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ORIGINAL ARTICLE

Neutrophil extracellular traps enriched with IL-1β and IL-17A participate in the hepatic inflammatory process of patients with non-alcoholic steatohepatitis

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Abstract

Neutrophil extracellular traps (NETs) are implicated in the pathogenesis of various non-infectious inflammatory and thrombotic diseases. We investigated the presence and possible associations of NETs with various histopathologic parameters in patients with non-alcoholic steatohepatitis (NASH). We retrospectively assessed 20 liver biopsy specimens from patients with non-alcoholic fatty liver disease (NAFLD), including 17 specimens with NASH, and 14 control specimens. NETs were identified with confocal microscopy as extracellular structures with co-localization of neutrophil elastase (NE) and citrullinated histone-3. Interleukin-1β (IL-1β) and IL-17A were assessed with the same methodology. Histologic features of NAFLD were semi-quantitatively evaluated, and correlated with presence of NETs, neutrophil density, and platelet density/aggregates (assessed by immunohistochemistry for NE and CD42b, respectively). NETs were identified in 94.1% (16/17) of the NASH biopsy specimens; they were absent from all other NAFLD and control specimens. The presence of NETs was strongly correlated with steatosis ($p=0.003$), ballooning degeneration ($p<0.001$), lobular inflammation ($p<0.001$), portal inflammation ($p<0.001$), NAS score ($p=0.001$), stage ($p=0.001$), and diagnosis of NASH ($p<0.001$). NETs were decorated with IL-1β and IL-17A. Platelet aggregates were much larger in NASH specimens, as compared to controls. In conclusion, NETs are implicated in the pathogenesis of NASH. Their associations with inflammation, ballooning degeneration (a hallmark of NASH), and stage emphasize their role in the disease process. In this setting, NETs provide a vehicle for IL-1β and IL-17A. In addition, platelet aggregation in hepatic sinusoids implies a role for thromboinflammation in NASH, and may explain the low peripheral blood platelet counts reported in patients with NASH.

Keywords Neutrophils · Liver · Steatosis · Steatohepatitis · Platelets · Interleukin 1β · Interleukin 17A

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Introduction

A significant subset of patients with non-alcoholic fatty liver disease (NAFLD), representing 20–25% of cases, develop hepatocellular injury and inflammation, often followed by fibrosis, i.e., features that histopathologically define non-alcoholic steatohepatitis (NASH) [9]. The clinical importance of NASH relies on the fact that it has been linked to increased risk for end-stage liver disease, liver failure, and hepatocellular carcinoma [9]. To date, the etiopathogenesis of NASH is incompletely understood, but appears to be associated with the proinflammatory microenvironment created by toxic lipid-induced hepatocyte injury, termed lipotoxicity [11, 19]. Exposure of hepatocytes to lipotoxic stimuli results in the release of extracellular vesicles and damage associated with molecular patterns, which induce inflammation, fibrosis, and angiogenesis [16, 19]. The inflammatory milieu during lipotoxic injury in NASH is characterized by the accumulation of monocytes/monocyte-derived macrophages and by an increased number of natural killer (NK) cells [21]. Kupffer cells and monocyte-derived macrophages play a key role in the progression of NASH and fibrosis [24] by initiating and orchestrating the inflammatory reaction through the recruitment of other monocytes and neutrophils into the liver [6]. The accumulation of macrophages has been correlated with the severity of histological activity in human NASH [19], while neutrophils are generally found to be fewer than in alcoholic steatohepatitis. However, experimental data obtained from mice lacking key neutrophilic enzymes, such as myeloperoxidase (MPO) or elastase, suggest that neutrophils are potentially key players in NASH progression [25, 37, 38].

There is increasing evidence that neutrophil-derived chromatin network filaments and proteins, known as neutrophil extracellular traps (NETs), play an important role in the pathogenesis of several non-infectious inflammatory or thromboinflammatory diseases [3, 20, 29, 33]. The process of NET production and release by neutrophils is called "NETosis" [3]. Neutrophils release NETs that are qualitatively variable and express disease-related bioactive proteins in accordance to the inflammatory environment, implying that the plasticity of neutrophils may also be reflected upon NETs [20, 29]. Elevated levels of MPO-DNA complexes, a NET marker, have been found in blood serum of patients with NASH [43].

Platelets also participate in the inflammatory microenvironment of NASH, playing a role in disease development [33]. Platelet number, adhesion, activation, and, in particular, platelet-derived glycoprotein Ibα chain are increased in NASH, while their liver colonization depends mainly on Kupffer cells [26, 36]. Miele et al. have recently demonstrated increased intrahepatic platelet accumulation in patients with NASH, which correlated with NAS and intrahepatic NET formation, assessed by immunohistochemistry [28]. Platelet-neutrophil interaction has been found to be important in NET-induced thromboinflammation, either in both infectious and non-infectious diseases [7, 8, 41]. Furthermore, after partial ligation of the suprahepatic inferior vena cava, sinusoidal neutrophil-platelet complexes are formed, leading to NET release [17].

In the present study, we investigated (a) neutrophil infiltration and NET formation in a series of liver biopsy specimens from patients with NAFLD; (b) possible associations of NETs with various histopathological parameters; (c) whether IL-1β and IL-17A, two key pro-inflammatory and pro-fibrotic cytokines, are components of NETs in NASH; and (d) possible associations of platelet density with histopathological parameters..

