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Altered clot microstructure detected in obstructive sleep apnoea hypopnoea syndrome

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ARTICLE INFO

ABSTRACT

Abnormal clot microstructure plays a pivotal role in the pathophysiology of thromboembolic diseases. Assessing the viscoelastic properties of clot microstructure using novel parameters, Time to Gel Point (T_{GP}), Fractal Dimension (d_f) and clot elasticity (G'_{GP}) could explain the increased cardiovascular and thromboembolic events in patients with Obstructive Sleep Apnoea Hypopnea Syndrome (OSAHS). We wanted to compare T_{GP}, d_f, and G'_{GP} and their diurnal variation in OSAHS and symptomatic comparators. thirty six patients attending a sleep disturbed breathing clinic with symptoms of OSAHS were recruited. T_{GP}, d_f and G'_{GP} were measured alongside standard coagulation screening, thrombin generation assays, and platelet aggregometry at 16:00 h and immediately after an in-patient sleep study at 07:30 h. OSAHS group had significantly lower afternoon d_f than comparators (1.705 ± 0.033 vs. 1.731 ± 0.031, $p < 0.05$). d_f showed diurnal variation and only in the OSAHS group, being significantly lower in the afternoon than morning ($p < 0.05$). Diurnal changes in d_f correlated with 4% DR, even after controlling for BMI ($r = 0.37$, $p = 0.02$). The lower d_f in the afternoon in OSAHS suggests a partial compensatory change that may make up for other pro-clotting abnormalities/hypertension during the night. The change to the thrombotic tendency in the afternoon is biggest in severe OSAHS. d_f Shows promise as a new microstructural indicator for abnormal haemostasis in OSAHS.

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Peer review under responsibility of Brazilian Association of Sleep.

1. Introduction

Obstructive sleep apnoea hypopnoea syndrome (OSAHS) affects 2–4% of middle-aged adults [1–3]. It is associated with various adverse cardiovascular outcomes [4,5] and appears an independent factor for arterial [6] and possibly venous thrombosis [7]. However, studies focusing on mechanisms to explain the dysfunctional haemostasis in OSAHS are scarce and inconsistent. Recent research highlights the role of static fibrin (clot) microstructure in abnormal clot development and its direct relation to the increased risk of cardiovascular events in OSAHS [8]. The formation of an initial fibrin network has been shown as the primary templating microstructural component of a blood clot formation (Incipient clot formation) [9,10]. The clotting process starts the activation of time-based coagulation pathways which result in the development of clot microstructural fibrinopeptide A and B cleaved by thrombin from fibrinogen, and the resulting fibrin monomers bind together to create oligomers and subsequently two-stranded protofibrils [11]. Protofibrils aggregate axially to build up a fibre that gels via lateral aggregation [12]. This cross-linking therefore determines the elasticity and mechanical strength of the developing fibrin architecture. Fibres become thicker due to lateral aggregation and branch that leads to a sample-spanning network (i.e., gel). Fibrin network conformation and fibrin fibre diameter also influence fibrinolysis speed. Therefore, fibrin clots built of thin fibres are dissolved at a slower rate than clots with the thick fibres [13], and the resulting formation of tight, rigid and space-filling fibrin network structures with small pores is associated with premature coronary artery disease (CAD) [14,15]. Furthermore, Mills et al. [16] found that healthy male relatives of patients with premature CAD had fibrin clots which polymerized more quickly, had thicker fibres and were less permeable than controls matched for traditional CAD risk factors [16]. The initial fibrin network acts as a template for how the clot develops and determines the clot's eventual physical properties. According to Wolberg [17], the conditions i.e., inflammatory and physiological alterations affect how fibrinogen is converted to fibrin determines fibre thickness, branching and network density of the clot development and morphology. Hence, measuring initial clot microstructure and its dynamic development would determine a meaningful marker of coagulation in pro-coagulable states. Incipient clot microstructure is associated with significant changes in blood viscoelasticity (a measure of a material's viscous and elastic properties). Recent studies of viscoelastic properties are among the most sensitive measures of fibrin polymerization and blood clot microstructure and its mechanical properties [18,19]. Fractal analysis is an established method that allows the testing of a viscoelastic fluid, such as blood by applying a stress (small amplitude oscillatory shear) that varies harmonically with time, and then measuring the response. This technique generates a phase angle (δ) which is a measure of the ratio between the material's viscous and elastic properties. An incipient clot is formed at the Gel Point (GP) [20,21] just as the blood turns from viscoelastic liquid to a viscoelastic solid. Therefore, rheological analysis explains in a dynamic way the process of clot initiation to clot formation.

