Challenging role of neutrophil extracellular traps in vascular complications of diabetes mellitus

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Abstract
Diabetes mellitus (DM) is a global metabolic disease with prevalence rates which have reached pandemic levels. This disease is strongly associated with vascular complications, even when the hyperglycemia is kept under control. The main complications of DM are stroke, myocardial infarction, and peripheral artery disease, and they often increase the risk of developing atherosclerosis, inflammatory angiopathy, and thrombosis. Angiopathy is commonly initiated by microvascular inflammation characterized by neutrophil extracellular traps (NETs) and metabolic abnormalities, and is controlled by (epi)-genetic events and immune / antigen-presenting cells. NETs play an important role in blood coagulation, activation of the innate and adaptive immune system, as well as vascular integrity and endothelial dysfunction. The aim of the review is to summarize the current knowledge about the role of NETs in the pathogenesis of vascular complications in DM.

Introduction
Diabetes mellitus (DM) is one of the most important causes of premature death, disability and vascular complications worldwide [1]. The prevalence of DM steadily rises in developing and developed countries and has been achieved epidemic level. On the one hand, the prevalence of vascular events (stroke, myocardial infarction, peripheral artery disease) in diabetics frequently corresponds to occurrence of cardiovascular (CV) risk factors, such as hypertension, overweight / obesity, smoking, and dyslipidemia. On the other hand, developing DM strongly associates with endothelial dysfunction, microvascular inflammation and accelerating atherosclerosis, which negatively impact on short-term and long-term prognosis and relate to worsening quality-of-life [2,3]. However, DM-induced angiopathy predominantly accompany with type 1 DM (T1DM) and type 2 DM (T2DM) and does not closely relate to chronic hyperglycemia, which represented by glycated hemoglobin and glycemic variability [4]. Developing angiopathy in DM associates with early accelerating of atherosclerosis, stroke, unstable angina / myocardial infarction, blinding, advanced limb ischemia, nephropathy, and thrombotic complications [5-7]. Moreover, the risk of death due to vascular causes dramatically rises up to eight fold in DM patients with angiopathy in comparison with those who had no this complication [8]. Additionally, angiopathy due to other metabolic and inflammatory causes exhibited a positive liner correlation to a risk of death due to CV disease [6,7].

In this context, the common factor that directly triggers angiopathy is microvascular inflammation, which corresponds to metabolic abnormalities and is under tight control of (epi)-genetic regulation and immune / antigen-presenting cells [9-11]. Indeed, hyperglycemia alone, metabolic abnormalities, oxidative stress components (reactive oxygen species, advanced glycation end products), chemokines, cellular adhesive molecule, hormones (endothelin-1, aldosterone, angiotensin-II) interact with neutrophils and macrophages via several intercellular signaling pathways (i.e. PI3K/Akt / eNOS / NF-xB and ERK1/2 / p38 MAPK-activated protein kinases) and recruit predominantly neutrophil subsets in the vasculature promoting inflammation through not just synthesis and releasing of pro-inflammatory cytokines (tumor necrosis factor-alpha, interleukin-[IL]-2, IL-8, adiponectin, vistafin), but shaping of neutrophil extracellular traps (NETs) [12].

Recently NETs are considered as potent anti-microbial, anti-fungi, anti-viruses and anti-parasitic mechanism occurs with releasing of decondensated chromatin with a wide range of granular and intracellular proteins from some populations of activated neutrophils [13]. Therefore, NETosis plays an important role in blood coagulation, activation of innate and adaptive immune system, as well as vascular integrity and endothelial dysfunction [14,15]. Previous studies yielded that NETs appeared to be a link between endothelium, inflammation and thrombosis and that NETosis is crucial for development of vasculopathy due to numerous causes including metabolic diseases [16, 17]. The aim of the review is to summarize current knowledge regarding the role of NETs in pathogenesis of vascular complications in DM.

Definition of neutrophil extracellular trap
The NETs or NETosis is unique form of cell death, which is a core component of innate immune system that has been actively investigated for the last two decades [13]. NETosis is web-like structures composed of nuclear material and neutrophil granular proteins, which directly relate to releasing of modified chromatin in extracellular space due to several factors, i.e. pathogens, lipopolysaccharide, thrombaxone A2, β-defensin-1, P-selectin/ P-selectin glycoprotein ligand-1, activated platelets, metabolic triggers (peptidyl-arginine deiminase 4 and nuclear

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DNA-binding protein of the high-mobility group box 1 (HMGB1), oxidative stress components (free radicals, oxygen ions, superoxide, hydrogen peroxide, and hypochloride), and even simple contact of various cells with NETosis-releasing cells [18].

