

# Characterizing the presence of neutrophil extracellular traps in neutrophilic dermatoses

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## Abstract

Neutrophil extracellular traps (NETs) are implicated in the pathogenesis of multiple inflammatory dermatoses. However, characterization of NETs in neutrophilic dermatoses was performed on very limited number of patients; this limitation precluded definitive conclusions. In this case series of 57 patients, we compared the amounts of neutrophils producing NETs in cutaneous lesions of different entities of neutrophilic dermatoses (17 with pyoderma gangrenosum, 37 with Sweet's syndrome and three with subcorneal pustular dermatosis). NETs were identified by double immunofluorescence on formalin-fixed paraffin-embedded skin biopsies using antibodies against elastase and citrullinated histone 3. Percentages of neutrophils showing NETs were high across all three entities (62.9% in PG, 48.5% in SS and 37.8% in subcorneal pustular dermatosis). The differences in mean percentages were significant between entities, with PG showing significantly superior percentage of NETs compared with SS. In our series, 15.8% of neutrophilic dermatoses were associated with malignancies, 10.5% with autoimmune diseases and 73.7% were idiopathic. Percentages of NETs were not statistically different between aetiologies. These findings suggest that NETs are abundantly produced in the various entities and different aetiologies of neutrophilic dermatoses. In comparison with SS, the superior percentage of NETs in PG is clinically mirrored in its greater ulceronecrotic nature.

## KEYWORDS

neutrophil extracellular traps, neutrophilic dermatoses, pyoderma gangrenosum, subcorneal pustular dermatosis, Sweet's syndrome

## 1 | BACKGROUND

Of the multiple agents involved in the innate immune response, neutrophils are the earliest and most abundant responders. Employing a vast array of antimicrobial granule proteins, neutrophils are highly adept in disposing of their microbial targets. Given the non-specific

destructive potential implicit in their molecular armamentarium, neutrophils achieve on-demand targeted elimination via degranulation and phagocytosis. A third mechanism of neutralization occurs via the deployment of neutrophil extracellular traps (NETs). First described in 2004 by Brinkmann et al.,<sup>1</sup> NETs are net-like structures composed of antimicrobial enzymes such as myeloperoxidase,

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elastase, cathepsin G and other granule proteins that overlie a scaffold of decondensed chromatin. Akin to the spider's web, NETs ensnare the microbial assailant in question and ensure its extracellular demise. In stark contrast to their antimicrobial function, NETs have been incriminated in the instigation of autoimmune responses. Histones and DNA, key in NET formation, double as autoantigens for multiple autoimmune diseases including systemic lupus erythematosus; furthermore, the combination of DNA and LL37, another of the antimicrobial constituents of NETs, confers a potent immunostimulatory effect in autoimmune diseases such as SLE and psoriasis by virtue of their positive effect on Type 1 IFN release by plasmacytoid dendritic cells.<sup>2-5</sup>

Neutrophilic dermatoses are a heterogeneous group of disorders affecting the skin and intermittently reflecting internal disease. They can be associated with malignancies, autoimmune diseases and a number of drugs. Despite the variety of aetiologies conducive to the appearance of neutrophilic dermatoses, the histopathologic findings remain uniform; pathology fails to allude to the underlying aetiology. These dermatoses are united by the histologic finding of a predominantly neutrophilic infiltrate within the various layers of the skin. The abundance of neutrophils and their central role in the pathogenesis of neutrophilic dermatoses presupposes a critical pathomechanistic role for NETs. To lend credence to the aforementioned, we have previously shown an increase in NETs expression and influence in several neutrophilic dermatoses including Behçet's disease<sup>6</sup> and neutrophilic cutaneous vasculitides such as urticarial vasculitis and erythema elevatum diutinum.<sup>7</sup> A recurring theme in NETs pathogenesis is the presence of low-density granulocytes (LDG). This proinflammatory subset of neutrophils expresses the terminal differentiation markers CD15<sup>high</sup>/CD14<sup>low</sup>/CD10<sup>+</sup>/CD16<sup>+</sup> and was initially attributed a role in SLE pathogenesis.<sup>8</sup> A salient feature of LDGs is their enhanced capacity to form NETs. In their seminal work on NETs in the neutrophil-mediated PAPA syndrome, Mistry et al.<sup>9</sup> uncover an increased level of circulating LDGs in subjects with PAPA syndrome; moreover, the LDG gene signature and increased NETs were observed in lesions of pyoderma gangrenosum but not in uninvolved skin of PAPA syndrome patients. NETs are thus an integral part of neutrophilic dermatoses pathophysiology, yet characterization of NETs in a number of neutrophilic dermatoses remains scarce at best. While increased NETs formation has been reported in skin of patients with neutrophil-mediated Schnitzler's syndrome, Sweet's syndrome and pyoderma gangrenosum, sample sizes of 4 patients for each of the Sweet's syndrome and pyoderma gangrenosum cohorts precludes drawing definitive conclusions surrounding NETs formation in these entities.<sup>10</sup>

