Can we use biomarkers of coagulation to predict which patients with foot and ankle injury will develop deep vein thrombosis?

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1. Abstract

Background

Our aim was to determine whether plasma levels of Tissue Factor (TF), Vascular Cell Adhesion Molecule 1 (VCAM-1), Interleukin 6 (IL-6) or D-dimer after foot and ankle injury could predict which patients would develop deep vein thrombosis (DVT).

Methods

Patients aged 18-60 years with acute foot and ankle injury had venous blood sample to measure TF, VCAM-1, IL-6 and D-dimer within 3 days of injury. Patients had bilateral lower limb venous ultrasound to assess for DVT on discharge from clinic.

Results

21 of 77 patients were found to have DVT (27%). There was no statistically significant association between levels of TF, VCAM-1, IL-6 or D-dimer and subsequent development of DVT.

Conclusion

Tissue Factor (TF), Vascular Cell Adhesion Molecule-1 (VCAM-1), Interleukin-6 (IL-6) and D-dimer levels were not associated with development deep vein thrombosis in patients with acute foot and ankle injury.

Keywords: Biomarkers, D-dimer, Tissue factor, Interleukin 6, Vascular Cell Adhesion Molecule 1, Venous thrombosis

2. Introduction

Patients with foot and ankle trauma treated with leg casts are at risk of venous thrombosis (VTE). Tissue injury results in activation of the coagulation cascade through initiation of the extrinsic coagulation pathway. The primary cellular activator of this process is tissue factor (also known as TF, Thromboplastin, Coagulation factor III), which is released by tissues in response to injury [1]. Tissue factor acts as the co-factor for factor VII. The combination of these results in activated VIIa, which activates factors X and IX [2]. Factors VIIa and Xa subsequently result in activation of prothrombin to thrombin, which subsequently results in formation of a fibrin clot from fibrinogen. Fibrin clots cause haemostasis, and are subsequently broken down by the action of plasmin into d-dimer products [3]. In a rabbit model, Himber et al demonstrated that inhibition of tissue factor inhibited venous thrombosis propagation [4]. However, despite these findings, there are limited numbers of studies which have investigated the association between TF and subsequent development of VTE. Similarly, many studies have shown that patients who undergo lower limb venous ultrasound and subsequently found to have deep vein thrombosis (DVT), also have significantly higher d-dimer levels than those with normal imaging [5].

Recently, blood tests including Inflammatory cytokines such as Interleukin 6 and adhesion molecules including Vascular cell adhesion molecule 1 (VCAM-1) have been found to be associated with development of venous thrombosis [3, 6]. However, it is not known whether this is a cause or a consequence of

thrombosis. Cheng et al found that IL-6 levels significantly increased on day 1 after total knee replacement when compared to pre-operative levels, suggesting that tissue injury activates inflammation [7]. In view that IL-6 creates a prothrombotic state by increasing the expression of tissue factor, it is logical to consider that tissue injury may result in venous thrombosis [8]. VCAM-1 levels are increased at sites of endothelial inflammation and are involved in leukocyte adhesion and migration across vascular endothelium.

Our aim was to determine whether tissue factor, interleukin 6, VCAM-1 or D-dimer in the early injury period could predict subsequent development of DVT in patients with acute foot and ankle injury treated with below knee cast.

3. Methods

As part of the Active Toe Movement study (AToM), adult patients presenting to the Emergency Department at University Hospital of Wales with lower limb trauma requiring treatment with a below knee non weight bearing cast for at least 1 week were assessed for eligibility [9]. Only patients considered low risk for VTE after assessment were eligible (Table 1).

All patients were recruited within 72 hours of injury, none were provided with chemical thromboprophylaxis. At time of enrolment to the study, 3.5ml of venous blood was taken using standard Vaccutainer technique into a 3.2% sodium citrate coagulation tube. This was centrifuged at 1500 rpm for 15 minutes and the supernatant plasma was frozen at -70 degrees centigrade

