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Circulating nucleosomes, neutrophil activation and development of venous thrombosis in patients with multiple myeloma

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Abstract

Background: Treatment of multiple myeloma (MM) is associated with a high risk of venous thromboembolism (VTE). Nucleosomes and neutrophil activation are associated with thrombus development in mouse models, and with VTE in patients. No data are available on the role of nucleosomes or neutrophil activation in patients with MM and VTE.

Methods: We assessed levels of nucleosomes and systemic neutrophil activation, by presence of elastase-α1-antitrypsin (EA) complexes, in 131 patients with newly diagnosed MM (HOVON-50 or HOVON-65 study). We used a nested case-control design to investigate their association with VTE, by calculating odds ratios (ORs) with corresponding 95% confidence intervals (CIs). A comparison of nucleosome and neutrophil activation levels was also made between MM patients, a cohort of patients with deep-vein thrombosis (DVT) of the leg with and without malignancy, and a cohort of DVT controls with and without malignancy.

Results: 19 of 131 MM patients (14.5%) developed VTE during MM treatment. No association was found between nucleosomes, neutrophil activation, and VTE (OR 0.5; 95% CI 0.2-1.6, and OR 2.1; 95% CI 0.5-9.7, respectively) in MM patients. A significant difference was found between median levels of EA complexes and higher MM disease stage (Kruskal Wallis test, p=0.03). Nucleosome levels were significantly higher in MM patients compared with DVT patients and DVT controls, irrespective of malignancy status, while levels of neutrophil activation were significantly lower in MM patients in all comparisons.

Conclusions: Elevated levels of nucleosomes were found in MM patients, but there was no association between circulating nucleosomes, neutrophil activation, and VTE in MM. Our results suggest that nucleosomes are a marker of plasma cell death rather than of neutrophil or coagulation activation in patients with MM. Future studies are necessary to investigate the source and clinical relevance of nucleosomes in MM.

Introduction

Patients with multiple myeloma (MM) are at a 9-fold increased risk of venous thromboembolism (VTE), with at least 10% of patients developing VTE during the course of the disease.[1] Mechanisms underlying the increased risk of VTE in MM are multifactorial, with contributions of patient-related factors, e.g., older age, as well as disease-related factors, e.g. blood hyperviscosity due to high levels of immunoglobulins and increased cytokine release.[2] Increased levels of interleukin-6 (IL-6) have been observed in patients with MM. IL-6 is involved in one of the major signaling pathways in the pathogenesis of MM,[3] but can also lead to a procoagulant state,[4] with elevated levels of factor VIII, von Willebrand factor, tissue factor and D-dimer.[5] However, an association between high levels of prothrombotic factors and VTE in MM has not been confirmed.[5-7]

Treatment agents for MM are also an important risk factor for VTE, and the incidence of VTE varies between different regimens.[8] The introduction of immunomodulatory drugs (IMiDs) thalidomide and its derivate lenalidomide has improved overall response rate and clinical outcome.[9, 10] However, use of IMiDs in combination with dexamethasone and chemotherapy has also significantly increased the risk of VTE during MM treatment.[11, 12] In contrast, use of bortezomib, a proteasome inhibitor introduced after the IMiDs, is associated with a lower risk of VTE in MM patients.[13]

Recently, activation of neutrophils, consequently leading to the formation of neutrophil extracellular traps (NETs), and the exposure of nucleosomes on these NETs, was shown to play a central role in coagulation activation in vivo and development and propagation of DVT in animal models.[14-18] Nucleosomes exposed on NETs are crucial in the prothrombotic potential of NETs. [15-17] Nucleosomes consist of a core octamer, with 2 copies each of histones H2A, H2B, H3, and H4, around which a stretch of helical DNA of 146 base pairs is wrapped.[19] Circulating nucleosomes detected in sepsis have been reported to correlate with markers for coagulation and neutrophil activation,[20] and were found a suitable marker for NET formation in plasma in baboons and humans.[21] In patients with VTE, increased nucleosome levels and markers for neutrophil activation were also demonstrated, supporting the role of NET formation in the development of VTE.[22] NETs have also been associated with cancer and cancer-related VTE.[23] Whether there is a role of nucleosomes or neutrophil activation in patients with MM and VTE, is currently unknown.

