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Review

Neutrophil extracellular traps: The core player in vascular complications of diabetes mellitus

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ABSTRACT

Diabetes mellitus (DM) is the most important metabolic disease with major threat for public health and increased risk of premature death. The prevalence of DM steadily rises in developing and developed countries achieving the epidemic level. Manifestation and progression of DM corresponds to developing vasculopathies, such as retinopathy, micro- and macro angiopathies, which negatively influence on clinical outcomes and quality-of-life. Although there are remarkable differences in the prevalence of vasculopathy in various types of DM, hyperglycemia and lipotoxicity are discussed as a major factors contributing to vascular complications partly through inducing neutrophil extracellular trap (NET). The NET or NETosis is unique form of cell death, which is an important core component of innate immune system. The review is dedicated the role of NET as a link between endothelium, inflammation and thrombosis that is crucial for development of DM-induced vasculopathy. It has suggested that NET formation could be not just a target for the DM care, but also a biomarker for stratification of DM patients at higher risk of vascular complications.

1. Introduction

Diabetes mellitus (DM) is the most important metabolic disease worldwide that characterizes a global threat to public health and economic burden for healthcare systems due to increased risk of premature death, disability and serious complications [1]. The prevalence of DM steadily rises in developing and developed countries achieving the epidemic level. In fact, the vascular complications of DH frequently corresponds to manifestation of vasculopathies that are combined into retinopathy, micro- and macro angiopathies associated with accelerating atherosclerosis, which negatively influence on both short-term and long-term clinical outcomes as well as relate to worse quality-of-life [2]. The risk of vasculopathies in DM patients was increased two to four fold in compared to none-DM population [3]. However, DM-induced vasculopathies predominantly accompany with type 1 DM (T1DM) and type 2 DM (T2DM) and do not closely relate to chronic hyperglycemia represented by glycated hemoglobin and glycemic variability [4]. Developing vasculopathy in DM associates with early 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 vasculopathy in comparison with those who had no these complications [8].

Although the molecular basis responsible for vasculopathy in DM has been deeply investigated, the innate mechanisms of the disease remain not fully clear [9,10]. It was well established that vasculopathy in DM is result in various processes, such as exaggerated oxidative stress, impaired vascular repair, endothelial dysfunction, systemic and microvascular inflammation, acceleration of atherosclerosis due to fluctuated hyperglycemia and remarkable lipotoxicity [10]. In this context, the common factor that directly triggers vasculopathy in different types of DM is microvascular inflammation, which corresponds to metabolic abnormalities and is under tight control of (epi)-genetic regulation and immune/antigen-presenting cells [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-κB 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

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pro-inflammatory cytokines (tumor necrosis factor-alpha, interleukin-[IL]-2, IL-8, adiponectin, vistafin), but shaping of neutrophil extracellular trap (NET) [12]. Recently NET is 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 NET appeared to be a link between endothelium, inflammation and thrombosis that is crucial for development of DM-induced vasculopathy [16,17]. The aim of the review is to summarize knowledge regarding the role of NET in pathogenesis of DM-induced vasculopathy and discuss the possibilities to implement of NET biomarkers in routine clinical practice to predict of vascular complications.

2. Definition of neutrophil extracellular trap

The NET or NETosis is unique form of cell death, which is an important core component of innate immune system that has been actively investigated for the last two decades [13]. NET is web-like structures composed of nuclear material and neutrophil granular proteins, which directly relates to releasing of modified chromatin in extracellular space due to several factors, i.e. pathogens, lipopolysaccharide, thromboxane A_2, β-defensin-1, P-selectin/P-selectin glycoprotein ligand-1, activated platelets, metabolic triggers (peptidyl-arginine deiminase 4 and nuclear 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 NET-releasing cells [18].

3. 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 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 mechanisms regulating cell death (Fig. 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 to destroy histones and then this process may be supported by MPO [23].

