# Neutrophil Extracellular Traps (NETs) as a Potential Target for Anti-Aging: Role of Therapeutic Apheresis



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## ABSTRACT

Neutrophil extracellular traps (NETs) are large structures composed of chromatin, histones and granule-derived proteins released extracellularly by neutrophils. They are generally considered to be a part of the antimicrobial defense strategy, preventing the dissemination of pathogens. However, overproduction of NETs or their ineffective clearance can drive various pathologies, many of which are associated with advanced age and involve uncontrolled inflammation, oxidative, cardiovascular and neurodegenerative stress as underlying mechanisms. Targeting NETs in the elderly as an anti-aging therapy seems to be a very attractive therapeutic approach. Therapeutic apheresis with a specific filter to remove NETs could be a promising strategy worth considering.

## Introduction

Neutrophil extracellular traps (NETs), discovered in 2004, are net-like complexes consisting of DNA, histones, and granule proteins, which are released by neutrophils to the extracellular space in a special form of programmed cell death (NETosis) [1, 2]. They represent an evolutionarily conserved element of the innate immune response and bind pathogens to prevent their spread. NETs formation is triggered by immune receptors through downstream intracellular mediators including reactive oxygen species (ROS), produced by mitochondria or NADPH oxidases, which in turn activate myeloperoxidases (MPO),

neutrophil elastase (NE) and protein-arginine deiminase type 4 (PAD4) to stimulate chromatin decondensation [3, 4]. Current data show that NETosis can be classified into two types: 1) 'suicidal', dependent on NADPH oxidase activity, ultimately leading to death of the neutrophils; and 2) 'vital', NADPH oxidase-independent, after which the cells are still alive [5]. Additionally, NETosis is regulated by the size of the microorganism, microbial virulence factors and cytokines [6–10] and the created NETs can have different forms, ranging from a band form, through a cloud-like shape, when the NET is fully hydrated, up to a network-like structure, exceeding 10–15 times the volume of the releasing cells [2, 11]. Recent studies revealed that NETs are branching filament networks, with a highly organized porous structure and with openings in the sizes corresponding to small pathogens [12].

However, in addition to current advances pointing to specialized protective functions of NETs, the list of medical conditions in which NETs are implicated as a pathogenic factor is continuously expanding. In addition, the role of NETs in healthy or disease-prone elderly is currently not well understood. Therefore, we aim to review and assess the current role of NETs in aging and to discuss the role of NETs as a potential target for an anti-aging therapy.

## NETs and the aging process: what do we currently know?

The process of aging is generally characterized by a gradual functional decline. In mammals it occurs in a heterogeneous manner across multiple tissues, which leads to a progressive deterioration and ultimately causes organ dysfunction. In consequence, advanced age is an independent risk factor for many diseases, including cardiovascular disease [13], dementia [14], osteoporosis [15], cancer [16], type 2 diabetes [17], idiopathic pulmonary fibrosis [18], glaucoma [19], and metabolic-associated fatty liver disease (MAFLD) [20]. Our understanding of aging remains limited, and its biological causes are largely unknown. What we do know, however, is that aging promotes chronic inflammation, which in turn is associated with the development of fibrosis and organ decline. The release of NETs initiated by protein-arginine deiminase type 4 damages cells and organs in various models of acute inflammation. For example, a reduction in fibrosis was demonstrated in the hearts and lungs of aged PAD4$^{-/-}$ mice compared with wild-type littermates. In addition, in an experimental model of cardiac fibrosis, pressure overload contributed to NETosis and significant platelet recruitment in myocardium from WT but not PAD4$^{-/-}$ mice [21, 22]. During the aging process, NETosis may be reduced [23–26] or increased [21, 27], but most importantly the NETs are functionally altered, significantly contributing to both elevated infection susceptibility and increased cardiovascular risk in the elderly. NETs are capable of targeting senescent vasculature for tissue remodeling in retinopathy [28]. Aged vasculature releases a secretome that attracts neutrophils and leads to NETosis. The NETs produced can remodel retinal vasculature through apoptotic elimination of endothelial cells. On the other hand, abundant NETosis may exacerbate vasculopathy by causing microvascular occlusion or small vessel vasculitis. These data are in line with the associations of diabetic retinopathy in patients with elevated neutrophil levels [29, 30] and the finding that immunodepletion of neutrophils preserves retinal microvasculature [31, 32]. Finally, chronic stress shifting normal circadian rhythm of neutrophils and leading to an increase in NETs formation may be related to the increased risk of metastases and worse cancer survival in the aging population [33].

