REVIEW

NETosis contributes to the pathogenesis of diabetes and its complications

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Abstract

NETosis, a novel form of neutrophil-related cell death, acts as a major regulator of diabetes and diabetes-associated complications. In this review, we show that the extrusion of neutrophil extracellular traps, termed NETs, plays an important role in the pathogenesis of type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and diabetes-induced complications. In T1DM, β -cell death induces the sequestration of neutrophils in the pancreas and seems to be correlated with increased NETosis. In T2DM patients, products of NETs release are significantly elevated. Increased levels of dsDNA are correlated with the presence of cardiovascular disease and diabetic kidney disease, further supporting the role of NETosis in the pathogenesis of other diabetes-induced complications such as impaired wound healing and diabetic retinopathy. NETosis is induced by high glucose through incompletely understood mechanisms, but it also appears to be elevated in patients with diabetes who have tightly controlled glucose levels. We hypothesize that hyperglycemia worsens the already elevated baseline of NETosis in diabetic patients to further increase its detrimental effects.

Key Words

- ▶ NETosis
- ▶ neutrophil extracellular traps
- ▶ diabetes mellitus
- ▶ diabetes-induced complications
- ▶ NADPH oxidases

*Journal of Molecular
Endocrinology*
(2020) **65**, R65–R76

Introduction

Polymorphonuclear neutrophils (PMNs), the most abundant leukocytes in the blood, are considered part of the first-line immune defense and play a central role during the innate immune response to protect the body against invading pathogens, such as bacteria, fungi, protozoa, and viruses (Rosales *et al.* 2017). Upon infection or inflammation, PMNs are the first leukocytes to migrate from the blood to injured or infected sites where they phagocytose invading microorganisms in order to eliminate them via different cytotoxic mechanisms (Rosales *et al.* 2017). Neutrophils target these invading

microorganisms through three different mechanisms: (1) phagocytosis and degradation through the synthesis of reactive oxygen species (ROS) in the phagolysosomes, (2) degranulation or the release of antimicrobial peptides, and, (3) as recently described, microbial trapping through the release of extracellular DNA traps termed neutrophil extracellular traps (NETs) (Papayannopoulos 2018).

NETosis was first described in 1996 by Takei *et al.* as a cell death pathway that differs from necrosis and apoptosis (Takei *et al.* 1996). Phorbol 12-myristate 13-acetate (PMA), a potent activator of neutrophils, is used to elucidate

the relationship between activation of neutrophils and their subsequent death. Clear morphological changes are observed in PMA-treated neutrophils, starting with a fusion of the multilobulated nucleus, followed by reduced chromatin condensation, and soon after, by a disruption of the nuclear envelope with cytoplasmic organelles remaining intact. This mechanism is described to be dependent on reactive oxygen species production (Takei *et al.* 1996). In 2004, Brinkmann *et al.* further interpreted this unusual cell death process and termed it NETosis (Brinkmann *et al.* 2004).

NETosis is an essential process of the innate immune response. It is triggered by both infectious and 'sterile' stimuli such as cytokines, cholesterol crystals, monosodium uric acid crystals, immune complexes, and autoantibodies (Keshari *et al.* 2012, Schorn *et al.* 2012, Behnen *et al.* 2014, Yalavarthi *et al.* 2015). Moreover, NETosis is shown to play a role in the adaptive immune response where neutrophils engage with T cells, B cells, and antigen-presenting cells (Mantovani *et al.* 2011). Also, NETosis is shown to stimulate the immune system during an inflammatory response (Garcia-Romo *et al.* 2011, Semeraro *et al.* 2011).

This process of NETosis leads to the release of neutrophil extracellular traps (NETs), which are large, web-like structures composed of decondensed chromatin associated with granules of degradative enzymes (Brinkmann *et al.* 2004). NETs prevent the migration of extracellular pathogens, and neutralize and kill bacteria, fungi, viruses and parasites (Papayannopoulos 2018). NETs are ultimately cleared either through the engulfment by macrophages through an active endocytic process or by DNase I, an endonuclease that cleaves chromatin within NETs (Hakim *et al.* 2010, Farrera & Fadeel 2013). Despite a beneficial effect of NETs production in pathogens clearance, there is increasing evidence that excessive NETs formation may contribute to the pathogenesis of autoimmune diseases including rheumatoid arthritis (Corsiero *et al.* 2016), systemic lupus erythematosus (Cooper *et al.* 1999), vasculitis, and, recently, diabetes (Wong *et al.* 2015, Corsiero *et al.* 2016, Qin *et al.* 2016). Herein, we describe and discuss the underlying mechanisms of NETosis and its role in the pathogenesis of diabetes and diabetes-induced microvascular complications.

