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Main title: The effect of tyramine infusion and exercise on blood flow, coagulation and clot microstructure in healthy individuals

Short title: The effect of catecholamine's on coagulation

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Abstract

Background The long term benefits of exercise on the cardiovascular status of a patient have been proven, however, their benefit/risk relationship with exercise intensity is unclear. Furthermore, many thromboembolic diseases such as myocardial infarction and ischaemic stroke are associated with profound catecholamine release. In this study we explore the relationship between catecholamine release and hemodynamic changes and their effect on coagulation.

Materials and Methods Twelve healthy recreationally active males were recruited. Local anaesthesia was given and catheters were placed under aseptic conditions, in the femoral artery and vein of the experimental leg. The first experiment involved tyramine infusion into the femoral artery at a dose of $1.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L leg volume}^{-1}$. The second experiment involved single leg knee-extensor exercise performed at 30 W for 15 min. Venous blood was collected at each time point to assess clot microstructure using the d_f biomarker.

Results and Conclusions Tyramine infusion causes a local noradrenaline release in the leg. The increase in noradrenaline was associated with a significant increase in clot microstructure formation (d_f increased from 1.692 ± 0.029 to 1.722 ± 0.047 , $p=0.016$). Additionally moderate intensity single leg knee extensor exercise, which minimally alters sympathetic activity, also induced an increase in d_f (from 1.688 ± 0.025 to 1.723 ± 0.023 , $p=0.001$). This suggests that exercise can alter clot microstructure formation both via an increase in catecholamine levels and by factors related to muscle activity per se, such as increased blood flow and consequent shear. These findings have implications for recommendations of exercise in patients at risk of cardiovascular events.

Keywords: catecholamine, clot microstructure, coagulation, exercise

Highlights

- **Exercise and thromboembolic diseases are associated with profound catecholamine release**
- **In this study we show for the first time how catecholamine release and exercise alters coagulation**
- **Both moderate exercise and catecholamine produce denser/compact clot microstructures**
- **These findings have implications for recommendations of exercise in patients at risk of cardiovascular events.**

Introduction

High intensity exercise induces physiological changes such as increases in skeletal muscle blood flow, altered blood volume distribution and shear stress blood profile as well as platelet reactivity.[1,2] All of which have been shown to be driving factors for hypercoagulability in healthy people performing exercise.[3,4] Accordingly, a previous study on exercise in healthy volunteers demonstrated that hypercoagulability was associated with exercise intensity.[5]

The hemodynamic changes that occur with exercise are controlled by the autonomic nervous system which increases both cardiac output and systemic vascular resistance. In the working muscle, the vasoconstriction is potently overruled by locally formed sympatholytic and vasodilator compounds, leading to marked increases in local blood flow and oxygen supply and demand.[6] The extent of the sympathetic drive is related both to exercise intensity and the size of muscle mass involved in the exercise.[7] The hypercoagulable state induced by exercise shown in previous studies [5,8] may therefore be associated, not only with the change in shear profile, but also with an increase in plasma catecholamine (norepinephrine) levels. The specific roles of shear profile and catecholamine release in exercise induced hypercoagulability have not been examined.

The roles of catecholamines and hemodynamic changes are important for two reasons: firstly, although the long term benefits of exercise on the cardiovascular status of a patient have been proven, their benefit/risk relationship with exercise intensity is unclear. A particular concern here could be the more widespread use of high intensity exercise which leads to high plasma catecholamine levels and potentially an acute increased risk of blood clot formation. [5] Secondly, many thromboembolic diseases such as myocardial infarction and ischaemic stroke are associated with profound catecholamine release.[9,10]

Thromboembolic disease is also strongly associated with altered blood flow and altered shear patterns due to underlying vascular endothelial damage and obstruction which are known to affect outcome and thromboembolic risk.[11,12] Furthermore, rehabilitation of the patient often involves a graded and prolonged exercise program to improve cardiac function and coronary blood flow. Therefore the exercise regime prescribed to cardiac patients, who may already have a compromised coagulation system, may place them in an unfavourable risk / benefit ratio.

