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Vitamin D is associated with enhanced neutrophil extracellular traps formation in type 2 diabetes: the effect of glycemic control

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Introduction

Neutrophil extracellular traps (NETs) composed of DNA, histones and granular proteins, play an important role in innate immunity [1], but also under chronic and low-grade inflammation [2]. NETs formation can be indirectly evaluated using circulating NETs-associated proteins

such as citrullinated histone 3 (citH3), cell-free DNA (cfDNA), myeloperoxidase (MPO), and neutrophil elastase (NE) [2]. Increased levels of NETs-associated proteins have been reported in a vast array of diseases, including type 2 diabetes (T2DM) [2,3]. Compelling basic and clinical data support enhanced NETs formation (NETosis) under high-glucose conditions and NETs-mediated harmful effects in T2DM, including their association with macro- and microvascular manifestations, impaired wound healing, and retinopathy [4-7]. Cardiovascular complications in patients with T2DM, such as myocardial infarction, have been attributed at least in part to increased NETosis, which renders fibrin clots more prothrombotic, while fibrinolysis impaired [3].

There is evidence that T2DM patients are often impacted by micronutrient deficiencies with the most prevalent being vitamin D deficiency, which affects around 60% of the diabetic population [8] and may impair metabolic control [9]. Both impaired pancreatic β-cell function and insulin resistance have been reported with low blood 25-hydroxyvitamin D concentrations [9]Kliknij lub naciśnij tutaj, aby wprowadzić tekst.. Vitamin D supplementation is recommended in adults with prediabetes to reduce the rate of progression to T2DM [9]. Although growing evidence has downplayed the role of vitamin D supplementation in T2DM [10], some studies have demonstrated a reduction in glycated hemoglobin (HbA_{1c}) following such an intervention [11]. It has been shown that in ex vivo high-glucose conditions, vitamin D may decrease NETs formation, suggesting the potential of vitamin D supplementation for the prevention of NETosis-associated adverse events [12]. Additionally, vitamin D was shown to positively affect wound healing in T2DM, at least in part by decreased NETs formation [13]. To our knowledge, no study has yet investigated the potential association between vitamin D status and NETs formation in patients with T2DM in relation to glycemic control. The aim of our study was to investigate whether vitamin D deficiency is associated with enhanced NETs formation in T2DM

patients. We also tested whether vitamin D concentrations might be linked to fibrin clot parameters and thrombin generation in T2DM.

Patients and methods

We included 115 patients diagnosed with T2DM in a tertiary-care center, Krakow, Poland between October 2016 and July 2017. This population was presented in detail in previous papers [3,14]. In the current analysis, we excluded 11 patients due to unavailable data on 25-OH vitamin D concentrations. Exclusion criteria were: advanced chronic kidney disease, autoimmune disorders, cancer, signs of acute infection, recent arterial or venous thromboembolic events. Microalbuminuria was defined as the urinary albumin/creatinine ratio within the range of 30-300 mg/g, whereas macroalbuminuria was reported above 300 mg/g. The study protocol was approved by the Jagiellonian University Medical College Bioethics Committee (1072.6120.40.2017). All participants provided informed written consent.

Fasting venous blood samples were collected between 8-10 AM. Routine laboratory investigations were performed at the hospital laboratory. HbA1c concentrations were analyzed using the Variant II Turbo analyzer (Hercules; reference range: 4.8-5.9%). All samples were stored in -80 °C until further analysis.

Serum 25-hydroxyvitamin D (25-hydroxyvitamin D, including vitamin D_2 and vitamin D_3) was measured using ELISA (Abcam). Patients with vitamin D deficiency (< 20 ng/mL) and insufficient vitamin D concentration (20 to <30 ng/mL) were categorized as patients with non-sufficient vitamin D concentrations, as opposed to patients with sufficient vitamin D concentrations (≥30 ng/mL) [15].

Among NETs-associated proteins, MPO, NE (Abcam), and citH3 (Cayman Chemical) were evaluated using ELISAs, while cfDNA levels were performed using a commercially available assay (Invitrogen, Life Technologies). The reference range for citH3 established in our

laboratory was 0.5-1.7 ng/ml. Interleukin-6 and 8 (IL-6 and IL-8) concentrations were also determined by ELISA (R&D Systems).

Thrombin generation potential was evaluated using previously described assay [3] and was expressed by area under curve (AUC). Fibrin clot characteristics were expressed as permeation coefficient (Ks) and clot lysis time (CLT), according to previously described techniques [3].

