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Inflammatory Diseases

Neutrophil Extracellular Traps Are Widely Distributed Across Lesional and Perilesional Hidradenitis Suppurativa Skin, and Elevated Serum NET Markers Associate With Moderate to Severe HS Disease

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ABSTRACT

Background: Neutrophils are scarce in healthy skin but infiltrate lesions of hidradenitis suppurativa (HS) patients. Activated neutrophils release proinflammatory neutrophil extracellular traps (NETs), which have been implicated in the pathophysiology of HS. This study aimed to describe the distribution of NETs relative to the features of HS skin lesions and reveal whether serum NET markers were elevated in association with disease activity.

Methods: Immunohistochemistry assessed the distribution of the key NET component citrullinated histone H3 (CitH3) in lesional, perilesional, and unaffected HS skin. Several markers of NETs (nucleosomes, calprotectin, and CitH3) were quantified in HS serum with ELISA.

Results: HS lesional skin biopsies showed increased CitH3-positive staining compared to unaffected skin. This signal was widely distributed across both lesional and perilesional regions of HS skin and was associated with HS structures such as the lining of epithelialized skin tunnels. Moreover, several NET-associated markers were elevated in the serum of HS patients compared to healthy volunteers and correlated with each other. Finally, serum NET markers showed significant elevation in patients with moderate to severe disease activity based on IHS-4 scores, compared to those with no or mild activity.

Conclusions: Elevated NET markers are widely distributed in HS skin and serum. These data indicate that NET-associated markers in serum are candidate biomarkers for HS disease severity. The results confirm the rationale for anti-inflammatory therapy targeting NETs in HS.

1 | Introduction

Hidradenitis suppurativa (HS) is a chronic, inflammatory, and proliferative skin disease characterized by recurrent, painful skin lesions, including inflammatory nodules and abscesses in the axillae, groin, and other flexural areas [1]. Irreversible tissue destruction and extensive scarring may result as the disease progresses. In Europe and North America, HS disproportionately

Summary

- Hidradenitis suppurativa (HS) is an inflammatory skin disease that poses significant unmet needs.
- We investigated abnormalities in HS skin and blood to identify new therapeutic approaches.
- During disease, cells release their DNA as inflammatory structures called neutrophil extracellular traps (NETs).
- We showed the enrichment of NETs in all layers of HS lesions and nearby skin with microscopy.
- Moreover, we found higher levels of NET markers in HS blood compared to healthy volunteers, which increased with more active disease.
- Our findings support the development of therapies targeting NETs in HS.

affects women and typically appears in early adulthood. Despite being a debilitating skin disorder, HS is often underreported and misdiagnosed [2] but affects approximately 1% of the European population, with rates varying in different regions worldwide [3, 4].

The primary pathogenic event in HS involves hair follicle occlusion due to hyperkeratosis and hyperplasia of the follicular epithelium, followed by cyst formation and eventual rupture [5]. The rupture of cysts, with the release of commensal microbiota and keratin, triggers inflammatory cell infiltration comprising neutrophils, lymphocytes, and macrophages [6]. This results in recurring, painful nodules and pus-containing abscesses which, in patients with severe disease, can progress into inflamed, pus-draining, connected epithelialized skin tunnels (previously also known as sinus tracts or fistulae) that are characteristic of HS [7]. These skin tunnels cause substantial pain, are predictors of poor response to therapy [8], and are associated with a more aggressive disease course [9]. In recent years, a detailed understanding of the features of HS lesions has translated into therapeutic approaches that have brought much-needed benefit to a subset of patients [10]. In addition, several studies propose a pathogenic role for neutrophils [11] with therapeutics targeting neutrophil chemotaxis [12] and neutrophil priming for activation [13] showing favorable responses in clinical studies.

