Microparticle-associated vascular adhesion molecule-1 and tissue factor follow a circadian rhythm in healthy human subjects

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Summary
An increased risk of death or severe injury due to late-morning thrombotic events is well established. Tissue factor (TF) is the initiator of the coagulation cascade, and endothelial stresses, coupled with production of pro-coagulant microparticles (MP) are also important factors in loss of haemostasis. TF and vascular cell adhesion molecule-1 (VCAM-1) -positive cell microparticles were assessed periodically over a 24-hour (h) period in healthy human subjects to ascertain if they followed a circadian rhythm. Eleven healthy male subjects were assessed in a temperature-controlled environment with dietary intake consistent between subjects. Blood samples were taken every 4 h by venipuncture, and TF and VCAM-1 positive microparticles were quantified by flow cytometry. A significant circadian rhythm was observed in VCAM-1 MP (p=<0.0001), and a trend was shown, although not statistically significant (p=0.065) in TF microparticles. A peak was observed at 9 a.m. for VCAM-1 positive MP, followed by a decrease and subsequent peak at 9 p.m. and a minimum at 5 a.m. TF-positive MP followed a strikingly similar trend in both variation and absolute numbers with a delay. A circadian rhythm was observed in VCAM-1 and less so TF-positive MP. This has significant implications in terms of the well known increased risk of cardiovascular thrombotic events matching this data. To our knowledge this is the first such report of quantified measurements of these MP over a 24-h period and the only measurement of a 24-h variation of in-vivo blood-borne TF.

Keywords
Circadian rhythm, microparticles, tissue factor, vascular cell adhesion molecule-1

Introduction
Circadian rhythms are important events in cardiology, whereby there is an increased incidence of myocardial infarction, stroke, complex arrhythmia, and sudden cardiac death in early waking hours. For example myocardial infarction was found to be four times more likely to occur between 8 a.m. and 9 a.m. than between midnight and 1 a.m. (1). A circadian variation in leukocyte activation and endothelial function has also been described (2, 3).

The association between cardiovascular events and time of day is well documented and shows a consistent well-defined peak in the hours after waking and also a second, smaller peak in the early evening (4–6). Circadian mechanisms for elevated risk include an increase in heart rate, blood pressure and platelet aggregation and decrease in fibrinolytic activity (7). Seasonal variations have also been observed with a significant increase in fatal events in the months of December and January compared to the summer months in a US population-based study (8). Circadian variation has also been reported in factor VIIa (7), with a peak in the morning. Factor VIIa is implicitly involved in complexing with tissue factor (TF) and the subsequent coagulation cascade, and so this variation, coupled with a noted decrease in prothrombin fragment F1+2 (7), lends evidence to the biological basis behind the increase in thrombotic events in the morning.

Maintenance of endothelial function has a preventative role in loss of homeostasis and subsequent cardiovascular risk factors. Flow-mediated endothelium-dependent vasodilation has been shown to be significantly decreased at 6 a.m. in humans (9). Following an endothelial insult, the endothelium may become activated, expressing vascular cell adhesion molecule-1 (VCAM-1), which has been shown to be significant in progression of vascular disease (10). VCAM-1 mediates rolling and tethering of circulating cells to the endothelium via integrin α4β1.

Damaged endothelium also results in increased availability of TF from sub-endothelial cells such as fibroblasts, pericytes and macrophages. Furthermore, microparticles (MP) are shed...
into the circulation from activated/damaged endothelium due to membrane remodelling (11).

MP are released into the circulation from many types of cells upon activation, apoptosis and under normal physiological conditions. MP have been identified from platelets (12), endothelium (13), leukocytes (14) and red blood cells (15). MP and their role in vascular disease (16) and homeostasis (11) are well described elsewhere. These particles carry the antigens particular to their cellular origin and with it an indication as to the state of that cell. VCAM-1 positive MP are released in vitro after tumor necrosis factor (TNF) stimulation of cultured vascular endothelial cells (16). Activated endothelium has been shown in many diseases, and so these MP can act as a marker (17), whether increased in number or altered antigen expression.