We have shown that incipient clots form in healthy whole blood within a narrow range represented by a fractal dimension (d_f) value derived from the GP and δ. A higher d_f is suggestive of a more pro-coagulable state. The measurement of d_f in a healthy state is maintained within remarkably narrow limits at 1.74 ± 0.07 [22]. Hypoxic events and diurnal changes in catecholamines and blood pressure can affect the mechanistic changes in clot structure thereby causing increased cardiovascular events in OSAHS. In the general population, myocardial infarction (MI) and sudden cardiac death peak in incidence between 06:00 and 12:00 [23] but the frequency of MI in people with OSAHS is significantly higher than in non-OSA patients (32% vs. 7%; $p = 0.01$) between 00:00 and 06:00 [8]. The purpose of this pilot study was to infer if T_{GP}, d_f and G'_{GP} are altered in OSAS patients compared to symptomatic comparators. Our secondary aim was to see if any diurnal variation existed in the standard laboratory screening tests in the above groups.

2. Methods

The study was approved by the local Research Ethics Committee and Institutional review boards and registered ClinicalTrials.gov Identifier: NCT01525160.

2.1. Participants

This was a prospective and cross-sectional study consisting of patients aged 18–80 years, attending our sleep-disordered breathing (SDB) clinic at a UK district general hospital. Patients were referred from primary and secondary care with varying symptoms suggestive of OSAHS (daytime sleepiness, snoring and/or nocturnal apnoeas). We excluded anyone with other diseases known to affect coagulation (e.g. cancer, hepatic and/or renal dysfunction, acute or chronic inflammatory conditions), those receiving antiplatelet or anti-coagulation treatment or with a personal or family history of bleeding or thromboembolic disorder. Thirty six Patients (50 ± 11 years, males ($n=31$), females ($n=5$) were recruited into the study, all underwent an in-patient overnight limited channel sleep study (Visilab, Oakwood Scientific, Oxford, UK). All video recordings and pulse oximetery waveforms were manually reviewed to confirm that episodes were predominantly obstructive rather than central apnoeas. According to the National guidelines to diagnose OSAHS, patients with a 4% dip rate (DR) >10 events/h were classified as OSAHS. Those with symptoms but a 4% DR <10 events/h were classified as comparators. To reduce the chance of false positives or false negatives we excluded borderline cases either side of the cut-off of 10 DR events/h (Table 1). To look for any biological signal we wanted to compare more extreme cases and therefore included patients with OSA who had a 4% DR of >10/h, and only included controls as true negatives if they had a 4% DR <10/h. Sleep tests were continued until we had 18 per group.

Table 1 – Patient characteristics for the OSAHS and comparator groups.

Demographics (mean ± SD)	Comparator $n=18$	OSAHS $n=18$
Male	15	16
Age (years)	45 ± 10	55 ± 11
Neck circumference (cm)	40.8 ± 2.9	44.3 ± 4.7
Body mass index (kg/m^2)	32 ± 7	36 ± 8
Systolic BP (mm Hg)	138 ± 26	125 ± 14
Smoking pack years	6 ± 10	19 ± 17
Exhaled carbon monoxide (ppm)	7 ± 10	11 ± 13
Epworth sleepiness score	12 ± 6	14 ± 6
4% DR (events/h)	5 ± 3	46 ± 23
% TST with SaO$_2$ <90%	0.5 ± 0.8	18.5 ± 16.1
Mean overnight SaO$_2$ (%)	95 ± 0.9	92 ± 2

2.2. *Procedure*

Blood sampling occurred at 16:00 h before the sleep study and at 07:30 h the following morning. Those with OSAHS had further blood sampling at 07:30 following their first night of continuous positive airway pressure (CPAP) and at 07:30 after a month of CPAP.