One pro-inflammatory action of neutrophils is the release of neutrophil extracellular traps (NETs) by a highly organized process characterized by citrullination (i.e., arginine deamination) of histones, chromatin reorganization within the nucleus, breaching of the nuclear membrane followed by chromatin decondensation within the cytoplasm, and finally plasma membrane rupture resulting in the release of chromatin in association with the granule contents into the extracellular space [14, 15]. The dysregulated release of cytotoxic and pro-inflammatory NETs is associated with the pathology of several immune-mediated inflammatory diseases [16], including HS [17]. Neutrophils from HS patients have an enhanced propensity to release NETs [18], HS patients have an impaired ability to degrade DNA [19], and higher tissue NET levels associate with more severely affected skin regions [18]. Furthermore, numerous genetic mutations in patients with HS have been described in subunits of γ-secretase [20], and the observation that NETs can activate γ-secretase to induce pro-inflammatory and fibrotic responses directly links NETs with disease pathogenesis [21].

The current study aimed to conduct an immunohistochemical analysis of different skin regions associated with HS lesions to show the presence and distribution of NETs and their association with other characteristic features of HS. In addition, we sought to compare serum levels of neutrophil activation markers across mild to severe HS disease activity with those of healthy volunteers.

2 | Materials and Methods

2.1 | HS Patient Samples

Two cohorts of HS patients from Cardiff University Biobank (CUB) and Wroclaw Medical University (WMU) were used for the analysis of biopsies from lesional, perilesional (2 cm away from involved skin in the region), or unaffected skin (at least 10 cm away) (Table 1). Samples were collected with 4 mm biopsy punches from surgically resected tissue, snap-frozen in liquid nitrogen, and stored at −80°C prior to analysis. Serum samples were obtained from three cohorts of HS patients from CUB, WMU, and Erasmus Medical Centre (EMC) (Table 1). Serum from two cohorts of healthy volunteers, matching in age average and range with the HS cohorts, were obtained from the Centre of Human Drug Research (CHDR, NL, $n = 50$) and Quest Pharmaceutical Services (QPS, NL, $n = 11$) as well as healthy serum from EMC ($n = 5$). Whole blood was collected in serum separator tubes and allowed to clot for 30 min at room temperature (RT). Next, the samples were centrifuged at 2000 ×*g* for 10 min and aliquoted before storage at −80°C. Ethical approval was provided by CUB (22/0004), WMU Ethics Committee (KB-901/2022 and KB-234/2023), EMC (MEC-2016-246), and for CHDR and QPS the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (NL77528.056.21). All participants provided informed written consent.

2.2 | Immunohistochemistry of HS Skin Tissue

Hematoxylin & eosin (H&E) staining and immunohistochemistry (IHC) on HS biopsies were performed by HistologiX Ltd. Sections of 6 μm were cut, air dried at RT for at least 30 min (min), and fixed in acetone for 10 min. For IHC staining, sections were rinsed in running tap water for 5 min, washed with Tris Buffered Saline (TBS) containing 0.1% (v/v) Tween 20, blocked with 2.5% normal donkey serum for 20 min, and incubated with isotype control (Abcam, ab172730) or recombinant rabbit anti-histone H3 (citrulline R17) antibody (CitH3; Abcam, ab219407) at 2.2 μg/mL for 60 min. After several washes, the sections were incubated with 10 μg/mL donkey anti-rabbit AlexaFluor Plus 647 (Invitrogen, A32795) for 30 min. The sections were washed and counterstained with 300 nM DAPI for 15 min, followed by several washes. H&E staining was performed to study the histological features of HS pathology. Stained whole sections were digitally scanned with a 20× objective using the Hamamatsu

TABLE 1 | Patient characteristics.

HS patients			**Not available (*n*)**
n (patients that donated samples)	Lesional Perilesional Unaffected skin Serum	$n=23$ $n=21$ $n=9$ $n=50$	
Gender	M F	$n=17$ $n=34$	
Age (mean and range in years)	37.7	16–65	
BMI (mean and range in kg/m^2)	30.9	19.8–46.3	3
Hurley stage	I II III	$n=4$ $n=29$ $n=15$	3
IHS-4 score (mean and range)	17.2	0–57	8
Patients with concomitant IMIDs (*n* and percentage)	2	5.6%	15
Smokers	Never Previous Current	10 12 27	2
Healthy volunteers			
n (volunteers that donated samples)	Serum	66	
Gender	M F	$n=45$ $n=21$	
Age (mean and range in years)	36	19–55	
BMI (mean and range in kg/m^2)	23.6	18.5–29.9	5

Note: Serum and skin biopsies were obtained from HS patients from three different institutes (Cardiff University Biobank [CUB], Wroclaw Medical University [WMU], and Erasmus Medical Centre [EMC]) after approval by affiliated ethical committees. Healthy control serum was obtained from the Centre of Human Drug Research (CHDR), Quest Pharmaceutical Services (QPS), and EMC. Anonymized clinical data was obtained, including gender, age, disease activity scores (Hurley staging and IHS-4 scores), body mass index (BMI), concomitant diseases, and smoking behavior.