Under normal physiological conditions the majority of blood-borne TF is believed to be associated with monocyte MP (18). TF is present in the circulation under normal physiological conditions but changes are observed in various disease states including those characterised by coagulation (19). TF is a receptor for factor VII/VIIa and has been demonstrated to be the initiator of coagulation within the circulation (20) and has a role in maintenance of haemostasis (21). Endothelial MP have also been shown to be able to induce TF driven coagulation in vitro (22).

We report here for the first time a circadian variation in VCAM-1 positive MP and TF-positive MP in healthy human subjects and discuss these findings in relation to the wealth of published data showing increased cardiovascular events in the later morning hours.

### Material and methods

#### Subjects

Eleven male non-smoking subjects participated in this 24-hour (h) study (mean ± SD height, body mass and age: 177 ± 6.4 cm, 75.7 ± 10.9 kg, 19.8 ± 4.3 years). None of the subjects had been training excessively or had been spending time in hot climate over the preceding six weeks. The subjects were moderately trained with an average training load of 5.9 ± 2.2 h/week. The local institutional ethics committee approved the study, and all the subjects provided written, informed consent.

#### Experimental protocol

The subjects abstained from alcohol, caffeine and exercise 48 h prior to the testing. On the day of testing, the subjects reported to a temperature-controlled laboratory (average Wet-Bulb Globe Temperature Index 18.1°C ± 0.9°C) at 08.00 a.m. for acclimatisation. Dietary intake was equivalent between subjects and food was provided at 09.30 a.m., 1.30 p.m. and 6.00 p.m.. During the study, subjects were asked to refrain from rigorous activities. They were asked to retire to bed at 11.30 p.m. and were woken up at 07.30 a.m. the following day. Blood samples were taken in the same order every 4 h from 09.00 a.m. to 09.00 a.m. the next day. Blood samples were drawn by a standard venipuncture technique from the antecubital vein into a tri-sodium citrate Vacuette tube (Vacuette®, Greiner Bio-one, Stonehouse, UK) for subsequent microparticle quantification. All samples were processed immediately. Additionally serum was isolated from serum separator tubes (Vacuette®).

#### Microparticle assay

Citrated blood was processed and microparticles (<1.5 μm) analysed according to a well-established method (23). Briefly, citrated tubes were centrifuged (10 minutes [min], 160 x g) and the platelet-rich plasma (PRP) was aspirated and further centrifuged (6 min, 1,500 x g). The now platelet-poor plasma (PPP) was analysed. Samples (25 μl) were incubated with equal concentrations of either isotype (IgG) matched negative control: FITC (AbD Serotec), anti-TF:FITC (American Diagnostics), anti-VCAM-1:FITC, or anti-annexin V:FITC (AbD Serotec) antibodies (all 80 μg/ml final concentration) for 30 min. Filtered (0.22 μm) phosphate-buffered saline (PBS) (100 μl) and counting beads (25 μl, Caltag Laboratories) were added immediately prior to analysis by flow cytometry (BD FACSCalibur). Positive MP were defined as an increase in mean fluorescence intensity over the isotype matched negative control and were quantified in relation to counting beads according to manufacturers’ instructions.

#### Oxidative stress

Serum was analysed using a commercially available thiobarbituric acid reactive substances (TBARS) kit (Zymo Research, US) according to manufacturer’s instructions. Results obtained are expressed as malondialdehyde equivalents.

#### Procoagulant microparticle ELISA

Citrated blood was collected from an expanded cohort of 17 subjects (including the original 11) at 9 a.m. and 1 p.m. Samples were centrifuged (1,500 x g for 15 min), the plasma removed and centrifuged again (13,000 x g for 2 min) and analysed immediately by a commercially available ELISA (Zymuphen MP-activity, Hyphen BioMed, Neuville-sur-oise, France) according to manufacturers’ protocol. At this time flow cytometry analysis was also repeated, with the inclusion of an annexin V:FITC antibody, as the ELISA is based upon annexin V positive MP capture.