Therefore, the primary aim of this study was to investigate whether circulating nucleosomes and systemic neutrophil activation, as evidenced by presence of human neutrophil elastase-α_1-antitrypsin (EA) complexes, are associated with VTE in patients with newly diagnosed MM. Secondly, levels of nucleosomes and neutrophil

activation in MM patients were also compared with a cohort with patients with objectified deep vein thrombosis (DVT cases) of the leg, with and without malignancy, and matched controls in whom DVT was objectively excluded (DVT controls), with and without malignancy.[22]

Materials and methods

Patients and controls

We used three different cohorts of patients for this study: 1) 131 patients with MM, of whom 19 with VTE and 112 without VTE, 2) 149 patients with DVT (DVT cases), of whom 26 with malignancy and 123 without malignancy, and 3) 181 controls without DVT (DVT controls), of whom 24 with malignancy and 157 without malignancy. DVT cases and DVT controls were matched for sex, age (±5-year intervals) at the time of blood sample collection, and time of DVT evaluation. DVT cases, controls and definitions for DVT, as well as the association between nucleosomes, neutrophil activation, and DVT have been described previously.[22] Patients with MM and DVT cases or controls were not matched.

Plasma samples of MM patients were obtained from 131 consecutive patients of age 65 years and younger, with newly diagnosed MM according to the Mayo Clinic criteria, who were included in the HOVON-50 MM or HOVON-65 MM / GMMG HD4 studies between November 2001 and November 2006 (ISRCTN06413384 and ISRCTN64455289).[24, 25] Patients were referred to the Department of Hematology of the Erasmus University Medical Center (EMC), Rotterdam, the Netherlands, for intensive chemotherapy, followed by stem cell mobilization with CAD (cyclophosphamide, doxorubicin, dexamethasone), and subsequent intensification with high dose melphalan, followed by autologous stem cell transplantation. For intensive chemotherapy, patients were randomized between receiving three courses of VAD (vincristine, doxorubicin, and dexamethasone) or TAD (thalidomide, doxorubicin, and dexamethasone; HOVON-50), and between VAD or BAD (bortezomib, doxorubicin, and dexamethasone; HOVON-65). Patients randomized to TAD received thromboprophylaxis (prophylactic dose low-molecular-weight heparin [LMWH]) during TAD. Disease stage was classified according to the International Staging System (ISS).

Plasma samples of the 149 patients with symptomatic DVT of the leg (DVT cases) and 181 matched controls with a clinical suspicion of DVT, but in whom DVT was objectively excluded (DVT controls), were obtained from patients who were referred for suspicion of acute symptomatic DVT of the leg to the Academic Medical Center (AMC) in Amsterdam, The Netherlands, between September 1999 and May 2006.

Malignancy status at the time of DVT evaluation was known for all DVT cases and DVT controls. Malignancy was defined as having an active malignancy or receiving treatment for a malignancy at the time of DVT evaluation. Patients were categorized accordingly; of 149 DVT cases, 26 had a malignancy and 123 did not, of 181 DVT controls, 24 had a malignancy and 157 did not.

Blood collection

Venous blood was collected after MM diagnosis but before start of treatment in MM patients, and upon DVT evaluation before start of anticoagulant treatment in DVT cases and DVT controls. Venous blood was drawn in citrate containing vials (0.105 M, Beckton-Dikinson, Plymouth, United Kingdom) and plasma was prepared by centrifuging twice at 4°C for 10 minutes at 2000g (EMC), or twice at room temperature for 15 minutes at 1500g (AMC). Plasma was aliquoted and stored at -80°C until used. Blood samples were taken after having obtained written informed consent. Collection of plasma was approved by the Medical Ethical Committee of the EMC, Rotterdam, and of the AMC, Amsterdam, respectively.

Symptomatic VTE

Symptomatic VTE events in MM patients were detected by close clinical monitoring of patients during the treatment period, and confirmed using appropriate objective radiographic tests. No definitions were stipulated for clinical presentation, which was left to the judgement of the attending physician. Symptomatic DVT in DVT cases was diagnosed when a proximal leg vein was not compressible on ultrasound or by the presence of an intraluminal-filling defect on venography. Proximal DVT was defined as a thrombus in the popliteal vein, superficial femoral vein, or common femoral vein. If compression ultrasound showed no venous thrombosis and the D-dimer plasma level was ≥0.5 mg/L, compression ultrasound was repeated after 7 days. DVT was ruled out in case of a Wells score ≤1 in combination with a low D-dimer plasma level (<0.5 mg/L). DVT was also ruled out in case of a normal venography or negative compression ultrasound in combination with a low D-dimer plasma level (<0.5 mg/L), and after a repeated negative ultrasound.

