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Graphical abstract

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Angiotensin II triggers neutrophil extracellular traps release linking thromboinflammation with essential hypertension

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Conflict of interest
Authors have no conflict of interest to declare.
Abstract

Innate immunity and chronic inflammation are involved in atherosclerosis and atherothrombosis leading to target organ damage in essential hypertension (EH). However, the role of neutrophils in EH is still elusive. We investigated the association between angiotensin II (Ang II) and neutrophil extracellular traps (NETs) in pathogenesis of EH. Plasma samples, kidney biopsies and surgical specimens of abdominal aortic aneurysms (AAA) from EH patients were used. Cell-based assays, NETs/human aortic endothelial cells co-cultures and in situ studies were performed. Increased plasma levels of NETs and tissue factor (TF) activity were detected in untreated, newly-diagnosed, EH patients. Stimulation of control neutrophils with plasma from untreated EH patients generated TF-enriched NETs promoting endothelial collagen production. Ang II induced NETosis in vitro via a reactive oxygen species (ROS)/peptidylarginine deiminase type 4 and autophagy-dependent pathway. Circulating NETs and thrombin generation levels were reduced significantly in EH patients starting treatment with Ang II receptor blockers, whereas their plasma was unable to trigger procoagulant NETs. Moreover, TF-bearing NETotic neutrophils/remnants were accumulated in sites of interstitial renal fibrosis and in the subendothelial layer of AAA. These data reveal the important pathogenic role of Ang II/ROS/NETs/TF axis in EH, linking thromboinflammation with endothelial dysfunction and fibrosis.

Keywords: Angiotensin II, neutrophil extracellular traps, tissue factor, thromboinflammation, fibrosis, essential hypertension.
Introduction

Essential hypertension (EH) is a major risk factor for cardiovascular diseases (CVD) and chronic kidney disease (CKD), constituting a leading cause of morbidity and mortality (1, 2). Endothelial dysfunction, oxidative stress and chronic low-grade inflammation have been documented to contribute in the initiation and maintenance of EH (3), leading to the prevalence of a pro-inflammatory and pro-thrombotic phenotype (4, 5). In these procedures activation of the renin-angiotensin-aldosterone system holds also a central pathogenetic role (6).

Importantly, recent emerging evidence has shown that in EH, an excessive or prolonged stimulation of innate immune cells mediates a chronic inflammatory state that promotes vascular injury and target organ damage (3). Neutrophils represent the most abundant innate immune cells and through the secretion of several pro-oxidant and proinflammatory molecules, are able to promote and facilitate immune-mediated inflammation at sites of tissue injury (7). However, even though epidemiological studies have correlated neutrophil count with increased risk of developing EH and kidney dysfunction (8, 9), the experimental and clinical evidence regarding their exact role in the pathogenesis and complications of EH is limited (10).

Increased attention has been currently paid to neutrophils activation in response to a wide variety of stimuli mainly including microbial factors, reactive oxygen species (ROS) and activated platelets, which leads to the unfurling of their DNA into the extracellular space, thereby forming neutrophil extracellular traps (NETs)/NETosis. NETs constitute a meshwork of extruded chromatin fibers decorated with various highly active neutrophil-derived granular and cytosolic proteins such as myeloperoxidase (MPO), elastase (NE) and citrullinated histones (11, 12). Apart from their protective antimicrobial role, excessive NETosis may have a detrimental effect to
the host by inducing autoimmunity, excessively activating innate and adaptive immune system and promoting endothelial damage and inflammation (12, 13). Most importantly, NETs may exert significant prothrombotic properties by presenting extrinsic neutrophil proteins, namely Tissue Factor (TF), which represents the main in vivo initiator of the coagulation cascade (14, 15). In this context, experimental data in patients with coronary thrombosis have shown that NETs expose a highly functional TF which is able to induce both thrombin generation and thrombin/protease-activated receptor 1 (PAR1)-mediated platelet activation (16). As such, NETs have been implicated as key drivers in atherothrombosis and atherosclerosis in cardiovascular disease (14, 17). However, the role of NETs in EH and related complications is largely unknown.

Herein, we identified neutrophils/NETs expressing TF as main components of EH pathogenesis. This study unveils an inflammatory effect of Angiotensin II (Ang II) through the induction of ROS/NETosis pathway leading to thromboinflammation and fibrotic damage in EH.
Results

Increased levels of circulating NETs in untreated newly-diagnosed EH patients

Several studies have demonstrated that NETs are implicated in atherosclerosis, fibrosis and thrombotic CVD (18–20). Thus, we first used ELISA to measure the levels of MPO/DNA complexes and citrullinated histone H3 (CitH3), well-defined circulating markers of NET release (14), in the plasma of EH treatment-naïve (untreated) patients. We detected significantly increased levels of these NETs-remnants in patients compared to healthy individuals (controls) of similar profile (Figure 1A,B). Moreover, in EH patients, CitH3 values were well-correlated with MPO/DNA complexes (Figure 1C).

In an effort to support our ex vivo observations, and concerning that distinct inflammatory mediators could drive the formation of NETs (12, 13), neutrophils isolated from healthy individuals (control neutrophils) were stimulated with plasma samples from treatment-naïve EH patients that had various levels of NETosis markers (Supplemental Table S1). We found that EH plasma-treated control neutrophils generated NETs, as assessed by immunofluorescence microscopy (Figure 1D,E) and MPO/DNA complex ELISA (Figure 1F). On the other hand, inhibition of NADPH oxidase using diphenyleneiodonium (DPI) or peptidylarginine deiminase 4 (PAD4) using Cl-amidine, prior to stimulation with EH plasma, prevented NET formation, suggesting the involvement of ROS and histone citrullination in this process (Figure 1D-F).

