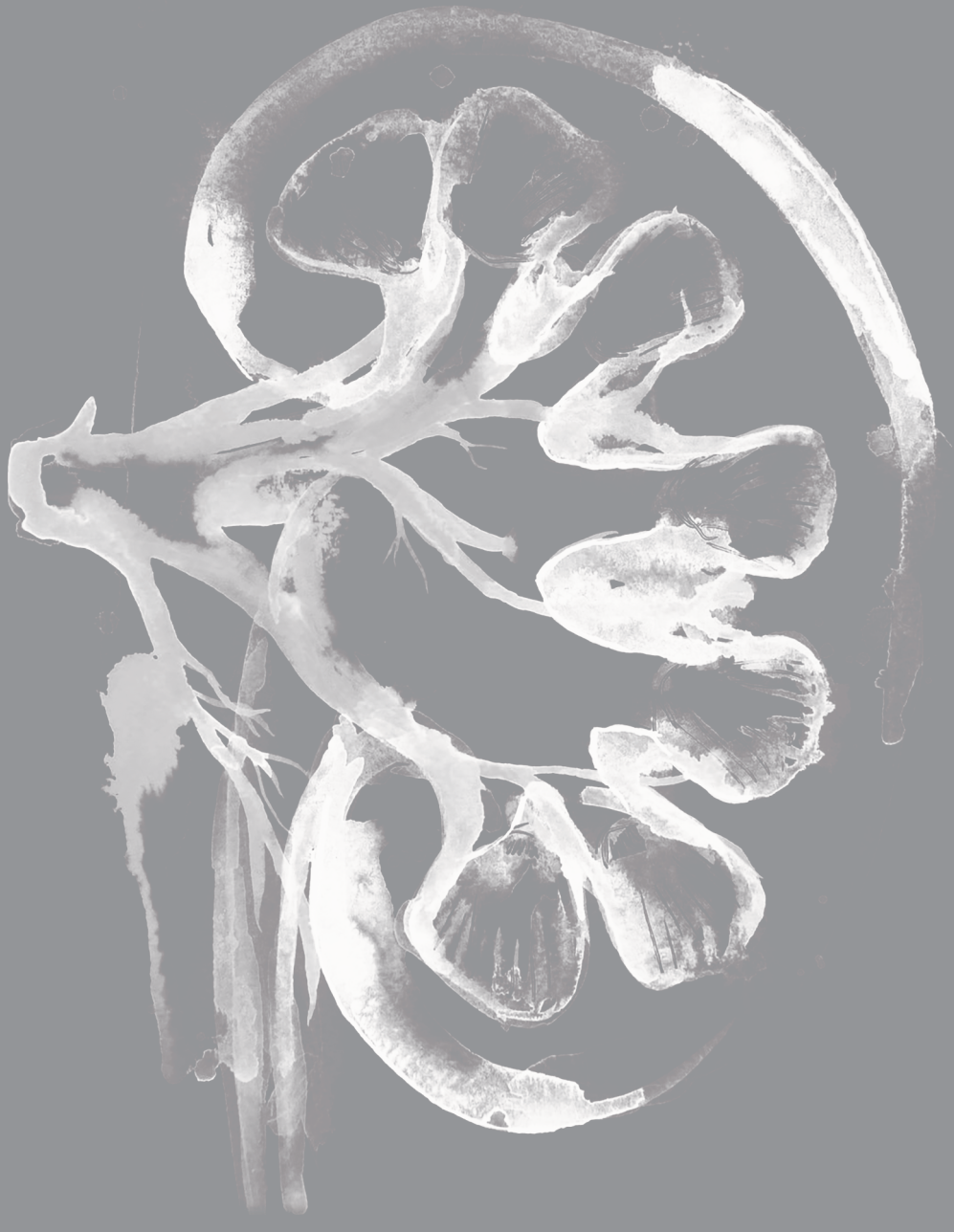

Chapter 7

Procoagulant tissue factor activity on microparticles is associated with disease severity and bacteremia in febrile urinary tract infections

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Abstract

Introduction

Inhibition of tissue factor, the primary initiator of coagulation in sepsis, attenuates morbidity in primates infused with *Escherichia coli*. In a human endotoxemia model, microparticles expressing procoagulant TF (MP-TF) are released in blood concurrently with markers of inflammation and coagulation. We investigated whether the release of MP-TF into blood is accompanied by procoagulant and inflammatory changes in patients with *E. coli* urinary tract infection.