Nucleosome ELISA

Nucleosome levels were assessed with an ELISA as recently described.[26] Briefly, monoclonal antibody CLB-ANA/60 (Sanquin, Amsterdam, The Netherlands), which recognizes histone 3 was used as a catching antibody. Biotinylated F(ab)2 fragments of monoclonal antibody CLB-ANA/58 (Sanquin, Amsterdam, The Netherlands), which recognizes an epitope exposed on complexes of histone 2A, histone 2B and dsDNA, in combination with poly-horseradish peroxidase-labeled streptavidin (Sanquin, Amsterdam, The Netherlands) was used for detection. As a standard we used

culture supernatant of Jurkat cells (1x10^6 cells/ml), cultured for an additional week, to obtain 100% apoptotic cells. One unit is the amount of nucleosomes released by approximately 100 Jurkat cells. The lower detection limit of the assay was 2.5 U/ml.[27] The reference range for circulating nucleosomes in our laboratory is <10.3 U/ml. The inter- and intra-assay coefficient of variation is 8.5% and 4.3%, respectively.

Neutrophil activation (as evidenced by presence of human neutrophil EA complexes)

EA complexes were measured by an ELISA that has been adapted from a previously described radioimmunoassay.[28] Briefly, plates were coated with a polyclonal rabbit anti-human neutrophil elastase antibody (1.5 µg/mL; Sanquin, Amsterdam, The Netherlands). Standard and samples were diluted in high-performance ELISA buffer (HPE; Sanquin, Amsterdam, The Netherlands) + 40 µg/mL bovine IgG. Bound complexes were detected by incubation with biotinylated monoclonal anti-α_1-antitrypsin antibody (1 µg/mL) in combination with poly-horseradish peroxidase-labeled streptavidin. Results were expressed in ng/mL by reference to a standard curve of normal human citrated plasma in which EA complexes were generated by incubating with porcine elastase (final concentration 2 µg/mL; Sigma Zwijndrecht, The Netherlands) for 15 minutes at room temperature. The lower detection limit of the assay was 2 ng/ml. The reference range for EA complexes in our laboratory is 8.5 to 55.7 ng/ml. The inter- and intra-assay coefficients of variation are 9.5% and 5.7%, respectively.

Statistical analysis

Descriptive statistics were used for analysis of patient characteristics of both MM patients as well as DVT cases and DVT controls. Levels of nucleosomes and EA complexes were described by medians and interquartile ranges (IQRs). The primary aim of this study was to investigate whether nucleosomes and neutrophil activation are associated with VTE in patients with newly diagnosed MM. For this analysis, we used a nested case-control design within the cohort of MM patients to compare MM patients with and without VTE. Mann-Whitney U, Kruskal-Wallis 1-way ANOVA and Chi^2-tests were used to analyze differences between MM patients with VTE and without VTE and with different ISS disease stage. Associations between nucleosomes, EA complexes, and development of VTE during MM treatment, were explored by means of logistic regression analysis and expressed as odds ratios (ORs) with corresponding 95% confidence intervals (CIs). These analyses were performed with nucleosomes and EA complexes as categorical variables, created by dividing patients into 2 groups based on the 80th percentile for MM patients without VTE. This was done for separately for nucleosome and EA complex levels, with ≤80th percentile being the reference category. Potential confounders for the associations between nucleosomes, neutrophil activation, and the development of VTE in MM patients (i.e., heavy chain

type [non-IgG versus IgG)], disease stage [ISS 3 versus 1 or 2] or treatment regimen [TAD versus VAD or BAD]) were evaluated using multivariable logistic regression models. Associations between nucleosomes, EA complexes, and the time to VTE event, were explored by means of Cox regression analysis and expressed as hazard ratios (HRs) with corresponding 95% CIs. The secondary aim of this study was to investigate whether nucleosomes and neutrophil activation are associated with MM or other malignancies, in addition to VTE. For this analysis, Mann-Whitney U tests were also used to analyze differences in levels of nucleosomes and EA complexes between MM patients, DVT cases and DVT controls with other malignancies, and DVT cases and DVT controls without malignancy. Statistical analyses were performed with IBM SPSS Statistics software for Windows (version 22; IBM, Armonk, NY, USA) and GraphPad Prism for Windows, version 5 (GraphPad Software Inc, San Diego, CA, USA, www.graphpad.com). Statistical significance in all analyses was set at $P<0.05$.

Results

Characteristics of patients with MM

Patient baseline characteristics of the 131 MM patients are shown in Table 1. 19 of 131 patients (14.5%) developed VTE during MM treatment. VTE events included DVT of the leg (n=8), pulmonary embolism (n=8), and upper limb venous thrombosis (n=3; Table 2). There was no difference in VTE incidence between patients randomized to VAD, TAD or BAD treatment. The prothrombin G20210A mutation was significantly more prevalent in MM patients with VTE than in patients without VTE during treatment (15.8% vs. 1.8%; p=0.023; Table 1).

