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Neutrophil extracellular traps in the cross-talk between periodontitis and chronic kidney disease

Shucheng Hu[1†], Ruhan Yang[1†], Wenying Yang[1], Jiaqi Tang[1], Weijun Yu[1], Dan Zhao[1], Lu Lin[1], Yuting Gu[1], Min Jin[1], Ziyuan Xu[1*], Qin Wang[2*] and Eryi Lu[1*]

Abstract

Background The objective was to evaluate the level of neutrophil extracellular traps (NETs) in patients with chronic kidney disease (CKD) and periodontitis, and to explore the relationship between NETs and both diseases.

Methods 63 CKD and 40 non-CKD participants were recruited and underwent periodontal examination, among which 35 early CKD patients underwent periodontal therapy. The concentrations of NETs were determined by dsDNA assay in gingival crevicular fluid (GCF) and plasma, and by flow cytometry or immunofluorescence assay in blood and gingival tissues. The correlations between NETs and clinical parameters were analyzed. The influence of periodontal therapy on periodontitis, CKD and NETs concentrations was also evaluated.

Results CKD patients had higher concentrations of NETs in plasma than non-CKD patients, and NETs concentrations were also increased in both GCF and plasma of patients with periodontitis than that of periodontally healthy patients. NETs concentrations were positively correlated with increased clinical parameters of CKD and periodontitis. The positive correlation between CKD and periodontitis was demonstrated. Moreover, periodontal therapy ameliorated periodontitis and CKD, and reduced NETs concentrations in GCF of patients.

Conclusions This study revealed that NETs might be a possible bridge between periodontitis and CKD, and suggested the potential target for therapy.

Keywords Neutrophil extracellular traps, Periodontitis, Chronic kidney disease, Periodontal therapy

Introduction

Initiated by the imbalance between subgingival communities and the host immune response, periodontitis is a chronic inflammation that leads to progressive loss of periodontium. It is the sixth most common human disease, affecting 45-50% of adults in the population [1]. Severe periodontitis affects 10-15% of the population, with the impact that is not limited to the periodontal tissues. It has been well demonstrated that the periodontal pathogens and inflammatory cytokines at the site of infection could affect the distant organ via the bloodstream, thereby inducing the systemic inflammatory

†Shucheng Hu and Ruhan Yang should be considered the co-first authors of this article.

*Correspondence:
Ziyuan Xu
xuziyuanrj@126.com
Qin Wang
qinwang_1975@126.com
Eryi Lu
lueryi222@outlook.com
[1]Department of Stomatology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 160 Pujian Road, Shanghai, China
[2]Department of Nephrology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 160 Pujian Road, Shanghai, China

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response and associating with some systemic diseases [2–4].

Chronic kidney disease (CKD), characterized by the decline in the glomerular filtration rate (GFR) and the abnormalities on the structure or the function of kidney [5], is a major health problem worldwide and affect 8-16% of the population [6]. Recently, interest in the relationship between periodontitis and CKD has increased given that they share the same genetic and environment risk factors [7]. Several studies have confirmed the epidemiological association between these two diseases [8, 9]. However, the mechanisms of the association have not yet been well defined.

There is ample evidence that the cross-talk of these diseases occurs through abnormal immune system function, with mechanisms including abnormal neutrophil activity and increased oxidative stress [10, 11]. Described as a suicidal tool used by neutrophils, NETs are induced by a large variety of stimuli and regulated by reactive oxygen species. It is a kind of meshed complex comprised of DNA and polymorphonuclear proteins such as myeloperoxidase (MPO), neutrophil elastase (NE) and citrullinated histones H3 (citH3). As reported, it plays a beneficial role during infections by trapping and degrading the invading pathogens. However, it also accelerates the inflammatory process via releasing active molecules and serves as the antigens in autoimmune diseases.

Emerging studies have extended our understanding of NETs as the regulator in several inflammatory diseases. It has been confirmed that NETs, elicited by periodontal bacteria, could reduce oral inflammation by restricting the spread of pathogens, whereas, their dysregulation disrupts homeostasis and causes tissue injury. Similarly, NETs also drove pathophysiological conditions associated with CKD. Current evidence suggested that neutrophils could infiltrate into kidney and release NETs [12]. Meanwhile, the imbalance between production and clearance of NETs caused kidney injury [13]. However, it is not clear whether NETs are involved in the interaction between periodontitis and CKD.

In this study, we hypothesized that NETs contributed to the pathogenic relationship between periodontitis and CKD. And we would like to develop a new direction to understand and manage these two diseases.

