Acquired coagulation abnormalities and thrombosis in Multiple Myeloma

Johannes J.A. Auwerda

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Stollings afwijkingen en trombose bij het Multiple Myeloom

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If you love something, let it go....
If it comes back to you, it's yours to have.
If not, it never was yours to begin with.

4M 41gels

CONTENTS

Chapter

General introduction

1.1 MULTIPLE MYELOMA

Multiple Myeloma (MM) is a malignant plasma cell disorder which accounts for approximately 10% of the malignant hematologic neoplasms(1, 2). In the pathophysiology of MM, the interaction between myeloma cells and the bone marrow microenvironment leads to a complex signalling network that sustains survival of the malignant cell and mediates tumour progression and drug resistance. Major signalling pathways involved are the IL-6R/STAT3, Ras/MAPK, PI3K/Akt, notch, WNT- and NF-κB pathways(3). The cytokine interleukin-6 is presumed to play a pivotal role in the pathogenesis and malignant growth of MM(4) and IL-6 levels are elevated in case of active disease(5). IL-6 also plays a stimulatory role in the coagulation mechanism(6). It has been shown to promote the transcription of the factor VIII gene(7), decrease protein S levels in canine models(8), induce ultra large and hyperreactive von Willebrand Factor(9), and inhibit the cleavage of ultra large von Willebrand Factor(6).

In search for the optimal treatment for MM, many regimens have been introduced during the past twenty years resulting ultimately in a significant improvement in survival(10). The first standard of care was a melphalan/prednison based regimen with complete remission rates of less than five percent. In the early eighties, high dose melphalan was introduced in combination with autologous stem cell infusion support(11). This resulted in a complete remission rate of up to 50 percent and a moderate increase in mean overall survival of less than three years to nearly 5 years. The high dose melphalan was usually preceded by multi-agent chemotherapy such as vincristin, doxorubicine and dexamethason (VAD) to reduce tumor burden. In search for newer strategies and therapy regimens, thalidomide was introduced, due to its anti-angiogenic effect, and exhibited exciting responses in refractory and/or relapsed MM(12, 13). The most recently developed and introduced treatment is the proteasome inhibitor Bortezomib(14). Bortezomib was introduced as a second or third line treatment option in refractory and or relapsed MM. Currently this drugs is investigated in the upfront setting in clinical trials. Novel agents including lenalidomide and anti interleukin-6 antibodies are also currently under investigation in MM.

The current standard of care for younger patients (<65years of age) with MM who are in good performance status is combination chemotherapy using non-alkylator-containing regimens such as VAD as an induction therapy followed by high dose melphalan in

combination with autologous stem-cell transplantation(15). VAD induction courses are now preferred over regimens containing melphalan because they are less toxic to bone marrow stem cells and allow stemcell harvesting prior to high dose therapy. Recently also thalidomide containing induction regimens have been introduced (thalidomide, doxorubicin and dexamethason: TAD). Patients treated for MM may encounter significant toxicity such as (catheter related) infection, sepsis and venous thrombosis. Especially doxorubicin containing chemotherapy regimens in combination with thalidomide have been shown to enhance the risk of venous thrombo-embolic events(16).

1.2 MALIGNANCY AND VENOUS THROMBOEMBOLISM

Deep vein thrombosis (DVT) and pulmonary embolism (PE), are manifestations of venous thromboembolism (VTE). The annual age- and sex-adjusted incidence of VTE in the general population, is $1^0/_{00}$, including $0.5^0/_{00}$ for DVT and $0.7^0/_{00}$ for PE(17). In cancer patients, the incidence is more than 7% with certain cancers even having a significantly higher risk of VTE (18). Factors that increase this risk include high age, recent surgery, recent trauma, hormonal therapy and long periods of immobility(19). The French physician Armand Trousseau was the first to recognize the association between malignancy and VTE(19). In patients with overt cancer, spontaneous venous thrombosis, thromboembolism after cancer surgery, thromboembolism during chemotherapy and thrombosis of central venous access lines occur in 10-15% as clinical manifestations of thrombosis(20). Advanced tumour stage, conventional chemotherapy, and immunomodulators in combination with chemotherapy increase the risk of VTE in cancer patients(21-23). Overall, cancer increases the risk of thrombosis more than four-fold and chemotherapy 6.5-fold(21).

Hereditary risk factors for VTE include the factor V Leiden mutation and the prothrombin 20210A mutation. In the general population the factor V Leiden mutation increases the risk of VTE by 3-8 fold(24). In cancer patients who are carriers of the factor V Leiden mutation the risk of VTE is increased to 12-fold compared to non cancer patients who are carriers(25). The protrombin 20210A mutation increases the risk of VTE approximately two-fold in the general population(26) and in cancer patients this mutation increases the risk of VTE similar to that seen in cancer patients with the factor V Leiden mutation(25).

1.3 INCIDENCE OF THROMBO-EMBOLIC COMPLICATIONS IN MULTIPLE MYELOMA

The first report regarding an increased incidence of VTE during or shortly after multi-agent chemotherapy in patients with newly diagnosed MM dates from 1999(27). In this report a VTE incidence of 10% was reported in newly diagnosed patients receiving VAD or high dose cyclophosphamide as induction chemotherapy to reduce tumour burden(27). Since then, many reports regarding the incidence of thrombo-embolic complications in patients with either newly diagnosed, refractory or relapsed MM have been reported (summarized in Table 1, 2a and 2b).

Among the reported VTE complications DVT of the lower extremity is the most frequent occurring events. Less frequently observed are central venous catheter-related thrombosis, pulmonary embolism and venous thrombosis of the arm(28, 29). More rarely observed thrombotic complications concern arterial thrombosis including myocardial infarction, intestinal ischemia, and ischemic stroke(30-32).

The incidence of VTE varies between the different regimens used to treat MM. Oral therapy with melphalan and prednisone (MP) is associated with the lowest incidence of VTE of 0-2%(31, 33). Thalidomide, an immunomodulatory drug, used as a single agent, is associated with a similar incidence of VTE of 2%(34). However, thalidomide in combination with dexamethasone only or in combination with cytotoxic chemotherapy regimens, increases the risk of VTE significantly(35). The combination of thalidomide and dexamethasone (thal/dex) seems to be associated with a VTE incidence of 7-26%(1, 13, 36-42). When combined with the oral MP regimen (MPT), incidences of 12-16% are reported in elderly patients(31, 43). The VTE incidence during regimens combining thalidomide with antracyclines based cytotoxic chemotherapy regimens ranges from 10-35%(16, 28, 44-51). Lenalidomide is a new immunomodulatory drug with a similar structure to thalidomide but functionally distinct. In a multicenter phase 3 trial in relapsed/refractory MM patients, thromboembolic events were reported in 4.5% and when lenalidomide was combined with dexamethasone this incidence increases to 8.5%(52). The recently introduced proteasome inhibitor Bortezomib seems to be less thrombogenic and up till now the reported incidence of thrombo-embolic complications remains low (0-1%), even when used in combination with dexamethasone(53-55).

TABLE 1. VENOUS THROMBO-EMBOLIC COMPLICATIONS IN PREVIOUSLY TREATED/RELAPSED/REFRACTORY MM

Study	Regimen	No. Pts.	Age (range)	Male	VTE incidence (%)
Single agent thalidomide					
Barlogie et al(34)	T	169	40% >60jr		2%
Streetly et al(56)	T	15	67 (55-81)	60%	20%
Schey et al(32)	T	69	62 (39-84)	46%	10%
Thalidomide and dexamethasone					
Tosi et al(57)	TD	65	63 (35-78)	71%	1.5%
Anagnostopoulos et al(37)	TD	47	48 (31-77)	?	8%
Thalidomide plus chemotherapy					
Urbauer et al(49)	DCEP + T	14	?	?	21%
Galli et al(30)	Chemotherapy* & Thal	199	66	?	9%
Schutt et al(48)	VED + T (60% + HDM)	31	57 (32-77)	68%	26%
Minnema(46)	Various chemotherapy +T	20	?	?	35%
Single agent Bortezomib					
Jagannath et al(53)	Bortezomib	256	61 (30-84)	?	<1%
Lenalidomide plus dexamethasone					
Dimopoulos (58)	LD	?	?	?	8.5%
Lenalidomide plus chemotherapy					
Morgan (59)	LCD	21	59 (34-76)	?	14%
Lenalidomide plus bortezomib					
Richardson(60)	LB	19	?	?	0%

MP = melphalan and prednisone, D = dexamethasone, T = thalidomide, TD = thalidomide and dexamethasone, MPT = melphalan, prednisone, and thalidomide, CCT = combination chemotherapy, VAD = vincristin, doxorubicin, and dexamethasone, HDM = high dose melphalan, DDT = doxorubicin, dexamethasone, and thalidomide, DCEP = dexamethasone, cyclophosphamide, etoposide, and cisplatin, LD = lenalidomide and dexamethasone, LCD = lenalidomide, cyclophosphamide and dexamethasone, LB = lenalidomide and bortezomib, *chemotherapy not specified

TABLE 2A. VENOUS THROMBO-EMBOLIC COMPLICATIONS IN NEWLY DIAGNOSED MM

Study	Regimen	No. Pts.	Age (range)	Male	VTE incidence (%)
Single agent dexamethasone					
Rajkumar et al(41)		102	65 (38-82)	59%	3%
Thalidomide and dexamethasone					
Osman et al(47)	TD	45	?	?	7%
Dimopoulos et al(13)	TD	44	67 (38-87)	73%	7%
Cavo et al(38)	TD	19	?	?	26%
Rajkumar et al(42)	TD	50	61 (33-78)	62%	12%
Abdelkefi et al(36)	TD (80% + OAC)	60	49 (33-60)	47%	3%
Palumbo et al(40)	TD	41	71 (61-82)	55%	20%
Cavo et al(39)	TD	100	54	?	15%
Palumbo et al(40)	TD	41	71 (61-82)	55%	20%
Cavo et al(39)	TD	100	54	?	15%
Rajkumar et al(41)	TD	102	65 (38-83)	51%	17%
Multiagent chemotherapy					
Rus et al(31)	MP	64	71,5	?	0%
Palumbo et al(43)	MP	126	72	?	2%
Barlogie et al(27)	VAD-HDM	231	51 (26-71)	62%	10%
Zangari et al(50)	DPACE	100	56 (32-71)	67%	4%
Zangari et al(16)	VAD/DCEP	134	?	65%	14%
	VAD/DCEP	62	?	57%	15%
	VAD/DCEP	68	?	?	15%
Cavo et al(39)	VAD	100	54	?	2%
Cavo et al(39)	VAD	100	54	?	2%
Barlogie(44)	VAD/CEPD	345	?	61%	30%
Minnema(29)	VAD	211	?	?	9%

MP = melphalan and prednisone, T = thalidomide, TD = thalidomide and dexamethasone,
MPT = melphalan, prednisone, and thalidomide, CCT = combination chemotherapy, VAD = vincristin, doxorubicin,
and dexamethasone, HDM = high dose melphalan, DDT = doxorubicin, dexamethasone, and thalidomide,
DCEP = dexamethasone, cyclophosphamide, etoposide, and cisplatin.

TABLE 2B. VENOUS THROMBO-EMBOLIC COMPLICATIONS IN NEWLY DIAGNOSED MM

Thalidomide plus chemotherapy					
Study	**Regimen**	**No. Pts.**	**Age (range)**	**Male**	**VTE incidence (%)**
Rus et al(31)	MPT	67	72	?	16%
Palumbo et al(43)	MPT	65	72	?	12%
Dimopoulos et al(33)	MDT	50	77 (75-85)	58%	9%
Zangari et al(50)	DPACE + T	100	56 (32-71)	67%	28%
Osman et al(47)	DDT	15	?	?	27%
Zangari et al(61)	CCT + T	62	?	58%	19%
Zangari et al(28)	DPACE + T	192	60	65%	16%
	DCEP + T	40	58	56%	2,5%
Zangari et al(16)	VAD/DCEP + T	122	?	?	34%
Zervas et al(51)	VAD + T	39	68 (43-75)	51%	10%
Hassoun et al(45)	AD-TD	42	59 (35-82)	62%	12%
Barlogie(44)	VAD/DEPD+T	323	?	58%	17%
Minnema(29)	TAD + LMWH	201	?	?	5%
Thalidomide plus lenalidomide					
Rajkumar(62)	Len/High dose dexameth	223	65	?	25%
Rajkumar(62)	Len/low dose dexameth	222	65	?	9%
Thalidomide plus Bortezomib and chemotherapy					
Jagannath(63)	TBDC	25	60	65%	0%
Lenalidomide plus dexamethasone					
Rajkumar(64)	LD (high dose Dex)	223	?	?	25%
Rajkumar(64)	LD (low dose Dex)	222	?	?	9%
Zonder (65)	LD	21	?	?	75%

MP = melphalan and prednisone, T = thalidomide, TD = thalidomide and dexamethasone, MPT = melphalan, prednisone, and thalidomide, CCT = combination chemotherapy, VAD = vincristin, doxorubicin, and dexamethasone, HDM = high dose melphalan, DDT = doxorubicin, dexamethasone, and thalidomide, DCEP = dexamethasone, cyclophosphamide, etoposide, and cisplatin.

1.4 HEMOSTATIC ABNORMALITIES IN MULTIPLE MYELOMA

The development of a thrombus depends on three elements which were first described by Virchow over 150 years ago (Virchow's triad)(66). These elements include stasis and reduced blood flow, abnormal blood coagulability and an altered blood vessel wall. The formation of a thrombus depends on the primary- and secondary haemostatic system. In the primary haemostasis circulating platelets interact with activated endothelial cells or the subendothelial matrix to form mural thrombi. The interaction of platelet GPIbα or GPVI with von Willebrand factor (vWF) and collagen, initiates activation of integrin αIIbβ3 that binds von Willebrand factor and fibrinogen. This eventually results in platelet-platelet interaction and platelet aggregation, finally resulting in the formation of a platelet plug (Figure 1)(67, 68).

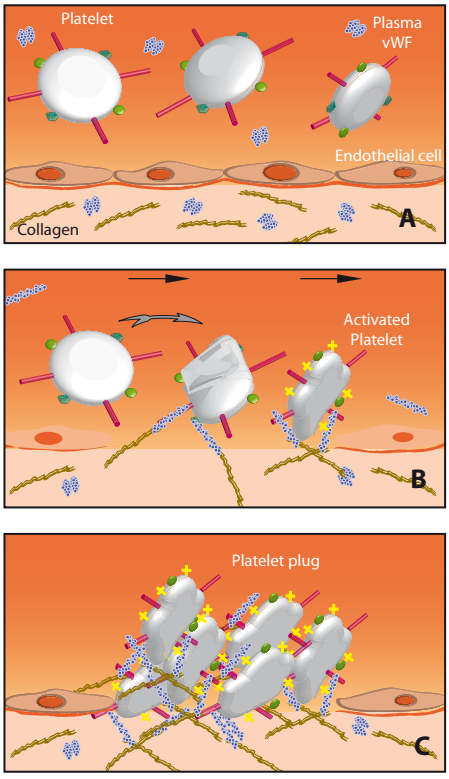

Figure 1. Primary haemostatic system.When the vessel wall is intact, plasma von Willebrand factor (vWF) that is present in a coiled structure and platelets coexist in circulating blood with minimal interactions (Panel A). In the damaged vessel wall (Panel B), collagen of the subendothelial matrix becomes exposed and binds to plasma von Willebrand factor which than uncoils and supports the adhesion of platelets and promote platelet adhesion and activation through a stepwise proces. This allows plates to bind to the vessel wall and forms a platelet plug (Panel C).

Secondary hemostasis is initiated by Tissue Factor (TF) and proceeds through a stepwise activation of proteases that eventually results in a fibrin network which stabilises the platelet plug and forms a thrombus (Figure 2)(69).

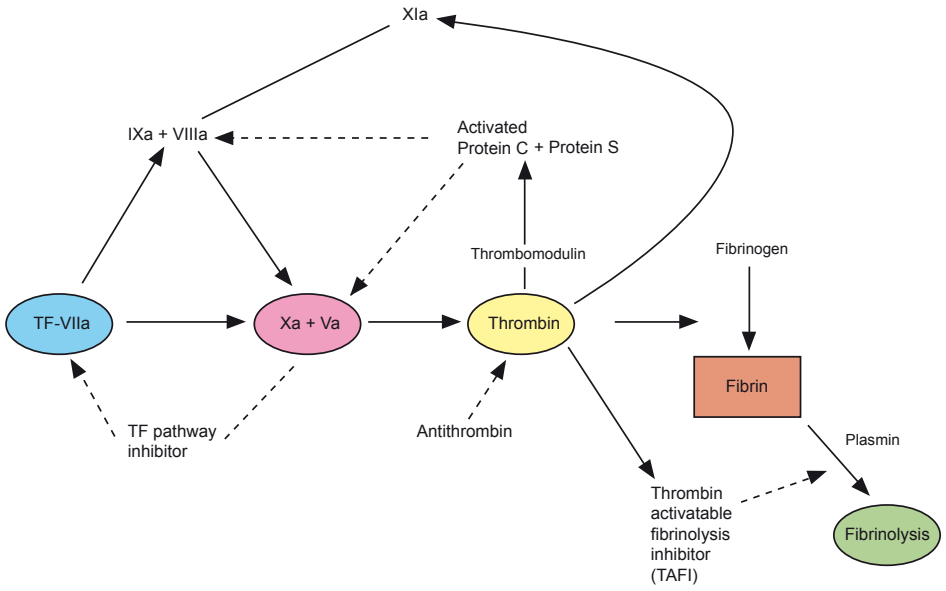

Figure 2. Secondary haemostatic system, inhibition (-------), activation (———). Plasmin induces the breakdown of fibrinogen.

In this cascade, tissue factor expression is a critical step in the initial formation of fibrin. The formation of the clot is highly regulated by natural anticoagulant mechanisms such as antithrombin, the protein C system and the fibrinolytic system. It is well established that patients with malignancy have a high risk of VTE which is suggested to be a result of an altered balance between the coagulation and fibrinolytic systems resulting in a prothrombotic or hypercoagulable state(70). In patients with malignancy, also other factors may increase the risk of thrombosis, including the use of central venous catheters, immobilisation and infections.

Well known additional risk factors for thrombo-embolic complications specifically encountered in patients with MM include increased age, use of anti-angiogenic drugs in combination with chemotherapy, and high levels of acute phase proteins(71). Recently, in

MM, new acquired coagulation changes have been identified that are associated with a hypercoagulable state. These changes involve factors that are related to both primary and secondary hemostasis such as increased FVIII levels and vWF levels and acquired resistance to activated protein C (APC)(35, 46, 72).

Most thrombo-embolic episodes are observed in the early course of treatment and it has been suggested that they may be related to a high tumor load(73). Furthermore, the release of inflammatory cytokines, especially interleukin (IL)-6, which plays a pivotal role in MM, may alter the coagulation mechanism into a hypercoagulable state(74). In contrast, inherited thrombophilic factors, including Factor V-Leiden mutation and the prothrombin gene mutation, seem to play only a marginal role(75).

1.5 PREVENTING THROMBO-EMBOLIC COMPLICATIONS IN MULTIPLE MYELOMA

The recognition of a hypercoagulable state in newly diagnosed MM, especially during multi-agent chemotherapy in combination with anti-angiogenic drug, has led to the introduction of prophylactic anticoagulant therapy. The use of either low molecular weight heparin or full-intensity warfarin (target INR: 2-3) has been shown to reduce the risk of thrombo-embolic complications(29, 76). A fixed low dose of warfarin however, seems to reduce this risk insufficiently(16). The use of a fixed low-dose aspirin as thrombo-phophylaxis in clinical trials has also been shown to reduce the incidence of venous thromboembolism in MM(77). The optimal antithrombotic regime in MM patients receiving induction treatment has yet to be established and is currently under investigation(78).

1.6 AIMS AND OUTLINE OF THE THESIS

As previously described, patients with untreated MM exhibit a hypercoagulable state which makes these patients susceptible to thrombo-embolic complications. Although some coagulation abnormalities have been reported in the literature, there are still many questions left unanswered and the mechanism of VTE in MM is still not yet fully understood. Therefore we initiated several studies to increase our knowledge regarding the hypercoagulable state in patients with MM.

Since most thromboembolic complications in untreated MM patients are observed during the induction phase of treatment, our first aim is to study the presence of coagulation abnormalities in patients with newly diagnosed MM who are eligible for intensive multi-agent chemotherapy **(chapter 2)**. Both the primary and the secondary hemostasis-related coagulation factors are determined prior to the start of intensive cytoreductive chemotherapy.

The second aim of the study is to monitor the disorders in the coagulation system during and after intensive chemotherapy. For this, we determined the plasma levels of various coagulation factors at start of treatment and at several time points during and after chemotherapy **(chapter 3)**.

Our third aim is to investigate the fibrinolytic system in these patients and determine alterations of fibrinolysis during treatment, since hypofibrinolysis is known to be associated with venous thrombosis **(chapter 4)**.

Recent data indicate that microparticle associated tissue factor (MP-TF) activity is increased in patients with adenocarcinoma and that there is an association with the development of thrombosis(79). Our fourth aim is to assess the level of MP-TF activity in patients with MM prior to and during chemotherapy **(chapter 5)** and to determine whether this is related to the subsequent development of VTE.

Recently, an increased thrombo-embolic incidence has also been reported in patients with other malignant and benign plasma cell disorders. Therefore we studied the levels of the above mentioned coagulation variables in patients with other plasma cell disorders such as Waldenstrom's macroglobulinemia (WM), systemic Amyloidosis (AL) and monoclonal gammopathy of uncertain significance (MGUS) and compared these with our findings in MM patients **(chapter 6)**.

