Diplomarbeit

Immobilization and Coagulation: Molecular mechanisms

eingereicht von

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Graz, 17/March 2014

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, 17/March 2014 *Sabine Wolf*

Preface

I talked several times with my friend's mother about geriatric medicine as she was working for many years in this field and has a lot of experience in it. Through our conversations I found out that immobility in old people seems to be a huge problem and thought it would be an interesting topic for my thesis. After my decision to write about immobility in the elderly and its consequences I was looking for a department and a supervisor who would be interested in this topic. Moreover I looked up topics in mugthesis, a pool for thesis topics often proposed by doctors. There I suddenly detected the topic „Immobilization and Coagulation". After having read the abstract I thought that this meets my interests very well. Therefore I immediately contacted Dr. Goswami, my present supervisor, for a meeting after which I knew I had found the perfect diploma thesis topic. Thanks to Dr. Goswami I had the opportunity to participate in a project of the European Space Agency and be a part of his research team.

Acknowledgement

I would like to give a thousand thanks to my mother who supports and motivates me with her love and positive thinking in all circumstances of life.

My best thanks also to my deceased godmother who always provided me with financial support and loved me like her own child.

I would also like to thank my sister for encouraging me to finish my diploma thesis and for being proud of myself and my success.

I want to thank my supervisor Dr. Nandu Goswami who led me through the diploma thesis and helped me at any time needed.

Many thanks also to Dr. Patrick De Boever who helped me to analyze aliquots.

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Glossary and acronyms

AT:	Antithrombin
BDC:	Baseline data collection
BR:	Bed rest
BR+EXC:	Bed rest and exercise
BR+NUT:	Bed rest and nutrition
CT:	Coagulation time
CFT:	Clot formation time
CVP:	Central venous pressure
DVT:	Deep vein thrombosis
e.g.:	Example given
ESA:	European Space Agency
FIII:	Factor III
FV:	Factor V
FVII:	Factor VII
FVIIa:	Activated factor VII
FVIII:	Factor VIII
FVIIIa:	Activated factor VIII
FIX:	Factor IX
FIXa:	Activated factor IX
FX:	Factor X
FXa:	Activated factor X
FXI:	Factor XI
FXIa:	Activated factor XI
FXII:	Factor XII

FXIIa: Activated factor XII

Fig.: Figure

MCF: Maximum clot formation

mRNA: Messenger ribonucleic acid

HDT: Head down tilt

HMWK: High-molecular-weight kininogen

ICU: Intensive care unit

PK: Pre-kallikrein

PAI-1: Plasminogen activator inhibitor 1

PC: Protein C

PCR: Polymerase chain reaction

PS: Protein S

R: Recovery

RNA: Ribonucleic acid

Scuba: Self-contained underwater breathing apparatus

sec.: Seconds

PE: Pulmonary embolism

TEM: Thrombelastometry

TAFI: Tissue factor inhibitor

TF: Tissue factor

TFPI: Tissue factor pathway inhibitor

TPA: Tissue pathway activator

t-PA: Tissue-type-Plasminogen activator

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Zusammenfassung

Hintergrund: Eine erhöhte Blutgerinnung und die damit in weiterer Folge riskanten Entstehung einer Thrombose werden als Folge von länger andauernder Bettruhe gesehen. Der molekulare Hintergrund mit einer möglich veränderten Genexpression von den beteiligten Faktoren wurde bis dato noch nicht näher untersucht. Mit der vorliegenden Arbeit sollen die Zusammenhänge zwischen Genexpression und veränderter Blutgerinnung dargestellt werden.

Material und Methoden: In einer randomisierten, cross-over Studie wurde die Genexpression der Gerinnungsparameter und der Faktoren die im Zusammenhang mit der Gerinnung stehen bei 12 männlichen gesunden Personen untersucht. Dies erfolgte im Bed rest Modell, wo die Probanden für 21 Tage in einer Kopftieflage von 6° verweilten. Zur Materialgewinnung dienten Blutproben vor, während und nach der Studie. Mittels Microarray-Technologie erfolgte die Ermittlung der Genexpression.

Resultate: Es zeigten sich bei den Studienteilnehmern durch die Immobilisation individuelle Reaktionen in der Genexpression betreffend das Gerinnungssystems. Lediglich für zwei Faktoren (Thrombin und Plasminogenaktivator) ergab sich beim t-test eine Signifikanz (p-Wert <0.05). Grundsätzlich ist aber festzuhalten, dass bei allen Studienteilnehmern die meisten Gene keine signifikante Veränderung in der Expression zeigten.

Diskussion: Die Ergebnisse dieser Studie zeigten, dass Bettruhe alleine zu keiner gesteigerten Genexpression in Bezug auf die Blutgerinnung führte. Wir sind der Meinung, dass zusätzliche Faktoren wie z.B. größere chirurgische Eingriffe für eine gesteigerte Gerinnung verantwortlich sind.

Abstract

Background: An increased blood clotting and its further complication thrombosis are associated with long-term Bed rest (BR). The molecular background with its possibility of changed expression in genes of the coagulation system has not been studied until now. In this study we want to show the gene expression and the connection between the bloods clotting system.

Materials and Methods: In this randomized, cross-over study we investigated the gene expression closely linked to coagulation in 12 healthy young men during long-term BR with head down tilt position 6° (HDT 6°). Blood samples were taken before, during and after BR. We used microarray technology to measure gene expression changes.

Results: All study subjects showed individual expression of coagulation genes due to immobilization. Only the expression of two genes (thrombin & plasminogen activator) were significant in t-test (p-value <0.05). Most of coagulation genes of study subjects did not change their expression significantly.

Discussion: The results of this study showed that bed rest itself does not result in increased gene expression concerning the coagulation system. We think that additional factors such as major surgery are reasons for increased coagulation.

I. INTRODUCTION

Blood clotting is associated with prolonged bed rest (BR). In previous literature there is a high correlation between immobilization and increased risk of blood clotting. It is shown that posture leads to hemodynamic changes (1-4) which support the development of a thrombus. A thrombus results of abnormal coagulation due to changes in vessel wall, hemodynamic disorders, defects of blood components or a combination of all (Virchow-Triad) (5-7). Depending on the localization of the thrombus (arterial or venous) further complication such as infarcts or embolism can occur (8). Thrombosis is one of the most feared complications in patients after major surgery primarily orthopedic surgery (9). Particularly old patients take longer time of remobilization after major surgery and are more often victims of immobility with its consequences (10). Further risk factors for developing increased intravascular coagulation with building of a thrombus are overweight, prolonged sitting position (e.g. oversea flights), oral contraceptives, smoking, malignant tumors or genetic disorders (11,12). Clinical symptoms of deep vein thrombosis are pain, swelling, heat, tenderness of lower extremity. To minimize the risk of developing thrombus it is state of the art to give anticoagulants after major surgery (e.g. femoral neck fracture) when patients might need to stay in bed for a longer period (9,13).

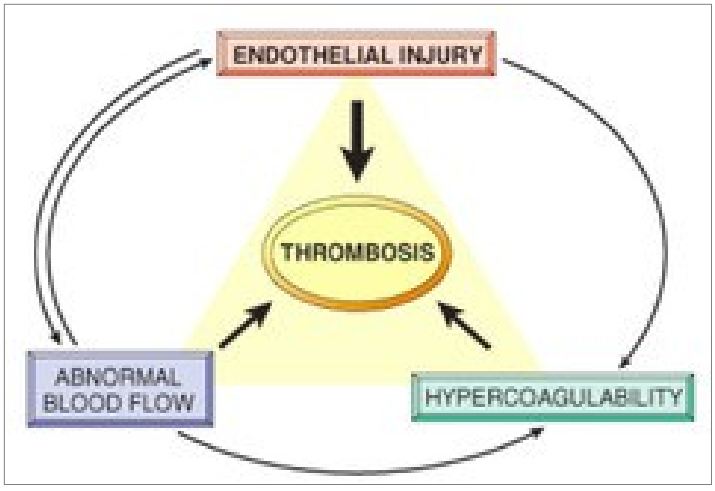

Figure 1: Virchow-Triad. Reproduced from http://quizlet.com/7053428/tb-grundlagen-pathologie-blutungenthrombose-embolie-flash-cards/

1 Immobilization

1.1 Definition

"A restriction of the movement of whole or part of the body by physical means (restraint, physical) or chemically by analgesia, or the use of tranquilizing agents or neuromuscular non depolarizing agents. It includes experimental protocols used to evaluate the physiologic effects of immobility." (14)

1.2 Physiology

Due to long-term immobilization the body shows changes on the cardiovascular, respiratory, digestive and renal system as well as on the immune system, on the locomotor apparatus and skin problems (10,15-17) .

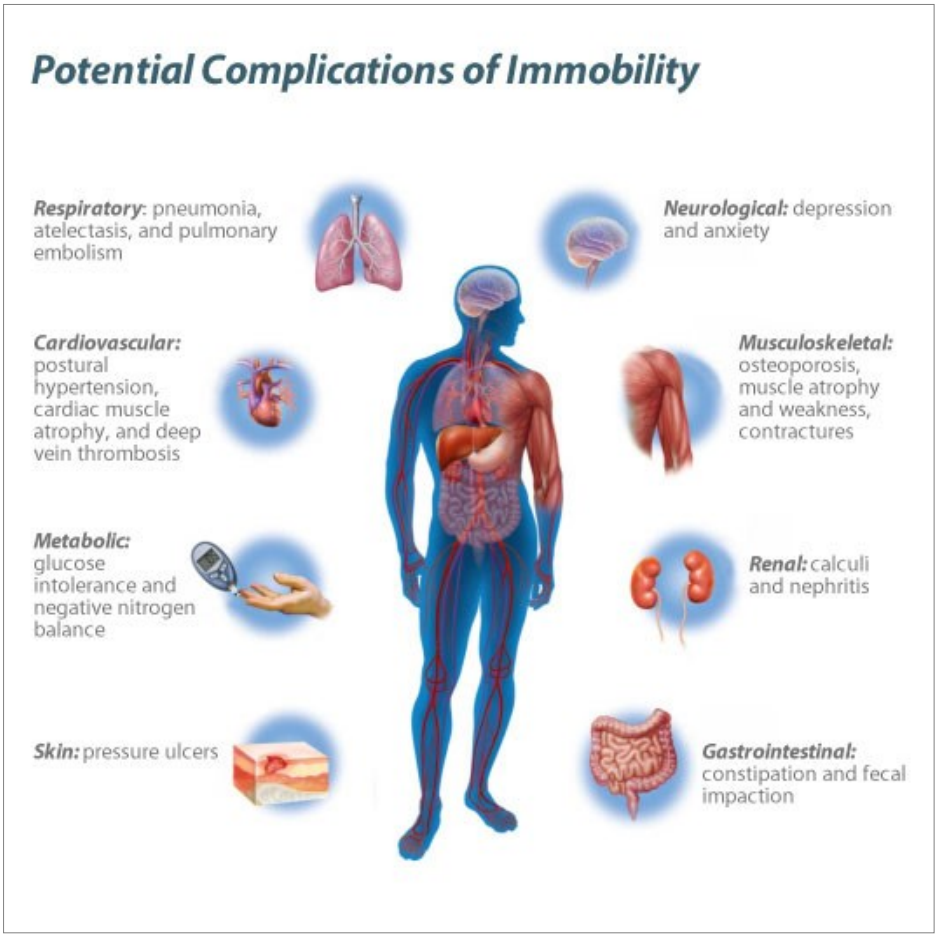

Figure 2: Systemic Effects of Immobility. Reproduced from http://www.hill-rom.com/usa/Clinical-Resources/Clinical-Focus-Areas/Safe-Patient-Handling/The-Challenge-of-Patient-Handling/The-Impact-on-Patients-/