Materials and methods

Subject recruitment

The Recruitment was started in January 2022 and was completed in December 2022 at the Department of Nephrology and Stomotology, Renji Hospital Affiliated to Shanghai Jiaotong University School of Medicine. CKD was diagnosed and staged by the clinical practice guidelines of CKD in 2020 [14]. Then, they were referred to the Department of Stomatology and underwent periodontal examination. The full-mouth periodontal examination was performed at six sites per tooth (excluding third molar). Inclusion criteria were as follows: (1) According to the consensus report of 2017 World Workshop [15], patients who met the following criteria were classified into periodontitis. 1.Interdental CAL is detectable at ≥2 non-adjacent teeth, or 2. Buccal or oral CAL≥3 mm with PD≥3 mm is detectable at ≥2 teeth but the observed CAL cannot be ascribed to non-periodontitis-related. (2) aged between 18 and 75. Exclusion criteria were as follows: (1) active malignancy (2) pregnant and lactating (3) taking antibiotics within the past three months. (4) systemic diseases associated with periodontal lesions, such as diabetes, cardiovascular disease, systemic lupus erythematosus, etc. The GCF and venous blood were collected for each subject at the first appointment. In total, 63 CKD and 40 non-CKD participants were recruited and underwent periodontal examination. Of the early CKD patients, 35 patients completed the non-surgical periodontal therapy (including oral hygiene instruction, supra-gingival and sub-gingival scaling) and the GCF, gingival tissues and clinical parameters were collected again after three months of the treatment.

Clinical parameters

The following periodontal clinical measurements were recorded at the first appointment and follow-up visit by a calibrated periodontist using the same type of periodontal probe (Hu-Friedy, Chicago, IL, USA): (1) probing pocket depth (PPD) (2) clinical attachment level (AL) (3) gingival index (GI) (4) plaque index (PI).

Sample collection

Gingival crevicular fluid (GCF) was collected by paper points as described before [16, 17]. Briefly, GCF samples were collected from Ramfjord index teeth (the maxillary right and mandibular left first molars, maxillary left and mandibular right first premolars, and maxillary left and mandibular right central incisors), the sterile paper point was gently inserted into the gingival pocket, removed after 25–30 s and placed into an Eppendorf tube. Normal tissues were obtained from impacted tooth extraction at baseline with periodontal healthy patients. Inflamed tissues were collected by curettage or microsurgical scissors during the subgingival treatment at baseline and at follow-up in some periodontitis patients. The gingival tissues were excised, washed and then fixed with 4% PFA. All samples were collected by an experienced investigator.

Flow cytometry

The whole blood from participants was obtained in EDTA tube. Within 4 h of collection, 100ul blood was transferred into the flow cytometry sample tube and then

2 ml red blood cell lysis buffer was added for the removal of erythrocytes. After washing and centrifugation, the cells were then incubated with antibody master mix for 20 min at 4℃ in the dark. The antibody master mix was prepared for cell surface marker staining and consisted of one μl of BV421-conjugated anti-CD15 antibody (BD), one μl of BV650-conjugated anti-CD16 antibody (BD), one μl of Alexa Fluor 700-conjugated anti-CD45 antibody (BD). All samples were stained with Live/Dead Aqua or Green (Invitrogen) to discriminate live and dead cells. Subsequently, samples were incubated with NE (Abcam) and MPO (Novus) in the dark for 20 min, followed by incubation with Alexa Fluor 647-conjugated anti-rabbit antibody (Invitrogen) and Alexa Fluor 555-conjugated anti-mouse antibody (Solarbio). Data were analyzed with FlowJo software v10.8.1.

Immunohistochemistry assay

Gingival tissues were obtained from participants and stored at -80℃ after OCT embedding. Sections were blocked with 3% BSA for 30 min and permeabilized with triton for 15 min. Gingival sections were then incubated with a rabbit antibody against citH3 (Abcam) and a mouse antibody against myeloperoxidase (Novus) overnight according to protocols. After rinsing three times for 5 min with PBS, the slides were incubated with Cy3 conjugated Goat Anti-mouse IgG (H+L) (Servicebio) for MPO detection and Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG (H+L) (Servicebio) for citH3 detection for 50 min at room temperature.

Quantitation of NETs

GCF was collected by the same examiner and put into sterile Eppendorf vials. The venous blood was collected into tubes containing EDTA and then immediately centrifuged at 2000 rpm for 10 min to obtain plasma. All samples were stored at -80 °C for temporary preservation.

The Quant-iT Picogreen dsDNA Assay Kit (Invitrogen) was used to quantify NETs according to the protocol [18]. In brief, 100 μl of DNA stock solutions or prepared supernatants was added to the flat-bottomed 96-well plates. Then, 10 μl of Picogreen reagent diluted with TE buffer was mixed with the samples. After 5 min of incubation with room temperature and dark conditions, the fluorescence values were recorded using a fluorescence microplate reader (SpectraMax® Gemini™ EM, Molecular Devices, USA) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Then, the extracellular DNA was quantified based on the DNA standard curve.