Primary analysis: Nucleosomes and neutrophil activation in patients with MM

We used a nested case-control design within the cohort of MM patients to compare MM patients with and without VTE, and to analyze the association between nucleosomes, neutrophil activation and VTE. Levels of circulating nucleosomes (median 39.5 U/mL; interquartile range [IQR] 7.9-156.8, versus median 25.6 U/mL; IQR 10.9-76.4; p=0.61) and neutrophil activation, as evidenced by presence of EA complexes (median 30.1 U/mL; IQR 22.6-39.3, versus median 31.6 U/mL; IQR 24.3-46.0; p=0.45), did not differ between patients with and without VTE during MM treatment (Table 1; Figures 1A and 1B).

There was no association between high levels of circulating nucleosomes and VTE (nucleosomes >80th percentile of non-VTE MM patients: OR 0.5; 95% CI 0.18-1.55; p=0.25), and neither between high levels of neutrophil activation and VTE (EA complexes >80th percentile of non-VTE MM patients: OR 2.1; 95% CI 0.45-9.67; p=0.35).

Table 1. Patient baseline characteristics of patients with multiple myeloma

	MM patients; total (N=131)	MM patients; with VTE (N=19)	MM patients; without VTE (N=112)	p-value*
Age at inclusion, median (IQR)	55.5 (29-65)	51 (46-57)	56 (49-60)	0.05
Male, n (%)	76 (58.0)	10 (52.6)	66 (58.9)	NS
Baseline hemoglobin; mmol/l, median (IQR)	6.9 (4.0-10.1)	7.4 (5.2-8.7)	6.8 (4.0-10.1)	NS
Baseline leukocytes; x10^9/l, median (IQR)	5.6 (0.9-17.8)	6.6 (0.9-10.7)	5.6 (1.3-17.8)	NS
Baseline platelets; x10^9/l, median (IQR)	242 (65-446)	259 (130-383)	236 (65-446)	NS
Monoclonal protein light chain				NS
kappa, n (%)	90 (68.7)	15 (78.9)	75 (67.0)	
lambda, n (%)	40 (30.5)	4 (21.1)	36 (32.1)	
missing, n (%)	1 (0.8)		1 (0.9)	
Monoclonal protein heavy chain				NS
IgG, n (%)	78 (59.5)	8 (42.1)	70 (67.0)	
non-IgG, n (%)	42 (32.1)	10 (52.6)	32 (28.6)	
missing, n (%)	11 (8.4)	1 (5.3)	10 (8.9)	
ISS stage				NS
1	41 (31.3)	10 (52.6)	31 (27.7)	
2	70 (53.4)	7 (36.8)	63 (56.3)	
3	20 (15.3)	2 (10.5)	18 (16.1)	
Treatment induction				NS
VAD, n (%)	73 (55.7)	9 (47.4)	64 (57.1)	
TAD, n (%)	42 (32.1)	8 (42.1)	34 (30.4)	
BAD, n(%)	16 (12.2)	2 (10.5)	14 (12.5)	
Thrombophilia				
Factor V Leiden mutation, n (%)	3 (2.3)	0	3 (2.7)	NS
Prothrombin G20210A mutation, n (%)	5 (3.8)	3 (15.8)	2 (1.8)	0.02
Nucleosome levels, median (IQR)	26.0 (10.8-78.7)	39.5 (7.9-156.8)	25.6 (10.9-76.4)	NS
EA complexes levels, median (IQR)	31.2 (24.3-45.5)	30.1 (22.6-39.3)	31.6 (24.3-46.0)	NS

BAD = bortezomib, doxorubicin, and dexamethasone; EA complexes = human neutrophil elastase–α1-antitrypsin complexes; MM = multiple myeloma; NS = not significant; TAD = thalidomide, doxorubicin, and dexamethasone; VAD = vincristine, doxorubicin, and dexamethasone VTE = venous thromboembolism. *of comparison MM patients with VTE versus MM patients without VTE.

Table 2. MM patients with VTE during treatment

Patient (sex; age)	VTE type	ISS stage	Treatment regimen	Timing of VTE event	Time to VTE from inclusion (days)
F; 51	PE	1	VAD	after induction / during HDM	233
M; 47	DVT	2	VAD	during induction	47
M; 39	DVT	1	VAD	before start of treatment	-34 (before treatment)
F; 61	DVT	1	VAD	during induction	78
F; 44	CVC	1	VAD	after induction / during HDM	154
F; 46	CVC	3	TAD	after induction / during HDM	213
M; 51	PE	2	TAD	during induction	78
M; 52	CVC	2	TAD	during induction	17
M; 42	PE*	1	TAD	during induction	98
M; 56	PE*	2	TAD	during induction	159
F; 54	DVT	1	TAD	after induction / during HDM	777
F; 46	DVT	1	TAD	during induction	61
M; 54	DVT	2	VAD	during induction	93
F; 58	PE	1	TAD	during induction	75
M; 60	PE	3	VAD	during induction	48
F; 51	DVT	1	VAD	before start of treatment	-28 (before treatment)
M; 38	DVT	2	BAD	during induction	57
M; 57	PE	1	BAD	during induction	9
F; 65	PE	2	VAD	during induction	57