20 ml of venous blood was collected from a peripheral vein (antecubital fossa) with a 21-gauge needle into a Vacutainer system. The first 2 ml of each sample was discarded to reduce any contamination with skin tissue and tissue factor. A 7 ml aliquot of whole blood was then immediately transferred (<30 s) into the double-gap concentric cylinder measuring geometry (340 μm shearing gap) of an AR-G2 controlled-stress rheometer (TA Instruments, Delaware, US). All measurements were performed as previously described by [Evans et al., 2008, 2010]. The following parameters were recorded: the time taken to reach the gel point (T_{GP}) d_f and G'. Sodium citrated blood (3.2% sodium citrate Greiner bio-one; ref: 454327) was used to measure Prothrombin time (PT), Activated Partial Thromboplastin Time (APTT), and Clauss fibrinogen (Fib) and was measured via a Sysmex CA1500 analyser within 2 h of collection by scattered light detection (percentage test endpoint method). Thrombin generation was performed using the Thrombin Generation Assay (Technoclone Diagnostics, Vienna, Austria) as described by Ay et al. [25]. Platelet function was assessed with hirudinated whole blood was kept at room temperature for 30 min before testing platelet aggregation [26] using the Multiplate impedance analyser (Dynabyte GmBH, Munich, Germany).

2.3. *Statistical analysis performed using SPSS version 20.0 software (SPSS Inc., Chicago)*

All data were checked for normality using the one sample Kolmogorov–Smirnov test. Between group differences were assessed using chi-square, two-sample independent t-test, Mann–Whitney U and within group differences were tested with a repeated measures ANOVA.

3. Results

Within the OSAHS group, d_f was significantly lower in the afternoon 1.705 ± 0.033 than the morning 1.737 ± 0.042 (Fig. 1) ($p<0.05$). The comparator group, however, showed no changes in d_f between the morning and afternoon measurements (Table 2).

There was a significant positive correlation between Δd_f (the difference between the mean morning and mean afternoon d_f), and 4% DR and Δd_f correlated positively with neck circumference and BMI across the whole study population. After controlling for BMI, the correlation between Δd_f and 4% DR remained significant ($r=0.37$, $p=0.002$).

No significant differences were found in T_{GP} or G' between and within the OSAHS and comparator groups. No significant differences were seen between any of the thrombin generation assays, platelet aggregometry and standard coagulation screening tests between the OSAHS and comparator groups (Table 3). There were no differences between the afternoon and morning measurements for these haemostatic variables in OSHAS or comparators.

4. Discussion

This is the first study to investigate dynamically and quantify the arrangement and mechanical properties of fibrin clot microstructure in OSAHS using fractal analysis. We found higher levels of d_f in the morning in the OSAHS group. In the OSAHS group, the afternoon d_f values were significantly lower when compared to their comparators. This reduction in d_f within the OSAHS group is indicative of a diurnal variation
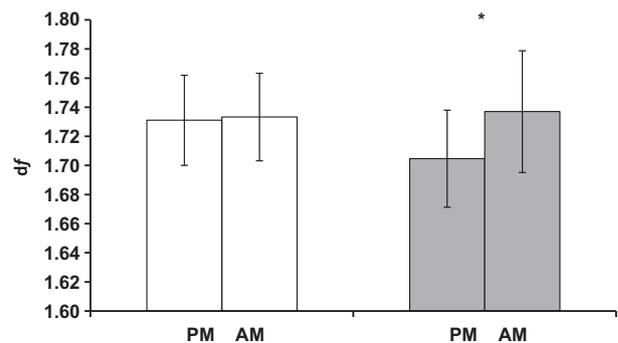
Fig. 1 – Afternoon and morning d_f in OSAHS and comparators * denotes $p<0.05$.