within 1 hour of blood being withdrawn. Participants were managed in the fracture clinic with lower limb cast immobilization according to their injury. On discharge from clinic, patients underwent bilateral lower limb ultrasound scan to assess for above and below DVT. Deep vein thrombosis at the level of the popliteal vein or more proximal was termed above knee, whereas thrombosis below the level of the popliteal vein was considered below knee. All assessments were performed by medical physicists who perform these venous ultrasound studies as part of their role in the National Health Service, all with a minimum of 5 years experience. After the last patient was discharged from clinic, blood samples were thawed and analysed. Plasma was tested for quantitative levels of Human Coagulation Factor III/Tissue factor, Interleukin 6 (IL-6), VCAM-1 and D-dimer. We used the Quanikine ELISA Immunoassay kits (R&D Systems) for each test according to the manufacturers instruction. All kits were stored between 2 and 8 degrees centrgrade and used before their expiry dates. Calibrators were used and diluted according to assay standard operating protocols in order to reference test results. After incubation with ELISA microplates, samples were analysed using an optical microplate reader. A standard curve was created using calibration diluent results, from which test sample results were calculated. D-dimer results were analysed in batch using a fully automated, bench-top, random access analyser (ACL TOP 700) after calibration and internal quality control using quality control plasmas at low and high control levels. All tests were performed with the assistance of 2 experienced laboratory technicians, who were blinded to the DVT status of the patient. Funding for consumables to conduct this study was provided by AO UK.

Statistical analysis

Test for normality were performed using Kolmogorov Smirnov test. Unpaired t-tests were used to test for statistical significance between Group 1 (DVT) and Group 2 (No DVT) where data was parametric. Mann Whitney U test was used for non parametric data.

4. Results

77 patients were recruited. The majority were male (n=50). Patient demographics and injury types are displayed in Table 2. 27% (n=21) of the 77 patients were found to have asymptomatic DVT on bilateral lower limb venous ultrasound scanning, all of which occurred in the lower limb that had been injured and treated in cast. 2 of the DVT's were above knee (prevalence 2.6%), the rest were below knee (25%).

Tissue factor was normally distributed, therefore unpaired students t-test was used to assess for statistically significant differences between Group 1 (No DVT) and Group 2 (DVT) (Mean 23.92 pg/mL v 20.33 pg/mL, p=0.422). 18 patients (23%) had TF levels >35pg/mL, 3 of these subsequently developed DVT. TF levels ranged from 0 to 68pg/mL. There was no significant difference in Interleukin 6 levels between Group 1 (median 3.91 pg/mL) and Group 2 (median 4.59 pg/mL), p=0.764), range 0 to 84.68 pg/mL). Median values for VCAM-1 were also similar between groups (552.98 v 496.84 ng/mL, p=0.111). VCAM-1 levels ranged from 412.63 to 823.15 ng/mL. Although there was a trend for median D-Dimer to be higher in those who subsequently sustained

DVT, this was also non significant (p=0.490). Results are displayed in Table 3.

5. Discussion

The prevalence of deep vein thrombosis in our study was 27% (n=21), all of which occurred in the lower limb that had been injured and treated in cast. 2 of the DVT's were above knee (prevalence 2.6%). This is higher than found in the recent study by Ho et al, which reported an overall DVT prevalence of 11% in non-surgically treated patients with foot and ankle fractures (1.4% above knee) [10]. None of the plasma biomarker levels tested in our study (Tissue Factor, Interleukin 6, VCAM-1 and D-Dimer) predicted subsequent development of DVT.

In our study, Tissue factor levels were 23.92pg/ml in patients who did not subsequently develop DVT, compared with 20.33pg/ml in those who did (p=0.422). However, in contrast to our study findings, Walenga et al (2014) found that those who developed VTE did have significantly higher tissue factor levels (median 49.05 pg/mL vs 14.86 pg/mL, p = 0.003). This difference was present at baseline, between 10 to 14 days post injury and at time of cast removal [11]. Interestingly, at time of injury, levels were not elevated above normal (<35pg/mL) (11). 18 patients (23%) in our study had TF levels >35pg/mL, 3 of which subsequently developed DVT.

In our study, median IL-6 levels were 3.91 pg/mL in those who did not subsequently develop DVT and were 4.59 pg/mL in those who did develop DVT (p=0.764) (range 0 to 84.68 pg/mL). In a study by Mosevoll et al (2015), the R&D systems Luminex assay kit was used to measure inflammatory markers in plasma of patients suspected of having lower limb DVT [6]. They found no significant difference between IL-6 levels in those with (1.240 pg/ml), compared to those without (2.020 pg/ml) DVT on subsequent venous USS. Furthermore, IL-6 levels in the 21 patients found to have DVT on USS, were not significantly higher than 20 normal control patients without DVT (1.240 pg/ml vs 3.470 pg/ml. p=0.1967) [6]. In contrast, in a series of 40 patients with phlebographically proven lower limb DVT, Roumen-Klappe et al (2002) measured IL-6 on day of presentation and compared levels to a group of 33 controls. They also measured IL-6 on the subsequent 5 days following DVT and found that IL-6 levels were significantly higher in the group with DVT (15pg/mL, range <3 to 70 pg/mL) as compared with the control group (<3 pg/mL, range <3 to 11 pg/mL), but subsequently decreased during the following 5 days, to 5.5 pg/mL by day 5 (p <0.01). This indicates that that the raised IL-6 levels were the result of thrombosis rather than the cause [12]. At 32 months after DVT, patients continue to have increased levels of IL-6 compared to controls, suggesting a persistent chronic sub-clinical response [13]. In view of that there were no significant differences in IL-6 between those who did and did not develop DVT in our study, this may represent that none had DVT at the time of measurement. We would agree with the aforementioned study authors that IL-6 levels are raised in response to DVT rather than being the cause.