In this study we explore the relationship between blood flow, shear stress, catecholamine release and coagulation in the human leg vasculature. We aim to use a biomarker of clot microstructure, fractal dimension at the Gel point, d_f , to evaluate these relationships.[13] We have chosen to investigate clot microstructure, as it has been shown to be of critical importance in the quality of the clot that is formed during coagulation and plays a vital role in pathophysiology and outcome of cardiovascular and thromboembolic disease.[14-17] How the microstructure of the clot is organised governs its mechanical properties and controls the rate of fibrinolysis.[15,18] The clot microstructure biomarker d_f has previously been successfully used to measure the effect on coagulation of several diseases; anticoagulation/antiplatelet treatment and high intensity exercise.[5,19-22] In the present study we performed two experiments in young healthy men. The first involved local infusion of tyramine to induce the release norepinephrine. The second involved single leg knee extensor exercise, engaging only a small muscle mass and with a consequent low degree of sympathetic drive. Clot microstructure, coagulation factors, noradrenaline and hemodynamic changes were assessed to explore the specific role of catecholamines and blood flow/shear in changing coagulation and clot microstructure formation.

Methods

Twelve healthy recreationally active males were recruited to participate in the study (Age: 23.1 ± 3.2 yrs; Height: 185 ± 7 cm; Weight: 80.7 ± 9.3 kg; VO_{2MAX} : 4151 ± 554 mL/min and BMI: 1.85 ± 0.07). The subjects underwent an initial screening consisting of: a medical examination; 12-lead electrocardiogram (ECG); and venous blood sampling for health related parameters. Exclusion criteria were history or symptoms of cardiovascular disease, renal dysfunction, insulin resistance, diabetes, or hypercholesterolemia, recent (<3 years) history of smoking and intake of prescription medicine. In addition, the participants performed an incremental bicycle ergometer exercise test to determine pulmonary VO_{2max} ($L \cdot min^{-1}$) (Oxycon Pro, Intramedic, Denmark). The study was approved by the Ethics Committee of the Capital Region of Copenhagen (H-15014050) and conducted in accordance with the guidelines of the *Declaration of Helsinki*. Written informed consent was obtained from all subjects before enrollment into the study.

Experimental protocol

The participants ($n=12$) were instructed not to ingest caffeine, alcohol, or to exercise for 24 h before each experimental day and to eat their regular breakfast. After a short rest in the supine position, local anesthesia (lidocaine, 20 mg/ml; Astra Zeneca, Denmark) was given and catheters (20 G; Arrow, Reading, PA) were placed under aseptic conditions, in the femoral artery and vein of the experimental leg 2 cm below the inguinal ligament. A three-port connector was placed in series so that infusion and blood pressure measurements could be performed simultaneously. Arterial and venous pressures were monitored by transducers positioned in the femoral artery and vein (Pressure monitoring it, Baxter, IL, USA). Leg vascular conductance was calculated from perfusion pressure (femoral arterial – femoral venous pressure) and blood flow.

Effect of elevated noradrenaline levels on clot structure: Tyramine (Sigma Aldrich, St. Louis, MO, USA) was infused into the femoral artery for 3 min at a dose of $1.0 \mu mol \cdot min^{-1} \cdot L$ leg volume⁻¹ with participants in supine position. Femoral arterial blood flow, intra-arterial and venous blood pressure were determined and venous and arterial blood samples were obtained prior to and during the last minute of infusion. Tyramine evokes norepinephrine release from neuronal vesicles and consequent release of norepinephrine out of nerve terminals. [23] 10 ml of venous blood was collected before and after tyramine infusion. Each

blood sample was divided into several aliquots. One aliquot of whole venous blood was immediately transferred and used for viscoelastic measurements. The remaining aliquots were used to perform standard coagulation screens and noradrenaline concentration.

Effect of exercise with a small muscle mass on clot structure. After 30 min of rest, the participants were seated in a semi-recumbent position with the hip-angle fixed at $\sim 120^\circ$. Knee-extensor exercise was performed with the experimental leg at 30 W and a frequency of 60 extensions per minute. The workload was sustained for 15 min. Venous and arterial blood samples were drawn prior to and at the end of the exercise session. 10 ml of venous and arterial blood was collected before and after the exercise program. Each blood sample was divided into several aliquots. One aliquot of whole venous and whole arterial blood was immediately transferred and used for viscoelastic measurements. The remaining aliquots were used to perform standard coagulation screens.