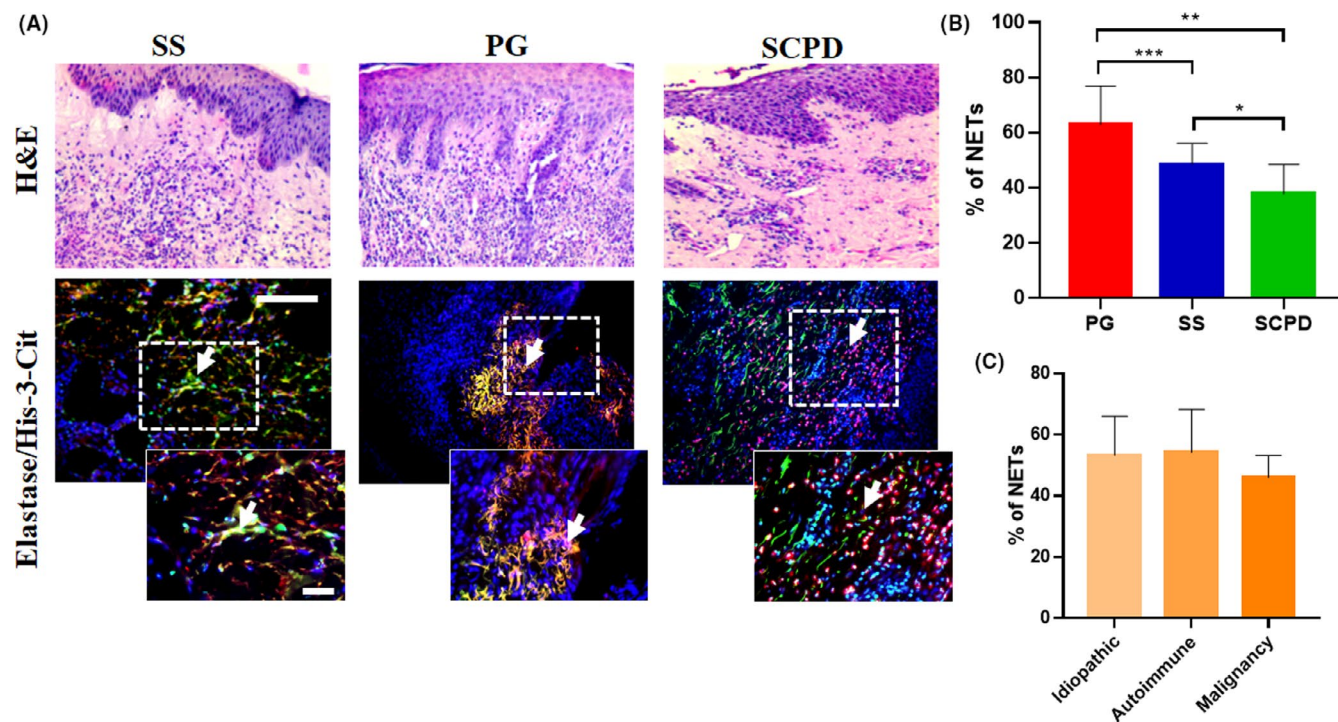
## 2 | QUESTIONS ADDRESSED

This study aims to characterize and to compare the number of neutrophils producing NETs in a large series harbouring patients with pyoderma gangrenosum, Sweet's syndrome and subcorneal pustular

dermatosis, three entities pertaining to the neutrophilic dermatoses. Furthermore, we wanted to quantify the effect of aetiology (idiopathic, malignancy-associated and autoimmune-mediated) on NETs formation and compare their means with one another to gauge the NET's potential as a diagnostic tool.