Following principle changes can be noticed due to immobilization:

- Loss of muscle mass
- Bone demineralization
- Loss of plasma volume
- Decrease of ventilation
- Pressure sore

Long-term immobilization (therapeutic or lifestyle-related) has shown changes in the production of stress hormones. This increased stress responses lead to changes in the glucose metabolism and fat metabolism and subsequently through these changes in the metabolic system to chronic diseases. Due to this processes which result from immobility, changes in the coagulation system appear with its unwanted effects such as hypercoagulability (18).

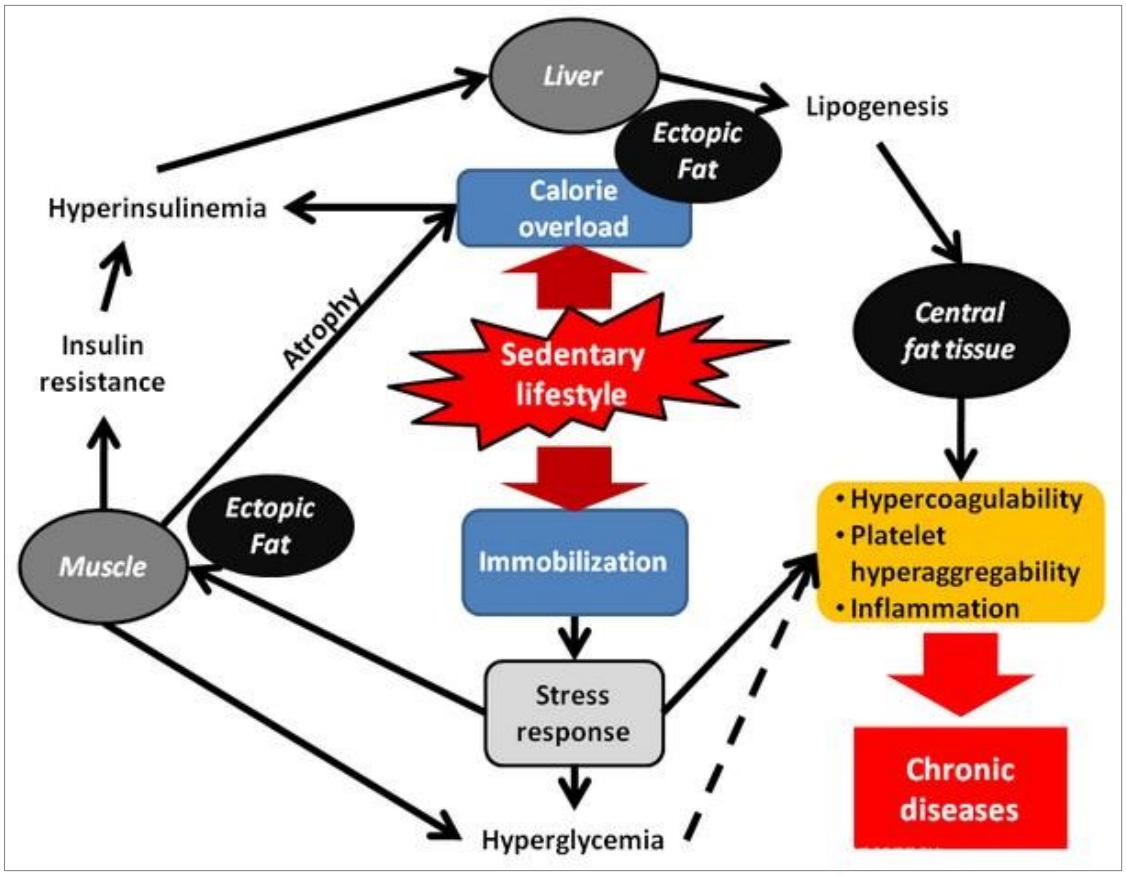

Figure 3: Consequences of Immobilization. Reproduced from http://www.discoverymedicine.com/Olivier-L-Charansonney/2011/09/09/physical-activity-and-aging-a-life-long-story/

1.3 Risk group

Mainly the listed persons are confronted with bed rest:

1. old people
2. orthopedic surgery patients
3. patients of intensive care units

1.4 Immobilization as a model

Beside the mentioned ways (see subheading risk group) which can lead to immobilization, in research especially in spaceflight medicine bed rest is a popular model. By this bed rest model physiological effects of spaceflight can be reproduced and show similarly effects like immobilization on earth. In the 1970 researchers discovered through sleeping problems of returned astronauts that the position of head down bed rest was alike being in space. They tested different angles of head down position and found out that 6° became the most comfortable position and serves as a reliable model (19) for spaceflight medicine.

1.5 Spaceflight deconditioning

The technological progress in space research and the fast growing and tremendous knowledge in space medicine of the last decades allow more and more long-term missions. Due to this longer stay in outer space and in microgravity the body of astronauts shows changes in their physiology. After space missions astronauts present loss of muscle mass (20-24), bone demineralization (22,24-27), problems in the vestibular system (24), changes in the cardiovascular system (24,28) and alteration in plasma volume (24). Main problems back on earth after long missions are orthostatic intolerance, which means difficulties in standing on back on earth, and weight loss, mainly as a result of demineralization and muscle loss. During spaceflight astronauts report of nausea, also described as space motion sickness, as a vestibular/central nervous problem. The loss of hydrostatic pressure, which helps to bring blood to the lower body on earth, shifts blood volume into the upper part of the body (29). As a result of this blood shift, which occurs due to microgravity and also in head-down tilt

position as a model of microgravity, central blood volume increases by approximately 400ml (2). Due to this blood volume shift the heart increases its stroke volume and cardiac output. Interestingly that measurement of central venous pressure (CVP) during microgravity and head-down tilt position showed different results. In microgravity values of direct CVP measurements decreased (30), whereas in investigations from bed rest analogs of space flight (head-down tilt position of 3°) CVP increased (2). As mentioned before a further changing in physiology during microgravity is plasma volume. Peter Norsk et al. showed that during a 12-h HDT position of 3° plasma volume increase because of volume shifts from the interstitial into the intravasal space (2). As a result of these mentioned changes, physiological characteristics especially characteristics of blood due to changes in the circulatory system are relevant in spaceflight medicine as well as in bedridden patients.

1.6 Ground analogues of microgravity

On earth signs of microgravity can be simulated by parabolic flight, water immersion or bed rest (31-34).

1.6.1 Parabolic flight

Parabolic flight is a model where test persons can undergo the feeling of weightlessness such as astronauts do when in space. Atmospheric aircraft maneuver are favored models in spaceflight research to investigate different physiological processes. On this method the airplane flies a parabolic trajectory (Fig.4) that can provide up to 40 seconds of free fall. The airplane accelerates from about 7000m at an angle of 45 degrees until it reaches an altitude of 10000m. In this phase hypergravity occurs (g level is at 1.8). Then the pilot turns of the machines and the aircraft starts to return to earth in freefall at 9,81m/s² (microgravity). At the end of microgravity hypergravity occurs again. To start the next parabola the pilot increases g-level from normogravity to hypergravity. This circle repeats 30 to 60 times with each 25 seconds of freefall (35).

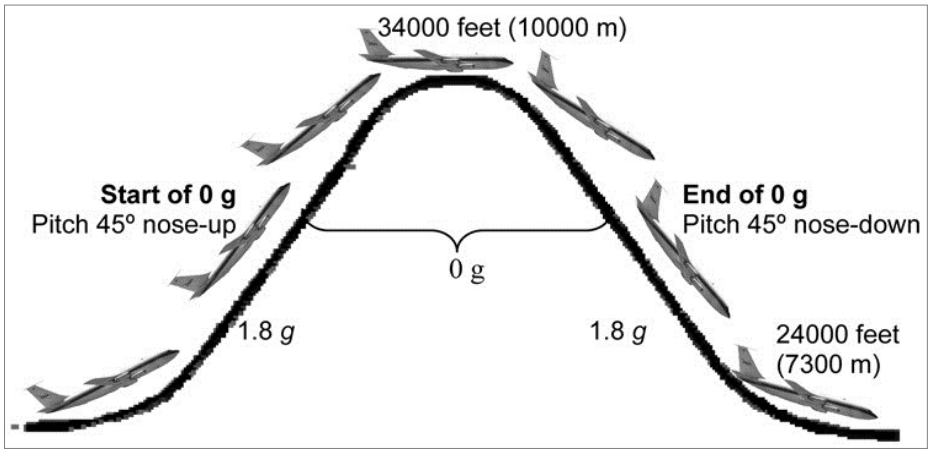

Figure 4: Parabolic flight model (35)

1.6.2 Water immersion

Water immersion is a further model to simulate hypogravity. Using this method subjects stay in a pool or in special designed immersion facility. The immersion can be whole body (with scuba) or head-out water immersion. The immersion media is mainly tap water, but some investigators also used 12% solution of sodium chloride or 0,9% saline. Water temperature should be at core temperature of the body. Changes in temperature showed significant influence on the investigation (e.g. on the circulatory system). By water immersion there is almost no force of gravity, so this method became an important tool of space research and space medicine. Studies showed that water immersion has effects on renal function and volume hemostasis such as it appears during space missions. This method makes it an important tool to gain better insight of physiological processes during microgravity and to gain further knowledge on this field and to develop different training programs. Furthermore studies showed that the physical fitness of subjects have another response on water immersion than untrained persons (34).

