DIPLOMARBEIT

The Seasonal Variation in the Effects of Central Hypovolemia Induced by Lower Body Negative Pressure (LBNP) on Coagulation

eingereicht von

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Graz, am 24.03.2020

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Graz, am 24.03.2020 *Alexander Andreas Taucher eh.*

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1 Abbreviations

Adenosine diphosphate **(ADP)**, activated Partial Thromboplastin Time **(aPTT)**, Antithrombin **(AT)**, activated protein C resistance **(APCR)**, α2-antiplasmin **(A2AP)**, calibrated automated thrombography **(CAT)**, clot formation time **(CFT)**, C-reactive protein **(CRP)**, coagulation time **(CT)**, deep vein thrombosis **(DVT)**, Dehydroepiandrosterone **(DHEAS)**, Nitric oxide synthase **(eNos)**, endogenous thrombin potential **(ETP)**, Endothelin 1,2 and 3 **(ET 1,2 and 3)**, prothrombin fragment **(F1+2),** Factor II, III, IV... **(FII, FIII, FIV...)**, follicle-stimulating hormone **(FSH)**,Glycoprotein **(GP)**, haematocrit **(Hct)**, haemoglobin **(Hb)**, International Normalized Ratio **(INR)**, lower body negative pressure **(LBNP)**, low density lipoprotein **(LDL)**, lipoprotein **(LP)**, lag time **(LT)**, maximal clot firmness **(MCF)**, mean corpuscular haemoglobin **(MCH)**, mean corpuscular haemoglobin concentration **(MCHC)**, mean corpuscular volume **(MCV)**, nictric oxide **(NO)**, plasminogen inhibitor **(PAI)**, Protein C **(PC)**, pulmonara embolism **(PE)**, platelet count **(Plt)**, protein S **(PS)**, prothrombin time **(PT)**, platelet poor plasma **(PPP)**, red blood cell counts **(RBC)**, standard deviation **(SD)**, thrombin activated fibrinolysis inhibitor **(TAFI)**, thrombin-antithrombin-complex **(TAT)**, tissue factor triggered thromboelastometry **(TEM)**, tissue factor **(TF)**, tissue factor pathway inhibitor **(TFPI)**, tissue plasminogen activator **(T-PA)**, time to peak **(ttPeak)**, urokinase type plasminogen activator **(u-PA)**, venonus thromboembolism **(VTE)**, von-Willebrand-factor **(vWF)**, whole blood **(WB)**, white blood cell counts **(WBC)**

2 Abstract

2.1 Abstract (german)

Einleitung: LBNP ist eine Methode um mittels einer Vakuumpumpe erzeugtem niedrigen zentralen Venendruck ähnliche physiologische Bedingungen zu orthostatischem Stress oder Blutungen simulieren zu können. Diese Diplomarbeit vergleicht sowohl die basalen Gerinnungsparameter als auch die Reaktion des Blutgerinnungssystems der ProbandInnen zwischen der kalten und warmen Jahreszeit.

Methode: Es wurde in einer kontrollierten Umgebung mit 23°C-24°C eine graduelle Applikation von LBNP (-10mmHg, -20mmHg, 30mmHg, -40mmHg) durchgeführt. Es wurden eine basale Blutabnahme, eine während laufendem LBNP sowie nach 10 min Erholung durchgeführt und anschließend ausgewertet.

Resultate: Die TeilnehmerInnen waren 18-35 Jahre alt, 160-180cm groß, NichtraucherInnen und ohne bekannte Auffälligkeiten im Gerinnungssystem.

Die basalen Werte von Faktor II, TF und die Parameter für Thrombingeneration F1+2 und TAT waren in der warmen Jahreszeit im Vergleich zur kalten erhöht. Faktor VIII, F1+2, TAT und CT zeigten einen signifikanten Anstieg durch eine Stimulation mit LBNP.

Im Sommer bewegten sich die durch CAT gemessenen Werte für Lag, ttPeak und Peak unter LBNP in Richtung eines gerinnungsfördernden Milieus.

Hct, begleitet von RBC, Hb, und WBC sank initial während laufendem LBNP gefolgt von einem anschließenden Anstieg in der Erholungsphase. PT sank im Winter unter LBNP, aPTT im Sommer.

Diskussion: Die gemessenen höheren basalen Werte von FII, TF, F1+2, TAT als auch die größere Reaktion von Messwerten der Thrombingeneration F1+2, TAT, Lag, Peak, und ttPeak auf LBNP deuten auf eine höhere plasmatische Koagulationsbereitschaft der ProbandInnen im Sommer. Diese Ergebnisse stehen im Gegensatz zur bekannten Literatur, da venöse thromboembolische Ereignisse im Winter deutlich häufiger sind.

Die normalisierten Verhältnisse des Testraumes und die im Vergleich zu den meisten Quellen jüngere ProbandInnengruppe, sowie individuelle Faktoren wie potentielle entzündliche Erkrankungen könnten diese Diskrepanz möglicherweise

erklären und legen den Verdacht nahe, dass Störfaktoren wie die eben genannten einen größeren Einfluss auf die Gerinnung haben und somit saisonale Tendenzen bei jungen Erwachsenen überdecken können.

2.2 Abstract (english)

Introduction: LBNP is a method to stimulate an artificial state similar to orthostatic stress and/or bleeding, reducing the central venous pressure by applying a vacuum at the lower body. This thesis aims to compare coagulation related baseline values as well as their response to LBNP in cold versus warm seasons.

Methods: LBNP was applied gradually (-10mmHg, -20mm Hg, -30mmHg, -40mmHg) in a controlled setting with a room temperature of 23°C-24°C. During the experiment three blood samples, one for baseline parameters, one at the point of applied LBNP, and one after a 10min recovery-phase were sampled and subsequently evaluated.

Results: The subjects were 18-35 years old, 160-180cm tall, non-smokers without known abnormalities in the coagulation system.

The baseline values of FII, TF and the markers for thrombin-generation TAT and F1+2 were increased during summer when compared to winter. Factor VIII, F1+2, TAT and CT showed a significant rise in response to LBNP. In summer some variables measured via CAT (Lag, ttPeak, Peak) shifted in a procoagulant manner during applied LBNP.

Hct showed an initial decrease with a subsequent rise in recovery phase accompanied by RBC, Hb and WBC.

In the examined subjects LBNP application reduced PT in winter and aPTT in summer.

Discussion: The higher baseline values of FII, TF, F1+2, TAT as well as the greater response to LBNP by thrombin-generation markers F1+2, TAT, Lag, ttPeak and Peak imply a higher plasmatic coagulation activity of the subjects during summer as. These results stand in contrast to the available literature describing incidents of VTE accumulating during winter.

The room temperature of the testing facility, the in comparison to other sources relatively young sample group, as well as individual contributing factors such as potential inflammatory processes could possibly explain this discrepancy and could imply that factors such as those mentioned above may influence the coagulation more profoundly than seasonal tendencies, overruling their possible effects in young adults.

3. Introduction

3.1 Cardiovascular system

The vascular system is comprised of arteries and veins. Arteries arise from the heart and function as transport path for blood into the periphery. There the blood vessels get continuously smaller as their numbers rise through branching. They end in a capillary network, where the substance and gas exchange between blood and tissue takes place. The venous system rises from this network of capillaries and leads the blood back to the heart. There are two connected circulations to be distinguished. (1)

Body circulation: Starting in the left ventricle, the heart pumps oxygenated blood into the periphery, where it gives off oxygen and in return takes in carbon dioxide. The oxygen-depleted blood then travels back through the venous system to arrive in the right atrium and subsequently in the right ventricle. (1)

Lung circulation: This part of the circulation functions as a mean to re-oxygenate the hemoglobin of the red blood cells. After the blood has reached the right ventricle, it gets ejected towards the lungs. In the capillary network in the lungs, the blood gives off carbon dioxide and gets re-oxygenated. This oxygen-enriched blood then re-enters the heart in the left atrium, from where the body circulation starts again. (1)

3.1.2 Endothelium

3.1.2.i Endothelial structure

The walls of blood vessels are made of extracellular matrix, smooth muscle tissue and endothelium. They can be subdivided into three concentric layers named tunica intima, tunica media and tunica adventitia. (1)

The endothelium is the inner cell layer located in the tunica intima, which has direct contact with the flowing blood and builds an interface between the fluid and the outer parts of the vessel wall. The squamous cells line in a single layer, which elongates along the blood flow and is situated on a basal lamina.(1)

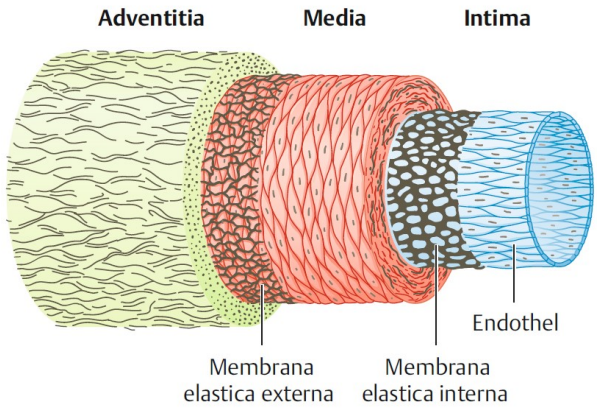

Fig. 1, Histology of blood vessels, taken from (1)

3.1.2.ii Endothelial barrier function

The endothelium serves as a regulative barrier between the intra- and extravascular space. The endothelial cells use two mechanisms to block unwanted outflow from the vessel. (2)

Firstly they build a **mechanical barrier**, which especially hinders the passive transport of bigger and hydrophilic molecules. (2) Specialized transmembrane proteins build close connections between the endothelial cells. These links called “tight junctions” seal the intercellular space and block the diffusion of non-permeable molecules. Depending on the function and needs of the surrounding tissue occurring tight junctions can be arranged in ways that differ greatly in their permeability (e.g. the very tight connection in brain vessels for a functioning blood-brain barrier). (3)

Secondly endothelial cells’ very active metabolism builds a **metabolic barrier** by interacting with and changing permeable substances. (2)

The endothelial cells possess specific transporters for a few select substances (e.g. glucose, amino acids), opening up a transcellular passage through the endothelium. (1)

3.1.2.iii Endothelial influence on perfusion

The vascular walls are influenced by endothelial cells via a variety of vasoactive factors affecting its smooth muscle. The most important mechanism resulting in **vasodilatation** is the release of nitrogen monoxide. The endothelial nitric oxide synthase (eNOS) builds NO in response to physiological agonists such as bradykinin or to shear stress caused by the blood flowing along the endothelial

surface and.(4) Endothelial cells that are stimulated this way additionally release vasodilating prostanoids. (5)

The then released NO diffuses out of the endothelium and activates the guanylate cyclase in neighbouring vascular smooth muscle that starts a biochemical cascade that results in the decrease of intracellular calcium levels, and subsequently the relaxation of those muscles. (4)

Endothelial cells can also initiate **vasoconstriction** via mediators such as thromboxane and endothelin. (6) Endothelin, from which we know three subtypes (ET1, ET2, ET3) (4) is the most effective vasoconstrictor known, (7) primarily acting in a paracrine/autocrine manner. (8)

However, endothelin doesn't take part in the short time regulation of the vascular tone, because of its long half-life. (4)

Endothelial tissue is not the only factor exercising influence on the vascular tone, but plays a part in a complex system along with hormones, the autonomic nervous system and other local biochemical influences, such as metabolic products or the level of oxygen. (4)

3.1.2.iv Endothelial pro- and antithrombotic factors

Under physiological circumstances the endothelial tissue has ***antithrombitic properties***, which mainly rely on four mechanisms.