## 3 | EXPERIMENTAL DESIGN

Formalin-fixed paraffin-embedded (FFPE) biopsies of cutaneous lesions from 57 patients (Table 1) with histologic and clinical evidence of either pyoderma gangrenosum, Sweet's syndrome or subcorneal pustular dermatosis were obtained from the dermatopathology data registry at the American University of Beirut Medical Center and inspected for neutrophil infiltration and the presence of NETs. The study was carried out in accordance with the Institutional Review Board of the American University of Beirut. Assessment of immunofluorescence using antibodies against elastase (10 µg/ml, Santa Cruz biotechnology, #NP57: sc-53388), a component of the neutrophilic granules, permitted ascertainment of NETs presence. As histone citrullination represents the first step of NETs formation, double immunolabelling of elastase and citrullinated histone 3 (His-3-Cit) (10 µg/ml, Abcam, #ab5103) was performed as previously described (Figure 1A).<sup>11</sup> The percentage of elastase-positive neutrophils expressing His-3-Cit was counted in an effort to quantify the proportion of infiltrating neutrophils showing NETs. Another technique employed to ascertain the presence of NETs in the samples examined was protein arginine deiminase 4 (PAD4, 10 µg/ml, abcam, ab128086) staining. The rationale behind using PAD4 as a marker of NETosis was brought to light by Thiam et al. In their seminal work, these authors demonstrated the localization of PAD4 in the nucleus and the vital roles that PAD4 plays in DNA decondensation, nuclear envelope rupture and extracellular DNA expulsion, all essential steps in NETosis.<sup>12</sup> All resultant test subject stains were compared to similar stains carried out on three subacute cutaneous lupus erythematosus patients, previously shown to exhibit very low NETosis (5%) by our team (Figure S1).<sup>11</sup> Subsequently, quantification was executed via ImageJ software.<sup>13</sup> Finally, we excluded subcorneal pustular dermatosis from the statistical analysis due to low sample size ( $n = 3$ ) and performed a statistical comparison between the mean percentages of the pyoderma gangrenosum and Sweet's syndrome samples using the Mann-Whitney test. Furthermore, the mean percentages of the three associations (idiopathic, malignancy-induced and autoimmune-mediated) were also compared using the Kruskal-Wallis one-way analysis of variance test. Statistical analysis was done via the GraphPad Prism 8 with a  $p$  value less than 0.05 considered significant. Moreover, functional immunohistochemical staining for IL-1 $\beta$  (10 µg/ml, abcam, ab2105) and TNF- $\alpha$  (10 µg/ml, ab66579, abcam) was carried out on three patients, one from each neutrophilic dermatosis group having a high percentage of NETosis, and superimposed on elastase staining (Figure S2).



**FIGURE 1** Identification and quantification of NETs in neutrophilic dermatoses. (A) Representative images of FFPE sections of Sweet's syndrome (SS), pyoderma gangrenosum (PG) and subcorneal pustular dermatosis (SCPD). H&E, haematoxylin and eosin staining; elastase/His-3-Cit, double immunofluorescence of elastase (red) and His-3-Cit (green) merged with Hoechst 33342 (blue); Colocalization of His-3-Cit and elastase was used to identify NETs (arrows) scale bar, 20  $\mu$ m. (B) Histograms showing the per cent of NETs-releasing neutrophils in the different entities of neutrophilic dermatoses described, calculated as the percentage of elastase-positive neutrophils expressing His-3-Cit. (C) Histograms showing the mean percentage ( $9 \pm$  SEM) of NETs-releasing neutrophils in the different associations implicated in the neutrophilic dermatoses described, calculated using the GraphPad Prism 8 software

## 4 | RESULTS AND DISCUSSION

Our cohort consisted of patients with pyoderma gangrenosum ( $n = 17$ ), Sweet's syndrome ( $n = 37$ ) and subcorneal pustular dermatosis ( $n = 3$ ). Demographic, clinical and histologic characteristics are outlined in Table 1. The mean percentages ( $\pm$ SEM) of elastase-positive neutrophils expressing NETs out of the total neutrophilic infiltrate were  $62.87\% \pm 3.40\%$  in pyoderma gangrenosum,  $48.52\% \pm 1.25\%$  in Sweet's syndrome and  $37.8\% \pm 6.14\%$  in subcorneal pustular dermatosis (Figure 1B). These results show significantly elevated NETosis when compared to a non-neutrophilic dermatosis such as subacute cutaneous lupus erythematosus, which was previously shown by our team to exhibit a very low percentage of neutrophils with NETosis at  $5\% \pm 5\%$ .<sup>11</sup> Interestingly, pyoderma gangrenosum had statistically significant higher percentages of neutrophils showing NETs than Sweet's syndrome ( $p < 0.0001$ ). We then asked whether the implication of NETs varies in the different aetiologies of neutrophilic dermatoses. Segregation of patients based on aetiology showed that percentages of neutrophils showing NETs were  $53.28\% \pm 1.97\%$  in the idiopathic group,  $54.2\% \pm 5.75\%$  in the autoimmune group (patients with systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease or inflammatory arthritis as neutrophilic dermatoses triggers) and  $46.06\% \pm 2.39\%$  in the malignancy group (Figure 1C). The difference between the