Materials and methods

Study design and participants

A retrospective study was conducted, including 20 liver biopsy specimens from 20 patients aged ≥ 18 years with newly diagnosed NAFLD from 2004 until 2020, retrieved from the archives of the Department of Pathology, School of Medicine, Aristotle University of Thessaloniki. Clinical data were obtained from the attending hepatologists at the First and Fourth Departments of Internal Medicine. On histopathological assessment, 17 of the 20 specimens were diagnosed as NASH using established criteria [22] (Supplementary Table 1). The non-alcoholic nature of the disease was clinically verified using a daily cut-off of < 20 g of alcohol intake for females and < 30 g for males within the past 5 years, as customarily used in NASH studies [46]. In addition, 7 liver biopsy specimens from age- and sex-matched patients with chronic viral hepatitis (B or C) and 7 liver biopsy specimens from age- and sex-matched individuals without significant pathologic changes were used as control samples for immunofluorescence (IF) and immunohistochemistry (IHC).

All biopsy specimens were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Three-micrometer-thick paraffin sections were used for hematoxylin-and-eosin stain (HE), IHC, and IF. Histological features (steatosis, ballooning degeneration, Mallory-Denk bodies, lobular and portal inflammation, NAS score, and stage) were semi-quantitatively assessed according to established criteria [22]. The evaluation of IHC and IF was performed by two pathologists working independently, who were blinded to the patient and outcome data.

The study protocol design is in accordance with the Declaration of Helsinki, and ethical approval was obtained from the appropriate institutional board (AHEPA University Hospital IRB No 509, 09/07/2016, modification 10/08/2019). This study is being reported according to the STROBE guidelines for observational studies [45]. Informed consent was obtained from all participants.

IF

Tissue sections were dewaxed and rehydrated in ethanol baths with descending concentrations (100%, 96%, and 70%). For heat-induced epitope retrieval, the sections were processed for 30 min in citrate buffer pH6 (DAKO, Agilent, Santa Clara, USA; K8005) heated in microwaves. After washes in phosphate-buffered saline (PBS), blocking was performed for 45 min with 5% goat serum (Invitrogen, Carlsbad, USA; 1:200 dilution) in PBS. Subsequently, the slides were incubated overnight at 4℃ with different primary antibodies (NE, anti-Histone H3 (citrulline R2 + R8 + R17), CD68, IL1β, and IL17A) (Supplementary Table 2). After washes in PBS, the secondary antibodies (Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse, Invitrogen, Carlsbad, USA) were applied for 45 min at room temperature, followed by washes in PBS and DAPI (4′,6-diamidino-2-phenylindole) counterstaining for 10 min. Sections were mounted and visualized using a confocal microscope (Spinning Disk Andor Resolution Confocal System, Ireland) with PLAPON 606O/TIRFM-SP (Numerical Aperture 1.45) and UPLSAPO Olympus × 100 (Numerical Aperture 1.4) objectives (Olympus, Hamburg, Germany). ImageJ Fiji software was used to analyze the data. NETs were visualized as extracellular co-localization of NE and citrullinated histone 3 (CitH3). The presence of NETs was scored as follows: 0: absence of NETs, 1: isolated NETs or in small traces, and 2: NETs in an aggregated pattern. "Negative controls" were obtained by omission of the primary antibody.

IHC

Immunohistochemical stains for neutrophil elastase (NE), MPO, CD68, and CD42b were performed using a Dako autostainer universal staining system (Supplementary Table 2). Two different chromogens for NE immunostaining were used, i.e., diaminobenzidine (DAB, brown color) and horseradish peroxidase (HRP) magenta (red color). Slides were scanned using an AxioScan.Z1 (Carl Zeiss, Jena, Germany) microscope and 20 × /0.8 Plan-APOCHROMAT objective and analyzed using ZEN 2.6 Blue software (Zeiss) on a desktop PC. Manual counting of NE-positive cells was performed in all examined cases; then, the number was divided by the surface area of each liver biopsy tissue and the density per square millimeter was calculated. The same method was used to evaluate the accumulation of platelets adherent to liver sinusoids. CD42b was used as a specific platelet marker and the values of platelets and their aggregates per square millimeter of liver tissue were determined. This method has been found to be more efficient and less time consuming for detailed counting of either NE-positive cells or platelets in the whole biopsy surface, as compared to the traditional manual counting under the light microscope [44].

Statistical analysis

Comparison of continuous variables between NAFLD/NASH patients and controls was performed using the Mann–Whitney *U* test in case that the normality assumption, as checked with the Shapiro–Wilk test, is violated; else, Student's *t*-test was alternatively preferred. Correlations between continuous variables were approached by Spearman's correlation coefficient ρ (rho) in case that either outliers are detected or normality was violated; else, Pearson's correlation coefficient was alternatively preferred. Benjamini–Hochberg correction was applied when multiple hypotheses were simultaneously tested. A univariate general linear model was introduced to detect any potential independent correlations of fibrosis, steatosis, ballooning degeneration, Mallory-Denk bodies, lobular inflammation, portal inflammation, and NAS ≥ 4 (independent variables) with NETs (dependent variable); in that model, group (NASH/NAFLD patients or controls) was considered as covariate. Ordinal regression was used to examine any potential independent correlations between stage, steatosis, ballooning degeneration, Mallory-Denk bodies, lobular inflammation, portal inflammation, and NAS ≥ 4 (independent variables) with NETs (dependent variable) within NAFLD-NASH patients (and vice versa); maximum iterations were set to 100, maximum step-halving to 5, log-likelihood convergence to 0, parameter convergence to 10^{-6}, delta to 0, and singularity tolerance to 10^{-8}. The level of statistical significance was set to $P = 0.05$. SPSS 26.0.0.0 (IBM ®) was used for statistical analysis. GraphPad Prism 9.3.1 was used to produce scattergrams and heatmaps for visualization purposes.