Various prophylactic regimens, in order to prevent thrombo-embolic complications during the course of either first line of second line of therapy for MM, have been investigated and described in the literature. There is however, still debate regarding which antithrombotic prophylaxis regimen should be used. Therefore a study is initiated to investigate thrombo-prophylaxis with low molecular weight heparin (LMWH) in patients with MM, who are treated with a combination of chemotherapy and thalidomide. The aim of this study is to determine whether LMWH can reduce the incidence of VTE during anti-angiogenic based chemotherapy **(chapter 7)**.

In chapter 8 we present a case history which illustrates how coagulation factor changes in multiple myeloma can alter the clinical phenotype of a patient with severe type 2A von Willebrand disease. In this case we studied the vWF/FVIII levels in a patient with von Willebrand disease who develops MM and experiences a temporary relief of bleeding symptoms during chemotherapy due to the increase of vWF levels **(chapter 8).**

Finally a case is presented of a patient with a plasma cell disorder suffering from therapy resistant ulcerating vasculitis due to a MGUS related cryoglobulinemia **(chapter 9)**. This patient developed a rare and severe complication as a result of frequent plasmapheresis with the plasma expander hydroxy-ethyl starch (HES). We extensively studied the accumulation of HES a group of patients undergoing plasmapheresis in whom HES was used as plasma substitution.

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Chapter 2

Prothrombotic coagulation abnormalities in patients with newly diagnosed Multiple Myeloma

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ABSTRACT

Background

A high incidence of thrombo-embolic complications has been reported in patients with Multiple Myeloma, especially when these patients receive multi-agent chemotherapy in combination with anti-angiogenic drugs. The mechanism is still poorly understood and studies regarding protrombotic risk factors are still limited. Therefore we studied various prothrombotic risk factors in large group of consecutive patients with newly diagnosed Multiple Myeloma. Patients were classified according to the recently introduced International Staging System.

Patients and Results

A total of 135 MM patients and 124 healthy controls were included in this study. Increased factor VIII:C (FVIII:C 2.11 ± 1.15 U/ml) and von Willebrand factor antigen (vWF:Ag 1.92 ± 1.13 U/ml) and ristocetin cofactor activity (vWF:RCo 1.78 ±1.08 U/ml) levels were observed compared to control subjects ($p<0.001$). The increase of FVIII and vWF levels were strongly related to the disease stage, with the highest levels in stage III. Protein S levels were significantly reduced in the most severe disease stage III compared to stage I.

Conclusion

Our study indicates that various prothrombotic abnormalities occur in patients with multiple myeloma, which may contribute to the increased the risk of venous thrombo-embolism observed in these patients.

INTRODUCTION

Patients with Multiple Myeloma (MM) are at a high risk for venous thromboembolism (VTE) with an incidence up to 30 percent, especially when receiving multi-agent chemotherapy and anti-angiogenic drugs (1-4). Several potential mechanisms have been suggested in the literature, such as increased viscosity as a result of high levels of circulation immunoglobulins, autoantibodies against natural anti-coagulants and increased cytokine levels(5). The first report on altered coagulation factors dates back to 1976 and concerned an observed increase in FVIII levels(6). However, only a few small-size studies on coagulation abnormalities in MM have been performed so far and these factors have yet not been studied in detail in a large group of untreated MM patients. Furthermore, it has not yet been elucidated what their contribution is to the development of VTE in patients suffering from MM. Recently, high levels of FVIII and vWF have been reported by Minnema et al. in patients with MM, which may contribute to the increased risk of thrombosis(7). Also, it has been postulated that acquired resistance to protein C, which was observed in up to 23 percent of patients with MM, may be associated with the increased VTE risk(8). Despite these reports, still only limited information is available on other prothrombotic coagulation abnormalities in MM patients. In order to further elucidate the underlying mechanisms for VTE in MM patients, we performed this prospective study to evaluate prothrombotic coagulation abnormalities in a large group of patients with newly diagnosed MM and whether this was related to disease status and the development of VTE.

MATERIALS AND METHODS

Patients

Consecutive patients with newly diagnosed MM according to the Mayo Clinic criteria(9) who were admitted to the department of Haematology of the Erasmus MC, an academic tertiary referral hospital, were included in the study. Patients who were eligible for intensified chemotherapy followed by high dose melphalan and autologous stem cell support. Their stage of disease was determined according to the recently introduced International Staging System (ISS)(10). Thromboprophylaxis consisting of low dose molecular weight heparin was given to the patients who received thalidomide based regimen. We also included a control group of 124 healthy controls, who were partners or friends of patients visiting the outpatient department of Haematology of our hospital. The Medical Ethical

Committee of the Erasmus MC approved the study and written informed consent was obtained from the patients before performing coagulation studies.

Coagulation variables

Venous blood was collected using a vacutainer system in citrate (0.105 M, Beckton-Dickinson, Plymouth, UK) and centrifuged at 4°C at 2.000 g for 10 minutes. The collected plasma was additionally centrifuged for 10 minutes at 20.000 g and stored in small aliquots at –70°C until use. Genomic DNA was isolated from the white cell fraction of citrated blood, using a standard salting out procedure. FVIII:C was measured by a one stage clotting assay using Platelin (Organon Teknika,Durham, USA) and factor VIII deficient plasma (Biopool, Ventura, USA). VWF:Antigen (vWF:Ag) was measured by an in-house sandwich enzyme linked immunosorbent assay (ELISA) using rabbit anti-human vWF and horseradish peroxidase conjugated anti-human vWF (DakoCytomation, Glostrop, Denmark). vWF collagen binding activity (vWF:CB) was measured by an in-house EIA using type I collagen (Sigma, StLouis, USA) and horseradish peroxidase conjugated anti-human vWF. vWF Ristocetin Cofactor activity (vWF:RCo) was measured by an aggregometric method using formaline-fixed platelets and ristocetin (Diagnostiga Stago, Asnieres, France). All assays were calibrated with pooled normal plasma (factor assay control plasma, George King Bio-Medical, Kansas, USA). Fibrinogen was measured as described by von Clauss(11). Screening for lupus anticoagulant (LAC) was performed essentially as recommended by the Subcommittee on Lupus Anticoagulant / Antiphospholipid antibodies(12), and included screening assays (aPTT, PT and dilute prothrombin time) as well as confirmatory procedures to demonstrate the phospholipid dependence. The presence of lupus anticoagulant was tested by an aPTT based assay and a diluted (1:10 and 1:100) PT assay. If the initial test was abnormal, both of them were performed in a 1:1 mixture of patient and normal plasma. The aPTT-based lupus anticoagulant assay was performed with Platelin®LS (Organon Teknika, Oss, the Netherlands) and considered positive when longer than 34 seconds after 1:1 mixture with normal plasma. The diluted PT-assay was performed with diluted Recombiplastin® (Instrumentation Laboratory, IJsselstein, the Netherlands). The test was considered positive if the ratio in a 1:1 mixture with normal plasma was above 1.20. Anticardiolipin antibodies (ACA) were tested by enzyme-linked immunosorbent sandwich assay (ELISA), using cardiolipin (Sigma, Zwijndrecht, the Netherlands) and horseradish peroxidase conjugated-rabbit

anti-human IgG and IgM (Dakopatts). Anticardiolipin titers were calculated and considered positive if titers were above 32 U for IgG or above 12 U for IgM. Patients were considered antiphospholipid antibodies (APA) positive if one or both tests for APA were positive, i.e. if LAC was present and/or ACA-IgG or ACA-IgM were positive. The within assay variation coefficient for the ACA assay was 10% for IgG and 11% for IgM.

Antithrombin activity levels were determined using a chromogenic substrate. For the measurement of protein S we used an assay (Staclot® Protein S, Diagnostica Stago) which is known not to interfere with FVIII when levels are lower than 250%(13). The prothrombin G20210A gene variant and the factor V Leiden mutation were identified simultaneously, using a multiplex PCR method previously described(14). Activated protein C (APC) resistance was determined on citrated plasma using an aPTT-based Food and Drug Administration-approved resistance assay in the presence of excess factor V-deficient plasma (Coatest APC Resistance, Chromogenix Nodia/Schmidt). The ratios between the aPTT with or without the presence of activated protein C were calculated and the patient was considered positive if the ratio was less than 0.8.

Statistical analysis

Statistical analysis consisted of basic descriptive statistics and the results are presented as median ± standard. Levels of prothrombotic variables in the different ISS groups were compared using ANOVA, which takes multiple testing into account (Bonferoni correction with mean correlation=0.40). A p-value below 0.013 was considered significant.

RESULTS

Patients

One hundred and thirty-five consecutive patients with untreated MM admitted to the Department of Hematology of the Erasmus Medical Center, and academic tertiary referral hospital, who were eligible for intensified chemotherapy followed by high dose melphalan and autologous stem cell support were included in this study, as were 124 sex- and age-matched, healthy controls. The characteristics are summarised in Table 1. The median age of the patients was around 60 years and similar to the control group. Most of the patients were male (58%). The majority of the MM patients had ISS stage I or II (31% and 56% respectively).

TABLE 1. PATIENTS CHARACTERISTICS		
	Control	**Multiple Myeloma untreated**
N	124	135
Age	57 (11.6)	58 (8.4)
Male gender (%)	53%	58%
B2M (mg/l)	*	2.88 (5.42)
Platelets(x109/l)	*	245 (79)
Albumin (g/l)	*	37 (6.8)
Calcium (mmol/l)	*	2.35 (0.24)
Hb (mmol/l)	*	6.8 (1.2)
ISS stage I	*	42 (31%)
ISS stage II	*	75 (56%)
ISS stage III	*	18 (13%)

median (±SD of the mean). * not available

Prothrombotic risk factors in MM

The results of the coagulation variables are presented in Table 2. The prevalence of factor V Leiden mutation and G20210A Prothrombin gene variant was similar in the MM patients and control group (Table 2). At baseline the incidence of lupus anti-coagulant (LAC) was 4% and for anti-cardiolipin antibodies (ACA) 6% in the MM patients. Anti-phospholipid antibodies (APA) were re tested after 3 months and had disappeared in all cases.

Von Willebrand Factor antigen levels were significantly increased in patients with MM (1.92 ± 1.13 U/ml) compared to the controls (1.17 ± 0.50 U/ml, p<0.0001). A similar increase in vWF:RCo and vWF:CB activity was also observed (Table 2). Furthermore, FVIII:C levels were significantly higher in MM patients compared to healthy controls (2.11 ±1.15 versus 1.13 ±0.46 U/ml, P<0.001). In contrast, protein S activity levels in MM patients were in the lower normal range (0.72 ±0.25 U/ml). When staged according to the ISS criteria a significant difference in vWF antigen and activity levels in the various ISS stages was observed, with the highest levels in stage III (Table 2). This was also observed for Factor VIII:C levels. In addition, significantly lower protein S levels were seen in stage III disease compared to stage I. Other prothrombotic coagulation factors, including antithrombin, fibrinogen levels and D-dimer levels, were similar in patients and control subjects, and did not differ significantly between the ISS stages.

Thrombo-embolic complications were observed in 14 patients (10%) and occurred

most frequently during the induction chemotherapy (Table 3). There was no correlation between the ISS disease stage and the development of a VTE.

TABLE 2. SUBJECTS CHARACTERISTICS AND COAGULATION VARIABLES

	Controls	Patients	P-value	ISS			P-value disease stage
				Stage I	Stage II	Stage III	
N	124	135		42 (31%)	75 (56%)	18 (13%)	
Age	57 (11.6)	58 (8.4)					
Male gender (%)	53%	58%					
vWF Ag (U/ml)	1.17 (0.50)	1.92 (1.13)	0.0001*	1.69 (1.05)*	1.97 (1.10)	2.94 (1.16)*	0.008*
vWF CB (U/ml)	1.27 (0.70)*	1.84 (1.10)*	0.01*	1.73 (1.01)*	1.91 (0.97)	2.72 (1.36)*	0.008*
vWF Rco (U/ml)	1.06 (0.47)*	1.78 (1.08)*	0.0001*	1.60 (0.75)*	1.84 (1.10)	2.60 (1.33)*	0.002*
Factor VIII (U/ml)	1.13 (0.46)*	2.11 (1.15)*	0.001*	1.83 (0.80)*	2.34 (1.17)*	3.17 (1,35)*	0.001*
Protein C activity (U/ml)	0.7-1.4§	0.95 (0.27)		0.98 (0.28)	0.93 (0.29)	0.88 (0.18)	n.s.
Protein S activity (U/ml)	0.7-1.4§	0.72 (0.25)		0.82 (0.24)*	0.68 (0.20)*	0.59 (0.32)	0.01*
LAC		6 (4%)					
APC (ratio)		1.12 (0.20)					
ACA IgG		6 (6%)					
ACA IgM		0 (0%)					
FV Leiden, n (%)	4 (3%)	3 (2%)					
FII variant, n (%)	5 (4%)	5 (4%)					

*P value (ANOVA), # median (±SD of the mean), ISS: International Staging System, § laboratory reference,
LAC: Lupus anticoagulansACA: Anticardiolipin antibodies, APC: activated protein C resistance

TABLE 3. THROMBO-EMBOLIC COMPLICATIONS DURING THERAPY

Patient	ISS stage	Therapy at time of thrombosis	Thrombotic complication
Female (62yrs)	I	VAD	DVT & PE
Male (49yrs)	II	VAD	DVT
Female (45yrs)	I	HDM	CVC thrombosis
Male (42yrs)	I	Radiotherapy*	DVT
Female (65yrs)	II	VAD	PE
Male (56 yrs)	II	VAD	DVT
Female (58yrs)	I	TAD	PE
Male (54yrs)	II	TAD	DVT
Male (52yrs)	I	TAD	PE
Female (48yrs)	I	TAD	DVT
Female (46yrs)	III	CAD	CVC thrombosis
Male (44yrs)	I	TAD	PE
Male (58yrs)	I	PAD	PE
Male (38yrs)	II	PAD	DVT

A= adriamycin, B= Bortezomib, C= cyclophosphamide, D= Dexamethasone,
T= thalidomide,V= Vincristine, HDM= high dose melfalan
CVC= central venous catheter, *Radiotherapy performed prior to induction chemotherapy

DISCUSSION

In our study of coagulation abnormalities in newly diagnosed untreated MM, FVIII and vWF antigen levels and activity were significantly higher in MM patients than in the controls. Furthermore, there was a significant correlation between prognostic disease stage according to ISS criteria and levels of FVIII and vWF, which were higher in stage III. The correlation with disease stage according to the Durie and Salmon classification system was however, less clear. The pathogenetic mechanism of these increased levels is yet unclear, but may be related to the neovascularisation in the bone marrow stroma, which is accompanied by increased vasculature(15). Because vWF is synthesised mainly in endothelial cells, these bone marrow changes may eventually lead to increased vWF levels in plasma. vWF is a carrier protein for FVIII and FVIII levels will therefore also increase in MM. Another mechanism for increased vWF levels may be the elevated interleukin-6 (IL-6) levels in serum of patients with MM. In the pathogenesis of Multiple Myeloma, IL-6 has been shown to play a pivotal role(16) and serum IL-6 levels are elevated in case of active disease(17). IL-6 plays a stimulatory role in the coagulation mechanism(18), because amongst others IL-6 promotes

the transcription of the Factor VIII gene(19). Recently it has been reported that IL-6, when in complex with the soluble IL-6 receptor, can induce hyperactive ultra large von Willebrand Factor (ULVWF)(20). IL-6 can also inhibit the cleavage of ULVWF resulting in an accumulation of this hyperactive ULVWF in plasma and on the surface of endothelial cells(18). This may also contribute to the increased vWF levels, and thereby of FVIII:C levels, in MM patients.

An interesting observation was a significant decrease in protein S activity levels, especially in patients with ISS stage III MM compared to stage I. These reduced levels may be also related to the increased IL-6 levels in MM, as IL-6 has shown to reduce protein S levels in a canine model(21). The reduced protein S level may contribute to the increased risk for thrombo-embolic complications in the more severe stages of MM. Previously, a relationship between IL-6 and plasma fibrinogen levels has been reported(22). High levels of fibrinogen are associated with an increased incidence of thrombo-embolic complications(23) as well as the risk of recurrent thrombosis24. However, in our study we did not observe a significant increase of fibrinogen levels in patients with MM compared to an age and sex matched control group.

In our patients with untreated MM the incidence of LAC and ACA were 4% and 6% respectively. This is higher than expected from the general population (0,9% and 1-5% respectively), and similar to the recently reported incidences in MM (24-26). This may also be related to the increased risk for thrombosis observed in patients with MM. These anti-phospholipid antibodies were retested after 3 months, as is recommended by the Sapporo criteria(12). In all cases these anti-phospholipid antibodies had disappeared after induction therapy. We found no relationship between positive APA tests and the presence of monoclonal IgG or IgA serum immunoglobulin, seen in the MM patients, which excludes the possibility that our findings are due to a cross reactivity of the monoclonal IgG and the APA tests.

During normal haemostasis, APC limits clot formation by proteolytic inactivation of factors Va and VIIIa. Acquired APC resistance is associated with a sevenfold increased risk for deep vein thrombosis(27). In our study we found APC resistance in only 2% of the patients. This is in contrast to the previously reported incidence of 23%(8). We could therefore not confirm this observation.

Thrombo-embolic complications were observed in 10% of the patients and occurred most frequently during the induction chemotherapy. Despite thromboprophylaxis consisting of low dose molecular weight heparin, which was given to the patients who

received the thalidomide based regimen, VTE was observed with similar incidences in the various regimens. There was no correlation between the ISS disease stage and the development of a VTE. Furthermore, the coagulation variables prior to chemotherapy did not differ significantly between the patients who did and who did not develop a VTE. However, the number of patients with VTE in our study is low and this research question should be addressed in larger cohorts of multiple myeloma patients.

CONCLUSION

Our study indicates that various prothrombotic abnormalities occur in patients with newly diagnosed untreated multiple myeloma, including an increase in vWF, Factor VIII, an increased incidence of anti-phospholipid antibodies and a slight decrease in protein S levels. The delicate balance between coagulation and fibrinolysis in multiple myeloma patients may be further disturbed by hypofibrinolysis, as has previously been reported(28), especially during chemotherapy(29). This results in a hypercoagulable state, which may promote the development of thrombo-embolic complications.

LITERATURE

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Chapter 3

Prospective evaluation of coagulopathy in multiple myeloma patients before, during and after various chemotherapeutic regimens

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ABSTRACT

Background

Venous thromboembolism (VTE) occurs frequently in multiple myeloma patients, especially during induction treatment with thalidomide in combination with anthracyclines and/or dexamethasone. Several coagulation abnormalities have been described in untreated myeloma patients, but these have not been prospectively evaluated during and after treatment.

Patients and Results

We performed a prospective study in 138 multiple myeloma patients in whom coagulation factor levels were evaluated longitudinally before, during induction and after intensification. Patients were randomized to induction treatment consisting of adriamycin and dexamethason, in combination with either vincristin (VAD), thalidomide (TAD), or bortezomib (PAD) followed by high dose melphalan and autologous stem cell transplantion (ASCT).

Factor VIII:C (FVIII:C) and von Willebrand factor (vWF) were significantly elevated before treatment (median FVIII:C 2.26 U/ml, vWF:Ag 1.95 U/ml). Irrespective of the type of induction regimen, these variables increased strongly during induction therapy (FVIII:C 2.55 U/ml and vWF:Ag 2.96 U/ml). Fibrinogen also showed a significant increase after induction therapy (3.5 g/l pre-treatment and 4.0 g/l after treatment, respectively, $p<0.001$). This was significantly higher in TAD than VAD treated patients. Three to six months after ASCT levels of vWF and FVIII:C had decreased to values lower than observed before treatment (1.71 U/ml and 1.67 U/ml respectively). There was no correlation between the increased levels at start and the response of multiple myeloma to treatment. High levels of vWF, fibrinogen and FVIII:C before start of treatment were significantly associated with mortality. Fourteen patients (10%) developed a venous thrombotic event (VTE). The coagulation factor abnormalities before and during treatment were not associated with the development of VTE.

Conclusion

During induction treatment several changes in coagulation factor levels are observed, which may result in a prothrombotic state. Larger studies are required to establish whether the changes in these coagulation factors during induction treatment contribute to the increased risk of venous thrombo-embolism in multiple myeloma patients.

INTRODUCTION

Multiple Myeloma (MM) is associated with an increased risk for venous thromboembolism (VTE). This risk is even higher when multi-agent chemotherapy and/or prednisone are combined with anti-angiogenic drugs, such as thalidomide(1-3). The combination of thalidomide and doxorubicin is associated with the occurrence of venous thrombosis in 10-30% of the patients, whereas venous thrombosis occurs in only 1-5% of the patients receiving more conventional medication(4-7). Low molecular-weight heparin has been shown to reduce effectively the risk of VTE during thalidomide treatment(8, 9). Treatment with the recently introduced proteasome inhibitor bortezomib has not been associated with an increased incidence of venous thrombosis(10). However, bortezomib has so far only been used as rescue or second line treatment, whereas an increased incidence of VTE is especially seen during induction treatment of newly diagnosed MM (11).

Multiple alterations in the hemostatic system promoting coagulation have been found in untreated myeloma patients, including elevated levels of factor VIII:C (FVIII:C) and von Willebrand factor (vWF), activated protein C (APC) resistance, and a hypofibrinolytic state (3, 11-15). Elice et al. studied APC resistance before and after treatment. Nine percent of the patients had a transient APC resistance, which was associated with an increased risk of VTE(16).