Blood tests

Viscoelastic Measurements: The viscoelastic measurements are based on attainment of the Gel Point (GP) from which the fractal dimension, d_f , is determined. The GP technique has been previously validated for use with blood in several studies.[5,19-22] Briefly, blood is placed within the double concentric measuring geometry of a controlled stress rheometer, AR-G2 (TA Instruments, New Castle, DE, USA) which is held a constant temperature of $37^\circ\text{C} \pm 0.1^\circ\text{C}$. Immediately after loading the blood into the AR-G2, viscoelastic analysis is performed using small amplitude oscillatory shear measurements at varying frequencies; 2, 0.93, 0.43 and 0.2Hz, with an applied peak stress amplitude of 0.03Pa. Repeatedly performing these measurements over time allows for the measurement of the GP. The GP marks the transition of the blood from a visco-elastic liquid to a visco-elastic solid, where the GP identifies the formation of the incipient blood clot or the first point which a sample spanning (haemostatic) structure can be identified. From the GP measurement we can quantify how the fibrin clot is organised by calculating its corresponding fractal dimension, d_f , clotting time (t_{gel}) and the elastic properties of the incipient clot (G'_{GP}).

Standard laboratory markers: A 3 ml aliquot of blood was drawn into the 3.2% sodium citrate vacutainers (Greiner Bio-One GmbH, Austria, REF: 454327) to assess standard coagulation markers: Prothrombin Time (PT), activated partial thromboplastin time (aPTT)

and Clauss fibrinogen. The standard coagulation markers were measured using a ACL TOP 750 cts Analyser by standard methods at The Department of Clinical Biochemistry, Rigshospitalet, Denmark.

Analysis of noradrenaline: A 2ml aliquot of blood was used to measure the plasma noradrenaline concentrations were determined with an immunoassay (Noradrenaline ELISA Fast Track, LDN, Nordhorn, Germany) according to the manufactures protocol.

Statistical Analysis: Differences between groups were compared using two sample t-tests for parametric data. Pearson correlation was undertaken to explore associations between d_f and physiological parameters, laboratory markers and platelet aggregometry. Statistical analysis was performed using Minitab version 15 software (Havertown, PA, USA) and deemed significant when $p < 0.05$.

Results

The characteristics of the participants are shown in table 1.

Effect of elevated noradrenaline levels on clot structure: With tyramine infusion, the d_f value increased from 1.692 ± 0.029 at baseline to 1.722 ± 0.047 ($p=0.016$). There was no significant change in the clotting time measurement of the GP (T_{GP}) (237 ± 56 secs vs. 235 ± 61 secs, $p=0.1$), however, there was a significant difference in the clot elasticity at the GP (G'_{GP}) results (0.032 ± 0.010 Pa vs. 0.046 ± 0.016 Pa, $p=0.01$) (see Fig 1). Venous plasma noradrenaline concentration increased in all volunteers following tyramine infusion (1.8 ± 0.7 vs. 10.0 ± 4.6 , $p > 0.001$). Leg blood flow (393 ± 156 mL/min vs. 81 ± 66 mL/min, $p=0.001$) and LVC (4.8 ± 2.1 mL/min/mmHg vs. 1.0 ± 0.8 mL/min/mmHg, $p=0.001$) were both reduced following tyramine infusion. In addition, tyramine infusion induced an increase in the mean arterial and venous pressures of all participants: MAP from 84.9 ± 4.4 mmHg to 88.0 ± 5.2 mmHg ($p=0.001$) and CVP from 3.7 ± 1.6 mmHg to 4.2 ± 1.6 mmHg ($p=0.001$). There were no significant changes in any of the standard laboratory markers between pre- and post- tyramine infusion (Table 1).

Effect of exercise with a small muscle mass on clot structure: Following moderate intensity knee extensor exercise there was an increase in d_f in the venous samples (from 1.688 ± 0.025 to 1.723 ± 0.023 , $p=0.001$). There was a decrease in the clotting time measurement of the GP (T_{GP}) (240 ± 76 secs vs. 204 ± 41 secs, $p=0.01$), and an increase in the clot elasticity at the GP (G'_{GP}) results (0.03 ± 0.01 Pa vs. 0.04 ± 0.02 Pa, $p=0.01$) (Fig 2). We found no significant changes between pre and post exercise in any of the standard laboratory markers of coagulation. Results for the pre and post knee extensor exercise experiment can be seen in Table 2. When comparing the venous results to the arterial results we found no difference in the samples before exercise (venous: $d_f = 1.688 \pm 0.025$ vs arterial: $d_f = 1.694 \pm 0.031$) or in the post exercise samples (venous: 1.723 ± 0.023 vs arterial: $d_f = 1.721 \pm 0.035$). No difference was detected for all of the other parameters tested (T_{GP} and G'_{GP}).