## Rational for targeting NETs in the elderly

The ability of NETs to damage tissue has been demonstrated both in infection and sterile inflammation. Specifically, NETs can directly kill epithelial cells [34], endothelial cells [35], and excessive NETosis injures the lung epithelium in fungal infections [6] and the endothelium in transfusion-related acute pulmonary injury [36]. What is more, emerging evidence revealed the role of NETs in many conditions usually associated with more advanced age, such as chronic inflammation, where they are implicated in various stages of atherosclerosis and respond to sterile inflammatory stimuli, such as lipoproteins and cytokines [37]. Furthermore, NETs promote immune thrombosis through interaction with vascular endothelial cells and platelets and have been linked to the progression of cardiovascular disease, including myocardial infarction and thrombosis [38, 39]. Moreover, NETs promote cancer-related inflammation and myocardial stress [40] and are implicated in interstitial lung disease and associated fibrosis in both acute and Long-COVID disease [41]. Finally, there is a growing body of evidence suggesting that dysregulated production of NETs significantly contributes to the pathogenesis of sepsis-induced multi-organ failure, including hypoxemia, arterial hypotension, coagulopathy, renal, hepatic and neurological dysfunction [42, 43]. Mechanistically, studies on antibody-mediated neutralization suggest that histones bound to NETs play a key role in NETs-mediated cytotoxicity [34]. Other NET proteins, for example, defensins, can permeabilize eukaryotic cells [44, 45], whereas neutrophil elastase targets extracellular matrix proteins, which disrupts cell junctions [46]. In addition to that, NETs are a source of autoantigens and may trigger autoimmunity leading to production of autoantibodies directed against NETs' components in rheumatoid arthritis or systemic lupus erythematosus (SLE) [47].

Given that the aging process is associated with cellular and immune senescence as well as chronic inflammation and vascular degeneration, targeting NETs as an anti-aging therapy could be an attractive strategy.

## Strategies for anti-NETs therapy and role of novel approaches for therapeutic apheresis

NETosis and NETs themselves have been emerging as a promising target for acute and chronic inflammatory disorders. In numerous preclinical disease models including wound healing, thrombosis, atherosclerosis, sepsis-induced endotoxic shock, colitis, fibrosis, and SLE blocking NETs exerted a positive effect [21, 48–58]

## PAD4 inhibition and DNase

Both DNase-driven removal and blockade of PAD4 have been evaluated as a potential NETs-targeting approach. For example, studies in ApoE-deficient mice fed a high-fat diet demonstrated that administration of a PAD inhibitor successfully blocked NETs gener-

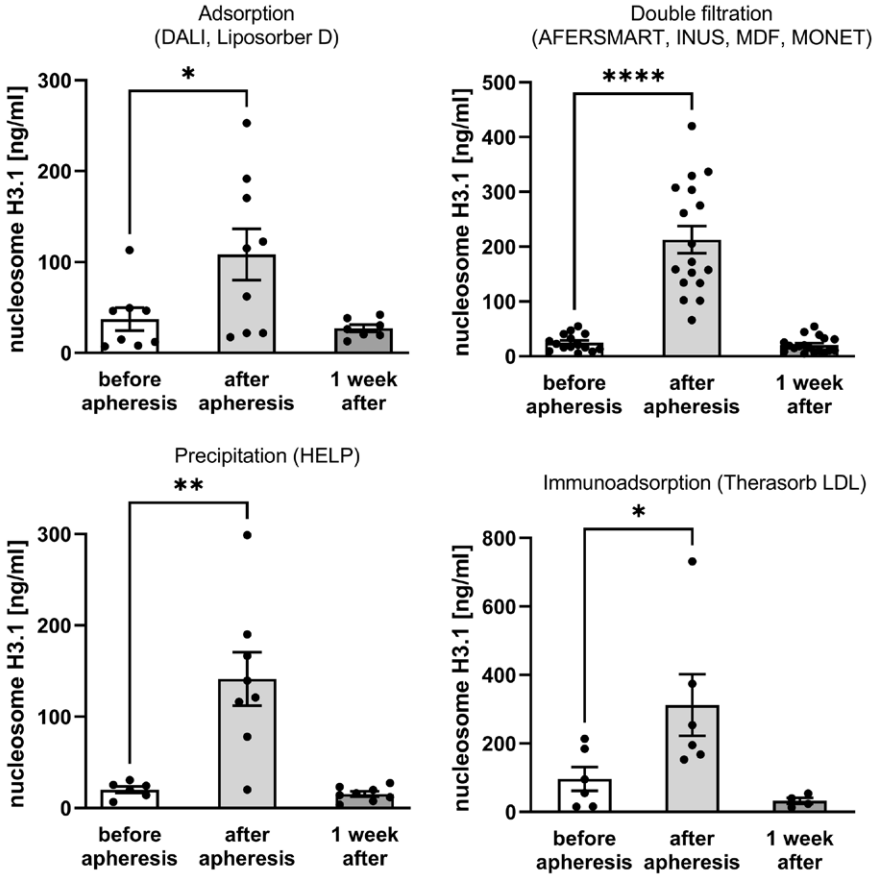
ation, decreased the size of atherosclerotic lesions, and postponed the onset of carotid thrombosis [59]. In acute carotid artery injury experiments, deficiency of PAD4 in the bone marrow-derived cells or DNase I treatment protected the mice from superficial plaque erosion [60]. Furthermore, in a mouse model of SLE, inhibition of PAD4 protected against kidney injury, vascular damage, and endothelial dysfunction [49], however, not against end-organ damage features of proteinuria [61]. In a rat model of ischemia-reperfusion injury, DNase I administration contributed to faster clearance of NETs and significantly improved microvasculature flow, which led to a diminished infarct size and ameliorated left ventricular remodeling [62]. However, DNase therapy is not free from disadvantages, as it removes only the DNA component of the NETs. The highly active enzymes, which were held by the DNA scaffold are released and still can damage healthy cells [63]. In addition to that, free circulating histones are cytotoxic, owing to their ability to compromise the integrity of cell membrane [64, 65]. Inhibition of PAD4 does not seem like a perfect solution either, given that even though heterozygous deletion of PAD4 in mice models of pneumonia improved survival, this was not the case in mice with total PAD4 deficiency [66]. Therefore, it would be very difficult to choose the correct dose of the drug that would have to be taken for a long time without resulting in an easily measurable readout that could be regularly monitored in the clinic.