Additionally, it was confirmed 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-alpha) and that fact has been emphasized the apoptotsis could be a sort of backup program for NETosis [24]. In this way, mitochondrial-induced ROS generation is probably not initial

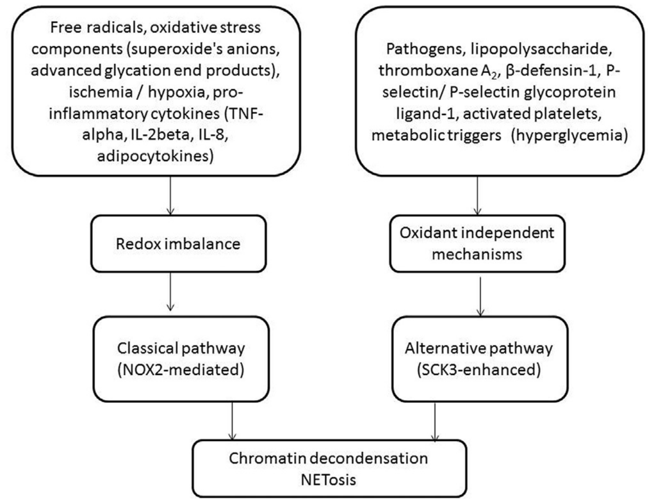

Fig. 1. Principal schema of two main pathways of NETosis. Notes: NOX2, NADPH oxidase 2; IL, interleukin; TNF, tumor necrosis factor; SCK3- small conductance potassium channel-3.

trigger of intracellular chromatin decondensation.

Alternate 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) (Fig. 2). Perhaps, MPO and neutrophil elastase play co-regulating role in this process acting as trigger 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 the dissociation of DNA from

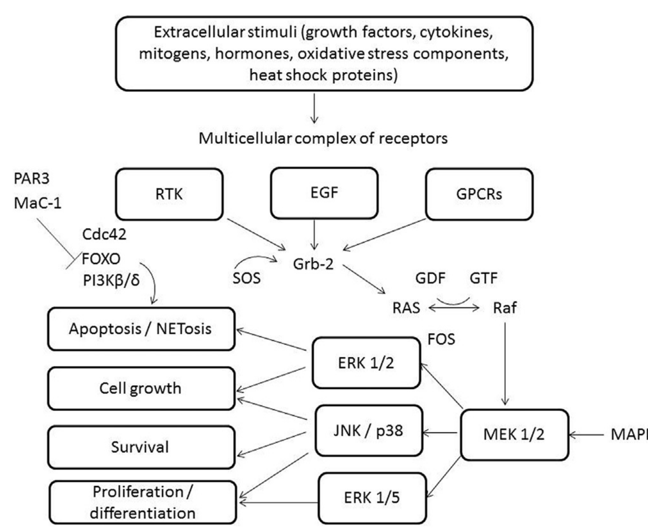

Fig. 2. Molecular mechanisms of receptor-enhanced NETosis. Notes: RTK, receptor tyrosine kinases; EGF, epidermal growth factor receptor; GPCRs, G protein-coupled receptors; SOS, guanine nucleotide exchange factor (son of sevenless); Grb-2, growth factor receptor bound protein; JNK, c-Jun-amino-terminal kinase; Raf, serine/threonine kinase; ERK, extracellular-signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; FOXO, forkhead box protein O transcription factors; PAR3, protease-activated receptor 3; Mac-1, CD11 b/CD18 integrins; FcγR, Fcγ-receptor; Cdc42 - Cell division cycle 42.