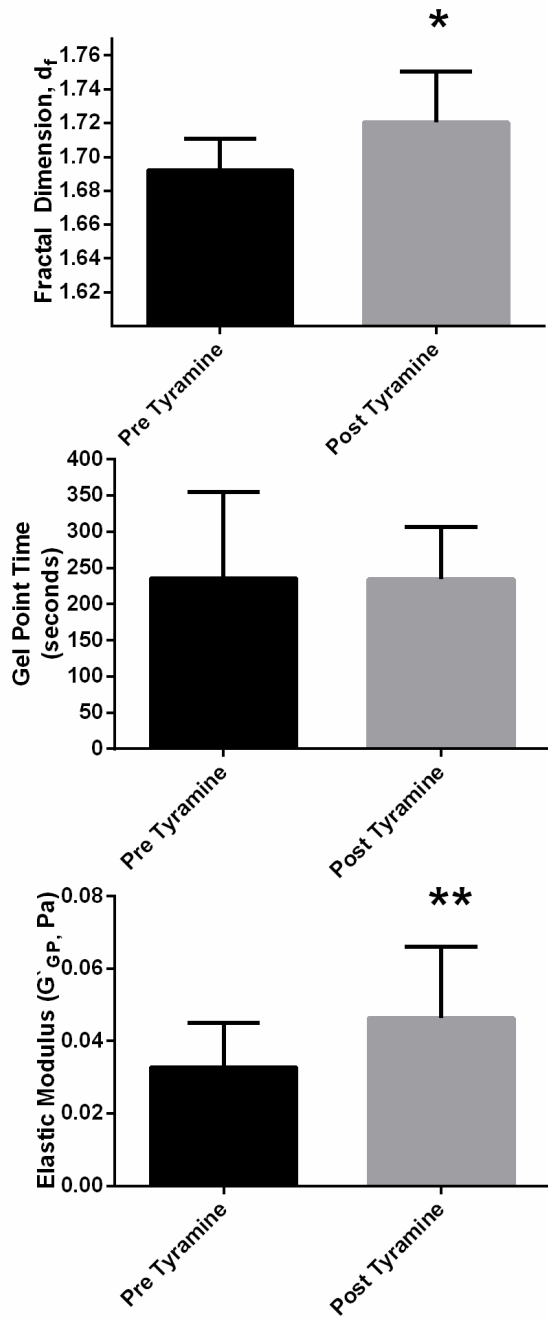


Figure 1: The effect of tyramine infusion on the rheological parameters of the gel point. This figure shows the change in the viscoelastic parameters for 12 healthy volunteers pre and post infusion with $1.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L leg volume}^{-1}$ of Tyramine. * denotes a significant change with a $p < 0.05$, ** denotes a significant change with a $p < 0.01$.

Table 1: Table showing results of the physiological measures for pre and post Tyramine infusion. This Table shows the change in the physiological parameters for 12 healthy volunteers pre and post infusion with $1.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L leg volume}^{-1}$ of Tyramine.

	Pre Tyramine	Post Tyramine	Significance
LEG BLOOD FLOW (mL/min)	393 ± 156	81 ± 66	0.001
MAP (mmHg)	85.5 ± 5.2	88.5 ± 5.2	0.001
CVP (mmHg)	3.7 ± 1.7	4.2 ± 1.6	0.001
LVC (mL/min/mmHg)	4.8 ± 2.1	1.0 ± 0.8	0.001
NOREPINEPHRINE (nmol/L)	1.8 ± 0.7	10.0 ± 4.6	0.001

Table 2: Table showing results of the standard laboratory markers for the Tyramine and single leg exercise experiments. This figure shows the change in the laboratory markers for 12 healthy volunteers pre and post infusion with $1.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L leg volume}^{-1}$ of Tyramine and pre and post single leg knee extensor exercise.