Because VTE occurs mainly during the induction treatment of multiple myeloma, it is of utmost interest to study the coagulation disorders during treatment. Therefore we evaluated coagulation factor levels longitudinally before, during and after different intensive chemotherapeutic regimens. The aim was to find coagulation factor abnormalities, which might correlate with an increased VTE risk and outcome of therapy. A second aim was to investigate whether the various chemotherapeutic regimens are associated with different coagulation profiles.

MATERIALS AND METHODS

Patients

Consecutive patients under the age of 65 years with newly diagnosed MM according to the Mayo clinic criteria who were admitted to the department of Hematology of the Erasmus MC Rotterdam or the University Medical Center Utrecht, both academic tertiary

referral hospitals in the Netherlands, were included in the study. Only patients who were eligible for intensive chemotherapy followed by high dose melphalan and autologous stem cell support were included. Informed consent was obtained from all patients. According to the Declaration of Helsinki, the protocol was approved by the Research Ethics Board of both participating hospitals. Patients were randomized to receive 3 courses of adriamycin, dexamethasone in combination with either thalidomide (TAD) or vincristine (VAD) (HOVON 50-study)(17) or in combination with bortezomib (PAD, 1.3mg/m2, day 1,4,8, and 11) (HOVON 65-study, ongoing) as induction treatment in all patients followed by stem cell mobilization with cyclophosphamin, adriamycin and dexamethasone (CAD) and intensive treatment with high dose melphalan (HDM, 200mg/m^2) and autologous stem cell transplantation as previously discribed(17). The starting dose of thalidomide was 100mg and could be escalated to 200mg. Thalidomide was stopped before stem cell mobilisation and re-administered after the autologous stem cell transplantation in a lower dose (50mg). Patients receiving thalidomide also received LMWH during the induction phase of treatment. Patients receiving VAD in the HOVON 50 trial also received IFN (3x 106 IU, 3 times weekly) as maintenance therapy after stem cell transplantation. Patients treated with bortezomib received bortezomib (1.3mg/m2, twice monthly) as consolidation.

Methods

Blood samples were collected at time of diagnosis before start of treatment (time point 1), directly after therapy with VAD, TAD or PAD (time point 2), and 3 to 6 months after high-dose melphalan (HDM) treatment (time point 3). Venous blood was collected using a vacutainer system in citrate (0.105 M, Beckton-Dickinson, Plymouth, UK) and centrifuged at 4° C at 2000 g for 10 minutes. The collected plasma was additionally centrifuged for 10 minutes at 2000 g and stored in small aliquots at –70°C until use. Genomic DNA was isolated from the white cell fraction of citrated blood, using a standard salting out procedure.

FVIII:C was measured by a one stage clotting assay using Platelin (Organon Teknika,Durham, USA) and factor VIII deficient plasma (Biopool, Ventura, USA) or a commercial coagulation method (Boehringer Mannheim, Mannheim, Germany). vWF: Antigen (vWF:Ag) was measured by using the LIA test (Boehringer Mannhein, Mannheim, Germany or an in-house sandwich enzyme linked immunosorbent assay (ELISA) using rabbit anti-human vWF and horseradish peroxidase conjugated anti-human vWF (DakoCytomation, Glostrop, Denmark).

vWF collagen binding activity (vWF:CB) was measured by an in-house EIA using type I collagen (Sigma, StLouis, USA) and horseradish peroxidase conjugated anti-human vWF. vWF Ristocetin Cofactor activity (vWF:RCo) was measured with an aggregometric method using formaline-fixed platelets and ristocetin (Diagnostiga Stago, Asnieres, France). All assays were calibrated with pooled normal plasma (factor assay control plasma, George King Bio-Medical, Kansas, USA). Fibrinogen was measured as described by von Clauss(18). Screening for lupus anticoagulant (LAC) was performed essentially as recommended by the Subcommittee on Lupus Anticoagulant / Antiphospholipid antibodies and was described earlier(19, 20). Anticardiolipin antibodies (ACA) were tested by enzyme-linked immunosorbent sandwich assay (ELISA), using cardiolipin (Sigma, Zwijndrecht, the Netherlands) and horseradish peroxidase conjugated-rabbit anti-human IgG and IgM (Dakopatts). Anticardiolipin antibodies (ACA) were tested by enzyme-linked immunosorbent sandwich assay (ELISA), using cardiolipin (Sigma, Zwijndrecht, the Netherlands) and horseradish peroxidase conjugated-rabbit anti-human IgG and IgM (Dakopatts). Anticardiolipin titers were calculated and considered positive if titers were above 32 U for IgG or above 12 U for IgM. Patients were considered antiphospholipid antibodies (APA) positive if one or both tests for APA were positive, i.e. if LAC was present and/or ACA-IgG or ACA-IgM were positive. The within assay variation coefficient for the ACA assay was 10% for IgG and 11% for IgM. Antithrombin activity levels were determined using a chromogenic substrate. Protein S was measured using an assay (Staclot® Protein S, Diagnostica Stago) which is known not to interfere with FVIII:C, when FVIII:C levels are lower than 2.5 U/ml (21). The prothrombin G20210A gene variant and the factor V Leiden mutation were identified simultaneously, using a multiplex PCR method previously described(22). Activated protein C (APC) resistance was determined on citrated plasma using an aPTT-based Food and Drug Administration-approved resistance assay in the presence of excess factor V-deficient plasma (Coatest APC Resistance, Chromogenix Nodia/Schmidt). The ratios between the aPTT with or without the presence of activated protein C were calculated and the patient was considered positive if the ratio was less than 0.8. Laboratory reference values were obtained from 40 healthy volunteers. Response to therapy was evaluated according to the EBMT criteria(23).

STATISTICAL ANALYSIS

Basic descriptive statistics are presented as median and interquartile range for continuous variables and as count (percentages) for categorical variables. Differences between groups and effects over time in the total group were tested using analysis of variance (ANOVA) and significant differences were further analyzed using Scheffé multiple-comparison post-hoc analysis. Effects over time between groups were compared using the linear mixed-effects model fit by REML(24), taking into account the course of the vWF and FVIII:C levels within the same patient. A two-sided value of $P<0.05$ was considered statistically significant. Statistical analysis was performed with SPSS for Windows, version 11.5 (SPSS Inc. Chicago, USA).

RESULTS

Patient characteristics

A total of 138 patients with multiple myeloma (MM) were included in this prospective study on coagulation variables, of whom 41 (30%) had stage I, 77 (56%) stage II and 20 (14%) had stage III disease according to the ISS criteria(25). The mean age was 55.2 years (range 22-71) and 57 % were males. Seventy-six patients received VAD, 45 TAD and 17 PAD. Baseline patient characteristics were not significantly different between the three treatment groups and are summarized in Table 1.

TABLE 1. PATIENT CHARACTERISTICS

		Therapy		
		VAD	**TAD**	**PAD**
N		76	45	17
Age (yrs)		56 (7.2)	54 (8.4)	52 (8.1)
Male gender (%)		61%	56%	65%
B2M (mg/l)		4.5 (6.4)	4.9 (4.4)	4.1 (2.0)
Platelets (x109/l)		248 (71)	229 (92)	221 (92)
Albumine (g/l)		36 (6)	35 (8)	35 (7)
Calcium (mmol/l)		2.31 (0.17)	2.38 (0.33)	2.37 (0.31)
Hb (mmol Fe/l)		7.0 (1.1)	6.6 (1.4)	6.4 (1.3)
ISS (n)	I	25	11	5
	II	43	25	9
	III	8	9	3

Numbers are median (IQR), ISS= international staging system, V= vincristine, A= adriamycine, T= thalidomide, B= bortezomib

Baseline coagulation variables

The baseline coagulation variables are summarized in Table 2. Screening for lupus anti-coagulant, the factor V Leiden mutation and the G20210A gene variant revealed a similar incidence compared to the normal population. FVIII:C and vWF:Ag were strongly elevated (2.26 [1.61] (median[IQR]U/ml and 1.95 [1.49] U/ml respectively) and a strong correlation between the vWF and FVIII:C levels was observed (r= 0.88, P<0.0001). VWF:Ag levels were strongly correlated with vWF collagen binding (vWF:CB) and ristocetin-cofactor activity (vWF:RCo) (r= 0.86 and r= 0.89 respectively, both P<0.0001).

Table 2. Coagulation variables at various time points in patients with Multiple Myeloma

	Laboratory reference	Time point			ANOVA*				
		t=1	t=2	t=3	1-2	1-3	2-3		
N		138							
FV Leiden, n (%)		3 (2%)							
FII variant, n (%)		5 (4%)							
Antiphospholipid	LAC positive	6 (4%)							
Antibodies	ACA IgG ≥32U	6 (6%)							
(LAC and/or ACL)	ACA IgM ≥ 12U	0 (0%)							
Antitrombin (U/ml)	0.8-1.2	0.94 (0.27)	1.15 (0.22)	1.01 (1.33)	X	X	X	<0.001	
APC ratio	<0.8	1.13 (0.29)	1.03 (0.35)	1.13 (0.32)		X	X	0.007	
D-dimer (mg/L)	<0.25	0.20 (0.60)	0.30 (0.50)	0.10 (0.10)		X	X	0.001	
Factor VIII:C (U/ml)	0.60-1.40	2.26 (1.61)	2.55 (1.78)	1.71 (0.67)	X	X	X	<0.001	
Fibrinogen (g/L)	1.5-3.5	3.50 (1.70)	4.00 (1.70)	3.70 (1.05)	X	X	X	<0.001	
Protein C act (U/ml)	0.7-1.4	0.92 (0.31	1.07 (0.29)	0.95 (0.26)	X		X	<0.001	
Protein S act (U/ml)	0.7-1.4	0.71 (0.31)	0.80 (0.39)	0.81 (0.30)	X	X		0.014	
vWF Ag (U/ml)	0.60-1.40	1.95 (1.49)	2.96 (2.00)	1.67 (0.94)	X		X	<0.001	
vWF CB (U/ml)	0.60-1.40	1.90 (1.22)	2.76 (1.74)	1.86 (0.95)	X		X	<0.001	
vWF RCo (U/mL)	0.60-1.40	1.78 (1.25)	2.46 (1.68)	1.65 (0.72)	X		X	<0.001	
APTT (sec)	29-39	31 (7.0)	28 (7.0)	30 (6.0)				0.44	

Numbers are median and interquatile range (IQR). t=1; before therapy, t=2 after induction chemotherapy, t=3 after high dose melphalan with autologous stemcell support. X= significant difference between two time points (*=P value).

Baseline values and the relationship with outcome.

Response to treatment was assessed after induction therapy and 3 to 6 months after autologous stem cell transplantation. At the end of treatment, most patients showed a remission (85%) of which 14% complete and 86% partial. In the total patient group we studied the association between coagulation abnormalities observed before start of treatment with outcome during and after treatment. We observed no association between pretreatment levels of vWF, FVIII:C and fibrinogen and the response at the end of treatment. Two patients died during the induction phase while receiving VAD or TAD. Two other patients that had received TAD or PAD died during the intensification phase. All these four patients were in the ISS stage III. The cause of death in all four patients was sepsis during neutropenia. The pretreatment levels of vWF:Ag, FVIII:C and fibrinogen in these patients were significantly higher than in those who survived (vWF:Ag; 3.64 [1.02] vs 2.07 [1.02] p=0.007 (median [IQR]), FVIII:C; 4.37 [1.42] vs 2.26 [1.00], p<0.001 (median [IQR]) and fibrinogen; 3.4g/l ±1.73 vs 5.35±4.3, P<0.002 (median [IQR]), for survivors and non-survivors respectively.

Coagulation factor levels during treatment

We compared levels of various coagulation factors during and after treatment in the total patient group. FVIII:C (p=0.003) and vWF:Ag (p=0.03) levels showed a parabolic course during the treatment phases with the maximal values after induction treatment in all three groups (VAD, TAD and PAD) (Table 2). This increase was significant for FVIII:C (from 2.26 [1.61] U/ml to 2.55 [1.78] U/ml) and vWF:Ag (from 1.95 [1.49] U/ml to 2.96 [2.0] U/ml) between time points 1 and 2. The decrease between time points 2 and 3 was also significant (Table 2). There was however, no difference between the type of induction regimen and the course of FVIII:C (Figure 1) nor vWF. Fibrinogen levels were also significantly increased (from 3.5 to 4.0g/l) during treatment and remained elevated at the end of treatment (3.70, P<0.001).

In the linear mixed-effects analysis of the three time points, fibrinogen levels were significantly different between the VAD and TAD group. The increase between time point 1 and 2 was higher in TAD group(t=1; 3.2g/L [IQR 0.9] and t=2; 4.3g/L [IQR 1.7]) versus the VAD group (t=1; 3.7g/L [IQR 1.3] and t= 2; 4.2g/L [IQR 1.4])(Figure 2). The PAD group consisted of 17 patients and was to small for subgroup analysis. Although antithrombin, protein C or

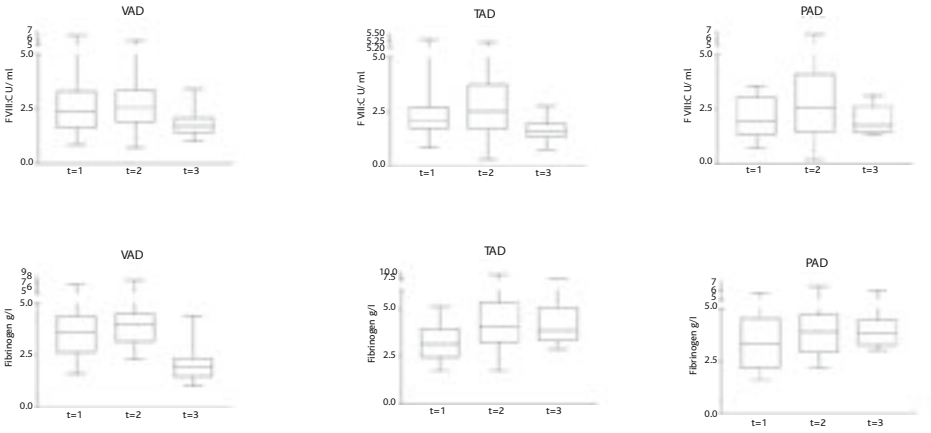

Figure 1. Levels of FVIII:C and fibrinogen at the three different time points during the VAD, TAD or PAD treatment. Shown are box and whisker plots in which the data in box represents the 25-75% range with the median shown as line. The minimum and maximum levels are given as high/low bars.

protein S levels increased slightly during induction chemotherapy, these levels remained within the normal range. D-dimer levels were decreased significantly at the end of treatment compared to base line.

Coagulation factor levels and VTE

Of the 138 patients included in this study, 14 patients experienced a venous thrombotic event. In one patient VTE occurred during radiotherapy prior to the start of chemotherapy. Thirteen patients developed a VTE during chemotherapy. These VTE were observed during all three chemotherapeutic regimens (VAD 8%, TAD 13% and PAD 6%) and were mainly observed during the induction phase (VAD n=4, TAD n=5 and PAD n=1). Two patients experienced a VTE during the CAD chemotherapy and one during the HDM treatment. We found no relationship between coagulation factor abnormalities before treatment, or after induction therapy and the risk of VTE (data not shown).

DISCUSSION

In accordance with previous studies, we found several coagulation abnormalities in patients with multiple myeloma before start of treatment. This study shows that these coagulation abnormalities worsen during induction treatment with combination chemotherapy.

In the VAD, TAD and PAD treated patients vWF and FVIII:C levels increased during chemotherapy, and decreased again after high dose melphalan treatment and ASCT. Most coagulation abnormalities exhibit a similar course during all three different treatment modalities except for fibrinogen, which was significantly higher in the TAD than in the VAD treated patients. Mortality during the induction treatment was significantly associated with high levels of FVIII:C, vWF and fibrinogen.

vWF and FVIII:C levels were strongly elevated in the patients at the time of diagnosis(11, 26). No difference was seen between the patients treated with PAD compared to the patients treated with VAD or TAD. vWF antigen levels correlated strongly with vWF functional activity measured by both ristocetin cofactor activity and collagen binding activity, indicating that the vWF in multiple myeloma patients is functionally normal. However, no differences in vWF and FVIII:C levels between the various regimens were observed. These results implicate that although increased FVIII:C levels may contribute to the increased risk of VTE in myeloma patients, these levels are unrelated to the increased thrombosis risk that is reported with TAD as compared to VAD or PAD. The patients who were treated with TAD also received LMWH as thrombo-prophylaxis. Previous studies have shown that vWF release may be influenced by unfractionated heparin, and to a lesser extent by LMWH (27, 28). This indicates that in the patients treated with TAD, the rise of vWF levels may be underestimated, due to the concomittant use of LMWH. In a previous study however, we have shown that levels in thalidomide treated multiple myeloma patients not receiving LMWH were similar to those in patients treated with other chemotherapeutic regimens (11). Similarly, we recently described an induction of hypofibrinolysis during treatment of multiple myeloma, which may contribute to the increased thrombotic risk, but also in this study, no differences between VAD and TAD treated patients were observed(12). Fibrinogen levels showed a significant higher increase after induction treatment compared to patients who received VAD or PAD. This might explain the higher risk of VTE in TAD treated patients, because high fibrinogen levels are related to an increased thrombotic risk(29). In our study LMWH was administered as thrombo-prophylaxis in the patients receiving TAD during the induction phase to reduce the incidence of VTE. Recent data however, also suggest a possible role for platelets in the pathogenesis of multiple myeloma associated VTE(30). Since the use of prophylactic fixed low-dose aspirin in patients treated with a thalidomide based

regimen has indeed been shown to reduce the VTE incidence(31).

Recently bortezomib was introduced as a new powerful treatment modality in multiple myeloma. It has been primarily used in relapsing of refractory multiple myeloma patients, and low incidences of VTE complications were reported(10). However no studies have been reported on VTE risk associated with bortezomib as induction treatment in untreated multiple myeloma patients. In our study we found similar coagulation disorders in patients treated with PAD compared with VAD. One patient treated with PAD developed a trombotic event during the induction treatment.

We found no relationship between coagulation parameters before and during treatment with the development of VTE. Fourteen patients (10% of our study population) developed a VTE, mostly during the induction treatment. Since all patients treated with TAD received thromboprophylaxis with LMWH, which has been shown to effectively reduce the incidence of thrombosis, it is difficult to assess the relationship between the coagulation abnormalities and risk of VTE in our study(8). Interestingly, we observed that pretreatment levels of FVIII:C, vWF and fibrinogen were associated with the risk of mortality. Patients who died during treatment exhibited the highest levels of these coagulation factors. Because of the limited number of patients this relationship remains speculative and this finding should be confirmed in larger cohorts. Mortality may be related to the severity of the disease stage since all these patients that died had ISS stage III. Furthermore, as was previously shown, the vWF and FVIII:C levels were significantly associated with the ISS stage(26).

CONCLUSION

In conclusion, this study shows that there is a significant increase in the levels of vWF, FVIII:C and fibrinogen during the induction therapy of multiple myeloma and that these levels, except for fibrinogen, decrease after the ASCT. Especially when treatment was combined with thalidomide, fibrinogen levels exhibited the highest increase during induction treatment TAD compared to the VAD regimen. Larger studies are required to establish whether the rise in these prothrombotic coagulation factors contributes to the increased risk of venous thromboembolism in multiple myeloma during induction treatment.

LITERATURE

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Chapter 4

Hypofibrinolysis during induction treatment of multiple myeloma may increase the risk of venous thrombosis

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ABSTRACT

Background

Patients with newly diagnosed untreated Multiple Myeloma (MM) are at high risk for thrombo-embolic complications, especially when they are treated with antracyclin based multi-agent chemotherapy in combination with anti-angiogenic drugs, i.e. thalidomide. The nature of this increased risk is yet poorly understood. In this study we analysed the longitudinal change in fibrinolytic activity in these patients before and after receiving induction chemotherapy followed by high dose melphalan (HDM) and autologous stem cell transplantation (ASCT).

Patients & Results

A total of 77 patients with newly diagnosed MM who were eligible for multi-agent chemotherapy followed by high dose melphalan with ASCT were analysed. The data were compared with a control group of 133 healthy volunteers. Patients received adriamycin and dexamethasone in combination with vincristin (VAD) or thalidomide (TAD) as induction therapy. Fibrinolytic activity was determined prior to and after induction therapy, as well as after high dose melphalan followed by ASCT. Clot lysis time was similar to the control subjects before treatment (controls vs patients: median 63 [44-91] minutes vs 65 [38-108] minutes). After induction chemotherapy the clot lysis time increased significantly in both the VAD and the TAD patients (68 [44-122] and 70 [49-107]. Following HDM and ASCT, clot lysis time returned to normal values.

Conclusion

In this study we observed hypofibrinolysis after induction chemotherapy in patients with MM irrespective of the type of induction therapy. These changes in fibrinolysis may contribute to the increased thrombosis risk in patient with multiple myeloma.

INTRODUCTION

Multiple myeloma is associated with an increased risk for venous thrombosis. The risk for venous thromboembolism is even higher in patients receiving multi-agent chemotherapy and/or prednisone combined with anti-angiogenic drugs. The combination of thalidomide and doxorubicin was reported to be associated with the occurrence of venous thrombosis in 10-30% of the patients, whereas venous thrombosis occurs in 1-5% of the patients receiving more conventional chemotherapeutic regimens(1, 2). Multiple hemostatic alterations promoting coagulation have been found in myeloma patients. These include high levels of factor VIII and von Willebrand factor, acquired activated protein C (APC) resistance, and the formation of procoagulant autoantibodies(3-5). Furthermore, it has been described that high levels of serum M-proteins and increased blood viscosity may interfere with fibrin polymerisation, resulting in a fibrin clot which is more resistant to fibrinolysis(6). Finally, high levels of plasminogen activator inhibitor type I (PAI-1) have been found in myeloma patients, which also results in inhibition of fibrinolysis(7).