1.6.3 Bed rest (BR)

By head down tilt (HDT) position blood volume from the lower extremities shifts towards thorax and head and increases thereby the volume in the upper body (36). This volume shift induces changes in the circulatory system, which includes the blood, the heart and the blood vessels. This results in an altered flow velocity and cardiac output as well as in an increased share-stress on the vessel walls (2,4,7). The clinical major symptoms of these afore-mentioned changes due to posture are headache as a result of an increased intracranial pressure (blood pooling) and polyuria resulting from the flooded heart. These symptoms occur particularly at the beginning of the head down tilt position until the body adapts to the posture and reaches a new steady state. In space flight the same changes in the cardiovascular system occurs due to the loss of gravity. Therefore the HDT position is a suitable model on earth to simulate weightlessness (37). Beside the named circulatory effects other symptoms arise during space flight missions, for example dizziness and nausea. These phenomena occur due to the loss of the force of gravity by what that part of the macula, which regulates the static balance, is disturbed. A further clinical symptom of long-term BR is the fact of losing muscle mass, which is also an additional symptom during microgravity.

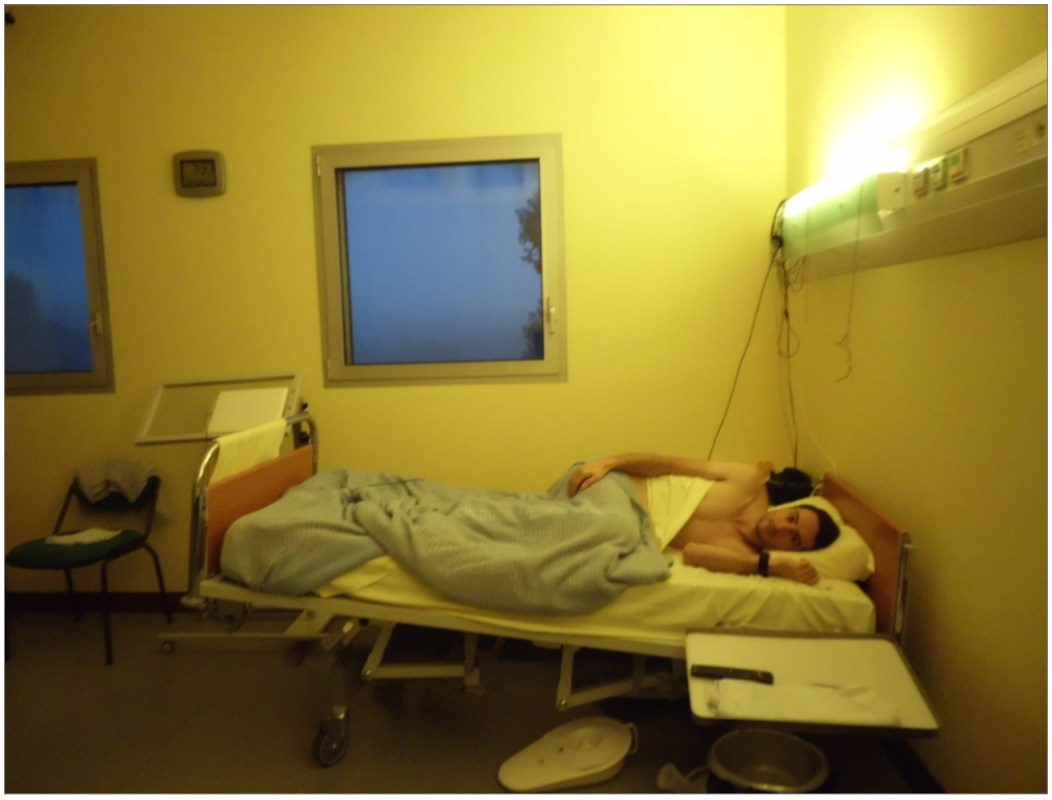

Figure 5: Head down tilt position 6° simulating microgravity

2 Coagulation system

2.1 Introduction

The purpose of the coagulation system is to protect the body of massive blood loss after injury. In the healthy body blood clotting occurs after an injury immediately within seconds and minutes. The coagulation process is a well-controlled system where the endothelium, blood cells, coagulation enzymes, co-factors as well as anticoagulant agents interact. Platelets are the first to react when there is an injured tissue (primary hemostasis) by adhesion. The primary hemostasis is supported by coagulation enzymes to build a fibrin clot (secondary hemostasis). Dysfunction in primary or secondary hemostasis leads to abnormal and prolonged bleeding or can increase coagulation (38).

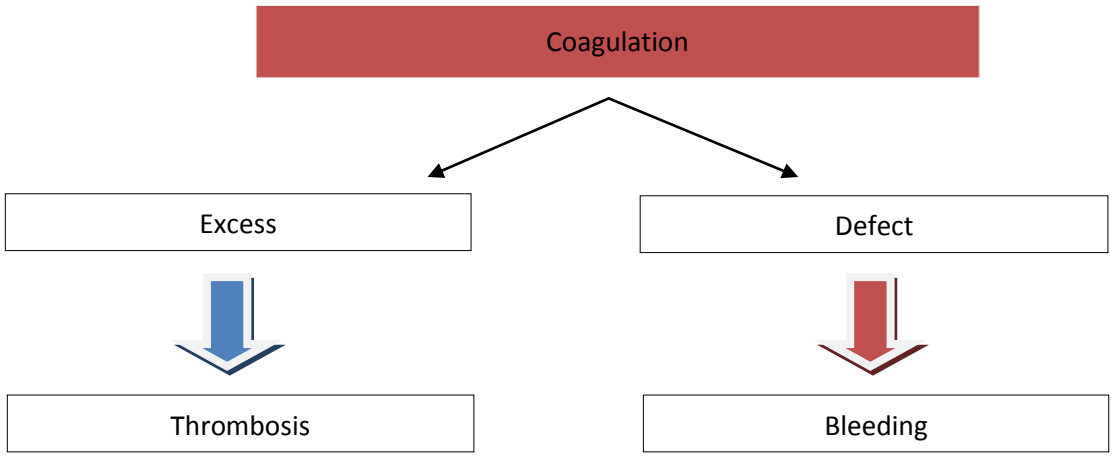

Figure 6: Dysfunction in Coagulation System

2.2 Endothelium

The Endothelium is the inner lining of blood vessels with many various functions. It is involved in vascular growth processes, in metabolism, intake and production of vasoactive hormones, in inflammatory processes, in the coagulation system as well as organ specific function (e.g. blood brain barrier) (39). The endothelium forms a barrier for in the blood existing dissolved substances, macromolecules and cells. The exchange of metabolism products or substrates between the lumen

and the interstitial space is active (40). The endothelium regulates the vascular tone by producing substances which can narrow (vasoconstrictors) or widen (vasodilatation) the blood vessels (see table 1).

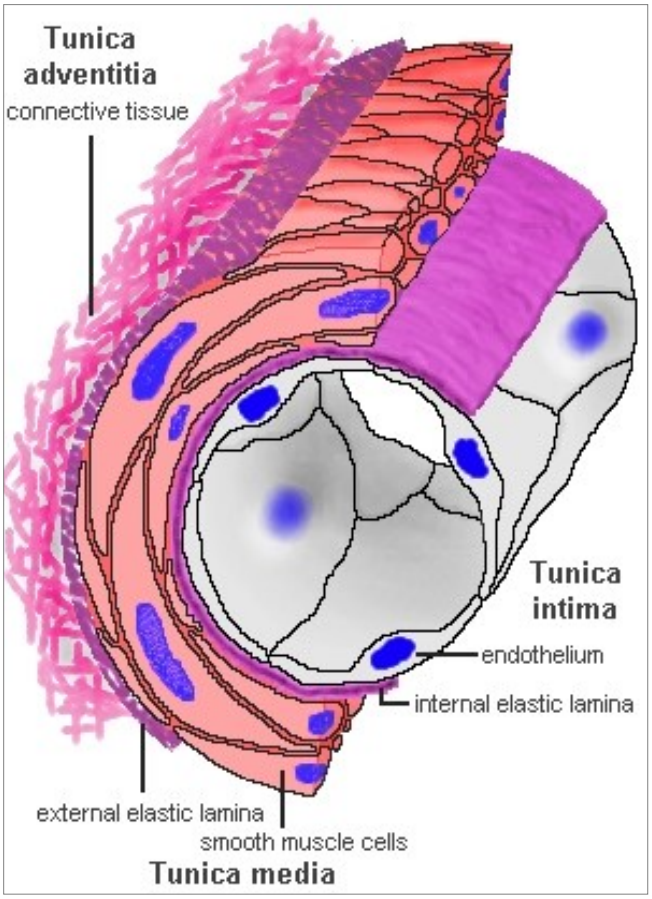

Figure 7: Structure of vessel wall. Reproduced from
http://www.lab.anhb.uwa.edu.au/mb140/corepages/vascular/vascular.htm

Table 1: Vasoactive endothelial mediated substances

Substance	Effect
Nitric oxide (NO)	Vasodilatation
Endothelin 1 (ET-1)	Vasoconstriction
Prostacycline (PGI_2)	Vasodilatation
Endothelin-derived hyperpolarizing factor (EDHF)	Vasodilatation
Endothelin-derived relaxing factor (EDRF)	Vasodilatation
Angiotensin converting enzyme (ACE)	Vasoconstriction
Natriuretic peptide C-Type (CNP)	Vasodilatation
Urotensin	Vasoconstriction
Endothelin-derived nitric oxide (EDNO)	Vasodilatation

The endothelium plays an important role in the coagulation system due to its pro- and anticoagulant function. In case of injury the endothelium starts blood coagulation via vasoconstriction. By exposure of transmembrane proteins such as von-Willebrand-Faktor it initiates the primary hemostasis (activation, adhesion and aggregation of thrombocytes) followed by the secondary hemostasis (activation of enzymes and co-factors) (41).