Collectively, our findings indicate that treatment-naïve EH patients exhibit high plasma levels of NETosis, while inflammatory environment of EH plasma is efficient to induce in vitro NET generation.
Plasma from patients with untreated EH induces the release of NETs carrying bioactive TF

EH is characterized by thrombotic complications. Moreover, NETs have been shown to drive thromboinflammation in several disorders (14, 21, 22). Hence, we measured the levels of thrombin-antithrombin (TAT) complexes in the plasma of untreated EH patients and significantly high TAT activity was detected in patient group compared to controls (Figure 2A), suggesting activation of the TF/thrombin axis in EH. In addition, NET release in plasma, as represented by the levels of CitH3, was significantly correlated with plasma TAT activity, suggesting the thrombogenic potential of NETs in EH (Figure 2B).

To further verify the thromboinflammatory aspect of the disease, in vitro stimulations were deployed. As indicated by TF qPCR and In-Cell ELISA, plasma from untreated EH patients was able to induce TF expression in control neutrophils (Figure 2C-D).

Considering that pathogenesis of neutrophil-mediated diseases is critically defined by the protein composition of NETs (12, 13), next we examined whether TF is externalized on NETs released from control neutrophils upon stimulation with treatment-naïve EH plasma samples having various levels of TAT activity (Supplemental Table S1). Indeed, we found that plasma-treated control neutrophils efficiently generated TF-bearing NETs, as assessed by immunofluorescence microscopy (Figure 2E).

Importantly, TF on NETs was bioactive, as indicated by TAT ELISA and TF activity quantitative assay (Figure 2F,G). On the other hand, dismantling NET structures with DNase I or neutralizing TF on NETs significantly reduced TAT levels and TF activity (Figure 2F,G).

Together, our findings indicate that NETs expressing TF are involved in thrombogenicity of treatment-naive EH patients.
Angiotensin II induces NET formation in a ROS/autophagy-dependent manner

It has been reported that Ang II exerts inflammatory effect on human neutrophils, through Ang II type 1 (AT1) receptor inducing neutrophil adhesion and production of ROS (23–26). Hence, we investigated the implication of Ang II in the formation of NETs. Control neutrophils treated with Ang II efficiently generated NETs in a dose-dependent manner (Figure 3A-B,G,H and Supplemental Figure S1A), while Ang II did not exhibit any apoptotic effect on human neutrophils as assessed by Annexin V/PI staining (Supplemental Figure S1B). On the other hand, pre-treatment of cells with irbesartan, an AT1 receptor blocker (ARB), significantly hindered the release of NETs mediated by Ang II (Figure 3C,G,H).

Next, we tried to elucidate the possible mechanisms through which Ang II induces NETosis. Distinct mechanisms could mediate and regulate the release of NETs, including reactive oxygen species (ROS), autophagy (27) and PAD4-mediated histone hypercitrullination (12, 27). Hence, prior to stimulation with Ang II, control neutrophils were treated with NADPH oxidase inhibitor DPI and a significant attenuation of Ang II-mediated NETosis was observed (Figure 3D, G-H). Furthermore, to investigate the role of autophagy, cells were pre-incubated with early (wortmannin) or late-stage (bafilomycin A1, hydroxychloroquine/HCQ) autophagy inhibitors. Both wortmannin and bafilomycin A1 markedly reduced NET formation (Figure 3E, G-H and Supplemental Figure S2), albeit not statistically significant for HCQ (Supplemental Figure S2).

Next, we addressed whether PAD4 citrullination of histones is involved in Ang II-induced NETosis. To this end, control neutrophils were treated with Cl-amidine, prior to incubation with Ang II, and a significantly reduction of NET formation was observed (Figure 3F-H).
Collectively, our findings demonstrate that Ang II enhances the formation of NETs \textit{in vitro} in a ROS/autophagy-dependent manner, and PAD4 histone citrullination is associated with Ang II-induced NETosis.

\textbf{Decreased levels of NETs and TF activity in EH patients treated with ARBs}

Prompted by our in vitro findings indicating that irbesartan reduces the release of Ang II-mediated NETs, we next performed a paired analysis in plasma derived from 12 EH patients who were sampled just before starting treatment with ARBs and 8 weeks later. Plasma obtained from EH patients under monotherapy with ARBs (treated EH patients) yielded significantly lower NET release compared to the same patients before the initiation of antihypertensive therapy, as assessed by CitH3 ELISA (Figure 4A). In addition, the subsequent evaluation of TF functionality showed that plasma derived from treated EH patients is characterized by diminished TF activity, as evidenced by TAT assay (Figure 4B).

These results were further reproduced by in vitro stimulations. Control neutrophils incubated with plasma from treated EH patients demonstrated a significantly diminished NET formation compared to plasma obtained from the same patients prior to the initiation of ARBs (Figure 4C). Subsequently, the amount of bioactive TF on NETs was found to be reduced in a similar manner, as indicated by TAT ELISA performed on NET structures (Figure 4D).

Taken together, blockage of Ang II signaling is associated with reduced release of NETs and their TF-mediated thrombogenicity in patients with EH.
TF-bearing NETs activate human endothelial cells toward collagen production

The association between endothelial dysfunction and EH is well established (28), however, the underlying mechanisms resulting in atherosclerosis are still unclarified. In parallel, the cross-talk between NETs and endothelial cells seems to deregulate endothelial function based on previous data (22, 29).

In this context, we deployed a co-culture system between human aortic endothelial cells (HAoEC) and disease NETs. As indicated by qPCR and In-Cell ELISA, NETs generated in vitro by control neutrophils exposed to plasma from treatment-naïve EH patients (hereafter EH-NETs) significantly up-regulated endothelial activation markers, compared to untreated HAoEC (Figure 5A,B). In contrast, NETs generated in vitro by control neutrophils exposed to plasma obtained from EH patients under ARBs treatment (hereafter ARB EH-NETs) were not able to trigger efficient activation of HAoEC (Figure 5A,B). This is also true, when HAoEC were stimulated with EH-NETs pre-incubated with DNase I disrupting the integrity of NET structures (Figure 5A,B).