We recently reported that overall plasma hypofibrinolysis, as measured by a tissue factor and tissue plasminogen activator (tPA)-induced clot lysis assay, constitutes a risk factor for venous thrombosis in otherwise healthy individuals(8). In this study, we measured plasma fibrinolytic potential in myeloma patients during the course of therapy including either Vincristine, Adriamycin, and Dexamethasone (VAD), or Thalidomide, Adriamycin, and Dexamethasone (TAD) to investigate whether chemotherapeutic treatment, especially in combination with thalidomide is associated with plasma hypofibrinolysis, and may therefore be an explanation for the increased incidence of venous thrombosis.

MATERIALS AND METHODS

We studied newly diagnosed multiple myeloma patients who were included in the HOVON 50 study, a prospective randomised phase III study on the effect of thalidomide combined with adriamycin, dexamethasone and high dose melphalan. The protocol has been described previously(9). From a subset of patients plasma was available. Blood samples from 77 patients taken at time of diagnosis, between the second and third course of induction therapy with VAD (45 patients) or TAD (32 patients), and from 35 patients after stem

cell transplantation (SCT) in the TAD arm and 47 after SCT in the VAD arm were studied. Clot lysis time was measured as described previously(8). As it is known that clot lysis time strongly increases with age, we used data obtained from 133 healthy controls above 55 years of age, which were recruited as part of the Leiden Thrombophilia Study (LETS). These data were published previously(8). The average age of our patient group was 57, while the age of the control group averaged 62. Statistical analysis was performed using the GraphPad InStat (GraphPad, San Diego, CA) software package. As there was a significant difference in standard deviations between the groups, we analysed data using the non-parametric Kruskal Wallis one-way analysis of variance (ANOVA) test. P values <0.05 were considered statistically significant.

RESULTS

Figure 1 shows clot lysis times of plasma samples obtained from myeloma patients at time of diagnosis, during VAD or TAD treatment, and after SCT, compared to clot lysis times from age-matched healthy controls. There was no significant difference in clot lysis time between the normal control group and the patients tested at time of diagnosis (controls median [range]: 63 [44-91] min vs patients 65 [38-108] min). However, there was a significant increase of clot lysis time during both VAD (median [range]: 68 [44-122] min) and TAD (median [range]: 70 [49-107] min) as compared to clot lysis times of the control subjects indicating the development of hypofibrinolysis during chemotherapy ($p<0.05$, Kruskal Wallis ANOVA with Dunn's post test). After SCT, the clot lysis times were again not different from the control group in both the VAD (median [range]: 67 [43-113] min) and TAD (median [range]: 65 [47-105] min) arm.

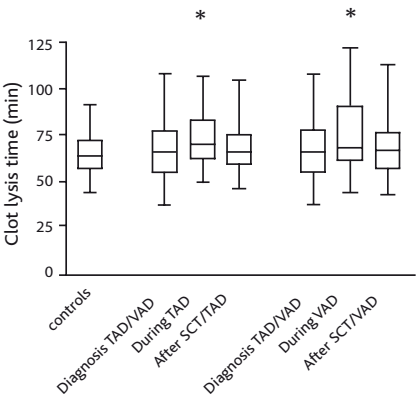

Figure 1. Box and whisker plot showing clot lysis times of plasma samples obtained from healthy controls compared to myeloma patients at time of diagnosis, during VAD or TAD induction therapy, and after SCT while receiving thalidomide (TAD) or interferon-alpha (VAD) maintenance therapy. The data in box represents the interquartile range with the median shown as line. The total range of data is demonstrated by the high/low bars. Groups significantly different to the control group are marked with * where $P < 0.05$. For easy comparison, the diagnosis group is shown twice in the Figure.

DISCUSSION

In this study we found no evidence of hypofibrinolysis in patients with multiple myeloma at the time of diagnosis. A hypofibrinolytic state did develop during both TAD and VAD therapy, and after SCT the hypofibrinolytic state was again resolved. The specific induction of hypofibrinolysis during chemotherapy might indicate that the increased thrombotic risk associated with chemotherapy might be explained in part by defective clot lysis. However, no increased hypofibrinolysis associated with thalidomide treatment was observed.

In this study we used a plasma-based clot lysis assay, which was previously demonstrated to be of clinical relevance, since hypofibrinolysis as detected with this assay was found to be a clear, and independent risk factor for the development of a first venous thrombosis in otherwise healthy individuals(8). As such, our overall assay is expected to yield more relevant information compared to the measurement of individual fibrinolytic factors. For example, the association of elevated levels of PAI-1, and decreased tPA levels with venous thrombosis has never been convincingly shown(10, 11). Also, other global tests of fibrinolysis such as the euglobulin clot lysis time and the dilute whole blood clot lysis time did not show a convincing association with the occurrence of venous thrombosis(12). Although the exact determinants of our clot lysis assay are still incompletely known, the available data suggest that the outcome of our test indeed reflects an overall plasma fibrinolytic potential(8, 13). An earlier study did show a hyperfibrinolytic state in multiple myeloma in a mixed population of which approximately half was sampled at the time of diagnosis(7), but this clot lysis assay employed citrated plasma, with an exogenously added fibrin clot, thereby obscuring the effects of thrombin generation and endogenous fibrin formation on the fibrinolytic potential. Furthermore, one study employing purified myeloma antibodies observed these antibodies to inhibit fibrinolysis in normal plasma(6). However, in our study, no correlation between serum M protein levels and clot lysis time could be demonstrated (data not shown).

The occurrence of hypofibrinolysis during chemotherapy is likely to contribute to the increased thrombotic risk in myeloma patients during this stage of treatment. However, we did not find a difference in the extent of hypofibrinolysis between patients receiving thalidomide and vincristine, indicating that the further increased thrombotic risk caused by administration of thalidomide is caused by alterations in a different part of the hemostatic system. Patients receiving thalidomide were also receiving low molecular weight heparin as

thromboprophylaxis, which could potentially have obscured clot lysis results(14). However, at the time of sampling, none of the patients had detectable plasma levels of low molecular weight heparin (as measured by an anti Xa assay, data not shown).

CONCLUSION

In conclusion, in this study we found an induction of hypofibrinolysis in patients with multiple myeloma during VAD and TAD treatment. These results may explain the elevated thrombotic risk in patients receiving induction chemotherapy with VAD or TAD. However, it does not explain the extra increased risk of VTE during thalidomide treatment, as hypofibrinolysis was observed both during VAD and TAD treatment. Presumably other hemostatic alterations are responsible for the thrombotic risk in myeloma patients during thalidomide therapy.

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Chapter 5

Microparticle-associated tissue factor activity and venous thrombosis in Multiple Myeloma

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Submitted for publication

Chapter

6

Prevention of venous thromboembolism with low molecular-weight heparin in patients with multiple myeloma treated with thalidomide and chemotherapy

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ABSTRACT

Background

An increased risk for venous thrombo-embolic (VTE) complications in patients with Multiple Myeloma (MM) has been reported, especially when these patients are treated with combination treatment including high dose dexamethason and doxorubicin. The addition of thalidomide can increase this risk up to 30%. Preventing these VTE complications and the choice of thromboprophylaxis remains a major issue. In this study we investigated the effectivenes of low molecular weight heparin (Nadroparin) in newly diagnosed MM patients who received multi-agent chemotherapy in combination with thalidomide.

Patients and Results

A total of 412 patients with newly diagnosed MM were included in this study. The patients were randomised to receive either the combination of vincristin, doxorubicin and dexamethasone (group A: VAD) without thrombo-prophylaxis or thalidomide, doxorubicin and dexamethasone (group B: TAD) in combination with LMWH nadroparine 2850 IE anti-Xa or 5700 IE anti Xa in case of weight above 90kg. Thirty cases of VTE were observed (7%; in group A 5% and group B 9%) and occurred especially during induction chemotherapy. Two patients in group B did not receive thromboprophylaxis at the time the VTE ocurred. No side effects were observed.

Conclusion

In conclusion, standard LMWH (Nadroparin) prophylaxis during chemotherapy is safe and effective in reducing the incidence of thalidomide-associated VTE in newly diagnosed MM patients.

INTRODUCTION

In multiple myeloma (MM) patients, treatment with thalidomide has proven its efficacy as monotherapy, but also combined with dexamethasone and chemotherapy. However, in combination with these drugs thalidomide may increase the incidence of deep venous thrombosis and pulmonary embolism up to 30%(1). The pathogenesis of these thrombotic events is poorly understood and it is also not known how to prevent thalidomide-associated venous thromboembolism (VTE)(2). In patients who were treated in the prospective, multicenter phase III HOVON-50/GMMH-HD3 study, we evaluated the incidence of VTE in newly diagnosed MM patients during induction therapy with thalidomide, doxorubicin and dexamethasone when using the low molecular-weight heparin (LMWH) nadroparin in prophylaxis dosage.

PATIENTS AND METHODS

Patients, age 18-65 years, with newly diagnosed MM, Salmon & Durie stage II or III, were eligible for inclusion. Informed consent was obtained from all patients. According to the Declaration of Helsinki, the protocol was approved by the Research Ethics Board of each participating hospital. Patients were randomly assigned to induction chemotherapy consisting of three cycles of vincristine (0.4 mg, i.v. on days 1-4), doxorubicin (9 mg/m2, i.v. on days 1-4) and dexamethasone 40 mg orally (days 1-4, 9-12, 17-20), (VAD) arm A. Patients assigned to arm B received thalidomide instead of vincristine (TAD). Cycle 2 starts at day 29, cycle 3 at day 57. Thalidomide was given as 200 mg orally, starting at day 1 of the first TAD cycle and was stopped 2 weeks before chemotherapy for stem cell mobilization was started. The thalidomide dose could be escalated to maximally 400 mg in case of good tolerability. Patients in arm B started with standard dosage thrombosis prophylaxis consisting of subcutaneously LMWH nadroparin 2850 IE anti-Xa or 5700 anti-Xa in case of weight above 90 kg. Prophylaxis was started at day 1 of the first TAD cycle until 1 week before start of chemotherapy for stem cell mobilization. Stem cells were mobilized after cyclophosphamide 1000 mg/m2 i.v. day 1, doxorubicin 15 mg/m2, i.v. on days 1-4, dexamethasone 40 mg orally on days 1-4 (CAD) and G-CSF 5 mg/kg twice daily until collection. After induction therapy all patients received one or two courses of high-dose melphalan (HDM) 200 mg/m2 with autologous stem cell rescue. Patients randomized to arm A received maintenance

therapy with α-interferon (3 x 10^6 IU, thrice weekly) and patients randomized to arm B received thalidomide 50 mg/day without VTE prophylaxis. The incidence of VTE was a secondary end point of the study and had to be reported directly by fax to the datacenters as a serious adverse event. The reporting hospitals were then contacted for further details. As control a separate questionnaire was sent to all participating hospitals. All types of venous thrombosis and pulmonary embolism were included and diagnosis was made by Doppler ultrasonography or spiral pulmonary computer tomography. The time to occurrence of the first VTE, T_{VTE} , was calculated from the date of randomization. Patients who died within 6 months without VTE were censored at the date of death. T_{VTE} was estimated with the actuarial method of Kaplan and Meier and 95% confidence intervals were calculated, Kaplan-Meier curves of T_{VTE} were generated to illustrate differences between the two treatment arms, and the log-rank test was used to compare the two curves. The reported P-values are two-sided, and a significance level of α=0.05 was used.

RESULTS

Inclusion started in November 2001 and as of May 1, 2003, 412 patients were included, 201 patients in Arm A and 211 patients in Arm B. The data were analyzed as of January 16, 2004. In all, 30 cases of VTE were reported in 412 evaluable patients (7%, 95% CI 5-10%) (Table 1).

Table 1. VTE incidence during chemotherapy and patients characteristics

Randomization arm	A	B	Total
Inclusion	201	211	412
VTE	11 (5%)	19 (9%)	30 (7%)
VTE during VAD/TAD	8 (4%)	17 (8%)	25 (6%)
VTE localization			
DVT leg	8	14	22
DVT leg+ PE	0	1	1
PE	1	3	4
DVT arm	2	1	3
Median days after start treatment (range)	79 (26-164)	60 (11-182)	
Male/Female	6/5	13/6	19/11
Age, median (range)	55 (46-62)	54 (41-64)	

VTE: venous thrombo-embolism, DVT; deep venous thrombosis, PE ; pulmonary embolism
VAD; vincristine, doxorubicin, dexamethasone, TAD; thalidomide, doxorubicin, dexamethasone

There were 22 lower extremity thrombosis (DVT leg), one DVT leg with pulmonary embolism (PE), four PE and three arm venous thrombosis (DVT arm). One arm venous thrombosis (arm A) was catheter related. In arm A, 201 patients were included of whom 11 patients developed VTE (5%, 95% CI 3-10%) at a median of 79 days (range 26-164) after randomization. VTE occurred in 19 patients in arm B (9%, 95% CI 6-14%), median 60 days (range 11-182) after randomization (P=0.15) (Figure 1).

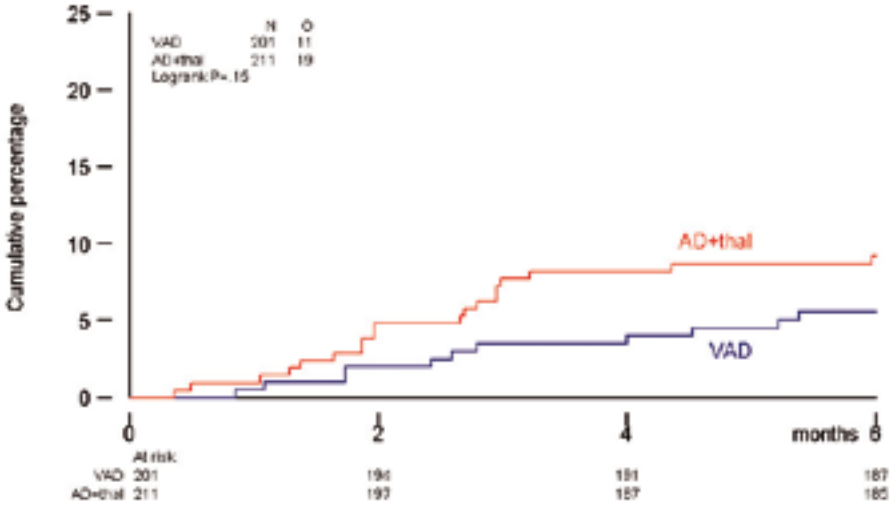

Figure 1. VAD; vincristine, doxorubicin, dexamethasone, AD+ Thal; thalidomide, doxorubicin, dexamethasone

Most cases of VTE occurred during induction chemotherapy, eight cases during VAD and 17 cases during TAD (P=0.08). In arm A, two cases were reported after CAD and one after HDM. In arm B, one patient developed a VTE after CAD and one patient after HDM. Two patients in arm B did not receive prophylaxis at the time of VTE while treated with TAD, in one patient prophylaxis was stopped to perform a bone marrow biopsy 1 week before VTE and one patient did not receive prophylaxis for unknown reasons. No side effects, especially bleeding, of nadroparine prophylaxis were reported.

DISCUSSION

This prospective study shows that with prophylactic dosage of the LMWH nadroparin the incidence of VTE during combined treatment of thalidomide, dexamethasone and doxoru-

bicin is about 10%. This incidence is comparable with the incidence of VTE during intensive chemotherapy in newly diagnosed MM patients without the use of thalidomide(3). In the standard VAD arm of this study the incidence of VTE with vigorous monitoring was 5%. For ethical reasons there was no randomization between patients receiving VTE prophylaxis, because previous publications demonstrated such a high incidence of VTE. Furthermore, in arm B vincristine was left out because of high risk of polyneuropathy when combined with thalidomide. Therefore, only a comparison can be made with historical controls. Zangari ***et al***(1) were the first to report the high incidence of thalidomide-associated VTE of 28% in the Total Therapy II trial during induction therapy with polychemotherapy consisting of vincristine, doxorubicin, dexamethasone, cyclophosphamide, etoposide and cisplatin. Osman ***et al***(4) reported an incidence of 27% in newly diagnosed patients using induction therapy consisting of thalidomide, doxorubicin and dexamethasone in almost similar dosages as in this study. A more recent study combined thalidomide with vincristine, doxorubicin and dexamethasone and reported four thrombotic events in the first 12 patients(5), that is, an incidence of 33% without the use of VTE prophylaxis, although Zervas ***et al***(6) reported an VTE incidence of 10% with the same regime. These high VTE-incidences are also found in thalidomide-based therapy regimes without anthracyclines and in patients with refractory or relapsed MM patients(7, 8). Others have tried fixed low-dose warfarin as VTE prophylaxis but reported conflicting results. Zamagni ***et al*** used 1.25 mg warfarin daily and demonstrated a decrease in VTE incidence of 26% to 9% when treating de novo MM patients with dexamethasone and thalidomide(9). However, others could not demonstrate a beneficial effect when using 1 mg fixed-dose warfarin, but also reported comparable VTE incidences with their control arm when changing to LMWH prophylaxis(10).

CONCLUSION

In conclusion, standard LMWH (nadroparine) prophylaxis during chemotherapy is safe and effective in reducing the incidence of thalidomide-associated VTE in newly diagnosed MM patients.

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Chapter 7

Prothrombotic coagulation abnormalities in patients with paraprotein producing B-cell disorders

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ABSTRACT

Background

An increased incidence of thromboembolic complications has been observed in Multiple Myeloma (MM), especially when patients are treated with antracycline-based chemotherapy. In MM patients, plasma levels of several prothrombotic coagulation factors are increased and this may contribute to the prothrombotic state of these patients. Recently, an increased thrombosis risk has also been described for other plasma cell disorders (PCD), such as Monoclonal Gammopathy of Uncertain Significance (MGUS) and systemic AL amyloidosis. The aim of this study was to analyse prothrombotic coagulation disorders in patients with paraprotein producing B-cell disorders, such as MGUS, AL amyloidosis and Waldenström's Macroglobulinemia (WM) and MM.

Patients and Results

A total of 198 patients with paraprotein producing B-cell disorders including MGUS, MM, AL amyloidosis and WM were included in this study. Different coagulation variables regarding the primary and secondary hemostasis were analysed as well as known prothrombotic gene mutations (factor V Leiden and G20210A variant) and screening for lupus anticoagulant / antiphospholipid antibodies.

An increase in factor VIII (FVIII) and von Willebrand Factor (vWF) was observed in patients with MGUS and AL amyloidosis that was similar to increases seen in patients with untreated MM. The highest levels were observed in patients with AL amyloidosis.

Conclusion

In conclusion, we observed several coagulation abnormalities in patients with different PCD. These prothrombotic changes in patients with MM, AL amyloidosis and Waldenström's Macroglobulinaemia may be causally related to the observed incidence of VTE in these forms of PCD

INTRODUCTION

Multiple Myeloma (MM) is associated with an increased risk of venous thrombo- embolism (VTE), especially in patients receiving multi-agent chemotherapy including anthracyclines in combination with anti-angiogenic drugs such as thalidomide(1, 2). Recently an increased incidence of VTE in other paraprotein producing B-cell disorders, such as Monoclonal Gammopathy of Uncertain Significance (MGUS) and systemic Amyloidosis (AL), has also been reported. VTE has been reported with an incidence of 6-7.5% in MGUS(3-5) and 11% in AL(6). In patients with MM, increased levels of Factor VIII (FVIII) and von Willebrand Factor (vWF) have been observed and are suggested to play an important role in the hypercoagulable state of these patients. Also acquired activated protein C resistance (APC) occurs in patients with multiple myeloma, which is related to the occurrence of VTE(7). The mechanism of VTE is however not fully understood and data regarding prothrombotic coagulation abnormalities in plasma cell disorders (PCD) other than MM are still limited. Therefore we compared prothrombotic coagulation factors in patients with various paraprotein producing B-cell disorders, including MGUS, AL, Waldenström's Macroglobulinemia (WM) and compared these with our observations in patients with newly diagnosed MM.

MATERIALS AND METHODS

Patients

198 consecutive patients with paraprotein producing B-cell disorders who were admitted to the department of Hematology of the Erasmus University Medical Center, an academic tertiary referral hospital, were included in the study. We also included a control group of 125 healthy individuals, who were partners or friends of patients visiting the outpatient department of Hematology of our hospital. The Medical Ethical Committee of the Erasmus MC approved the study and written informed consent was obtained before performing coagulation studies.

Coagulation variables

Venous blood was collected using a vacutainer system in citrate (0.105 M, Beckton-Dickinson, Plymouth, UK) and centrifuged at 4° C at 2000 g for 10 minutes. Plasma samples were obtained at time of diagnosis while the patients were not reveiving chemotherapy.