2.3 Thrombocytes

Thrombocytes are small, acaryote, disc-shaped blood cells with a diameter of 1-2 micrometer. They appear as fragments of megalokaryocytes and have a life cycle of 5-11 days. In the healthy body 150-400x10^9 cells exist. Thrombocytes are important cells in the initial phase of the coagulation system. They interact with the endothelium and help to stop bleeding within the first minutes (thrombocyte adhesion and thrombocyte aggregation) (42).

Figure 8: Electron microscope photograph of thrombocyte in inactive (left) and active (right) shape. Reproduced from http://anaesthesieintensivmedizin.charite.de/forschung/arbeitsgruppen/haemostaseologie/

Active thrombocytes release substances which help to build and stabilize blood clots (see table 2).

Table 2: Platelet release

Some of the major substances in thrombocytes and their effects	
Thromboxan A2	Aggregation, Vasoconstriction
Serotonin	Vasoconstriction
Catecholamine	Vasoconstriction
Thrombospondin	Irreversible Aggregation

2.4 Primary hemostasis

Blood vessels and thrombocytes are the two components which initiate the coagulation system after a lesion. While vasoconstriction is started by blood

vessels, adhesion, aggregation and release of thermostatic parts are the functions of thrombocytes.

When injury of blood vessels occurs, endothelial and subendothelial substances are exposed to the circulating thrombocytes. By exposure of this glycoprotein and collagen parts thrombocytes start to bind (adhesion). Through this interaction thrombocytes change their structure and simultaneously release components (activation) to draw further thrombocytes and agglutinate with them (aggregation) (Fig.9) (41,42).

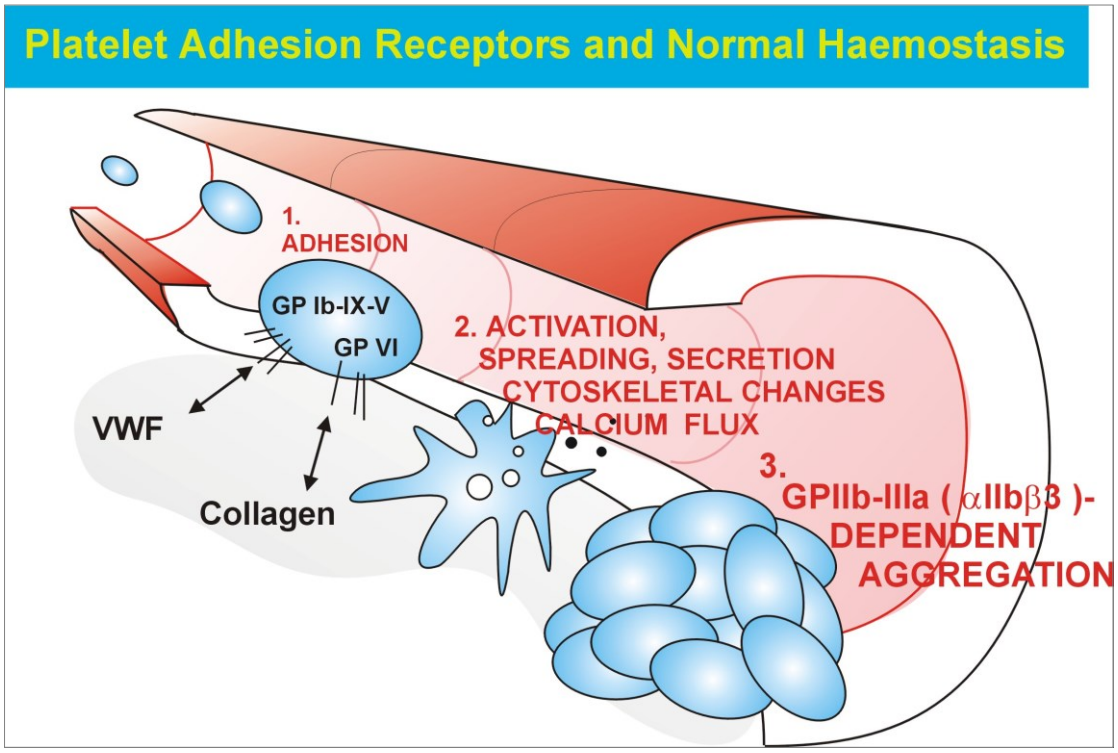

Figure 9: Order of Activation of Thrombocytes. Reproduced from http://www.acbd.monash.org/research/systemshaematology.html

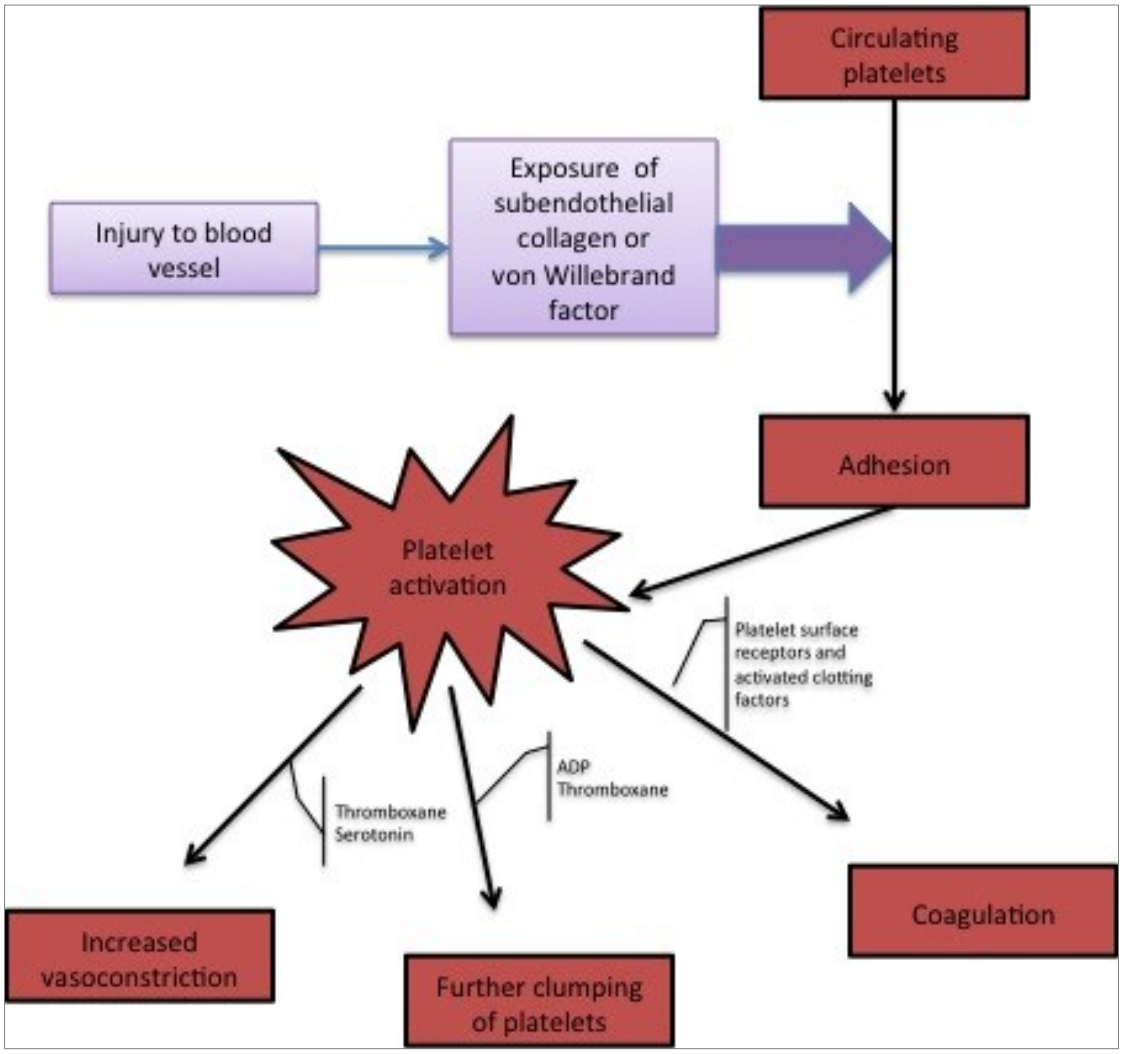

Figure 10: Flow chart of Primary Hemostasis. Reproduced from http://www.cpd4nurses.co.nz/Member/ViewArticle/1?pageNumber=2

2.5 Secondary hemostasis

Purpose of the secondary hemostasis is to make the initial blood clot more stable. This happens through number of coagulation factors which circulate in the blood, preliminary in an inactive form (see table 3) (38,41,42). There are 2 ways to activate the coagulation factors:

- Intrinsic pathway
- Extrinsic pathway

Table 3: Coagulation factors	
Factor number	Factor name
I	Fibrinogen
II	Prothrombin
III	Tissue factor
V	Labile factor
VII	Proconvertin
VIII	Antihemophilic factor
IX	Christmas factor
X	Stuart power factor
XI	Plasma thrombin antecedent
XII	Hageman factor
XIII	Fibrin-stabilizing factor

2.5.1 Intrinsic and Extrinsic pathway

When blood comes in contact with an exogenous surface (e.g. catheter) it starts to coagulate (intrinsic pathway). The intrinsic pathway or also named endogenous pathway is of secondary importance in daily life of healthy, active persons. In contrast: the exogenous pathway (extrinsic pathway) of the coagulation system is of the greatest import (41,42).

The aim of the coagulation cascade is to build thrombin, which itself leads to form the insoluble fibrin formation.