Results

Association of NETs with various histopathological parameters in NASH

NETs, as determined by extracellular co-localization of NE and CitH3, were identified in all but one (16/17, 94.1%) of the biopsy specimens with NASH, and in none of the other 3 cases of NAFLD or 14 control specimens. In 7 of the 16 NET-positive specimens (43.75%) with NASH, NETs were present in an aggregated pattern (score 2) (Fig. 1), mainly

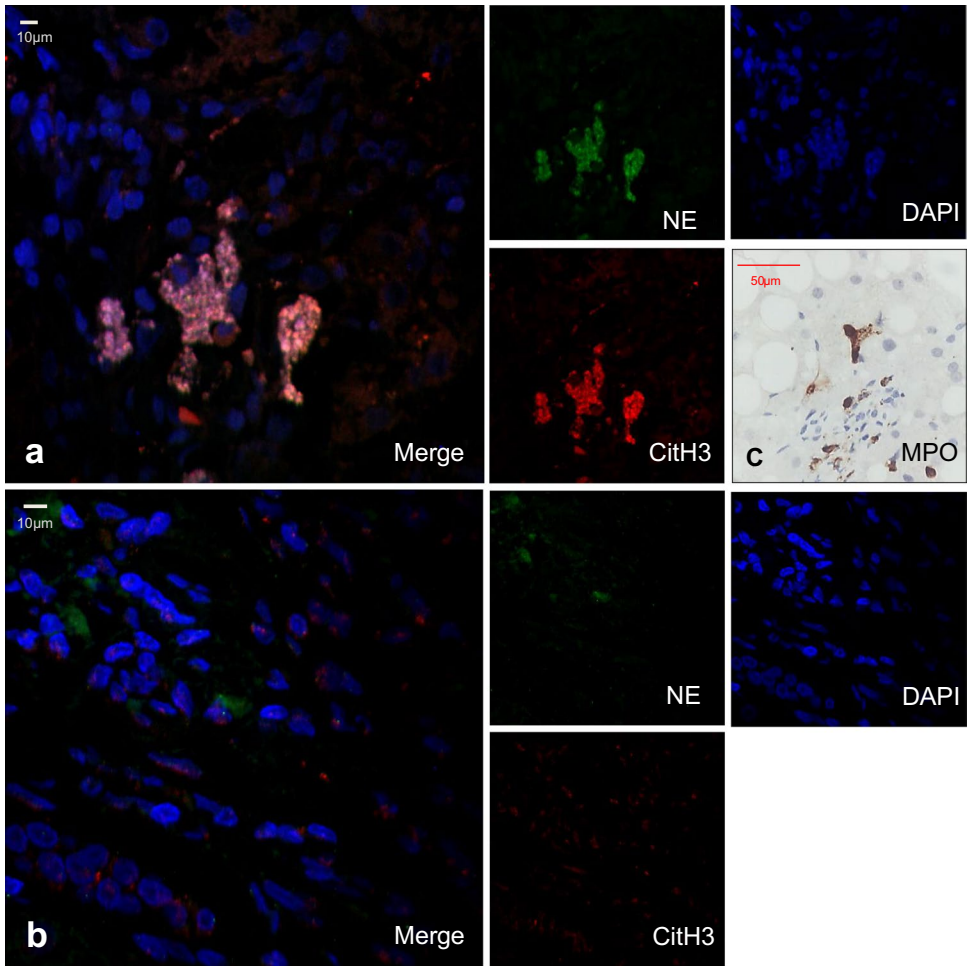

Fig. 1 Presence of NETs and neutrophils in liver biopsy specimen from patient with NASH but not in control biopsy specimen. **a** A representative case with NETs in an aggregated pattern visualized by immunofluorescence as extracellular structures with colocalization of NE and CitH3 immunostains (IF/confocal microscopy × 63; scale bar — 10 μm; green: NE, red: CitH3 and blue: DAPI) (IHC × 600). **b** Absence of NETs in liver biopsy from patient with chronic hepatitis B (negative control) (IF/confocal microscopy × 63; scale bar — 10 μm; green: NE, red: CitH3 and blue: DAPI). **c** Neutrophils and a structure with network-like features are seen with MPO immunostaining (IHC × 400). Abbreviations: NETs, neutrophil extracellular traps; NASH, non-alcoholic steatohepatitis; NE, neutrophil elastase; CitH3, citrullinated histone H3; DAPI, 4′,6-diamidino-2-phenylindole; MPO, myeloperoxidase; IF, immunofluorescence; IHC, immunohistochemistry

within areas of lobular and portal inflammation and/or fibrosis. In the remaining 9 specimens (56.25%), NETs were scarce (score 1). NETs were frequent in areas of ballooning degeneration. The semiquantitative assessments of the histologic findings of the specimens with NASH ($n = 17$) are summarized in Supplementary Table 3, whereas the associations between NETs and the histologic findings are presented in Fig. 2a. NETs were strongly correlated with steatosis ($p = 0.003$), ballooning degeneration ($p < 0.001$), lobular inflammation ($p < 0.001$), portal inflammation ($p < 0.001$), NAS score ($p = 0.001$), stage ($p = 0.001$), and diagnosis of NASH ($p < 0.001$) (Fig. 2a). The associations of NETs with steatosis, ballooning degeneration, portal inflammation, and NAS score > 3, were independent of diagnostic group assignment (Supplementary Table 4a). Considering NASH patients, steatosis, ballooning degeneration, portal inflammation, NAS score, and NAS score > 3 were independently and positively correlated with NETs, thus serving as potential prognosticators of NETosis (Supplementary Table 4b, c).