NETosis a neutrophil-specific cell death process

NETs are produced through a cell death process termed NETosis. To date, three models of NETosis

have been described: suicidal NETosis, vital NETosis with the release of nuclear DNA, and vital NETosis with release of mitochondrial DNA (Delgado-Rizo *et al.* 2017).

Conventional suicidal NETosis

Suicidal NETosis, which lasts 2–4 h (Pilszczek *et al.* 2010), remains the most elucidated model. In this model, neutrophils are first activated by ligand binding to neutrophil toll-like receptors and receptors for IgG–Fc, complement, or cytokines (Yang *et al.* 2016). Upon activation, the endoplasmic reticulum releases its stored calcium ions into the cytoplasm (Yang *et al.* 2016). This increase in cytosolic calcium cations then activates the protein kinase C (PKC)/Raf/MEK/ERK pathway, leading to gp91phox phosphorylation for the activation of NADPH oxidase (NOX) complex and the subsequent generation of reactive oxygen species. Cytosolic Ca²⁺ cations also act as cofactors for peptidyl arginase deaminase 4 (PAD4), an enzyme that catalyzes the hydrolysis of peptidyl-arginine to form peptidyl-citrulline on histones. This process, termed histone citrullination, promotes the loss of positive charges required for the interaction of histones with DNA, and, hence, leads to the decondensation of chromatin (Li *et al.* 2010, Lewis *et al.* 2015). In suicidal NETosis, reactive oxygen species behave as second messengers promoting neutrophil elastase (NE) and myeloperoxidase (MPO) translocation from azurophilic granules to nucleus. NE proteolytically processes histones to disrupt chromatin packaging, while MPO binds chromatin and synergistically with NE, promotes chromatin decondensation (Papayannopoulos *et al.* 2010). This enhances the gradual separation and loss of the nuclear membrane, which disintegrates into smaller individual vesicles. Decondensed chromatin then associates with cytoplasmic proteins and granule toxins. Finally, the DNA is released as extracellular traps (ETs) secondary to cellular lysis, ultimately entrapping exogenous bacteria (Brinkmann & Zychlinsky 2007, Neeli *et al.* 2008, 2009, Li *et al.* 2010, Lewis *et al.* 2015). Conventional suicidal NETosis differs from programmed cell death or apoptosis. In the event of apoptosis, DNA condenses first then fragments (Hamam & Palaniyar 2019b). It is suggested that post-translational modifications (PTMs) of histones including acetylation appear to play a central role in regulating neutrophil death (Hamam & Palaniyar 2019b). Histone acetylation was recently found to promote NETosis at baseline when induced by NOX-dependent or NOX-independent pathway agonists in human neutrophils (Hamam *et al.* 2019). In general, histone acetylation results in chromatin

decondensation. However, increased levels of histone acetylation was shown to switch neutrophil death from NETosis to apoptosis by activating the cleavage of caspase-3 (Hamam & Palaniyar 2019a). Interestingly, in the presence of NETosis-promoting stimuli, high levels of histone deacetylase inhibitors (HDACis) inhibit both NETosis and apoptosis. Taken together, both histone citrullination and acetylation of histones have been found to alter NETosis and apoptosis with the net outcome depending upon the degree of PTMs of histones and the presence or absence of NETosis-inducing stimuli (Hamam & Palaniyar 2019b).

Vital NETosis

In *vital* NETosis, also termed nonlytic NETosis, neutrophils release NETs without losing their plasma membrane. It happens within 5–60 min in response to *Staphylococcus aureus* (Pilszczek *et al.* 2010) and *Candida albicans* (Byrd *et al.* 2013) and does not require the generation of reactive oxygen species (Delgado-Rizo *et al.* 2017). The sequential steps of *vital* NETosis occur as follows: (1) recognition of the stimuli through toll-like receptors (TLRs) and the complement receptor for C3 protein (Clark *et al.* 2007, Byrd *et al.* 2013); (2) loss of the lobular and multinucleated shape of the nucleus; (3) nuclear envelope disruption and budding of vesicles; (4) vesicles containing DNA filaments approach the intact plasma membrane; (5) release of DNA as ETs through a small area in cell surface and release of some cytoplasmic granules into the extracellular space to associate with DNA (Delgado-Rizo *et al.* 2017). In *vital* NETosis, the release of nuclear content does not affect the lifespan of the neutrophils. In fact, these neutrophils retain their ability to phagocytose pathogens (Yipp & Kubes 2013).

Additionally, a second type of *vital* NETosis was described. It involves the release of mitochondrial DNA instead of nuclear DNA and is dependent on ROS production. The use of diphenyleneiodonium (DPI), an inhibitor of NADPH oxidases, resulted in a complete block of mitochondrial DNA release from neutrophils, suggesting that this type of NETosis is also dependent on NADPH oxidases. This process results in NETs formation within 15 min through the recognition of C5a or lipopolysaccharide (LPS) (Yousefi *et al.* 2009).