The plasma was collected and centrifuged for an additional 10 minutes at 2000 g and stored in small aliquots at –70°C until use. Genomic DNA was isolated from the white cell fraction of citrated blood, using a standard salting out procedure. FVIII:C was measured by a one stage clotting assay using Platelin (Organon Teknika, Durham, USA) and factor VIII deficient plasma (Biopool, Ventura, USA). vWF:Antigen (vWF:Ag) was measured by an in-house sandwich enzyme linked immunosorbent assay (ELISA) using rabbit anti-human vWF and horseradish peroxidase conjugated anti-human vWF (DakoCytomation, Glostrop, Denmark). VWF collagen binding activity (vWF:CB) was measured by an in-house EIA using type I collagen (Sigma, StLouis, USA) and horseradish peroxidase conjugated anti-human vWF. VWF Ristocetin Cofactor activity (vWF:RCo was measured by an aggregometric method using formaline-fixed platelets and ristocetin (Diagnostiga Stago, Asnieres, France). All assays were calibrated with pooled normal plasma (factor assay control plasma, George King Bio-Medical, Kansas, USA). Fibrinogen was measured as described by von Clauss(8). Screening for lupus anticoagulant (LA) was performed essentially as recommended by the Subcommittee on Lupus Anticoagulant / Antiphospholipid antibodies(9), and included screening assays (aPTT, PT and dilute prothrombin time) as well as confirmatory procedures to demonstrate the phospholipid dependence. Anticardiolipin antibodies were tested by enzyme-linked immunosorbent sandwich assay (ELISA), using cardiolipin (Sigma, Zwijndrecht, the Netherlands) and horseradish peroxidase conjugated-rabbit anti-human IgG and IgM (Dakopatts). Patients were considered aPL positive if one or both tests for aPL were positive. The within assay variation coefficient for the aCL assay was 10% for IgG and 11% for IgM.

Antithrombin activity levels were determined using a chromogenic substrate. Protein S activity was determined using the Staclot® Protein S assay (Diagnostica Stago) which is not influenced by high FVIII(10). The prothrombin 20210GA gene variant and the factor V Leiden mutation were identified(11). Activated protein C (APC) resistance was determined on citrated plasma using an aPTT-based resistance assay in the presence of excess factor V-deficient plasma (Coatest APC Resistance, Chromogenix Nodia/Schmidt). The ratios between the aPTT with or without the presence of activated protein C were calculated and the patient was considered positive if the ratio was less than 0.8 D-dimer levels were measured using the Biopool AutoDimer assay and expressed mg/l. Reference values in our laboratory were obtained from 40 healthy volunteers.

STATISTICAL ANALYSIS

Statistical analysis consisted of basic descriptive statistics and the results are presented as median with interquartile range. Differences between groups were analysed using a Kruskal-Wallis test and for those variables where differences exist among the means, post hoc range tests (Tukey) were used to identify which means differ. The statistical analysis was performed with SPSS software, version 11.

RESULTS

A total of 198 patients with paraprotein producing B-cell disorders and 125 control subjects were included in this study. The patient characteristics are summarized in Table 1. The median age was around 60 years and did not vary significantly between the different patient groups. Baseline genetic and plasma risk factors for thrombosis in the different groups of patients with PCD are presented in Table 2.

Table 1. Patients characteristics

	Control	MGUS	Multiple Myeloma	Amyloidosis	Waldenstrom's
N	125	25	145	20	8
Age*	55 (11.6)	64 (11.5)	56 (8.1)	57 (9.6)	66 (12.3)
Male gender (%)	53	64	58	40	88
B2M	#	1.68 (1.4)	3.08 (2.8)	2.00 (1.79	2.87 (1.3)
Platelets	#	262 (108)	243 (102)	311 (224)	190 (95)
Albumine	#	41 (4)	37 (9)	43 (13)	33 (5)
Calcium	#	2.35 (.016)	2.34 (.024)	2.30 (0.36)	2.27 (0.22)
Hb	#	8.6 (1.1)	6.8 (1.7)	8.8 (2.5)	6.5 (1.8)

* mean (SD), # not available. Other variables are median (interquartile range)

Antigen and activity levels of von Willebrand Factor (vWF) were significantly increased in patients with plasma cell disorders compared to the healthy control group. Subgroup analysis revealed that vWF antigen and activity levels were similarly increased in the patients with MM, MGUS and AL amyloidosis, but not in patients with WM(Figure 1). FVIII levels were also significantly increased in the total group of patients with PCD compared to the control group. The highest FVIII levels were seen in patients with AL amyloidosis (Figure 1). Although protein S activity was within the lower range of normal in all forms of

Table 2. Coagulation variables in newly diagnosed Multiple Myeloma and other plasma cell disorders

	Laboratory reference	Control group	MGUS	Multiple Myeloma	Primary Amyloidosis	Waldenstrom's Macroglobulinemia	P-value (ANOVA)
N		125	25	145	20	8	ns
FV Leiden, n (%)		4/124 (3%)	1/22 (5%)	3/132 (2%)	0/4 (0%)	0/3 (0%)	ns
FII variant, n (%)		5/124 (4%)	0/22 (0%)	5/132 (4%)	0/4 (0%)	0/3 (0%)	ns
Antiphospholipid LAC positive	nd	1/22 (5%)	6/115 (5%)	2/6 (33%)	2/8 (25%)	ns	
antibodies	ACA IgG ≥32U	nd	0/25 (0%)	7/145 (5%)	0/20 (0%)	1/8 (13%)	ns
(LAC and/or ACL)	ACA IgM ≥12U	nd	0/25 (0%)	1/145 (1%)	0/20 (0%)	2/8 (25%)	ns
Antitrombin (U/ml)	0.8-1.2	nd	1.00 (0.24)	0.93 (0.26)	0.86 (0.11)	0.83 (0.21)	ns
APC ratio	<0.8	nd	0/14 (0%)	4/80 (5%)	1/3 (33%)	0/4 (0%)	ns
D-dimer (mg/L)	<0.25	nd	0.10 (0.8)	0.20 (0.6)	0.55 (0.53)	0.20 (0.60)	ns
Factor VIII:c (U/ml)	0.60-1.40	1.13 (0.51)1,4	1.77 (0.78)2,4	2.14 (1.70)1	2.68 (1.24)1,2,3	1.64 (1.33)3	0.0011,2 0.0024
Fibrinogen (g/L)	1.5-3.5	3.4 (0.7)1	3.7 (1.3)2	3.5 (1.7)3	4.3 (1.4)1,2,3	4.4 (1.8)	0.0011 0.042 0.0033
Protein C act (U/ml)	0.7-1.4	nd	1.08 (0.32)	0.93 (0.30)	0.70 (0.80)	0.84 (0.23)	ns
Protein S act (U/ml)	0.7-1.4	nd	0.80 (0.28)	0.71 (0.32)	0.85 (0.34)	0.78 (0.56)	ns
vWF Ag (U/ml)	0.60-1.40	1.17 (0.68)1	1.51 (0.99)	1.92 (1.57)2	3.69 (2.09)1,2	1.68 (1.40)	0.0011, 0.0022
vWF CB (U/ml)	0.60-1.40	1.27 (0.95)1	1.69 (0.96)2	1.88 (1.20)1	2.46 (1.80)1,2	2.04 (2.14)	0.0011, 0.022
vWF RCo (U/mL)	0.60-1.40	1.06 (0.68)1	1.42 (0.84)1	1.72 (1.22)1	2.34 (2.15)1,2	1.98 (1.68)2	0.0011, 0.012
APTT (sec)	29-39	30 (5.0)	31 (9.8)	31 (7.0)	32 (2.5)	35 (15.3)	ns

Numbers are median (interquartile range). LAC: Lupus anticoagulans, ACA: Anticardiolipid antibodies APC: % of patients with acquired APC resistance

PCD, there was no significant difference between the various groups. Fibrinogen levels were significantly higher in the patients with Amyloidosis compared to the other PCD. The frequencies of the Factor V Leiden mutation and the prothrombin 20210A gene variants were similar in patients with PCD and control subjects. Acquired APC resistance was observed in only a few patients. The incidence of lupus anticoagulant varied between 5 and 33 percent (MGUS and AL Amyloidosis respectively). None of the tested patients had anti-cardiolipin IgM antibodies whereas IgG was observed in only a few of the patients suffering MM and WM (Table 2).

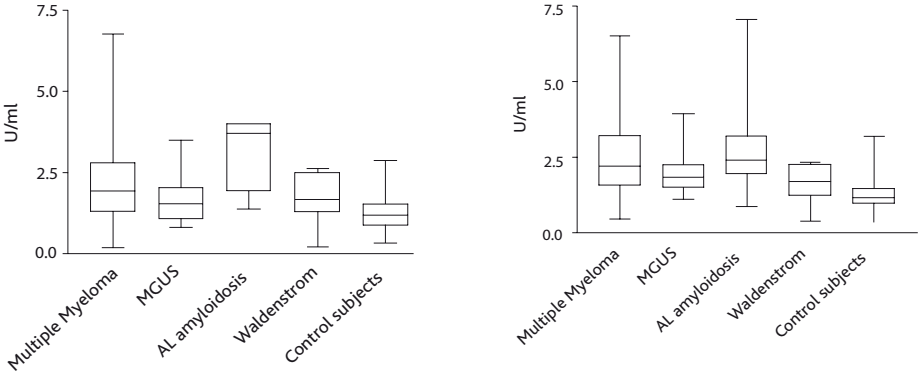

Figure 1. vWF and Factor VIII levels and activity in various plasma cell disorders.

DISCUSSION

Recently, we reported increased levels of FVIII and vWF in patients with untreated MM and that there was a significant difference between the various ISS stages(12). In the present study we also observed an increase in the level and activity of FVIII and vWF in patients with other PCD.

Previous studies have indicated that angiogenesis is increased in MM and that the extent of angiogenesis has prognostic value in the disease(13). Since vWF is mainly synthesized and stored in endothelial cells, an increase in bone marrow neovascularisation is suggested to contribute to this increase in vWF activity. It has been shown that bone marrow angiogenesis progressively increases along the spectrum of PCD from the more benign MGUS stage to advanced myeloma(14). This would suggest an increase of vWF along the same scale. We did observe that vWF is also increased in other PCD and that there was no difference between the various forms of PCD. The highest levels were observed in patients with AL

amyloidosis. However, it has been reported that in these patients, bone marrow angiogenesis seems to be normal(14). Furthermore, in patients with WM only a minority exhibit increased angiogenesis(15). This suggests that other mechanisms, such as inflammation, might also be involved in the pathogenesis of increased levels of vWF. IL-6 plays a stimulatory role in the coagulation mechanism(16), because IL-6 promotes the transcription of the Factor VIII gene(17). Recently it has been reported that IL-6, when in complex with the soluble IL-6 receptor, can also induce hyperactive ultra large von Willebrand Factor(18). Furthermore, Zezos ***et al*** observed a correlation between vWF activity and acute phase reactants (APRs), such as the erythrocyte sedimentation rate and C-reactive protein in patients with inflammatory diseases(19). Elevated vWF activity might therefore also represent an endothelial component of the acute-phase response(20, 21). In our study we mainly focussed on the coagulation system and did not measure markers of APRs, besides fibrinogen.

During normal hemostasis, activated protein C (APC) limits clot formation by proteolytic inactivation of factors Va and VIIIa. APC resistance may be acquired in patients with malignancy(22). In our patients we observed acquired APC resistance in only a few patients with untreated MM and in other PCD. In previous studies, acquired APC resistance was observed in 6 to 23 percent of patients with MM (7, 23, 24). Furthermore, acquired APC resistance has recently been shown to be associated with a increased risk for deep vein thrombosis in patients with MM and it tends to disappear after treatment(24). We have no explanation for the difference between our observation and that reported in the literature, but it may be caused by the different assay used in our study.

Patients with AL amyloidosis are known to have various coagulation abnormalities. Some rare encountered coagulation abnormalities in AL amyloidosis are hyperfibrinolysis or factor X deficiency which can result in a severe bleeding tendency(25, 26). This is only observed in a minority of the AL amyloidosis patients and is associated with a specific coagulation factor disorder, for instance due to absorption of factor X by amyloid fibrils or increase of urokinase type plasminogen activator levels. On the other hand, an increased incidence of VTE (11%) in amyloidosis has recently been reported(6). Our data from a group of 20 patients revealed that FVIII and vWF levels were both strongly increased which may result in a hypercoagulable state.

Analysis on antiphospholipid antibodies revealed an incidence of 12% lupus anticoagulant in the untreated MM patients, which is a higher incidence than recently reported by

Hugo *et al*(23). Anticardiolipin IgG antibodies were detected in a small number of patients with newly diagnosed MM (6%) and MW (13%) whereas no ACL IgM antibodies were observed. Recently similar low incidences have been reported by Zangari *et al*(7).

It would be of interest to analyse the association between the thrombophilia markers and risk of thrombosis in our group of patients. However, only three patients (2 MGUS and 1 AL amyloidosis) and fourteen patients (10%) with newly diagnosed MM who received anthracyclin based multi-agent chemotherapy developed a thrombo-embolic complication. The venous thrombotic events included central venous catheter associated thrombosis of the arm in 14%, deep venous thrombosis of the lower extremity in 50% , and pulmonary embolism in 37% of the events. The number of patients with VTE in our study is limited and larger studies are required to study the relationship between coagulation abnormalities in PCD and the development of VTE.

CONCLUSION

In conclusion, we observed several coagulation abnormalities in patients with different PCD. No major differences were observed for the different subgroups. The thrombophilic state in patients with MM, AL amyloidosis and Waldenström's Macroglobulinaemia may be causally related to the observed increased incidence of VTE in these forms of PCD.

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Chapter

Temporary relief of symptomatic Von Willebrand Disease by Multiple Myeloma

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INTRODUCTION

Multiple Myeloma can be associated with various hemostatic abnormalities which predispose the patient to either venous thromboembolism or hemorrhage. Recently Minnema ***et al*** reported increased levels of von Willebrand factor and factor VIII in patients with multiple myeloma which may result in a prothrombotic state(1). In this chapter we report a patient with type 2A von Willebrand disease who experienced relief of hemorrhagic symptoms due to the development of a multiple myeloma.

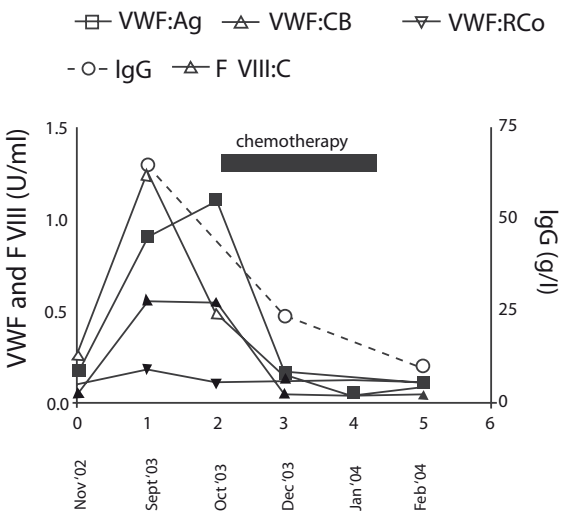
CASE REPORT

Recently a 55-year old male patient was referred to our hospital for treatment of multiple myeloma. In his youth he presented with a bleeding diathesis, due to type 2A Von Willebrand Disease (vWD). Laboratory investigation in the past showed FVIII:C of 0.23 U/ml (normal 0.6-1.4), Von Willebrand Factor: Antigen (vWF:Ag) 0.12 U/ml (normal 0.6-1.4 U/ml), vWF:Ristocetin cofactor activity (vWF:RCo) <0.10 U/ml (normal 0.6-1.4), vWF:Collagen binding activity (vWF:CB) <0.05 U/ml (normal 0.6-1.4). Multimer analysis performed by SDS gel electrophoresis, showed absence of high molecular weight vWF multimers. He had recurrent episodes of epistaxis for which he was treated regularly with tranexamic acid. In addition he underwent several surgical interventions after infusion of FVIII/vWF concentrate (Haemate P, ZLB Behring, Zaventem, Belgium), with normal recovery of FVIII and vWF. In his family several other members were diagnosed with vWD also, including his father, brother, son and daughter, and two grand-children, indicative of an autosomal dominant trait. This confirms the hereditary nature of vWD and excludes an acquired von Willebrand syndrome.

In 2003 he noticed that the frequency of epistaxis diminished while symptoms of fatigue and fever occurred. Laboratory investigations revealed an increased ESR (67mm/hr) and a serum monoclonal IgG-kappa (65 g/l). In the bone marrow aspirate infiltration by monoclonal plasma cells (>60%) confirmed the diagnosis multiple myeloma. Laboratory investigations at that moment revealed increased plasma levels of FVIII:C of 1.29 U/ml, vWF:Ag of 0.9 U/ml, and vWF:CB of 0.56 U/ml. vWF:RCo level remained low (0.18 U/ml). The patient received three courses of Thalidomide, Adriamycin and Dexamethasone according to the prospective phase III HOVON 50/GMMG-HD3 study. During treatment IgG-kappa levels

decreased to 9 g/l, resulting in a good partial response. This co-incided with a decrease of FVIII:C and vWF antigen and activity to pre-existing levels (Figure 1). In addition he again encountered several episodes of epistaxis.

Panel A



Panel B

Figure 1: Plasma levels of vWF, Factor VIII and IgG during the course of treatment (panel A) and SDS agarose gel electrophoresis of vWF multimers pattern (panel B)

DISCUSSION

Multiple myeloma has primarily been associated with a bleeding tendency due to acquired von Willebrand syndrome, factor X deficiency or thrombocytopenia(2, 3). Recently however, an increased risk of venous thromboembolism (VTE) has been reported in patients with multiple myeloma in particular during treatment with doxorubicin and thalidomide(4, 5). The mechanism of the increased thrombotic tendency has not yet been fully elucidated. In multiple myeloma patients high levels of FVIII:C have been reported and a causal relation has been suggested(1). It is already known from population based studies that high FVIII:C levels may be associated with an increased risk of VTE(6). The increase in FVIII:C in multiple myeloma is probably caused by an increased half-life due to high levels of vWF, the carrier protein of FVIII.

It has been hypothesised that bone marrow microvessel density increases due to neo-

vascularization in multiple myeloma(7, 8), which in turn results in an increase in vascular endothelium. Because endothelium is the main source of vWF in the circulation(9), this may explain increased vWF levels in multiple myeloma. However, it remains to be proven whether a direct relation of neovascularization with increased levels of vWF exists. Interestingly, we analysed in retrospect a serum sample of 1 year before the diagnosis of multiple myeloma, and found a M-protein IgG-kappa of < 5 g/l, indicating a pre-existing MGUS. This was not accompanied with changes in FVIII:C or vWF:Ag levels compared to laboratory results obtained in the past. This demonstrates that the transformation of the MGUS to multiple myeloma resulted in the increased FVIII:C and vWF:Ag levels.

In our patient with inherited type 2A vWD, vWF:Ag and FVIII:C levels increased to normal levels at the time of diagnosis of multiple myeloma. vWF:RCo however remained low due to the underlying qualitative disorder of the vWF molecule. The multimeric pattern before and after start of therapy exhibited predominantly low molecular weight multimers, indicative of active proteolysis of vWF (Figure 1). The rate of proteolysis of vWF may be influenced by plasma ADAMTS13 activity which is a recently discovered metalloprotease. In our patient ADAMTS13 activity was 0.63 U/ml (normal 0.6-1.4) and did not change significantly during treatment (at diagnosis of multiple myeloma 0.51, after treatment 0.51). In patients with vWD the bleeding tendency is not only related to vWF levels in plasma, but is also dependent on FVIII:C levels. This is examplified in patients with vWD treated with FVIII/vWF concentrate, in whom FVIII:C levels are of main importance for clinical efficacy(10). Therefore we suggest that in our patient increased FVIII:C and vWF associated with multiple myeloma resulted in relief of symptoms of vWD and that the successful treatment of multiple myeloma re-induced the bleeding tendency related to vWD.

We believe this to be the first report of a temporary relief of symptomatic vWD due to multiple myeloma. This report increases the understanding of hemostatic alterations in multiple myeloma, and may provide additional insight in the thrombotic tendency seen in these patients.

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Chapter 9.1

Foamy Macrophage Syndrome due to Hydroxyethyl Starch Replacement: A severe side effect in plasmapheresis

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INTRODUCTION

In this journal we present a patient with Monoclonal Gammopathy of Uncertain Significance (MGUS) who developed a serious life threatening complication due to massive exposition to hydroxy-ethyl starch (HES) which was used as a plasma expander during repeated plasmapheresis.

CASE REPORT

A 38-year-old woman had received stanozolol, cyclosporine, and frequent plasmapheresis since 1997 because of IgG lambda monoclonal gammopathy with cryoglobulinemia-associated ulcerating leukocytoclastic vasculitis. Initially, gelatin-based plasma expanders and pasteurized human albumin were used. Beginning in February 1999, plasma volumes were exchanged at a 1:1 ratio with undiluted 6% hydroxyethyl starch (HES, Fresenius-Kabi, Hamburg, Germany) because of the reported advantages of HES.

In August 2000, the patient developed severe weight loss, sensory polyneuropathy of the legs, and deteriorating eyesight. On examination, she was malnourished (body mass index, 17.6 kg/m^2) with hepatosplenomegaly and ascites. She had normocytic anemia (hemoglobin level, 7.1 mmol/L; mean corpuscular volume, 82 fL), thrombocytopenia (platelet count, 121 × 10^9 cells/L), low serum creatinine concentration (40 µmol/L [0.45 mg/dL]) and albumin level (26 g/L), and slightly elevated serum IgG level (15.9 g/L) and aminotransferase levels (aspartate aminotransferase level, 62 U/L; alanine aminotransferase level, 52 U/L). Magnetic resonance imaging of the brain revealed vasculitis of the choroid plexus with hydrocephalus and pituitary stalk edema. Histologic examination of bone marrow, skin, duodenal mucosa, liver, peritoneum, and dura mater and cytologic examination of the liver revealed massive infiltration with typical foamy macrophages (Figure). Hematoxylin-eosin, periodic acid Schiff, and oil red O staining showed empty vacuoles. Mycobacterium avium infection was ruled out. Enzyme analysis excluded Wolman, Niemann-Pick, and Gaucher diseases (all lysosomal storage diseases). Immunoelectron microscopy with polyclonal rabbit anti-HES serum, performed by Dr. S. Ständer (Westfälische Wilhelm-Universität, Munster, Germany), confirmed massive tissue storage of HES, especially in the vacuoles of the macrophages (Figure). Although plasmapheresis with conventional expanders was continued, the patient improved only slightly. After 8 months, a bone marrow biopsy still revealed

massive foamy cell infiltration.