Statistical analysis

GraphPad Prism 9.0 statistical software package was used for statistical analysis. The results were shown as mean ± standard deviation (SD). Mann-Whitney test was applied to evaluate the expression levels of NETs in different subgroups, Wilcoxon test was performed to assess the variation in clinical parameter between baseline and follow-up, and Spearman's correlation analysis was conducted to evaluate the correlations between NETs concentration and clinical parameters. $^{*}p<0.05$ was considered to be statistically significant. Significance was expressed as: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

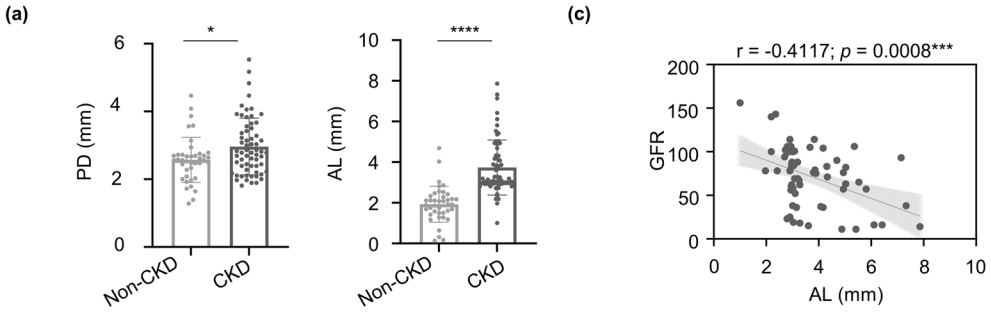
Results

The CKD severity was correlated with periodontitis status

A total of 240 participants were screened. Of these, 113 patients were refused to participate in the study for various reasons and 24 patients were excluded because of the systemic diseases associated with periodontal lesions (such as diabetes, cardiovascular disease, systemic lupus erythematosus, etc.) and the edentulous. Finally, 103 patients were enrolled, including 63 patients with CKD and 40 non-CKD patients. According to the clinical practice guidelines of CKD, the diagnosis of CKD requires GFR < 60 mL/min/1.73 m^2 for more than 3 months, and it's also the boundary between stage II and stage III. Therefore, on the basis of the GFR at enrollment, these patients were divided into early CKD ($n=43$) and late CKD ($n=20$) group. At first, periodontal examination was performed on all volunteers. It was revealed that the CKD patients have poor periodontal condition than healthy controls (Fig. 1a). Moreover, all enrolled CKD patients had periodontal problems to some extent. To further explore the relationship between periodontitis and CKD, the participants were grouped, as shown in Fig. 1b. And the flow of the participants throughout the study was presented in Fig. 1d. As shown in Fig. 1b, the results of the chi-square test showed an association between periodontitis and CKD ($p=0.0000296^{****}$). Furthermore, Spearman correlation analysis showed that there was a negative correlation between AL and GFR (r = -0.4117, $p=0.0008^{***}$), which confirmed the correlation between these two diseases and inspired us to explore the underlying mechanism.

The NETs concentration was correlated with renal condition

As previously reported, NETs are the major source of circulating dsDNA, and circulating dsDNA has always been used as a surrogate for NETs levels [19, 20]. The Quant-iT Picogreen dsDNA Assay Kit was performed in all participants for quantifying NETs in GCF and plasma. As shown in Fig. 2a, NETs concentrations in plasma were significantly higher in CKD group compared with non-CKD group ($p < 0.0001^{****}$). However, no significant difference was observed in two groups in terms of GCF ($p=0.4635$). In addition, the relationship between



(b)

CKD	Periodontal status		Sum up	*p*
	No detectable periodontitis	Periodontitis		
Non-CKD	10	30	40	
CKD	0	63	63	0.0000296***
Sum up	10	93	103	