	APTT (s)	Pt-time (s)	Fibrinogen ($\mu\text{mol/L}$)
Pre Tyramine	32.9 ± 6.3	24.4 ± 1.5	7.0 ± 0.7
Post Tyramine	30.6 ± 2.5 (NS)	24.8 ± 1.4 (NS)	6.8 ± 0.7 (NS)
Pre Exercise	32.8 ± 9.3	24.4 ± 1.5	7.3 ± 0.8
Post Exercise	32.3 ± 8.0 (NS)	24.0 ± 1.2 (NS)	7.6 ± 0.5 (NS)

(NS is an indication no significant change)

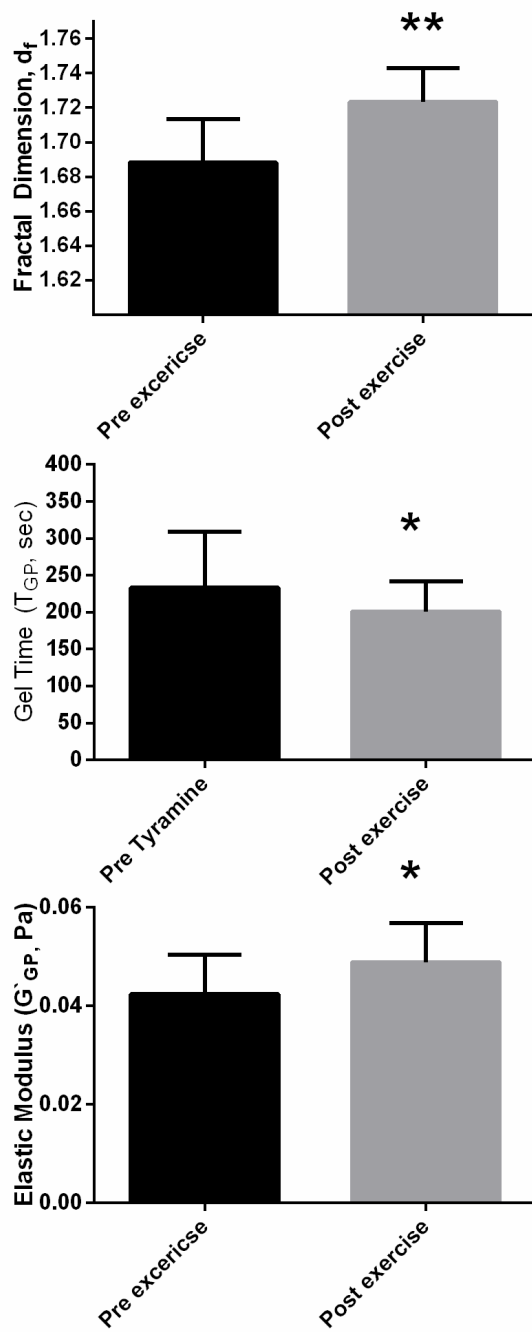


Figure 2: The effect of exercise on the rheological parameters of the gel point. This figure shows the change in the viscoelastic parameters for 12 healthy volunteers pre and post single leg knee extensor exercise. * denotes a significant change with a $p < 0.01$, ** denotes a significant change with a $p < 0.001$.

Discussion

It has been reported that exercise influences the balance in coagulation in normal subjects and also in patients with an increased risk of thrombo-embolism.[24] In a previous study using the d_f biomarker to explore the systemic effect of exercise on coagulation we showed that, as exercise intensity was increased, so was the effect on coagulation.[5] Whereas exercise intensity was increased, the tendency was for the blood to produce clots with a significant increase in d_f indicating denser, more compact and mechanically stronger clot microstructures being produced. However, controversies exist concerning the mechanisms behind exercise induced alterations in the coagulation process. In the present study, we show for the first time that an experimentally induced increase in noradrenaline by intra-arterial tyramine infusion results in denser more compact clot microstructures being produced as evidenced by an increase in d_f in healthy volunteers. By use of a small muscle mass exercise model we, moreover, show that exercise can influence clot microstructure most likely via a change in blood flow and thus shear.