Materials and methods

In a multicenter cohort study, we determined clinical disease severity using APACHE II scores and measured plasma MP-TF activity, TAT, sE-selectin, sVCAM-1, procalcitonin and monocyte count in blood of 215 patients with community-acquired febrile *E. coli* urinary tract infections.

Results

Plasma MP-TF activity on admission corresponded with clinical disease severity (APACHE II score; P=0.006) and correlated significantly but weakly with plasma markers of disease severity (sE-selectin, sVCAM-1, procalcitonin). Additionally, median plasma MP-TF activity was higher in patients than in healthy controls (197 vs. 79 fM Xa/min; P<0.0001), and highest in bacteraemic patients (325 fM Xa/min). MP-TF activity showed a weak inverse correlation with monocyte count (rs -0.22; P=0.016) and a weak correlation with TAT (rs 0.23, P=0.017). After 3 days of antibiotic treatment, upon resolution of the infection, plasma MP-TF activity and TAT concentrations declined.

Conclusions

Microparticle-associated procoagulant tissue factor activity is related to disease severity and bacteremia in febrile *E. coli* UTI patients and may contribute to the prothrombotic state in gram-negative sepsis.

Introduction

Sepsis is associated with activation of the coagulation cascade, which may range from subclinical to widespread microvascular thrombosis and disseminated intravascular coagulopathy. Blood microparticles are highly mobile carriers of pro-inflammatory mediators and procoagulant proteins and could play a major role in the onset of sepsis-related morbidities and mortality.
Tissue factor (TF), the primary initiator of coagulation *in vivo*, is thought to play an important role in sepsis. In primates, inhibition of the tissue factor pathway with anti-TF monoclonal antibodies, tissue factor pathway inhibitor (TFPI) or active site-inactivated FVIIa attenuated coagulopathy and prevented acute lung injury, renal failure and mortality in septic shock caused by *Escherichia coli* [1-3]. Furthermore, in human endotoxemia models, coagulopathy improved following infusion of TFPI or recombinant human IL-10 (known to inhibit LPS-induced TF activity and monocyte TF-expression, respectively) [4, 5].
In a kinetic study of healthy volunteers challenged intravenously with purified *E. coli* lipopolysaccharide, we demonstrated that microparticles bearing functional procoagulant TF (MP-TF activity) are concurrently released with markers of inflammation and coagulation [6]. Interestingly, the subject with the most prominent clinical response to endotoxin also had the highest MP-TF activity. The role of microparticles expressing tissue factor (MP-TF) in the pathogenesis of sepsis has not yet been elucidated.
As inflammatory conditions caused by an intact pathogen may similarly induce shedding of MP-TF, we investigated the relation between MP-TF activity, bacteremia and clinical disease severity using the APACHE II score in a large cohort of patients with febrile *E. coli* urinary tract infection (UTI). In addition, we assessed whether MP-TF activity decreased upon resolution of the infection and explored the association between MP-TF activity, markers of disease severity (sE-selectin, sVCAM-1, procalcitonin) and blood monocyte count. Finally, we examined levels of thrombin-antithrombin complex (TAT) as a marker of coagulation in a subset of patients.

Materials and methods

Study design

From January 2004 to December 2009, we enrolled 787 consecutive patients presenting with community-onset febrile urinary tract infections at the emer-

gency departments of seven hospitals and 35 affiliated primary healthcare centers in the western part of The Netherlands [7]. This multicenter cohort study was approved by the Medical Ethical Committees of the participating centers and all patients gave written informed consent.