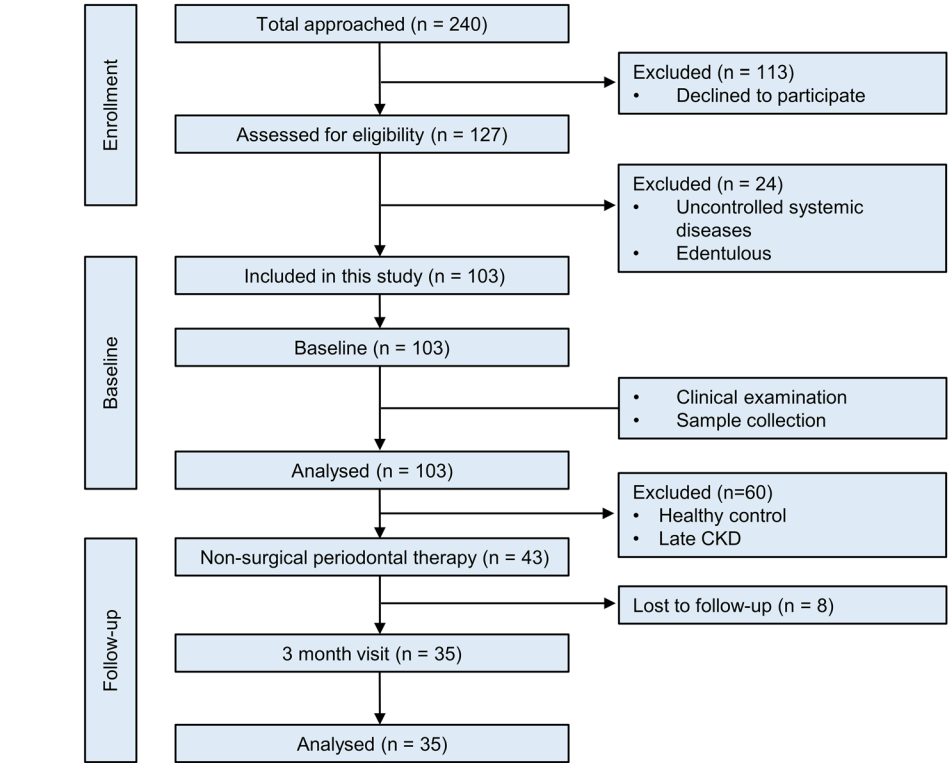


Fig. 1 CKD severity was correlated with periodontitis status. (**a**) The periodontal status (PD and AL) of all participants. (**b**) Study design and grouping of all participants. (**c**) Correlation between AL and GFR of all CKD patients ($n=63$). (**d**) Flow diagram displaying the process of patient selection and design of the project. Significance was defined as *$p<0.05$, ***$p<0.001$ and ****$p<0.0001$

NETs concentrations and the severity of CKD was analyzed. Here, GFR was used for indicating the severity of CKD. Consistent with the results of t test, NETs concentration was significantly and negatively correlated with GFR in plasma (r = -0.3972, p=0.0013**). But no significant correlation was observed in GCF (r = -0.1686, p=0.1865). These results suggested that renal condition was obviously related to NETs in plasma. To confirm the above results, the levels of NETs in the blood of CKD patients were qualified by flow cytometry. The neutrophil