- The endothelial cells help to suppress the production and function of **thrombin**, which induces platelet-activation, by releasing **heparan- and dermatan-sulfate**. (9,10)
- The **extrinsic pathway** is inhibited by the expression of the **"Tissue Factor Pathway Inhibitor"** (TFPI) (9), which is interfering in the initial coagulative phase by inhibiting the TF-$FVII_a$ complex in its role of activating of FX. (11)
- The **intrinsic pathway** is inhibited through the involvement of endothelial receptors (thrombomodulin) in generating **activated Protein C**, which blocks the activation of Factor V and Factor VIII of the coagulation cascade. (9,12)
  This process is initiated by the interaction of thrombomodulin and thrombin and catalyzed by the cofactor Protein S. (13) Because of its location on the endothelium thrombomodulin suppresses coagulation in areas with intact vessel walls. (14)

- The endothelial cells express the protein **tissue-plasminogen-activator** (tPA) that supports the transformation of plasminogen to plasmin which can dissolve a clot by degrading fibrinogen and fibrin, (9) a process called fibrinolysis. (10)

The before mentioned NO as well as endothelium derived prostacyclin directly inhibit the activity of the blood-platelets as well. (10)

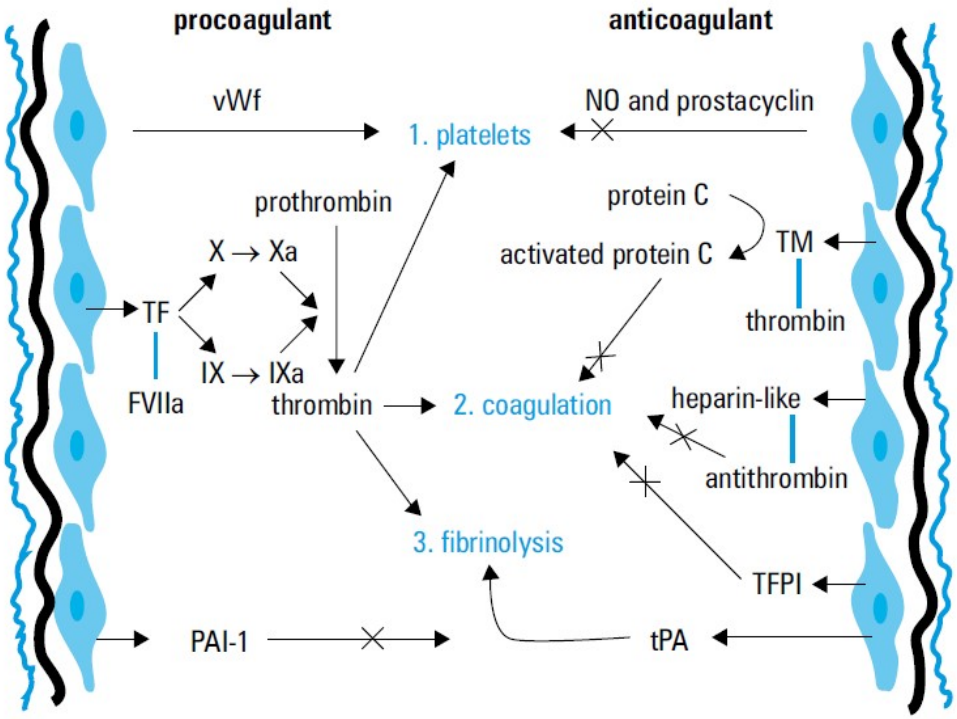

Figure 2: procoagulant and antigoaculant endothelial factors, taken from (9)

Prothrombotic endothelial factors are the expression and release of plasminogen-activator-inhibitor type 1 (PAI1), as well as the von-Willebrand-factor (vWf). (9)

The **von-Willebrand-Factor** plays a role right at the beginning of the hemostasis. If endothelium is injured vWF gets released and builds a connection between during injury exposed subendothelial collagen fibrils and a glycoprotein-complex (GPIb-V-IX complex) present on thrombocytes, therefore playing a major part in their adhesion process. (13)

The von-Willebrand-Factor also builds a complex with coagulation factor VIII, enhancing its half-life by impeding its protein C mediated degradation. (15)

The endothelium builds an important barrier between the circulating factor VII and the **tissue factor** (factor III), that is expressed on the outside of subendothelial

barrier, and also gets exposed when a vessel wall is damaged. If these two factors come in contact they start the extrinsic coagulative pathway. (13)

Plasminogen-activator inhibitor type 1 blocks the pathway of before mentioned fibrinolysis (9) by inhibting tissue type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), (16) the two proteases primarily transforming plasmin to plasminogen. (17)

3.2 Hemostasis

The first step of clotting is the primary hemostasis describing the process of thrombocytes building adhesive connections to injured parts of the vessel wall, and between each other. The end-result of the platelets actions is a so called “white thrombus” (because it doesn’t yet contain erythrocytes). During this process the platelets activate, and release various substances that induce the coagulation cascade. (13)

This is the start of the secondary hemostasis. The complex system of coagulation factors results in a net made of fibrin, stabilizing the now-called “red thrombus”, which contains erythrocytes as well. (13)

3.2.1 The primary Hemostasis

During the primary hemostasis, more and more platelets aggregate at the site of an injury and seal it. For a small wound this process takes about 1-3 minutes. The end result of the primary hemostasis is the so called white thrombus. (13)

3.2.1.i Thrombocytes

Thrombocytes are cells without a nucleus that are essential for the primary hemostasis. They are built by the fragmentation of megakaryocates in the red bone marrow, and cycle in the bloodstream for about ten days, before they get dismantled in liver and spleen. They are 2-3 µm in diameter, and their concentration is about 250.000/µl. (18)

Inside the platelets are granules that are released when the thrombocytes are activated. These substances recruit additional platelets, help connecting them with one another and collagen fibrils, and constrict the vessel. Various glycoprotein-complexes, located in the cellular membrane build the docking-point for those

connections and are therefore essential for both the adhesion and aggregation. (18)

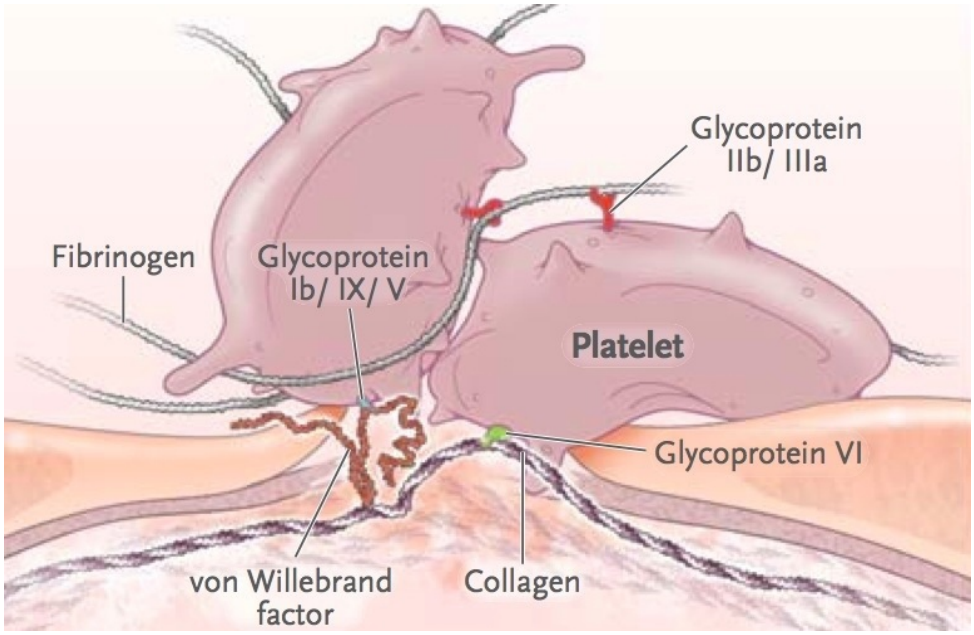

Fig. 3, Adhesion and aggregation of thrombocytes, taken from: https://usmle287.wordpress.com/2012/09/25/platelet-adhesionaggregationactivation/ (01.08.2019)

3.2.1.ii Adhesion of thrombocytes

When, on the basis of an injury, collagen fibrils are exposed to platelets, endothelial derived **von-Willebrand-factor** that is activated by thrombocytes, forms a connection between the subendothelial collagen and a glycoprotein-complex **(GP Ib/IX)** expressed on the platelets' surface. (13)

Other thrombocytic glycoprotein complexes **(GP Ia/IIa and GP VI)** can interact with the collagen directly, without any further linking factors. When the vWf attaches to the GP Ib/IX, the glycoprotein complex initiates an intracellular cascade increasing the cytoplasmic calcium concentration resulting in the activation of the platelet. (13)

3.2.1.iii Activation and aggregation of thrombocytes

The reaction, initiated by the connection between the vWf to GP Ib/IX entails three aspects.