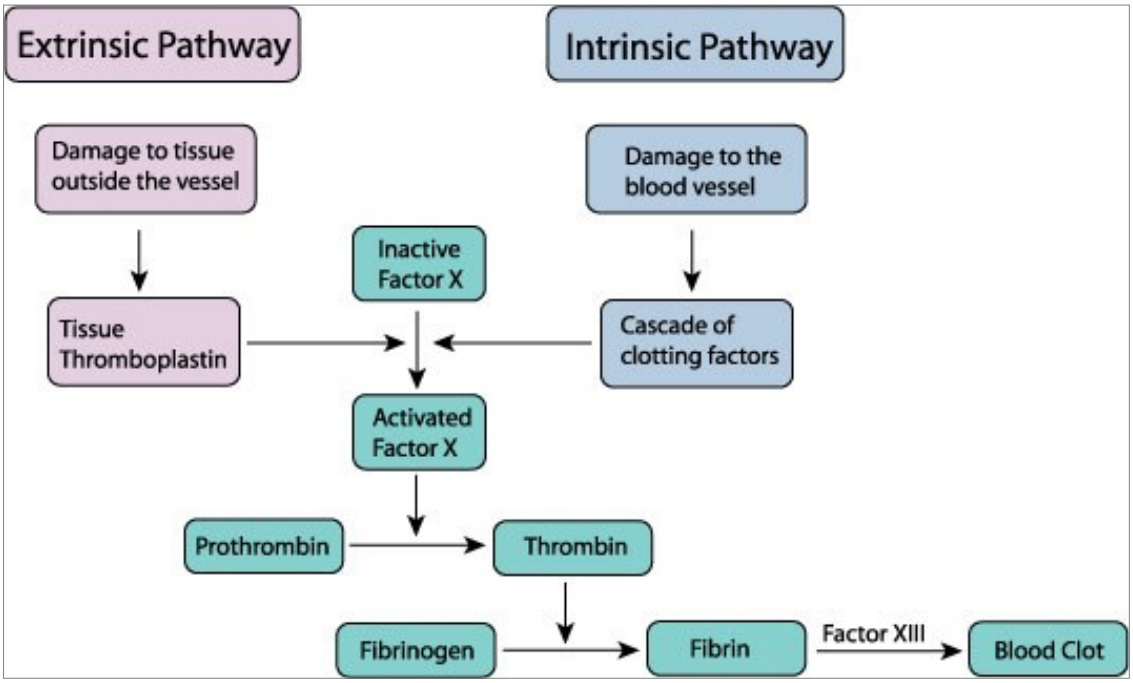

Figure 11: Overview of intrinsic and extrinsic pathway with the target to build a blood clot. Reproduced from http://healthyprotocols.com/2_bc.htm

Extrinsic pathway

The extrinsic pathway starts when blood comes in direct contact with cells expressing tissue factor (TF), also called thromboplastin or factor III (FIII). TF is an integral membrane protein normally protected via the endothelium, the inner lining of vessels and is therefore not exposed. TF exists in cells of organs where bleeding might be a great problem. High concentration of TF can be found in brain, heart, lung, uterus and placenta.

If blood vessels get injured, TF is immediately exposed and starts to bind with factor VII (FVII), a circulating seine protease. FVII is produced in the liver and a vitamin K-dependent protein. The complex of TF and FVIIa (a=activated) activates factor X (FX) and factor IX (FIX). Activated factors (FXa) activate further enzymes or increase the reaction cascade (FIXa) until the target component thrombin respectively fibrin is built. The coagulation cascade continues by a positive feedback of thrombin on factor XI (FXI). Reactions of this cascade are calcium

dependent and occur on phospolipid surfaces. To maintain the fibrin clot, thrombin activatable fibrinolysis inhibitor (TAFI) will be expressed at high levels (38).

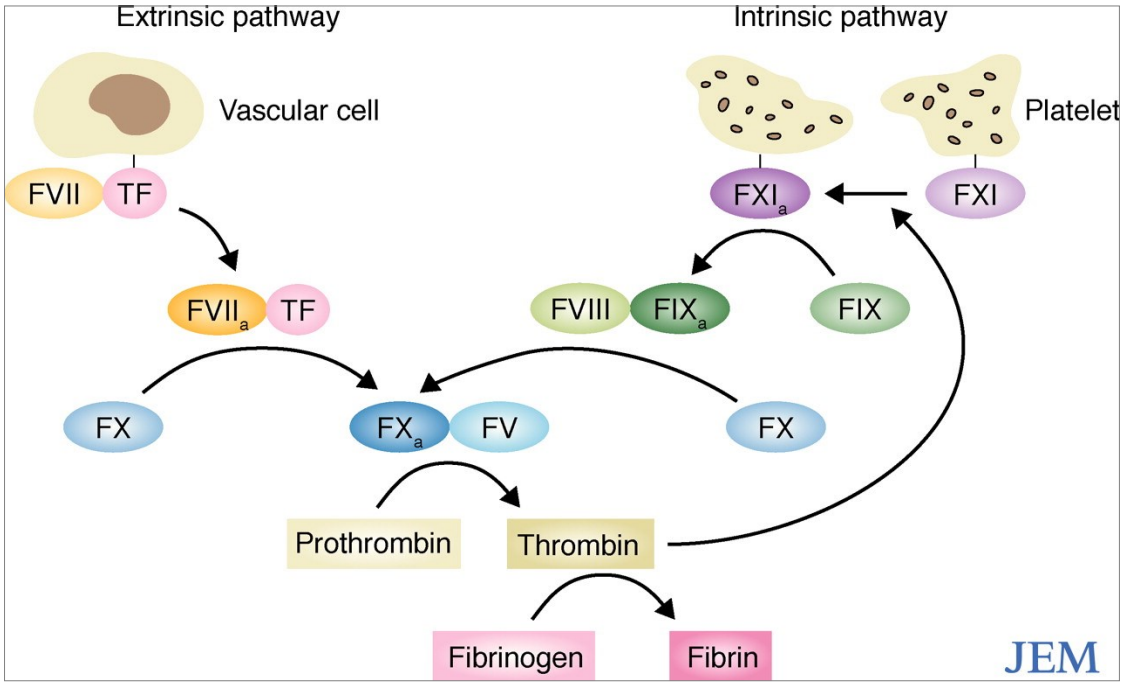

Figure 12: Coagulation Cascade Model with Intrinsic and Extrinsic pathway. Reproduced from http://jem.rupress.org/content/203/3/493/F1.expansion.html

Intrinsic pathway

Factor XII (FXII), pre-kallikrein (PK) and high-molecular-weight kininogen (HMWK) are the essential members of the intrinsic pathway, also named exogenous or contact pathway or kallikrein-kinin pathway. The first reaction of the intrinsic pathway is in contrast to the extrinsic pathway non calcium depended. FXII is activated by kallikrein or plasmin or can be activated through negatively charged surfaces like extracellular ribonucleic acid (RNA), collagen or polyphosphate. Activated factor XII (FXIIa) activates FXI with the help of HMWK. Activated factor XI (FXIa) activates FIX (FIXa) and FIXa activates FX. The latter activation already requires calcium, phospholipids and activated FVIII (FVIIIa). The activation of FX is from where the intrinsic and extrinsic pathways then have the same way to build the end product fibrin (43).

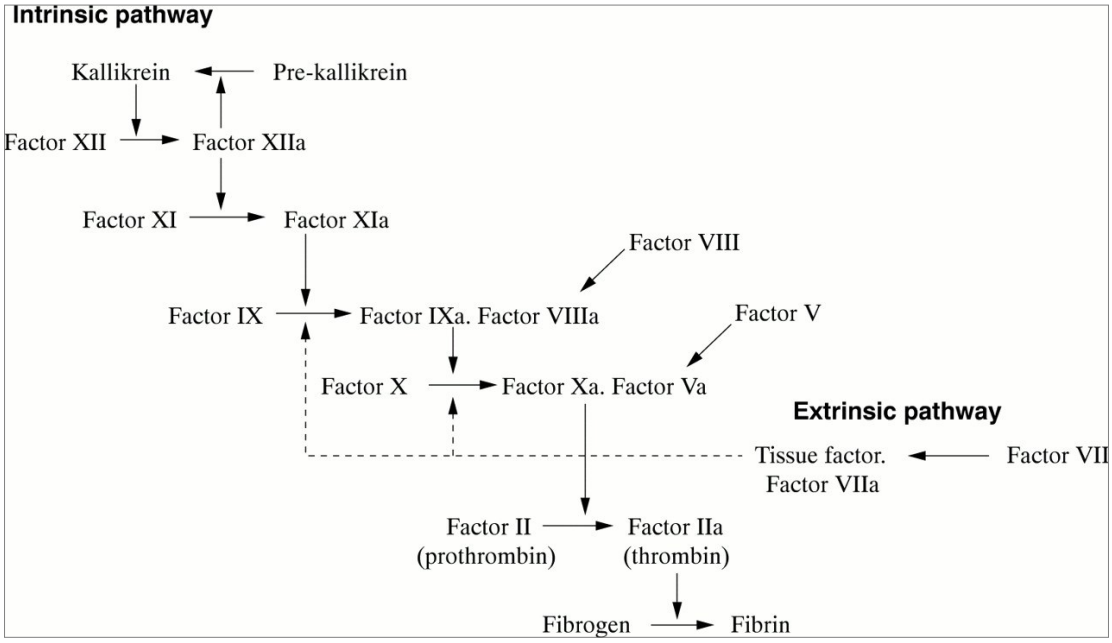

Figure 13: Coagulation Cascade Model showing the Intrinsic pathway to Clot formation. Reproduced from http://mp.bmjjournals.com/cgi/content-nw/full/55/2/127/F1

2.6 Fibrinolysis

To prevent of uncontrolled clotting, anticoagulant agents (fibrinolytic system) help to keep the balance and solubilize blood clots after the coagulation process. Tissue factors (t-PA, tissue-type-Plasminogenactivator) and plasma factors (e.g. $FXII_a$) convert plasminogen into plasmin, which is the essential substance for this process. Plasmin is a proteolytic enzyme, which has strong affinity to fibrin and stops its effect (blood clot). Moreover plasmin inhibits the effect of fibrinogen, prothrombin and some coagulation factors. Hence this system not only leads to dissolution of blood clots it also reduces the effect of the coagulation cascade (42).

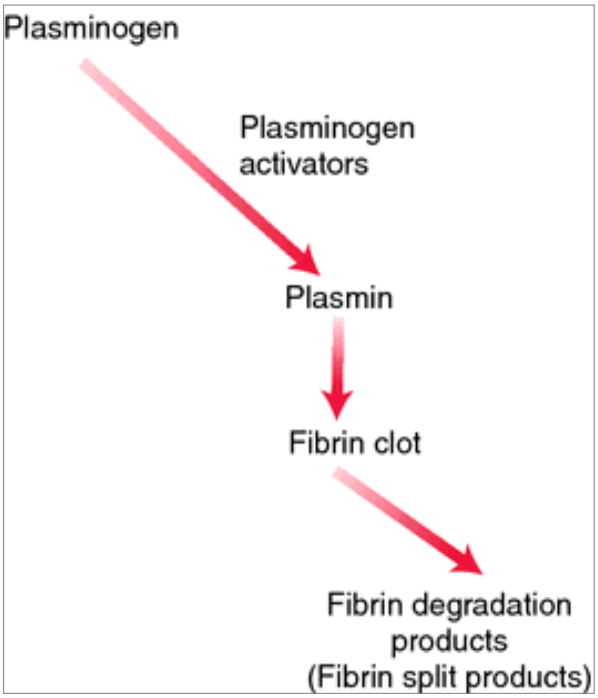

Figure 14: Fibrinolysis. Reproduced from http://medical-dictionary.thefreedictionary.com/Fibrinolysis+system

3 Molecular mechanisms

The investigation of genes is predominantly common in the field of prenatal diagnosis or to confirm certain diseases. But in the last decades it became more and more a tool to reveal the effects of environmental exposure (44). Emotional stress can influence the expression of genes. Changes in gene expression due to exogenous stress factors have shown changed responses in the immunological system (45).