Statistical analysis Statistical analysis was conducted using IBM SPSS Statistics for Windows, version 31 (IBM Corp.). Categorical data are presented as number (percentage), while continuous variables as median (interquartile range; IQR) or mean (standard deviation; SD) for non-normally and normally distributed variables, respectively. Statistical significance of intergroup differences in qualitative parameters was evaluated using Fisher's exact test (when expected count in any cell of a contingency table was below 5) or χ^2 test with Yates' correction (correction applied if all expected counts were above 5, while observed value in at least one cell was below 10). Normality of distribution of continuous variables was tested using Shapiro–Wilk test and homogeneity of variance using Levene's test. Differences in continuous data between groups were assessed using Mann–Whitney test and Student t-test for normally distributed and non-normally distributed variables, respectively. Evaluation of correlations between the two parameters was performed using Spearman's correlation coefficient with nonparametric curve fitting if at least one of tested variables was non-normally distributed. A two-sided *P* value <0.05 was considered statistically significant.

Results

A total of 102 patients at a mean (SD) age of 64.4 (8.2) years, mostly males (n = 55, 54%), with median (IQR) HbA_{1c} of 6.90 (6.00-8.05) % were studied. A median (IQR) vitamin D level in the whole population was 27.5 (23.5-32.0) ng/mL. Most of the patients (n = 68, 66.7%) had non-sufficient vitamin D concentration (Supplementary material, *Table S1*), with a median

(IQR) level of 25.3 (21.5-27.6) ng/mL, while the remainder had sufficient vitamin D concentration (33.6 [32.0-38.6] ng/mL). Vitamin D status showed no associations with demographic and clinical variables (Supplementary material, *Table S1*). Antidiabetic medications were not associated with vitamin D status (Supplementary material, *Table S1*). Patients with non-sufficient vitamin D had 20.3% higher HbA1c and 18.7% higher serum glucose concentrations (Supplementary material, *Table S1*). Moreover, vitamin D correlated negatively with HbA_{1c} (Figure 1A) in the whole group as well as in patients with non-sufficient (Figure 1A) and sufficient (R = -0.460, P = 0.006) vitamin D concentration. Vitamin D correlated with serum glucose solely in the whole group (R = -0.311, P = 0.001).

Patients with non-sufficient vitamin D concentrations had 47.1% higher citH3 levels, 32.4% higher cfDNA, and 13.6% higher MPO concentrations when compared with the remainder (Supplementary material, *Table S1*). The groups did not differ in circulating NE levels (Supplementary material, *Table S1*). Moreover, patients with non-sufficient concentration of vitamin D had 47.4% higher IL-6, while IL-8 concentrations did not differ (Supplementary material, *Table S1*).

NETs-related proteins, except for NE, were associated with vitamin D in the whole T2DM population (Figure 1B–1D) and among individuals with non-sufficient vitamin D concentrations (Figure 1B–1D). Similarly, we observed associations of vitamin D with IL-8 and IL-6 in the whole population and in deficient patients (Figure 1E and 1F).

There were no differences in thrombin generation potential and Ks regarding vitamin D status. Patients with non-sufficient vitamin D concentration had 13.7% prolonged CLT as compared to those with sufficient vitamin D concentration (Supplementary material, *Table S1*). We found weak associations of vitamin D with both Ks and CLT in the whole group (R = 0.248, P = 0.01 and R = -0.392, P <0.001, respectively) and in patients with non-sufficient vitamin D

concentration (R = 0.326, P = 0.007 and R = -0.298, P = 0.01, respectively). Vitamin D did not correlate with thrombin generation potential (P >0.05).

Discussion

This study demonstrates for the first time that vitamin D status may contribute to the modulation of NETosis in patients with T2DM. Importantly, glycemic control was also associated with NETs-related proteins. Our findings extend beyond NETosis and suggest that vitamin D deficiency may affect fibrin clot phenotype, particularly fibrinolysis. Patients with lower vitamin D concentrations formed fibrin networks that were relatively resistant to lysis, despite no differences in fibrinogen levels or thrombin generation in T2DM.