NanoZoomer 2.0 HT. Histopathology and CitH3 staining in HS biopsies were assessed in a blinded manner by a pathologist.

Histological sections from skin biopsies were screened for quality and completeness of the epidermis (proportions of stratum corneum, stratum granulosum, and stratum basale) and dermis. H&E sections were then assessed for key diagnostic features of HS: follicular hyperkeratosis, psoriasiform epidermal hyperplasia, perifolliculitis, and skin tunnels. Histological analysis of CitH3 signal in the epidermis and dermis compartments was assessed using the following semiquantitative grade scale: 0: no signal; 1: minimal, focal signal in one zone; 2: minimal, focal signal in several zones; 3: moderate, focal, or multifocal signal involving several anatomical structures; 4: marked, focal or multifocal signal involving multiple anatomical structures.

2.3 | Nucleosome Quantification in Serum

The Cell Death Detection ELISA PLUS kit (Roche, 11920685001) was used to determine nucleosome content with an adapted protocol. The positive control of the kit was used to generate a standard curve for interpolation. The incubation buffer was used as a blank. Samples and calibrators were prepared and incubated on the plates according to the manufacturer's instructions. The nucleosome content in undiluted HS and healthy serum was determined by interpolating the sample optical density signal using a nonlinear 5PL fit with GraphPad Prism software. The lower limit of detection (LOD) was determined at 2.0 mU.

2.4 | Calprotectin and Citrullinated Histone H3 Quantification in Serum

Calprotectin was measured as a neutrophil activation marker in 500 times diluted HS and healthy serum using the Quantikine Human S100A8/S100A9 Heterodimer Immunoassay (R&D Systems, DS8900) according to the manufacturer's protocol. The LOD was determined at 62.5 ng/mL. CitH3 levels were measured as a marker for NETs in 5 times diluted HS and healthy serum using a CitH3 (Clone 11D3) ELISA kit (Cayman Chemical, 501620) according to the manufacturer's protocol. The LOD was determined at 0.1 ng/mL.

2.5 | Subgrouping According to Disease Activity

To further understand the relationship between NET markers in HS patient serum and disease activity, the HS cohorts were subgrouped into mild, moderate, and severe HS based on their IHS-4 scores [22]. Briefly, an IHS-4 score of 0–3 points was classified as mild HS, 4–10 points as moderate, and severe HS as an IHS-4 score of 11 points or higher.

2.6 | Statistics

Generation of graphics and statistical analyses were performed using GraphPad Prism 10 software (version 10.2.3). The quantitative results are reported as means with standard deviation or as the median when using a logarithmic scale. Differences were considered significant at $p<0.05$. Data below the LOD were set at the value of LOD. Results were first tested for normal distribution using the Shapiro–Wilk test. Where data were not normally distributed, two-tailed, unpaired nonparametric tests were used for assessing significance (Mann–Whitney test for single comparisons and Kruskall–Wallis test with Dunn's post hoc test for multiple comparisons). Spearman's rank correlation coefficient investigated the correlation between serum NET markers. Missing data (8 out of 50 IHS4 scores) were omitted from the analysis.

3 | Results

3.1 | NETs Are Present in Lesional and Perilesional Regions of HS Skin

To study the distribution of NETs in HS lesions, biopsies of lesional, perilesional, and unaffected skin were collected from 27 HS patients, the characteristics of whom are described in Table 1. Biopsies presented with a wide range of tissue lesions, but not all biopsies presented with all lesion types. Figure 1 demonstrates the staining of lesional, perilesional, and unaffected skin for CitH3. Unaffected skin from HS patients showed an intracellular, punctate CitH3 staining mainly in the basal layer and epidermis (Figure 1a, white arrowheads), which is not NET-related but reflects the typical physiology of maturing keratinocytes [23]. NETs were identified as a diffuse extracellular CitH3-positive signal (yellow arrowheads) present in the lesional epidermis with HS-related psoriasiform epidermal hyperplasia (Figure 1b). The perilesional dermis was marked with HS structures such as follicular hyperkeratosis and dilation with perifolliculitis, and CitH3-positive diffuse staining was also observed in this region (Figure 1b). These data demonstrate that NETs are enriched in lesional and perilesional HS skin and co-localize with the histological features of HS.