Elimination of HES depends on distribution and back-diffusion from body tissues and metabolism (α-amylase), followed by urinary excretion(1). Persistent pruritus due to dose-dependent and time-related skin deposits has been the most frequently reported complication of HES(2, 3). Skin deposits can be detected up to 4 years after the final administration of HES(2, 4). In our patient, HES exposure (130 L within 20 months) led to symptomatic, massive, diffuse tissue infiltration with HES-laden foamy macrophages.

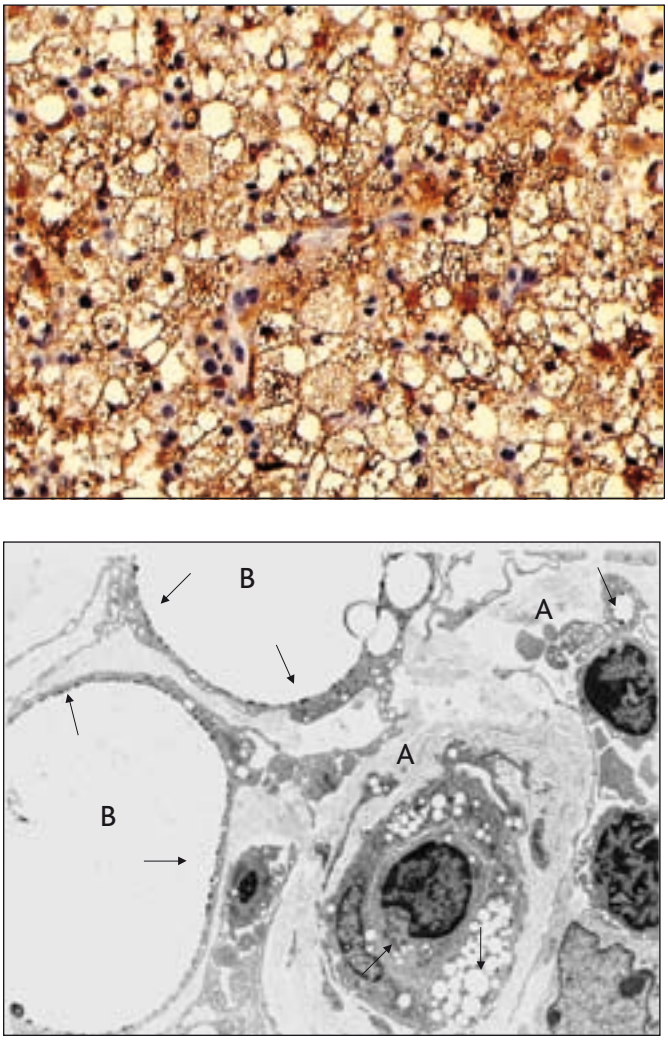

Figure. Microscopic images. Top. Light microscopic image of a bone marrow biopsy revealing massive infiltration with CD68+ foamy macrophages. (Original magnification, ×200) Bottom. Immunoelectron microscopic image of endothelial cells with many small vacuoles (A) and two large vacuoles in the cytoplasm of histiocytes (B). The vacuoles express the characteristic peripheral amorphous material that is reactive for the anti-HES antibody (arrows). (Original magnification, ×2200.)

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Chapter 9.2

Acquired lysosomal storage caused by frequent plasmapheresis with Hydroxyethyl Starch

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Transfusion 2007;49:1330-1331

ABSTRACT

Background

Hydroxyethyl starch (HES) solutions have largely replaced conventional plasma expanders such as human albumin and colloidal fluids. Only a few side effects have been reported and mainly concern pruritus or blood coagulation disorders. Excessive HES exposure can result in diffuse tissue storage and accumulation with foamy appearing macrophages which produce the enzyme chitotriosidase (CT). In case of massive tissue storage this enzyme activity can reach levels comparable to Gaucher's disease.

Study design and methods

In this singel center retrospective analysis of eleven consecutive patients receiving large amounts of HES for chronic plasmapheresis we investigated plasma CT activity. Five patients receiving chronic intermittent plasmapheresis with conventional plasma expanders served as a control. Plasma CT activity was measured and plotted against creatinine clearance. Where available bone marrow aspirate was analysed with light microscopy to detect foamy macrophages. One patient developed a lysosomal storage disease and was examined extensively.

Results

Conventional plasma expanders did not alter plasma CT activity. In patients with impaired renal function, frequent plasma replacement with HES resulted in an increase in plasma CT activity. In the patient with the acquired lysosomal storage disease massive tissue infiltration with activated foamy macrophages was observed. The phagocytic capacity in this patient however did not seem to be altered.

Conclusion

Patients with impaired renal function receiving large amounts of HES exhibit an increase in plasma CT activity. Because excessive HES exposure can result in an acquired lysosomal storage disease this should be avoided in chronic plasmaphereses.

INTRODUCTION

Since 1990, synthetic plasma expanders have replaced conventional, bovine gelatin-based, plasma expanders because they lack the risk of prion transmission, have no bacterial or viral contamination and their use is associated with fewer adverse reactions. Hydroxyethyl Starch products (HES, Fresenius-Kabi, Hamburg, Germany) are synthetic plasma expanders that are frequently used to maintain and restore intravascular volume, stabilize hemodynamic conditions and improve tissue perfusion in ischaemic tissues(1-3). The most frequent reported side effects concern persistent pruritus of the skin and coagulation disorders. Especially due to the blood coagulation disorder the clinical use of HES is limited and maximum daily doses of HES solutions have been recommended (25-50ml/kg)(4). Because of the reported advantages the use of HES products have also been proposed as a standard replacement therapy in apheresis. Data reporting on the side effects during long term use of HES products is however scarce and concern mainly therapy resistant pruritus of the skin due to tissue storage of HES. Large amount of HES however, can even result into massive tissue infiltration with activated foamy macrophages reflecting an acquired lysosomal storage disease(5). Activated foamy macrophages produce the enzyme chytotriosidase (CT) which can be used to monitor the effect of therapy in case of a lysosomal storage disease. In this study we analyzed plasma CT activity in eleven patients who had been exposed to large amounts of HES during frequent plamapheresis.

PATIENTS & MATERIALS

Patients and plasmapheresis technique

Eleven patient from one center were collected who had received more than 10 liters of HES 200/0,5 (EloHAES ® 6%, Fresenius Kabi, Germany), during a sequence of plasmapheresis using a single apheresis machine (GAMBO COBE type Spectra, Zaventem, Brussels, Belgium). During standard plasmapheris a total of 2500ml HES was used as a replacement during the procedure. When the plasma viscoicity was above two times normal a total of 2000ml of HES was used together with 500ml normal saline (0.9% NaCl). The frequency of the procedures was based on the underlying disease in the patients and varied between once a week to three times a week.

Plasma samples were taken one month after cessation of plasmapheresis for determi-

nation of plasma CT activity. Since elimination of HES products mainly depends on renal clearance, the estimated creatinine clearance was calculated from serum creatinine levels by the Cockcroft and Gault formula(6) and plotted against the plasma CT activity. When available bone marrow aspirate morphology was examined by light microscopy. To determine the effect of conventional plasma expanders on the plasma CT activity a group of five patients with Thrombotic Thrombocytopenic Purpura (TTP) who underwent frequent plasmapheresis with replacement by conventional gelatin based plasma expanders (2500ml per session, Gelofusine, Braun) were used as controls. Plasma samples in these control subjects were obtained prior to and after several sessions of plasmaphereses. Informed consent was obtained according to the declaration of Helsinki.

Determination of plasma chitotriosidase

Plasma CT activity was measured by incubating 5µl of EDTA plasma with 100µl of 0.022 mM 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotriose (4 MU-chitotrioside; Sigma Chemical Co., St Louis, MO) as substrate in citrate/phosphate buffer (0.1/0.2 M), pH 5.2 at 370 C, essentially as described previously(7).

Microscopy

Light-microscopic histological analysis of available tissue biopsies was performed using Hematoxylin-Eosin, Periodic Acid Shiff and Oil Red O staining. For electron microscopically investigation, biopsies were fixed in Karnovsky's fixative, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Semi-thin sections were cut with glass knives on an ultramicrotome and stained with toluidine blue. For routine electron microscopy, ultra thin sections were cut with diamond knives, mounted on copper grids, and stained with uranyl acetate and lead citrate.

One patient received excessive amounts of HES and was therefore extensively examined. In this patient various tissue biopsies were obtained and tissue storage was confirmed with immuno-electron microscopy utilizing polyclonal rabbit anti-HES antibodies(8). Pre-incubation with millipore filtered 4% ovalbumin for 10 minutes was followed by overnight incubation with the primary polyclonal antibody (diluted 1:4000 in phosphate-buffered saline: PBS)(9) . The ultra-thin specimens were then examined using a Philips CM10 electron microscope. Fluorescent-activated cell sorter (FACS) analysis of peripheral blood

mononuclear cells (PB-MNC's) was performed with a FACS Calibur Becton-Dickinson (BD) using the following monoclonals: CD15, CD16, CD13, HLADR, CD11b and CD45 (BD, San Jose, California). For this purpose cryopreserved PB-MNC's were sorted by FSC and SSC. (DIVA BD, San Jose, California). The phagocytic activity was tested using Phagotest (Orpegen Pharma, Heidelberg, Germany). In short, 100 µl whole blood or thawed peripheral blood MNC's were incubated for 0,10, 20 and 30 minutes with opsonized E-Coli FITC. After incubation cells were put on ice water and stained with quenching solution to quench bacteria outside the phagocytic cell. DNA staining was performed to discriminate between bacteria and phagocytes. The percentage of cells with phagocytosed bacteria was measured using a FACS Calibur (BD, San Jose, Ca).

STATISTICAL ANALYSIS

This study was a retrospective analysis of eleven consecutive patients receiving large amounts of HES for chronic plasmapheresis in our hospital. Although the sample size was small, when possible statistical analysis was performed using the one-tailed Wilcoxon signed rank test and the one-tailed Spearman correlation coefficient test.

RESULTS

Plasmapheresis with HES

The characteristics of the patients are summarized in Table 1. Plasmaphereses were performed in six patients with hematological disorders, three patients with neurological disorders, one patient with a dermatological disorder and one patient with a benign recurrent intrahepatic cholestasis syndrome.

Table 1. Patients and control subjects

Patient (M/F)	Age (yrs)	Underlying disease	HES liters/month (total)	Creatinine clearance (ml/min)	Plasma chitotriosidase (nmol/h/ml)	Bone marrow Aspirate
1 (F)	43	Monoclonal gammopathy of uncertain significance, vasculitis	6,5 (130)	61	6130	Foam cells Massive tissue infiltration
2 (F)	54	Haemorrhage, mixed cryoglobulinemia	4,7 (14)	84	242	Foam cells present
3 (M)	54	Hyperviscocity M. Waldenström	3,6 (18)	88	422	n.a.*
4 (M)	68	Hyperviscocity M. Waldenström	3,2 (16)	73	307	Foam cells present
5 (F)	54	Polyneuropathy	1,4 (31,5)	70	165	n.a.*
6 (M)	66	Hyperviscocity M. Waldenström	1,1 (25,5)	85	60	Foam cells present
7 (M)	75	Hyperviscocity M. Waldenström	10 (10)	119	93	Foam cells present
8 (F)	34	Myasthenia gravis	4,5 (31,5)	116	59	n.a.*
9 (M)	51	Pemphigus vulgaris	4,1 (33.1)	112	200	n.a.*
10 (M)	23	Benign recurrent intrahepatic cholestasis	2,2 (42,5)	120	206	n.a.*
			Conventional plasma expander (liters)		Plasma chitotriosidase (nmol/h/ml)	
Controls					Before plasmaperesis	After plasmapheresis
1 (F)	29	TTP	20	112	45	46
2 (F)	39	TTP	17,5	183	52	43
3 (F)	35	TTP	35	216	94	38
4 (M)	41	TTP	22,5	113	86	76
5 (F)	34	TTP	17,5	114	12	15
11 (M)	31	Myasthenia gravis	1,6 (67,5)	187	78	n.a.*

* Not available for examination.

In patients with impaired creatinine clearance (patients 1 to 6), a high HES exposure (liters/month) was associated with an significant increase in plasma CT activity above the normal range (Wilcoxon signed rank test P=0,02 Spearman correlation coefficient 0,8586). In patients with a normal creatinine clearance (7 to 11) however, high doses of HES were not associated with a plasma CT activity above the upper normal limit. Although the sample size is small, these results suggest that plasma CT activity is increased in patients with impaired renal function who were exposed to a high dose of HES. The relation between plasma CT activity and renal function is plotted in Figure 1.

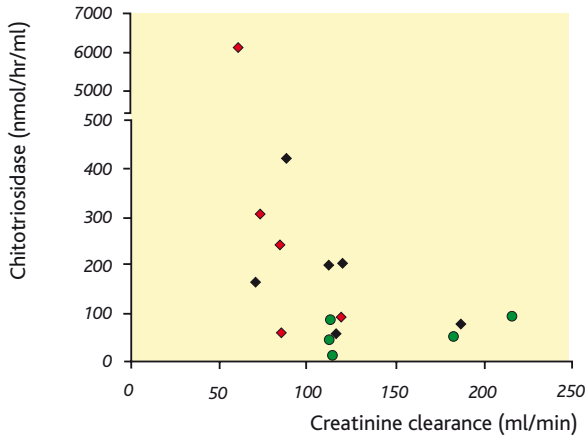


Figure 1. Plasma CT level and renal function in patients with (♦♦) and without (●) HES exposition. Patients with foamy macrophages in bone marrow aspirate (♦). Normal plasma CT level range: 4-195 nmol/ml/hr.

Bone marrow aspirates were available for examination in five patients (patients 1, 2, 4, 6 and 8). Despite normal plasma CT activity in two of these patients, foamy macrophages were observed in five patients even after a cumulative HES dose of less than 10 liters. Patient 1 received the highest cumulative dose and exhibited massive bone marrow infiltration with foamy macrophages (Figure 3). This patient developed a severe acquired lysosomal storage disease (ALSD) with plasma CT activity within the range of Gaucher disease (GD). The extent of marrow infiltration with foamy macrophages in the other patients was however much less than in the patient with the ALSD.

Plasmapheresis with conventional expanders

Plasma CT activity was within normal range in all patients in the control group. In these subjects, frequent plasmaphereses with conventional expanders did not alter the plasma

CT activity significantly (Table 1). These results support the hypothesis that plasmapheresis with conventional expanders do not alter plasma CT activity.

Acquired lysosomal storage disease

One patient (no 1) received a high dose of HES (130 liters) and developed a lysosomal storage disease. This patient, who was an outlier compared to the other patients, was diagnosed suffering MGUS with type IgG kappa cold agglutinin disease. Chronic plasmaphereses was started because of severe disabling skin necrosis due to therapy resistant leucocytoclastic vasculitis. At that time plasma CT activity was normal and a bone marrow biopsy revealed no foamy macrophages at all. This patient received during the first six months of phereses the combination of gelatin based plasma expander (36.5 liters) and pasteurized human albumin (25.5 liters, Cealb, Sanquin) without altering plasma CT activity (Figure 2). The following twenty months a cumulative dose of 166,5 liters of Gelofusine (Braun) was used as a single agent. Again plasma CT activity was unaltered and remained normal (47 nmol/h/ml). A bone marrow biopsy performed at that time did not show foamy macrophages. Next, the conventional plasma expanders were replaced by HES. Serum CT activity increased exponentially and reached the level comparable to that found in Gaucher Disease after a cumulative dose of 100 liters over a time period of ten months (Figure 2).

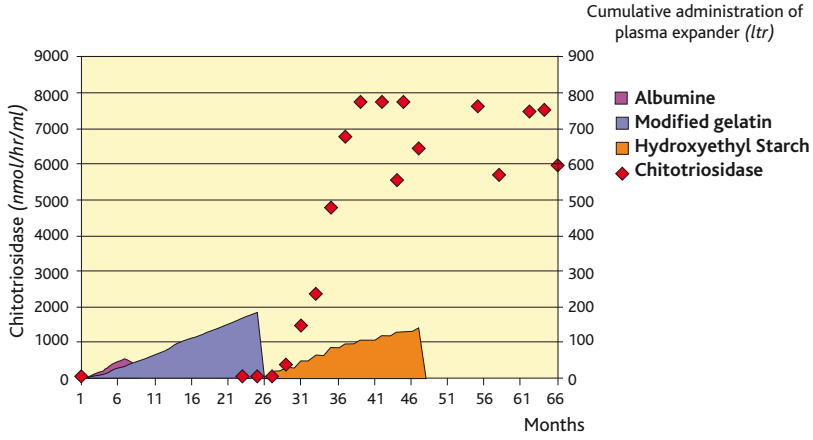


Figure 2. Plasma chitotriosidase level in a patient with acquired lysosomal storage disease following plasmapheresis.

At the same time this patient developed symptoms of a lysosomal storage disease i.e., severe weight loss, organomegaly with ascites, myelofibrosis, polyneuropathy and hydrocephalus with vasculitis of the choroid plexus and pituitary stalk edema as a result of exces-

sive tissue infiltration with foam cells (Figure 3). Bone marrow aspirate morphology confirmed these cells to be of macrophage origin. Similar massive foamy macrophages infiltration was observed in biopsies taken from the skin, liver, duodenal mucosa, peritoneum and dura mater. Typical HES storage within the vacuoles in the cytoplasm of skin macrophages and endothelial cells was confirmed with immuno-electron microscopy. In this patient, cultured fibroblasts exhibited a normal alpha-glucosidase activity. After confirming the relation between excessive HES exposition and ALSD, plasmapheresis was continued with a change to conventional expanders in order to facilitate a gradual clearance of HES. This approach did not result in a significant decrease of plasma HES concentrations or plasma CT activity (Figure 2). One year after cessation of HES, the plasma CT activity was still within the range of GD (higher than 7000 nmol/hr/ml) and in the bone marrow morphology massive foamy macrophage infiltration was still present.

In this patient, the phagocytic capacity of monocytes (85%) and granulocytes (95%) after thirty minutes was within normal limits. However, the thawed peripheral blood mononuclear cells (PB-MNC's) contained a subpopulation of granulocytes, which was absent in a control subject (Figure 4). These cells were resistant to freezing and thawing and had a lower phagocytic capacity (max 38% after 20 minutes). On light microscopy however, these cells had a normal morphology without the typical foamy appearance.

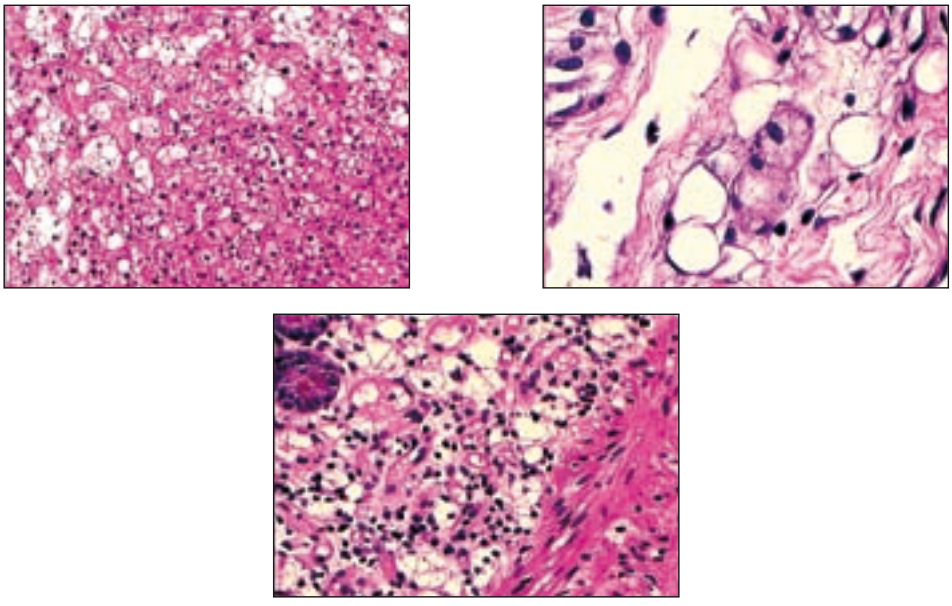
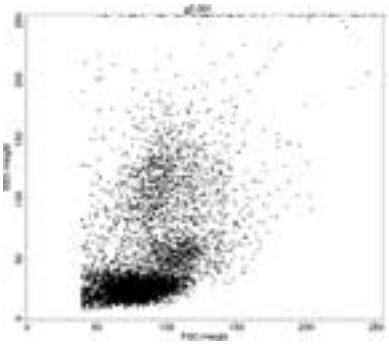


Figure 3. Light microscopic image of liver (A, 200x), peritoneum (B, 400x) and the duodenal mucosa (C, 200x) of the patient with the acquired lysosomal storage disease.

Panel A



Panel B

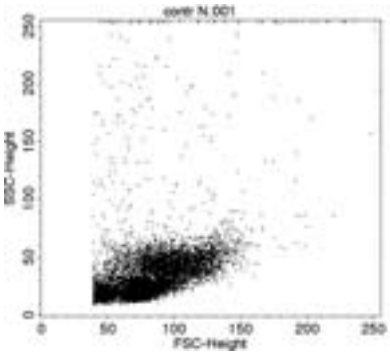


Figure 4. FACS staining of peripheral blood mononuclear cells revealing the extra population (Panel A) in the SALSD patient.