- The platelets undergo a **form change**. The initially biconcave configured thrombocytes build pseudopodia, which amplify the adhesion to one another as

well as to the endothelium. In addition phospholipids, an important cofactor for the coagulation cascade are revealed on their membrane. (13)

- Furthermore, the change in the cytoskeleton makes the **receptor GP IIb/IIIa**, appear on the cell membrane. (10) This glycoprotein complex binds fibrinogen, a dimer, that forms intercellular links between the platelets, resulting in a stable aggregation (19)
- Activated thrombocytes release **mediators** from their granules. These various substances help with aggregation (e.g. fibrinogen, thromboxane a2), adhesion (vWf) and the activation of additional platelets (ADP, calcium, serotonin), include growth factors, coagulation factors (V and VIII) as well as vasoconstrictors (serotonin, thromboxane a2). (13)

  Thromboxane A2, one such mediator, is produced and released by the platelets when activated. It works as vasoconstrictor and enhances aggregation. (13) It is synthesized from prostaglandin H2, whose production depends on the cyclooxigenase 1. Acetylsalicylic acid owes its effect on the blockage of that enzyme. (20)

The product of the above mentioned actions is the formation of a **white thrombus**, the end result of the primary hemostasis. (13)

Tab. 1, List of thrombocytes' mediators made on the basis of Behrends JC et al. Physiologie [Internet]. 3. Ausgabe. 2017. 191 p

Mediators in thrombocytes' granules	Function
Von-Willebrand-factor	Adhesion to collagen
Fibrinogen (Coagulation factor I)	Platelets' aggregation via GPIIb/IIIa and coagulation
Fibronektin	Adhesive protein
Thrombospondin	Irreversible linkage of platelets
Growth factors	Stimulate wound healing
Coagulation factors V and VIII	Part of coagulation cascade
ADP	Activates thrombocytes
Calcium	Co-factor for platelet-activation and plasmatic coagulation
Serotonin	Platelet-activation, Vasoconstriction

3.2.2 The secondary hemostasis

The second part of the coagulation is characterized by the coagulation cascade, where step by step clotting factors are activated in order to transform fibrinogen to insoluble fibrin, which builds a net stabilizing the clot of platelets. (13,21)

3.2.2.i The Clotting Factors

Most clotting factors are proenzymes that circulate in the blood. When activated they have proteolytic properties and build the core of the coagulation cascade. They are numbered in Roman numeral and their activated forms are usually depicted by the suffix “a”. (21)

Tab. 2, List of clotting factors and their function, on the basis of Tab. 6.8 of (13) *and Tab. 3 of* (21)

Factor	Name	Function of active form
I	Fibrinogen	Clot formation.
II	Prothrombin	Activates FI, FV, FVII, FVIII, FXI, FXIII, as well as platelets and protein C
III	Tissue Factor	Co factor VII
IV	Ca^{2+}	Co factor in activation process of most coagulation factors
V	Proaccelerin	Builds complex with FX that converts prothrombin to thrombin.
VI	non-existent	
VII	Proconvertin	Activates FX while in a complex with TF (FIII)
VIII	anti-hemophilic factor	Builds complex with FIX that activates FX.
IX	Christmas Factor	Together with FVIII converts FX into its active form
X	Stuart-Prower Factor	Works in a complex with V to converts prothrombin to thrombin
XI	Plasma-Thromboplastin-antecendent (PTA)	Transforms FIX into its active form
XII	Hageman Factor	Activation of FXI and FVII, converts prekallikrein to kallikrein,
XIII	Fibrin Stabilizing Factor	links fibrinogen strands, stabilizing the clot

3.2.2.ii The Coagulation Cascade

The coagulation cascade is traditionally subdivided in extrinsic, intrinsic and common pathway. While this model proves very useful in understanding in vitro tests, in vivo the pathways overlay and interact with one another in multiple ways. (21)

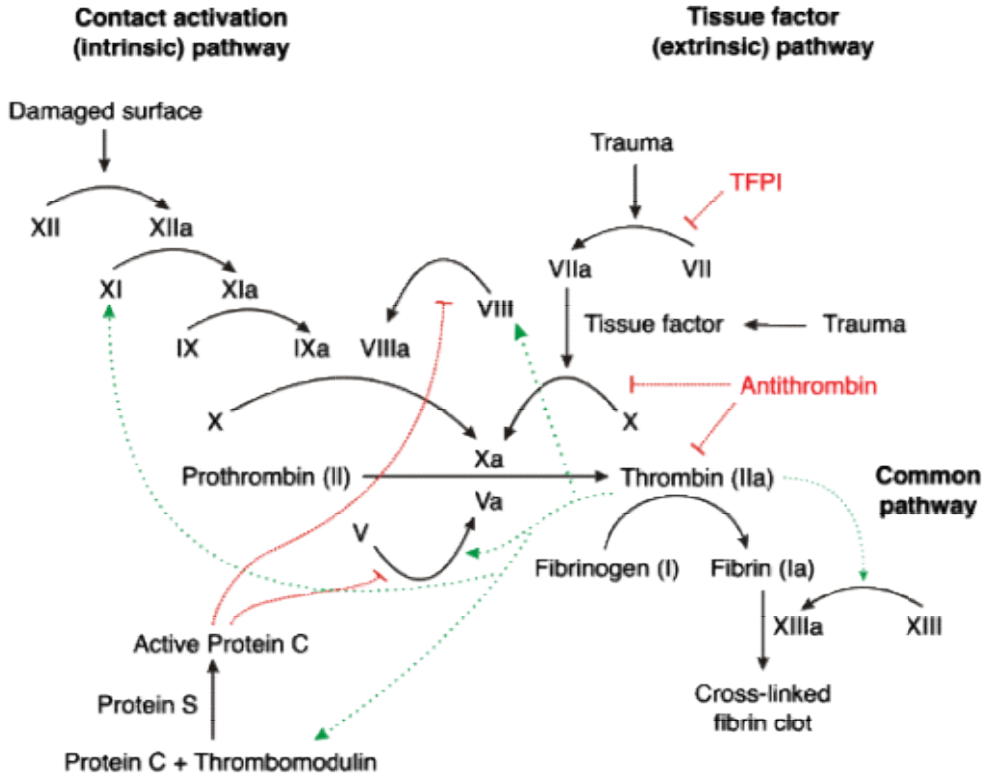

Fig. 3, the coagulation cascade, taken from: https://step1.medbullets.com/hematology/111004/coagulation-cascade (07/17/2019)

- **The extrinsic pathway:** This process is initiated when the subendothelial tissue factor (FIII/TF) gets in contact with the activated $FVII_a$, which is circulating in the blood. This happens when the endothelial integrity is damaged. (21) These two coagulation factors form a complex with phospholipids expressed on the surface of activated thrombocytes under the mediation of calcium. (13) This **TF-$FVII_a$-Ca^{2+}-P-Lip-complex** now activates FX, the beginning point of the common pathway (13) and FIX building a bridge to the intrinsic pathway. (21)

  Thrombin generation via FX_a as a result of the extrinsic pathway is limited, due to an interference by the tissue-factor-pathway-inhibitor (TFPI) early after its

initiation. The deciding factor if positive feedback loops are activated sufficiently to sustain the coagulation process is the amount of already produced Thrombin. (10)

- **The intrinsic pathway:** It starts when FXII makes contact to negative surfaces, like exposed subendothelial collagen fibrils after an injury. Positively influenced by high molecular kininogen, kallikrein FXII activates FXI, which in return activates FIX. (21) When calcium is present FIX_a builds a complex with phospholipids of activated platelets, and Factor $VIII_a$. This **$FVIII_a$-FIX_a-Ca^{2+}-P-Lip-complex** can activate factor X. (10)

- **The common pathway:** The activation of FX is the end result of both the intrinsic and extrinsic pathway. (10) In the presence of calcium FX_a builds a complex with FV_a, and phospholipids of activated platelets. This **FX_a-FV_a-Ca^{2+}-P-Lip-complex**, also referred to as prothrombinase complex, transforms prothrombin (FII) to **thrombin** (FII_a). (13,21)

Thrombin keeps the coagulation process going via a positive feedback loop that activates **FVIII and FXI**, resulting in the creation of more of FX_a via the intrinsic pathway. Additionally Thrombin activates **FV** which, in association with FX_a, forms the before mentioned prothrombinase complex that directly transforms more prothrombin (FII) to thrombin (FII_a). (10)
Besides its feedback-role in the coagulation cascade, thrombin is also vital for forming a stabile clot. It forms insoluble **fibrin** monomers from before soluble fibrinogen, which then polymerize.(22) Another function of thrombin lies in its activation of Factor XIII which stabilizes the clot by forming covalent bonds between these fibrin strands. (10,22). The last part of the secondary hemostasis is the **retracting of the fibrin net**. The fibrin strands bind the receptor GP IIb/IIIa on thrombocytic surfaceses. These platelets then contract under the promoting influence of thrombin, hardening and further stabilizing the clot, forming the final product of the secondary hemostasis.(10)

3.2.2.iii Inhibiting factors on coagulation

There is a further mechanism inhibiting the coagulation in addition to anticoagulant properties of the endothelial tissue mentioned in chapter 3.1.2.iv listed here:

- production of heparan and dermatan-sulfate
- expression of the “tissue factor inhibitor”
- expression the receptor thrombomodulin and its activation of the anticoagulant Protein C

Antithrombin (AT or AT III) is the most important thrombin inhibitor. Its activity in binding the active forms of FIX, FX, FX and FXII in addition to thrombin is increased by heparin. These complexes are then removed from circulation. Further thrombin inhibiting substances to be mentioned are α1-antitrypsin, α2-macroglobulin and heparin cofactor II. (21)

3.3 Fibrinolysis

The formation of a clot is followed by the initiation of fibrinolysis. Plasminogen is transformed into its active form plasmin, which dissolves fibrin (as well as FV, FVIII and fibrinogen). In healthy individuals exists a physiological balance between a small but steady production of fibrin and its dissolution by plasmin. (10)

Fibrinolysis is regulated by a number of cofactors and inhibitors:

3.3.1 Fibrinolysis activating factors:

Tissue plasminogen activator (t-PA) produced by endothelial cells and **urokinase type plasminogen activator** (u-PA) synthesized by macrophages, monocytes and urothelial cells are converting plasminogen to plasmin.