Inclusion criteria were age ≥18 years, ear temperature ≥38.0°C or a history of fever and rigors within 24 hours prior to presentation, at least one symptom of UTI (dysuria, frequent or urgent urination, perineal pain, flank pain or costovertebral tenderness), and positive nitrite dipstick test or leukocyturia. Leukocyturia was defined as a positive leukocyte esterase dipstick test or the presence of >5 leucocytes per high-power field in the urine sediment. Exclusion criteria were current treatment for urolithiasis or hydronephrosis, hemodialysis, kidney transplantation or polycystic kidney disease.

From the initial cohort of 787 patients, we selected all 420 patients in whom *E. coli* was cultured from the urine sample obtained upon admission. We excluded 156 patients with pre-existing disorders or severe co-morbidity, i.e. cancer, autoimmune disease, diabetes, cerebrovascular accident or heart failure, because elevated plasma levels of microparticles and MP-TF activity have been reported in patients with these disorders [8-12]. This exclusion criterion resulted in a cohort of 264 fairly healthy patients at the onset of their urinary tract infection, since abovementioned disorders and comorbidities were more prevalent amongst the majority of the critically-ill patients with the highest APACHE II scores. Another 49 patients were excluded because blood cultures or frozen plasma samples could not be retrieved, rendering a final study cohort of 215 patients.

As required by the study protocol, all patients received empirical intravenous or oral antibiotic treatment upon admission according to local hospital policy. Antimicrobial therapy consisted of intravenously administered cefuroxime (n=89; of which 39 in combination with gentamicin) or oral treatment with ciprofloxacin (n=106). A small number of patients (n=20) was treated with other oral or intravenous antibiotic agents directed against gram negative micro-organisms.

Healthy volunteers without history of fever or infectious disease were recruited in the participating primary healthcare centers and amongst laboratory staff. Age and gender were similar to that of the study patients (63% female, median age 59 years [range 24-76]), as well as the protocol for blood collection and processing.

Procedures and definitions

Clinical data and routine laboratory measurements were collected by the clinical investigators and qualified research nurses. Baseline data of the patients were obtained within 24 hours using a standardized questionnaire and by reviewing medical records. Double data entry was performed by two independent data managers and both entries were compared for discrepancies.

We calculated the most commonly used clinical disease severity score for septic patients, the Acute Physiology and Chronic Health Evaluation II (APACHE II) score. Patients were allocated to previously reported score categories, allowing assessment of the severity of the disease and providing an estimate of in-hospital mortality risk (an APACHE II score of 1-4 corresponded with a 4% mortality rate, whereas APACHE II scores of 5-9, 10-14, and 15-19 translated into an observed mortality rate of 8%, 15% and 25%, respectively) [13]. Clean midstream-catch or catheter-port urine were collected upon admission and cultured using local standard microbiological methods. Urine samples were considered infected in case of bacterial growth $>10^3$ CFU per ml urine or a bacterial monoculture $>10^2$ CFU per ml urine in the presence of leukocyturia.

Disease burden was systematically quantified in all 215 patients by taking blood cultures on admission. Patients were considered to have bacteremia when *E. coli* was cultured.

At baseline (day 0) and three days thereafter (day 3), venous blood samples were collected into EDTA [ethylenediaminetetraacetic acid] BD Vacutainer tubes (Franklin Lakes, NJ, USA) applying minimal venostasis and discarding the first tube. Plasma was prepared by removing cells through a single centrifugation step at 3500 x *g* (5 minutes at room temperature). Aliquots were transferred immediately to polypropylene tubes and frozen at -80°C to enable future simultaneous analysis of day 0 and 3 samples. Sample processing time from venepuncture to storage at -80°C was less than an hour for the majority of study patients and well within two hours for all patients as required by the study protocol. Plasma samples remained deep-frozen until analysis.