Diabetes mellitus and the widespread consensus on tight glycemic control

While NETosis was first described not much more than two decades ago, research on diabetes mellitus (DM)

was initiated about a century ago. DM is defined as a group of metabolic diseases clinically characterized by hyperglycemia that results from defects in insulin action, insulin secretion, or both. Despite the different etiologies of type 1 (insulin deficiency) and type 2 diabetes (insulin resistance), both are associated with ‘macrovascular complications’, including cardiovascular disorders, heart failure, atherosclerosis, and cerebrovascular events, as well as with ‘microvascular’ complications, such as diabetic neuropathy, diabetic retinopathy, and diabetic kidney disease (Forbes & Cooper 2013). Herein, we will be focusing on the diabetes-induced microvascular complications.

Achieving optimal glycemic control as early as possible in the course of the disease has always been the ultimate goal of diabetes management. In type 1 diabetic subjects, rapid-acting and long-acting insulin analogues improve glycemic control. In type 2 diabetes, there are a range of pharmacological agents used to attain normoglycemia including insulin-sensitizing agents such as metformin and thiazolidinediones, drugs that stimulate the release of insulin from the pancreas, namely sulfonylureas, glucagon-like peptide-1 (GLP-1) agonists, and dipeptidyl peptidase-IV (DPP-IV) inhibitors, as well as sodium-glucose transport protein 2 (SGLT2) inhibitors, which prevent reabsorption of glucose in the kidneys (Forbes & Cooper 2013).

Randomized controlled trials (RCTs) emphasize the significance of tight glycemic control in preventing the onset or progression of diabetes-associated complications. The Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) trial in type 1 diabetes have shown that tight glycemic control ameliorated but did not halt the progression of the microvascular diabetic complications (Skyler 2004, Nathan & DCCT/EDIC Research Group 2014). In type 2 diabetes, the RCTs were equivocal. While the UK Prospective Diabetes Study (UKPDS) showed a significant risk reduction in diabetic kidney disease and diabetic retinopathy after 10 years of intensive glycemic control (King *et al.* 1999), other RCTs questioned the implication of tight glycemic control in all diabetic patients. For example, The Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease (ADVANCE) and Veteran Affairs Diabetes Trial (VADT) all showed that tight glycemic control does not reduce macrovascular disease in older patients with diabetes who are at risk for or have cardiovascular disease, and improvement in microvascular complications were relatively modest and

limited to reduced proteinuria (Terry *et al.* 2012). In fact, intensive glycemic control was associated with increased mortality in the ACCORD study, and with more frequent and severe episodes of hypoglycemia in the ACCORD and VADT trials (Terry *et al.* 2012). Collectively, these RCTs have shown that it is important to reevaluate the well-known consensus on tight glycemic control to prevent diabetes-associated microvascular and macrovascular complications. More importantly, these trials highlight that glucose alone is not accountable for the pathogenic manifestations seen in diabetes mellitus, paving the way for identifying adjunct therapy to halt the development or progression of diabetes-induced complications, or even reverse their outcomes.

The role of NETosis in the pathogenesis of type 1 diabetes mellitus

Neutrophils and NETs were recently shown to be involved in the pathogenesis of type 1 diabetes mellitus (Table 1). The inhibition of neutrophil function and NETs formation attenuates the development of type 1 diabetes (Diana *et al.* 2013). In experimental models of type 1 diabetes, physiological β -cell death induced the recruitment and activation of B-1a cells, neutrophils

and plasmacytoid dendritic cells (pDCs) to the pancreas. Subsequently, dsDNA-specific IgGs were secreted from activated B-1a cells, stimulating neutrophils to release DNA-binding cathelicidin-related antimicrobial peptide (CRAMP), which binds self-DNA. Self-DNA, dsDNA-specific IgGs and CRAMP peptide then activated pDCs leading to interferon- α production. Also, histological analysis confirmed the presence of NETs associated with CRAMP release (Diana *et al.* 2013). In that same spirit, a study conducted on 107 adult patients with newly diagnosed type 1 diabetes and 21 children with persistent islet autoantibodies and a family history of type 1 diabetes revealed a significantly lower neutrophil count compared to age and gender-matched control subjects. Notably, all subjects had no laboratory signs of acute infection (Harsunen *et al.* 2013). Further studies aiming to understand the mechanism behind the perturbation of neutrophil homeostasis in type 1 diabetes have shown that the circulating neutropenia was not caused by impaired neutrophil output from the bone marrow or by impaired differentiation, but rather by an increased sequestration of the neutrophils in the pancreatic tissue (Valle *et al.* 2013). In an effort to correlate NETosis with β -cells death in type 1 diabetes, it was reported that the circulating protein levels and enzymatic activities of proteinase 3 (PR3) and

Table 1 Neutrophil extracellular traps (NETs) in patients with type 1 diabetes mellitus.