histones and thereby attenuate NETosis [27]. Although the activation of Akt and p38 mitogen-activated protein kinases (MAPK)-mediated signaling pathway is essential in both pathways, non-classical NETosis in contrast to classical cellular redox imbalance-induced NETosis could be activated through oxidant-independent mechanisms [28] and realize through Raf-MEK-extracellular signal regulated kinase 1/2 [29]. Finally, chromatin decondensation, which is core element of NETosis, dependent upon the activity of NADPH oxidase in classic pathway, otherwise neutrophil elastase and MPO is crucial in alternate pathway of NETosis [30,31]. However, NOX-related and MPO-derived ROS production, but not mitochondrial ROS shaping are extremely important for and down-regulation of anti-apoptotic proteins and the release of NETosis [32]. Nevertheless, unlike PMA calcium- and protein arginine deiminase 4-dependent NETosis is initiated without previous neutrophil activation and degranulation. Additionally, there is sufficient difference between both types of NETosis in involvement of several molecular mechanisms activating neutrophil cell surface receptors. In fact, canonic Ras/Raf-1/MAPK/ERK kinase (Mek)/extracellular signal-regulated kinase (Erk) pathway is essential in spontaneous NETosis, while PI3K-dependent, non-canonical, pro-apoptotic FcγR-PI3Kβ/δ-Cdc42-Pak-Mek-Erk signaling pathway may play a pivotal role in immune-complex-driven human neutrophil activation [33,34]. All these facts suggested that NETosis is potentially regulated receptor-mediating process. Indeed, prostaglandin E2, protein C can inhibit NETosis via its cognate receptor or through cooperation with protease-activated receptor 3 (PAR3) and CD11 b/CD18 (Mac-1) integrins. In contrast, hypoxia, 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 microdomains, 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 vacuolization, and turn into extracellular space and eventually shapes NET. NET contains 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. Thus, NETosis is typical immune process that protects against invasion of hosts, but also it able to coordinate immune response with an activation of endothelial cells, coagulation and thrombosis, and microvascular inflammation.

4. Biological role of NETosis

NET has been implicated in numerous infections, trauma, sepsis, eclampsia, inflammation, thrombosis, autoimmune reactions, and malignancy [39]. In fact, chromatin modifications and NET formation is mutually co-regulating processes that are coordinated by means gene transcription and RNA polymerase II elongation [40]. Consequently, deiminated 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, NET 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].

5. NETosis in DM

Previous studies have shown that the neutrophils received from peripheral blood of diabetics exhibited spontaneous NETosis and that NETs are enriched several components, such as elastase, histones, NADPH oxidase and protein kinase C [44]. Hyperglycemia demonstrated an ability to induce NETs 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. (2016) [46] reported that hyperglycemia was controlled in 6 months of metformin treatment, although 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. (2016) [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 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 DM may relate to different molecular pathways.

6. NETosis in DM-induced atherosclerosis

Interestingly, activated macrophages the role of which in pathogenesis of atherosclerosis and peripheral artery disease (PAD) has now well established could maintain inducible NETosis of neutrophils is T2DM and thereby influence on accelerating atherosclerosis [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 in ones that lead to aggravation of atherosclerotic lesion 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, Pertiwi KR et al. (2018) [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 PAD. Fig. 3 is reported the role of NETosis in vascular impairment in diabetic population.

Clinical studies have shown that elevated levels of serum dsDNA as a marker of NETosis was correlated with the presence of cardiovascular (CV) disease, atherosclerosis, nephropathy, PAD in patients with T2DM [54,55]. There is evidence that T2DM-induced vasculopathy 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 [59]. Probably, spontaneous/inducible NETosis in T2DM could be a biomarker of higher risk of microvascular inflammation, endothelial dysfunction, thrombotic complication and atherosclerosis in T2DM [60]. Whether NETosis is target for T2DM medical care with aim to prevent vascular complications is not clear and requires investigation in large clinical trials.