In the present study, we utilized femoral arterial tyramine infusion to induce a local noradrenaline release in the leg, amounting to a femoral venous concentration of ~ 10 nmol/L. This concentration corresponds to plasma noradrenaline levels during moderate to high intensity cycling exercise.[25] The increase in noradrenaline in the present study was associated with a significant increase in the d_f value (Fig. 1). An increase in d_f corresponds to a more tightly packed, highly branched clot that is less permeable and stronger, clot characteristics that are associated with thromboembolic or hypercoagulable disease.[19-22] The magnitude of the change in d_f in this study is comparable to the change seen in healthy people pre and post aspirin.[22] Furthermore there was a significant increase in the G'_{GP} value after tyramine infusion, a measure of clot elasticity/strength (Fig. 1). The results indicate that a physiologically relevant increase in noradrenaline *in vivo* produces a hypercoagulable effect in healthy volunteers. Interestingly, it has been shown previously in an *in vitro* study that catecholamines cause an increase in platelet activity.[26,27] This could be a possible rationale as to why in the present *in vivo* study catecholamine release induces this hypercoagulable effect. Unlike d_f , the measure for clotting time using viscoelastic analysis, T_{GP} , did not change pre and post tyramine ($p=0.34$, Figure 1). This is also shown in the time based standard markers of coagulation PT and aPTT, both of which were not

significantly altered (Table 1). This observation agrees with previous studies from our group, showing that rate based assessments of coagulation such as PT, aPTT and T_{GP} are often insensitive to changes in coagulation induced by disease and treatment, unlike measures of clot microstructure and strength which do detect changes.[28,29]. Interestingly we found no difference in the viscoelastic measurements when comparing arterial and venous samples, suggesting that there is no difference in coagulability in blood between these two systems.

In a previous study investigating the systemic effect of exercise we showed that a graded exercise program to exhaustion causes a hypercoagulable response that results in an increase in clot microstructural properties as measured by the df biomarker.[5] To assess whether the previously observed effect of exercise on clot microstructure was caused by catecholamine release, we investigated the effect of moderate intensity knee extensor exercise which engages a small muscle mass (approx. 2.5 kg of muscle). Due to the small muscle mass recruited, this exercise form induces only a minor increase in sympathetic activity in young healthy individuals, as evidenced by small increases in plasma noradrenaline and by minor increases in HR and MAP.[30-32] In contrast, local blood flow during moderate knee extensor exercise is markedly (~10 fold) increased leading to an enhanced level of vascular shear stress.[33] We observed a smaller magnitude of change in df during knee extensor exercise when compared to the high intensity cycling exercise in the previous study (about half as large 0.03 vs 0.06).[5] Combined with the current finding that tyramine infusion results in an increase in the value of df , our results suggest that both noradrenaline and exercise related factor(s), including the increase in blood flow with consequent increases in vascular shear stress, contribute to changes in clot microstructure.[33] Additionally, it has previously been shown that the effect of acute exercise on platelet function depends on the intensity of the exercise, with only strenuous exercise temporarily increasing platelet reactivity and moderate exercise being shown to suppress platelet reactivity.[34-36] Therefore, whereas the acute high intensity cycling exercise trial in our previous study likely would have resulted in an increase in platelet reactivity, the small muscle mass moderate level exercise undertaken in this study would most likely not.[34-36]

In this study we show for the first time how the systemic effect of increased levels of noradrenaline alters clot microstructure formation and can promote abnormal clot formation. In healthy people, abnormal clot formation and its progression is prevented by factors such as healthy endothelium, an effective fibrinolytic pathway, and other inhibitory and protective pathways in the blood. However, patients with arteriosclerotic disease such as myocardial infarction will have abnormal and damaged endothelium which leads to an altered blood flow pattern corresponding to a change in shear rate which promotes and enhances a hypercoagulable state.[16,20] In acute vascular disease these changes, in combination with catecholamine release, promote atherothrombotic risk. At present these changes have not been determined in either normal or abnormal vessel disease alongside both the intensity and long term risk of exercise. The present study has explored the mechanisms of blood flow, catecholamine release, and exercise to show that all these factors have potential to modify the coagulation status of the individual and alter clot quality. Considering these effects, particular caution should be taken in introducing high intensity exercise with large muscle mass for individuals at risk. Further work is now required to investigate the mechanisms linking catecholamine release to abnormal clot formation and determine the risk/benefit ratio in patients with aeropathic disease and its rehabilitation through exercise.

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Authors' Contributions

MJL & GRD: study design and analysis, data collection (rheology), drafting of the article; MN: volunteer recruitment, revising the article for scientific and intellectual content, interpretation of the data; PR, VE & JW: revising the article for scientific and intellectual content, interpretation of the data; YH & PAE: Idea initiation, study design and data analysis,

final approval of the version to be published. All authors read and approved the final manuscript.

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