#### Statistical analysis

Analysis was carried out by a professional statistician using statistics package R (24). ANOVA and quadratic terms were analysed. Tests for increases to a peak or decreases to a trough followed by a change in the reverse direction were made by adding quadratic terms to the statistical model and testing the statistical significance of these terms. Quadratic trends indicate a statistically significant but non-linear trend in the data over time.

#### Results

Of the 11 subjects who began the trial nine completed the 24 h with two terminating the trial after the 1 a.m. sample was taken. Flow cytometry profiles of MP and their staining with both negative control, VCAM-1- and TF-specific antibodies can be seen in Figure 1. Average VCAM-1-positive MP numbers were seen to decrease linearly from 9 a.m. to 5 p.m. (Fig. 2), followed by a slight increase to a minor peak at 9 p.m., then decreased to a minimum at 1 a.m., followed by a linear increase through 5 a.m. back to a peak at 9 a.m. with absolute count reaching levels observed 24 h previously. The trend was found statistically signifi-
cant, with a quadratic trend (non-linear) observed (p =< 0.0001). Strikingly TF-positive MP followed the same variation, albeit with a 4 h lag (Fig. 3), although not significant (quadratic trend p = 0.256). It is thought that this is probably due to a relatively high inter-subject variability; performing a square-root transformation to account for this resulted in a trend towards significance (p = 0.065). Both these markers on MP were observed to return to the levels of 24 h previously, suggesting a possible causal link between endothelial function and coagulation. Inter-subject variation was observed, as would be expected in human subjects, with a mean coefficient of variation of 0.47 observed for VCAM-1-positive MP, with the highest variation observed at the two 9 a.m. sampling times. The peak-average absolute count of VCAM-1 positive MP was 1,552 MP/μl PPP (9 a.m.), 838 MP/μl PPP (9 p.m.) and 1,643 MP/μl PPP (9 a.m. +24 h) for VCAM-1 MP, with a minimum of 547 MP/μl PPP at 1 a.m. TF-positive MP was observed to peak in number at 1 p.m. (1,719 MP/μl PPP), with a minor peak at 1 a.m. (781 MP/μl PPP) and a minimum observed for the 24-h cycle at 5 a.m. (479 MP/μl PPP).

An expanded cohort of subjects (n=17) were re-tested (9 a.m. and 1 p.m.) at a later date, using an alternative MP preparation method (49) in accordance with manufacturers' instructions for a

![Flow cytometry profile showing gated microparticles (MP) (top left) and MP incubated with either negative control antibody (bottom left) or VCAM-1 specific antibody (top right). Gate +VCAM-1 indicate MP stained positive for VCAM-1. Gate +TF indicates MP stained positive for tissue factor (bottom right). Within gates B1 and B2 are counting beads used to quantify MP numbers.](image1)

![Average (± SD) total count of VCAM-1 positive microparticles (MP) over 24 h. PPP, platelet-poor plasma.](image2)
commercially available procoagulant MP ELISA. Samples were analysed by flow cytometry and by ELISA within 1 h of collection. VCAM-1 positive MP were shown to have a mean decrease of 14.5% from 9 a.m. (2,362 MP/μl PPP) to 1 p.m. (2,019 MP/μl PPP), within standard deviation of the data obtained here with the method of Jiminez et al. (23). TF-positive MP showed a mean increase of 24.6%, again (1,390 to 1,732 MP/μl PPP) within error reported for the data obtained via the original method. Also annexin V MP were analysed by flow cytometry and showed no overall change in these subjects from 9 a.m. (415 MP/μl PPP) to 1 p.m. (414 MP/μl PPP). Results from the procoagulant ELISA are shown in Figure 4. Mean procoagulant MP decreased slightly, from 2.66 nM (± 0.50) to 2.45 nM (± 0.55), in agreement with the data obtained from flow cytometry. The ELISA method is based on annexin V MP and so MP derived from cell activation rather than apoptosis would not be captured by this assay. Oxidative stress may be a mechanism of cell activation-derived MP, especially VCAM-1 MP released from activated endothelium. To assess oxidative stress, a commercial thiobarbituric acid reactive substances (TBARS) kit was used to determine serum concentrations. The results obtained showed relatively small changes in daytime hours, followed by a decrease from 9 p.m. to 1 a.m., a minimum observed at 5 a.m. followed by an increase again at 9 a.m. the following day (Fig. 5). This trend was found significant, using repeated measures ANOVA (p=<0.001).