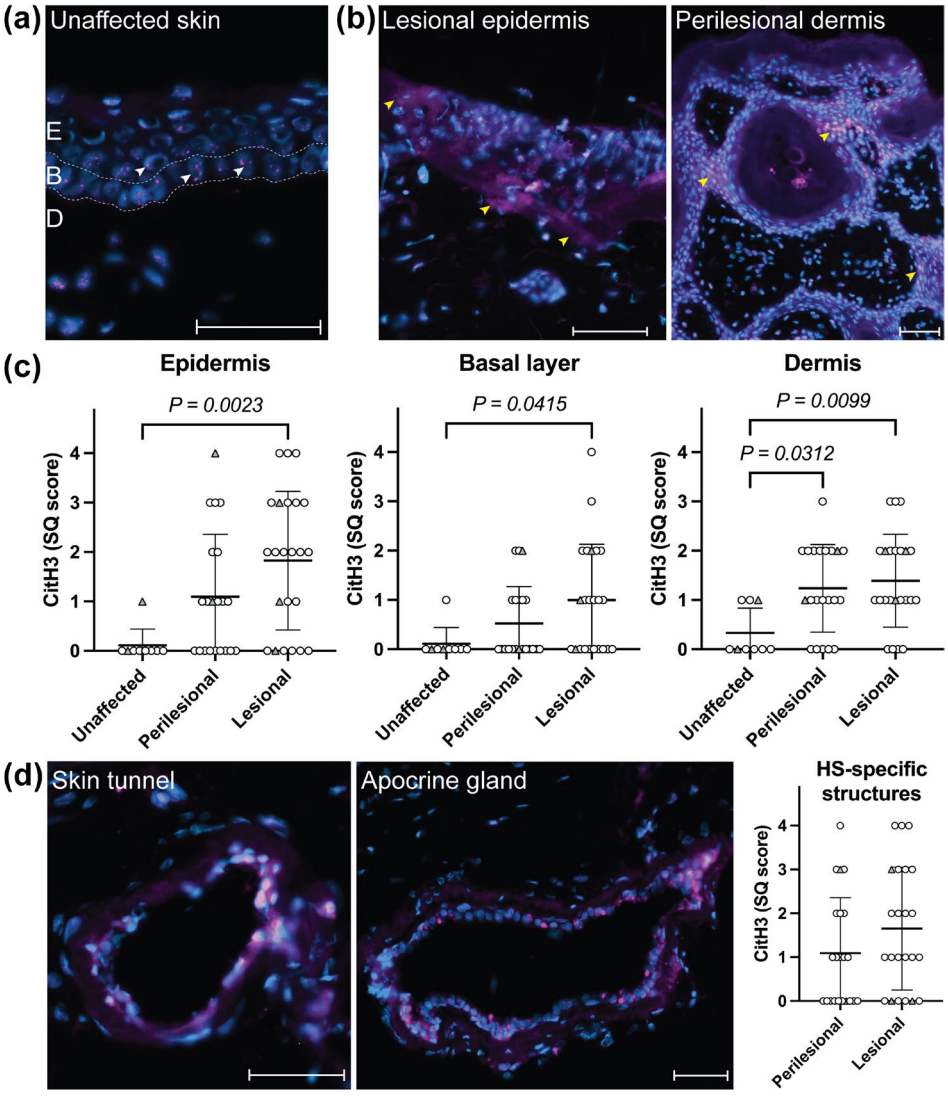

FIGURE 1 | NETs are widely distributed across HS lesional and perilesional skin. (a) Immunohistochemical staining of unaffected skin for citrullinated histone H3 (CitH3; pink) and DNA (blue) showed punctate intracellular CitH3 (white arrowheads) in the epidermis (E), basal layer (B), and dermis (D). (b) Hidradenitis Suppurativa (HS) lesional and perilesional skin CitH3 staining shows a diffuse extracellular signal, indicating neutrophil extracellular traps (NETs; yellow arrowheads). (c) CitH3 staining was scored semiquantitatively (0–4) in the epidermis, basal layer, and dermis of unaffected skin biopsies ($n = 9$) and perilesional ($n = 21$) and lesional ($n = 23$) HS skin biopsies. (d) Staining of HS lesional and perilesional skin for CitH3 aligns tunnel epithelium and apocrine glands with a positive signal. Alignment of the CitH3 signal with HS-specific features was scored semiquantitatively (0–4) in perilesional ($n = 22$) and lesional ($n = 23$) HS skin biopsies. Magnifications are indicated with scale bars of 50 μm in (a), (b), and (d). Statistical differences were analyzed using Kruskall-Wallis and Dunn's post hoc tests for multiple comparisons. Gray triangles in (c) and (d) indicate skin specimens from HS patients with IHS4 scores of 0 points. Lines and error bars in (c) and (d) indicate the mean with standard deviation. SQ = semiquantitative.

3.2 | NETs Are Widely Distributed Across HS Lesional and Perilesional Skin

Compared to unaffected skin, CitH3 in the lesional epidermis and basal layer was elevated, whereas the perilesional epidermis and basal layer showed a trend toward elevated NET levels (Figure 1c). In the deeper-located dermis, the elevation of CitH3 levels was prominent in both the lesional and perilesional HS skin. Surprisingly, in biopsies taken from noninflamed scar tissue or papules of patients with inactive disease (IHS-4 score of 0 points), detectable CitH3 levels were also found in different layers of lesional and perilesional skin (Figure 1c, gray triangles). Regarding the association of NETs with characteristic features of HS histopathology, marked diffuse