Study reference	Subjects	Findings pertinent to NETosis
Harsunen <i>et al.</i> 2013	<ul style="list-style-type: none">107 adult patients with newly diagnosed T1DM21 children with persistent islet autoantibodies and a family history of T1DM	<ul style="list-style-type: none">Decreased neutrophil count in adult patients with newly diagnosed T1DM and in children with persistent islet autoantibodies
Valle <i>et al.</i> 2013	<ul style="list-style-type: none">1238 age and gender matched control subjectsPediatric patients with T1DM at onsetHealthy pediatric controlsAdult patients with T1DM at onsetAdult patients with long-standing T1DM and T2DMHealthy adult controlsRelatives of patients with T1DM	<ul style="list-style-type: none">Peripheral neutropenia precedes and accompanies the onset of T1DMNeutrophils were sequestered in the exocrine pancreas of multiorgan donors with T1DM (at onset and at later stages of the disease)
Wang <i>et al.</i> 2014	<ul style="list-style-type: none">A total of 149 children with T1DM and 77 age- and sex-matched healthy control subjects	<ul style="list-style-type: none">Circulating protein levels and enzymatic activities of PR3 and NE were significantly elevated in patients with T1DMCirculating NE and PR3 levels were positively correlated with autoantibodies against β-cell antigensA marked increase in circulating MPO-DNA complexes was observed in patients with T1DM
Wong <i>et al.</i> 2015	<ul style="list-style-type: none">Neutrophils isolated from patients with T1DM	<ul style="list-style-type: none">Expression of PAD4 was elevated in neutrophils from individuals with diabetes
Qin <i>et al.</i> 2016	<ul style="list-style-type: none">Forty-four subjects with T1DM with a median age of 26.5 years and median disease duration of 2.2 years and 38 age-matched controls	<ul style="list-style-type: none">NE and PR3 were significantly reduced in patients with T1DM, particularly in subjects within 3 years of diagnosis.A decrease in neutrophil counts in recent-onset T1DM was observedNE and PR3 levels were correlated with absolute neutrophil counts

neutrophil elastase (NE) were significantly elevated in patients with type 1 diabetes, especially in patients with disease duration of less than 1 year, compared to age and gender-matched control subjects. The increase in NE and PR3 was positively correlated with the increase of the positive numbers and titers of the autoantibodies against β -cell antigens. However, it was noted that an elevation was also observed in autoantibody-negative patients. In line with the increased NE and PR3 levels, a marked increase of circulating MPO-DNA complexes was seen in patients with type 1 diabetes as compared to healthy subjects. Taken together, these observations suggest that the increase in circulating NE and PR3 protein levels may be associated with enhanced neutrophil extracellular traps formation (Wang *et al.* 2014). To further investigate the link between NE, PR3, and the onset of type 1 diabetes, changes in the expression of NE and PR3 were determined in non-obese diabetic (NOD) mice from 2 to 30 weeks of age. It was shown that the enzymatic activities of circulating NE and PR3 were significantly elevated and sustained before the onset of diabetes. Intriguingly, the NE/PR3 activities in diabetic mice gradually decreased to baseline levels, most likely due to the termination of autoimmune response after complete β -cell destruction (Wang *et al.* 2014).

These findings support the role of NETosis in the pathogenesis of β -cell autoimmunity and highlight NE and PR3 as sensitive biomarkers for the diagnosis of type 1 diabetes. Nevertheless, in a recent study, it was stated that both NE and PR3 were significantly reduced in patients with type 1 diabetes, particularly in subjects within 3 years of diagnosis. This is associated with the known decrease in neutrophil counts in recent-onset type 1 diabetes (Qin *et al.* 2016). Therefore, circulating levels of PR3 and NE generally reflect neutrophil counts. Even if found to be low, they should not rule out the presence of NETosis in type 1 diabetes.

The role of NETosis in the pathogenesis of type 2 diabetes mellitus

In line with the results obtained in type 1 diabetes, increasing evidence has shed light on the role of neutrophils and NETosis in the pathophysiology of type 2 diabetes (Table 2). Considering that NETosis occurs during inflammation and that patients with type 2 diabetes are characterized by a state of low-grade chronic inflammation, NETosis was evaluated in the sera of 38 patients with type 2 diabetes and 38 age and gender-matched non-diabetic

Table 2 Neutrophil extracellular traps (NETs) in patients with type 2 diabetes mellitus.