**Discussion**

The increased incidence of detrimental cardiovascular events in waking hours is established. TF is inherently involved in initiation of coagulation and thrombus progression via attachment to platelets (25) and TF-positive MP have been demonstrated in the current study to follow a circadian rhythm, peaking in the waking hours, and thus offering a potential role in the increased cardiovascular risk observed in the hours following sleep.

Pericardial blood from patients undergoing coronary bypass surgery has been shown to have a dense population of procoagulant MP derived from various cells, and more importantly these MP were proposed to support coagulation via MP-TF dependent factor VII pathway (26). It may be assumed that the presence of heparin in such surgery prevents thrombosis.

Endothelial MP have been shown to be associated with TF; however, whether this TF is of endothelial cell origin, or from bound monocyte-derived TF is unclear (18). Upon activation, platelets are known to bind monocyte MP and thus acquire TF, which could become active (27). Endothelial cells have been shown to be able to produce TF in vitro (28), however, only following severe treatment, i.e. extraction and stimulation. Furthermore, it has been proposed that platelet functionality may also follow a circadian variation (29). This could offer an explanation as to the lag of TF in the circadian cycle, i.e. platelet binding of monocyte MP thus reducing the measured TF on MP in this study.

Many coagulation factors have previously been demonstrated to follow circadian rhythms, including prothrombin (7), factors VII (7), X (30) and tissue factor pathway inhibitor (TFPI) (31). The circadian rhythm in factor VII and TFPI has been...
postulated to maintain haemostasis (31) as TFPI negatively influences the coagulation cascade. Circadian variations also have been shown to exist in endothelial function mediators, endothelin-1 (ET-1) and, to a lesser extent nitric oxide (NO) (32). ET-1 concentration peaked at 8 a.m. and 8 p.m. with a minimum observed at 4 p.m., in contrast NO levels peaked at 8 p.m. with minimal levels at 4 a.m. and again at 8 a.m. Although there have been studies on circadian variations in endothelial function, no MP correlation has ever been established.

Pro-coagulant MP have been demonstrated to be highly relevant biological markers of the state of the vasculature in vivo and in particular as a prognostic indicator of the likelihood of thrombotic events (33). VCAM-1-positive MP are exclusively of activated endothelial origin and endothelial MP have been widely demonstrated to be a marker of endothelial state in vivo, and increased numbers have been observed in many disease states linked to loss of vascular homeostasis (16). Endothelial function has been demonstrated to be impaired in the hours following waking, as measured by flow-mediated vasodilation (9, 32) and is thought to be a result of a surge in blood pressure upon waking. A study of MP in hypertension (34) found a positive correlation with blood pressure, which itself follows a circadian variation (35); therefore it appears highly likely MP may follow a similar pattern. A study of serum markers of endothelial state showed a statistically significant circadian variation in serum ICAM-1 and E-selectin (p<0.0001) in healthy male volunteers (36). Soluble ICAM-1 peaked at midday and decreased to a minimum at 4 a.m., whereas soluble E-selectin concentration peaked again at midday but decreased to a minimum at midnight. The data may also point to a role of endothelial activation in the timing of symptoms of cardiovascular events. However, another study showed that serum levels of ICAM-1 and L-selectin do not show circadian variations in healthy subjects (37). VCAM-1 and ICAM-1 have been widely studied in relation to cardiovascular disorders, both prescriptive and descriptive (38–42). VCAM-1 is expressed upon endothelial activation and mediates cell adhesion to the endothelium, in this state it appears likely that monocyte MP are able to bind to the endothelium (18), thus reducing the circulating pool of blood-borne TF. Upon return of endothelial function to a daily normal level, less monocyte MP would bind, thus leading to an increase in monocyte derived blood-borne TF, offering an alternative explanation for the observed lag between VCAM-1 and TF positive MP reported here. Although these MP may be being cleared by phagocytosis (43–44), as newly formed MP are shed into the circulation, however this would assume variations in either rates of cell MP shedding or phagocytosis, or both.