To further underline the key role of NETs in the activation of HAoEC, NET structures were treated with neutralizing antibodies against main protein components of NETs (12), including cathelicidin LL-37, neutrophil elastase (NE), myeloperoxidase (MPO) or citrullinated histone 4 (H4Cit3). These inhibitions significantly attenuated ICAM1 and VCAM1 mRNA expression in HAoEC (Supplemental Figure S3A-B). These findings suggest that NETs generated in EH environment may be potent activators of vascular endothelium.

Considering the interplay between NETs and HAoEC activation, as well as recent data suggesting that coiled-coil domain containing protein 25 (CCDC25) acts as NET-DNA sensor in cancer cells (30), we investigated whether CCDC25 expression is altered in HAoEC upon stimulation with EH-NETs. As evidence by qPCR and In-Cell ELISA,
no significant effect in CCDC25 expression was observed, compared to untreated cells (Supplemental Figure S4A-B).

Vascular and renal fibrosis are main components of EH-mediated tissue damage. CCN2 is tightly associated with fibrotic response in various tissues and is a well-known marker of fibrosis (31). Previous evidence has supported that endothelial cells express high levels of CCN2 (32) that is critically involved in the development and progression of atherosclerosis (33). Moreover, endothelial cells can be activated by TF/thrombin pathway through protease-activated receptor-1 (PAR1) receptor (34).

We observed that incubation of HAEoC with NETs generated in vitro by control neutrophils that had been exposed to plasma isolated from treatment-naïve EH patients (EH-NETs) significantly enhanced CCN2 mRNA and protein expression (Figure 5C-D), as well as collagen production (Figure 5E). This was not true for plasma obtained from ARB-treated patients which is characterized by diminished levels of NETs (Figure 5C-E). To highlight the significant role of NET structure integrity on HAOEC activity, next EH-NETs were destabilized with DNase I and a marked decrease of CCN2 expression and collagen release was detected (Figure 5C-E). To further investigate the interplay between the protein components of NETs and the fibrotic potential of HAOEC, EH-NETs were pre-incubated with a monoclonal antibody against TF or HAOEC were pre-treated with the FLLRN peptide (PAR-1 receptor blocker) to hinder thrombin signaling. These inhibitions on TF/thrombin axis resulted in a significant attenuation of endothelial fibrotic markers (Figure 5C-E), indicating a specific effect of disease TF-bound NETs in HAOEC function.

Together, our findings indicate that bioactive TF on NETs induced by EH environment may promote endothelial cells dysfunction switching them to a pro-fibrotic phenotype.
Presence of NETotic neutrophils expressing TF is observed in the fibrotic renal and aneurysmal aortic tissue of patients with EH

To gain further insights into the role of NETs in the pathogenesis of EH, we investigated their presence in kidney biopsies from patients with hypertensive nephropathy and in multiple representative tissue sections of abdominal aortic aneurysms (AAA) obtained from patients with EH. Of note, we observed NETotic neutrophils and deposition of NET remnants mainly in the renal interstitium, which is commonly characterized by fibrosis in hypertensive nephropathy (35). NETosis was detected as colocalization of neutrophil elastase with CitH3 (Figure 6A), in contrast to their absence in biopsies from normotensive patients suffering from minimal change disease (MCD, Supplemental Figure S5A), which was used as a non-inflammatory control renal disorder. Moreover, the NETotic structures in biopsies from hypertensive nephropathy patients were found to express TF (Figure 6A, Supplemental Figure S5C-D), in contrast to biopsies from MCD patients (Supplemental Figure S5B). As expected, patients with hypertensive nephropathy were characterized by renal interstitial fibrosis, as indicated by Masson’s Trichrome and Hematoxylin & Eosin staining (Figure 6B-C).

NET remnants were also detected in tissue sections from AAA, mainly in subendothelial layer in the weakened AAA wall in close proximity with the disrupted elastic lamina (Figure 6D-E). Similar to kidney biopsies, these NETotic remnants were decorated with TF (Figure 6E, Supplemental Figure S5E).

Taken together, the above findings suggest that TF-expressing neutrophils/NETs are present in tissues that are severely affected by EH exhibiting fibrotic and aneurysmal lesions.
Discussion

This study describes a pathogenic role of Ang II in EH that links neutrophils/NETs with thromboinflammatory tissue injury. Environment of EH triggers the release of NET-bound TF which exhibits both thrombogenic and profibrotic activity. Ang II emerges as an inducer of the ROS/NETosis pathway promoting collagen production by activated endothelial cells, vascular injury and interstitial renal fibrosis.

Neutrophils and NETs have been recently recognized as essential players for the initiation and propagation of thromboinflammation in CVDs such as coronary arterial disease and stroke, which represent well-defined complications of EH (16, 21, 36, 37). We identified that untreated EH patients were characterized by elevated levels of circulating NETs, which were significantly correlated with high thrombogenic plasma activity, as observed by TAT assay. Of note, prothrombotic capacity of EH plasma-induced NETs relied on the delivery of bioactive TF. This is in accordance with recent studies demonstrating EH as a prothrombotic state characterized by increased thrombin generation (5, 38). Moreover, previous research indicated neutrophil functional plasticity leading to TF-bearing NETs as a common pathogenic mechanism in several immunothrombotic conditions (14, 15, 22). Increased NET formation was detected in newly-diagnosed, without any hypertension-related complication patients, suggesting that thrombogenic NETosis might be commenced early during EH development. However, these pathologic events were subsided after the initiation of anti-hypertensive treatment with ARBs.