Study reference	Subjects	Findings pertinent to NETosis
Joshi <i>et al.</i> 2013	<ul style="list-style-type: none">Patients with T2DM ($n = 18$)Healthy donors ($n = 17$)	<ul style="list-style-type: none">Neutrophils from diabetic subjects make inadequate and inactive NETs.
Menegazzo <i>et al.</i> 2015	<ul style="list-style-type: none">38 T2D and 38 age- and sex-matched non-diabetic individuals	<ul style="list-style-type: none">T2D patients showed increased neutrophil elastase, mono- and oligonucleosomes, and cell-free dsDNA as compared to the control subjectsA positive correlation was observed between HbA1c and mono- and oligonucleosomesdsDNA was positively correlated with the presence of nephropathy and cardiovascular diseaseSerum IL-6 concentrations were higher in T2D patients as compared to control subject and correlated with serum dsDNA levels
Wong <i>et al.</i> 2015	<ul style="list-style-type: none">Neutrophils isolated from patients with T2DM	<ul style="list-style-type: none">Expression of PAD4 was elevated in neutrophils from individuals with diabetes
Carestia <i>et al.</i> 2016	<ul style="list-style-type: none">Recently diagnosed T2DM patients and after 6 and 12 months of metformin treatment (500–2500 mg/daily) ($n = 25$)Healthy Donors ($n = 25$)	<ul style="list-style-type: none">Basal levels of NETs in patients with recently diagnosed T2DM patients were higher compared to healthy donors.Neutrophils from T2D patients did not respond to further stimulation by PMA or TNFαNET formation, nucleosomes, and NE-dsDNA complexes were still elevated in the plasma of patients with T2D after 6 months of treatment.Neutrophil functional responses were restored to normal after 12 months of treatment
Menegazzo <i>et al.</i> 2018	<ul style="list-style-type: none">Pre-diabetes patients randomized to receive metformin or placebo for 2 monthsPatients with T2DM before and after treatment with insulin or dapagliflozin	<ul style="list-style-type: none">Metformin significantly decreased the concentrations of neutrophil elastase, PR3, histones and dsDNA, whereas insulin or dapagliflozin had no significant effect.

control subjects (Menegazzo *et al.* 2015). Type 2 diabetic patients showed increased NETosis products, including neutrophil elastase, mono- and oligonucleosomes, and cell-free double stranded DNA (dsDNA), as compared to the control subjects. More importantly, a positive correlation was observed between HbA1c and mono- and oligonucleosomes, while dsDNA was correlated with the presence of nephropathy and cardiovascular disease. Also, serum concentration of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α), markers of systemic low-grade inflammation, which are also known to be NET inducers, were shown to be elevated in patients with type 2 diabetes as compared to the control subjects, and to be directly correlated with dsDNA concentrations (Menegazzo *et al.* 2015). It was also reported that high glucose (25 mM) significantly increased the rate of NETosis and release of NETs *in vitro*, with and without PMA, as compared to 5 mM glucose and 25 mM mannitol (Menegazzo *et al.* 2015). To assess the effect of metabolic control, NETosis was re-evaluated in diabetic patients after metformin treatment (500–2500 mg/daily) (Carestia *et al.* 2016). Despite the restoration of glucose and HbA1c levels to normal levels after 6 months of metformin treatment, NET formation, nucleosomes, and NE-dsDNA complexes were still present in the plasma of diabetic patients. However, these neutrophil functional responses were restored to normal levels after 12 months of treatment with no change in neutrophil count across this period. Indeed, neutrophils from diabetic patients at time of diagnosis had elevated levels of basal NET production compared to control subjects. Interestingly, neutrophils from diabetic patients did not respond to further stimulation by PMA or tumor necrosis factor alpha (TNF α) as did the neutrophils of healthy donors. This supports the fact that neutrophils from diabetic patients already possess increased basal NET production and are unable to further undergo NETosis (Carestia *et al.* 2016). This study shows, for the first time, that despite the normalization of glucose levels, NETosis remained elevated, suggesting that increased NET formation is not a consequence of impaired glycemic control. To further assess the effect of metformin on NETosis, NET products were assessed in the plasma of patients with pre-diabetes who were randomly assigned to receive either metformin or placebo for 2 months. Additionally, in order to control for the effect of glucose, NET components were also evaluated in the plasma of patients with type 2 diabetes before and after treatment with dapagliflozin or insulin (Menegazzo *et al.* 2018). After 2 months of treatment, patients in the metformin group showed a significant decrease in the concentrations