Isolation of microparticles and MP-TF activity assay

After thawing of deep-frozen EDTA-anticoagulated plasma samples, microparticles were pelleted and repeatedly washed with pH 7.45 filtered 0.32% citrate/PBS buffer (30 minutes at 18,890 x *g* with minimum brake, 20°C) thus diluting plasma constituents and EDTA more than 200-fold. The mic-

roparticle suspension was subsequently recalcified and incubated in a 1:5 ratio (v/v) with 10 mM pH 7.45 Hepes, 137 mM NaCl, 4 mM KCl, 5 mg/ml ovalbumin, 50 nM hirudin, 6 mM $CaCl^2$ and 25 μM negatively charged phospholipid vesicles (dioleoylphophatidylserine/dioleoylphosphatidylcholine 1/9). TF/FVII complex formation was initiated by the addition of FVII (Kordia, The Netherlands). The reaction was started by the addition of S2765 (Chromogenix, Italy) and FX (Kordia, The Netherlands). Subsequently, cleavage of the chromogenic substrate S2765 by the generated FXa was recorded during 90 minutes (absorbance at 405 nm). Parallel experiments were performed in the absence of FVII and in the presence of excess polyclonal sheep anti-human TF-IgG (Affinity Biologicals Inc., Canada) to demonstrate FVII and TF-dependency, respectively. MP-TF activity, defined as FVII- and TF-dependent FXa formation, was calculated as previously described [8] and expressed as fM Xa/min in plasma assuming a 100% microparticle recovery. None of the plasma samples used for isolation of microparticles had been thawed before and all samples were analysed within one year after completion of the study. In previous experiments on plasma samples stored for more than 15 months, we did not observe degradation of active TF on microparticles after prolonged frozen storage; the same MP-TF activity was found in aliquotted samples from 16 patients after prolonged frozen storage.
Nineteen healthy volunteers were recruited to establish reference values for MP-TF activity, although power calculations indicated that a sample size of merely 9-10 for each group would suffice to detect a statistical significant difference. We defined elevated plasma MP-TF activity as levels >172 fM Xa/min, indicating the 99^{th} percentile of MP-TF activity of these 19 healthy controls (e.g. mean MP-TF activity + 2 SD).

Other assays

Endothelial activation and leukocyte recruitment play a key role in inflammation and are characterized by ectodomain shedding of adhesion molecules. As levels of soluble adhesion molecules have been related to disease severity [14], soluble E-selectin and VCAM-1 levels were measured by ELISA according to the manufacturer's protocol (Diaclone, Besançon, France). Procalcitonin, a sensitive biomarker differentiating bacterial infection from non-infectious inflammation [15], was routinely measured using a Time Resolved Amplified Cryptate Emission technology assay (TRACE®, Kryptor compact, PCTsensitive; Brahms AG; Hennigsdorf, Germany). C-reactive protein (CRP) levels were not measured considering the higher diagnostic accuracy of procalcitonin, which is already peaking at 8 hours following an infectious stimulus, in

contrast to 36 hours for CRP. To detect whether (subclinical) coagulation had occurred *in vivo*, thrombin-antithrombin complex, was quantified using an enzyme immunoassay according to the manufacturer's protocol (Enzygnost, Malburg, Germany). TAT levels of > 4.2 µg/L were considered to be elevated.

Statistical analysis

As the distributions of biomarkers were skewed, univariate analysis was performed using the Mann-Whitney *U*-test. Additionally, (M)ANOVA was used to compare between APACHE II disease severity groups after correction for the skewed distribution by log-transformation of data. When appropriate the paired-samples T-test was performed on log-transformed data. Correlations were assessed using the Spearman's rho (*rs*) test. *P*-values <0.05 were considered significant. For all analyses, SPSS Statistics software (version 20.0; IBM Corporation, Armonk, NY, USA) was used.

Results

Patient characteristics

The median age in the study population of 215 patients with febrile *E. coli* UTI was 51 years (range 18-96 years) and 73% were female. Almost all patients were capable of daily self-care activities. The majority of patients (70.1%) had signs of pyelonephritis, 64.6% presented with rigors, while only 22.3% had bacteremia confirmed by blood culture testing, and 37.1% reported vomiting. Relevant comedication consisted of antihypertensive agents (23.3%) and agents to reduce the heart rate (10.2%). A large proportion of patients (63.3%) took antipyretics at the time of admission. Antibiotic treatment was already started in 34 of 215 (15.8%) patients for a median period of 2 days (range 1-10 days) prior to inclusion, and switched upon inclusion in 32 of 34 patients.