Fibrinsurfaces play a key role in this process as well as initiation sites for fibrinolysis. When t-PA binds to fibrin, plasminogen activity increases about 500-fold. (17)

In addition to its role in initiating the intrinsic pathway of the coagulation (chapter 3.2.1.ii), circulating kallikrein can activate plasminogen to plasmin. Factor XII plays a role in this system by activating it from prekallikrein. (10)

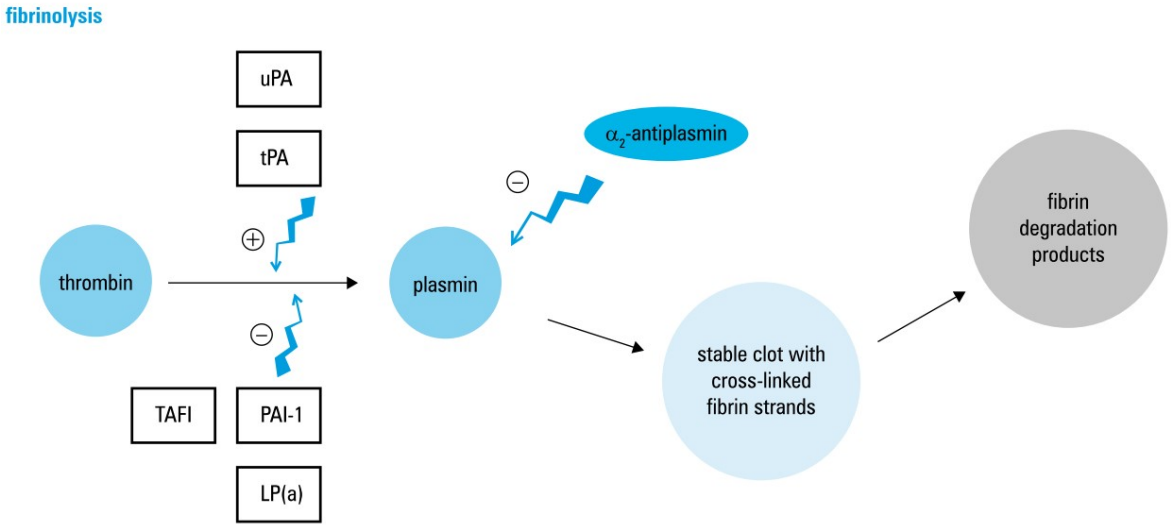

Fig. 4, Overview of fibrinolysis, taken from (9)

3.3.2 Fibrinolysis inhibiting factors:

The two enzymes tPA and uPA are most importantly inhibited by the serpins **plasminogen inhibitor 1 and 2** (PAI-1 and PAI 2). Serpins inhibit their target enzymes by covalently binding them. Another serpin, **α2-antiplasmin** (A2AP) binds plasmin directly. Fibrin can interfere in this process. When plasmin is bound to fibrin, for example at the site of a clot, it is protected from A2AP, and can stay active. (9)

Thrombomodulin not only has anticoagulant properties as described in chapter 3.1.2.iv, it also decreases fibrinolysis. When associated with thrombin it activates the **thrombin activated fibrinolysis inhibitor** (TAFI) that removes fibrinic binding sites for plasminogen, resulting in a slowdown of fibrin mediated plasmin activation. (9,17)

These inhibiting factors prevent unregulated plasmin formation and activity (17)

3.4 Thrombosis

Thrombosis is the pathological coagulation of blood forming a blood clot in either arteries or veins. Acute arterial thrombosis and its resulting diseases (e.g. heart attack and stroke), are the leading cause of death in the first world. Thromboembolic incidents in the venous system are on the third place causing cardiovascular-associated decease. The mechanisms of thrombosis differ in arteries and veins. (23)

Venous thrombosis is usually triggered by endothelial damage, arterial thrombosis by atherosclerosis. (24) Venous blood clots, so called red clots, consist of more fibrin and erythrocytes compared to the arterial ones that have a higher platelet density and are called white clots. Therefore the treatment is different as well. While arterial thrombosis is treated with platelet aggregation inhibitors, the medication for venous thrombosis targets the plasmatic coagulation.(23)
Both, arterial as well as venous thrombosis, have common risk factors such as obesity, smoking, chronic inflammation and high age. (24)

3.4.1 Virchofs triad

"Virchow's triad" describes three common causes contributing to the pathogenesis of thrombosis.

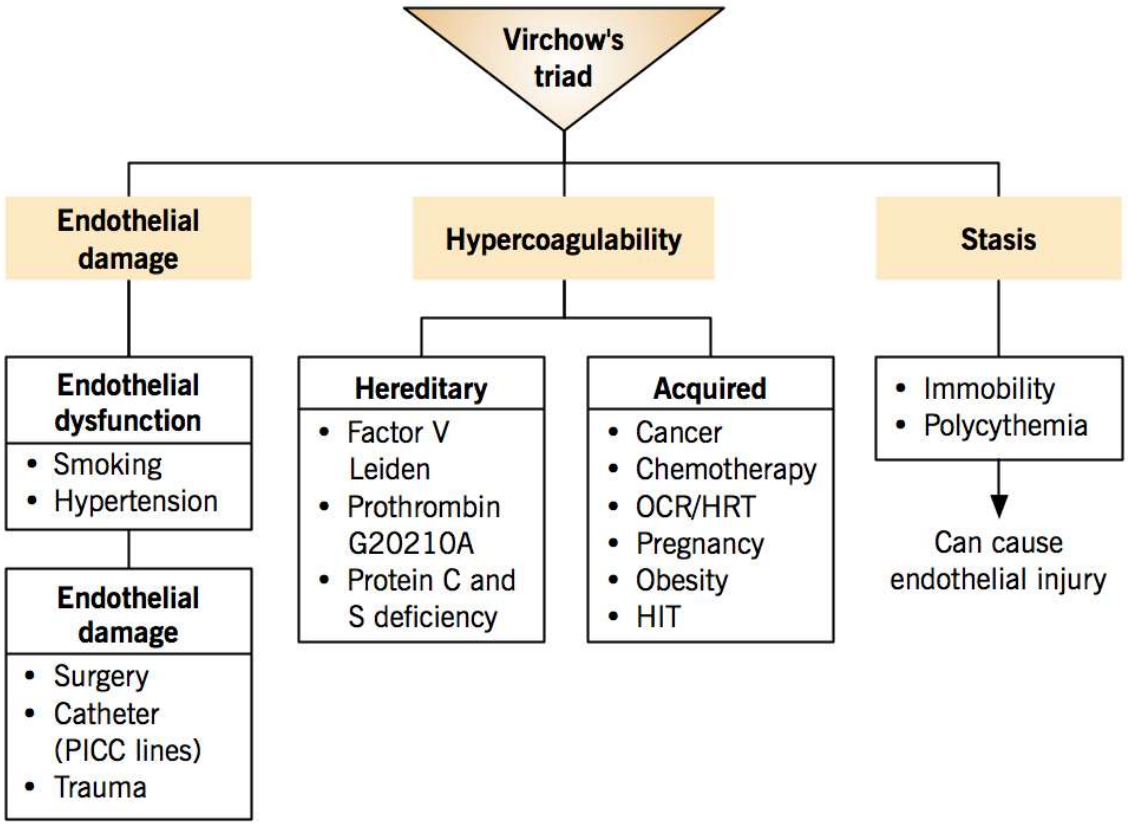

Fig.5: Overview of Virchow's triad, taken from: http://www.pathophys.org/vte/pe-virchow-2/ (01.08.2019)

- Damage to the endothelium (e.g. by trauma or a catheter)
- Stasis of the blood (e.g. immobility, or hindered blood flow by a previous thrombosis)

- Hypercoagulalibility

  This category of the Virchow's triad includes factors that increase the concentration of circulating prothrombotic components. Inflammatory factors, prothrombotic proteins, the viscosity of the blood as well as the functionality of our endogenous anticoagulant blood components have an influence on the coagulability of the blood. (24)

  Diseases such as antithrombin III, Protein C or S deficiency, as well as activated protein C resistance (APCR) also promote a hypercoagulable state. (24)

3.4.2 Venous Thrombosis

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are summarized under the term venous thromboembolism (VTE). (PE) that occurs when parts of a thrombus, most often located in the deep veins of the lower limbs, break away and are transported to the pulmonary arteries, disrupting the proper circulation in the organ. (23)

The conventional idea is that when a vessel wall is damaged, subendothelial tissue factor gets exposed, prothrombotic cytokines are produced and there is an increase in platelet activation. These cytokines lead to the expression of prothrombotic adhesion molecules via an increase in interaction between leukocytes and endothelial cells.(24)

Besides thromboembolic incidents linked to surgery, examined veins in the area of the thrombosis seldom show injuries, therefore it is believed, that VTE often is initiated at the sites of venous valves, where hypoxic areas with stasis may occur.

Besides the decline in oxygen, slower blood flow is also linked to a higher hematocrit (25,26), one of the determining factors for an increased blood viscosity. (27) Hypoxia likely reduces the concentration of important anticoagulant proteins and in return increases the expression of some procoagulants like P-selectin. When expressed by the endothelial cells, P-selectin recruits leukocytes and microparticles derived from leukocytes containing tissue factor, and therefore can be the starting point of a thrombosis. (25,26)

3.4.2 Arterial Thrombosis

Arterial thrombosis typically occurs on the grounds of atherosclerosis, more specific when an atherosclerotic plaque ruptures. Platelets interact with, through

vascular injury exposed, van Willebrand factor and collagen fibrils. Platelets are recruited to the site, activated and form a platelet-rich thrombus. The coagulation cascade is activated by the contact between the blood and exposed tissue factor as well. (23)

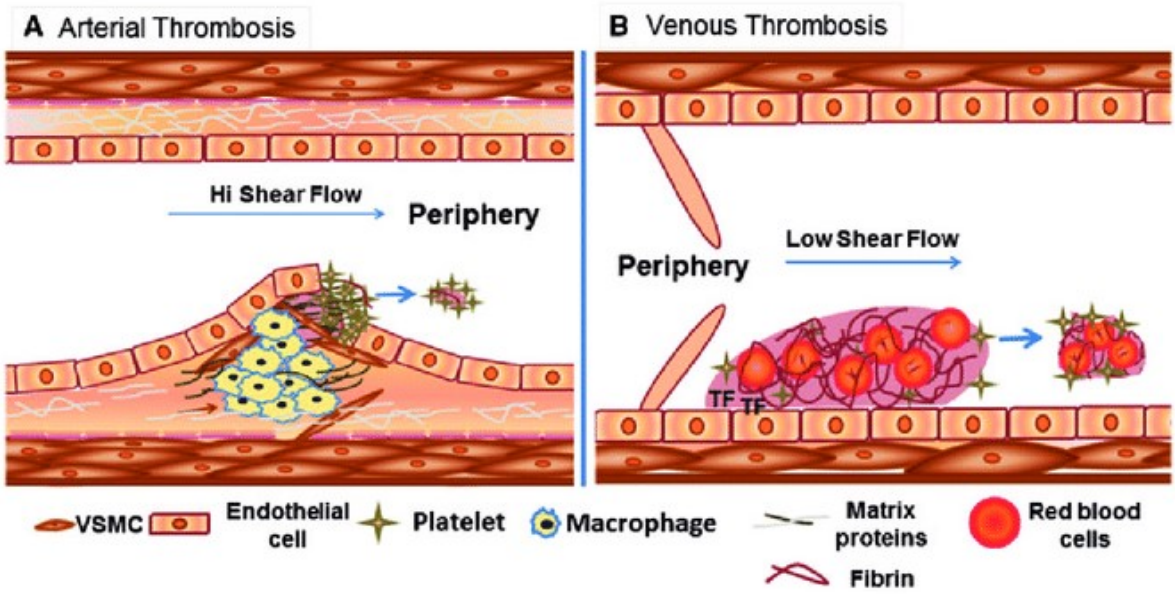

Fig. 6, Difference between arterial and venous thrombosis, taken from: https://www.researchgate.net/figure/Major-differences-between-arterial-and-venous-thrombosis-A-Arterial-thrombosis-occurs_fig1_311986292 (02.08.2019)

3.5 Lower Body Negative Pressure (LBNP)

LBNP is a method that reduces central venous pressure by redistributing the blood towards the lower body via the use of a vacuum chamber that is sealed at hip level. Since the 60s LBNP has been used in medical research to simulate and examine the physiological responses to conditions of blood loss and orthostatic stress, in particular that associated with flight in zero gravity. Studies with LBNP bear advantages such as the prompt initiation, recovery and controllability of the level of central hypovolemia. Furthermore the experiments are non-invasive, repeatable, reproducible and safe. Most studies use a graded application of LBNP, with a gradual increase of the negative pressure. (For example in this experiment with -10mmHg, -20mmHg, -30mmHg and -40mmHg) The subjects are in supine position providing the benefit of an examination of the physiological response independent from vestibular and muscle pump activity. (28)

Tilting the device in a heads-up position can further enhance its effect and reduce the negative pressure needed. (29)

3.5.1 Effects of LBNP on the cardiovascular system

The dip of the blood towards the lower body diminishes the amount of venous blood arriving at the heart, decreasing the central venous pressure. (30) Therefore LBNP induces a reduction in preload that significantly lowers the stroke and end-diastolic volume. (31) in linear relationship with the degree of negative pressure applied (28).