extracellular CitH3 staining was detected in the lining of skin tunnels and apocrine glands (Figure 1d), with no observed differences between lesional and perilesional biopsies. In 74% of the HS patients from which we obtained biopsies, NETs were detected in the lesional epidermis, 83% in the lesional dermis, and 76% in the perilesional dermis. Only 11% of the patients from which we obtained biopsies showed an absence of CitH3 staining, and their IHS4 scores varied between 2 and 10 points.

3.3 | Several NET Markers Are Elevated in a Correlative Manner in HS Patient Serum

The comparison of circulating levels of nucleosomes, calprotectin, and CitH3 in the serum of HS patients and healthy controls is provided in Figure 2a. All three markers were increased in HS patient sera compared to healthy volunteers. Gray triangles indicate samples from patients with inactive disease, showing moderate NET marker levels. A strong correlation between CitH3 and nucleosomes was found ($r = 0.767$, $p = 1.291\text{e-}10$) and between CitH3 and calprotectin ($r = 0.590$, $p = 8.143\text{e-}6$), indicating that the principal source of these markers in serum is NET release (Figure 2b). To a lesser extent, a correlation was observed between calprotectin and nucleosomes ($r = 0.490$, $p = 3.566\text{e-}4$).

3.4 | Levels of Serum NET Markers Correlate With Disease Severity

Figure 3 shows NET marker levels in HS serum subgrouped by disease activity. Despite the limited subgroup sizes, HS patients with moderate or severe disease activity had elevated levels of nucleosomes and CitH3 in serum compared to healthy volunteers. Additionally, severe HS patients had increased levels of nucleosomes and CitH3 in serum compared to mild HS patients. For calprotectin, only severe HS patients had elevated levels in serum compared to healthy volunteers (Figure 3).

Two out of four serum samples from HS patients with inactive disease (gray triangles) showed elevated serum nucleosomes and CitH3 compared to the median of the healthy volunteers, and one out of four showed increased calprotectin levels. This indicates that although the IHS4 score may suggest low disease