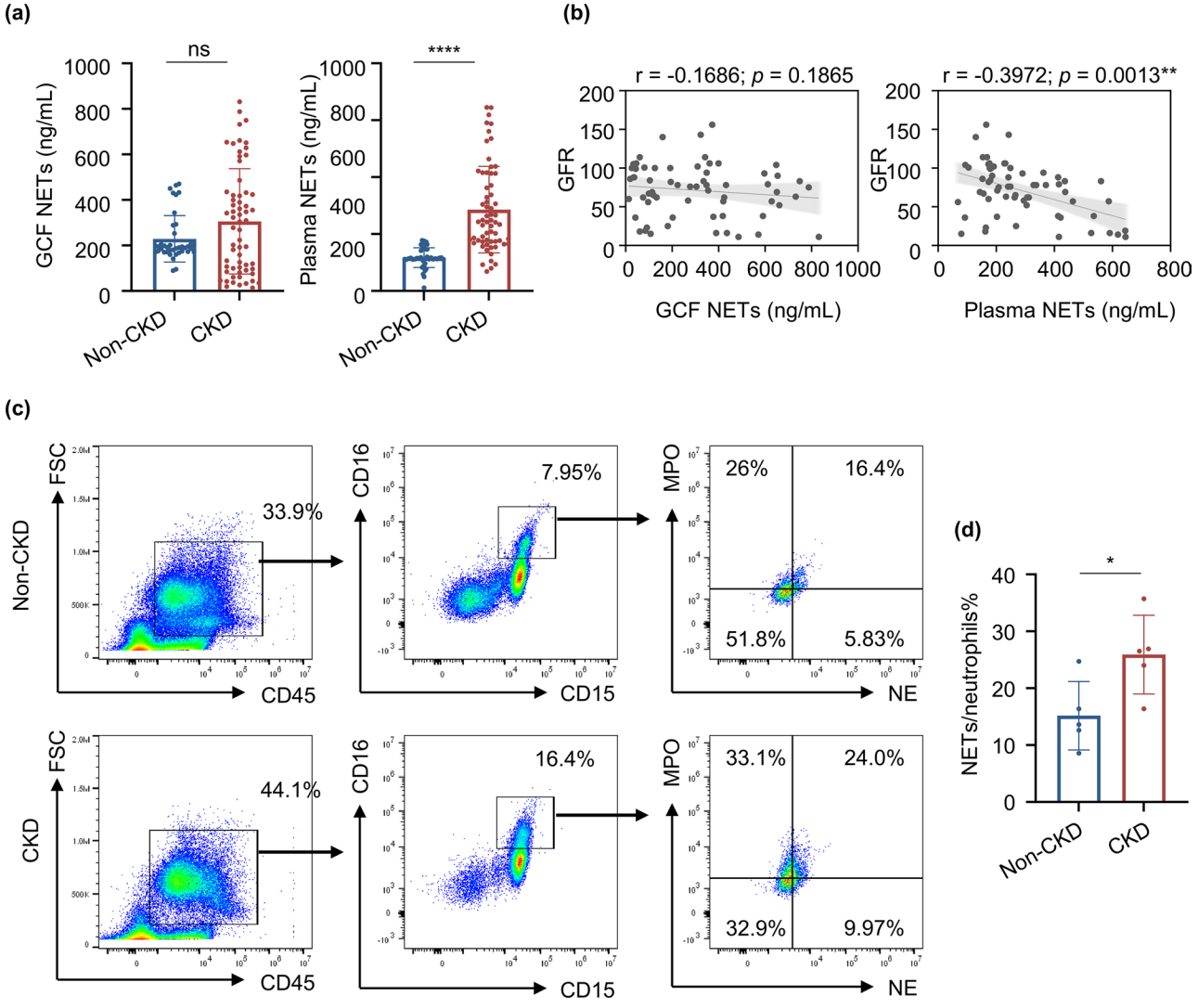


Fig. 2 Upregulated NETs concentration was correlated with renal condition (**a**) The level of NETs in GCF and plasma of patients from non-CKD participants ($n=40$) and CKD participants ($n=63$) was detected using the dsDNA assay kit. (**b**) The scatter plot showed the correlations between NETs concentration and GFR of patients ($n=63$). (**c**) Representative flow cytometry analysis of neutrophils ($CD15^+$ $CD16^+$) and NETs (NE^+ MPO^+) in the blood of non-CKD and CKD participants. (**d**) Quantification of NETs as percentages of neutrophils in samples from non-CKD ($n=5$) and CKD participants ($n=5$). The data were presented in the form of mean ± SD. Significance was defined as $^*p<0.05$, $^{**}p<0.01$ and $^{****}p<0.0001$

population is gated based on the dual positive surface expression of CD15 and CD16. The NETs were identified by the dual positivity of NE and MPO, and the thresholds were determined by the fluorescence levels of isotype control. As shown in Fig. 2c and d, there was a significant increase in NETs in patients with CKD compared with non-CKD ($p=0.0397^*$). The results suggest that the progression of CKD might be influenced by NETs in the blood. Therefore, further studies need to be carried out to investigated the factors causing the alteration of NETs in the blood.

The NETs concentration in CKD patients was correlated with periodontal status

As shown in previous study [21], the increased level of circulating NETs, accompanied with the propensity for increased NETs formation and delayed NETs clearance, was observed in periodontitis. To explore the effect of periodontitis on CKD patients, the patients were grouped according to periodontal status and then NETs levels were analyzed. As expected, patients with periodontitis had a significantly higher NETs concentration in both GCF ($p=0.0355^*$) and plasma ($p<0.0001^{****}$) when compared to periodontally healthy participants (Fig. 3a). To further validate the results of dsDNA, the levels of NETs were determined by flow cytometry and immunohistochemistry. As expected, patients with periodontitis had