three aetiologic groups was not significant ( $p = 0.3064$ ). Finally, we asked whether cytokines that are implicated in the pathogenesis of neutrophilic dermatosis are associated with NETs. Indeed, IL-1 $\beta$  and TNF- $\alpha$  staining demonstrated the presence of both proinflammatory cytokines with partial colocalization to areas of NETosis (Figure S2).

To our knowledge, this study has employed the largest cohorts of pyoderma gangrenosum and Sweet's syndrome to date. Moreover, it is the first series of its kind to attempt to characterize the presence of NETs formation in a cohort of patients with subcorneal pustular dermatosis. The successful demonstration of high NETosis across the board further solidifies their role in the pathogenesis of the neutrophilic dermatoses under study. Furthermore, pyoderma gangrenosum demonstrated a superior percentage of NETs as compared to Sweet's syndrome. This interesting finding alludes to greater neutrophilic activation and damage in pyoderma gangrenosum and suggests a tentative rationale for the greater ulceronecrotic tendency pyoderma gangrenosum lesions exhibit compared to Sweet's syndrome. The role of NETs in our study might be partially extrapolated from the role of NETs in PAPA syndrome, another neutrophil-predominant autoinflammatory disorder.<sup>9</sup> NETs derived from neutrophils obtained from PAPA patients induced the release of IL-6 by macrophages; this alludes to a vicious cycle whereby NET formation is triggered by proinflammatory cytokines which themselves are induced by NETs.<sup>9</sup> While the autoimmune group displayed a slightly higher percentage

TABLE 1 Demographic, clinical and histologic characteristics as well as NETs quantification of neutrophilic dermatoses patients