Since neutrophils are not always easily discernible in HE-stained sections, we assessed their presence and density by IHC. MPO staining, performed in selected cases, revealed positivity in a greater number of cells than NE, including structures with network-like features, suggestive of NETosis (Fig. 1c). Neutrophils were apparent in portal inflammation (Fig. 3a), around fat droplets (Fig. 3b, c), and in areas of hepatocellular ballooning (Fig. 3d). Neutrophil numbers were greater in NASH than in control liver biopsy specimens (Fig. 3e, f). However, neutrophil density was not statistically associated with NASH ($p = 0.505$). In addition, no association was evident between neutrophil density and NETs ($p = 0.309$) in NAFLD biopsies.

Regarding clinical and standard laboratory tests, we found that ALT and AST values were associated with lobular inflammation, ballooning degeneration, and NAS score, while ALT was also associated with steatosis. There was no statistically significant association between NETs, neutrophil density, and platelet density, with other clinical or laboratory data (Supplementary Table 5).

Assessment of platelets

Recent evidence supports that platelets play a crucial role in NASH progression [26]. Since platelets also are well-known

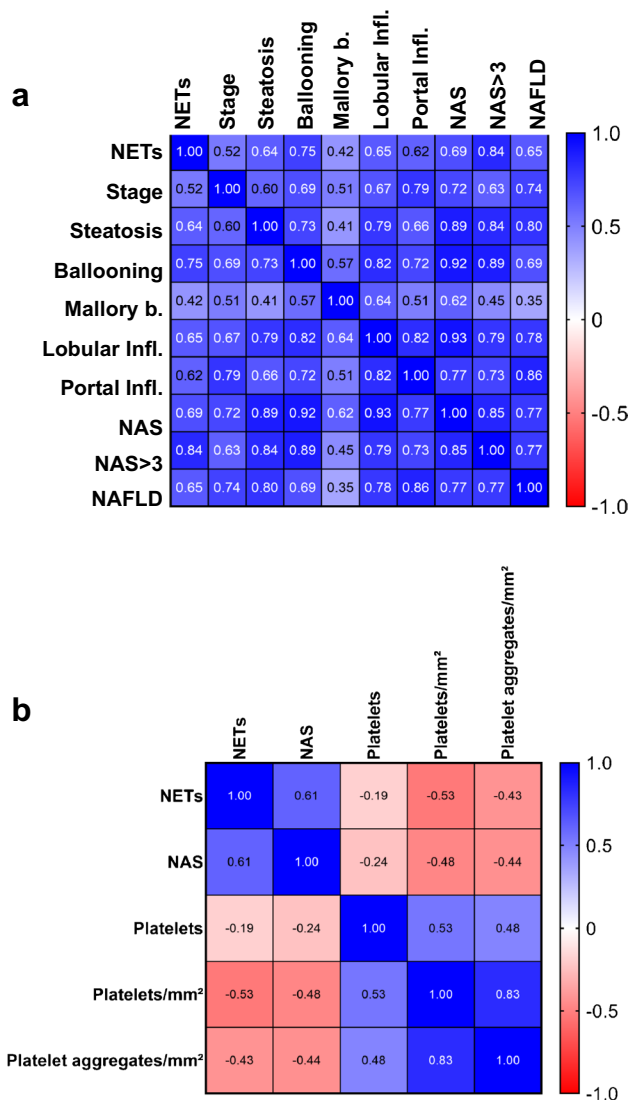

Fig. 2 **a** Heatmap depicting correlations between NETs and histologic parameters of interest using non-parametric Spearman's correlation coefficient ρ (rho). **b** Heatmap depicting correlations between platelets, platelets/mm^2, platelet aggregates/mm^2, NAS, and NETs using non-parametric Spearman's correlation coefficient ρ (rho)

initiators of NET release, and NETs induce platelet aggregation [50], we explored their presence by CD42b immunostaining. The median number of isolated platelets in biopsy specimens of patients with NASH was 84 (5–233), whereas that of the control group was 138 (68–190). Similarly, the median number of platelet aggregate density of patients with NASH was 1 (0–13), whereas in the control group was 4 (3–18). A negative, moderate association between NETs and platelets/mm^2 ($p = 0.008$) or platelet aggregates/mm^2 ($p = 0.038$) was found (Fig. 2b). However, platelet aggregates were significantly larger in specimens with NASH, as compared with those of the control groups (Fig. 3g, h).

NETs co-localize with IL-1β and IL-17A in NASH

Depending on the inflammatory microenvironment, NETs display the plasticity to express disease-related bioactive proteins. In this content, NETs are decorated with IL-1β in patients suffering from neutrophil-driven inflammatory flares [1, 41]. Moreover, the inflammasome, a key molecular complex of hepatic inflammation and liver fibrosis participating in NASH, promotes the production of caspase-1-depedent IL-1β [23]. Hence, we investigated the expression of IL-1β, which was detected mainly on NETs, as demonstrated by co-localization of NE with IL-1β (Fig. 4a). We also examined the expression of interleukin 17A on NETs, since this protein appears to have a crucial role in NASH progression [14, 15], and is upregulated by IL-1β in human NETs [32]. NETs in our cases were decorated with IL-17A, as assessed by double IF (Fig. 4b). Taken together, our data demonstrate that NETs provide a vehicle for IL-1β and IL-17A in NASH, supporting their potential involvement in the inflammatory and fibrotic process.