3.1 DNA

Desoxyribonucleid acid (DNA) is a double stranded helix made of base pairs (adenine, thymine, guanine and cytosine), pentose sugar (desoxyribose) and 3',5'-phosphodiester bond (46). The DNA contains the total genetic information and the molecular biologist Griffith Frederick found in the year 1928 out that this information can be copied and transferred. Griffith took non-virulent bacterials and brought them together with pathogenetic ones and observed a transformation into

virulent bacterials (47). The DNA is so to speak the storage room with the information of protein synthesis.

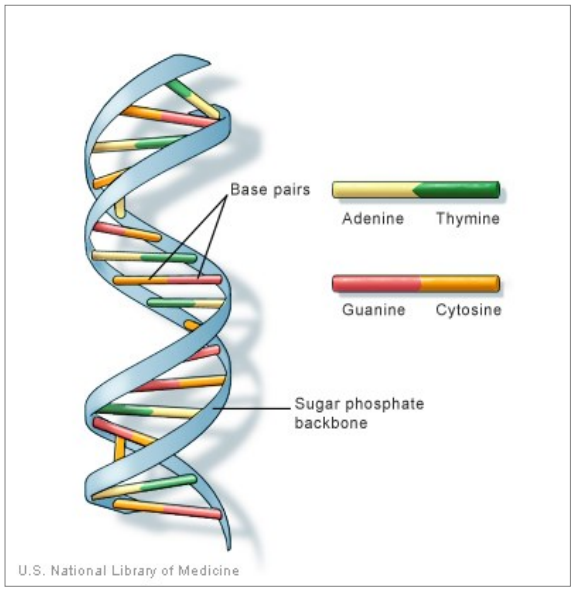

Figure 15: Structure of DNA. Reproduced from http://ghr.nlm.nih.gov/handbook/basics/dna.

3.2 RNA

Ribonucleic acid (RNA) has similar components as the DNA molecule. RNA is made of base pairs (adenine, uracil, guanine and cytosine), pentose sugar (ribose) and 3',5'-phosphodiester bond. RNA can be found as double stranded structure but usually appears as single stranded structure (47). RNA serves as a copy of the genetic information of the DNA. The process of copying the DNA information is named transcription. Transcription is followed by the post-transcriptional modification of the RNA. In doing so either messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) or other classes of RNA are going to be built (48). By using mRNA genetic information from the DNA is carried to ribosomes to do protein biosynthesis (=translation). The evidence of mRNA gives information of the current activity in cells respectively the expression of genes.

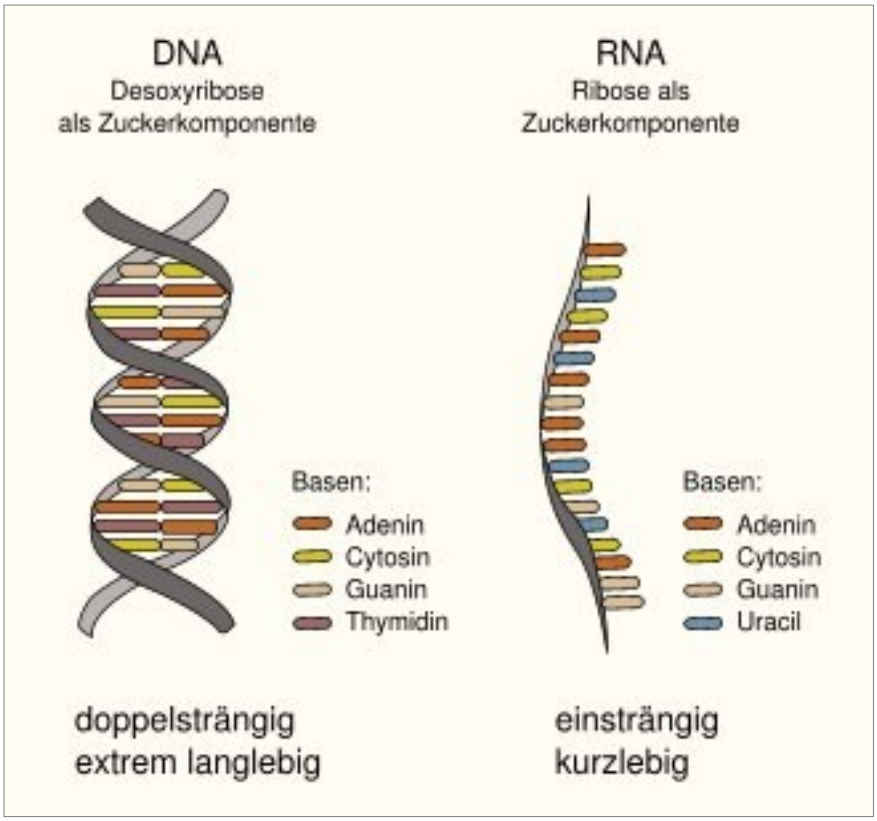

Figure 16: Differences between DNA and RNA: double stranded vs. single stranded, Thymidine vs. Uracil, Desoxyribose vs. Ribose. Reproduced from http://www.wissensschau.de/genom/rna_genom.php

3.3 Proteins

Proteins are molecules produced by translating mRNA. This procedure happens on ribosomes, the so called factories of protein synthesis situated on the endoplasmic reticulum. Proteins are important molecules as they build the fundament of all other molecules, because they are the ones through which the whole genome can be distributed to cells. A protein molecule is made of different combinations of the so called 21 proteinogenic amino acids. Proteinogenic amino acids are those which are involved in the production of protein molecules. The smallest proteins contain a number of 100 of the proteinogenic amino acids (48).

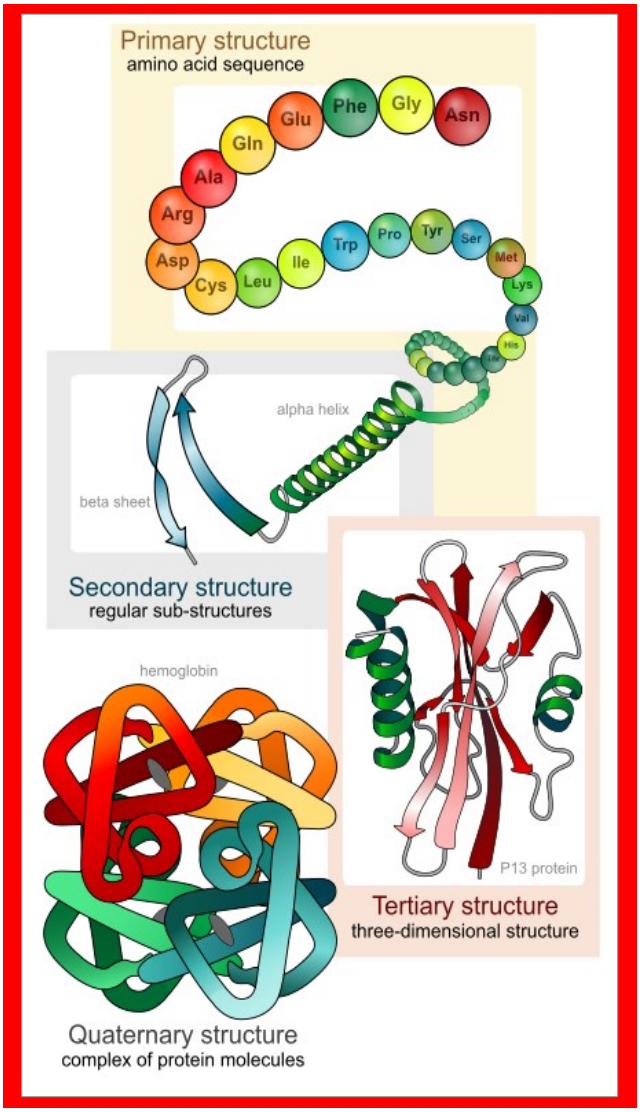

Figure 17: Protein structure. Reproduced from
http://en.wikipedia.org/wiki/Protein_structure

3.4 Central Dogma

The Central Dogma first described by Francis Crick states that the flow of genetic information is from DNA via RNA to Proteins (Fig. 18). This principle is basically seen as still valid but some authors say it is not an absolute principle and that the other way round is not totally impossible but has not been seen yet in evolution (49).

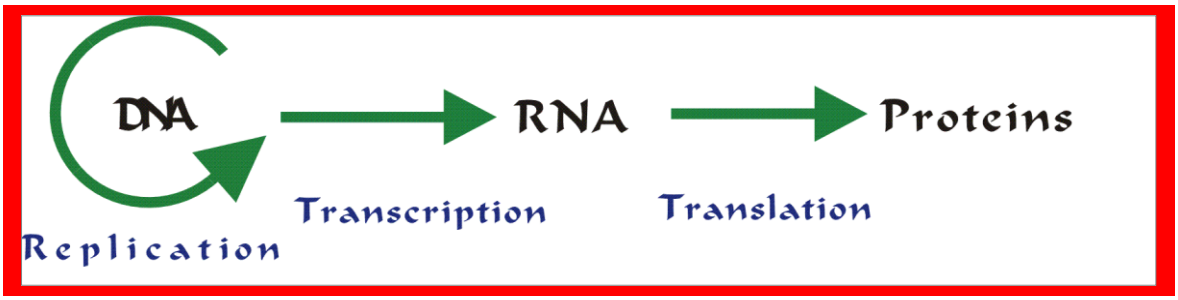

Figure 18: Central Dogma. Reproduced from
http://library.thinkquest.org/C0122429/intro/genetics.htm

3.5 OMICS - Transcriptomics

By continuous development of measuring techniques the technology of OMICS arose. OMICS is a suffix and means the investigation of large sets of biological molecules. The idea is to look at all biological molecules as a whole script to get a complete picture of gene expression pattern. Currently the most important OMIC technologies are genomics (sets of whole genes), transcriptomics (sets of RNA molecules), proteomics (sets of proteins) and metabolomics (sets of small molecules) (50). In this diploma thesis we focus on the investigation of transcriptomics. The molecules can be obtained from blood or saliva whereas saliva is mainly used in periodontal diseases but also to investigate cardiovascular diseases (51). The knowledge of those molecules, their correlation and reaction to exogenous stress factors could help to use them as a monitoring of diseases and to act earlier to subclinical signs. Gene expression of peripheral blood cells, as an easy accessible material, shows a high correlation with gene expression of other tissues and may therefore serve as a prognostic factor of biological changes (52). Thus researchers started to investigate genetic expression of pharmacological effects considering toxicity. Damages could be detected at molecular levels even before pathohistological evidence of the tissue was shown (53).