## Th2 cytokines

Interestingly, patients suffering from allergic diseases like atopic dermatitis have reduced numbers of neutrophils in the skin compared to healthy people and diminished NETosis [67]. Experimental evidence indicates that Th2 cytokines including IL-4 and IL-13 are involved in this phenomenon, rising the intriguing possibility of utilizing them in a NETs-targeting therapy, however, more research is needed to investigate the clinical benefits of this approach [68].

## Hydroxychloroquine

Hydroxychloroquine (HDQ) is a drug used primarily for treating malaria, but also SLE and RA [69, 70]. Interestingly, studies have reported that HDQ can also reduce NETosis by suppressing PAD4 expression [71]. The utility of this approach was shown in a mouse model of ischemia/reperfusion injury [72]. Furthermore, HDQ was




▶ **Fig. 1** NETs levels in patients referred for lipoprotein apheresis before the treatment, immediately after and 1 week after the treatment, divided according to apheresis method. The bars represent mean ± SEM. Data were analyzed using repeated measures ANOVA, or, if missing data is present, using mixed-effects analysis. The Geisser–Greenhouse correction was used to adjust for the lack of sphericity and the Sidak correction was used to adjust for multiple comparisons. *p<0.05; **p<0.01; ****p<0.0001.

demonstrated to prevent neutrophils from absorbing tumor-derived extracellular vesicles, which contribute to NETs formation [73]. However, there is also contradictory experimental evidence available, showing no effect of HDQ on NETosis [74]. Therefore, the therapeutic potential of HDQ remains unclear.

## Molecular hydrogen

Recently, the therapeutic potential of molecular hydrogen ($H_2$) to inhibit formation of NETs has been demonstrated [75]. In vitro experiments on human neutrophils showed that $H_2$ can suppress neutrophil aggregation and subsequent NETosis. In vivo, inhalation of $H_2$ limits NETs formation in the pulmonary arteries of a lipopolysaccharide-induced sepsis model of aged mini pigs [76].