Possible macrophage clearance of NETs in NASH

In three cases, in addition to extracellular co-localization of NE with CitH3, we also observed in areas of inflammation occasional cells with macrophage features, which showed cytoplasmic co-localization of NE with CitH3 in a granular pattern. To further clarify whether these cells were neutrophils in the process of NETosis or macrophages with entrapped NETs through efferocytosis, we performed double immunofluorescence staining with NE and CD68. The co-localization of NE with CD68 was suggestive of efferocytosis (Fig. 5a). The presence of small numbers of macrophages with abundant cytoplasm in these areas was further confirmed by IHC for CD68, performed on sequential paraffin sections (Fig. 5b). On the other hand, immunostaining for NE, performed in parallel, showed only positivity in neutrophils and not in macrophages. IHC also highlighted tiny dispersed positive dots (Fig. 5c), either isolated or in small clusters, possibly representing NETs, in areas of ballooning degeneration and portal tracts, in close proximity to the CD68 positive cells. NE staining was repeated with a second chromogen (HRP magenta) and provided the same histological findings excluding the possibility of non-specific brown pigment (Fig. 5d).

Discussion

In NASH, the participation of neutrophils in the liver inflammatory cell infiltrate has been well documented; however, little is known about their role in the disease process [25, 38]. This study demonstrates for the first

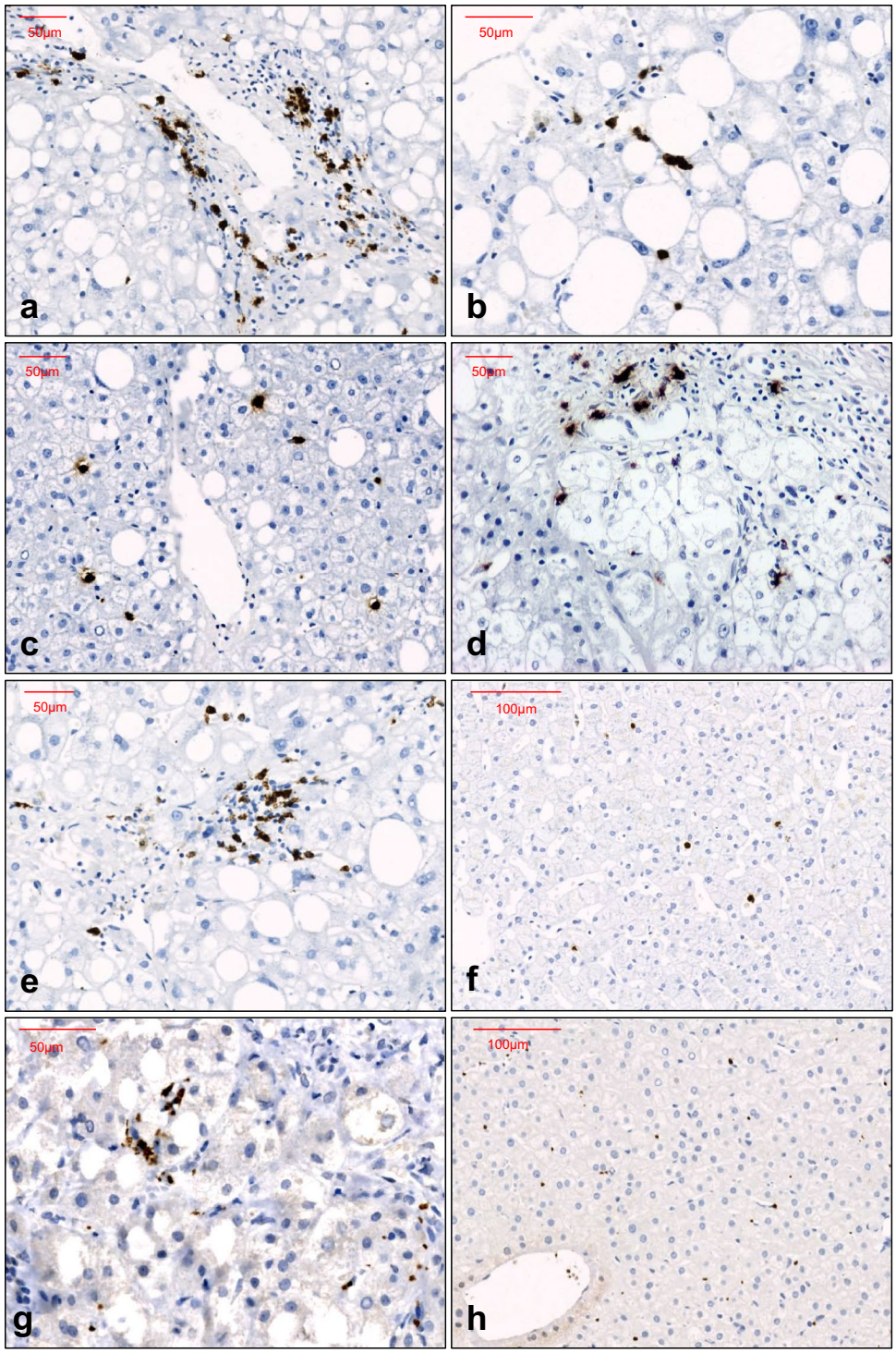

Fig. 3 Assessment of neutrophil density and platelets. **a** Immunohistochemical positivity for NE is observed in neutrophils located in a portal tract with inflammation and fibrosis, **b**, **c** around fat droplets, and **d** in an area of hepatocellular ballooning. **e** Immunohistochemistry for NE shows significantly increased neutrophil density in a liver biopsy specimen with NASH **f** as compared to a normal liver biopsy specimen. **g** Immunohistochemistry for CD42b shows increased platelet aggregates in sinusoids of a liver biopsy specimen with NASH **h** as compared to a normal liver biopsy specimen (**a**–**e**, **g**: IHC×400; **f**, **h**: IHC×200). Abbreviations: NETs, neutrophil extracellular traps; NASH, non-alcoholic steatohepatitis; NE, neutrophil elastase; DAB, diaminobenzidine; HRP, horseradish peroxidase; IHC, immunohistochemistry