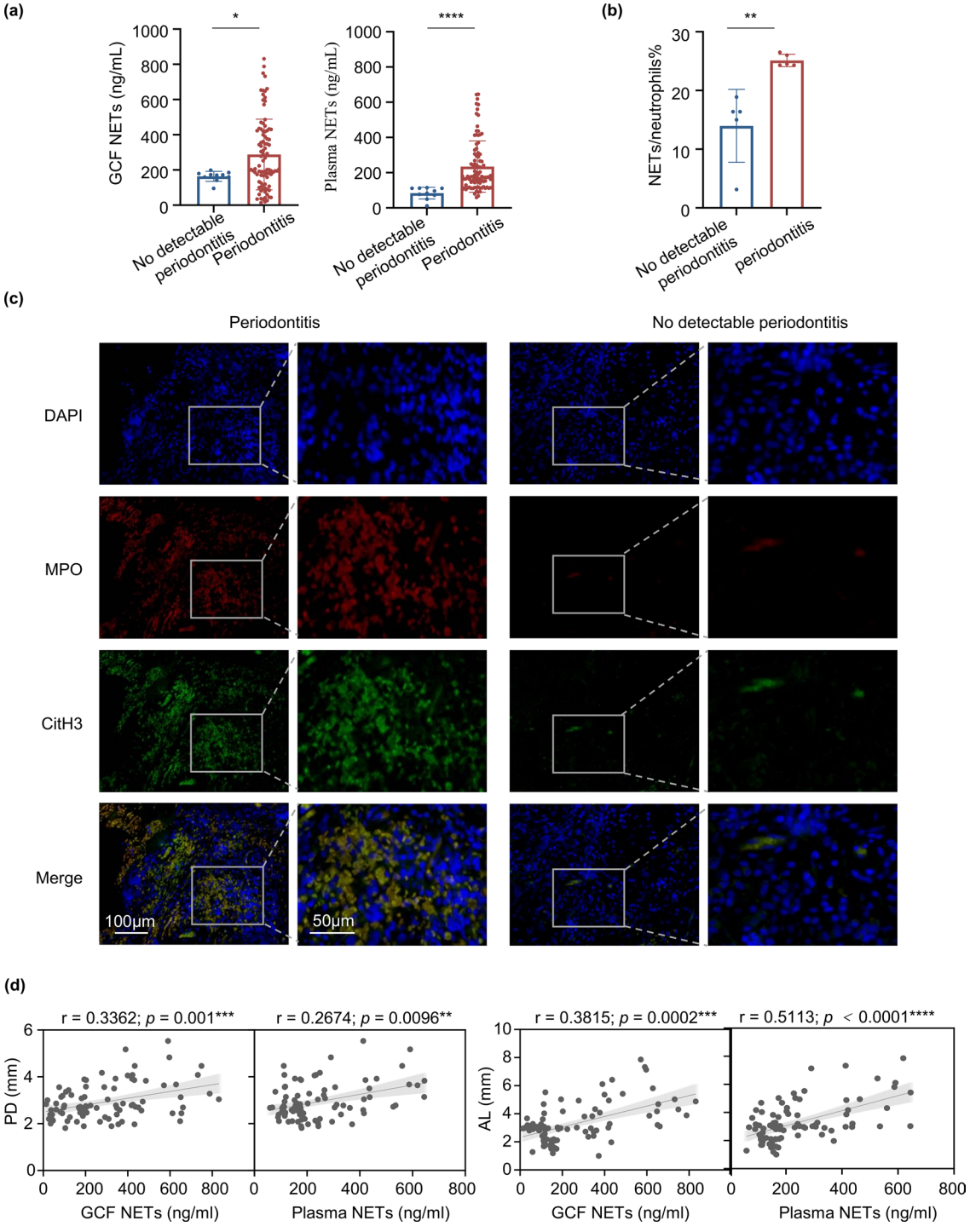


Fig. 3 Upregulated NETs concentration was correlated with periodontal status (**a**) The level of NETs in GCF and plasma of participants who have no periodontal disease ($n=10$) and periodontitis participants ($n=93$) was detected using the dsDNA assay kit. (**b**) Quantification of NETs as percentages of neutrophils in samples from periodontally healthy participants ($n=5$) and periodontitis participants ($n=5$). (**c**) Representative immunofluorescence of gingival tissues in participants who have no detectable periodontal disease and participants who have periodontitis showed MPO, citH3 and DAPI staining. (**d**) The scatter plot showed correlations between NETs concentration and clinical periodontal parameters (PD and AL) in GCF and plasma of patients ($n=93$). The data were presented in the form of mean ± SD. Significance was defined as $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$

higher levels of NETs in peripheral blood than those periodontal healthy participants (Fig. 3b, $p=0.0079^{**}$). In addition, the distribution and expression of NETs in local inflammation site were analyzed by immunohistochemistry. Immunofluorescence staining of the gingival tissues was shown in Fig. 3c. Compared with patients who have no detectable periodontal disease, the biopsies of the patients with periodontal disease showed greater citH3 staining and MPO staining in the extracellular medium.

Spearman's correlation analysis was then performed to further evaluate the association between the NETs concentration and clinical periodontal parameters in the patients. As shown in Fig. 3d, there was a noteworthy and positively correlation between NETs concentrations and PD ($r=0.3362$, $p=0.001^{***}$) and AL ($r=0.3815$, $p=0.0002^{***}$) in GCF. Similarly, the positive correlation was also observed between NETs concentrations and PD ($r=0.2674$, $p=0.0096^{**}$) and AL ($r=0.5113$, $p<0.0001^{****}$) in plasma, indicating the possible systemic effect of periodontitis in those patients. These results suggested that NETs, associated with periodontitis, might have some systemic effects in CKD patients.

Periodontal therapy significantly reduced NETs concentrations and improved clinical parameters of periodontitis and CKD

Considering the significant correlation between NETs concentrations and periodontal clinical parameters in CKD patients, we investigated if periodontal therapy could impact NETs concentration and reduce disease severity. Considering the physical condition of enrolled patients, the early CKD patients ($n=43$) were included in the treatment, with 35 out of the 43 participants successfully completing one month of periodontal therapy. In the patients who had received periodontal treatment, the distribution and the dose of CKD medication remained unchanged between the baseline and reassessment. As expected, periodontal treatment significantly reduced the values of periodontal parameters, including the BOP ($p<0.0001^{****}$), PD ($p<0.0001^{****}$), AL ($p<0.0001^{****}$), PI ($p<0.0001^{****}$), GI ($p<0.0001^{****}$), as well as the levels of NETs in GCF ($p<0.0001^{****}$, Fig. 4a). Immunofluorescence images verified a marked decrease of citH3 and MPO in gingival tissues of patients treated with periodontal therapy (Fig. 4b). Furthermore, the GFR of these patients was remarkably increased after the treatment (Fig. 4a), implying the benefits of periodontal treatment on periodontitis and CKD.