Table 2 – Rheological parameters for the OSAHS and comparator groups.

Variable	Sampling time	Comparator	OSAHS
d_f	PM	1.731 ± 0.031	1.705 ± 0.033
T_{GP} (s)		228.7 ± 90.8	276.1 ± 107.1
G' (TGP 2.0 Hz)		0.036 ± 0.012	0.031 ± 0.011
d_f	AM	1.733 ± 0.03	1.737 ± 0.042
T_{GP} (s)		193.5 ± 54.2	249.3 ± 114.1
G'' (TGP 2.0 Hz)		0.037 ± 0.011	0.037 ± 0.015

Table 3 – Coagulation screening, inflammatory markers, platelet function and thrombin generation for the comparator and OSAHS group.

Variables	Comparators		OSAHS	
	PM	AM	PM	AM
HB (g/l)	15.1 ± 1.2	15.2 ± 1.3	14.8 ± 1.2	14.9 ± 1.3
HCT (l/l)	0.44 ± 0.02	0.44 ± 0.03	0.43 ± 0.03	0.44 ± 0.03
Platelets ($\times 10\widehat{9}$/l)	278.8 ± 54.3	244.9 ± 74.4	273.0 ± 59.6	263.2 ± 54.1
PT (s)	10.3 ± 0.4	10.3 ± 0.4	10.3 ± 0.4	10.3 ± 0.4
APTT (s)	25.08 ± 2.09	25.7 ± 1.7	24.4 ± 1.8	24.7 ± 1.7
Fibrinogen (g/l)	3.3 ± 0.5	3.4 ± 0.5	3.1 ± 0.4	3.2 ± 0.4
CRP (mg/l)	5.58 ± 6.02	5.03 ± 4.68	5.0 ± 3.5	4.4 ± 2.7
VCAM-1	327.6 ± 80.1	312.9 ± 62.3	349.6 ± 79.8	336.8 ± 74.5
ICAM-1	286.8 ± 65.6	279.7 ± 50.9	282.7 ± 82.7	286.5 ± 82.3
Peak thrombin (nm)	338.9 ± 97.4	355.6 ± 78.0	430.0 ± 104.3	335.2 ± 79.0
SAA (µg/ml)	10.4 ± 13.5	6.8 ± 5.8	5.3 ± 5.1	5.6 ± 4.7
ADP (AUC)	81.6 ± 20.0	81.8 ± 24.3	72.8 ± 22.1	65.8 ± 24.3
ASPI (AUC)	92.6 ± 20.8	98.7 ± 27.2	91.1 ± 20.0	86.1 ± 23.2
Collagen (AUC)	81.3 ± 21.8	87.8 ± 17.4	80.5 ± 22.1	71.6 ± 14.0

that was not evident in the comparator group. To our knowledge, only one study has reported fibrin clot formation in OSAHS. McEwen et al. [27] investigated fibrin polymerisation indirectly via turbidimetry across the sleep–wake cycle in 25 patients with severe OSAHS (AHI ≥ 25). They reported that compared to 06:00 and 09:00 peak values, fibrin generation parameters were significantly lower at midnight and 03:00, suggesting as in our study that there is a prothrombotic shift n subjects with OSAHS [27].

Although necessary for clot microstructure, it is important to note that fibrinogen (FIB) concentrations explain only up to 18% of the variation in clot permeability [28]. The fibrinogen levels in our study did not show any circadian variation nor differed between groups. In contrast, Mehra et al. [29] found a positive linear relationship between severity of OSAH, and fibrinogen levels but only for mild to moderate disorders. They observed that morning fibrinogen level increased on average by 14.4 mg/dl per 5-unit AHI increase until an AHI of 15. However, no significant incremental changes in morning fibrinogen were observed with increasing AHI above 15 suggesting a threshold effect [29]. Our OSAHS group had more severe OSA, with a mean 4% DR of 46 ± 23 events/h. It could be that other factors or compensatory mechanisms are activated above a certain DR threshold.