time that NETs are present in human liver biopsies from patients with NASH, by using confocal microscopy. The importance of NET involvement in the pathogenesis of this disease is underlined by the statistically significant associations identified between NETs and the components of the NAS score, including ballooning degeneration, which is a hallmark of NASH, as well as between NETs and stage. The immunofluorescence and immunohistochemical findings of our study indicate that NETs, independently of neutrophil density, represent active participants in the inflammatory milieu of NASH. Along this line, mechanistic studies have revealed that excessive activation of neutrophils induces liver damage, mainly through the release of proteases into the extracellular environment [4].

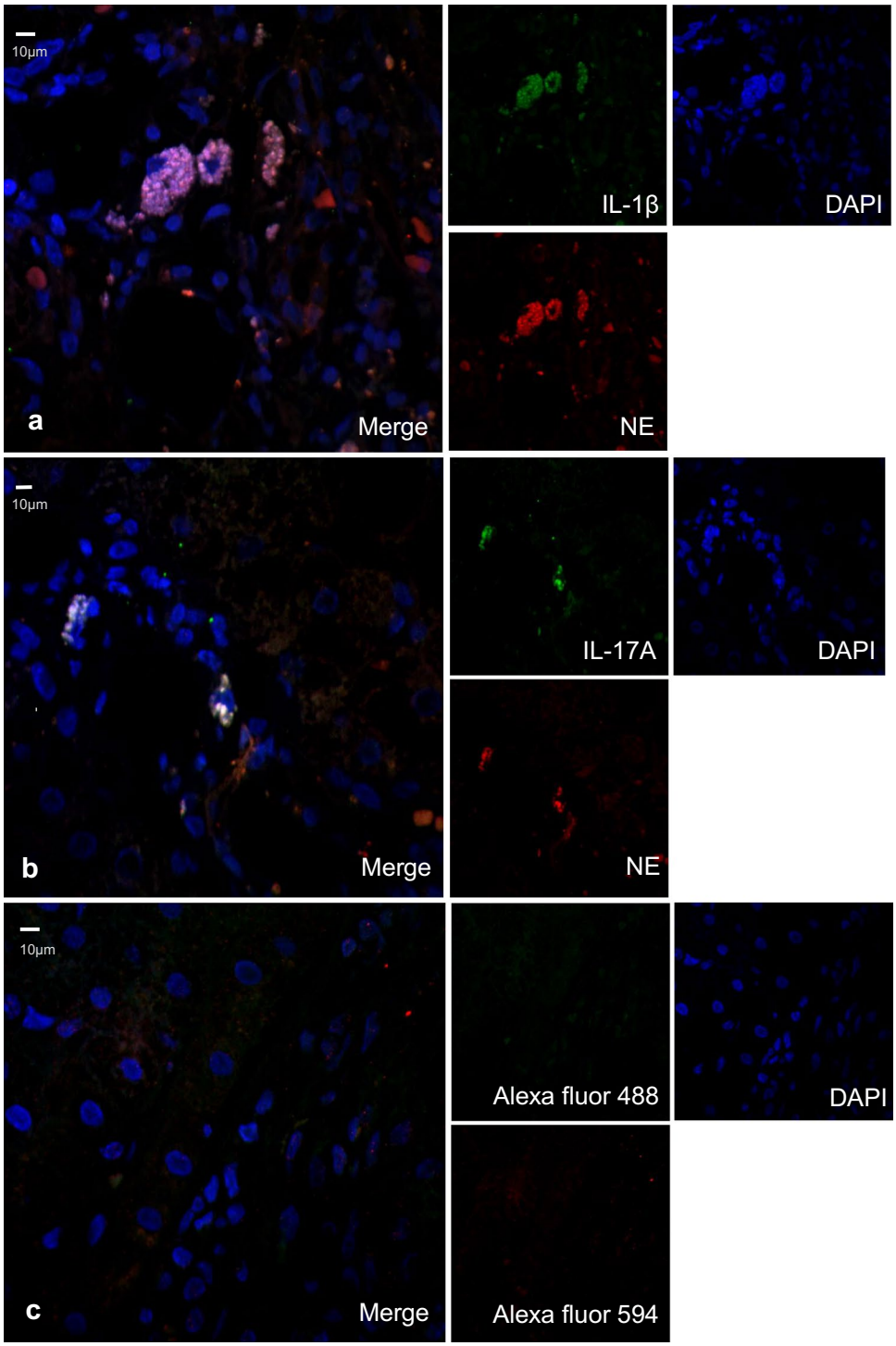

Fig. 4 NETs are decorated with IL-1β and IL-17A. **a** Co-localization of IL-1β and NE **b** as well as IL-17A and NE is identified in NET-positive biopsies from patients with NASH (representative cases are shown in **a** and **b**, and negative control in **c**) (IF/confocal microscopy × 63; green: IL-1β in **a**, IL-17A in **b**, red: NE and blue: DAPI). Abbreviations: NETs, neutrophil extracellular traps; IL-1β, IL-17A, interleukins 1β and 17A; NE: neutrophil elastase; NASH, non-alcoholic steatohepatitis; IF, immunofluorescence; DAPI, 4′,6-diamidino-2-phenylindole