**Molecular mechanisms of neutrophil extracellular trap**

Although extracellular factors are essential for initiating of NETosis, it is suggested that this process is under direct regulation of phagocyte Nox2 and it can be related to either redox imbalance or receptor-depending pathway [19]. Consequently, there are two major types of canonical NETosis, which distinguish one another with mechanisms regulating cell death (Figure 1). Classical canonical NETosis is mediated by NADPH oxidase 2 (NOX2), which is a highly regulated membrane-associated multiple protein complex that produce reactive oxygen species (ROS) including superoxides, hypochlorite, and peroxides leading to an oxidative stress, mitochondrial dysfunction, massive nuclear vacuolization and eventually intracellular chromatin decondensation and plasma membrane disintegration. Recent studies have shown that peptidyl arginine deiminase 4 (PAD4), elastase and myeloperoxidase (MPO) play a pivotal role in classical NETosis acting as mutually interplayed triggers [20-22]. For instance, PAD4 is able to catalyze histone citrullination and thereby induces chromatin decondensation, which may also be activated by MPO and elastase. After initiation of NETosis, elastase migrates to the nucleus and destroy histones and then this process may be supported by MPO [23].

Additionally, it was confirmed with the results of studies regarding pharmacological suppression of phorbol myristate acetate (PMA)-induced NADPH oxidase activity that prevents intracellular chromatin decondensation and consequently NET formation [24]. On the other hand, autophagy is not fully controlled by pharmacological inhibition of NADPH oxidase and tumor necrosis factor-alpha (TNF-α) and the fact has emphasized the apoptosis could be a sort of back-up program for NETosis [24]. In this way, mitochondrial-induced ROS generation is probably not initial trigger of intracellular chromatin decondensation.

Alternative type of NETosis is called NOX-independent NETosis and it closely associates with opening of a voltage-dependent calcium-activated small conductance potassium (SCK) channel member SCK3 that directly control apoptosis [25]. It is confirmed the fact that neutrophils can produce NETs without previous activation of the NADPH oxidase complex with tight relation to several extracellular stimuli (growth factors, cytokines, mitogens, hormones, oxidative stress components, heat shock proteins). Perhaps, MPO and neutrophil elastase play co-regulating role in this process acting as triggers of delivery of ROS into mitochondria and thereby mediating mitochondrial dysfunction, accelerating ROS shaping, inducing cell death and NET formation [26]. Additionally, MPO may directly lead to dissociation of DNA from histones and thereby attenuate NETosis [27].

![Figure 1: Diagram illustrating the two main pathways of NETosis](image)

Notes: NOX2, NADPH oxidase 2; IL, interleukin; TNF, tumor necrosis factor; SCK3- small conductance potassium channel-3.
ischemia, bicarbonate levels) may activate NETosis, while there is not strong evidence regarding the role of hypoxia-inducing factor 1-alpha in this way. Probably, hypoxia / ischemia are able to increase the content of membrane-associated cholesterol, which could affect cell signaling loci, lipid micro domains, membrane rigidity and macrophage activation [35]. Thus, canonical ERK-1/2 signaling pathway was found as trigger of both responses, e.g. attenuation of cell survival by the post-translational modification of pro-survival genes as well as inactivation of a component of the apoptosis. On the other hand, none-canonical ERK-1/2 signaling pathway was rather apoptosis-enhanced machinery that pro-survival mechanism.

Finally, regardless of initial stimuli and pathways decondensed nuclear chromatin mixes other cytoplasmic components and granular membranes of cells, which is appeared due to massive vaculization, and turn into extracellular space and eventually shapes NETs. NETs contain proteases (proteinase-3, neutrophil elastase), oxidases (MPO, NADPH oxidase, superoxide dismutase), wide range of bactericidal peptides, several post-translational modifications of histone / DNAs and ribonucleoproteins, which play a pivotal role in the trap and killing of hosts, but an exaggerated NETosis may directly lead to tissue damage [36]. Interestingly, that prior to plasma membrane rupture and NETosis the generation of many vesicles in neutrophils was found [37]. These vesicles have a double phospholipid bilayer and are believed to originate from the nuclear envelope [38], which disintegrates during NET cell death.

NETs have been implicated in numerous infections, trauma, sepsis, eclampsia, inflammation, thrombosis, autoimmune reactions, and malignancy [39]. In fact, chromatin modifications and NETs formation is mutually co-regulating processes that are coordinated by means gene transcription and RNA polymerase II elongation [40]. Consequently, deaminated chromatin is internalized on surface of the endothelium and mediates its biological effect through externalization of modified autoantigens, producing of type I interferon, stimulating the inflammasome shaping, and activating both the classic and alternative complement pathways [41,42]. Finally, NETs influence negatively on an ability of progenitor endothelial cells to proliferation, migration, differentiation and thereby it plays a pivotal role in impairment of vascular repair accompanying endothelial dysfunction, atherosclerotic plaque accumulation, and thrombosis [43]. Thus, NETosis is typical immune process that protects against invasion of hosts, but also it is able to coordinate immune response with an activation of endothelial cells, coagulation, thrombosis, and microvascular inflammation.