of dsDNA, neutrophil elastase, proteinase-3, and histones as compared to patients in the placebo group. Notably, no changes were observed in glucose levels or insulin sensitivity. Moreover, in patients who initiated basal insulin therapy or dapagliflozin, circulating biomarkers were not significantly reduced after 3 months of treatment as compared to baseline. These observations suggest that the observed effects of metformin on NETosis were independent of glucose control. *In vitro*, metformin significantly blunted NETosis in PMA and ionomycin-stimulated neutrophils. Also, metformin prevented membrane translocation of protein kinase C-beta II isoform (PKC- β II), which is a mediator of hyperglycemia-induced chronic complications, and activation of NADPH oxidase in neutrophils, which is required for neutrophil oxidative burst and is a critical step of NOX-dependent NETosis, both of which decreased NETosis response (Menegazzo *et al.* 2018). These findings highlight that the inhibition of NETosis is a new promising pharmacological effect that can be exerted by metformin. More importantly, expression of peptidylarginine deiminase 4 (PAD4), a crucial enzyme in chromatin decondensation and in mediating NETosis, was shown to be significantly elevated in patients with type 1 or type 2 diabetes as compared to healthy controls (Wong *et al.* 2015). Additionally, basal calcium levels were markedly elevated in patients with type 2 diabetes, and intracellular calcium levels were found to be positively correlated with fasting serum glucose levels (Alexiewicz *et al.* 1995), reminding us of the role of calcium in NET formation as it induces the generation of reactive oxygen species and PAD4-mediated histone citrullination and subsequent chromatin decondensation. Furthermore, NETosis was shown to metabolically require glucose (Rodriguez-Espinosa *et al.* 2015), therefore, elevated glucose is associated with NETosis at many levels. In contrast to these observations, Joshi *et al.* have previously shown that high glucose impairs and delays NETs formation as T2DM neutrophils possessed elevated basal NET formation and are unable to undergo further NETosis in response to external stimuli (Joshi *et al.* 2013). These differences may be attributed to the nature of the stimuli used in each study.

The role of NETosis in the pathogenesis of diabetes-induced microvascular complications

In addition to its potential role in the development of type 1 and type 2 diabetes, NETosis is also thought to be involved in promoting diabetes-induced complications (Fig. 1)

(Table 3). Indeed, NETs are implicated in diabetic wound healing, frequently associated to diabetic patients with distal symmetric polyneuropathy (DSP) (Forbes & Cooper 2013, Callaghan *et al.* 2015, Wong *et al.* 2015, Fadini *et al.* 2016, Volmer-Thole & Lobmann 2016, Das *et al.* 2018). When exposed to excisional skin wounds, diabetic mice had higher levels of citrullinated histone H3 (H3Cit), a marker for NETosis, in their wounds, and healing was delayed when compared to WT normoglycemic mice. DNase I, an enzyme involved in the disruption of NETs, accelerated wound healing in diabetic and WT mice. To further establish the role of NETosis in wound healing, protein arginine deiminase knockout (*Padi4*^{-/-}) mice were used.

In contrast to the robust increase of H3Cit signals that were detected in the WT wounds, no H3Cit was observed in wounds from *Padi4*^{-/-} mice, despite normal neutrophil recruitment. Notably, *Padi4*^{-/-} mice did not possess clinical signs of infection and their wound healing was accelerated. This may be explained by the fact that, in *Padi4*^{-/-} mice, in addition to a decrease in NETosis, other neutrophil functions such as phagocytosis remained intact. Intriguingly, accelerated wound healing was not compromised by inducing type 1 diabetes in *Padi4*^{-/-} mice, suggesting that diabetes delays wound healing by activating NETosis. PAD4 inhibition and DNase 1 may thus serve as potential therapeutic approaches to

Figure 1

Schematic diagram representing the key role of NETosis in the development and/or progression of diabetes-induced complications. Hyperglycemia increases the production of free radicals resulting in oxidative stress. It is also associated with elevated levels of both proinflammatory cytokines and autoantibodies. This, in turn, promotes neutrophil recruitment and activation. An increase in cytosolic calcium cations activates NADPH oxidases and acts as a cofactor for PAD4. PAD4 catalyzes histone citrullination inducing chromatin decondensation. Increased reactive oxygen species behave as second messengers promoting NE and MPO translocation to the nucleus and enhancing chromatin decondensation. The DNA is then released with toxic granules and cytoplasmic proteins as extracellular traps secondary to cellular lysis, eventually leading to diabetes-associated complications. PAD4, peptidylarginine deiminase 4; MPO, myeloperoxidase; NE, neutrophil elastase. A full-colour version of this figure is available at <https://doi.org/10.1530/JME-20-0128>.

Table 3 Neutrophil extracellular traps (NETs) in patients with diabetes-induced complications.