The interpretation of associations between NETs-related proteins with vitamin D in T2DM patients remains complex, and may involve at least two mechanisms. One possibility is a direct effect of vitamin D on NETosis. Another explanation is indirect and related to glycemic control. Patients with non-sufficient vitamin D concentrations had increased HbA_{1c} levels, supporting the hypothesis that suboptimal vitamin D status contributes to impaired glycemic control [11], which in turn may exacerbate NETosis [5]. Our research group previously showed that HbA1c predicts citH3 concentration in T2DM [3]. The current findings expand this observation by placing vitamin D within the network of processes associated with NETosis in T2DM.

However, the data showing that vitamin D supplementation inhibits NETosis in response to high glucose concentration [12,13], points towards the hypothesis of a direct impact. In line with this data, we observed increased concentrations of citH3, cfDNA and MPO in patients with non-sufficient vitamin D concentration. Importantly, a similar vitamin D cut-off had previously been linked to increased cardiovascular disease and T2DM [16]. Therefore, enhanced NETosis associated with vitamin D deficiency may represent a previously unrecognized factor contributing to cardiovascular complications in T2DM. Moreover, our study showed that only

cfDNA correlated with HbA_{1c}, whereas other NETs-related proteins did not. This discrepancy suggests that circulating cfDNA in T2DM may not originate exclusively from NETosis. Other sources, such as endothelial injury, may also contribute. Endothelial dysfunction is a hallmark of diabetes and is driven by chronic hyperglycemia and oxidative stress [4].

Our findings also support the hypothesis that vitamin D deficiency may contribute to prothrombotic alterations mainly via impaired fibrinolysis. This is with line with previous study by Varjú and Longstaff [17] who have demonstrated that extracellular DNA and histones can directly modulate fibrin clot structure and clot lysis time. Their study provides a mechanistic link between NETs components and unfavorably altered clot properties [17]. Evidence regarding the role of vitamin D in fibrin clot properties is very limited. Previous interventional studies showed that vitamin D supplementation can modulate thrombin generation and fibrin clot architecture, leading to the formation of less dense and potentially less thrombogenic fibrin networks. However, these changes were not consistently accompanied by alterations in CLT [18]. In contrast, Elbers et al [19] failed to show significant effects of vitamin D supplementation on fibrinolysis. In our cohort, patients with non-sufficient vitamin D concentrations had prolonged CLT despite comparable concentrations of fibrinogen, C-reactive protein, and activities of alpha-2-antiplasmin and plasminogen, compared with the remainder. IL-6 was the only parameter that differed between the groups and was increased in patients with non-sufficient vitamin D concentrations. Interestingly, vitamin D has also been associated with lower oxidative stress markers in T2DM patients [11]. This highlights the complexity of the mechanisms linking vitamin D, NETosis, and neutrophil activation. Our data provide a complementary mechanism connecting vitamin D deficiency with enhanced NETosis and impaired fibrinolysis in T2DM. In our opinion, the association between vitamin D and CLT may be particularly relevant in T2DM because hyperglycemia-induced IL-6 secretion may

promote NETosis. Further studies are needed to determine whether vitamin D supplementation may inhibit NETosis in the diabetic milieu and improve clot properties.

The potential influence of pharmacotherapy should also be considered. Previous studies reported reductions in NETs-associated proteins with high-dose statin therapy [20]. We did not observe such effects, likely due to lower statin doses in our patients. Similarly, antidiabetic therapies were not associated with vitamin D status or NETs, although subgroup analyses were limited by the small sample size.

This study has several limitations. First, the sample size was relatively small and the presented associations do not necessarily mean the cause-effect relationship. Second, seasonal variation in vitamin D concentrations across the calendar year was not considered and may have affected the results. Future studies should include repeated measurements across seasons with different levels of sun exposure [21]. Third, the molecular mechanisms underlying vitamin D-mediated modulation of NETs formation were beyond the scope of this study. Additionally, no follow-up was conducted, so we could not determine whether the observed differences predispose patients to arterial or venous thromboembolism. Finally, it remains unclear whether vitamin D supplementation can reduce NET formation independently of glycemic control. Therefore, the present study should be considered hypothesis-generating, and its findings interpreted cautiously.

Conclusions Non-sufficient concentrations of vitamin D in T2DM patients were associated with enhanced NETosis detectable in circulating blood and with impaired fibrinolysis. This finding suggests that vitamin D may partly modulate thrombotic risk in T2DM. Further mechanistic and interventional studies are needed to determine whether vitamin D supplementation can attenuate NETs formation and improve fibrinolytic efficiency in T2DM.

Article information

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Conflict of interest None declared.

AI statement Artificial intelligence was not used in the preparation of this manuscript.