**NETosis in diabetes mellitus**

Although the molecular basis responsible for angiopathy and atherosclerosis in DM has been deeply investigated, the innate mechanisms of the disease remain not fully clear [9,10]. It was well established that angiopathy in DM is result in various processes, such as exaggerated oxidative stress, impaired vascular repair, acceleration of atherosclerosis, altered endothelial function, systemic and microvascular inflammation due to fluctuated hyperglycemia and remarkable lipotoxicity [10]. Previous studies have shown that the neutrophils received from peripheral blood of diabetics exhibited spontaneous NETosis and that NETosis is enriched several components, such as elastase, histones, NADPH oxidase and protein kinase C [44]. Hyperglycemia demonstrated an ability to induce NETosis and associated with increased levels of circulating markers of NETosis including free cell DNA, elastase, mono- and oligonucleosomes [45]. In fact, though basal levels of NETs in patients with established T2DM were higher compared to healthy volunteers and related to fasting hyperglycemia, an attenuation of glycemia status with metformin was not completely associated with suppressed activity of TNF-alpha- and PMA-induced NETosis [46]. Indeed, Carestia A, et al. [46] reported that hyperglycemia was controlled in 6 months of metformin treatment, although a basal level and stimulated form of NETosis exhibited normal values in 12 months together with circulating biomarkers of NETosis, such as IL-6, TNF-alpha, cell-free DNAs. Joshi MB, et al. [47] have yielded a results regarding a formation of NETosis in neutrophils exposed to high glucose level as well as homocysteine and, IL-6. Authors believed that hyperglycemic conditions closely related to homocysteine, but homocysteine led to accelerated NETosis via both NADPH oxidase dependent and independent mechanisms in glucose interdependent manner. Thus, it has been suggested that increased level of NETosis in patients with T2DM did not occur to an impaired glycemic control but rather related to inflammation. In this context, spontaneous and inducible NETosis in T2DM may relate to different molecular pathways.

**NETosis in vascular complications**

It is well known that activated macrophages plays a pivotal role in pathogenesis of atherosclerosis supporting inflammatory reaction in plaque and leading to plaque instability [17]. Previous studies have now well established that activated macrophages are able to induce NETosis and that this mechanisms can tie atherosclerosis and intra plaque thrombosis [17,48]. Additionally, numerous non-specific stimuli, i.e. ischemia / hypoxia, oxidized lipoproteins, free fatty acids, necrotic cells, and modified histones, may be triggers for neutrophils to release NETs via represent an endogenous danger signal, which are able to activate stress-responsive transcription factor Nrf2 and regulate synthesis of inflammasome with pro-inflammatory cytokines (IL-1 alpha and IL-1 beta) [49-51]. Additionally, NETs are essential components of plaques and probably they contribute to generation of autoantibodies that lead to an aggravation of inflammation-related injury of vasculature. There is evidence that atherosclerosis may be accelerated by augmenting NETs formation within the plaque beyond autoantibodies’ shaping throughout activation of peripheral blood polymorphonuclear myeloid-derived suppressor cells, but not of inflammatory monocytes / macrophages [51,52]. Interestingly, Pertivi KR et al. [53] have found that neutrophils and NETs were more frequently present in non-organized acute thrombi. Authors concluded that NETosis could be a prominent pro-thrombotic player in all distinct types of atherosclerosis and thrombosis, which facilitate the progression of vascular complications [53] and thus the onset of ensuing clinical peripheral artery ischemic syndromes. Thus, the molecular mechanisms of cell programmed death leading to apoptosis and also NETosis link inflammation and atherosclerosis, contribute to accumulation of pro-atherogenic, thrombotic factors and cell debris in circulation and thereby mediate progression of the peripheral artery disease (PAD).

Clinical studies have shown that elevated levels of serum dsDNA as a marker of NETosis was correlated with the presence of CV disease, atherosclerosis, nephropathy, and PAD [54,55]. There is evidence that angiopathy that appeared prior to atherosclerotic plaque accumulation and associated with endothelial dysfunction was closely linked to apoptotic endothelial cell originated chromatin-contained micro vesicles, which are trigger of NETosis [56,57]. Furthermore, cell free dsDNA levels associated positively with morphological evidence of plaque destabilization, severity of PAD and the risk of limb ischemia, as well as CV mortality rate [57,58]. Additionally, cell free DNA has not just cytotoxic effect, but can be a trigger of pro-thrombotic responses.
In conclusion, in patients with DM vascular injury, endothelial dysfunction, accelerating atherosclerosis could be induced by metabolic signals that correspond to both spontaneous and inducible NETosis for neutrophils. Probably, neutrophils could be a target for personified medical care in diabetics with higher risk of NETs-related vascular complications. However, the predictive value of NETs-related organ damages in DM requires to be investigated in large clinical trials in the future.

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**References**


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