Category	Case	Gender, age	Duration <sup>a</sup>	Location	Morphology	Histology	Aetiology	NETs <sup>b</sup> (%)
PG	1	Male, 17	3 w	Scrotum	Ulcer	Ulcer, SPLNVI	Malignancy	35
	2	Male, 38	1 y	N/A	10 × 15 cm ulcer with violaceous borders	Ulcer, SPVNI, DPVNI, INI, lymphos, ERBCs	Autoimmune	59
	3	Male, 11	6 m	Abdomen & LE	Ulcers	Ulcer, SPVNI, DPVNI, INI,	Autoimmune	32
	4	Female, 25	N/A	N/A	Ulcers	Ulcer, SPVNI, DPVNI, INI, lymphos	Autoimmune	67
	5	Male, 48	N/A	LE	Ulcers	Ulcer, SPVNI, DPVNI, INI, DF, lymphos, ERBCs	Idiopathic	50
	6	Male, 29	3 m	LE	Ulcers	Ulcer, SC, SPVNI, DPVNI, INI, lymphos, DF	Idiopathic	49
	7	Female, 16	N/A	N/A	N/A	N/A	Idiopathic	60
	8	Male, 85	N/A	LE	Ulcers	Ulcer, epidermal hyperkeratosis, FP, DF, SPVNI, DPVNI, INI, lymphos	Idiopathic	71
	9	Male, 45	N/A	LE	Ulcers	Ulcer, epidermal hyperkeratosis, SPVNI, DPVNI, INI, lymphos	Idiopathic	74
	10	Male, 22	N/A	LE	Ulcer	Ulcer, epidermal hyperkeratosis, FP, SPVNI, DPVNI, INI, lymphos	Idiopathic	76
	11	Male, 57	N/A	LE	Ulcers	Ulcer, SPVNI, DPVNI, INI, lymphos, ERBCs	Idiopathic	76
	12	Female, 38	N/A	UE	Ulcers	Ulcer, Epidermal hyperkeratosis, SPVNI, DPVNI, INI, lymphos	Idiopathic	75
	13	Male, 42	N/A	Dorsa of foot	Ulcers	Ulcer, SPVNI, DPVNI, INI, endothelial hyperplasia	Idiopathic	70
	14	Female, 50	N/A	N/A	Bullae	SPVNI, DPVNI, INI, lymphos, vasculitis	Idiopathic	69
	15	Male, 52	N/A	LE	Ulcers	Ulcer, epidermal hyperkeratosis, FP, SPVNI, DPVNI, INI, lymphos	Idiopathic	70
	16	Female, 45	N/A	Breast	Ulcer	Ulcer, SPVNI, DPVNI, INI, lymphos	Idiopathic	60
	17	Female, 24	N/A	LE	Ulcer	Ulcer, Epidermal hyperkeratosis, FP, PDE, SPVNI, DPVNI, INI	Idiopathic	77
SS	18	Female, 70	3 d	LE	Erythematous indurated plaques	Epidermal hyperplasia, focal spongiosis, PDE, ERBCs, DPVNI, INI, lymphos, leucocytoclasia	Malignancy	42
	19	Male, 5	3 w	LE	Painful nodules	SPVNI, DPVNI, INI, SubqNI	Malignancy	46
	20	Female, 73	1 w	Head & UE	Indurated pseudo-vesicular papules & nodules	SPVNI, DPVNI, INI	Malignancy	51
	21	Male, 64	6 m	Generalized	Erythematous papules	SPVNI, DPVNI, INI, SubqNI	Malignancy	38
	22	Female, 35	4 d	Trunk	Violaceous indurated plaques with fever	SPVNI, DPVNI, INI, SubqNI	Malignancy	56
	23	Female, 33	N/A	Scalp & UE	Indurated erythematous plaques	SPVNI, DPVNI, INI, SubqNI	Malignancy	56
	24	Male, 12	3 d	LE	Erythematous papules	SPVNI, DPVNI, INI, SubqNI	Malignancy	46
	25	Female, 49	N/A	Trunk, UE, & LE	Plaques with overlying pustules	SPVNI, DPVNI, INI, SubqNI	Malignancy	46
	26	Female, 21	6 y	Generalized	Bullae and infiltrated papules	SPVNI, DPVNI, INI	Autoimmune	69

(continues)

TABLE 1 (continued)

Category	Case	Gender, age	Duration <sup>a</sup>	Location	Morphology	Histology	Aetiology	NETS <sup>b</sup> (%)
	27	Female, 23	1 m	LE	Large erythematous and tender infiltrated plaques	SPVNI, DPVNI, INI, lymphos	Autoimmune	45
	28	Male, 29	2 w	LE	Painful progressive subcutaneous nodules	SPVNI, DPVNI, INI, lymphos, eos, SubqNI, septal fibrosis	Autoimmune	53
	29	Female, 39	5 d	N/A	Papules, burning pain, & arthralgias	PDE, DPVNI, INI	Idiopathic	55
	30	Male, 20	1 w	Dorsa of feet & palms	Symmetric erythematous papules	PDE, DPVNI, INI, Eos	Idiopathic	42
	31	Female, 50	3 d	Trunk	Erythematous urticarial plaques	PDE, DPVNI, INI	Idiopathic	56
	32	Male, 62	10 d	Trunk	Erythematous plaques + fever	FP, SC, irregular epidermal hyperplasia, focal keratinocyte necrosis, PDE, DPVNI, INI, SubqNI	Idiopathic	45
	33	Male, 53	1 y	LE	Itchy papules	SPVNI, DPVNI, INI, SubqNI	Idiopathic	54
	34	Female, 49	N/A	LE	Erythematous itchy petechial macules & papules	SPVNI, DPVNI, INI, lymphos, eos	Idiopathic	50
	35	Male, 65	1 m	Left LE	Itchy indurated scaly plaques	SPVNI, DPVNI, INI, lymphos, eos	Idiopathic	65
	36	Female, 69	2 d	Back & LE	Erythematous pseudo-vesicular papulonodules	SPVNI, DPVNI, INI, lymphos, eos, endothelial cell swelling	Idiopathic	54
	37	Male, 39	1 w	Nose & perioral area	Erythematous papules and nodules	FP, SC, PDE, SPVNI, DPVNI, INI, endothelial cell swelling	Idiopathic	52
	38	Male, 69	2 w	LE	Erythematous subcutaneous papules and nodules	DPVNI, INI, SubqNI, vasculitis	Idiopathic	60
	39	Male, 51	20 d	UE	Erythematous infiltrated papules with fever + chills	SPVNI, DPVNI, INI, SubqNI	Idiopathic	48
	40	Male, 49	N/A	N/A	N/A	SPVNI, DPVNI, INI, SubqNI	Idiopathic	33
	41	Male, 33	2 d	Head & neck	Violaceous plaques	SPVNI, DPVNI, INI, SubqNI	Idiopathic	41
	42	Male, 65	2 d	UE	Erythematous papulonodules	SPVNI, DPVNI, INI, SubqNI, eos	Idiopathic	37
	43	Female, 45	3 y	Neck, UE, & LE	Targetoid bullae and patches	SPVNI, DPVNI, INI, eos, nuclear debris	Idiopathic	51
	44	Female, 41	1 m	Head, neck, & UE	Itchy burning erythematous infiltrated papulovesicles	SPVNI, DPVNI, INI, SubqNI	Idiopathic	59
	45	Female, 43	2 w	Dorsa of right hand	Erythematous papulovesicles	SPVNI, DPVNI, INI, SubqNI	Idiopathic	42
	46	Male, 74	2 d	Dorsa of hands	Erythematous papulonodules	SPVNI, DPVNI, INI, SubqNI	Idiopathic	48
	47	Female, 74	N/A	Trunk	Erythematous indurated papules	SPVNI, DPVNI, INI, nuclear debris	Idiopathic	46
	48	Female, 41	1 w	Torso, chin, & UE	Papulovesicles and nodules	PDE, DPVNI, INI	Idiopathic	46
	49	Male, 74	N/A	UE	Erythematous plaques with overlying vesicles	PDE, SPVNI, DPVNI, INI, lymphos, eos, nuclear debris	Idiopathic	41
	50	Female, 69	1 w	Dorsa of hands	Erythematous papules	SPVNI, DPVNI, INI, SubqNI	Idiopathic	45