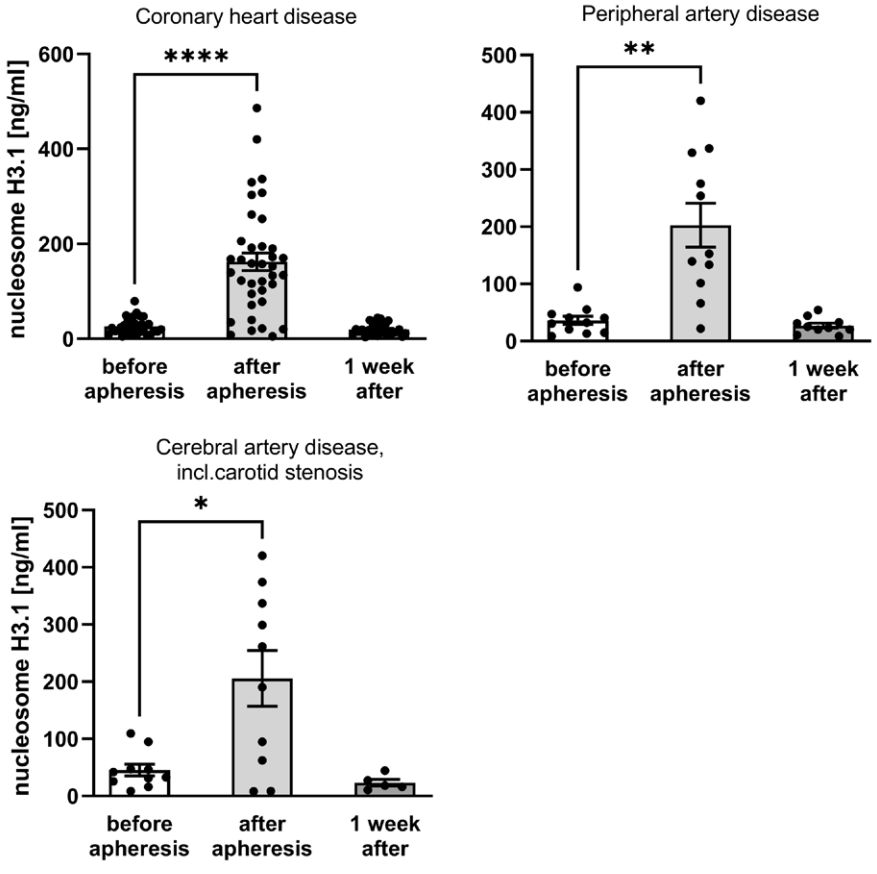
## *N*-Acetylcysteine

*N*-Acetylcysteine (NAC), is an antioxidant used in the treatment of chronic obstructive lung disease, acetaminophen overdose, bronchiectasis, and other diseases associated with ROS [77–80] Intriguingly, the drug has also been reported to dose-dependently reduce formation of NETs in human neutrophils stimulated with phorbol-12-myristate-13 acetate (PMA) [81]. It also has been shown to limit NETosis in primary human neutrophils extracted from patients with chronic hematologic malignancies and healthy individuals [82].

## Antibiotics

In addition to their antibacterial properties, some antibiotics can also be used as immunomodulators, as they are able to interact with various immune cells, including neutrophils [83, 84]. Following this observation, pretreatment of human PMA-stimulated neutrophils with azithromycin and chloramphenicol has been demonstrated to reduce NETs formation [85].

In summary, targeting specific steps of NETosis or NETs themselves can offer therapeutic benefits in NETs-associated pathologies. However, all the approaches considered so far are not free from severe side effects, such as increased susceptibility to infections and weakened immune system, which can be particularly detrimental in the elderly. Additionally, it has to be taken into account that most of the data on the potential anti-NETs approaches have been generated using PMA, a plant-derived organic compound, as a neutrophil stimulant. This substance is inducing only one of many molecular pathways involved in NETosis [3]. It cannot be excluded



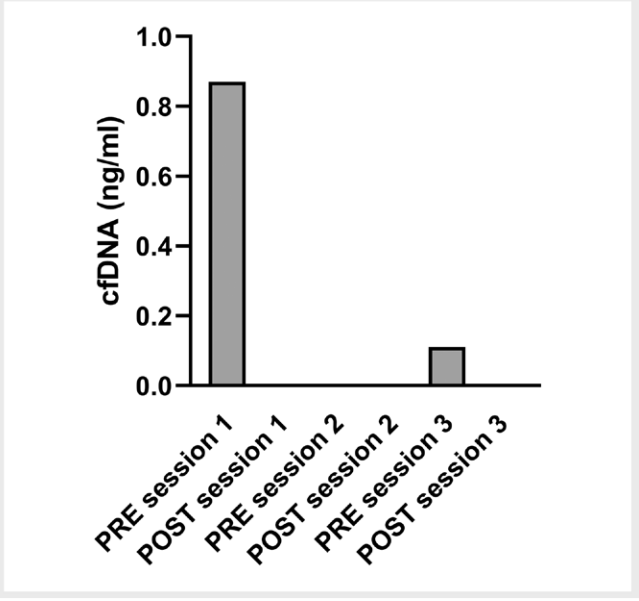

▶ **Fig. 2** NETs levels in patients referred for lipoprotein apheresis before the treatment, immediately after and 1 week after the treatment, divided according to the concomitant disease. The bars represent mean ± SEM. Data were analyzed using repeated measures ANOVA, or, if missing data is present, using mixed-effects analysis. The Geisser–Greenhouse correction was used to adjust for the lack of sphericity and the Sidak correction was used to adjust for multiple comparisons. *$p<0.05$; **$p<0.01$; ****$p<0.0001$.