* = also arterial thrombotic event (after VTE).
BAD = bortezomib, doxorubicin, and dexamethasone; CVC = central venous catheter-related VTE; DVT = deep vein thrombosis of the leg; EA complexes = human neutrophil elastase-α1-antitrypsin complexes; HDM = high dose melphalan treatment; MM = multiple myeloma; PE = pulmonary embolism; TAD = thalidomide, doxorubicin, and dexamethasone; VAD = vincristine, doxorubicin, and dexamethasone; VTE = venous thromboembolism.

Adjustment for heavy chain type non-IgG, ISS stage 3, or TAD treatment, did not alter these results. An association between elevated levels of nucleosomes or EA complexes (>80th percentile of non-VTE MM patients), and time to VTE, could also not be found (nucleosomes: HR 0.5; 95% CI 0.13-1.68; EA complexes: HR 1.4; 95% CI 0.30-6.28).

A significant difference was observed between median levels of EA complexes and the three ISS disease stages (Kruskal-Wallis 1-way ANOVA, p=0.03), while a similar, non-significant, trend was seen for median levels of nucleosomes and higher ISS disease stage (p=0.11; Figures 2A and 2B). No association was found between ISS stage and VTE (ISS 1 vs. ISS 3 OR 0.3; 95% CI 0.07-1.75, ISS 2 vs. ISS 3 OR 1.0; 95% CI 0.19-5.24).

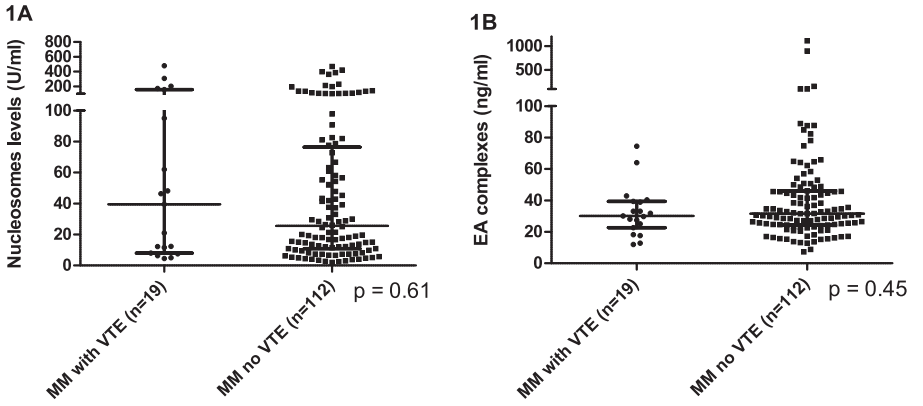

Figure 1. *Levels of nucleosomes and human neutrophil elastase-α1-antitrypsin (EA) complexes in patients with multiple myeloma, with and without VTE*
Median levels of circulating nucleosomes (**1A**) and neutrophil EA complexes (**1B**) in patients with multiple myeloma, with and without VTE. Bars represent interquartile ranges (IQR).

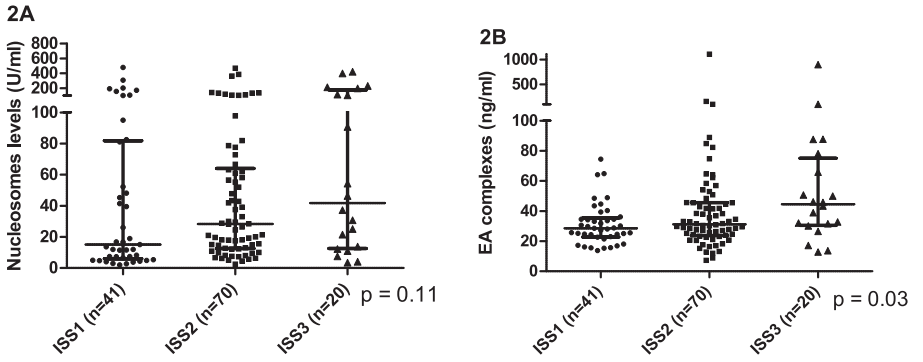

Figure 2. *Levels of nucleosomes and human neutrophil elastase-α1-antitrypsin (EA) complexes in patients with multiple myeloma, by International Staging System (ISS) disease stage.*
Median levels of circulating nucleosomes by ISS disease stage 1, 2 or 3 (**2A**), and of neutrophil EA complexes by ISS disease stage 1, 2 or 3 (**2B**), in patients with multiple myeloma. Bars represent interquartile ranges (IQR).