The pro-inflammatory neutrophil action findings are reinforced by a recent study, in which high levels of MPO-DNA (a marker for NETs) were identified in sera of patients with NASH [43]. In addition, the authors used the animal model of STAM mice, where NASH was induced by neonatal streptozotocin administration and high fat diet, and they observed an early neutrophil infiltration and NET formation in the liver [43]. Furthermore, in the STAM animal model, an important role of NETs has been demonstrated in the development of NASH-associated hepatocellular carcinoma, possibly due to their activity in generating an immunosuppressive microenvironment [47]. Although using animal models is an established approach to investigate human NASH by inducing steatosis or inflammation, they do not take into account some key features of human disease, such as the progression of fibrosis in the course of years or decades,

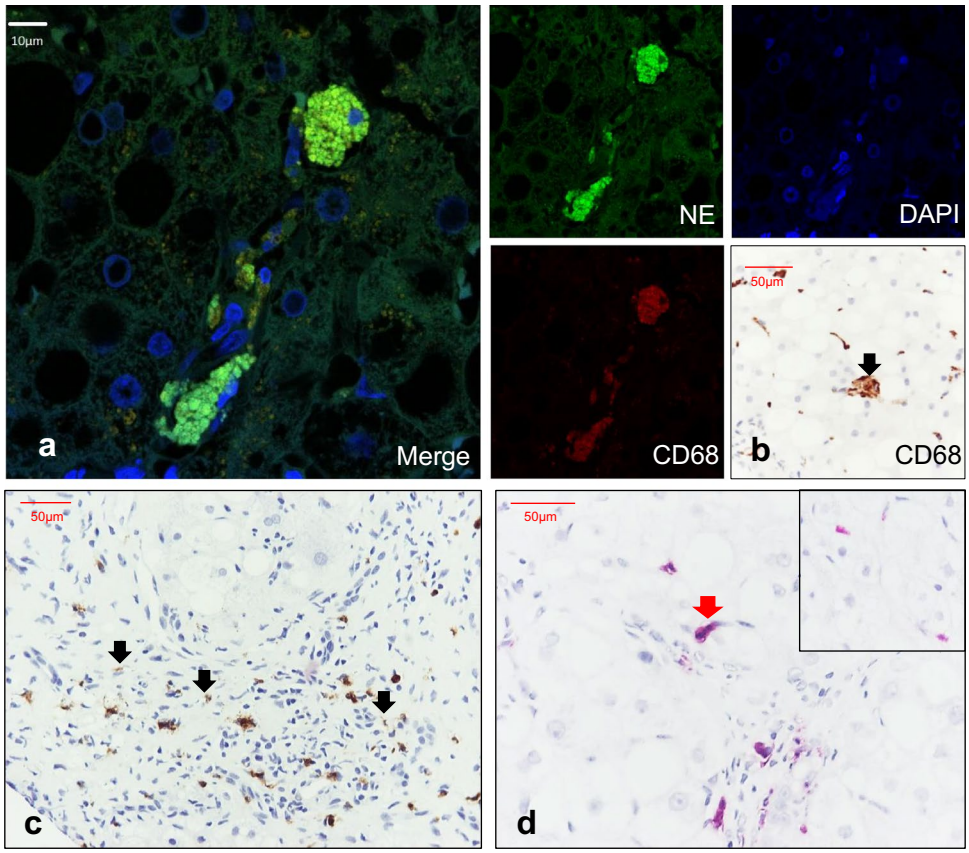

Fig. 5 Possible macrophage clearance of NETs in NASH. **a** Macrophages with abundant granular cytoplasm, positive for NE and CD68 antigens, suggestive of efferocytosis, as revealed by co-localization of NE and CD68 antigens (IF/confocal microscopy × 63; green: NE, red: CD68 and blue: DAPI). **b** Immunohistochemistry for CD68 reveals similar macrophages with abundant granular cytoplasm (black arrow), among positive Kupffer cells (IHC × 400), **c** which were non-reactive to NE antibody. **c**, **d** Immunohistochemistry for NE reveals neutrophils and tiny dispersed positive dots, either isolated or in small clusters, apparently representing NETs (positive dots, **c**: black arrows, **d**: inset) by using two different chromogens, namely **c** DAB and **d** HRP magenta. **d** A positive cellular structure with features suggestive of NET and an elongated pyknotic nucleus is indicated with a red arrow (**c**, **d**: IHC × 400). Abbreviations: NETs, neutrophil extracellular traps; NASH, non-alcoholic steatohepatitis; NE, neutrophil elastase; DAPI, 4′,6-diamidino-2-phenylindole; IF, immunofluorescence; IHC, immunohistochemistry

as well as the systemic insulin-resistant state observed in NASH [11, 39].