that the results would have been different with a more physiologically relevant stimulus. What is more, proteomic analysis revealed that NETs induced by various stimuli are highly heterogeneous in terms of post-translational modifications and protein composition. This data strongly suggest that NETs induced in different conditions may exert different biological functions [86].

## Therapeutic apheresis?

Therapeutic apheresis (TA) is an extracorporeal treatment method, which selectively removes cells or molecules from the blood, which are contributing to or directly cause certain pathologies. Given the involvement of NETs in many pathologies described above and the lack of specific, approved and safe approaches to inhibit the formation of or to remove NETs, we raised the intriguing question, whether therapeutic apheresis may have a non-specific, additional effect of removal of NETs. With this goal in mind, we collected blood samples from 52 patients treated with lipoprotein apheresis at the Lipid Center of the University Hospital in Dresden, Germany, just before and after a single treatment session and one week later (ethics vote: EK403102014). Apheresis was performed using methods based on double-filtration, precipitation or (immuno) adsorption and NETs in plasma were measured using a chemiluminescence immunoassay including labeled anti-nucleosome and anti-histone modification antibodies. Surprisingly, all apheresis methods investigated led not to a decrease, but an increase in the levels of circulating NETs right after the treatment session. One week later the plasma levels of NETs were back to baseline. We observed the same pattern of response regardless of the apheresis method used or the concomitant disease (▶ **Fig. 1, 2**). Our results suggest the possible benefit of adding an additional NETs-specific filter to remove the excess of NETs before reinfusion of plasma in patients undergoing apheresis. However, at this point we do not know what the physiological consequence of the abrupt increase in the NETs levels after apheresis is or how long does it last. Understanding the mechanisms responsible for the elevated NETs levels after apheresis requires further research, but one possible explanation might be that cell injury in the extracorporeal circuits leads to release of NETs.

In the next step, we tested the efficiency of the NucleoCapture column (Santersus) to remove NETs from circulation. A patient with systemic lupus erythematosus and severe exacerbation (SLEDAI-2K – 32) was treated with 3 sessions of apheresis with the NecleoCapture column with one-day interval between procedures together with the standard therapy (6-methylprednisolone at a dose of 16 mg/day). The treatment not only led to almost complete removal of cell-free DNA (▶ **Fig. 3**), but also to a reduction of the SLEDAI-2k score to 12 right after the procedure and to 6 three months later. Even though the patient received a standard therapy, which potentially could affect the NETs levels, the effect of steroids on NETs is complex and current findings seem to be conflicting. While in vitro data show little effect on NETs, there is recent data showing that endogenous glucocorticoids via activation of the hypothalamic-pituitary-adrenal axis may induce NETosis [33]. However, high dose of external glucocorticoids has been reported to decrease NETs formation in vivo in an equine model of asthma [87].




▶ **Fig. 3** Cell-free DNA in a systemic lupus erythematosus patient after 3 sessions of apheresis with the NucleoCapture column.

## Conclusions

Targeting NETs and their removal in elderly patients could potentially be an intriguing anti-aging strategy capable of alleviating at least some of the diseases associated with advanced age. Currently considered approaches compromise the defensive functions of neutrophils, which may be particularly detrimental in the elderly. We have demonstrated that usual lipoprotein apheresis, contrary to our initial hypothesis, leads to an increase in the levels of circulating NETs. Our observations suggest the potential benefit of adding an additional NETs-specific filter to remove the excess of NETs before reinfusing plasma in patients undergoing lipoprotein apheresis. Special attention will have to be paid not to introduce this therapy in circumstances where NETs are beneficial, like acute infection. After all, it is all about right timing.

## Limitations

The study is observational only, we did not study the mechanism of increase in NETs after the conventional apheresis methods.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## Conflict of Interest

The authors declare that they have no competing financial interest in relations to this work. KVB, RS and YK work for for the INUS clinic and AM and PM work in the Ayus clinic. AA is the Chief Medical Officer in Santersus, which developed NucleoCapture column to remove NETs from circulation.

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