Secondary analysis: Patients with MM, DVT cases and DVT controls with other malignancies, and DVT cases and DVT controls without malignancy

Of 149 patients with symptomatic DVT of the leg (DVT cases), 26 patients had a malignancy and 123 did not. Of 181 matched controls without DVT (DVT controls), 24 had a malignancy and 157 did not. Table 3A shows baseline characteristics of the

Table 3A. Characteristics of DVT cases, with malignancies and without malignancy

	All DVT cases N=149	With malignancy N=26	Without malignancy N=123	p-value*
Median age upon DVT evaluation, years (IQR)	57 (45-70)	64 (55-74)	55 (43-68)	0.026
Male sex, n (%)	69 (46.3)	11 (42.3)	58 (47.2)	NS
Type of malignancy				
Hematological malignancy, n (%)		2 (8.3)		
Breast cancer, n (%)		5 (20.8)		
Gastrointestinal cancer, n (%)		5 (20.8)		
Pancreatic cancer, n (%)		2 (8.3)		
Lung cancer, n (%)		1 (4.2)		
Prostate cancer, n (%)		1 (4.2)		
Skin cancer, n (%)		1 (4.2)		
Other, n (%)		6 (25.0)		
Unknown, n (%)		1 (4.2)		
Nucleosome levels, median (IQR)	17.0 (8.5-35.0)	35.5 (12.5-55.8)	16.0 (8.0-32.0)	0.004
EA complexes levels, median (IQR)	53.0 (42.6-71.0)	59.0 (46.0-72.3)	51.0 (41.8-71.3)	NS

DVT = deep vein thrombosis of the leg; EA = human neutrophil elastase-α1-antitrypsin; IQR = interquartile range; NS = not significant. *of comparison DVT patients with malignancy versus DVT patients without malignancy.

Table 3B. Characteristics of DVT controls, with malignancies and without malignancy

	All DVT controls (N=181)	With malignancy N=24	Without malignancy N=157	p-value*
Median age upon DVT evaluation, years (IQR)	56 (47-71)	64 (54-78)	55 (45-69)	0.011
Male sex, n (%)	81 (44.8)	13 (54.2)	68 (43.3)	NS
Type of malignancy				
Hematological malignancy, n (%)		1 (4.5)		
Breast cancer, n (%)		4 (18.2)		
Gastrointestinal cancer, n (%)		2 (9.1)		
Pancreatic cancer, n (%)		-		
Lung cancer, n (%)		4 (18.2)		
Prostate cancer, n (%)		5 (22.7)		
Skin cancer, n (%)		2 (9.1)		
Other, n (%)		1 (4.5)		
Unknown, n (%)		3 (13.6)		
Nucleosome levels, median (IQR)	9.0 (5.0-17.0)	11.0 (5.8-28.8)	8.0 (5.0-16.5)	NS
EA complexes levels, median (IQR)	44.0 (33.0-55.0)	47.0 (30.5-62.5)	44.0 (33.0-55.0)	NS

DVT = deep vein thrombosis of the leg; EA = human neutrophil elastase-α1-antitrypsin; IQR = interquartile range; NS = not significant. *of comparison DVT controls with malignancy versus DVT controls without malignancy.

149 DVT cases with malignancy and without malignancy, and Table 3B of the 181 DVT controls with malignancy and without malignancy.

Patients with MM and VTE had similar levels of nucleosomes compared with DVT cases with other malignancies, and significantly higher levels compared with DVT cases without malignancy. MM patients without VTE had significantly higher levels of nucleosomes compared with DVT controls, irrespective of malignancy status (Figures 3A and 3B). In contrast, patients with MM, irrespective of VTE, had significantly lower levels of EA complexes compared with both DVT cases and DVT controls, irrespective of malignancy status (Figures 4A and 4B).