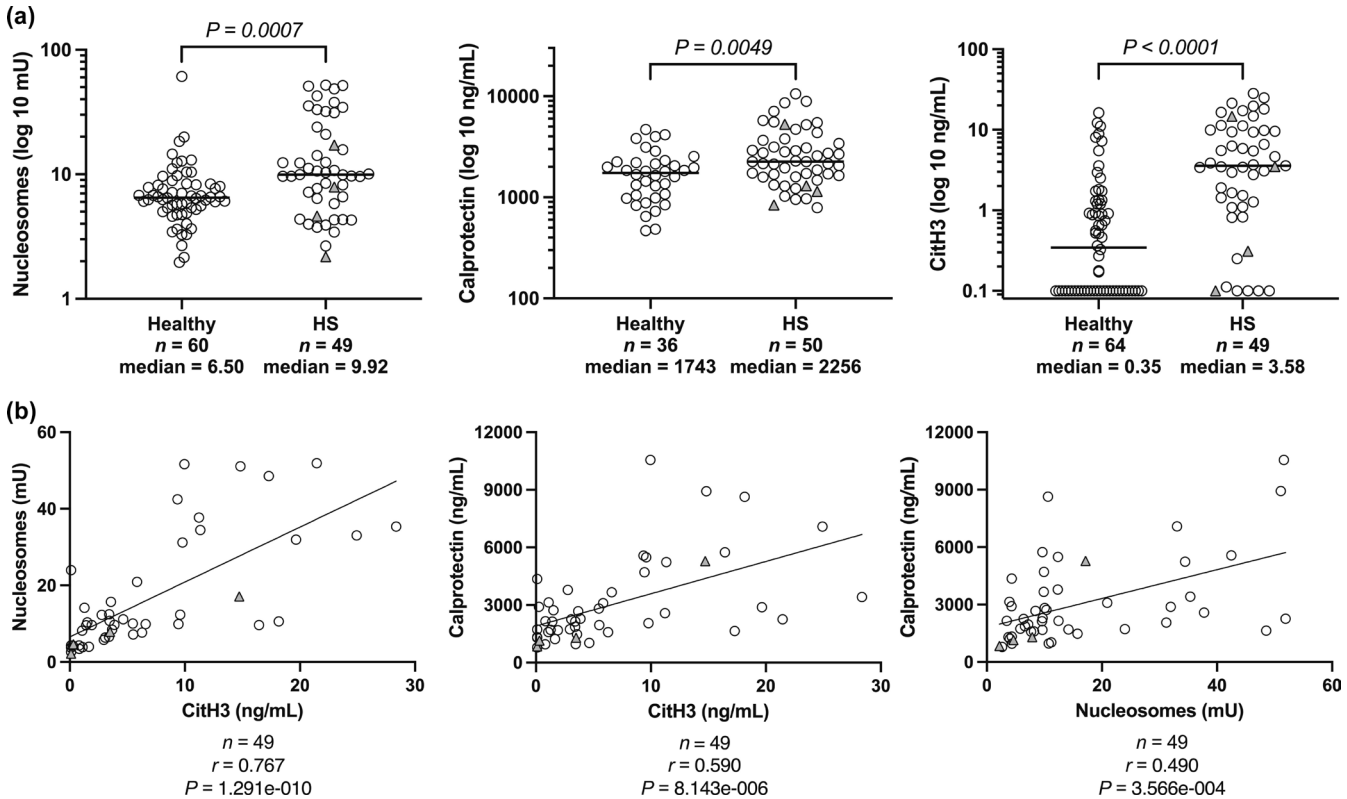

FIGURE 2 | Several NET markers are elevated in a correlative manner in HS patient serum. (a) ELISA quantified nucleosomes, calprotectin, and citrullinated histone H3 (CitH3) in Hidradenitis Suppurativa (HS) patients and healthy volunteer serum. Horizontal lines indicate the medians and statistical significance was assessed using the nonparametric Mann–Whitney test. (b) The nonparametric Spearman's rank correlation coefficient assessed correlations between HS patient serum levels of nucleosome, calprotectin, and CitH3. Lines indicate simple linear regressions. Gray triangles in (a) and (b) indicate serum specimens from HS patients with IHS4 scores of 0 points.