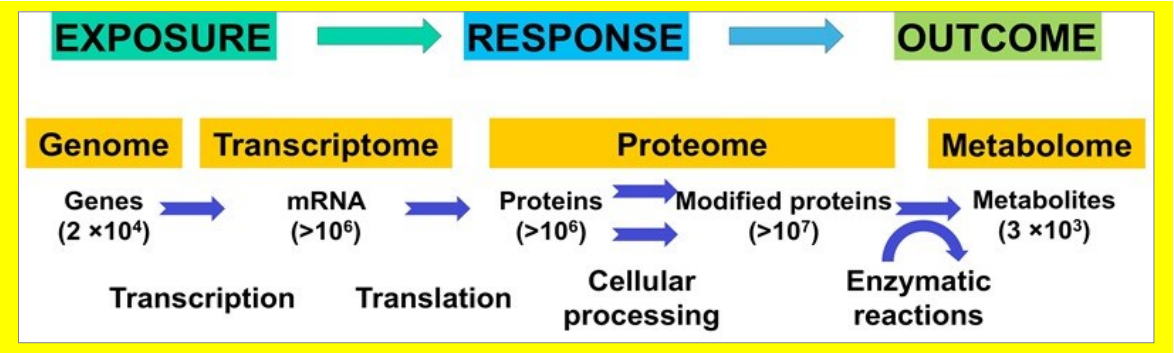

Figure 19: Molecular Response to Environmental Influence provided from Dr. Goswami Nandu

II. AIMS AND OBJECTIVES

Many patients have to stay in bed for weeks to recover from a major surgery or fracture. Old people are also quickly bedridden after an injury particularly in the last years of their life. As a side effect thrombosis might be an unwelcome problem of the situation. Therefore patients receive anticoagulant medication from the first day of bed rest to avoid the complication of thrombosis. At the moment bed rest is associated with changes in the coagulatory system and seen as a high risk of developing deep vein thrombosis. Nevertheless not everybody who is confined to bed must develop blood clotting with its further complications and therefore receive thrombosis prophylaxis. We hypothesize that bed rest reveals additional effects such as it does in short-term and is not the main reason of developing blood clotting. We think that the body will find a new steady state between the pro- and anticoagulatory system when changing posture for a longer period. Aim of this study is to show if bed rest has an influence on the coagulation system and if this can be seen on the molecular level. By this study we want to find out what happens to the expression of the coagulation genes during long-term bed rest. We think that our results are useful for patients who are confined to bed for longer periods but also in aviation and spaceflight medicine as the body develops similar signs as in bedridden persons.

III. MATERIAL AND METHODS

1. Experimental set up

The cross-over, randomized study started in November 2012. The study was divided into three campaigns. Each campaign took twenty-one days of experiments. In between the campaigns study subjects had a three months long break. First campaign of the study started in November 2012. We used peripheral blood as material of investigation withdrawn from an upper arm veins. Blood collection was performed by medical stuff persons of MEDES (Institute for Space Medicine and Physiology in Toulouse, France) between 7.00 and 8.30 a.m. on seven blood collecting time points within the twenty-one days campaign (see table 4). Check of body weight, monitoring of blood pressure and heart rate were done every morning before experiments.

2. Subject selection

In this pilot study we investigated twelve healthy, non-medicated men aged between twenty and fourty-five years. Subjects were recruited by French media and website of ESA (European Space Agency).

Subjects were familiarized with the test protocol and gave written informed consent. They were allowed to exit the test protocol at any time for any reason. Any acute, chronic or psychological disease which could be a risk during the experiment needed to be excluded. Each subject underwent a comprehensive medical examination before participating in the study. Subjects were also asked to avoid strenuous exercise in the week prior the study. Furthermore subjects were requested not consuming coffee or other stimulants within fourty-eight hours prior the trial. The protocol was approved by the MEDES Ethics Board in Toulouse and performed in accordance with the 1989 WMA Declaration of Helsinki.

3. Inclusion criteria

- Informed consent before trial-related activities
- Healthy male aged 20-45 years
- Body Mass Index 20-26 kg/m²
- Body Height 158-190 cm
- Aerobic endurance (measured on the basis of VO2 max)

  Age < 35yrs: 35 – 60 ml/min/kg

  Age > 35yrs: 30 – 60 ml/min/kg
- No orthopedic, musculoskeletal or cardiovascular disorder
- Non-smoker
- No alcohol, no drug dependence, no medical treatment
- Covered Social Security system
- Free of any engagement during the planned protocol
- Successful completion of the medical screening

4. Exclusion criteria

- Cardiac rhythm disorders
- Past record of orthostatic intolerance
- Chronic back pain
- History of hiatus hernia or gastro-esophageal reflux
- Past records of thrombophlebitis, family history of thrombosis or positive thrombosis screening procedure

- Abnormal result for lower limbs echo-doppler
- History of active claustrophobia
- History of genetic muscle or bone disease of any kind
- Bone mineral density: T-score <1.5
- Osteosynthesis material, presence of metallic implants
- History of knee problems or joint surgery/broken leg
- Poor tolerance to blood sampling
- Blood donation (more than 8ml/kg) less than 8 weeks before study start
- Special food diet, vegetarian or vegan
- History of intolerance to lactose or food allergy (milk proteins)
- Positive reaction to HVA IgM (hepatitis A), HBs antigen (hepatitis B), anti-HVC antibodies (hepatitis C), anti-HIV_{1+2} antibodies
- Echocardiography: inappropriate thoracic acoustic window
- Subject already participating or in the exclusion period of a clinical research
- Incarcerated persons
- Persons who might be non-compliant or unable to cooperate because of a language problem or poor mental development
- Subject who has received more than 4.500 Euros within 12 months for being a research subject
- Subject under guardianship or trusteeship

5. Bed rest model

For this study testing persons were put in head down tilt position 6° for twenty-one days (1 campaign). After this period of bed rest testing persons had a three months recovery period before the next bed rest campaign started. Testing persons were put in three groups (A, B, C). Testing persons of group A were put in a head down tilt position of 6° only. Testing persons of group B were put in a head down tilt position of 6° and had a defined exercise training program, also in head down tilt position of 6°. Group C was put in a head down tilt position of 6° and received additional protein nutrition. After a bed rest session of twenty-one days testing persons changed group in the next session.

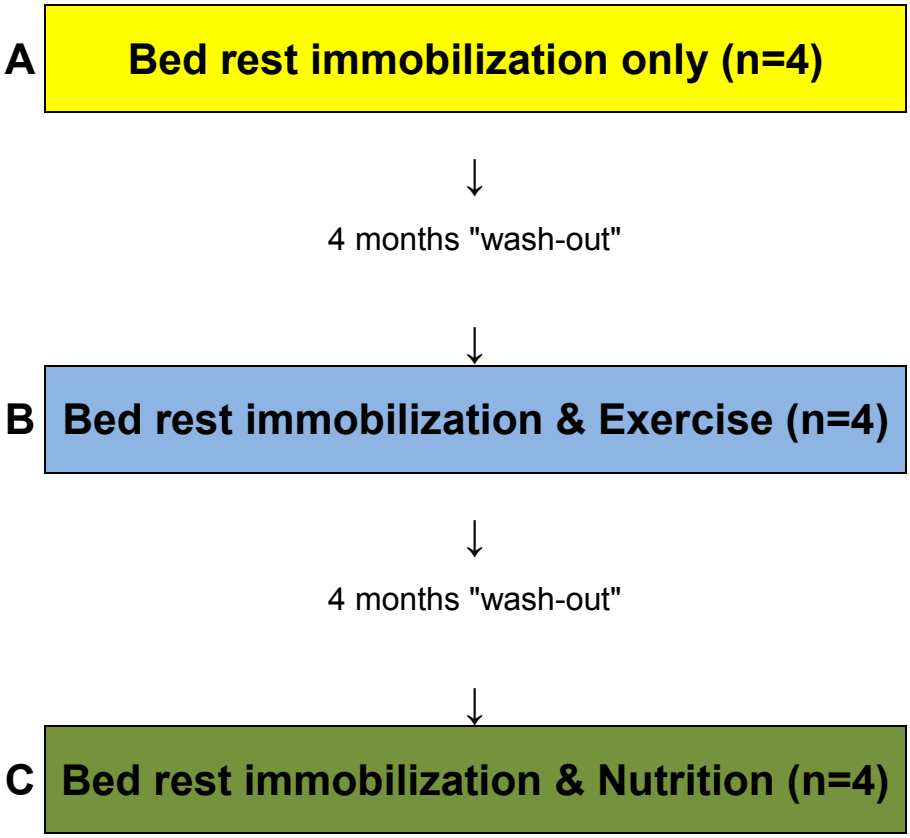

Figure 20: Cross-over, randomized study with wash-out period

6. Sample collecting time points

Test persons stayed five days in supine position prior head down tilt position 6°. First blood samples were taken at this time point (BDC-5). Further blood samplings were done within a following three week bed rest and post bed rest (recovery period=R). Blood was sampled on day 2 (HDT 2), day 7 (HDT 7), day 14 (HDT 14), day 21 (HDT 21) of head down tilt position 6°. Further blood sampling were done at the end of head down tilt (R 0) and day 2 of recovery (R 2).

Table 4: Timetable of blood collection before, during and after bed rest. The last line displays the labeling abbreviation.