Discussion

In this study, we found for the first time that NETs may be the link for the cross-talk between periodontitis and CKD physiopathology. Moreover, non-surgical periodontal treatment proved to be effective in reducing NETs concentration, and helping to alleviate periodontitis and CKD.

Periodontitis and CKD share a lot of common risk factors, including age, smoking and diabetes. It has been well confirmed that there is a bidirectional relationship between periodontitis and CKD based on biological hypotheses. Kshirsagar showed that periodontitis could induce the dysfunction of kidney through the dissemination of the bacteria and inflammatory cytokines from local inflamed tissues [22]. The large longitudinal CKD cohort also demonstrated that periodontal inflammation and renal function are causally linked [23]. Consistently, the Spearman correlation analysis showed the correlation between periodontitis and CKD, which deserve further investigation. Emerging evidence have shown that periodontal inflammation represented an occult source of oxidative stress in patients with CKD [24]. In addition, the source of the reactive oxygen species as well as plasma protein and lipid damage in periodontitis is due to neutrophil activation, which is closely related to the induction of NETs. Thus, we hypothesized that NETs contribute to the dialogue between periodontitis and CKD.

NETs constitute a highly conserved antimicrobial strategy that is caused by inflammatory immune cells releasing the de-condensed nuclear chromatin into the extracellular space, thereby immobilizing and killing pathogens [25]. It has been established that the number of circulating neutrophils and NETs formation was markedly increased in CKD patients [26]. Our results showed a significant negative correlation between NETs concentration and GFR, suggesting that NETs may play an important role in the occurrence and development of CKD pathological process. Indeed, there has been growing interest in the role of NETs in CKD. Basal NETs formation was reportedly increased in CKD patients on maintenance hemodialysis, which was related to the increased basal autophagy activity [26]. In addition, NE activity and inflammation were shown to increase in the last stage of chronic kidney disease [27]. As CKD progresses and renal function declines, the number of circulating neutrophils increases, accompanied by the dysfunction of neutrophil, causing damage to the kidney. Therefore, the level of NETs in circulating blood might affect the progression of the CKD.

Previous research has shown that microorganisms and their products activate oral polymorphonuclear neutrophils to secrete NETs, which can maintain the inflammatory state [28]. We assumed that the NETs concentration in CKD patients was correlated with their periodontal status. As expected, we found that there was a significant difference of NETs concentrations between two groups divided by periodontal status. In addition, our results showed that the concentrations of NETs in GCF and

(a)

Parameter	Baseline (n = 35)	Follow-up (n = 35)	*p*
Age	47	47	ND
Female (%)	60	60	ND
Smoker (%)	20	11	ND
BOP (%)	86	40	****
PD (mm)	2.84	2.48	****
AL (mm)	3.55	3.18	****
GI	1.95	0.79	****
PI	2.03	0.93	****
NETs level (ng/ml)	253.8	189.2	****
GFR	87.68	90.57	****

(b)