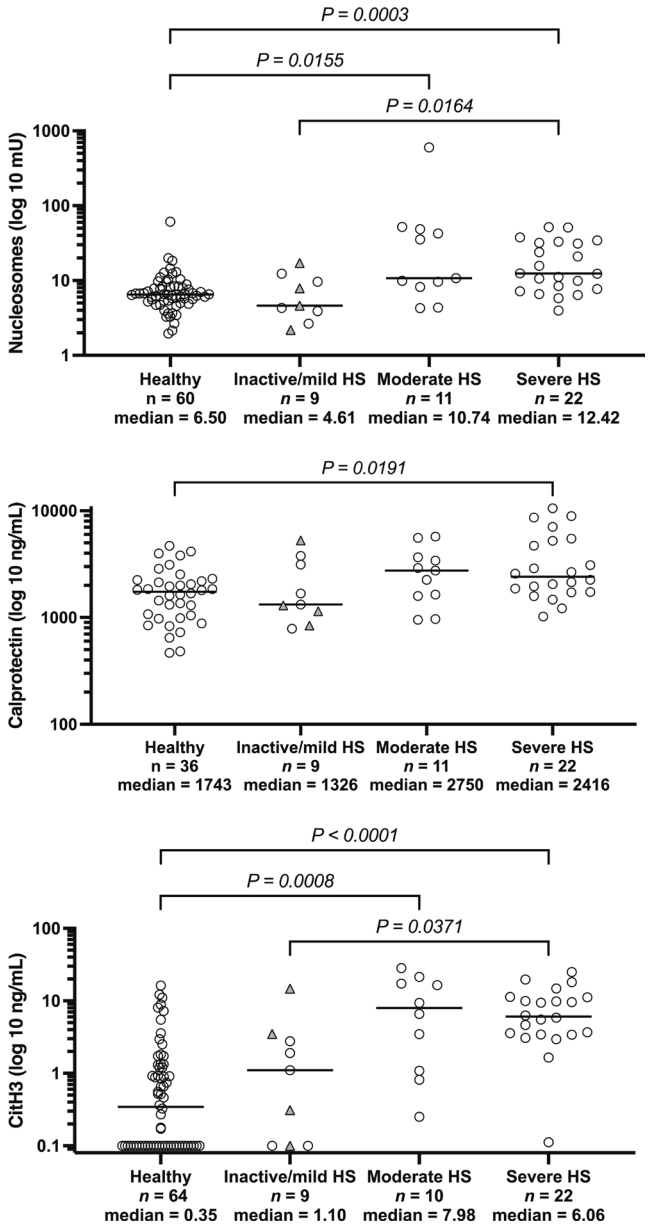

FIGURE 3 | Levels of serum NET markers correlate with disease severity. Endpoints from Hidradenitis Suppurativa (HS) patient serum samples were grouped into three classifications based on their IHS4 scores and compared with measurements from the serum of healthy volunteers. Classifications were inactive/mild (IHS4 = 0–3 points), moderate (IHS4 = 4–10 points), and severe (> 10 points) HS. Data from eight subjects was omitted as their IHS4 score was unknown. Statistical differences were analyzed using Kruskall-Wallis and Dunn's post hoc tests for multiple comparisons. Lines indicate the medians. Gray triangles indicate serum specimens from HS patients with IHS4 scores of 0.

activity, subclinical signs of ongoing inflammation can be detected in these patients.

4 | Discussion

In this study, we show positive diffuse extracellular staining for CitH3, a marker for NETs, across the epidermis, basal layer, and dermis of HS lesions and perilesional regions, with little or no extracellular staining in unaffected skin from HS patients (Figure 1a–c). This suggests the involvement of all skin layers in HS inflammation. Furthermore, NET staining was detected in the lining of epithelialized skin tunnels and inflamed apocrine glands (Figure 1d). In addition, the analysis of all HS patients as a single category showed elevation of several serum NET markers relative to healthy volunteers (Figure 2), with a positive correlation with increasing IHS4 scores (Figure 3).

The presence of cytotoxic, proinflammatory NETs in inflamed tissue has been reported in numerous chronic inflammatory disease settings [16], including HS, where NETs have been detected in resected tissue from HS lesions and positively correlated with increasing Hurley scores [18]. Moreover, elevated levels of cell-free DNA in the serum of HS patients are inefficiently cleared due to the impairment of nuclease activity by autoantibodies [19, 24].