7. NETosis and thrombotic complications

Previous studies have shown that during NETosis activated neutrophils can promote hypercoagulation and be an active part of the thrombus formation [61,62]. Indeed, activated complement proteins activate NET formation and interact as inflammation and coagulation [63]. Developing DM closely associates with low-grade inflammation, which is maintained with oxidative stress components and activated macrophages. Interestingly, the promoting effect of activated macrophages and bone marrow-derived cells on vascular complications in DH is not obvious and it seemed to be reported controversially in several studies. For instance, PAD4 produced by bone marrow-derived neutrophils and previously reported as a main trigger of NETs did not promote plaque in chronic experimental model of atherosclerosis, whereas it remained a leading cause in acute thrombus shaping in intimal lesions [63]. Authors concluded that the thrombotic complications in the plaque could be related to NETsis, but atherosclerosis did probably not [63]. On the other hand, cell-death-associated nuclear factors, such as DNAs, histones and nucleosomes, are actively released into the extracellular space by neutrophils through NETosis and directly contribute to factor VII activating protease acting as pro-coagulants [64]. On the other hand, cell-death-associated nuclear factors predominantly histones and cell-free DNAs were involved indirectly in coagulation cascade after releasing of factor XII from injured vascular wall [65]. Finally, factor VII activating protease together activated protein C are able to suppress histone cytotoxicity and thereby neutralizes or diminishes damaging effect of NETosis on vasculature and prevent coagulation [66,67]. Nevertheless, these effects were supported by hemodynamic forces, such as shear stress, they were not abrogated by inhibitors of PAD4, cyclooxygenase, platelet-neutrophil adhesion, high-mobility group protein box 1-receptor for advanced glycation end products interaction, and ATP/ADP [68,69]. Thus, in DM patients NETosis plays a pivotal role in pro-thrombotic state, while relationship between an ability of neutrophils to shape NET-related pro-coagulation state depending on hyperglycemia control requires to be investigated in detail in the future.

8. NETosis and diabetic retinopathy

Retinopathy is early complication of T1DM and accompanies to advance T2DM frequently out of close relation to glycated hemoglobin level and daily variability of glucose concentration. Previous studies have reported that developing and progression of DH-induced proliferative retinopathy is neutrophils were able to exert retinal vein occlusion through direct damage of vasculature and via NET-depending mechanism mediating thrombosis of central vein [70,71]. Additionally, activation of neutrophils was associated with capillary closure in experimental model of DM [72].

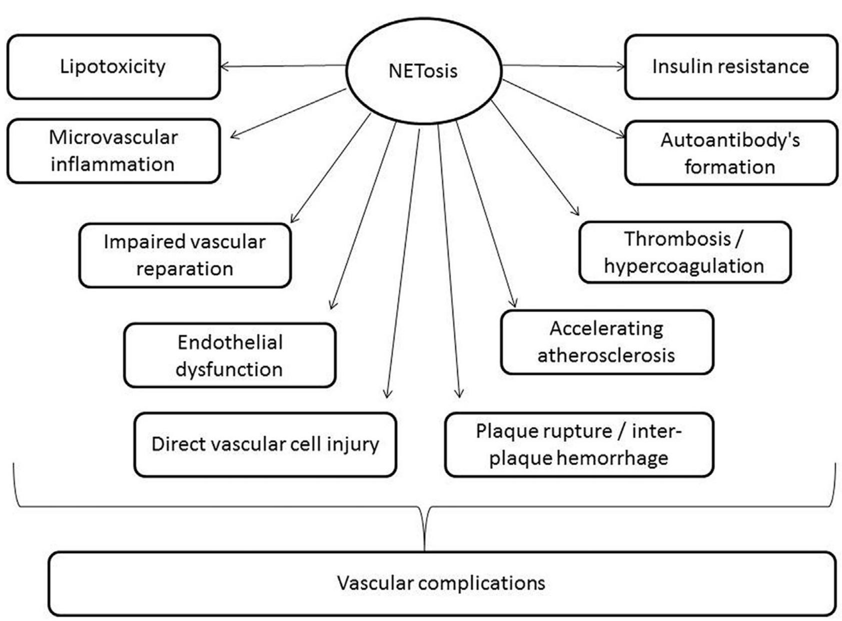

Fig. 3. Importance of NETosis in developing of vascular complications in DM patients.