In a study of 135 patients suspected of having DVT, Bozic et al (2002) used R&D Systems quantitative ELISA to measure plasma VCAM-1. The 39% percent of patients who were subsequently found to have DVT on lower limb doppler ultrasound, had significantly higher VCAM-1 levels (392 micrograms/litre vs 417 (p=0.03). However, VCAM-1 was not as accurate as D-dimer in diagnosis using ROC analysis (0.6 vs > 0.8 depending on D-dimer method used) [14]. In a similar recent study of 89 patients suspected of having DVT, Mosevoll et al (2015) measured VCAM-1 using R&D systems Luminex assay. VCAM-1 levels were significantly higher in the 21 patients who were subsequently found to have DVT on lower limb ultrasound (850.161 ng/ml, range 104.311 to 1571.607 vs 635.436, range 290.605 to 2793.862, p=0.0009). Furthermore, in comparison to 20 control patients, VCAM-1 levels were also significantly higher in patients with DVT [6]. In our study, median VCAM-1 levels were 552.98 ng/mL in those who did not develop DVT, as compared with 496.84 ng/mL in those who did (p=0.111). In our study, VCAM-1 levels ranged from 412.63 to 823.15 ng/mL. In a recent mouse model, thrombin was shown to induce the expression of VCAM-1, suggesting that VCAM-1 is increased prior to fibrin clot formation [15]. Levels of VCAM-1 may therefore increased before levels of D-Dimer rise. The difference with our study is that we measured VCAM-1 within 3 days of injury, as opposed to at time of diagnosis of DVT. This may have been too early, before a pro-thrombotic state had occurred.

In our study, D-Dimer levels were only 203.5 ng/mL in those who did not develop DVT and 236 ng/mL in those who did (p=0.490). Levels ranged from 31 to 1184 ng/mL. Although it is not possible to draw direct comparisons

between our absolute D-dimer levels and those found by others due to differences in methods used to quantify D-dimer, it is evident that levels in our study are relatively low [16]. Many studies have shown that patients who undergo lower limb venous ultrasound and subsequently found to have deep vein thrombosis (DVT), also have significantly higher d-dimer levels than those with normal imaging [14]. Recently, a d-dimer result of <500ng/ml was shown to have a negative predictive value of 99.48% irrespective of clinical suspicion of DVT [17]. In patients who have undergone surgery, cut off levels for excluding DVT are higher. Abraham et al (1999) found that a d-dimer cut off level of <2808ng/mL on day 1 post total hip or knee arthroplasty was associated with a significantly lower incidence of subsequent asymptomatic DVT (USS proven) on postoperative day 7 (8% vs 21.4%) [18]. Yoo et al measured d-dimer on day 3 following total hip replacement or surface replacement in 221 patients and found a significant correlation with the finding of DVT on ultrasound/venogram at day 7 postoperative. A cut off value of 2640 ng/mL had a negative predictive value of 98.8% [19]. Sudo et al (2009) suggested a cut off level of 17700 ng/mL after hip or knee arthroplasty [20].

One explanation for the comparatively low levels of d-Dimer found in our study is that our blood samples may have been taken prior to the prothrombotic state occurring. For example, in a study of 99 patients who underwent THR or TKR, d-dimer was measured pre and postoperatively. Patients also had DVT ultrasound scan at day 4 and 10 postoperatively, with 15% being found to have a DVT. D-dimer levels were significantly higher postoperatively as compared to pre-operative levels and those who were