Our findings describe the NET distribution in the different compartments of the skin and its association with characteristic pathologic features of HS. The detection of perilesional NETs in this study identifies an additional feature of subclinical inflammation in HS perilesional regions [6]. Interestingly, CitH3-positive staining was also pronounced in the lining of epithelialized tunnels, strengthening the idea that tunnels are active contributors to HS pathology, preventing its resolution or even amplifying disease activity [7, 25]. This active role for epithelialized tunnels is further supported by evidence that the invasive proliferative gelatinous mass found in the lumen of HS tunnels also contains NETs [26].

Despite numerous studies describing increased levels of circulating inflammatory mediators in HS, there is currently no blood biomarker with sufficient clinical validation for routine use in the clinical setting [27, 28]. Our data offer candidate serum biomarkers for disease activity associated with neutrophil activation and NETosis for future study.

The enrichment of NETs in HS skin and serum is consistent with the pathogenesis of HS, with neutrophils being the most abundant IL-17-positive cell infiltrate in HS lesions [11, 29, 30], IL-17 enhancing neutrophil mobilization to affected tissue [31], and elevated TNFα shown to enhance NET release [32–34]. Additionally, NETs are linked to various exaggerated innate and adaptive immune features of HS, such as NLRP3 inflammasome activity [11, 35], type I interferon pathway activation [18], and autoantibody production [18, 36].

5 | Limitations

Other white blood cells can also release extracellular traps (ETs) [37]. Infiltrating monocytes, macrophages, mast cells, dendritic cells, eosinophils, and B and T cells are described in HS tissue, making it possible that ETs measured in this study originate from more than one cell type [6, 38]. However, in a correlative study, the significant association between serum CitH3 and calprotectin suggests a predominant role for the neutrophil [39]. Our data also identify the accumulation of NETs as an additional feature of perilesional subclinical inflammation, but further analysis is required to assess if this is a bystander effect or

represents expanding inflammation with ongoing neutrophil recruitment and activation. Finally, increasing the sample sizes to achieve a balanced distribution of the disease severities and the availability of more extensive patient histories would also increase the strength of our conclusions. In the current sample set, one HS patient reported the use of adalimumab for inflammatory bowel disease, and one patient used secukinumab for an undisclosed condition. Endpoints associated with these patients were at the lower end of the HS population and could be affected by their use of immunomodulating therapy.

6 | Conclusion

The widespread accumulation of cytotoxic, proinflammatory NETs in lesional and perilesional regions of HS skin supports the rationale for neutrophil and NET-targeting anti-inflammatory therapeutic approaches in HS [15, 40]. Results from the current study demonstrate a gradient of NET levels present in HS skin, with the highest level of NET staining found in lesional skin, followed by perilesional biopsies, and little or no NETs in unaffected regions. Serum levels of NET markers are significantly elevated in HS patients and correlate with each other and with increasing disease severity.

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Ethics Statement

Ethical approval for the use of patient material for these studies was provided by CUB (22/0004), WMU Ethics Committee (KB-901/2022 and KB-234/2023), EMC (MEC-2016-246), and for CHDR and QPS the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (NL77528.056.21).

Consent

All participants provided informed written consent.

Conflicts of Interest

Citryll employees have financial interests. MLF: is a consultant to Citryll B.V. PKK: performs clinical trials for Celltrion, InfraRX, Janssen-Cilag, BMS, and UCB. He is a speaker at UCB and Axxon. KRvS: has received consultancy fees or honoraria from Novartis and UCB. EP: Consultant, advisory board member, speaker for, and received honoraria from Almirall, Janssen-Cilag, GSK, MoonLake Immunotherapeutics, Novartis, and UCB Pharma. The department has received investigator-initiated grant support from CHDR, Citryll, Janssen-Cilag, Kymera, MSD, and UCB Pharma. JRI: received a stipend as Editor-in-Chief of the British Journal of Dermatology and an authorship honorarium from UpToDate. He is a consultant for Abbvie, Boehringer Ingelheim, ChemoCentryx, Citryll, Insmed, Kymera Therapeutics, MoonLake, Novartis, UCB Pharma, UNION Therapeutics, and Viela Bio. He is a cocopyright holder of HiSQOL, Investigator Global Assessment, and Patient Global Assessment instruments for HS. His department receives income from the copyright of the Dermatology Life Quality Index (DLQI) and related instruments.

Data Availability Statement

All data are available under a material transfer agreement with Citryll B.V.

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