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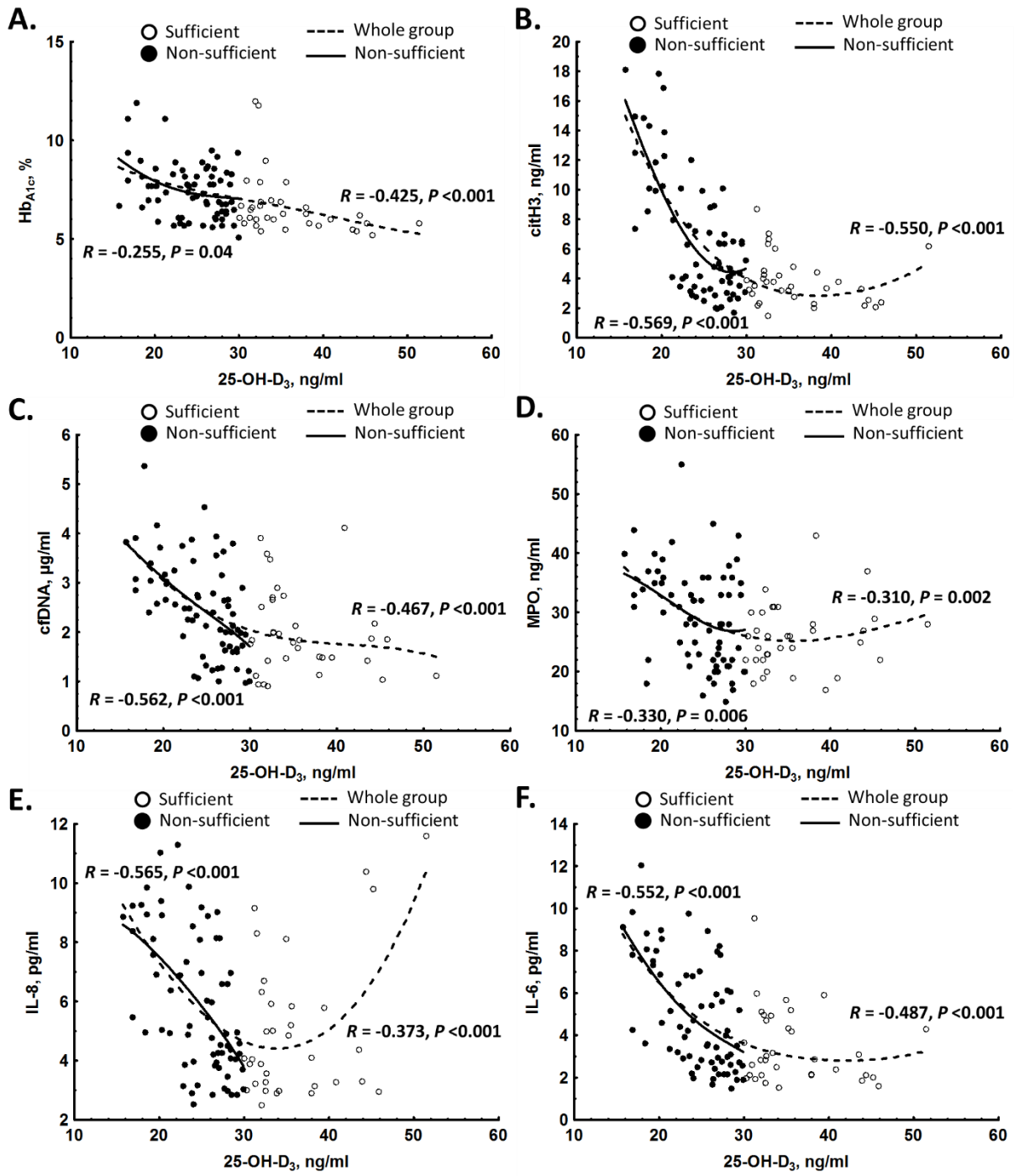

Figure 1 Linear correlations of vitamin D with glycated hemoglobin (**A**), citrullinated histone 3 (**B**), circulating free DNA (**C**), myeloperoxidase (**D**), interleukin 8 (**E**), interleukin 6 (**F**) in 102 patients with type 2 diabetes. Full circles denote patients with non-sufficient vitamin D concentration, open circles patients with vitamin D within the reference range.

Short title: Vitamin D is associated with enhanced NETosis in type 2 diabetes