The generation of thrombin is another fundamental part of the clotting cascade [30] and pivotal in the clot development in vascular diseases. We found no significant differences in thrombin generation between and within groups, but there was a trend of higher thrombin generation levels noticed in the AM measurement OSAHS indicating perhaps a sign of an underlying effect of prothrombotic predisposition. Recurrent upper airways obstruction is causing alternating cycles of hypoxia/re-oxygenation with a rapid re-oxygenation of transiently ischaemic tissues leads to tissue injury and release of reactive oxygen species (ROS) [31]. Transient oxidant formation is thought to occur during MIs and stroke and acute inflammation, but more prolonged and recurrent oxidant generation has been proposed in chronic inflammatory conditions such as OSAHS [32]. ROS start an inflammatory cascade via activation of transcription factors and downstream genes such as inflammatory cytokines and adhesion molecules [33]. ROS that are not destroyed can be toxic because of their propensity to react with

lipids, proteins and DNA. Some enzymatic and non-enzymatic oxygen free radical-generating systems can catalyze the oxidative modification of proteins causing gross structural change [32]. Such modified proteins can undergo spontaneous fragmentation or can exhibit substantial increases in proteolytic susceptibility [34]. Faure et al. [35] reported that OSAHS patients have significantly fewer albumin thiol groups than healthy controls, indicating greater protein oxidation. Moreover, the number of albumin thiol groups was inversely correlated with AHI and mean nocturnal SaO_2 [35]. Free radicals and ROS produced during oxidative stress also attach very easily to FIB to decrease the rate of clot formation [36]. Oxidative modification of purified human FIB leads to an exposure-dependent loss of thrombin-induced clot formation. Thrombin-catalyzed fibrinopeptides release is normal indicating that the inhibition of clotting activity is due to impaired fibrin monomer polymerization rather than increased breakdown [37].

Oxidized fibrinogen also impairs the microrheological properties of the blood, it sharply reduces erythrocyte deformability, modifies blood viscosity and reduces the suspension stability of the blood. These physical changes play a significantant role in the development of atherosclerosis [38]. Glycated FIB leads to denser fibrin clots that are stiffer and more resistant to fibrinolysis thus leading to an increased thrombotic burden [39]. It is also possible that subjects with untreated OSAHS develop other mechanisms that alter clot microstructure independent of FIB, thrombin such as erythrocytosis that contributes to thrombotic events. Our study does present certain limitations as the patients were primarily from a SDB clinic rather than from the general population. Our sample size is also relatively small and the comparators not fully matched; finding sleepy obese people with the same BMI and cardiovascular risk factors without OSAHS is difficult. We applied limited channel sleep studies not full polysomnography to diagnose OSAHS, as is our current (The UK and European) standard clinical practice. We did not measure the effect of treatment with CPAP and its associated benefits to infer if any diurnal variation existed in d_f as this was a pilot study. We however, recommend that any future work should consider this. We therefore present this evidence as pilot work and propose that more research is

needed to identify the pathophysiological mechanisms that are associated with clotting abnormalities in OSAHS. We are also mindful that the routine coagulation tests have limitations in their ability to predict the relationship between coagulation pathways and clot outcome regarding functionality. Therefore, we propose that the understanding of clot microstructure offers a unique way of comprehending the complex mechanisms associated in the development of coagulopathy in sleep apnoea.

Conflict of interest

Funding

Author's contributions

LD: draughting of the article, data analysis, statistical analysis and literature review; MW: subjects recruitment, data collection and interpretation, literature review; KL: study design, revising the article for scientific and intellectual content, interpretation of the data (Sleep apnoea); ML: data collection (rheology); KH: revising the article for scientific and intellectual content, interpretation of the data (rheology); RW: revising the article for scientific and intellectual content (rheology); SS: data collection, revising the article for scientific and intellectual content (laboratory markers); SD: revising the article for scientific and intellectual content, interpretation of the data (laboratory markers); KM: revising the article for scientific and intellectual content and statistical analysis; AE: Idea initiation, study design and data analysis, final approval of the version to be published. All authors read and approved the final manuscript.

Acknowledgements

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