isolated white blood cells, we believe that the presence of TNF inhibition therapy affected the neutrophil responsiveness to TNF $\alpha$ .

#### Neutrophil apoptosis in SAPHO syndrome

Neutrophils are short-lived cells and spontaneous apoptosis is of importance to maintain homeostasis. Under inflammatory conditions the longevity of neutrophils has been reported to increase [14, 19] or decrease [31–33]. We tested whether the neutrophils from patients with SAPHO syndrome showed any tendencies of altered apoptosis in a 20 h *in vitro* assay. Spontaneous apoptosis was lower in the inflammatory neutrophil sample from patient B (Fig. 4, B1), in accordance with the highly inflammatory status of this patient [30, 31]. The neutrophils in this sample responded readily to pro-apoptotic (FASL) and anti-apoptotic stimuli (LPS) (Fig. 4), indicating normal apoptosis regulation. Neutrophils from patient A (Fig. 4) showed normal spontaneous apoptosis and responded to the anti-apoptotic stimulus LPS, although this effect was slightly less apparent in these cells compared with controls. The pro-apoptotic effect induced by FASL in these samples, as well as in sample B2, was very low, most probably due to the already quite high spontaneous apoptosis in these samples after 20 h incubation. Patients C and D showed normal apoptosis regulation

(data not shown). Taken together no signs of dysregulated neutrophil apoptosis could be associated with SAPHO syndrome.

### SAA is a more sensitive inflammation marker than CRP during resolution and relapse in SAPHO syndrome

For patients A and B, both SAA and CRP were markedly increased during the acute onset of disease (Fig. 1). Interestingly, SAA was consistently higher during the follow-up period and began the decrease back to baseline at a later time point than CRP in both patients. During relapse in patient B, SAA increased earlier and rose substantially more than CRP (Fig. 1B). In addition, patient D, who was in remission at the time of sampling, showed increased SAA levels. As she was sampled 5 weeks after infliximab treatment and just prior to receiving the next dose, we anticipate that SAA could be a sign of re-activation of the inflammatory response. Thus, SAA can be considered a more sensitive monitoring acute-phase reactant in these patients as compared with CRP, the more widely used inflammation marker.

## Discussion

The inflammatory mechanisms underlying SAPHO syndrome are largely unknown, but neutrophil activation is suggested as part of disease pathophysiology [34]. The interesting study by Ferguson and co-workers [13] investigating a family with SAPHO syndrome-like phenotype, featuring pustulosis, severe acne and osteitis, has fostered an intense discussion regarding the pathogenesis of SAPHO syndrome [22, 35, 36]. A main finding of that study was that intracellular ROS production was defective in the patient neutrophils, suggesting that this feature is associated with the hyperinflammatory phenotype of SAPHO syndrome. Specific lack of intracellular ROS production has also been linked with atypical chronic granulomatous disease [21], featuring hyperinflammation. Both these findings indicate a regulatory role for intracellular ROS in aseptic inflammation.

The generalizability of decreased intracellular neutrophil ROS production as part of SAPHO syndrome pathogenesis has so far not been investigated, which is why we analysed four patients diagnosed with the disease, with involvement of both bone and skin. We chose to include patients of relatively different characteristics, but within the diagnostic boundaries. In this small cohort, we were, however, unable to verify the neutrophil phenotype presented by Ferguson and coworkers; intracellular ROS production was normal in neutrophils from all patients in our cohort. Not only did the patient neutrophils produce normal levels of intracellular ROS, but the intracellular ROS production was also increased during inflammatory periods as compared with periods of remission in both patients for whom such samples were available. In fact, this is in accordance with our recently published data on patients with periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis syndrome [30]. Neutrophils from patients with periodic fever, aphthous stomatitis,

pharyngitis and cervical adenitis produced increased amounts of intracellular ROS during fever flares compared with periods of remission, suggesting that increased intracellular ROS is associated with the proinflammatory episodes also of that disease. Whether this is a generalizable effect in autoinflammatory disease, now supported by our findings in SAPHO patients, remains to be investigated.

The inter-individual variation in neutrophil ROS production is in our experience quite large and the use of one or two paired controls for comparison with a patient sample, as employed in many studies, can be misleading when drawing conclusions on results being out of range. We therefore compiled a larger set of control data from analyses done in our laboratory over time, for comparison with the SAPHO syndrome samples (and their paired controls). The data showed considerable inter-individual variability (Fig. 2), and compared with this large control cohort, neutrophils from all patients generated normal levels of ROS, both intra- and extracellularly and at all sampling occasions. However, we can draw the conclusion that patients A and B vary in their ROS production between the two sampling times (inflammatory and non-inflammatory phase), as intra-individual variation in neutrophil ROS production is low (A. Karlsson and J. Bylund, unpublished data).

We also performed a general characterization of neutrophil function in the SAPHO syndrome cohort. Neutrophils exist in different priming and activation states depending on the inflammatory status of their milieu. Priming of neutrophils is mainly due to upregulation of new receptors such as CD35 and CD11b from intracellular granule stores, but is also accompanied by shedding of CD62L from the surface. This process renders the cells more responsive to inflammatory stimuli when needed, but leaves the cells inactive, and thus less dangerous to their immediate surroundings. The priming process involves phenotypic changes such as decreased spontaneous apoptosis [37] and altered expression of surface receptors [38]. In neutrophils from patients with SAPHO syndrome, the responsiveness to the priming stimulus TNF seen as degranulation-dependent receptor upregulation and CD62L shedding was normal, as was the regulation of apoptosis in response to pro- and anti-apoptotic stimulation. We found neutrophils from two of the patients in an inflammatory phase of SAPHO syndrome to be slightly more easily primed than healthy controls (and patient samples taken in the non-inflammatory phase), that is, they were pre-primed. The presence of such pre-primed neutrophils in peripheral blood could possibly increase the risk for unnecessary inflammatory activation, as they are more easily triggered and may be activated prematurely, in the wrong place at the wrong time.

Finally, SAA appeared to be a more sensitive biomarker during SAPHO syndrome improvement and relapse. In fact, SAA was significantly elevated on several occasions when CRP was normal. We therefore suggest that SAA may be a valuable inflammatory indicator for monitoring disease progression and treatment response in SAPHO syndrome, in particular during circumstances where CRP is only slightly (or not at all) increased.



Taken together, neutrophils from patients with SAPHO syndrome showed a normal neutrophil phenotype with regard to functional parameters such as ROS production, degranulation, priming and apoptosis regulation. We conclude that a deficient NADPH oxidase-dependent ROS production in neutrophils is not a general feature associated with SAPHO syndrome, as has previously been suggested, and the pathological mechanisms behind this disease are still a matter of uncertainty.

## Acknowledgements

The authors thank participating patients and families. We also acknowledge the physicians and nurses providing clinical care of the patients, in particular Drs Eva Karnå, Ann Olsson, Karin Rydenman and Håkan Östberg. We thank Eva Olsson for coordination of the clinical aspects of the study and for providing logistic support.

**Funding:** This work was supported by the Swedish Medical Research Council, the Swedish Rheumatism Foundation, the King Gustaf V 80-year Memorial Foundation, the Fyrbodals Research and Development Council, Region Västra Götaland, the Department of Research and Development NU-Hospital Organisation, the Clas Groschinsky Foundation and the Swedish government under the agreement concerning research and education of physicians (ALF).

**Disclosure statement:** The authors have declared no conflicts of interest.

## Supplementary data

Supplementary data are available at *Rheumatology* Online.

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