Deregulation of renin-angiotensin-aldosterone system is thought to be critical for the development of EH, thus classical anti-hypertensive treatments target Ang II axis to effectively control blood pressure (BP) (6). In the present study, Ang II was found to be a stimulant of NET formation, while recapitulating our in vitro findings, EH patients
significantly reduced peripheral blood NETs upon treatment with ARBs and their plasma abolished the capacity to stimulate procoagulant NET release. Given that EH-patients under ARBs treatment were normotensive, we cannot exclude that BP control per se could also contribute to these events. Moreover, ARBs such as irbesartan might affect neutrophils through additional to Ang II type 1 receptor (AT1 receptor), pleiotropic, modes of action (39). Nevertheless, it appears that Ang II induces NETosis during the EH-related inflammatory response, beyond its classical effects in regulating vascular tone and BP. In a similar way, we have previously described that another BP regulator, epinephrine, is able to stimulate NET formation linking sympathetic system and adrenergic stress with neutrophil-mediated autoinflammation (40). These results suggest an additional anti-inflammatory action for ARBs and further support the association among neuroendocrinical axis, immune system and stress-induced inflammation (40, 41).

Previous studies have indicated that oxidative stress and autophagy are core and interconnected regulators of NET formation in health and disease (12). Here, we showed that Ang II-mediated NETosis is a ROS-dependent phenomenon, further supporting the role of oxidative stress in EH (42, 43). Furthermore, Ang II-induced NET formation it appears to be associated with autophagy machinery and histone citrullination through PAD4. These results are complementary to previous studies demonstrating that Ang II can stimulate neutrophils through AT1 receptors inducing NADPH-oxidase, ROS production, and adhesion to endothelial cells (24, 25). Further studies, that will analyse the Ang II-induced intracellular molecular pathways in-depth, may provide additional pieces for the role of Ang II in neutrophils.

Atherosclerosis is characterized by uncontrolled collagen accumulation that leads to arterial stenosis (44). Activated vascular endothelial cells are among cellular
populations that contribute to collagen production in atherosclerotic lesions (45). Moreover, atherosclerosis has been associated with NETosis in experimental and human studies (18). This study indicates that thromboinflammatory NETs-delivering TF are able to activate in vitro HAoEC, thereby inducing significantly CCN2 expression and endothelial collagen production. In a recent COVID-19 study, we found that NET-bound TF activates endothelial cells increasing their thrombogenicity (22). Here, we further demonstrate a profibrotic effect of NETs/TF/thrombin axis on endothelium. Taken together and considering our previous work, we propose that TF on NETs may trigger both immediate (immunothrombosis of infection or vascular thrombosis in atherothrombosis) and long-term (vascular fibrosis in atherosclerosis) events. This could be related to the magnitude of inflammatory response (i.e. acute high-grade vs chronic low-grade) or/and the total amount of exposed TF. Experiments with DNase I indicated that integrity of NET structure itself may also be crucial for maintaining the bioactivity of TF, as has already been described for other effective NET components in various neutrophil-mediated disorders (12, 13). In a similar way, our previous research showed that NETs promote the in vitro differentiation of mesenchymal cells to collagen-producing myofibroblasts, while NETs and TF/thrombin signaling can accelerate the fibrotic response in patients with systemic autoimmunity (19, 46, 47). As regards EH, experimental studies have indicated that Ang II promotes vascular fibrosis through direct effects on vascular smooth muscle cells or through the induction of TGF-beta and CCN2 (48, 49). This study further suggests a profibrotic role of Ang II by fueling NET-mediated endothelial collagen production.

In order to confirm ex vivo our in vitro observations we examined biopsies from target tissues affected by long-term EH. Hypertensive nephropathy is among the most
important late complications of EH, and is frequently characterized by renal interstitial fibrosis directly correlated to progression of CKD (35). Renal interstitial fibrosis is characterized by imbalance between synthesis and degradation of extracellular matrix constituents leading to excess collagen accumulation (35). Of note, we detected NETotic neutrophils/remnants decorated with TF mainly in sites of interstitial fibrosis. We can assume that TF-bearing NETs facilitate progressively the differentiation of normal resident renal fibroblasts to myofibroblasts accelerating their fibrotic potential, as already has been described in other clinical disorders (19, 47).

Similarly, we identified NET remnants expressing elastase and TF in the subendothelial layer of AAA tissue specimens obtained from patients suffering longstanding EH. Experimental and clinical evidence have demonstrated that increased turnover and dysfunctional deposition of collagen, in combination with elastin fragmentation were associated with the onset and progression of AAA (50, 51). Indeed, the observed presence of NETs expressing elastase and TF in the AAA tissue wall supports a pathogenic role of NETosis that probably leads to aneurysmatic vascular remodeling through both elastin degradation (elastase) and collagen production (TF). Accordingly, these results come in agreement with recent data describing an association between inflammatory NETs and AAA formation (52).

In conclusion, this study adds important context regarding the pathogenic role of NETs in EH. Neutrophils activated by the environment of EH expose active TF through NETs. Instigation of NET/TF/thrombin axis further amplifies the prothrombotic state of EH promoting vascular damage and renal fibrosis. Mechanistically, Ang II-induced oxidative stress, autophagy and histone citrullination regulate this axis, suggesting further anti-inflammatory and anti-fibrotic actions for drugs targeting renin-angiotensin-aldosterone system. Considering that thrombosis and atherosclerosis are
largely driven by innate immunity and inflammation, protection from NETosis appears to be an attractive candidate for therapeutic interventions against EH complications.
Methods

Patients
We prospectively recruited 55 adult patients with untreated newly-diagnosed EH and 26 age- and gender- matched healthy individuals (HI) which served as controls (Supplemental Table S2). Hypertensive patients were eligible to participate provided that they fulfilled the following criteria: (i) age≥18 years, (ii) absence of secondary causes of hypertension verified through medical history, physical examination and appropriate laboratory tests in cases of high suspicion, (iii) absence of pregnancy, clinically overt cardiovascular disease, diabetes mellitus, renal disease, any infectious, inflammatory or neoplastic disease or other significant comorbidities, (iv) absence of current use of antihypertensive or any other cardiovascular medication, (v) no vaccination at least 4 weeks before. Within the group of EH patients, 12 patients were also evaluated 8 weeks after starting antihypertensive monotherapy with an angiotensin II receptor blocker (irbesartan or olmesartan at maximum doses) being well-controlled in terms of blood pressure (BP). Renal biopsies were retrospectively analysed from 3 patients suffering from hypertensive nephropathy and 2 normotensive patients suffering from minimal change disease (Supplemental Table S3). Moreover, vascular specimens from abdominal aortic aneurysms due to hypertension, which were obtained after surgical resection from 3 patients, were prospectively analyzed (Supplemental Table S3).