In STAM mice, the recruitment of neutrophils was found to be followed by an influx of monocyte-derived macrophages, as a consequence of NET formation [43]. Neutrophil-monocyte/macrophage interactions have been previously shown to be crucial for NASH development, and this cross-talk can be facilitated by NETs [48]. Macrophages have their own role in hepatic fibrosis, since their activation promotes HSC differentiation into collagen-secreting myofibroblasts contributing to hepatic fibrosis [47]. In the present study, apart from resident and recruited macrophages in fibrotic areas, macrophages with abundant cytoplasm showing clearance of NETs were also identified, suggestive of efferocytosis. Efferocytic activity in macrophages may be prevented by changes in neutrophil activity or NET molecules, such as high mobility group box 1 protein [18]; this fact possibly explains why efferocytosis was detected in a minority of our cases. It is becoming clear that there is a complicated interplay between neutrophils/NETs and monocyte/macrophages in several autoimmune and inflammatory diseases [5, 35], which may be critical for the development of new therapeutics in the future. Along the same line, recent evidence suggests that cholesterol-induced M2-like macrophages and Kupffer cells undergo M4-like polarization, recruiting neutrophils and inducing NETosis [27]. Also of note is that NET blockade in mice significantly changed the pattern of liver inflammation by reducing monocyte-derived macrophage infiltration [43].

Another complicated cellular interaction in inflammatory processes is the one between neutrophils and platelets, which are considered a key initiator of NET release [50]. Subsequently, NETs directly feedback onto platelets, inducing their activation and aggregation. In our study,

a negative association between NETs and platelets was found, in contrast to what has already been reported [28]. However, the observed aggregates were significantly larger in biopsies from NASH patients, as compared to the control group. It should be kept in mind that defining platelet macro-aggregates as just above 2 positive dots in an immunostain specific for platelet antigens [31] makes a great difference in comparison to counting only larger aggregates (e.g., > 10 dots). After all, quantification is a difficult or impossible task when platelets are aggregated; therefore, methodological differences may account for the apparent discrepancy between our findings and those of other studies [31, 43]. Platelet aggregation in the liver, as shown in our study, may explain the low peripheral platelet counts observed in some patients with early stages of NASH [39, 48].

Interleukins have their own role in sterile inflammation and are implicated in NETosis [2]. For instance, IL-1β seems to be produced by neutrophils and to be released through NETosis in several autoinflammatory diseases [1, 40]. In NASH, IL-1β is produced by both macrophages and necroptotic hepatocytes [12, 27]. Likewise, evidence has emerged that plasma levels of IL-6 and TNF-α are positively correlated with circulating NET markers and that NETs in NASH potentially play an important role in the procoagulant activity, which could be reversed through DNase I pretreatment [10]. Recent studies in mice have revealed that hepatocyte inflammasome activation and IL-1β expression that promote pyroptosis may provide a link between the initial metabolic stress and subsequent hepatocyte death and stimulation of fibrogenesis in NASH [30, 42]. Moreover, the IL-1 pathway has been associated with liver inflammation and serum ALT elevation in patients with NASH [34].

In the present study, we demonstrate that NETs are decorated with both IL-1β and IL-17A. Although we did not study mechanistically the neutrophil/NETs/IL-1β/IL-17A axis and its regulation, a dual origin of neutrophil IL-1β and IL-17A (produced and acquired) cannot be excluded. Additionally, the effectiveness of neutrophil-derived cytokines could be significantly amplified due to their dense concentration in the meshwork of NETs [20]. In a recent study, neutrophils that express IL-17A were described in progressed NASH, significantly associated with the stage of fibrosis, the grade of ductular reaction, and the presence of portal inflammation [13]. It is known that IL-17A-expressing neutrophils can activate stellate cells promoting fibrosis [49] and IL-17A may also contribute to inflammation [14, 15]. Furthermore, suppression of Th17 cell differentiation or blocking IL-17A signaling in mice prevents NASH and subsequent hepatocellular carcinoma development [14]. Therefore, further investigation of the neutrophil/NETs/IL-1β/IL-17A axis is warranted both in innate and downstream of polarized Th17 cell-driven adaptive immune responses.

The main limitation of our study is the relatively small number of patients. However, the identification of NETs as an important pathogenic factor should prompt additional studies to address this issue. Another limitation relates to the retrospective nature of our analysis. Hopefully, future prospective clinical and mechanistic studies will solidify our findings.

In conclusion, our study provides evidence that neutrophils are implicated in the pathogenesis of human NASH, both in the form of intact cells and NETs that are not apparent on routine histologic examination. The associations of NETs with inflammation and ballooning degeneration, a hallmark of NASH, as well as with stage, emphasize their role in this disease. We also found that NETs are decorated with pro-inflammatory and pro-fibrotic interleukins, which possibly contribute to disease progression. Furthermore, our finding of platelet aggregation in hepatic sinusoids implies a role for thromboinflammation in NASH, and may explain the low peripheral blood platelet counts found in some patients. Overall, our data suggest an interplay between platelets-NETs-macrophages, which warrants further investigation through mechanistic studies in order to elucidate the pathogenesis of NASH and contribute novel therapeutic strategies.

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Author contribution SA, TK, GG, PH: conceptualization, methodology, writing original draft, writing review and editing, final approval. KA, VP: statistical analysis, writing review and editing, final approval. ES, EA, GG: clinical data collection, final approval. PS, KR: methodology, editing, final approval. GG, PH: supervision, project administration.

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Declarations

Ethics approval and consent to participate The study protocol design is in accordance with the Declaration of Helsinki, and ethical approval was obtained from the appropriate institutional board (AHEPA University Hospital IRB No. 509, 09/07/2016, modification 10/08/2019). This study is reported according to the STROBE guidelines for observational studies (see reference 45). Informed consent was obtained from all participants.

Conflict of interest The authors declare no competing interests.

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