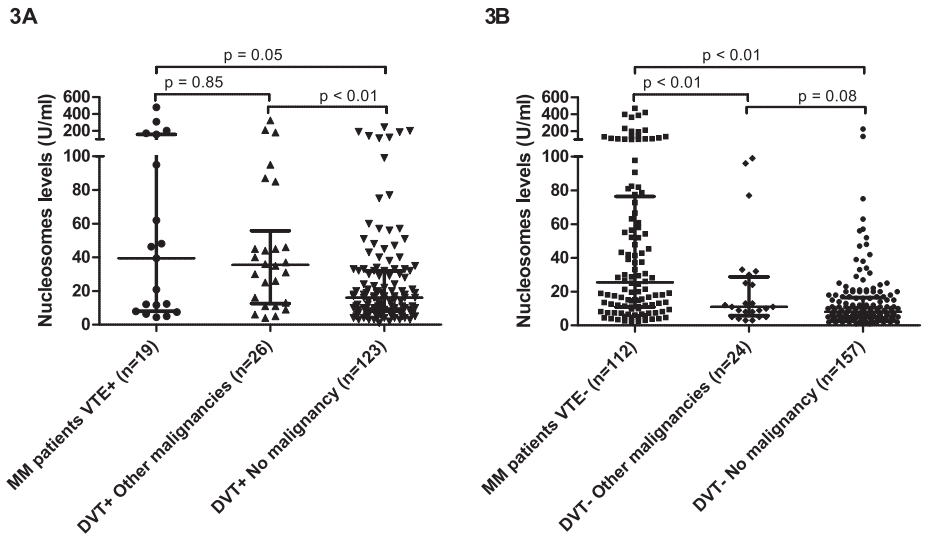

Figure 3. *Levels of nucleosomes in patients with multiple myeloma (MM) with and without venous thromboembolism (VTE), compared with deep vein thrombosis (DVT) of the leg cases with other malignancies or without malignancy, and DVT controls with other malignancies or without malignancy*
Median levels of circulating nucleosomes in patients with MM with VTE during treatment, compared with DVT cases with other malignancies or without malignancy (**3A**), and in patients with MM without VTE during treatment, compared with DVT controls with other malignancies or without malignancy (**3B**). Bars represent interquartile ranges (IQR).

Discussion

In this study, we observed elevated levels of nucleosomes in patients with MM in comparison with DVT cases with other malignancies and without malignancy, and DVT controls with other malignancies and without malignancy, while levels of neutrophil activation were significantly lower in all comparisons.

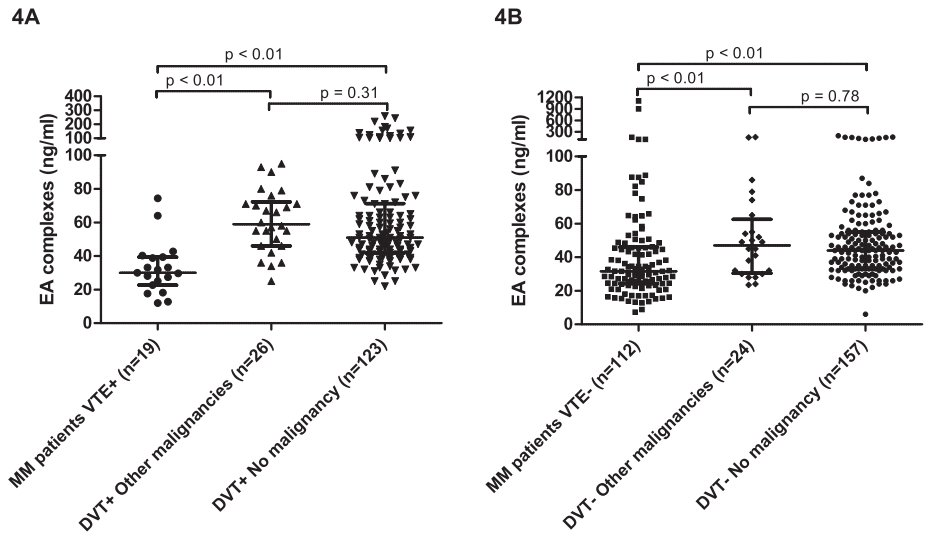

Figure 4. *Levels of neutrophil activation, as evidenced by presence of human neutrophil elastase-α1-antitrypsin (EA) complexes, in patients with multiple myeloma (MM) with and without venous thromboembolism (VTE), compared with deep vein thrombosis (DVT) of the leg cases and DVT controls, with other malignancies or without malignancy*

Median levels of EA complexes in patients with MM with VTE during treatment, compared with DVT cases with other malignancies or without malignancy (**4A**), and in patients with MM without VTE during treatment, compared with DVT controls with other malignancies or without malignancy (**4B**). Bars represent interquartile ranges (IQR).