Assessment of office BP
Initially, office BP was measured after 5 minutes in the sitting position using a validated oscillometer device (Microlife Exact BP, Microlife AG, Widnau, Switzerland)
according to the standard recommendations of the European Society of Hypertension (ESH) for office BP measurement (1). The mean of the second and third value of three consecutive measurements with a 2-minute interval in the arm with the higher BP was considered as the patients’ office BP.

Assessment of ambulatory BP

For all participants, ambulatory BP monitoring (ABPM) was performed in the non-dominant arm, with an appropriately sized cuff using the validated Mobil-O-Graph-NG (IEM, Stolberg, Germany) device. The device was set to obtain BP values at 20-min intervals during the day (07:00-22:59 h) and at 30-min intervals during the night period (23:00-06:59 h) which were further rearranged according to the actual sleeping hours (from the time the patient went to bed until awaking) reported by each participant. Patients were instructed to maintain their usual activities, avoid strenuous exercise and keep the arm still at the time of measurements. Measurements were used for the analysis only if >70% of the recordings were valid and were analyzed to obtain average 24h, day- and nighttime systolic BP (SBP) and diastolic BP (DBP) values. Hypertension was defined according to the standard ESH guidelines as office BP ≥140/90 mmHg and ambulatory daytime BP ≥135/85 mmHg (1).

Laboratory measurements

For all participants plasma glucose, lipids [total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides] and kidney function were determined using routine laboratory techniques under 12h fasting conditions.


**Serum and plasma collection**

To isolate plasma, venous blood was collected in BD Vacutainer® EDTA tubes. Blood was centrifuged at 500x g for 15 min and then plasma samples were stored at -80°C until analyzed (16, 22).

**Neutrophil isolation**

Peripheral neutrophils were isolated from heparinized blood by double-gradient density centrifugation (11191 and 10771, Sigma-Aldrich, St Louis, MO, USA; 30 min, 700x g, at 20-25°C) according to the manufacturer’s instructions (53). The cell purity was ≥98%.

**Human aortic endothelial cell culture**

Human aortic endothelial cells (HAoEC) were purchased from PromoCell (C-12271, PromoCell, Heidelberg, Germany). Cells were cultured at 37°C and 5% CO₂ in endothelial cell growth medium MV2 (C-22020, PromoCell) and passaged after reaching confluence. Cells from passages between 3–6 were used in the study.

**Stimulation and inhibition studies in peripheral neutrophils**

Neutrophils isolated from healthy individuals (“control neutrophils”) were cultured at 37°C and 5% CO₂ in Roswell Park Memorial Institute (RPMI) medium (21875, Thermo Fisher Scientific, Carlsbad, SA, USA) supplemented with 2% heterologous healthy donor serum. To reproduce the *ex vivo* findings, control neutrophils were stimulated with plasma derived from EH treatment-naïve patients or EH patients treated with angiotensin II receptor blockers (ARBs), at a final concentration of 4% in RPMI.
To evaluate the role of Angiotensin II (Ang II) in the release of NETs, control neutrophils were incubated with the peptide hormone (0.1nM; A9525, Merck KGaA, Darmstadt, Germany), following appropriate dose experiments (Supplemental Figure S1). To attenuate Ang II signaling, control neutrophils were pre-treated (30min) with Irbesartan - an angiotensin II receptor blocker (1umol/L; I2286, Merck KGaA) (54). To inhibit late-stage autophagy (47), control neutrophils were incubated (30min) with hydroxychloroquine sulfate (HCQ; 50uM; H0915, Merck KGaA) or Bafilomycin A1 (30nM; SML1661, Merck KGaA). Moreover, neutrophils were treated with wortmannin (100 nM; W3144, Merck KGaA), an early-stage autophagy inhibitor. To hinder ROS production (55), control neutrophils were treated (30min) with diphenyleneiodonium (DPI; 10uM; D2926, Merck KGaA), an inhibitor of NADPH oxidase. To inhibit protein arginine deiminases (PADs), neutrophils were incubated with Cl-amidine (100 uM; 10599, Cayman Chemical, Michigan, USA). In inhibitions studies, neutrophils were pre-treated with inhibitory agents for 30min. In in vitro stimulations, control neutrophils were cultured for 3h to evaluate NET formation and 90min to study mRNA expression or In-Cell ELISA. Iomomycin-treated neutrophils (3uM; I3909, Merck KGaA) were used as positive control.