As classified by the APACHE II disease severity score, most patients had a low mortality risk. Only thirty-four patients (15.8%) had an APACHE II score of 10-14, whereas 10 patients (4.7%) had an APACHE II score of 15-17 corresponding with a mortality risk of 15% and 25%, respectively. None of the patients had an APACHE II score ≥ 18. Approximately half of the patients (n=107) was admitted to the hospital, from whom only one patient was directly admitted to the intensive care unit (ICU). None of the 215 patients died from urosepsis. Furthermore, none of the 215 patients was diagnosed with clinically manifest thrombosis.

Disease severity and plasma MP-TF activity at baseline

Median plasma MP-TF activity was higher in patients with febrile *E. coli* UTI than in healthy subjects (197 fM Xa/min [IQR 113-398] vs. 79 fM Xa/min [IQR 57-126]; Fig. 1A). Patients with proven bacteremia had higher median plasma MP-TF activities (325 fM Xa/min [IQR 166-641 fM Xa/min] than patients with localized infection and negative blood cultures (186 fM Xa/min; IQR 110-324; Fig. 1B). Plasma MP-TF activity also corresponded with clinical disease severity as determined by APACHE II scores (Fig. 1C). Patients with APACHE II scores of 0-4 had a median plasma MP-TF activity of 152 fM Xa/min [IQR 98-280], whereas patients in higher APACHE II score categories had higher median MP-TF activities: 227 fM Xa/min [IQR 123-501] for patients with an APACHE II score of 5-9, 259 fM Xa/min [IQR 144-599] for patients with an APACHE II score of 10-14, and 471 fM Xa/min [IQR 180-848] for patients with an APACHE II score of 15-17. The differences between the four APACHE groups as analyzed by ANOVA after log-transformation of mean MP-TF activity revealed a significant increase in differences between the four categories (P=0.006). Within each of the APACHE II score categories, the mean MP-TF activity was higher in patients with bacteremia than in those without. Overall, this effect of bacteremia on MP-TF activity was significant (P=0.031 in MANOVA).

Plasma disease severity markers sE-selectin and sVCAM-1 correlated moderately with APACHE II score (rs 0.35 and 0.39, respectively; P<0.0001), but only weakly with MP-TF activity (rs 0.28 and 0.30, respectively; P<0.0001; n=213). Plasma procalcitonin showed a slightly better correlation with MP-TF activity (rs 0.35, P<0.0001).

MP-TF activity, TAT levels and monocyte count at baseline

As monocytes have been shown to be the sole hematopoietic source of TF and activated monocytes shed microparticles expressing procoagulant tissue factor [16, 17], we examined the correlation between MP-TF activity and blood monocyte count. From 117 of 134 patients (87.3%) presenting with febrile *E. coli* UTI at one of the emergency departments, monocyte counts were available. Monocyte counts showed a weak inverse correlation with plasma MP-TF activity (rs -0.22; P=0.016). Of 106 patients, plasma samples were available for determination of TAT levels. MP-TF activity correlated weakly with TAT (rs 0.23, P=0.017; n=106). Interestingly, monocyte count showed a moderate inverse correlation with TAT (rs -0.40, P=0.003; n=55). In contrast, MP-TF activity did not correlate with total leukocyte count (which in septic patients mainly consists of neutrophils).