(continues)

TABLE 1 (continued)

Category	Case	Gender, age	Duration <sup>a</sup>	Location	Morphology	Histology	Aetiology	NETs <sup>b</sup> (%)
	51	Female, 62	15 d	Dorsa of hands	Violaceous plaques with overlying pustules	SPVNI, DPVNI, INI	Idiopathic	41
	52	Female, 50	1 w	Left hand	Erythematous indurated plaque	SPVNI, DPVNI, INI	Idiopathic	46
	53	Female, 32	1 w	Dorsa of left hand	Pseudovesicular papules	SPVNI, DPVNI, INI, SubqNI	Idiopathic	47
	54	Female, 51	1 d	UE	Erythematous and indurated papulonodules	SPVNI, DPVNI, INI, nuclear debris, endothelial cell swelling	Idiopathic	49
SCPD	55	Female, 35	7 m	Trunk	Recurrent pruritic vesicles	SCP (PMNs & eos), acantholysis, FPE, SPVNI, lymphos, eos	Idiopathic	28
	56	Male, 32	3 y	Trunk	Recurrent pruritic vesicles	SCP (PMNs & eos), acantholysis	Idiopathic	49
	57	Female, 64	N/A	Inframammary area	Erythematous crusted plaques with minute pustules	SCP (PMNs & eos), acantholysis	Idiopathic	36

Abbreviations: DF, dermal fibrosis; DPVNI, deep perivascular neutrophilic infiltrate; eos, eosinophils; ERBCs, extravasated red blood cells; FP, focal parakeratosis; INI, interstitial neutrophilic infiltrate; LE, lower extremities; lymphos, lymphocytes; PDE, papillary dermal oedema; PMNs, polymorphonuclear cells; SC, scale crust; SCP, subcorneal pustule; SPVNI, superficial perivascular neutrophilic infiltrate; SubqNI, subcutaneous neutrophilic infiltrate; UE, upper extremities.

<sup>a</sup>Duration of cutaneous manifestations (d = days, m = months, w = weeks, y = years).