Study reference	Subjects	Findings pertinent to NETosis
Fadini <i>et al.</i> 2016	<ul style="list-style-type: none"> Control subjects without diabetes Patients with diabetes Patients with DFUs 	<ul style="list-style-type: none"> NET components were increased in nonhealing human DFUs Increased concentrations of NE in the wound was correlated with infection and worsening of the ulcer Elastase, histones, neutrophil gelatinase-associated lipocalin, and PR3 levels were increased in the blood of patients with DFUs Circulating elastase and PR3 were associated with infection Serum elastase predicted delayed wound healing Neutrophils isolated from patients with DFU possessed increased spontaneous NETosis but impaired inducible NETosis
Yang <i>et al.</i> 2019	<ul style="list-style-type: none"> 14 diabetic foot patients (seven males and seven females) 14 healthy volunteers (seven males and seven females) 	<ul style="list-style-type: none"> dsDNA and NE were significantly elevated in diabetic foot patients
Park <i>et al.</i> 2016	<ul style="list-style-type: none"> Of 88 patients with T2DM, 26 patients with diabetic retinopathy and 62 patients without diabetic retinopathy 28 healthy controls 	<ul style="list-style-type: none"> Circulating DNA-histone complex and polymorphonuclear NE levels were significantly increased in patients with diabetic retinopathy compared with those without retinopathy DNA-histone complex and NE were independent risk factors of retinopathy
Wang <i>et al.</i> 2018	<ul style="list-style-type: none"> Healthy controls ($n = 27$) Patients with T2DM ($n = 90$) Seventy-five T2DM patients were divided into three groups: diabetes without retinopathy (DWR) ($n = 30$), non-proliferative diabetic retinopathy (NPDR) ($n = 29$), and proliferative diabetic retinopathy (PDR) ($n = 31$) 	<ul style="list-style-type: none"> NETs formation was increased in the sera of patients with diabetic retinopathy irrespective of the disease stage
Miyoshi <i>et al.</i> 2016	<ul style="list-style-type: none"> 11 well-controlled patients with T2DM patients 13 healthy volunteers 	<ul style="list-style-type: none"> Serum MPO-DNA complex levels were significantly increased in well-controlled patients with T2DM and positively correlated with clinical/laboratory parameters which are known to be risk markers for diabetic nephropathy or diabetic kidney disease

wound healing (Wong *et al.* 2015). PAD4 inhibition with Cl-amidine reduced NETs formation and rescued wound healing in diabetic mice (Fadini *et al.* 2016), and the inhibition of PKC β II also prevented exaggerated NET production in diabetic mice and promoted accelerated wound healing (Das *et al.* 2018). Of interest, PKC β II is tightly correlated to phosphorylation of the p47^{phox} thus activating Nox2 and leading to ROS production (Fontayne *et al.* 2002). To validate these pre-clinical findings, the effect of NETosis on delayed wound healing was established in patients with diabetes. Using proteomics, NET products were found to be elevated in nonhealing human diabetic foot ulcers (DFUs). Moreover, since DFUs are associated with systemic inflammation, NETosis biomarkers were assessed in the bloodstream of diabetic patients with DFUs compared with matched diabetic patients without DFUs. NET components, including elastase, histones, neutrophil gelatinase-associated lipocalin,

and proteinase-3, were found to be elevated in blood of patients with diabetic foot ulcers. Additionally, a high concentration of circulating elastase and proteinase-3 was shown to be associated with infection, while serum elastase predicted delayed healing (Fadini *et al.* 2016). Cumulatively, these data imply that NETosis delays DFU healing. Consistent with these findings, a recent study revealed high levels of NET products in the sera of diabetic foot patients (Yang *et al.* 2019). Also, wound healing was significantly delayed in diabetic mice lacking leptin receptor (*LepR^{db/db}*), accompanied with high NETs levels, as compared to normoglycemic control mice (*LepR^{+/db}*). Furthermore, dsDNA, PAD4 and citrullinated histone H3 were all significantly elevated in an *in vitro* PMA-induced NETosis model. Importantly, for the first time, the study demonstrated that treatment with hydrogen sulfide (H₂S), an endogenous signaling molecule with antioxidant effects, significantly inhibited NETosis and

lead to accelerated wound healing in *LepR^{db/db}* mice. The inhibitory effect of H₂S on NETs production was attributed to the blockage of ROS-induced activation of mitogen-activated protein kinase (MAPK), ERK1/2 and p38 signaling pathways is involved in NETosis (Yang *et al.* 2019).

Beside neuropathy, NETosis is thought to be associated with the development of diabetes-induced retinopathy, since inflammation has been described as a key player in the onset of this microvascular complication. In fact, a case-control study investigated this hypothesis and results showed that NET formation markers, including circulating DNA-histone complex and polymorphonuclear neutrophil elastase levels, were elevated in patients with diabetic retinopathy as compared to diabetic patients without retinopathy. Interestingly, a multivariable logistic regression analysis, adjusted for fasting blood glucose and HbA1c levels, revealed that NET formation markers were significant independent risk factors of retinopathy (Park *et al.* 2016). These observations highlight the importance of NETs as a potential therapeutic target in diabetic retinopathy.