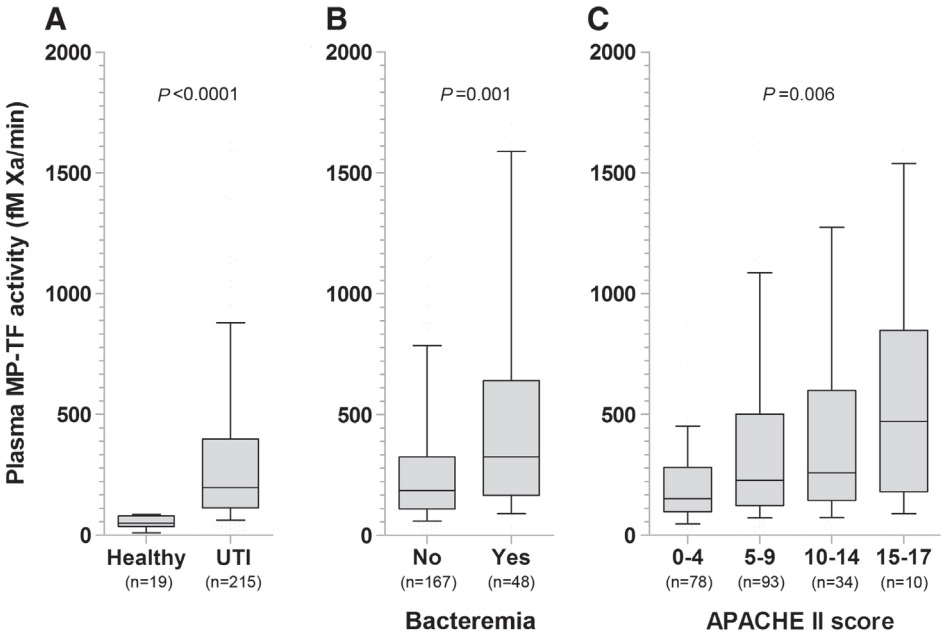

Figure 1. Boxplot showing plasma MP-TF activity in (A) 19 healthy volunteers and 215 patients with febrile *E. coli* UTI, (B) patients with localized disease and no bacteremia (n=167) vs. patients with bacteremia (n=48), and (C) patients with different APACHE II scores. Whiskers indicate the 10^{th} and 90^{th} percentile. Depicted *P*-values were calculated using the Mann-Whitney *U*-test for panels A and B, and ANOVA after log-transformation of data for panel C.

MP-TF activity and TAT on days 0 and 3

As all patients received antibiotic treatment upon admission, we examined MP-TF activity on day 3. In 98 patients, EDTA-anticoagulated plasma samples collected on day 3 were available allowing paired analysis of MP-TF activity on days 0 and 3 (Fig 2A). Overall, mean MP-TF activity declined with 95 fM Xa/min over 3 days of antibiotic treatment (P=0.0003; Paired-Samples T-test after log-transformation). Although the overall MP-TF activity decreased, the MP-TF activity on day 3 showed a 1.2 to 22-fold increase in ten patients. Six of these patients remained febrile or showed signs of inflammation on day 3. Interestingly, the patient who experienced the highest increase in blood MP-TF activity from 183 to 3928 fM Xa/min was an elderly patient with proven bacteremia, who deteriorated clinically despite adequate oral antibiotic treatment. His urine contained $>10^5$ CFU's and the time to positive blood culture was 12 hours. Although vomiting and diarrhea were not reported, this patient had prolonged fever and malaise. As for the eleven patients with the highest MP-TF activities (i.e. > 1500 fM Xa/min), symptoms improved

promptly upon antibiotic treatment. Nine of the eleven patients had to be hospitalized for a median period of 7 days (range 2-28). From four of these eleven patients with MP-TF activities > 1500 fM Xa/min, day 3 samples were available showing a marked decrease in MP-TF activity to 98-265 fM Xa/min. In 106 patients, TAT levels were determined. TAT ranged from < 2.0-145 µg/l and was elevated in approximately 50% of patients. From 11 patients with MP-TF activities > 1500 fM Xa/min, TAT levels were available for 5 patients, all being elevated, the patient with the highest MP-TF activity (3597 fM Xa/min) having one of the highest TAT levels (133 µg/ml). Of 40 patients, plasma was available for paired analysis of TAT on day 3 (Fig 2B). Mean TAT declined with 7.6 µg/l (*P*=0.0096; paired-samples T-test after log-transformation). Interestingly, the patient with prolonged fever who showed the highest increase in MP-TF activity also showed one of the highest increases in TAT from 5.0 to 24.5 µg/ml. The second patient with an increase in TAT on