The body physiologically compensates this effect with an increase in heart rate (although modest up to the state of presyncope), as well as peripheral, renal, and splanchnic vasoconstriction triggered by cardiopulmonary and carotid baroreceptors (28,32,33) ensuring sufficient perfusion of vital organs. (28) The changes induced by the baroreceptors come about through their modulation of the autonomic nervous system. (34,35) If these compensation mechanisms fail, the arterial blood pressure can fall to the point of syncope.(29) Paul M. Stevens et al describe increased frequencies of syncopes in relation to the applied LBNP with no syncopes at -25mmHg up to a 100 percent rate thereof at -80mmHg in their study published in 1965. (30)

3.5.2 Effects of LBNP on the coagulation system

LBNP and orthostatic stress have an activating effect on the coagulation system (36–38). The pressure gradient created by LBNP causes a migration of intravascular fluid to the lower body's extravascular compartment, causing a rise in hemoconcentration. As a result blood viscosity and plasma protein concentration rise, increasing the interactions between procoagulant cellular factors and coagulation factors therefore creating a procoagulant environment. (39). Shear stress caused by the increased viscosity through LBNP could be another mechanism promoting coagulation by interfering with the balance of anticoagulant and procoagulant factors exhibited by endothelial cells under unstressed conditions. (38,40) Shear stress also promotes the pathogenesis of arterial thrombi, by increasing the concentration of platelets at the endothelium, promoting their activation. (41) Zaar M. et al describe LBNP induced platelet activation, reflected by an increase of active glycoprotein IIb/IIIa and platelet counts in a study done 2014. (42)

LBNP has also been found to promote fibrinolysis similar to hemorrhage. The sympathic response to LBNP induced central hypovolemia might contribute in part to the release of factors (e.g. vWF, t-PA) promoting both coagulation and fibrinolysis. (42)

LBNP studies have already helped to gain new insights in areas like gendered differences in blood pressure regulation, the effects of space flight, autonomic dysfunctions, orthostatic hypotension, central hypovolemia and more. (43)

3.6 Climatic and seasonal influences on the human body

3.6.1 Body temperature, its effect and regulation

Human internal temperature is set to a narrow range. Core and skin thermoreceptors monitor the internal and surface temperature and send the information to the thermoregulatory center in the hypothalamus, that responds to changes in ambient temperature or heat production that would cause deviations from the internal temperature of about 37°C. (44)

The main response to the disturbance of the heat balance by cold environment can be categorized in two main patterns: metabolic and insulative physiological adjustments. (45) The metabolic rate of cellular oxidation, the main source of body heat production increases, accompanied by peripheral vasoconstriction. (44,45) The insulative vasomotor response decreases heat conduction from the core to the surface, decreasing the temperature of the skin and preventing body heat loss. (45) The effect of these mechanisms can be expanded by muscular shivering mechanically producing heat. (44,45)

Required heat dissipation relies on an increased blood flow towards the body's surface and the initiation of sweating. (44)

Increased body temperature is linked to rise in respiratory and heart rates, as well as vasodilatation of superficial vessels. (46) Zwierzina et al have described an activation in blood coagulation after induced hyperthermia (38,2-39,9°C) reflected in a rise of factor IX, XII, fibrinogen as well as the maximal amplitudes of the subjects' thrombelastograms. (47)

Lowered body temperature at 35°C has been described to have no significantly altering effect on the coagulation cascade. While hypothermia between 33°C and 35°C only mildly negatively impacts the platelet function in some patients,

temperatures below 33°C are required to impact coagulative processes such as the function and synthesis of clotting enzymes, creating an anticoagulant environment. (48)

3.6.2 Seasonal variation in the cardiovascular system

PJ Brennan et al describe a significant higher arterial blood pressure in winter compared to summer with older people more affected than younger ones. The effect was dependent on the daily temperatures, with colder air temperature being associated with higher blood pressure and vice versa. (49) Other authors have found corresponding results, describing a negative relationship between air temperature and blood pressure. (50,51)

While some authors did not find any significant seasonality in heart rate (49,52), Yuda E. et al have described a significant but small rise in median and basal heart rate during the cold months (either < 2 beats per minute). (53)

The heart rate variability describes the physiological changes in pulse frequencies to adapt to physical and psychological challenges under the influence of the autonomic nervous system (54) Kristal-Boneh E. et al describe a seasonal fluctuation with a decrease in heart rate variability during the winter as compared to summer. Low heart rate variability correlates with an increased occurence of arrhythmic incidents and cardiac death (52) and could be linked to the increased cardiovascular mortality in winter according to some studies. (55,56)

3.6.3 Seasonal variation in coagulation parameters

Knowledge about biological fluctuations in seasonal variation of hemostatic parameters is mostly based on studies with a small sample size and still limited. (57) M Fröhlich et al found significantly elevated levels of plasma and whole blood viscosity, hematocrit, hemoglobin, platelet count, plasminogen activator inhibitor 1 as well as fibrinogen in a study during the winter months in young healthy adults. (n=16) (58). Other authors also describe a rise of fibrinogen winter (59–61) and factor VII (59) during winter. Van der Bom JG. et al. corroborate this result, finding elevated fibrinogen level, implying a more procuagulant milieu, during winter in a relatively big study (n=2325). (60,62) There has also been described a negative relationship between fibrinogen level and both, environmental and core body temperature in a study on elderly people (n=100) with an overall rise of 23% in

fibrinogen in winter. (61) The susceptibility to seasonal influences on fibrinogen levels seems to be most pronounced in elderly people. (60)

D-dimer, a marker for fibrinolysis that usually rises in response to increased coagulation activity has been found to be higher during winter as well. (56,63) The excess of admission for (64–66) and mortality of myocardial infarction (67), as well as the increase in VTE incidents (68,69) could be reflected in these observations.

3.6.4 Seasonal variation in hormone regulation

Vitamin D that acts like a steroid hormone is produced in a UVB-dependent manner in the skin and plays part in the regulation of the calcium homeostasis and a functioning bone metabolism. (70,71) Seasonal variation levels of vitamin D coinciding with those of HbA1c, circulating lipids and blood pressure, could indicate a possible association of vitamin D deficiency and metabolic disease. There has also been described an increase in systemic inflammation markers during winter, most pronounced in subjects with insufficient vitamin D levels. (72) Inflammation in return creates a procoagulant milieu, promotes clotting, impaires the function of the fibrinolytic system and other anticoagulant mechanisms (73) and can induce thrombosis. (74)

Sexual hormones are barley influenced by the seasons. Bjørnerem A. et al have found that the season dependent fraction of physiological fluctuation in estradiol is below 1% in both man and women. DHEAS and FSH didn't show any seasonality at all. (75)

Melatonin is a pineal hormone that under normal conditions is only produced and secreted during the night while daylight suppresses its production. Although the knowledge of the function of Melatonin is largely based on correlation between its secretion and clinical observations, evidence suggests that it plays a role in the organization of the sleep-wake rhythm and its coupling with other circadian rhythms (e.g. that of core temperature). (76) As days in winter become shorter the hormone's production increases. In combination with lower levels of serotonin in people with seasonal affective disorder, the higher production of Melatonin likely negatively affects the patients' symptoms like sleepiness and lethargy. (77)

3.6.5 Seasonal variation in lifestyle

Although the variations are small, physical activity, caloric intake and even body weight are described to be influenced by the seasons. Ma Y. et al describe higher

bodyweight by about 0.5 kg in winter as compared to summer. In winter physical activity was observed to be the lower than in the other seasons, (78,79) commonly accompanied by a decrease of muscle strength and maximal oxygen intake (79), an indicator of aerobic fitness. (80)

4 Aims and Objectives

The aims of this study were…

1. …to identify seasonal patterns in hemostatic baseline parameters.
2. …to examine the difference in the coagulative response to applied LBNP when comparing the cold and warm seasons.

The knowledge of the seasonal dependency of the coagulation system is still limited and mostly based on studies with a small number of subjects. (57) This project aims to provide further insight into the seasonal variability of the coagulation system, which could help to identify seasonal bias in future studies, better the understanding of factors influencing the pathogenesis of coagulation related incidents, and have clinical implications in relation to those diseases.
The literature research done did not find studies comparing the seasonality of the coagulation response to either LBNP or application of orthostatic stress.

4.1 Hypotheses

Hypothesis #1: This thesis hypothesizes that the baseline coagulation values present a more procoagulant pattern during the cold season, as in addition to the increased thromboembolic incidents some authors have described a rise of factors promoting coagulation (e.g. fibrinogen, plasma viscosity) during the winter months (58,60).

Hypothesis #2: This thesis hypothesizes that seasons do affect the coagulation related response to LBNP, with a higher activation of the coagulation system in winter on the basis of previously published studies describing an excess in venous thromboembolism (68,69), admission of cardiac infarction (64,65) and cardiovascular mortality (55,56) during the winter months.

5 Methods

The Ethics Committee of the Medical University of Graz provided an approval in advance of the study. All subjects signed informed consent beforehand.

5.1 Subjects

The criteria for inclusion were: males and females 18-35 years of age, and 160-180 cm.

Criteria for exclusion were: smoking, pregnancy, orthostatic intolerance, endurance athletes, coagulation disorders, thrombosis, subjects who suffer from cardiovascular diseases or have a family history thereof, and coagulation altering medication with the exception of oral contraceptives.

For this set of data, we have chosen those participants that had at least on measurement in each the cold (November-April) and warm months (May-October), which we divided based on a paper by Trozic I. et al (81). If there was more than one measurement in either of the seasons, we selected one by random.

5.2 Sample size:

Preceding studies examining coagulation parameters during exposure to central hypovolemia were used as the basis to determine the number of subjects needed to state statistical significance. (37,82,83) Sample size was calculated using an average effect size of 0.5 (d), power of 0.80 (1-β) and an error probability of 0.05 (α). (82)

The sample consists of 13 subjects, 7 of which are male, 6 are female.