MP have been implicated in coagulation (27). Furthermore, VCAM-1-positive MP have been observed within the milieu of atherosclerotic plaques; however, circulating VCAM-1 was not shown to be associated with coronary heart disease or carotid artery atherosclerosis, but it was proposed that VCAM-1 may play a role in early atherogenesis (38). In a more recent study, patients with coronary artery disease were shown to have significantly higher VCAM-1 than patients with normal arterial function and healthy controls, and so VCAM-1 was proposed as a marker of endothelial injury in patients (45). Additionally, decreased endothelial function was suggested to potentially have a role in the observed incidence of increased cardiovascular events in the waking hours (9). It was also shown that the increase in VCAM-1-positive MP was due to activation, rather than apoptosis (23). The functionality of antigens carried by MP is questionable and problematic to quantify; however, they are acknowledged as a sensitive marker of the state of the endothelium in vivo. Therefore, data presented here indicates endothelial function improves in the hours after waking and may become activated upon waking, probably caused by the well-established known surge in blood pressure at this time.

The measurement of MP has been the subject of much debate (46–52). Two sequential centrifugations have been shown to be most effective at sample preparation for MP analysis (53), avoiding platelet contamination. Storage of plasma for MP analysis has also been shown to result in significant reductions in both number of MP and TF-MP, regardless of temperature and duration of storage (53). In addition, freezing of plasma has also been shown to alter counts and certain antigen properties (52).
and also result in non-specific loss of endothelial-derived MP (54). Considering the nature of this study in terms of sampling and throughput without storage, and in light of the above observations, the method of the group of Jy was adopted (23). As previously discussed, the exact nature and functionality of TF-bearing MP is questionable, thus here we reported changes in both TF and VCAM-1 MP over a 24-h period in a controlled environment. A procoagulant ELISA on 17 subjects showed no overall change from 9 a.m. to 1 p.m.; however, this assay does rely on the capture of annexin V-positive MP, which are released from apoptotic bodies, and may or may not express TF. The increase in TF-MP from 9 a.m. to 1 p.m. may therefore be assumed to originate from cellular activation. Assessing the functionality of TF-bearing MP from plasma is fraught with difficulty. Isolation of MP via specific antigens, such as TF, requires the use of antibodies, followed by purification using, for example sorting flow cytometry or immunomagnetic separation (IMACS). Both these techniques have the potential to alter antigen activity, due to antibody binding. Removal of antibody, following IMACS results in loss of MP (unpublished data).

Similar trends were observed from 9 a.m. to 1 p.m., in VCAM-1- and TF-positive MP, using the methods of Jimenez et al. (23) and Hugel et al. (49). The lack of any increase in annexin V MP from 9 a.m. to 1 a.m., as measured by flow cytometry and ELISA, demonstrates that any increase in TF-MP is probably due to cell activation rather than apoptosis. The observed increase in oxidative stress as measured by TBARS may be an indicator of potential endothelial activation upon waking, leading to TF release. This could correlate with the known surge in blood pressure upon waking and may be involved in the mechanism behind elevated VCAM-1 MP, as the endothelium is known to be sensitive to oxidative stress and shear rate leading to vascular remodelling (55–56).

The normal physiological state described here shows a circadian rhythm following waking and subsequent increase in VCAM-1-positive MP, accompanied by corresponding trough and peak in TF-positive MP a period later. The changing numbers of these circulating MP described here allow an insight into the in-vivo state of both endothelium and monocytes and have been demonstrated to have a late morning peak, indicating a more pro-thrombotic state (11) and thus a potential role in the increased occurrence of adverse cardiovascular events within this time period as well as a role in maintenance of haemostasis.

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