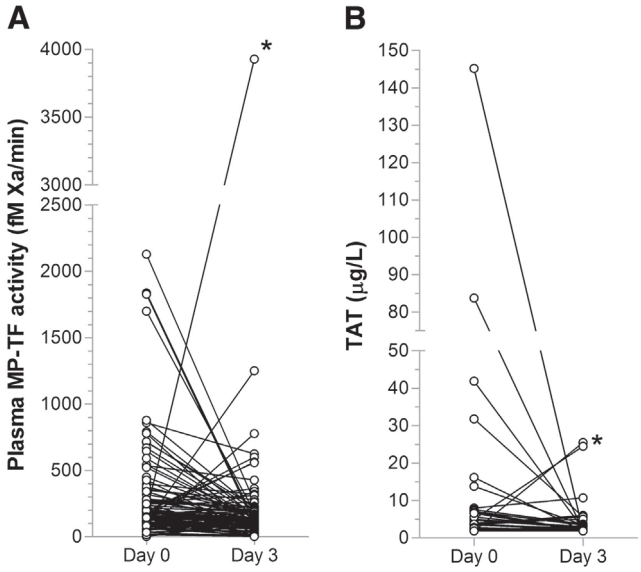

Figure 2. MP-TF activity and TAT at baseline and on day 3 following antibiotic treatment. (A) Median MP-TF activity at baseline (183 fM Xa/min) was higher than on day 3 (129 fM Xa/min; *P*=0.002; n=98). Ten patients showed an increase in MP-TF activity on day 3. In the remaining 88 patients, MP-TF activity decreased or varied within the normal range (< 172 fM Xa/min). Interestingly, the patient with the highest increase in MP-TF activity on day 3 had deteriorated clinically despite antibiotic treatment (*). (B) Median TAT levels at baseline (3.7 µg/l) were higher than on day 3 (2.3 µg/l; *P*=0.003; n=40). The majority of patients showed a decline in TAT following resolution of the infection. Interestingly, the patient with the highest increase in MP-TF activity also exhibited one of the highest increases in TAT (*). Finally, paired-samples T-test after log-transformation of data showed significant decrease in mean MP-TF activity and TAT levels (*P*=0.0003 and *P*=0.0096, respectively).

day 3 (1.9 to 25.5 µg/ml) similarly showed a concurrent increase in plasma MP-TF activity from 261 to 365 fM Xa/min. This elderly lady had been treated with oral antibiotics for recurrent urinary tract infections in a nursing home. She presented with an APACHE II score of 17 and a deteriorating localized urinary tract infection despite antibiotic treatment.

Discussion

In this large cohort study of 215 patients with febrile *E. coli* UTI, which reflects daily clinical practice and entails the most commonly cultured pathogen from urine (i.e. over 70%), we demonstrate that functional procoagulant tissue factor on blood microparticles (MP-TF activity) is higher in patients than in healthy individuals and is associated with disease severity and the presence of bacteremia. After three days of antibiotic treatment, MP-TF activity declined coinciding with defeverescence and resolution of the infection. In a minority of patients, mostly with prolonged fever and inflammatory symptoms, an increase in MP-TF activity was observed on day 3. Interestingly, one bacteraemic patient with delayed resolution of his illness and another patient who deteriorated despite antibiotic treatment which was started prior to admission showed a marked increase in MP-TF activity on day 3 and a concurrent increase in TAT levels. These observations illustrate that height and kinetics of MP-TF activity reflect disease severity and clinical deterioration in sepsis and underscores an association of plasma MP-TF activity with coagulation *in vivo.*

There was a large interindividual variation in MP-TF response to *E. coli* and a considerable number of patients (42%) did not have elevated MP-TF activities on day 0. This may reflect differences in the genetic ability of patients to respond to the pathogen (responders and non-responders) [6, 18], differences in plasma endotoxin concentrations [19] and differences in the time interval between the onset of *E. coli* infection and the time of inclusion into the study. Also, the exclusion of patients with co-morbidity may have resulted in the inclusion of a subgroup of patients who were less prone to bacteremia and severe inflammatory disease.