5.3 LBNP protocol

The subjects were asked to abstain from drinking coffee or alcohol for 24 hours beforehand. The test site was a quiet darkened room kept at 23°C-24°C temperature and 55%-60% humidity.

The procedure started with the instalment of cardiovascular monitoring via electrodes while the subjects were lying on their back for 30 min. Then followed graded application of LBNP, starting at a negative pressure of -10mmHg, which was increased every 5 minutes by further -10mmHg-intervals, up to a peak negative pressure of -40mmHg.

5.4 Blood Sampling

The blood was collected from veins in the antecubital area at 3 points: At the beginning there was taken a baseline sample, then one at the end of the phase with the peak LBNP of 40mmHg, and a last one after a 10 min recovery phase.

The blood was gathered into citrated Vacuette® tubes (Austria, Greiner Bio-one GmbH). The aggregation of platelets, TEM, and WB were then analyzed within the first three hours after the sampling, followed by the extraction of PPP via the centrifugation of some of the blood at 1200g for 15 min at room temperature. Pro- and anticoagulant proteins, common clotting times, markers of thrombin generation and its curve were determined from the obtained PPP. An automated Haematology Analyzer was used to measure blood cell counts, Hct, Hb, MCV, MCH and MCHC.

5.5 Hematocrit, blood cell counts and common coagulation tests

The Automated Haematology Analyzer Sysmex KX-21 N was used to measure Hematocrit, blood cell counts, Hemoglobin, MCV, MCH and MCHC.

Protein C and S, FII, FVII and FVIII, activated partial thromboplastin time and prothrombin time were measured by Analyzer BM/Hitachi 917.

5.6 Thrombin generation and endothelial activation measurement

The thrombin generation curves were observed via the method of calibrated automated thrombogram (Thrombinoscope B.V., CAT). Plasma thrombin generation was determined by measuring the endogenous thrombin potential (ETP), Lag time (LT), peak thrombin generation (Peak), time to Peak (ttPeak), the thrombin generation peak rate (Velocity Index), and the time in which free thrombin is no more detectable (Start Tail).

Thrombin/antithrombin complexes (TAT) and prothrombin fragments 1+2 (F1+2) were determined by ELISA. (Germany, Behring Diagnostics GmbH)

Tissue factor (TF) was quantified via ACTICHROME Tissue Factor ELISA assay (Germany, American Diagnostica) the concentration of tissue Plasminogen Activator (t-PA) by the use of IMUBIND t-PA ELISA kit (Germany, American Diagnostica).

5.7 Tissue factor triggered thromboelastometry (TEM)

The thrombelastometer was used to determine, the latency to the initiation of fibrin formation (Coagulation Time, CT), the time between the start of the clotting until the measured amplitude of the trace gets to 20 mm (Clot Formation Time, CFT), the maximal amplitude of the clot firmness trace (Maximum Clot Firmness, MCF) (84) and the Alpha angle, the angle between the time axis and the tangent to the TEM trace at 2mm amplitude. (85)

5.8 Statistics

All statistical calculations and analyses were done by using GraphPad 7.0. All values are represented with their standard deviation (±SD). The difference in the effect of LBNP on the subjects' coagulation measured at three points (baseline, LBNP at -40mmHg, and after 10 min recovery phase) between two timeframes (seasons divided in cold and warm months) was analyzed via repeated measures ANOVA. repeated measurements with two variables. First variable: comparison between seasons (cold vs. warm month); the second variable deals with the effects of LBNP on coagulation values following the Dunn's post-test. The Mann Whitney U-test was applied to analyze the seasonal variation in the baseline coagulation values. The limits of statistical significance were established as followed: $p>0.5$, $p\leq0.05$*, $p\leq0.01$** and $p\leq0.001$***

6 Results

The subjects of this study consist of seven men and six women. None of the participants had to quit the LBNP-protocol early.

6.1 Conventional coagulation measurements

The **baseline level** of factor II showed a significant higher concentration during summer (Fig. 7). APTT, PT, Factor VII, VIII as well as PC and PS showed no difference in baseline levels in the two seasons. The Application LBNP caused a shortening of PT during winter (p=0.028) and aPTT in summer (p=0.018) (Tab. 3, Tab. 4)

FVIII showed a pronounced rise in response to **LBNP** in both seasons (summer p=0.021, winter p=0.0053). (Tab. 3, Tab 4, Fig. 8). Factor II, VII, PC and PS aren't influenced by LBNP in the subjects.

The estimated seasonal influence in above mentioned variables, calculated by means of **ANOVA** including all 3 measurements in both winter and summer, only reaches the threshold for significance for FII (p=0.0001).

All figures and tables displayed in the chapter "Results" are reflecting the data as mean±SD and p≤0.05, p≤0.01**, p≤0.001***, p≤0.0001****.*

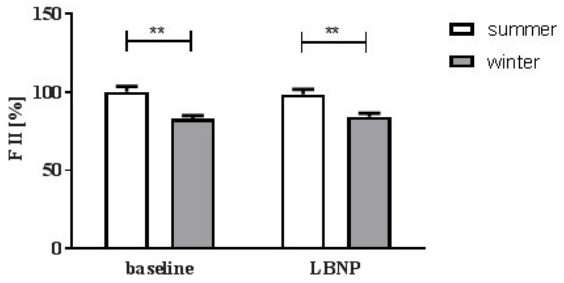

Fig. 7, Graph for Factor II

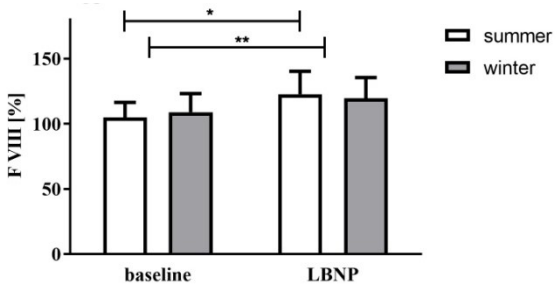

Fig. 8, Graph for Factor VIII

Tab. 3, **Summer** *LBNP results standard coagulation parameters*

Variable	Baseline	LBNP	Recovery	P
aPTT [s]	34.67 ± 2.78	32.30 ± 3.09	33.25 ± 4.47	**0.018**
PT [%]	103.92 ± 13.10	107.23 ± 13.73	104.08 ± 15.31	0.17
F II [%]	103.31 ± 15.72	102.23 ± 17.14	103.67 ± 15.50	0.91

FVII [%]	105.77 ± 35.15	107.85 ± 36.79	107.42 ± 37.44	0.61
F VIII [%]	105.00 ± 36.63	122.62 ± 55.92	126.42 ± 64.26	**0.021**
PC [%]	97.23 ± 31.31	101.00 ± 27.83	98.50 ± 31.77	0.56
PS [%]	85.85 ± 16.26	85.15 ± 22.70	91.92 ± 20.88	0.35

Tab. 4, **Winter** *LBNP results standard coagulation parameters*

F	Baseline	LBNP	Recovery	P
aPTT [s]	35.62 ± 6.05	33.69 ± 4.62	36.98 ± 16.16	0.71
PT [%]	103.85 ± 11.86	101.62 ± 10.88	106.00 ± 11.18	**0.028**
F II [%]	85.69 ± 10.65	84.25 ± 7.52	85.17 ± 5.26	0.46
FVII [%]	107.62 ± 22.75	108.17 ± 23.30	109.00 ± 24.17	0.69
F VIII [%]	107.77 ± 45.85	119.58 ± 52.92	124.75 ± 54.96	**0.0053**
PC [%]	94.15 ± 17.42	95.58 ± 15.11	95.08 ± 19.41	0.98
PS [%]	83.23 ± 23.48	88.00 ± 25.73	83.33 ± 16.04	0.61

Tab. 5, **Seasonal influence** *on standard coagulation parameters, calculated by means of ANOVA*

F	P_{Time} (ANOVA)
aPTT [s]	0.056
PT [%]	0.72
F II [%]	**0.0001**
FVII [%]	0.93
F VIII [%]	0.36
PC [%]	0.62
PS [%]	0.4

6.2 Hematocrit, blood cell count, haemoglobin, MCV, MCH and MCHC

The **baseline levels** of Hct, WBC, RBC, Hb, MCV, MCH, MCHC were the same for both seasons.

Hct showed the same pattern in both seasons with a reduction during **LBNP** application with a subsequent rise (winter p=0.0039, summer p=<0.0001) accompanied by WBC, RBC, Hb showing the same pattern. MCHC, MCH and MCV were not altered by LBNP. (Tab. 6, Tab. 7)

The **ANOVA**-calculated seasonal comparison on the above mentioned variables implies a seasonal influence only on Plt (p=0.022, Tab.8). While in summer Plt showed the same pattern as Hct with marginally missing statistical significance (p=0.056), in winter only a slight non-significant rise already measurable during LBNP-application and lasting to recovery could be observed (p=0.64). (Tab. 6, Tab. 7)

Tab. 6, **Summer** *LBNP results Hct, blood cell count, Hb, MCV, MCH and MCHC*

Variable	Baseline	LBNP	Recovery	P
WBC [103/µL]	5.11 ± 0.98	4.78 ± 1.11	5.43 ± 1.40	**0.0001**
Plt [103/mL]	203.15 ± 55.14	198.40 ± 54.00	208.58 ± 56.35	0.063
RBC [10^6/mL]	4.33 ± 0.40	4.26 ± 0.40	4.38 ± 0.40	**0.0001**
Hct [%]	37.12 ± 3.31	36.52 ± 3.36	37.44 ± 3.31	**<0.0001**
Hb [g/dL]	12.72 ± 1.38	12.58 ± 1.39	12.89 ± 1.46	**<0.0001**
MCHC [g/dL]	34.24 ± 1.12	34.40 ± 1.17	34.36 ± 1.24	0.35
MCH [pg]	29.37 ± 1.38	29.53 ± 1.33	29.38 ± 1.47	0.26
MCV [fL]	85.78 ± 2.77	85.77 ± 2.69	85.50 ± 2.70	0.94

Tab. 7, **Winter** LBNP results *Hct, blood cell count, Hb, MCV, MCH and MCHC*

Variable	Baseline	LBNP	Recovery	P
WBC [10^3/µL]	4.76 ± 0.77	4.23 ± 0.59	4.93 ± 0.79	**<0.0001**
Plt [10^3/mL]	192.54 ± 42.19	195.08 ± 38.50	195.62 ± 36.90	0.64
RBC [10^6/mL]	4.27 ± 0.33	4.25 ± 0.37	4.33 ± 0.35	**0.0036**
Hct [%]	36.72 ± 2.56	36.54 ± 2.91	37.26 ± 2.64	**0.0039**
Hb [g/dL]	12.69 ± 1.07	12.64 ± 1.22	12.89 ± 1.15	**0.0088**
MCHC [g/dL]	34.54 ± 1.01	34.54 ± 1.04	34.56 ± 1.00	0.97
MCH [pg]	29.73 ± 0.99	29.74 ± 0.97	29.76 ± 1.00	0.91
MCV [fL]	86.09 ± 2.43	86.10 ± 2.33	86.12 ± 2.27	0.98

Tab. 8, **Seasonal influence** *on Hct, blood cell count, Hb, MCV, MCH and MCHC, calculated by means of ANOVA*

Variable	P_{Time} (ANOVA)
WBC [10^3/µL]	0,12
Plt [10^3/mL]	**0.022**

RBC [10^6/mL]	0.29
Hct [%]	0.36
Hb [g/dL]	0.56
MCHC [g/dL]	0.26
MCH [pg]	0.13
MCV [fL]	0.21

6.3 Thrombin generation

The baseline levels of F1+2 and TAT were both higher during the warm season and strongly increased in response to LBNP. (Fig. 9, Fig. 10)

The base measurements of thrombin generation achieved via CAT (ETP, LT, Peak, ttPeak, Velocity Index, StartTail) and thrombelastometry (MCF, CT, CFT and alpha angle) didn't show a seasonal tendency.