DISCUSSION

Chitotriosidase (CT) is an enzyme that plays a role in the degradation of chitin-containing pathogens(10). In humans this enzyme is almost exclusively synthesized by activated macrophages(10). Chitotriosidase is a very stable enzyme and its activity can be tested in cryopreserved plasma samples for up to 8 years(11). The chitotriosidase activity increases with age and normal values range from 4 to 195 mmol/hr/ml(11). Elevated plasma CT levels serve as a diagnostic hallmark in patients with GD and the plasma level is correlated with the extent of tissue infiltration by foamy macrophages(7). Elevated plasma CT activity however, is not correlated with specific clinical signs and symptoms(11). Our study supports the assumption that plasma CT activity is not influenced by plasmaphereses with conventional expanders such as modified gelatin or human albumin. This suggests that these conventional expanders are not stored into macrophages in tissues.

HES is a synthetic colloid plasma volume expander which has been used as an alternative for albumin and gelofusine in patients undergoing plasma exchange. The kinetics of its elimination is complex. Smaller molecules (Mw<50.000 Da, T½ of 0,4 to 6,4 days) are readily excreted in the urine. Elimination of larger molecules depends on: (a) distribution into body tissues; (b) back-diffusion from tissues into intravascular spaces; and (c) metabolism by α-amylase in blood, tissues, and the reticuloendothelial system, followed by urinary and biliary excretion with a T½ ranging from 72 to 240 days(12, 13). Tissue storage in cells of various organs, predominantly affecting the mononuclear phagocyte system (MPS) has been demonstrated previously(14). This is a dose-dependent and time related phenomenon

and marked signs of storage can be detected up to 4 years after the final administration(15, 16). Patients on hemofiltration or dialysis exhibit an even more prolonged half-life due to incomplete HES elimination(17). In these patients, even small quantities of HES can cause hepatosplenomegaly, portal hypertension and ascites(18).

A relation between HES storage in macrophages and plasma CT activity has not yet been documented. Our data suggest that chronic intermittent administration of HES during plasmapheresis in patients with impaired renal function will result in tissue storage, especially within the MPS. These macrophages are unable to further digest the HES molecules. The continuous accumulation within the vacuoles will result in the foamy appearance. These stressed macrophages produce CT through a mechanism that is yet unknown. However, when sufficient tissue infiltration with activated foamy macrophages occurs, this will result in an increase of plasma CT activity. Since two patients (pt 5 & 6) receiving high dose of HES exhibited only a slightly elevated CT activity additional factors such as the time period in which the large amount of HES was administered may also be of importance. These patients (pt 5 & 6) received their HES during a prolonged period of time (1,1 – 1,4 ltr/month) whereas the other patients were exposed to higher doses over a shorter period of time (2,2-10 ltr/month). However, based on the limited number of patients this remains speculative.

In GD the level of plasma CT activity reflects the extent of tissue infiltration with activated foamy macrophages. The first patient exhibited massive tissue infiltration with foamy macrophages whereas in bone marrow biopsies of the other patients (when available), moderate infiltration was observed. We therefore suggest that the CT activity can serve as an indirect diagnostic tool to monitor the extent of tissue infiltration with HES. Because there is no evidence regarding this assumption we cannot confirm this statement and further in vitro studies exposing cultured macrophages to HES might be helpful in confirming this hypothesis. Since plasma CT activity directly reflects the extent of foamy macrophage infiltration in lysosomal storage disease, we suggest that in case large amounts of HES have been administered (> 30 liters) and plasma CT activity rises above the upper limit of normal, HES substitution should be discontinued. Brecher reviewed the literature on the use of large volumes of HES and found that is was well tolerated without any serious complications(2). However, in one case the patient developed normocytic normochronim anemia and bone marrow replacement with "lipid laden" macrophages(19).

One patient developed a severe ALSD due to massive tissue infiltration with these

foamy macrophages as a result of chronic plasmaphereses with HES (cumulative dose: 130 liters within 20 months). This resulted in severe weight loss, organomegaly with ascites, myelofibrosis, polyneuropathy and hydrocephalus with vasculitis of the plexus choroideus. Interestingly this patient did not complain of persistent pruritus, which has been reported to be the only major side effect of HES administration. This could be due to the polyneuropathy that she developed due to the massive tissue storage. Retrospective analysis of the plasmapheresis history of this patient revealed that conventional plasma expanders did not alter the plasma CT activity at all. After a cumulative dose of 20 liters of HES, plasma CT activity increased exponentially to finally reach the level of GD. When the relation between HES storage and plasma CT activity was confirmed, HES re-infusion was stopped, and plasmaphereses were continued with conventional expanders. Although this corrective action resulted in a clinical improvement, plasma CT activity remained high even after two years. Furthermore, a bone marrow biopsy performed after one year still revealed massive infiltration with foamy macrophages.

Recent clinical investigations suggest that the phagocytotic capacity and signal transduction of monocytes is unaltered by intracellular colloid storage after incubation with HES resuscitation fluids in vitro(20-23). However, there is no literature regarding the effect of long-term in vivo exposure to HES and the effect on the PB-MNC's. In our patient, the phagocytic capacities of the peripheral blood monocytes and granulocytes were within normal limits. However, thawed peripheral blood MNC's contained an extra population (CD15+, CD16+, CD13+, HLADR negative, CD11b+ and CD45 dim), which was absent in control patients. These granulocytes were resistant to freezing and thawing and had a lower phagocytic capacity. Light microscopic analysis of these granulocytes revealed no typical vacuolization. We assume that these granulocytes had already phagocytosed small amounts of HES before migrating into the tissues, and therefore exhibit a reduced phagocytic activity in the phagocytic test. In contrast, unaffected phagocytosis of HES by monocytes/macrophages in the tissues may result in prolonged storage of HES within the vacuoles, ultimately leading to the foamy appearance of these macrophages. The possible mechanism of the acquired lysosomal storage disease might be that HES molecules can only be hydrolysed and degraded by the enzyme alpha-glycosidase into smaller molecules. Urinary elimination is biphasic with an initial half-life of 3-4 days (small molecules) and of 48 days (larger molecule hydrolization)(24). The remaining HES molecules are resistant to cleavage and can

be detected several months after transfusion. These larger molecules are phagocytosed and stored in macrophages. Excessive exposure to these cleavage resistant HES molecules may result in extensive tissue accumulation with foamy macrophages. This may affect various organs and results in organ dysfunction and an acquired lysosomal storage disease. We believe that all forms of HES products despite its various forms, can result in tissue accumulation when substituted in large amounts(25).

CONCLUSION

We conclude that in patients with pronounced tissue accumulation and infiltration with HES containing foamy macrophages, plasma CT activity might serve as a diagnostic tool to monitor the extent of tissue infiltration. Furthermore, chronic intermittent plasmapheresis with excessive replacement with HES products might result in a severe ALSD. Based on this severe side effect and the reported effects of HES on blood coagulation(26)and renal function(27) we believe that HES products should be used with caution during chronic plasmapheresis, especially in patients with impaired renal function.

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Chapter 10

Summary and general discussion

SUMMARY

Thrombo-embolic complications occur frequently in patients with malignancy. In patients with multiple myeloma, incidences of up to 28 percent are reported. Most thromboembolic complications are observed during the induction phase of multi-agent chemotherapy. The combination of antracyclines and dexamethasone with anti-angiogenic drugs such as thalidomide, increases the incidence considerably. Although several studies have been performed on this topic in the past years, the mechanism of the increased thrombotic risk in patients with multiple myeloma remains largely unraveled.

The main objective of this thesis was to analyze different aspects of the hemostatic system in patients with multiple myeloma in order to increase our knowledge of hemostatic abnormalities and to determine their role in the development of venous thrombo-embolic complications.

The current literature on the relationship between multiple myeloma and thrombosis was summarized in **chapter 1**. In addition the aims of the studies described in this thesis were mentioned.

The first aim was to analyse baseline hemostatic variables in untreated patients with multiple myeloma. The results are presented in **chapter 2**. A total of 135 patients with newly diagnosed multiple myeloma were studied for this analysis. Factor VIII:C and von Willebrand Factor antigen (vWF:Ag) were strongly elevated. A strong correlation between vWF and FVIII:C levels was observed. Furthermore, vWF:Ag levels correlated strongly with vWF collagen binding (vWF:CB) and ristocetin-cofactor activity (vWF:RCo). Factor VIII and vWF levels correlated with the prognostic International Scoring System (ISS) disease stage with the highest levels in stage III. When staged according to the Salmon and Durie classification system, the correlation with disease stage was less clear. Protein S levels were in the low normal range in patients with multiple myeloma and were significantly lower in the higher ISS stages compared to stage I. Other coagulation parameters, including antithrombin, fibrinogen and D-dimer levels, were not significantly different compared to healthy volunteers. During the course of treatment, thrombo-embolic complications were observed in 10% of the patients and occurred most frequently during induction chemotherapy. However, no single prothrombotic abnormality at baseline was significantly associated with the development of a thrombo-embolic complication. In conclusion, this study indicates that various prothrombotic abnormalities occur in patients with newly diagnosed untreated

multiple myeloma, such as strongly increased vWF and Factor VIII levels, and a decrease of protein S levels. Furthermore, the level of these coagulation variables seem to correlate with the ISS stage of disease.

Subsequently we analyzed coagulation variables during several phases of treatment in patients with multiple myeloma who were treated with multi-agent chemotherapy followed by high dose melphalan (HDM) with autologous stem cell transplantation (ASCT). The results are presented in **chapter 3**. Response to treatment was assessed after induction therapy and 3 to 6 months after autologous stem cell transplantation. At the end of treatment, most patients showed a remission (85%), of which 14% complete and 86% partial. We compared levels of various coagulation factors during and after treatment in the total patient group. FVIII:C and vWF:Ag levels showed a parabolic course during the treatment phases with the maximal values after induction treatment. FVIII and vWF levels at the end of treatment, after high dose melphalan followed by autologous stem cell support, were significantly decreased compared to the levels during treatment. Fibrinogen levels significantly increased during treatment and remained elevated after the HDM with ASCT. In the linear mixed-effects analysis of the three time points, fibrinogen levels were significantly different between the VAD and TAD group with a higher increase in the TAD group versus the VAD group. Although antithrombin, protein C or protein S levels increased slightly during induction chemotherapy, these levels remained within the normal range. There was no relationship between coagulation parameters before, during and after treatment and the development of VTE. In conclusion, this study shows that there is a significant increase in the levels of vWF, FVIII:C and fibrinogen during the induction therapy of multiple myeloma and that these levels decrease after the ASCT.

In **chapter 4** the plasma fibrinolytic potential was measured in 77 patients with multiple myeloma patients who were treated with multi-agent chemotherapy followed by HDM and ASCT. Fibrinolysis was assessed before, during and after chemotherapeutic treatment. These patients received induction therapy with either VAD of TAD. There was no significant difference in clot lysis time between age matched control subjects and the patients prior to the start of induction chemotherapy. However, there was a significant increase of clot lysis time during both VAD and TAD as compared to clot lysis times of the control subjects, indicating the development of hypofibrinolysis during chemotherapy. After high dose melphalan with autologous stem cell support, the clot lysis times normalized and were similar to the control

group in both the VAD and TAD treated patients. It is concluded that hypofibrinolysis, which is known to be associated with VTE (1), occurs during induction chemotherapy in multiple myeloma patients.

Tissue factor is the principal initiator of the coagulation cascade and can be demonstrated on circulating microparticles that are released from cells following activation or during apoptosis. Recent studies have indicated an association between microparticle Tissue Factor (MP-TF) activity and thrombosis in adenocarcinoma(2). We analyzed MP-TF activity in multiple myeloma patients who were treated with multi-agent chemotherapy and presented this in **Chapter 5**. Prior to the start of chemotherapy, the median MP-TF activity level in multiple myeloma was significantly higher than in healthy volunteers (17.8 fM Xa/min [IQR 8.8-32.8] versus 4.7 fM Xa/min [IQR 2.3-6.6], P<0.001). Lambda type multiple myeloma exhibited higher MP-TF activity than kappa type multiple myeloma. The severity of disease, according to the Salmon and Durie criteria and the prognostic ISS score was not correlated with the levels of MP-TF activity. MP-TF activity decreased significantly after three courses of induction chemotherapy and the reduction was irrespective of the type of induction chemotherapy used. VTE incidence was 8% in the VAD treated patients, 11% in the TAD treated patients and 7% in the PAD treated patients. In patients who developed VTE, MP-TF activity remained increased after the induction therapy. In conclusion, MP-TF activity levels are increased in patients with untreated multiple myeloma and decrease significantly after induction chemotherapy. Our results may indicate that persistently increased MP-TF activity levels in plasma of multiple myeloma patients during induction treatment may be of pathogenetic importance in the development of VTE. Larger studies are required to investigate whether increased MP-TF activity levels are causally related to the occurrence of VTE in multiple myeloma patients.

The prevention of VTE, especially in multiple myeloma patients receiving multi-agent chemotherapy in combination with anti-angiogenic drugs is still a major issue and the optimal antithrombotic regime in multiple myeloma patients receiving induction treatment has yet to be established. We performed a prospective study to investigate the prevention of VTE with the low molecular weight heparin (LMWH) nadroparin in patients receiving multi-agent chemotherapy in combination with thalidomide included in the HOVON-50 study and presented the data in **chapter 6**. In this large prospective intervention study we observed a VTE incidence of 10% in the patients treated with thalidomide, dexamethasone

and doxorubicin who received prophylactic nadroparin. This incidence is comparable with the incidence of VTE during intensive chemotherapy in newly diagnosed multiple myeloma patients, without the use of thromboprophylaxis reported previously in the literature. In our prospective study the incidence of VTE in patients receiving VAD without thrombo-prophylaxis was 5%. It can be concluded that the use of LMWH is a safe when patients receive a thalidomide combination-based chemotherapeutic regimen and that the incidence of VTE is reduced to an incidence comparable to standard chemotherapy with antracyclin.

Recent reports suggest an increased VTE incidence in patients with other malignant plasma cell disorders, including AL-amyloidosis (VTE incidence 7%) and Monoclonal Gammopathy of Undetermined Significance (MGUS) (VTE incidence 11%) (3-6). We therefore analyzed coagulation variables in patients with other paraprotein producing B-cell disorders, such as MGUS, AL amyloidosis and Waldenstrom's Macroglobulinemia (WM). The results are presented in **chapter 7**. In this study we observed that vWF and factor VIII levels are not only increased in patients with multiple myeloma, but also increased in the other plasma cell disorders (PCD), with the highest levels in patients with AL amyloidosis. Protein S activity was within the lower range of normal in all forms of PCD, and no significant difference between the various groups was found. In patients with AL amyloidosis, fibrinogen levels were significantly higher compared to control subjects and the other PCD. In conclusion, we observed similar coagulation abnormalities in patients with different PCD, as previously found in patients with multiple myeloma. The highest levels of FVIII and vWF were observed in patients with AL amyloidosis.

In **chapter 8** we studied a patient with inherited type 2A von Willebrand disease (vWD) who developed multiple myeloma. This study shows that the clinical phenotype of vWD can be altered by the multiple myeloma associated increase of vWF and FVIII. In this patient vWF:Ag and FVIII:C levels increased to normal levels at the time of diagnosis of multiple myeloma and resulted in a temporary relief of bleeding symptoms especially during chemotherapy. vWF activity levels however remained relatively low due to the underlying qualitative disorder of the vWF molecule. The multimeric pattern before and after start of therapy exhibited predominantly low molecular weight multimers, indicative of active proteolysis of vWF. Treatment of multiple myeloma resulted in a decrease of FVIII and vWF to pre-existing levels and re-induced the bleeding tendency related to vWD in this patient. In conclusion this observation stresses the clinical importance of coagulation factor changes

in altering the hemostatic balance in patients with multiple myeloma. This provides more insight in the pathogenetic mechanism, underlying the VTE complications in multiple myeloma patients.

In **chapter 9** a patient with therapy resistant ulcerating vasculitis due to a MGUS related cryoglobulinemia was studied. In this patient, chronic plasmapheresis with the plasma expander hydroxy-ethyl starch (HES) was initiated because of severe disabling skin necrosis due to therapy-resistant leucocytoclastic vasculitis. This patient developed a rare and severe complication as a result of frequent plasmapheresis with HES. This resulted is diffuse systemic infiltration with foamy appearing activated macrophages and plasma chitotriosidase (CT) activity within the range of Gaucher's disease. In an additional study in 15 patients receiving chronic intermittent administration of HES during plasmapheresis we found that in patients with impaired renal function, this will result in tissue storage, especially within the mononucleair phagocyte system. Although the sample size of our study is small, these results suggest that plasma CT activity is increased in patients with impaired renal function who were exposed to a high dose of HES. It can be concluded that the administration of large amounts of HES as a plasma expander in plasmapheresis can result in tissue accumulation of HES especially in patients with impaired renal function.

GENERAL DISCUSSION

Thrombo-embolic events in multiple myeloma

Multiple myeloma accounts for approximately 10% of all hematologic malignancies with an annual incidence of 3-4 cases per 100,000 and is still considered incurable. The introduction of high-dose therapy with stem cell support and new antimyeloma agents has improved the overall survival from a median of 24-30 months obtained with conventional therapy to over 5 years nowadays. In recent years it has become evident that a major complication observed in patients with multiple myeloma is the development of venous thrombo-embolism (VTE). The overall incidence reported in untreated patients with multiple myeloma is appoximately 10% after the initiation of standard multi-agent chemotherapy, such as VAD or thalidomide(6, 7). This incidence of VTE strongly dependends upon the disease status (newly diagnosed, refractory or relapsing) and the kind of treatment the patient receives. When the anti-angiogenic drug thalidomide is added to chemo-therapeutic

regimens, VTE incidences up to 28% have been reported(8). This might be explained by the observation that the combination of doxorubicin with thalidomide may induce endothelial cell dysfunction(9, 10). In refractory and/or relapsed patients the incidence of VTE is lower. When treated with conventional chemotherapy the overall VTE incidence is 5% and increases to 12% when thalidomide is added to the regimen. The recently introduced thalidomide derivate lenalidomide is also associated with an increase in VTE risk, especially when combined with dexamethasone(11-14). On the other hand, the newly introduced proteasome inhibitor bortezomib is associated with a lower incidence of VTE(15, 16). The low VTE incidence during bortezomib treatment may in part be explained by the fact that bortezomib has so far predominantly been used in refractory patients, who, as mentioned above, have a lower incidence compared to previously untreated myeloma patients. However, in vivo and in vitro studies have shown an inhibitory effect of bortezmib on platelet aggregation induced by ADP which suggests an antithrombotic effect of bortezomib and might therefore also explain the lower incidence of VTE in these patients during multi-agent chemotherapy(16, 17). In our studies the incidence of VTE in a cohort of newly diagnosed multiple myeloma patients during chemotherapeutic treatment was 10%, which is similar to the data presented in the literature(18). The VTE incidence observed in our patients receiving the combination of thalidomide and multi-agent chemotherapy was also 10%. This lower incidence than previously reported in the literature, is due to the LMWH prophylaxis that these patients received during therapy(19).

Changes in the hemostatic system in Multiple Myeloma

Several independent risk factors have been reported in the literature to be associated with the development of VTE in multiple myeloma patients, including newly diagnosed status; the use of thalidomide/doxorubicin regimen; presence of chromosome 11 abnormalities; light-chain disease and elevated C-reactive protein (20).

Multiple myeloma is also associated with a hypercoagulable state. Treatment with chemotherapy with or without anti-angiogenic agents may aggravate this hypercoagulable state and may trigger the development of thrombosis. Previously reported factors that may contribute to the increased risk of VTE include acquired abnormalities in platelet function, secondary hemostasis and fibrinolysis. For instance acquired resistance to APC has been associated with the risk of VTE in multiple myeloma patients(20, 21 - 26). The fact that bor-

tezomib treatment is associated with a lower risk of VTE may be due to the anti-aggregating effect on platelet function(17, 27, 28). In a recent study by Zangari et al, platelet function was assessed pre- and post infusion of bortezomib. They found a significant decrease in epinephrine and ristocetin induced platelet aggregation. Also the expression of P-selectin at the platelet surface was decreased after bortezomib infusion (29). The addition of high dose dexamethasone may also be of pathogenetic importanve in the occurrence of VTE, since it has been shown that high dose dexamethasone increases circulating P-selectin and vWF and may also result in higher FVIII levels(30).

Recently, high plasma levels of soluble P-selectin in solid cancer-associated VTE was found to be a possible predictor for the development of VTE(31). The role of P-selectin in multiple myeloma associated VTE however has not yet been studied.

In this thesis we have shown that in newly diagnosed multiple myeloma, before treatment is initiated, several coagulation abnormalities are observed. During the induction phase with multi-agent chemotherapy these abnormalities in both coagulation and fibrinolysis deteriorate substantially, resulting in an even more severe hypercoagulable state(25, 32). The alterations in secondary hemostasis observed in our studies in a large group of MM patients are depicted in Figure 1.