In parallel, NETs formation is considered to be associated with NADPH oxidases-induced ROS generation. In fact, neutrophils isolated from healthy individuals were exposed to increasing concentrations of glucose preloaded with 2',7'-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE). Upon high glucose stimulation, neutrophils displayed increased ROS levels supporting the fact that NETs formation is associated with hyperglycemia-induced overproduction of ROS. Accordingly, pre-treatment with either DPI or apocynin, both inhibitors of NADPH oxidase, reduced NETs formation and the release of extracellular DNA in neutrophils exposed to high glucose (Wang *et al.* 2018). Furthermore, NETs were present in the sera of patients with diabetic retinopathy irrespective of the disease stage (Wang *et al.* 2018). As observed in previous studies on wound healing, these findings suggest that NADPH oxidases are crucial in high glucose-induced NET production in diabetes-induced retinopathy.

Besides the role of NETosis in wound healing and retinopathy, increasing evidence highlights NETs formation as a risk factor for other microvascular complications. In fact, a recent study has shown that serum MPO-DNA complex level, a marker of NETs, was elevated in well-controlled patients with type 2 diabetes treated with several (≥ 3) antidiabetic drugs as compared to patients treated with less (< 3) antidiabetic drugs and

Figure 2

Proposed crosstalk between hyperglycemia and NETosis in diabetes-induced complications. A full-colour version of this figure is available at <https://doi.org/10.1530/JME-20-0128>.

healthy controls. More importantly, the serum MPO-DNA complex levels were positively correlated with clinical/laboratory parameters, which are known to be risk markers for microvascular complications including diabetic nephropathy or diabetic kidney disease including BMI and urinary albumin (Miyoshi *et al.* 2016). These findings suggest that NETs formation may serve as a risk marker for diabetes-induced renal complications in well-controlled patients. Despite these observations, further research is needed to better elucidate the role of NETs in diabetic nephropathy. However, based on data from our group and others, the role of NADPH oxidases in diabetes-induced microvascular complications, specifically diabetic nephropathy, has been well documented (Eid *et al.* 2009, 2010, 2013a,b, 2016, 2020). Since NETosis and NETs formation were shown to be highly dependent on NADPH-induced ROS production in neuropathy and retinopathy, one can advance the hypothesis that NETosis may be considered as a key player in the onset and development of diabetic kidney disease. However, it is important to mention that the role of NADPH oxidases is site and disease specific. For instance, it has been previously shown that NADPH oxidase 4 (NOX4) mediates protection against chronic load-induced stress in the heart and is essential for full development of eccentric cardiac hypertrophy and remodeling of the heart during chronic volume overload (Zhang *et al.* 2010, Schnelle *et al.* 2019). Furthermore, NOX4 has been shown to have an anti-atherosclerotic effect; hence, NOX4 inhibitors should be carefully assessed for cardiovascular side effects (Schürmann *et al.* 2015).

Conclusion

This review highlights the important role that NETosis plays in the pathogenesis of diabetes and advances its role in diabetes-induced microvascular complications (Fig. 2). NETosis, or the process of NETs formation, has been shown to be activated by a wide array of proinflammatory cytokines and reactive oxygen species, notably in diabetes, which is characterized by a state of low-grade chronic inflammation. It has also been reported that a number of redox-sensitive pathways, including the NADPH oxidases pathways, orchestrate the key pathological events related to diabetes-induced microvascular complications, thus recognizing the role of NADPH oxidases-induced ROS as the possible bridge between NETosis and diabetes. Further studies aiming to better understand the exact mechanisms behind hyperglycemia-induced NETosis would be an essential step not only toward designating NETosis as a possible marker for the progression of diabetes, but also toward uncovering the likely therapeutic benefits of targeting NETosis in controlling the progression of diabetes-induced microvascular complications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

R N is funded by the American University of Beirut Faculty of Medicine Biomedical Sciences graduate program. S T A, H K K and A A E are supported by the American University of Beirut Medical Practice Plan regular research grant.

Author contribution statement

A A E and H K K conceived and designed the review article and approved the final version to be submitted. R N, W S A and A H F performed the literature review. R N and W S A analyzed and interpreted the data; wrote multiple subsections of the manuscript; and revised the manuscript for intellectual content. S T A reviewed and improved the entire manuscript.

Acknowledgement

Figures created with BioRender.com.

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Received in final form 30 July 2020

Accepted 3 September 2020

Accepted Manuscript published online 4 September 2020