Our findings are in accordance with the observation by Aras *et al.* as well as our own previous observation that intravenous administration of *E. coli* LPS to healthy volunteers results in a marked but transient increase in procoagulant tissue factor activity of blood microparticles isolated from EDTA and citrated plasma, respectively [6, 20]. Monocytes are thought to be the

sole hematopoietic source of TF and show significantly elevated levels of TF mRNA *in vitro* upon stimulation with *E. coli* LPS [16, 21]. It is highly likely, that the inflammatory response induced by invasion of an intact pathogen into urinary tract tissues similarly results in early activation of monocytes and the release of microparticles bearing TF [6], even though we only found a weak correlation between MP-TF activity and monocyte count. In our study, 70% of the patients had symptoms of pyelonephritis and since glomeruli exhibit strong TF expression [22], apoptotic glomerular cells may hypothetically form an alternative source of MP-TF activity. Provided the availability of a monoclonal antibody able to reliably assess tissue factor on microparticles, multicolor flowcytometry experiments could aid in establishing the cellular source of MP-TF. However, only limited amounts of plasma were available from patients participating in this multicenter study. Since MP-MP fusion events are likely to occur *in vivo*, thus complicating the correct identification of the cellular source of MP-TF, and flowcytometry does not detect the smallest microparticles due to limitations of the 488 nm laser light wavelength used, we did not further explore this.

Plasma MP-TF activity correlated weakly with TAT levels. This is not surprising as TAT concentrations *in vivo* are subject to the dynamics of TAT formation and degradation. Furthermore, the formation of TAT occurs downstream in the coagulation cascade and is influenced by multiple other factors such as TF on activated monocytes, levels of procoagulant phospholipids on microparticles and activation of the intrinsic route of coagulation [23, 24]. As yet, it is not known whether other forms of TF in plasma increase in infectious disease. Previously, we showed that in a human endotoxemia model the rise and fall of plasma TF antigen formed a mirror image of the monocyte count and that plasma TF antigen peaks earlier than plasma MP-TF activity [6]. Stimulation of monocytes may result in proteolytic cleavage of TF from the cell membrane (increase in soluble truncated TF), an increase in alternatively spliced TF and the shedding of MP-TF. However, truncated TF and alternatively spliced TF have strongly reduced (if at all) procoagulant activity. MP-TF, as measured in our assay, may therefore be the most important procoagulant form of TF in plasma.

Nonetheless, procoagulant microparticles have been shown to contribute to thrombus formation *in vivo* [25, 26]. In agreement herewith, consumption of procoagulant microparticles was suggested in a small study of septic ICU patients with multiple organ dysfunction syndrome and coagulopathy [27]. In addition to their procoagulant properties, microparticles may also function as highly mobile carriers of TF and may transfer TF to other cells through

membrane fusion [16]. MP-TF may furthermore contribute to local containment of the infection through capture and even killing of fibrinogen binding bacteria within microthrombi [28].
In conclusion, we demonstrated that inflammatory conditions caused by an intact pathogen can induce the release of MP-TF into the circulation and that plasma MP-TF activity levels are associated with disease severity and bacteremia. Considering the link between inflammatory and TF-related pathways, future studies of the pathophysiological role of MP-TF in critically ill patients with severe sepsis, septic shock, multiple organ dysfunction syndrome and disseminated intravascular coagulopathy are warranted. Moreover, microparticles carrying functionally active tissue factor may well be desirable targets for therapeutic purposes as, hypothetically, inhibition of procoagulant tissue factor on circulating blood microparticles could abort disease progression and even lethality from *E. coli* septicemias.

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