**Stimulation and inhibition studies in HAoEC**

To investigate the cross-talk between the TF-bearing NETs and endothelial cells, HAoEC were treated with in vitro generated NET structures (0.5 ug DNA/ml) (22). To destroy DNA scaffold, NET structures were incubated with DNase-I (1U/ml; EN0525, Thermo Fisher Scientific, Waltham, MA, USA), anti-myeloperoxidase (MPO; sc-52707, Santa Cruz Biotechnology Inc, Dallas, Texas, USA), anti-neutrophil elastase (NE; sc-25621, Santa Cruz Biotechnology Inc), anti-histone H4 citrulline 3 (H4Cit3;
07-596, Merck KGaA) or anti-LL37 (sc-166770, Santa Cruz Biotechnology Inc), according to manufacturer’s instructions. To hinder protease-activated receptor-1 (PAR-1) signaling, HAoEC were treated with the FLLRN peptide (500 uM; AS-60678, Anaspec, Fremont, USA). To inhibit TF signaling, NETs were treated with an IgG1 goat anti-human tissue factor (TF) polyclonal antibody (10 ug/ml; 4501, Sekisui Diagnostics, Burlington, Massachusetts, USA), having neutralizing effect. In inhibitions studies, HAoEC or NET structures were pre-treated with the abovementioned inhibitory agents for 30min. For mRNA studies and In Cell ELISA, HAoEC were incubated with NETs for 3h or 6h, respectively, at 37°C, 5% CO2. For collagen assay, cells were stimulated with NETs for 24h, at 37°C, 5% CO2, and cell culture supernatants were then collected. The concentrations and time points used to examine neutrophils and HAoEC were optimized before the experiments. All substances used in the study were endotoxin-free, as determined by a Limulus amebocyte assay (E8029, Sigma-Aldrich).

**Hematoxylin & Eosin, Masson’s Trichrome and Immunofluorescent staining**

Control neutrophils were seeded onto lysine-coated glass coverslips (Neuvitro; H-12-1.5-PDL) and procedures were performed as previously described (16, 22). In brief, samples were stained using a mouse anti-TF monoclonal antibody (1:200 dilution, sc-59714, monoclonal IgG1, Santa Cruz Biotechnology Inc), a rabbit anti-neutrophil elastase (NE) polyclonal antibody (1:200 dilution, sc-25621, polyclonal IgG, Santa Cruz Biotechnology Inc), a mouse anti-neutrophil elastase (NE) polyclonal antibody (1:200 dilution, sc-55548, polyclonal IgG, Santa Cruz Biotechnology Inc), or a rabbit anti-citrullinated H3 (R2+R8+R17) polyclonal antibody (1:200 dilution, ab5103, polyclonal IgG, Abcam, Cambridge, United Kingdom). A rabbit IgG polyclonal
antibody (Isotype control, ab171870, Abcam) or a mouse IgG polyclonal (Isotype control, ab37355, Abcam) were used as controls. DAPI (D9542, Sigma-Aldrich) was used for DNA staining. Visualization was performed using a fluorescence microscope (OLYMPUS BX51) with a fixed NIKON camera (model DS-Fi1). The percentage of NET-releasing cells was determined by examining 100 cells in a double-blind experimental procedure.

Cross-sections (4μm thickness) from renal biopsies tissues and mounted whole tissue sections of abdominal aortic aneurysms were stained with hematoxylin and eosin (H&E) to assess tissue morphology, using Axio Scan.Z1 (Carl Zeiss) microscope (20×/0.8 Plan-APOCHROMAT objective) and analyzed using ZEN 2.6 Blue software (Zeiss). To evaluate renal fibrosis, Masson’s trichrome staining was further performed in renal biopsies. The histological examination was performed by two-independent experienced pathologists. Moreover, all tissue sections were stained with the appropriate antibodies (referred above) by double immunofluorescence (19) and visualized using a motorized inverted confocal microscope (Zeiss, LSM710 AxioObserver, Plan-Apochromat 63x/1.40 Oil DIC M27).

**NET isolation**

In brief, 1.5×10⁶ purified neutrophils were seeded in six-well culture plates (Corning Incorporated), in RPMI culture medium, for 4h at 37°C and 5% CO2. Following incubation, the culture medium was removed and each well was washed twice with pre-warmed RPMI. To isolate *in vitro* generated NET structures, 750ul of fresh RPMI were added in each well and NETs adherent to the plate were collected after vigorous agitation. The medium was centrifuged at 20x g for 5 min and supernatant phase, containing NETs, was collected and stored at -20°C until use (16, 56).
**Thrombin-anti-thrombin (TAT) complex ELISA**

The thrombin concentration was measured in: (a) EDTA plasma or (b) *in vitro* generated NET structures. In case of NETs, the isolated structures were introduced in healthy plasma at a final concentration of 20%. Then, samples were incubated for 10 min at 37°C and immediately transferred on ice, to stop further thrombin activation. The procedure was performed according to the manufacturer’s instructions (ET1020-1, Assaypro, St Charles, MO, USA) and as previously described (16, 22). Isolated NET structures, treated for 30min with either DNaseI (1U/ml; EN0525, Thermo Fisher Scientific) or anti-TF polyclonal neutralizing antibody (10 ug/ml; 4501, Sekisui Diagnostics), were used as internal controls.

**Tissue factor activity assay**

Tissue factor activity was measured in *in vitro* generated NET structures, using Tissue Factor Human Chromogenic Activity Assay Kit (ab108906, Abcam) in accordance to the manufacturer’s instructions. DNaseI (1U/ml; EN0525, Thermo Fisher Scientific) or anti-TF polyclonal neutralizing antibody (10 ug/ml; 4501, Sekisui Diagnostics) were used as inhibitors.