CAT: In summer LT (p=0.0028) and ttPeak (p=0.0025) decreased during applied **LBNP** (with a subsequent rise in recovery) whilst Peak (p=0.014) and Velocity Index (p=0.036) showed the inversed pattern. (Tab. 9, Tab. 10)

In winter none of the CAT measured variables were affected significantly by LBNP. StartTail, and ETP were not altered in either season. (Tab. 9, Tab. 10)

TEM: CT was reduced in both seasons (winter p=0.042, summer p=0.023). The rest of the TEM-measured values, with the exception of Alpha (increase in winter p=0.035), were not influenced by **LBNP**. (Tab.9, Tab.10)

The statistical analysis of seasonal dependency via the means of **ANOVA** only showed significant results in F1+2 (p=0.0023) and TAT (p=0.0001) as opposed to markers measured via CAT and TEM, that didn't show any significant seasonal variation. (Tab. 11)

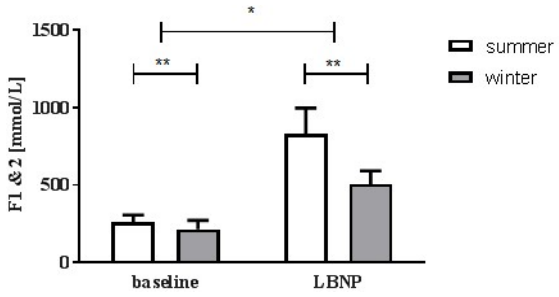

Fig. 9, Graph for F1+2

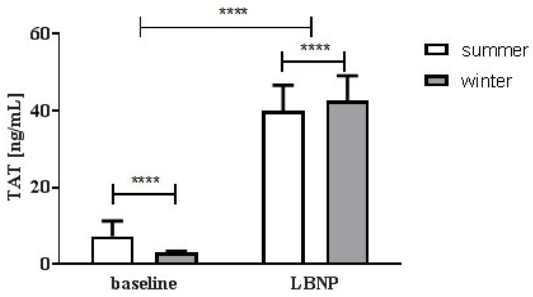

Fig. 10, Graph for TAT

Tab. 9, **Summer** *LBNP results Thrombin generation*

Variable	Baseline	LBNP	Recovery	P
F1+2 [pmol/L]	263.7 ± 142.6	776.3 ± 567.3	1078.4 ± 837.4	**0.0027**
TAT [ng/mL]	7.26 ± 13.36	37.21 ± 23.23	42.02 ± 23.62	**0.0004**
LT [min]	2.36 ± 0.30	2.31 ± 0.24	2.46 ± 0.27	**0.028**
ETP [nM•min]	1487.5 ± 449.3	1529.0 ± 417.3	1501.9 ± 484.7	0.20
Peak [nM]	273.30 ± 94.39	287.03 ± 77.09	263.55 ± 101.01	**0.014**
ttPeak [min]	5.61 ± 0.83	5.38 ± 0.57	5.92 ± 0.97	**0.0025**
VelIndex [nm/min]	91.54 ± 43.94	97.38 ± 35.92	85.53 ± 46.76	**0.036**
StartTail [min]	21.18 ± 1.73	21.29 ± 1.50	21.82 ± 1.79	0.27
CT [s]	199.38 ± 46.33	172.92 ± 33.57	188.83 ± 32.53	**0.023**
CFT [s]	151.23 ± 74.70	121.23 ± 38.37	142.67 ± 59.64	0.27
MCF [mm]	56.77 ± 6.73	58.00 ± 5.72	56.67 ± 5.93	0.49
Alpha [°]	63.00 ± 9.58	66.69 ± 6.19	64.17 ± 8.91	0.27

Tab. 10, **Winter** *LBNP results Thrombin generation*

Variable	Baseline	LBNP	Recovery	P
F1+2 [pmol/L]	208.85 ± 182.6	507.17 ± 278.9	613.75 ± 346.0	**0.011**
TAT [ng/mL]	3.10 ± 0.83	42.65 ± 21.52	44.45 ± 22.50	**0.0001**
LT [min]	2.35 ± 0.28	2.39 ± 0.34	2.39 ± 0.31	0.67
ETP [nM•min]	1310.15 ± 352.37	1302.17 ± 343.14	1304.09 ± 320.86	0.96
Peak [nM]	226.87 ± 57.02	220.37 ± 64.41	216.48 ± 49.51	0.27
ttPeak [min]	5.74 ± 0.63	5.83 ± 0.69	5.92 ± 0.67	0.19
VelIndex [nm/min]	69.57 ± 24.44	67.62 ± 27.56	63.89 ± 21.13	0.19
StartTail [min]	21.35 ± 0.97	21.67 ± 1.28	21.72 ± 1.18	0.25
CT [s]	197.92 ± 50.95	182.00 ± 42.16	179.23 ± 63.87	**0.042**
CFT [s]	173.77 ± 50.51	159.77 ± 56.81	138.54 ± 42.98	0.079
MCF [mm]	55.08 ± 5.15	55.00 ± 4.79	56.15 ± 4.87	0.48
Alpha [°]	58.46 ± 7.13	61.08 ± 7.95	63.85 ± 6.26	**0.035**

Tab. 11, **Seasonal influence** *on Thrombin generation, calculated by means of ANOVA*

Variable	P_{Time} (ANOVA)
F1+2 [pmol/L]	**0.0023**
TAT [ng/mL]	**0.0001**
LT [min]	0.31
ETP [nM•min]	0.11
Peak [nM]	0.13
ttPeak [min]	0.61
VelIndex [nm/min]	0.16
StartTail [min]	0.48
CT [s]	0.55
CFT [s]	0.22
MCF [mm]	0.38
Alpha [°]	0.12

6.4 Endothelial activation

The **baseline** tissue factor concentration was significantly higher during summer in comparison to winter (Fig. 12) while t-PA baseline levels didn't differ during the seasons.

Neither of those variables was significantly influenced by the use of **LBNP**.

The statistical analysis via the means of **ANOVA** implies a probable seasonal influence on TF (p=0.0083).

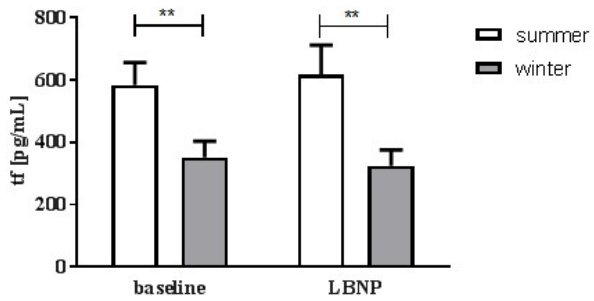

Fig. 11, Graph for TF

Tab. 12, **Summer** *LBNP results for parameters for endothelial activation*

Variable	Baseline	LBNP	Recovery	P
TF [pg/mL]	583.3 ± 241.9	593.7 ± 314.2	575.3 ± 169.0	0.63
tPA [ng/mL]	2.12 ± 1.96	1.45 ± 1.09	1.58 ± 0.85	0.25

Tab. 13, **Winter** *LBNP results of parameters for endothelial activation*

Variable	Baseline	LBNP	Recovery	P
TF [pg/mL]	351.1 ± 165.9	324.2 ± 170.4	377.92 ± 137.9	0.49
tPA [ng/mL]	1.88 ± 1.66	1.71 ± 0.99	1.59 ± 0.85	0.8

Tab. 11, **Seasonal influence** *on parameters for endothelial activation, calculated by means of ANOVA*

Variable	P_{Time} (ANOVA)
TF [pg/mL]	**0.0083**
tPA [ng/mL]	0.53

7 Discussion

The study found a higher base level (**FII, TF, F1+2, TAT**) as well as a more pronounced LBNP-induced pro coagulative impact on some of the measured coagulation related variables, especially those measured by CAT (**LT, Peak, ttPeak, Velindex**) during summer. In winter none of the measured coagulation related variables showed a significant greater base level. The only variables changing significantly in response to LBNP exclusively in winter were **PT** and **Alpha**.

These findings could imply a greater response of the coagulation system to central hypovolemia in warmer seasons, as the **Lag Time** reflects the delay of initial thrombin generation, **ttPeak** refers to the time it takes the blood to reach the point of maximum velocity of thrombin generation (**Peak**) (86) measured via CAT and thrombin generation being widely recognized as a reflection of the haemostatic/pro-thrombotic state of the blood due to its central role in coagulation with no by-passing pathways. (87) Although the statistical analysis with ANOVA does not conclude a seasonal influence on any of these single data points, it seems reasonable to deduce such, as all those measurements responded to LBNP significantly in a procoagulant way during summer in accordance.

CT, expressing the lag between the start of the test to the beginning of clotting measured via TEM (88) reflecting the start of thromboplastin activation shortened significantly in response to LBNP in both winter and summer.