However, in DM patients vitroretinal pathologies and NETosis tightly accompany one another. Indeed, Barliya T et al. (2017) [73] reported that presence of NETs complexes in DM patients with proliferative diabetic retinopathy positively correlated with the severity of the disease. Moreover, ability of neutrophils to realize NETs corresponded to increased level of pro-angiogenic vascular endothelial growth factor, chemokine (C-C motif) ligand 2 (CCL2), which recently were found as a core players in pathogenesis of DM-induced proliferative retinopathy [74,75]. Additionally, it is not known whether NETosis is cooperated with other growth factors (insulin-like growth factor I, pigment epithelium derived factor, hepatocyte growth factor, basic fibroblast growth factor, platelet derived growth factor), active molecules (monocyte chemo-attractant protein-1), pro-inflammatory cytokines (IL-1beta) and hormones (endothelin-1), which are also involved in the pathogenesis of DM-induced proliferative retinopathy as a modulators of retinal neo-angiogenesis. However, several factors contributing retinal proliferation (transforming growth factor beta, secretogranin III, thrombospondin, apelin and somatostatin) could be related to NET formation, because they are locally synthesized and their releases is under control of activated mononuclears/macrophages [76,77]. It has suggested that recurrent retinal ischemia and macular edema in retinal vein occlusion could be a result of NETosis initiation due to DM-induced pro-inflammatory cytokines overproduction [78]. Consequently, dysregulated angiogenesis and thrombus formation in micro- and macro vessels may be considered as both faces of one pathological state contributing to retinal disorders in DM. In this context, better understanding of innate molecular mechanisms of these processes including NETosis allows identifying the biological targets for personified therapy of the proliferative retinopathy in DM [15].

9. NETosis and diabetic nephropathy

Evidence regarding the interrelationship of NETosis and DM nephropathy is sufficiently limited. Although roles of various inflammatory molecules in relation to NETs in acute and chronic kidney disease associated with autoimmune disorders, vasculitis, connective tissue diseases, have quite well established [79,80], the pathogenesis of NETs-related cascades in diabetic nephropathy remains to be complicated and elusive [81]. However, it has been postulated that the imbalance between NET formation and degradation might increase direct microvascular injury, initiate a proliferative response of epithelial cells and mesangial cells in kidney, modify antigens in circulation, activate complement, pentraxins, and natural antibodies and mediate NET-induced kidney damage [82]. NET also activates human glomerular endothelial cells through stimulating secretion of the pro-inflammatory cytokines IL-6 and IL-8 [83]. Whether does NET-induced nephropathy in DM closely correspond to glycated hemoglobin level or fasting glucose level is not fully clear and requires to be deeply investigated. However, they can predispose that NETs-associated nuclear factors (histones, micro-vesicles, elastase, mono- and oligonucleosomes and cell-free DNAs) could be biological markers of higher risk of kidney damage in DM probably with independent predictive value for declining kidney function beyond glycaemia control.

10. In conclusion

In patients with DM developing of vasculopathy could be induced by metabolic signals that corresponding to both spontaneous and inducible NETosis for neutrophils. The biological markers of NETosis including histones, micro-vesicles, elastase, mono- and oligonucleosomes and cell-free DNAs have exerted strong positive associations with atherosclerosis, thrombotic complications, CV risk and mortality rate. 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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Conflicts of interest

Not declared.

List of abbreviations

Akt	RAC-alpha serine/threonine-protein kinase
Cdc42	Cell division cycle 42
CV	cardiovascular
DM	diabetes mellitus
DNA	deoxynucleic acid
EGF	epidermal growth factor receptor
eNOs	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FcγR	Fcγ-receptor
GPCRs	G protein-coupled receptors
Grb-2	growth factor receptor bound protein
HMGB1	high-mobility group box 1
IL	interleukin
JNK	c-Jun-amino-terminal kinase
Mac-1	integrins CD11 b/CD18
MAPK	mitogen-activated protein kinases
MEK	mitogen-activated protein kinase kinase
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-beta
NOX2	NADPH oxidase 2
PAD	peripheral artery disease
PAD4	peptidyl arginine deiminase 4
PAR3	protease-activated receptor 3
PI3K	phosphatidylinositol 3-kinase
PMA	phorbol myristate acetate
Raf	serine/threonine kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RTK	receptor tyrosine kinases
SCK channel	small conductance potassium channel
SOS	guanine nucleotide exchange factor (son of sevenless)
T2DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TNF	tumor necrosis factor

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