Although high levels of nucleosomes in MM patients may indicate a hypercoagulable state, our results also showed that there was no association between circulating nucleosomes, neutrophil activation, and VTE in MM patients. In addition, there was no difference in median levels of nucleosomes or neutrophil activation between MM patients with or without VTE during treatment. These results are in line with previous studies on prothrombotic abnormalities and VTE in patients with MM. High levels of von Willebrand factor, factor VIII, D-dimer and tissue factor have been observed in patients with MM, but no association could be found between high levels of these biomarkers and VTE during MM treatment.[5-7, 29]

The analyzed plasma samples were taken before start of MM treatment, and not at the acute moment of VTE diagnosis. Therefore, it is possible that we may have missed an important elevation of nucleosomes and neutrophil activation at the acute moment of VTE onset, and thereby an association between nucleosomes, neutrophil activation and VTE. The lack of association between elevated levels of nucleosomes, or coagulation factors, and VTE suggests that VTE cannot be predicted upon diagnosis in MM patients, or that VTE during MM treatment is not causally associated

with elevated levels of nucleosomes or coagulation factors. The latter suggestion is supported by the fact that aspirin is similarly effective for primary prevention of VTE during MM treatment compared with LMWH or vitamin K antagonists.[30, 31] These observations propose that other mechanisms, e.g. platelet activation and aggregation, may be of greater importance for MM-related VTE than procoagulant mechanisms.

A significant difference was found between median levels of neutrophil activation (EA complexes) and higher ISS disease stage, together with a non-significant trend between higher median levels of nucleosomes and higher ISS disease stage. This is in line with previous studies, in which higher levels of von Willebrand factor and factor VIII were demonstrated with more severe disease stage of MM.[4, 5] Mechanisms underlying higher levels of these biomarkers with more severe MM could be related to increased cytokine release in patients with more severe disease stage.[4] Indeed, IL-6 levels in patients with MM have been shown to reflect disease severity.[3] IL-6 can also activate neutrophils; hence, the observed increase in median levels of neutrophil activation with higher ISS disease stage may be a reflection of higher IL-6 levels according to disease severity. We did not find an association between ISS disease stage and VTE in our study. Further studies are therefore needed to determine the role of activated neutrophils in the pathogenesis of MM.

The source and clinical relevance of high levels of nucleosomes in patients with MM require further investigation. As levels of EA complexes were contrastingly low, circulating nucleosomes in MM patients do not seem directly correlated with neutrophil activation. Hence, nucleosomes in MM patients likely derive from other sources than activated neutrophils or NETs formation, such as death of endothelial cells, parenchymal cells and /or tumor cells, which implies plasma cells in our study population.[32, 33] This is in line with studies that have demonstrated presence of cell-free (tumor) DNA in plasma of cancer patients.[34] In studies with colorectal cancer or lymphoma patients, cell-free DNA was used as prognostic marker and for disease monitoring.[35, 36] Cell-free DNA in cancer patients is suggested to derive from active release by highly proliferating cells and deceased cells (after apoptosis), as well as from interactions between tumor and adjacent non-tumor cells.[34, 37, 38] Indeed, protease mediated nucleosome release from apoptotic cells by factor VII activating protease (FSAP), together with DNAse I from necrotic cells, has been observed in human plasma.[39, 40] Hence, elevated levels of nucleosomes in patients with MM may be a marker of plasma cell injury and plasma cell death rather than of coagulation activation. This would explain the absence of an association between nucleosomes, neutrophil activation and VTE during MM treatment. Whether nucleosomes are also suitable as prognostic marker, or for disease monitoring in patients with MM, requires further research.

We observed significantly higher levels of nucleosomes in patients with MM compared with DVT cases without malignancy, as well as with DVT controls with other malignancies and without malignancy. High levels of circulating DNA have previously been described in patients with MM in comparison with patients with colorectal cancer or healthy individuals.[41, 42] It is possible that plasma cells in MM more actively undergo apoptosis, and/ or more actively release nucleosomes in comparison with other types of cancer cells. However, additional research is needed to confirm this.

Further studies are also required to clarify the significantly lower levels of neutrophil activation observed in patients with MM compared with DVT cases and DVT controls, irrespective of malignancy status. This could be due to the different timing of blood collection; blood was collected before start of treatment in MM patients and not upon VTE diagnosis, whereas it was collected at the acute moment of DVT evaluation in DVT cases and controls. However, neutrophil activation may also be impaired in MM patients. Previous studies have described attenuation of neutrophil activation in MM due to the use of thalidomide,[43] and defective neutrophil maturation due to bone marrow infiltration in MM.[44]

In conclusion, elevated levels of circulating nucleosomes were found in patients with MM, but there was no association between nucleosomes, neutrophil activation, and VTE during MM treatment. Our results suggest that nucleosomes in MM are a marker of plasma cell death rather than of neutrophil or coagulation activation. However, further studies are needed to investigate the source and clinical and prognostic relevance of nucleosomes in MM.

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