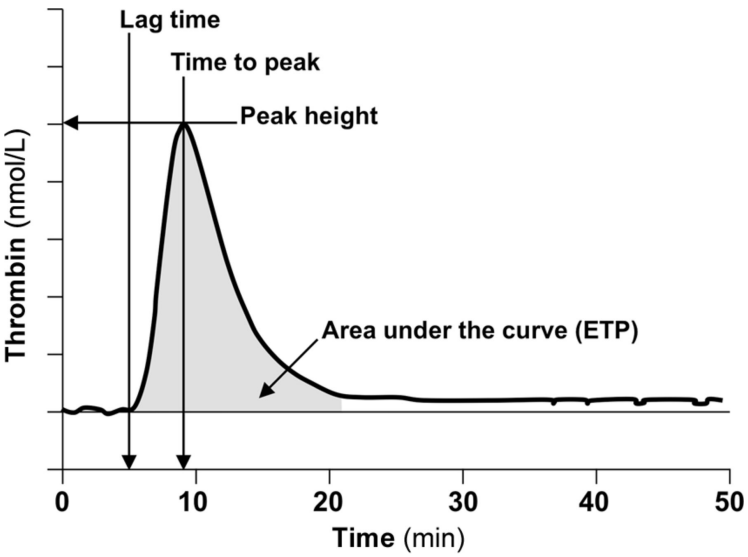

Fig. 12: Diagram of a thrombogram, taken from (89)

The **higher base concentrations** of **FII, TF, F1+2, TAT** further hint at a greater coagulability of this study's subjects during the summer, due to the variables' roles in the coagulation process:

As described in chapter 3.2.2.ii **prothrombin (FII)** is the inactive form of thrombin (FII_a), which is endresult of both extrinsic and intrinsic pathway. FII_a represents an integral part in the coagulation process transforming fibrinogen (FI) to fibrin (FI_a) and catalyzing the coagulation through a positive feedback loop activating FV, FVIII and FXI.
High levels of FII have been found to promote the pathogenesis for venous thrombosis (90–92). This is probably due to the direct relation between prothrombin levels and thrombin generation reflected in an increase in initial rate, peak and generated thrombin amount as described by Wolberg AS et al. Elevated FII levels also go along with an altered structure of the resulting fibrin clots, which could also promote thromboembolic incidents (92).

The study also found higher base concentrations of **tissue factor (TF)** during summer. TF is a marker for endothelial activation, and as described in chapter 3.2.2.ii initiates coagulation via the extrinsic pathway when it comes in contact with Factor VII. Its coagulation starting properties are so pronounced it is frequently used as part of coagulation triggering solutions in laboratory tests like thrombograms and thrombelastometries. (87,88)
Findings in the last two decades have added to this traditionally limited role, and described TF not only to occur separated from the blood through the endothelial barrier, but also circulating on microparticles, platelet- and white cell-associated or in a soluble form. (93) Even if not stimulated monocytes express small amounts of tissue factor, and could be the main source of those microparticles (94). This process has been described to be stimulated by oxidized LDL (95) that induces pro-inflammatory processes in macrophages (96) and is associated with genesis of atherosclerosis. (97) It has been suggested that this so called "blood-bourne tissue factor" is associated to myocardial infarction (98,99) and other thrombotic incidents(100).

A multitude of immunological and inflammatory processes have been described to increase the TF expressed by monocytes and endothelial tissue transiently. Surgical procedures were observed to stimulate the synthesis of TF in monocytes as well. (101)

Rietveld I. M. et al have found that the concentration of TF needed to initiate the formation of thrombin depends on the amount of anti- and procoagulant factors such as elevated levels of prothrombin. They describe the role of its threshold as a mediator between higher concentrations of procoagulant factors, and the resulting risk for VTE. (102)

There seems no literature available comparing TF levels during different seasons, or a conclusive explanation for the discrepancies between the seasons thereof in this study's subjects, as WBC, a reliable marker for inflammation (103) did not hint to inflammatory processes as a probable reason. CRP has not been included in the measurements.

This study showed a great LBNP triggered rise in **F1+2** and **TAT** during both seasons, with averages of both parameters increasing manifold. While the levels of TAT were similar during winter and summer, except for a higher baseline level in summer (Tab. 9, Tab. 10, Fig. 10), F1+2 not only presented a significantly higher baseline during summer (Fig. 9) but also a stronger response to LBNP (F1+2 level during *recovery phase* in summer p=1078.4±837.4 pmol/L, winter p=613.75±346.0 pmol/L).

Prothrombinfragment 1+2 (F1+2) and thrombin/antithrombin complexes (TAT) (and D-dimer) are markers for **thrombin generation** (104).

In the coagulation process the prothrombinase complex converts prothrombin to its active form thrombin by splitting off F1+2 that can then be detected in the serum. TAT are complexes that form when antithrombin inhibits the generated thrombin, and is a marker that can be used to detect hypercoagulability (105). High levels of (F1+2), thrombin-antithrombin complex (TAT) and D-dimer and have been described to be significantly higher in subjects with thrombosis (90). Ay C et al have conducted a cohort study (n=821) on cancer patients and found that elevated levels of F1+2 and D-dimer, could predict the occurrence of thromboembolic incidents. (15,2% cumulative probability of VTE after 6 months in the group with risen F1+2 and D-dimer levels, as compared to 5.0% in the rest of

the patients.) (106). While F1+2 levels seem to have a significant correlation with venous thromboembolism, a significant connection with major coronary events has not been established. (107) This is likely to be linked to the different pathogenesis of arterial and venous thrombosis, as in animal experiments other risk factors for VTE like elevated prothrombin levels have been shown to have no significant effect on arterial thrombosis as well. (108)
This measured rise in F1+2 and TAT in both seasons probably reflects the already known hypercoagulable state triggered by LBNP described Cvirn G et al and multiple other authors (36–39).
It should be mentioned that F1+2 and TAT had great interpersonal differences reflected by high values for standard deviations in both seasons.
Although the statistical analysis via ANOVA indicates a probable seasonal influence for both variables, further research with bigger sample sizes would be needed to further substantiate these findings regarding these tendencies.

Factor VIII shows a similar baseline level and pattern in both seasons significantly rising in response to LBNP.
The rise in factor VIII could be interpreted as an indirect sign of endothelial activation, since it is mainly produced and released into circulation by the liver and endothelium (109) or the adrenergic activation of the autonomous nervous system triggered by LBNP described by Franke WD et al (35). Adrenergic physiological stress reactions among other causes like pregnancy, liver diseases or advanced age raise the FVIII concentration. Furthermore as an acute phase protein FVIII also increases during infection triggered inflammatory processes, cancer and trauma. Elevated FVIII levels have been found to dose-dependently increase the risk of a VTE incident. (110)
It stands to reason to suggest that elevated FVIII, could be one of the pro-thrombotic mechanisms contributing to the higher VTE risk observed in cancer patients (111), patients with inflammatory diseases or post surgery (112). The ANOVA analysis did not indicate any seasonal influence either.

The standard coagulation test PT (or the INR calculated on the basis of PT) measures the function of the extrinsic pathway, testing for FII, FV, FVII, FX and fibrinogen (113,114) and **aPTT** measuring the effectiveness of the intrinsic

pathway by activating FXII (115) didn't show any significant difference in base levels between the seasons. In winter PT shortened during applied LBNP with a rise in recovery while aPTT was not significantly affected, in winter it was the other way around. Both tests, while only significant in one season, especially regarding the other data further demonstrate the shift towards a procoagulant environment by applied LBNP.

Hct, RBC, Hb and WBC demonstrate the same pattern during both seasons with an initial decrease during applied LBNP and consecutive increase. The results of the RBC and WBC could probably be explained when associated with the Hct. The fluidal shift from intracellular to extracellular mentioned by Zaar et al (42) would provide a conclusive explanation for these results. **Platelet count (Plt)** was the exception to this cell count related pattern, as its response did not reach the threshold for significance.
Zaar M et al found a rise in Plt in response to LBNP and suggests that the rise of Plt after applied LBNP might be in part due to adrenergic-mediated splenic release of platelets (42) as authors have described a rise of both FVIII and platelet count in vivo after adrenalin application only occurring in patients that still have a spleen. (116). Zaar M et al however describe the suction triggered shift of fluid to the extravascular space as the main contributor in their LBNP study. It is important to note that they have collected their blood samples before and 5 minutes after the application of LBNP, so there is no comparison with this study's measurements taken while under the effect of LBNP. (42) The opposite effects of adrenergic driven splenic release of platelets and the fluid shift especially at the second blood sample taken during applied LBNP could interfere with the expected attainment of statistical significance, and maybe explain this discrepancy between literature and this study.

To summarize the results; these findings could be interpreted as a more active plasmatic coagulation in summer while cellular components seem not to be affected.

When compared the results seem to stand in contrast to most of the literature comparing coagulation during different seasons. As mentioned in chapter 3.6.3

multiple authors have found risen contributors to plasmatic coagulation during winter (e.g. fibrinogen levels) as compared to summer. (58–61)
One difference between those studies and this one was the application of LBNP, as previous studies found only compare the coagulation system without any stimulation via LBNP or other methods, only enabling the comparison of this study's baseline coagulation values to other sources. Even this comparison of baseline coagulation is difficult due to a lack of literature concerning coagulation alterations during the seasons.

Furthermore it is important to mention that in the available literature fibrinogen levels were examined the most, but were not included in this study's measured parameters. Since elevated fibrinogen levels have been well-established as a risk factor for VTE in a concentration depend manner (117), described rise in fibrinogen in found studies as a reflection of a procoagulant state will be used here in order to indirectly compare their results with this study.

Another difficulty comparing this study to other sources is caused by the fact that out of the found literature only Fröhlich et al have had a group of subjects consisting of young and healthy people comparable to this study's, but with a relatively small sample size (n=16). (58)
The other three studies found had age ranges starting at 55 and above. (59–61) Van der Bom JG et al describes an age dependent pattern with the season associated variation in coagulation reflected by a rise in fibrinogen during winter being most observable in the elderly (60), which implies that the seasonal variation of coagulation might be comparably harder to measure in this study's subject group of young adults.

Another reason why the results might differ from what would be expected regarding the available literature might be rooted in the choice of testing site. Stout RW et al have described a negative correlation between risen fibrinogen levels and core body as well as environmental temperature. (61)
This could imply that the seasonal change observed in coagulation, even those reflected in the variables we have measured, could be significantly altered by the body temperature at the time of the blood sampling. In this experiment the

subjects were in a controlled environment with the same room temperature during summer and winter. The delay to the taking of the first blood sample in the 23°C-24°C room, as well as the temperature of the place the subjects were in before, and how long they've stayed there may have significantly impacted the test results.

Van der Bom JG et al describe no such correlation between outdoor temperature and fibrinogen levels, while still finding the highest fibrinogen levels in winter. This could very well be due to the different designs of those studies. Stout RW et al visited their subjects at their place of residence to collect the blood, whereas subjects of the Rotterdam study have had multiple examinations for the duration of about 5h in addition to the blood sampling in a research facility. (61,118)

If body temperature directly affects the coagulation system it stands to reason that the correlation between environmental temperature and coagulation could be more observable in subjects at their residences, where temperatures may fluctuate more, than inside the controlled environment of a research facility.

In conclusion the hypotheses based on the researched literature predicting a more procoagulant environment and a stronger activation of the coagulation system in response to LBNP in this study's subjects during winter could not be upheld. The suspected influence of the seasons may be covered by other influences like room temperature and individual factors like inflammatory processes or may simply be hard to detect in a sample group of young healthy adults.

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