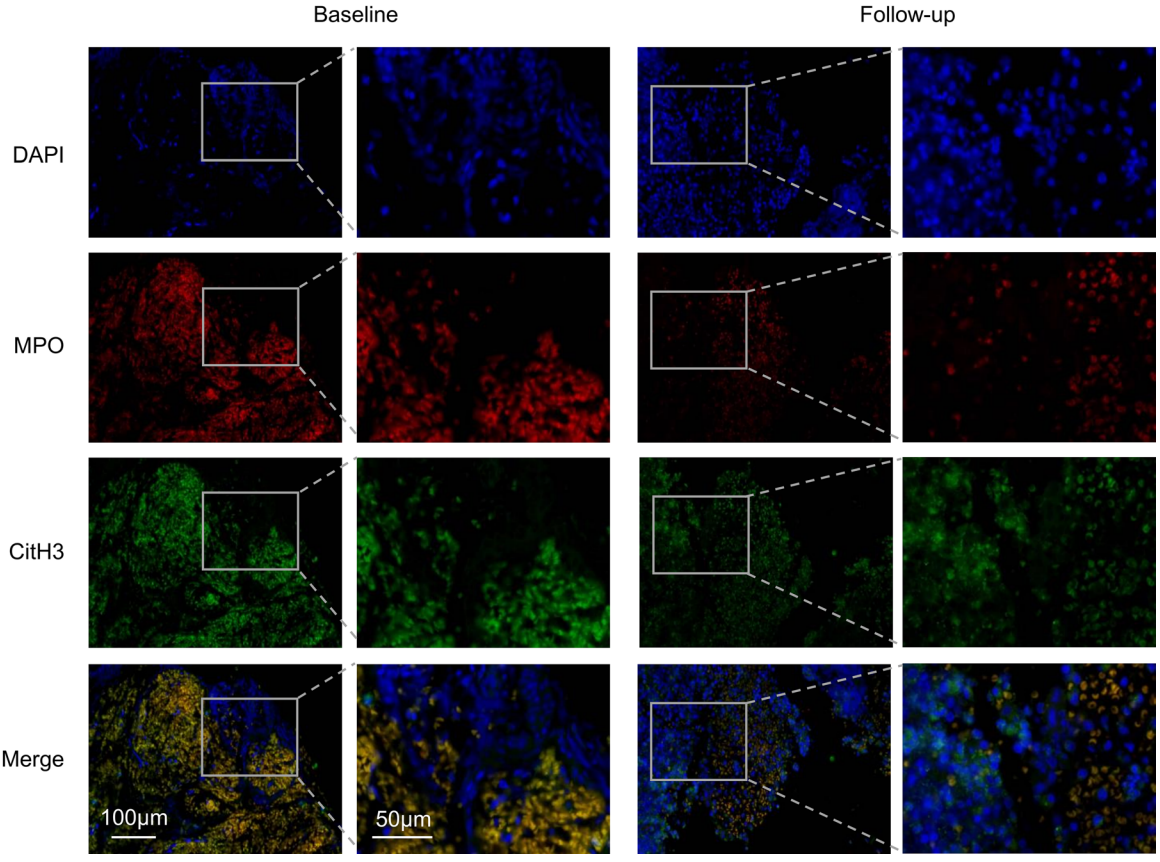


Fig. 4 The effects of periodontal therapy (**a**) Variations in the demographic parameters, clinical parameters and the decrease of the NETs concentrations. ($n = 35$). (**b**) Representative immunofluorescence of gingival tissues acquired from baseline and follow-up. Significance was defined as $^{**}p < 0.01$ and $^{****}p < 0.0001$. ND: not determined

plasma of these participants were positively correlated with periodontal parameters, PD and AL. The increased NETs formation was further confirmed using flow cytometry and immunofluorescence staining. These results suggested that NETs might be the link for the cross-talk between periodontitis and CKD. The results of periodontal intervention showed that the levels of NETs in CKD patients with periodontitis were significantly decreased after periodontal non-surgical treatment. This indicates that periodontitis might increase serum NETs levels, and

might be related to the chronic systemic inflammatory burden in CKD. However, future studies are needed to confirm the causal relationship between NETs and two diseases.

In fact, neutrophils have long been recognized as the predominant inflammatory cells and have been shown to exhibit hyperactivity and hyperreactivity in periodontitis [28]. They enhanced their antibacterial properties by releasing NETs. NETs were detected in blood and were regarded as the link between periodontitis and other diseases [29]. De Pablo showed high level of serum autoantibodies of NET-derived citrullinated in periodontitis patients, implying the systemic impact of NETs formation [30]. Thus, NETs might be the target in the management of periodontitis and related diseases. In the present study, we evaluated the influence of periodontal treatment on the levels of NETs in GCF of patients with CKD and periodontitis. In agreement with other studies [31–34], the result showed that non-surgical periodontal therapy significantly reduced NETs concentration and improved periodontal status and renal condition. That indicated a new direction for co-management of periodontitis and CKD.

In the present study, two kinds of biological sample, GCF and plasma, were chosen to detect the level of NETs concentration. GCF is a kind of oral bio-fluid which was in close proximity to the gingival tissues. There are various cytokines derived from the interaction between bacteria and host. Therefore, a growing number of studies use GCF as a diagnostic tool to detect the minute changes in the periodontitis progression [35, 36]. In our study, GCF was collected to detect NETs concentrations at the site of local inflammation, and plasma was used to examine their systemic effect. As shown, both GCF and plasma NETs concentrations were associated with periodontal parameters. Meanwhile, there was a more significant correlation between NETs concentration in plasma and GFR. The levels of NETs in GCF and blood well reflected their local and systemic effect.

Further research is needed to refine the study. Large cohort studies are needed to confirm and extend current observations. And animal experiments are needed to explore deeper mechanisms of interaction between NETs and these two diseases.

Conclusions

Our findings revealed that NETs concentrations were associated with periodontal status and renal condition and acted on the progression of both chronic inflammatory diseases. Non-surgical periodontal treatment was proved to be efficient in reducing NETs concentrations and in improving the clinical parameters of these illnesses. NETs may contribute to the dialogue between periodontitis and CKD, and NETs inhibition therapy may be a good way for the management of periodontitis and CKD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12903-024-05071-2.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

All authors have made substantial contributions to conception and design of the study. S.H., R.Y., E.L. designed the research; Y.G., W.Y., D.Z. administrated the project; W.Y., J.T., W.Y., L.L. acquired data, Z.X., Q.W., E.L. supervised the research; R.Y., M.J. analyzed data; S.H., R.Y. write the manuscript; Q.W., E.L., Z.X reviewed and edited the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (KY2021-196-B) and performed according to the Declaration of Helsinki. All patients gave their informed consent prior to study inclusion.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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