**In-cell ELISA**

In-cell ELISA was performed in confluent monolayers of (a) HAoEC to measure surface ICAM1, VCAM1 or CCDC25, (b) HAoEC to measure intracellular CCN2 and (c) control neutrophils to measure intracellular TF expression. HAoEC, in 96-well microplates, were incubated with *in vitro* generated NET structures for 6 hours. Control neutrophils were stimulated with serum (4% final concentration) derived from EH
treatment-naïve patients. In all conditions, cells were fixed with 8% paraformaldehyde for 30 minutes. Blocking was performed using 2x blocking solution (ab111541, Abcam) for 2 hours. In case of CCN2 (HAoEC cell culture) and TF (neutrophil cell culture), 1x permeabilization buffer was added in cells for 30 minutes. After thorough washing with PBS-1X, cells were incubated with primary anti-human antibodies against ICAM1 (5 ug/ml; ab2213, Abcam), VCAM1 (5 ug/ml; BBA5, Santa Cruz Biotechnology Inc), CCDC25 (5 ug/ml; sc-515201, Santa Cruz Biotechnology Inc), CCN2 (10ug/ml; sc-14939, Santa Cruz Biotechnology Inc) or TF (10ug/ml; sc-59714, Santa Cruz Biotechnology Inc) at 4°C overnight. Next, horseradish peroxidase conjugated rabbit anti-mouse IgG (1:2000 dilution, HAF007, R&D Systems, Minneapolis, USA) or rabbit anti-goat IgG (1:2000 dilution, HAF109, R&D Systems) was added in cells, at room temperature, for 1 hour. After washing with PBS-1X, 100 µl of TMB substrate was added till blue color development. Microplates were measured at 650 nm. The corrections were done by subtracting the signal of the wells incubated in the absence of primary antibody.

**Myeloperoxidase (MPO)/DNA complex ELISA**

NET-specific myeloperoxidase (MPO)-DNA complexes were measured in: (a) EDTA plasma or (b) *in vitro* generated NET structures. The method was conducted in accordance to the manufacturer’s instructions (Cell Death Detection ELISA Kit, 11544675001, Merck, Kenilworth, New Jersey, USA) and as previously described (57).
**Citrullinated histone H3 ELISA**

Citrullinated histone H3 (CitH3) levels on EDTA plasma were quantified using an H3Cit ELISA kit, according to the instructions of the manufacturer (501620, Cayman Chemical, Ann Arbor, Michigan, USA).

**RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR)**

Procedures were performed both in neutrophils and HAoEC, as previously described (16, 22). GAPDH or ribosomal protein L13A (RPL13A) were used as an internal control gene. Further details regarding the primers and conditions of qPCR are given in Supplemental Table S4. The data were analyzed using the 2^{-ΔΔCt} mathematical model (58).

**Collagen measurement**

The soluble collagen types (I–V) were determined using a Sircol Collagen Assay Kit, according to the instructions of the manufacturer (S1000, Biocolor Ltd, Carrickfergus, County Antrim, United Kingdom). Collagen release was measured in culture supernatants of HAoEC, based on optimization experiments.

**Analysis of neutrophils viability**

Apoptosis/necrosis was analyzed in neutrophil population by flow cytometry (CyFlow Cube 8, PARTEC, Norderstedt, Germany), as previously described (19). In brief, neutrophils were stained with a FITC-annexin V antibody (an apoptotic marker, BD Biosciences, Wokingham, Berkshire, United Kingdom) and propidium iodide (a late
apoptotic/necrotic marker, Sigma-Aldrich), after 2h of treatment with Ang II. Data were analyzed by FCS Express 4 Flow Cytometry software.

**Statistical Analysis**

Univariate comparisons between EH patients and HI (controls) were performed with the use of Pearson’s $\chi^2$ or a two-sample independent t-test (Student’s t-test, two-tailed) in case of categorical and continuous variables respectively. To check the homogeneity of variance, we used Levene’s test. The nonparametric Wilcoxon test for paired samples was used to compare two groups. For comparisons among more groups, the nonparametric Friedman test was used. Bivariate correlation analysis was performed using Pearson correlation coefficient test (at 95% confidence intervals). The level of statistical significance was set to $p=0.05$. Means are accompanied by their 95% confidence intervals (CI), unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 6 and SPSS 26.

**Study approval**

The study protocol design was approved by the Local Scientific and Ethics Committees of the University Hospital of Alexandroupolis, and Papageorgiou Hospital of Thessaloniki, Greece. All subjects provided informed consent in accordance with the principles expressed in the Helsinki Declaration.
**Author contributions**

AC drafted the manuscript, designed and conducted experiments, and analyzed data; EG, AL, provided clinical samples, analyzed data and contributed to writing; SA contributed to tissue data analysis and writing; PP provided clinical samples and contributed to tissue data analysis; MN, AM, conducted in vitro experiments; CA, CAr, GSG provided clinical specimens and analyzed data; VP review data and performed statistical analysis; AG review tissue specimens and analysed data; KR contributed to writing and critically review the manuscript; PS contributed to writing, conceived, designed and supervised the study. All authors have read and approved the final manuscript.