<sup>b</sup>Percentage of neutrophils showing NETs.

of NETs than the idiopathic and malignancy groups, differences in NETs expression between the aetiologies were not significant. This could be attributed to the low power of the analysis brought about by the imbalance in subject numbers between the three aetiologies (42 subjects in the idiopathic group vs. 6 in the autoimmune group vs. 9 in the malignancy group). As such, future research with larger and more balanced cohorts could provide better aetiologic comparisons and potentially elaborate an aetiology-dependent role for NETs formation in the different neutrophilic dermatoses. Owing to the overactivation of the innate immune system with an increase in the production of interleukin-1 (IL-1) family members and the sterile neutrophil-predominant characteristic infiltrate, pyoderma gangrenosum and Sweet's syndrome have recently been touted as auto-inflammatory disorders.<sup>14</sup> True to form, IL-1 $\beta$  and TNF- $\alpha$  have been ascribed a vital role in the pathogenesis of both entities vis-à-vis an overrepresentation of both cytokines in both neutrophilic dermatoses. In his seminal work on elucidating the autoinflammatory nature of pyoderma gangrenosum and Sweet's syndrome, Marzano et al.<sup>15</sup> describe an overrepresentation of IL-1 $\beta$  and TNF- $\alpha$  in tissue samples from both entities when compared to healthy controls. The centrality of IL-1 $\beta$  is further ascertained in the resolution of steroid-refractory pyoderma gangrenosum and steroid-refractory Sweet's syndrome on the IL-1 antagonist canakinumab<sup>16</sup> and the IL-1 receptor antagonist anakinra,<sup>17</sup> respectively. Likewise, TNF- $\alpha$ 's role in both entities was also corroborated by resolution of steroid-refractory pyoderma gangrenosum and steroid-refractory subcutaneous Sweet's syndrome on the TNF- $\alpha$  antagonists infliximab<sup>18</sup> and adalimumab,<sup>19</sup> respectively. In our study, cytokine staining did identify IL-1 $\beta$  and TNF $\alpha$  to further strengthen the notion of an autoinflammatory rationale for both pyoderma gangrenosum and Sweet's syndrome; furthermore, both cytokines were partially colocalized with areas of NETs formation, alluding to the possible role these proinflammatory cytokines play in tandem with NETs. NETs were previously found to elicit IL-1 $\beta$  production by macrophages.<sup>20</sup> While macrophages producing IL-1 $\beta$  were co-stimulated by lipopolysaccharide in the aforementioned experiment, it is possible that IL-1 $\beta$  release occurs in the context of NETosis and a similar unknown trigger in the neutrophilic dermatoses under study. Conversely, IL-1 $\beta$  and TNF- $\alpha$  were both found to induce NETosis in patients with systemic inflammatory response syndrome; IL-1 $\beta$ - and TNF- $\alpha$ -induced NETs could then exert their destructive effects via other mechanisms. Nevertheless, our findings of cytokine-NETs colocalization pave the way for future research with a much larger number of test subjects to derive important conclusions with regard to the role of proinflammatory cytokines in NETosis. Beside the imbalance in subject number per cohorts described above, other major limitations of this study include its retrospective and descriptive nature and the lack of clinical data in a select number of subjects.

## 5 | CONCLUSIONS

Neutrophil extracellular traps are massively produced in the different entities and various aetiologies of neutrophilic dermatoses.

Pyoderma gangrenosum lesions showed superior degree of NETosis compared to Sweet's syndrome, possibly providing a rationale for the greater ulceronecrotic tendency seen in pyoderma gangrenosum. Proinflammatory cytokines such as IL-1 $\beta$ - and TNF- $\alpha$  are expressed in areas of NETs deposition. Our findings point towards a central role of NETs in the pathogenesis of neutrophilic dermatoses.

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#### CONFLICT OF INTEREST

The authors have declared no conflicting interests.

#### AUTHOR CONTRIBUTION

EE collected FFPE tissues and the clinical data, analysed the results and wrote the manuscript; RS performed the experiments and analysed the results; GEH performed the experiments; OA and AGK performed histopathological analysis; and DN supervised the project and analysed the results. All authors read and approved the final manuscript.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**Figure S1.** Representative images of PAD4 immunolabelling (red) in Sweet's syndrome (SS), pyoderma gangrenosum (PG), subcorneal pustular dermatosis (SCPD) and subacute cutaneous lupus erythematosus (SCLE) patients. Scale bar, 20  $\mu$ m.

**Figure S2.** Identification of cytokines associated with NETs in neutrophilic dermatoses using double immunofluorescence for elastase (red) and either TNF $\alpha$  (green) or IL1  $\beta$  (green). Arrows point to the colocalization between elastase and either TNF $\alpha$  or IL-1 $\beta$ . Scale bar, 20  $\mu$ m.

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