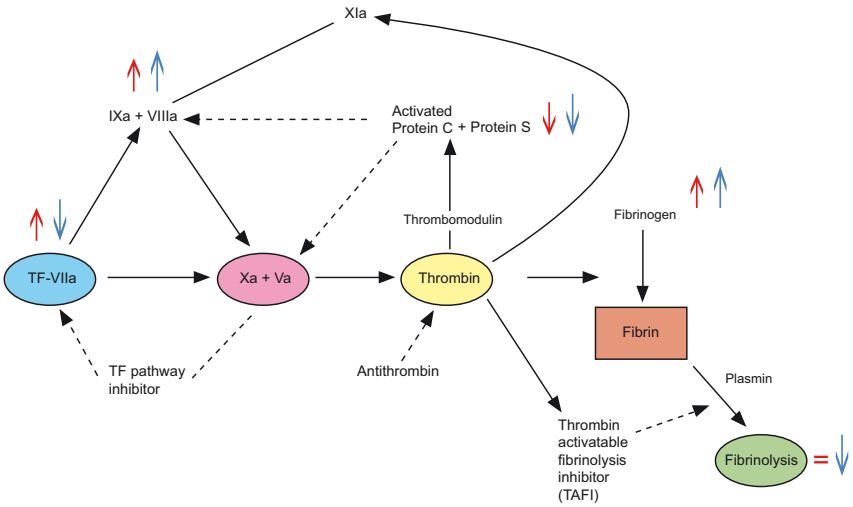

Figure 1 Alterations in the secondary haemostasis in multiple myeloma at diagnosis (red) and after multi-agent chemotherapy (blue).

Although the data from our studies, as well as the data recently presented in the literature, provide more insight into the coagulation abnormalities in patients with multiple myeloma, no single coagulation variable can yet be used to predict whether a patient will develop a VTE. We believe that the altered coagulation abnormalities in combination with other well known risk factors such as immobility, surgery and indwelling central venous catheters, may result in an additionally increased risk for VTE. Although we included a large cohort of multiple myeloma patients for our longitudinal coagulation studies, the number of patients that developed VTE was limited. Therefore we were not able to establish an association between coagulation disturbances and the development of VTE. Our studies however, show that increased FVIII and vWF levels, hypofibrinolysis and increased MP-associated TF activity occurs in patients with multiple myeloma and that this may contribute to the hypercoagulable pro-thrombotic state and the development of VTE. Larger studies however, are needed to provide more insight in the mechanism of thrombosis and the possible predictive role of the above-mentioned variables in multiple myeloma patients.

Prevention of VTE in multiple myeloma

The recognition of a hypercoagulable state in newly diagnosed multiple myeloma, especially during multi-agent chemotherapy in combination with anti-angiogenic drugs, has

led to the introduction of prophylactic anticoagulant therapy. There is however, still debate regarding the optimal antithrombotic regimen in multiple myeloma patients receiving induction treatment with anti-angiogenic drugs. The results of various thromboprophylactic regimens have recently been reported (Table 1) and several trials are ongoing to investigate which regimen is optimal to prevent VTE in patients with multiple myeloma treated with multi-agent chemotherapy.

Table 1. VTE incidence (%) with thromboprophylaxis during treatment of MM

	thromboprophylaxis				
Therapy	**None**	**aspirin**	**LMWH**	**LD-VKA**	**VKA**
Newly diagnosed MM					
Melphalan/thal/prednisone	12-21*(33-35)*	3-7.1*(33, 34)*			
Thal/Dex	12-26*(36-41)*		7*(41)*	25*(41)*	7-13*(36-38, 41)*
Thal/chemo (+antracyclines)	16-27*(8, 42)*		9-15*(19, 43)*	31*(43)*	12*(44)*
Thal/chemo (+antracyclines)	34*(45)*		24*(45)*		
Len/Dex		75*(46)*	3-18.5*(46-48)*		
Len/Dex (low dose dex)		5.4*(47)*			
Melphalan/prednisone/Len		2.1*(34)*			
Len/cyclophosphamide/Dex		16*(49)*			
Previously treated/relapsed/refractory MM					
Thal/chemo (+antracyclines)	15*(50)*		10*(15)*		
Bort/Thal/chemo (+antracyclines)			0*(15)*		
Chemo (+antracyclines)		9*(51)*			

Thal= Thalidomide, Bort= Bortezomib, Dex= Dexamethasone, chemo= multi-agent chemotherapy, Len= lenalidomide, LMWH= low molecular weight heparin, LD-VKA= low dose vitamin K antagonist, VKA= vitamin K antagonist *(references)*

Intervention with vitamin K antagonists seems reasonable because of the observed alterations in secondary hemostasis in multiple myeloma. However, a fixed low dose warfarin (1mg/day) is insufficient in reducing the VTE incidence(43). Full-intensity warfarin (target INR: 2-3) or low molecular weight heparin, reduces the risk of thrombo-embolic complications, but may be associated with increased bleeding(19, 34, 41, 52). In a sub study of the HOVON-50 study LMWH was used to prevent VTE in patients receiving a thalidomide-based regimen as induction therapy. This resulted in a VTE incidence of 10%, which is similar to that reported in the literature when multi-agent chemotherapy was given in combination with thalidomide and thromboprophylaxis(19). Aspirin is effective in preventing arterial thrombosis (e.g. myocardial infarction, stroke) and it has been recently been suggested that aspirin is safe and effective in preventing VTE in multiple myeloma(24, 26, 32). In refractory

patients receiving lenalidomide in combination with dexamethason aspirin (80-325mg/day) reduced the incidence of VTE significantly(47). Recent clinical trials confirmed the successful use of aspirin as thromboprophylaxis in untreated multiple myeloma patients receiving multi-agent therapy(22, 34, 53). In a randomised controlled trial, which was recently presented, in which various thalidomide containing chemotherapeutic regimens were used, LMWH was equal to aspirin or low dose warfarin in preventing VTE. However LMWH was associated with fewer bleeding complications(54). So far, except for the study presented by Palumbo ***et al***, few data are available regarding the use of thromboprophylaxis during treatment with the recently introduced new anti-myeloma agents such as bortezomib and lenalidomide. However, based on the available data, bortezomib seems to be associated with a low VTE incidence(55). Furthermore, the combination of lenalidomide with bortezomib without thromboprophylaxis is also associated with a low VTE incidence(54). Perhaps the possible inhibitory effect of bortezomib on platelet aggregation might provide some protection by interfering with primary hemostasis, as mentioned above(17). This phenomenon and its clinical implication need further clinical investigation. More prospective controlled trials should be conducted to answer the question which thromboprophylaxis should be used in various chemotherapeutic regimens as well as the duration of prophylaxis. Recently Palumbo ***et al*** presented their recommendations regarding the choice of thromboprophylaxis in myeloma patients receiving thalidomide or lenalidomide in combination with steroids of chemotherapy(56). The choice should be tailored to the presence of standard individual risk factors that may increase the risk of VTE (obesity, age, history of VTE, central-venous catheter, comorbidities, surgical procedures and inherited thrombophilia), myeloma related risk factors (previously untreated myeloma and/or hyperviscosity) and therapy related risk factors (dexamethasone and/or doxorubicin). Based on our observations and these recommendations aspirin could be used in low-risk patients, such as those with no risk factor or one individual/myeloma-related risk factor. In these patients interfering with primary hemostasis might be sufficient to prevent VTE. In the presence of more than one individual or myeloma-related risk factor, a more intensive thromboprophylaxis is warranted. In these patients LMWH should be recommended especially when high-dose dexamethasone or doxorubicin or multiagent chemotherapy are administered. In patients who receive multiagent chemotherapy in combination with bortezomib, the recommendations are still unclear and need to be investigated further.

Future studies

A variety of coagulation abnormalities in multiple myeloma patients have now been characterized that contribute to a state of hypercoagulability. However, a direct relationship with the development of VTE is not yet established. Therefore larger studies are required. Instead of focussing on single prothrombotic changes in MM patients , future studies should include global coagulation tests to assess the combined effect of these coagulation abnormalities. For this purpose thrombo-elastography, thrombo-elastrometry and thrombin generation assays can be used. Furthermore, the relationship between coagulation and inflammation could be further characterized, since in general inflammation also plays an important role in the development of thrombosis(57). Genetic predisposition due to single nucleotide polymorphisms (SNPs) or certain haplotypes may also be a risk factor for the development of thrombosis in VTE patients. A recent studie in a large cohort of multiple myeloma patients treated with thalidomide has identified several SNPs in genes and pathways important in drug transport, drug metabolism, DNA repair and cytokine balance that were associated with the risk of VTE (data unpublished). Remarkably in this analysis no significant association of VTE and SNPs within the coagulation or prothrombotic pathways was found. This suggests that VTE risk in multiple myeloma patients is mediated by alternative mechanisms, such as response to DNA damage and cytokine mediated apoptosis. In the same study it was shown that there are a limited number of SNPs, that can predict the risk of VTE, when analysed together. However this should first be validated in large prospective clinical trials(58). Future studies using global coagulation tests and studies on genetic factors that predispose for VTE will further unravel the pathogenesis of venous thrombosis in patients with multiple myeloma. This may be helpful in predicting which patients are most susceptible to develop a VTE complication during combination chemotherapeutic regimens. In turn this may lead to more appropriate antithrombotic prophylaxis to prevent VTE.

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Nederlandse samenvatting

Het multiple myeloom is een maligne plasmacel aandoening van het beenmerg en vormt ongeveer 1% van alle maligniteiten en 10% van de hematologische maligniteiten. Patienten met een multiple myeloom hebben een hoge incidentie van veneuze trombose, die varieert tussen de 2% en 29%. Opvallend genoeg worden de meeste veneuze thrombotische complicaties gezien in de eerste maanden van behandeling, met name bij antracycline bevattende chemotherapie in combinatie met angiogenese remmers. Algemene factoren waarvan bekend zijn dat deze een belangrijke rol spelen bij het ontstaan van veneuze trombose zijn onder andere hoge leeftijd, immobilisatie, chirurgische ingrepen, infecties en de aanwezigheid van een een centraal veneuze lijn. Over mogelijke stollingsafwijkingen die een pathofysiologische rol kunnen spelen bij de verhoogde thrombose neiging bij patiënten met een multiple myeloom is nog weinig bekend. Recent onderzoek heeft aangetoond dat verworven afwijkingen, zoals resistentie tegen geactiveerd proteine C en een verhoogd factor VIII, kunnen optreden. De studies beschreven in dit proefschrift zijn verricht om meer inzicht te krijgen in afwijkingen in het stollingsmechanisme bij het multiple myeloom en in de mogelijke rol van die afwijkingen bij het ontstaan van veneuze trombose.

In **hoofdstuk 1** wordt een overzicht gegeven van de wetenschappelijke literatuur over de incidentie van thrombo-embolische complicaties bij patiënten met een multiple myeloom en de beschikbare kennis over de primaire en secundaire hemostase bij patiënten met een multiple myeloom. Hiernaast wordt kort ingegaan op thromboprophylactische mogelijkheden ter reductie van de thrombose incidentie.

In **hoofdstuk 2** wordt een een studie beschreven naar afwijkingen in het stollingsmechanisme in een groep van 135 patienten met een onbehandeld multiple myeloom. Bij deze patiënten worden sterk verhoogde concentraties van factor VIII en von Willebrand Factor (vWF) gevonden. Deze waarden correleren sterk met het prognostische Internationale Scorings Systeem (ISS), waarbij de hoogste concentraties van FVIII en vWF gezien worden in het hoogste stadium. Proteine S activiteit is gerelateerd aan het ISS stadium waarbij de laagste activiteit gemeten wordt in patiënten met het hoogste ISS stadium. Tijdens de behandeling met chemotherapie trad bij 10% van de patienten een thrombotische complicatie op. Er werd geen associatie gevonden tussen de afwijkingen in de stollingsparameters bij diagnose en het optreden van veneuze trombose.

In **hoofdstuk 3** wordt een studie beschreven waarbij stollingsonderzoek werd verricht voor de start van behandeling, na inductie chemotherapie en 3 tot 6 maanden na intensieve behandeling met hoge dosis melphalan (HDM) gevolgd door autologe stamcel transplantatie (ASCT) bij patiënten met een multiple myeloom. Factor VIII en vWF vertonen een parabolisch verloop met een significante stijging van de afwijkingen na de inductie chemotherapie. Na de intensieve behandeling dalen deze factoren tot onder de uitgangswaarde van voor de aanvang van behandeling. Dit verloop wordt bij elke vorm van inductie theapie gezien. Fibrinogeen stijgt ook tijdens de behandeling, en blijft vervolgens verhoogd na afsluiten van de intensieve behandeling met HDM en ASCT. In de patienten die behandeld worden met thalidomide in combinatie met adriamycine en dexamethason (TAD) vertoonde het fibrinogeen een significant hogere stijging ten opzichte van de patienten die vincristine in combinatie met adriamycine en dexamethason (VAD) kregen toegediend. De afwijkingen die werden gevonden tijdens en na behandeling waren niet geassocieerd met het ontstaan van veneuze trombose.

In **hoofstuk 4** is een onderzoek beschreven naar de fibrinolyse bij patiënten met een multiple myeloom. Recent onderzoek heeft aangetoond dat de kans op trombose is toegenomen bij individuen met een lage fibrinolytische capaciteit. De fibrinolytische capaciteit werd gemeten in plasma van 77 patienten met een multiple myeloom voor, tijdens en na inductie chemotherapie met VAD of TAD. De tijd die nodig is om een stolsel op te lossen (stolsel lysis tijd, clot lysis time) is voor de inductie therapie vergelijkbaar met gezonde controle personen. Tijdens de inductie chemotherapie verlengt de lysis tijd significant ten opzichte van die van de controle personen ten teken dat er sprake is van hypofibrinolyse tijdens de therapie. Na de intensieve behandeling met hoge dosis melphalan, gevolgd door autologe stamceltransplantatie daalt deze weer tot normaal. De verminderde fibrinolytische capaciteit, die ontstaat tijdens behandeling van patiënten met een multiple myeloom, kan bijdragen aan de verhoogde kans op veneuze trombose.

Weefsel factor (Tissue Factor, TF) wordt gezien als de belangrijkste initiator van de stolling. Eerder onderzoek heeft aangetoond dat er een associatie is tussen micro-partikelgeassocieerde tissue factor activiteit (MP-TF) en het optreden van trombose bij patiënten met een adenocarcinoom. In **hoofdstuk 5** wordt de plasma MP-TF activiteit gemeten zowel

voor als na inductie chemotherapie in 123 patienten met een multiple myeloom. Voor de inductie therapie is de MP-TF activiteit significant hoger dan in gezonde vrijwilligers (17.8 fM Xa/min [IQR 8.8-32.8] versus 4.7 fM Xa/min [IQR 2.3-6.6], P<0.001). Er is geen relatie tussen MP-TF en de ernst van de ziekte, geclassificeerd met de Salmon en Durie criteria, noch met de prognostische ISS score. Na de inductie chemotherapie daalt de MP-TF activiteit significant. Trombose werd gezien in 8% van de patienten behandeld met VAD, 11% van de patienten behandeld met TAD en 7% van de patienten behandeld met PAD. In de patienten die een trombose ontwikkelen bleef na inductie therapie de MP-TF activiteit hoog. MP-TF activiteit na inductie chemotherapie is significant geasscocieerd met het risico op trombose met een 4% stijging voor iedere unit van TF activiteit (95% CI 1-8%; p=0.03). Dit suggereert dat MP-TF activiteit een rol speelt in de ontwikkeling van trombose bij patienten met een multiple myeloom.

Vanwege de hoge trombose incidentie, met name tijdens de inductie chemotherapie met thalidomide, is er momenteel veel aandacht voor medicamenteuze trombose profylaxe bij deze patientengroep. Bij multiple myeloom patienten die behandeled worden met chemotherapie in combinatie met thalidomide worden trombose incidenties tot 29% gemeld. In **hoofstuk 6** wordt het effect van laag moleculair gewichts heparine (LMWH) bestudeert op de trombose incidentie. In een groep van 412 patienten met een multiple myeloom die inductie chemotherapie kregen met TAD, werd tromboprofylaxe met LMWH toegepast. De incidentie van trombose bij de VAD patienten was 5% en bij de TAD patienten 10%. Deze studie toont aan dat toediening van LMWH aan patiënten met multiple myeloom, die chemotherapie in combinatie met thalidomide krijgen veilig is en de trombose incidentie reduceert.

In **hoofdstuk 7** werden verschillende stollingsparameters onderzocht bij patienten met andere vormen van plasmacel ziektes, zoals monoclonale gammopathie of undetermined significance (MGUS), AL-amyloidose en de ziekte van Waldenström. Factor VIII en vWF zijn ook bij deze patienten duidelijk verhoogd, waarbij de hoogste concentraties gemeten worden in de patienten met AL-amyloidose. De concentratie van proteine S is bij alle vormen van plasmacel ziektes verlaagd, terwijl het fibrinogeen gehalte bij de patienten met AL amyloidose significant hoger is ten opzichte van de andere plasmacel aandoeningen. Geconclu-

deerd kan worden dat bij andere plasmacel aandoeningen vergelijkbare stollingsafwijkingen worden gevonden, als bij het multiple myeloom.

In **hoofdstuk 8** wordt een patient beschreven die bekend is met een erfelijke type 2A von Willebrand ziekte, die een multiple myeloom krijgt. In het plasma van deze patient werd een sterke stijging van het factor VIII en vWF gezien tov eerdere metingen. Dit leidde tot een vermindering van zijn pre-existente bloedings neiging. De behandeling van het multiple myeloom resulteerde in een daling van factor VIII en vWF. Hierbij ontstonden ook weer klachten van een verhoogde bloedingsneiging.

In **hoofdstuk 9** wordt een patiente beschreven met een therapie resistente leukocytoclastische vasculitis ten gevolge van een MGUS. Deze patiente werd behandeld met frequente plasmaferese, waarbij Hydroxyethyl zetmeel (HES) werd toegediend ter vervanging van plasma. Door de frequente plasmafereses werd HES in grote hoeveelheden aan de patiente toegediend. Deze patiente ontwikkelde vervolgens een ernstige stapelingsziekte, waarbij diverse weefsels massaal geinfiltreerd waren met geactiveerde schuimachtige macrofagen. Aanvullend werd een onderzoek verricht naar mogelijke HES stapeling bij andere patiënten die eveneens frequente plasmafereses hadden ondergaan met HES als substitutie. De met HES beladen schuimmacrofagen produceren het enzym chitotriosidase. De concentratie van dit enzym, gemeten in het bloed, kan gebruikt worden om de ernst van de stapelingsziekte te meten. Uitgebreide analyse van een groep van 12 patienten laat zien dat met name bij patienten met een pre-existente nierfunctie stoornis, de toediening van dit product leidt tot stapeling van HES, hetgeen tot uiting komt in een stijging van het enzym chitotriosidase.

Hoofdstuk 10 bevat tenslotten een algemene samenvatting en discussie waarin de resultaten van dit proefschrift worden samengevat en bediscussieerd in het licht van recent ander onderzoek en de huidige inzichten.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 26 maart 1963 te Gouda. Zijn jeugd bracht hij door op Aruba alwaar hij het VWO-diploma behaalde aan het Colegio Arubano. In 1982 werd gestart met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In de laatste fase van zijn doctoraal liep hij onderandere stage in Hongarije en Kenia en in 1987 behaalde hij het doctoraal examen. Het arts examen werd afgerond in 1989 waarna hij aansluitend werkte als officier arts bij de Koninklijke Marine te Den Helder aan boord van de Philips van Almonde en op de Centrale Ziekenboeg. Vervolgens werkte hij, eerst als onderzoeker en later als assistent, op de afdeling Chirurgie in het Erasmus Universitair Medisch Centrum te Rotterdam. In 1996 werd gestart met de opleiding tot internist in Rotterdam (Ikazia Ziekenhuis, Medisch Centrum Rijmond Zuid en het Erasmus Universitair Medisch Centrum). In 2001 starte hij met de opleiding tot hematoloog (opleider Prof.dr. B. Löwenberg). In 2002 behaalde hij een oorkonde op de Nederlandse Internisten dagen met het onderzoek waarvan hoofstuk 9 een stille getuige is. In 2003 behaalde hij zijn aantekening tot hematoloog. Gedurende de laatste fase van de opleiding werd een aanvang gemaakt met het in dit proefschrift beschreven onderzoek. Gedurende 2003 - 2007 was hij werkzaam als internist-hematoloog in het Ikazia ziekenhuis te Rotterdam. Vervolgens was hij een periode waarnemend internist-hematoloog in het Meander Medisch Centrum te Amersfoort. Vanaf 2008 is hij werkzaam als internist-hematoloog in het Zuiderzee ziekenhuis te Lelystad. Naast algemene interne geneeskunde en hematologie verricht hij tevens diagnostische endoscopieen.

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ABBREVIATIONS

ACA	anticardiolipin antibodies
AL	amyloidosis
ALSD	acquired lysosomal storage disease
APA	antiphospholipid antibodies
APC	activated protein C
ASCT	autologous stem cell transplantation
CAD	cyclophosphamin, adriamycin and dexamethasone
CT	chitotriosidase
DVT	deep vein thrombosis
FVIII	factor VIII
GD	gaucher's disease
HDM	high dose melphalan
HES	Hydroxy-ethyl starch
IL-6	interleukin-6
ISS	International Staging System
LAC	lupus anticoagulant
LMWH	low molecular weight heparin
MGUS	monoclonal gammopathy of uncertain significance
MM	multiple myeloma
MNC's	mononuclear cells
MP	melphalan and prednisone
MPS	mononuclear phagocyte system
MP-TF	microparticle tissue factor
PAD	bortezomib, doxorubicin and dexamethasone
PAI-1	plasminogen activator inhibitor type I
PB-MNC's	peripheral mononuclear cells
PCD	plasma cell disorders
PE	pulmonary embolism
TAD	thalidomide, doxorubicin and dexamethasone
TP	tissue factor
t-PA	tissue plasminogen activator
ULVWF	ultra large von Willebrand Factor
VAD	vincristine, doxorubicin and dexamethasone
VTE	venous thromboembolism
VWF	von Willebrand Factor
VWF:Ag	von Willebrand Factor antigen
VWF:Cb	von Willebrand Factor collagen binding activity
VWF:RCo	von Willebrand Factor ristocitin cofactor
WM	Waldenström's macroglobulinemia

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