subsequently found to have DVT had statistically significantly higher d-dimer levels than those who did not, at days 4, 7, 10 and 14 postoperatively. Interestingly, there was no significant difference in d-dimer levels on day 1 postoperative between those who subsequently developed DVT and those that did not [20]. An et al measured d-dimer in 177 patients who underwent THR or TKR and found that d-dimer levels peaked 2 weeks postoperatively [21]. Similarly, Yoshioka et al (2010) found that d-dimer measurements within the first 3 days following spinal surgery were not predictive of findings of DVT on screening USS between days 7-10 postoperative. Interestingly, there was a statistically significant difference in d-dimer levels between those who did and did not have DVT, when measured on day 7 postoperative due to a rise in d-dimer in those with DVT. These studies suggests that there is a delay in prothrombotic state and subsequent rise in d-dimer following surgery [22]. In a study by Walenga et al, patients with lower limb cast treatment for soft tissue injury or fracture had blood samples were taken at baseline (time of randomisation), between day 10 and 14 after injury and again at time of cast removal. 18.6% of 188 patients who did not receive thromboprophylaxis developed VTE, which is similar to our findings. All DVT's occurred in the injured leg [11]. In view of this, it appears that deep vein thrombosis is influenced most strongly by either the injury or the cast itself, as opposed to general hypercoagulability. Interestingly, thrombin-antithrombin complex (TAT), which represents thrombin generation, was normal at time of injury and not significantly different at baseline between patients who went on to develop VTE compared with those who did not. However, when compared at between day 10-14 post injury, it was significantly higher in the group who were

subsequently found to have DVT [11].

Limitations of our study

In this proof of concept study we measured quantitative levels of Tissue factor, IL-6, VCAM-1 and D-dimer. We acknowledge that study may be underpowered to detect statistically significant differences between groups, however the results may assist in planning of larger confirmative studies. For some of these tests, such as for Tissue factor it would have been useful to measure activity, because levels and activity may be independent. Also, we did not measure Tissue factor pathway inhibitor (TFPI), so it is possible that the thrombogenic effect of exposed subendothelial tissue factor secondary to injury may have been prevented by TFPI [23]. We only took blood samples at time of recruitment i.e. within 3 days of injury. It may have provided additional understanding if we had taken further samples at intervals, which would have enabled calculation of trends in levels of biomarkers assessed.

6. Conclusion

In this study of patients with acute foot and ankle trauma, we were unable to find an association between levels of plasma Tissue factor, IL-6, VCAM-1 or D-dimer and subsequent development of DVT. Deep vein thrombosis only appears to occur in the lower limb that has been injured and treated with cast. Further larger study is required to determine which biomarkers of thrombosis can be used and when these should be measured in order to identify patients that will subsequently develop DVT.

Acknowledgements

We are grateful to AO UK for funding this study. We are also grateful to all patients who participated and staff at University Hospital of Wales who assisted with this study.

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Table 1 – Exclusion criteria

Age >60 years
Use of Hormone Replacement Therapy or Oral Contraceptive Pill
Personal or First degree relative with history of Venous Thrombosis
Hospital admission of major surgery within 3 months
Pregnancy or within 6 weeks postpartum
Any serious medical co-morbidity
Extensive varicosities
Active cancer
Body Mass Index >30 kg/m^2
Known thrombophilia
Achilles tendon rupture

1 Table 2: Patient Demographics and injury types

	No DVT (Group 1)	DVT (Group 2)
Number of patients	56	21
Males	36	14
Age	37 (18-60)	36 (20-53)
BMI	25 (19-31)	25 (20-32)
Injuries		
Ankle fracture –Weber A	13	1
Ankle fracture - Weber A and 5th Metatarsal fracture	0	1
Ankle fracture - Weber A and undisplaced Talus fracture	1	0
Ankle fracture – Weber B	18	9
Ankle fracture - Weber B and Cuboid fracture	1	0
Ankle fracture - Weber B and 5th Metatarsal fracture	1	0
Ankle fracture - Weber C	1	0
Ankle sprain	4	2
Anterior process of calcaneus fracture	1	0
Cuboid fracture	2	0
Dorsal talonavicular ligament avulsion	2	1
Fifth metatarsal fracture	3	0
Lateral process of talus fracture	1	1
Lisfranc injury	0	1
Medial malleolus fracture	5	4
Navicular fracture	1	1
Posterior malleolus fracture	1	0
Talar neck and Dorsal Talonavicular ligament avulsion	1	0

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Table 3: Blood results

	No DVT (Group 1)	DVT (Group 2)	
Tissue Factor (pg/ml)	23.92 (SD 17.52)	20.33 (SD 16.83)	p=0.422
Interleukin 6 (pg/ml)	3.91 (SD 13.07)	4.59 (SD 7.03)	u=561.5, z=0.29, p=0.764
VCAM 1 (ng/ml)	552.98 (SD 92.59)	496.84 (SD 114.02)	u=448.5, z=1.58, p=0.111
D-Dimer (ng/ml)	203.5 (SD 225.27)	236.0 (SD 262.95)	u=527.5, z=-0.68, p=0.490