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References


Figures

**Figure 1.** Markers of NETs are detected both in plasma of patients with essential hypertension (EH) and control neutrophils treated with EH plasma. (A) Myeloperoxidase (MPO)-DNA complex levels and (B) citrullinated histone 3 (CitH3) levels representing NET release in plasma from healthy individuals (controls, n=26) and EH patients (n=55). For (A) and (B), lines represent means accompanied by their ±95% CI, Student’s t-test (two-tailed). (C) Correlation between MPO-DNA levels and CitH3 levels in EH patients, Pearson correlation test. (D) Fluorescence microscopy images showing neutrophil elastase (NE)/CitH3 staining (Blue: DAPI, green: NE, red: CitH3, original magnification 1000x) and (E) percentage of NET release as assessed by immunofluorescence, in control neutrophils incubated with EH plasma and inhibited using diphenyleneiodonium (DPI; NOX-2 inhibitor) or Cl-amidine (pan PAD inhibitor). For (D), a representative example of six independent experiments is shown. (F) MPO-DNA complex levels in NETs isolated from control neutrophils treated with EH plasma and inhibited with DPI or Cl-amidine. Red and green dots indicate values yielded from control neutrophils that have been incubated with EH-plasma samples with higher or lower levels of NET markers, respectively. For (D)-(F), ionomycin stimulated neutrophils were used as positive control. For (E) and (F), data are from six independent experiments (mean ± SD Friedman test.). All conditions were compared to controls/untreated (statistically significant: p < 0.05, n.s.: not significant).
Figure 2. NETs in essential hypertension (EH) express tissue-factor (TF). (A) Thrombin-antithrombin (TAT) complex levels in plasma from healthy individuals (controls, n=26) and EH patients (n=55). Lines represent means accompanied by their ±95% CI, Student’s t-test (two-tailed). (B) Correlation between citrullinated histone 3 (CitH3) representing NET release and TAT levels in EH patients, Pearson correlation test. TF expression in control neutrophils treated with EH plasma as assessed by (C) qPCR or (D) In-Cell ELISA. For (C) GAPDH was used to normalize gene expression. For (C) and (D), Wilcoxon test for paired samples. (E) Fluorescence microscopy images showing TF/neutrophil elastase (NE) staining (Blue: DAPI, green: TF, red: NE, original magnification 1000x) in control neutrophils incubated with EH plasma. A representative example of six independent experiments is shown. (F) and (G) TAT levels and TF activity in in vitro isolated NET structures, respectively. NETs were obtained by control neutrophils incubated with EH plasma, and subsequently inhibited by DNase I or anti-TF neutralizing antibody, Friedman test. In (F), red and blue dots indicate values yielded by incubation of control neutrophils with EH-plasma samples that had higher or lower TAT levels, respectively. For (C), (D), (F) and (G), data are from six independent experiments (mean ± SD). All conditions were compared to controls/untreated (statistically significant: p< 0.05, n.s.: not significant).
Figure 3. Angiotensin II (Ang II) induces NET formation. (A)-(F) Fluorescence microscopy images showing neutrophil elastase (NE)/citrullinated histone 3 (CitH3) staining (Blue: DAPI, green: NE, red: CitH3, original magnification 1000x) in control neutrophils incubated with 0.1 nM Ang II and inhibited with Irbesartan (Ang II receptor blocker), diphenyleneiodonium (DPI; NOX-2 inhibitor), Wortmannin (early-stage autophagy inhibitor) or Cl-amidine (pan PAD inhibitor). A representative example of six independent experiments is shown. (G) Percentage of NET release assessed by immunofluorescence and (H) MPO-DNA complex levels in *in vitro* isolated NET structures. For (G) and (H), data are from six independent experiments (mean ± SD), Friedman test. Inhibitions are performed as described in (A)-(F). All conditions were compared to untreated (statistically significant: p< 0.05, n.s.: not significant).
Figure 4. Angiotensin II receptor blockers diminish NETs and thrombin-antithrombin (TAT) activity in essential hypertension (EH). Paired analysis of (A) citrullinated histone 3 (CitH3) levels and (B) thrombin-antithrombin (TAT) complex levels in plasma which was obtained from the same EH patients before and under treatment with Ang II receptor blockers (ARB) (n=12). For (A) and (B), lines represent means accompanied by their ±95% CI, Wilcoxon test for paired samples. (C) Myeloperoxidase (MPO)-DNA complex levels in NETs isolated from control neutrophils stimulated with plasma obtained from EH patients before (EH-plasma) and under treatment with ARB (ARB EH-plasma). (D) TAT levels in control plasma incubated with NET structures that were isolated from control neutrophils treated with EH-plasma or ARB EH-plasma, as described in (C). For (C) and (D), data are from six independent experiments (mean ± SD), Friedman test. All conditions were compared to untreated (statistically significant: p< 0.05, n.s.: not significant).
Figure 5. Human aortic endothelial cells (HAoEC) acquire a pro-fibrotic phenotype upon incubation with the thromboinflammatory NETs of essential hypertension (EH). (A) Relative fold expression of mRNA assessed by qPCR and (B) surface protein expression assessed by In-Cell ELISA for intercellular adhesion molecule1 (ICAM1) and vascular cell adhesion molecule1 (VCAM1). HAoEC were incubated with NETs released from neutrophils upon stimulation with plasma from EH patients before (EH-NETs) and after treatment with Angiotensin II receptor blockers (ARB EH-NETs). DNase I was used to dismantle NETs. (C) Relative fold expression of mRNA for connective tissue growth factor (CCN2), (D) CCN2 protein expression assessed by In-Cell ELISA and (E) release of collagen in HAoEC incubated with NETs, as described in A-B. To hinder tissue factor (TF)/thrombin axis, HAoEC were pretreated with FLLRN (PAR1 receptor inhibitor) or EH-NETs pre-incubated with a neutralizing antibody against TF. For (A) and (C), RPL13A was used to normalize gene expression. For (A)-(E), data are from four independent experiments (mean ± SD), Friedman test. All conditions were compared to untreated (statistically significant: p<0.05, n.s.: not significant).
Figure 6. NETotic neutrophils expressing tissue factor (TF) are identified in kidney biopsies and abdominal aortic aneurysm (AAA) specimens from patients with essential hypertension (EH). (A) NETotic neutrophils/remnants, visualized in renal specimens from a patient with hypertensive nephropathy by co-staining with neutrophil elastase (NE) and citrullinated histone 3 (CitH3) (Confocal microscopy; Blue: DAPI, green: NE, red: CitH3, original magnification 630x), express TF (Confocal microscopy; Blue: DAPI, green: TF, red: NE, original magnification 630x). White arrowheads indicate the renal tubules (either proximal or distant). Renal biopsy is characterized by interstitial fibrosis, as assessed by (B) Masson's trichrome staining and (C) hematoxylin and eosin staining (light microscopy, original magnification 1000x). For (A), (C), representative data from one of three patients is shown. (D), (E) NETs were identified in AAA specimens from patients with EH (Confocal microscopy; Blue: DAPI, green: NE, red: CitH3, original magnification 400x), bearing TF as indicated in (E) (Confocal microscopy; Blue: DAPI, green: TF, red: NE, original magnification 630x). For (D), (E) representative data from two of three patients is shown. Yellow (*) indicates the luminal site of the AAA and white (*) indicates disrupted elastic lamina.