Blood collecting time points						
Pre-bed rest	***Bed rest***				***Post-bed rest***	***Post-bed rest***
Day -5	*Day 2*	*Day 7*	*Day 14*	*Day 21*	*Day =0*	*Day =+2*
BDC-5	*HDT2*	*HDT7*	*HDT14*	*HDT21*	*R0*	*R+2*

For purposes of this thesis, data from pre-bed rest (BDC-5) and post-bed rest (R+2) of subjects doing BR only were examined

7. Sample collection

Blood as a material of investigation is a relatively easy accessibly substance and gives a wide range of information concerning physiology and pathophysiology. Because blood as a material is permanently in contact with its surrounding tissue reflects the cell metabolism as well as the transcriptome which means the current produced genes as well as RNA molecules as a sign of transcription activity (54). We investigated gene expression changes in whole blood. Therefore we collected

blood samplings by using butterfly catheter and Tempus Blood RNA tubes (Life Technology, Halle Belgium). Per blood collecting time point 6 ml blood was taken. After blood sampling blood tubes were ten times gently mixed and frozen within twenty minutes in a -80°C freezer. RNA sample analysis was done in Belgium by the Flemish Institute for Technological Research under supervision of Patrick De Boever.

8. RNA processing and quantification

To isolate RNA from whole blood tissue we used Tempus Spin RNA Isolation kit (Applied Biosystems). The system consists of Tempus blood RNA tubes and Tempus Spin RNA Isolation kit. Tempus blood RNA tubes contain 6 mL of Applied Biosystems Stabilizing Reagent which inactivates RNAse. RNA, gDNA and proteins as target products remain.

Blood from Tempus blood RNA tubes was transferred into 50-ml tubes and diluted with 1 x phosphate buffered saline (PBS), spun intensive for at least thirty seconds to bring up the lysate. Then centrifuged at 4°C at 3000 x g for thirty minutes and poured off supernatant. Resuspended RNA was transferred into purification filter and by micro centrifuge purified and eluted (Tempus Blood RNA Tube and Tempus Spin RNA Isolation Kit Protocol). RNA yield were checked by NanoDrop Spectrophotometer (Isogen Liefe Science, PW De Meern, the Netherlands) a medical device which can measure RNA assay from a very small sampling (0.05 - 2 µL) (www.nanodrop.com). By Ambion Globinclear kit (Applied Biosystems) globin mRNA was depleted from total RNA for increased transcript detection sensitivity. We determined the leftover globin-depleted RNA with Agilent 2100 Bioanalyzer using RNA 6000 Chips (Agilent Technologies, Diegem, Belgium) and stored samples at -80°C.

9. RNA amplification and labeling

By Low Input Quick Amp Labeling kit (one color) from Agilent Technologies globin-depleted RNA was amplified and labeled to produce a complementary RNA (cRNA). We reverse transcribed 100 to 200 ng of RNA into complementary DNA

(cDNA) applying T7-promotor primer and MMLV reverse transcriptase. T7 RNA Polymerase Blend amplifies target material and incorporate Cyanine 3-CTP (One-Color Microarray-Based Gene Expression Analysis, Protocol for use Agilent Gene Expression oligo microarrays, Version 6.6 Sept 2012) (see Fig. 20). We then transcribed cDNA into cRNA with cyanine 3-CTP. Using Qiagen's Rneasy mini spin columns (Qiagen, KJ Venlo, Netherlands) we purified the single-stranded, labeled cRNA. By using NanoDrop spectrophotometer we defined quantity and specific activity.

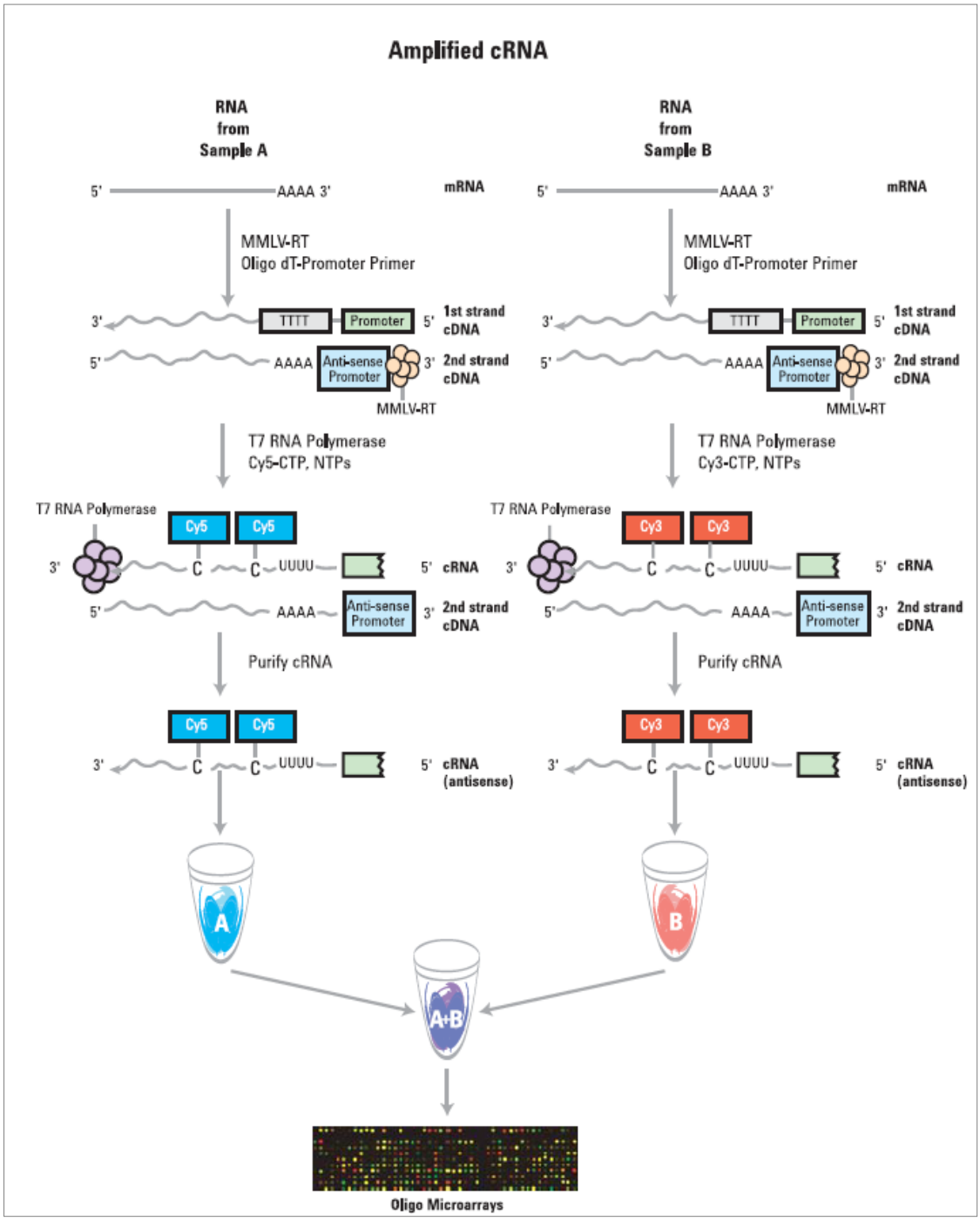

Figure 21: Process to replicate cRNA. Reproduced from http://www.genomics.agilent.com/article.jsp?pageId=2431

10. Microarray procedure and statistics

We used Tecan HS 4800™ pro microarray hybridization station to handle microarray slides. Therefore we put for a period of seventeen hours 1.65µg of cRNA to 4x44 Agilent Whole Human Genome microarray slides according to manufacturer's instruction. We then scanned all arrays by Agilent DNA microarray scanner (G2565BA) and processed with Agilent Feature Extraction Software Version 10.7. We used Agilent protocol GE-10.7-SEP09 to transform recorded images (tagged image file format) into text files. For every probe we used gProcessedsignal. For statistical analysis of signals we used Agilents's GeneSpring 12.1 software. Signals were quantile normalized and log_2-transformed. Copied regions on the array were saved as median signals. We collected all data files with expression signal of 41 000. We then filtered material according to expression intensity filter and matched to an approved HUGO gene symbol.

11. Statistical analysis

As a statistical hypothesis test we used paired sample t-tests for each examination. To correct multiple comparison of p-value we used Benjamini Hochberg false discovery rate. Signification was given when false discovery rate $p< 0.05$ and an absolute fold change of >1.5.

IV. RESULTS

1. Subject characteristics

In this study we investigated in twelve healthy, non-medicated male subjects the expression of genes correlated to coagulation during twenty-one days of bed rest. Study subjects were aged between 20 and 45 years old (average 34.3 years SD 8.3). Weight of study subjects was between 58 and 83 kg (average 69.8 kg SD 8.0). Height of study subjects was measured between 158 and 190 cm (average 176 cm SD 0.06) and BMI between 20 and 26 kg/m^2 (average 22.4 kg/m^2 SD 1.7).

Table 5: Anthropometric measurements of the subjects

Subject ID	Age [yrs]	Weight [kg]	Hight [cm]	BMI [kg/m2]
1	44	76.3	175	25
2	40	61.1	169	21
3	42	78.5	177	25
4	36	80.7	190	22
5	41	59	172	20
6	41	62.8	169	22
7	40	71.2	177	23
8	20	65	177	21
9	24	64.2	175	21
10	25	71.8	174	24
11	29	81.8	184	24
12	29	65.1	175	21

2. Microarray data generation

With microarray method we determined up- and/or down regulations in expression of coagulation genes during head down tilt position 6°. Therefore we extracted RNA from whole blood. Blood samples were taken from before and after HDT. Subjects showed different gene expression response during the three weeks recumbent position (see Fig.21 and Fig. 22). An up- respectively down regulation with a fold change of more than 1,5 indicates that the gene expression increased or decreased (indicated by a minus) by more than a half of the baseline value.

The following figure (Fig. 21) shows an up- and down regulation of 30.000 genes (red, yellow and blue lines) of three study subjects during bed rest (BR), three study subjects during bed rest combined with defined exercise (BR+EXERC) and another three study subjects during bed rest with additional protein nutrition (BR+NUT). The y-axis represents the level of fold change. Positive numbers indicate an increase, negative numbers a decrease of gene expression. The x-axis represents the study subjects. In this figure gene expression of nine subjects (three subjects per group) is displayed. In this diploma thesis only gene alterations during bed rest (BR), which is first graphic representation in figure 21, is considered.

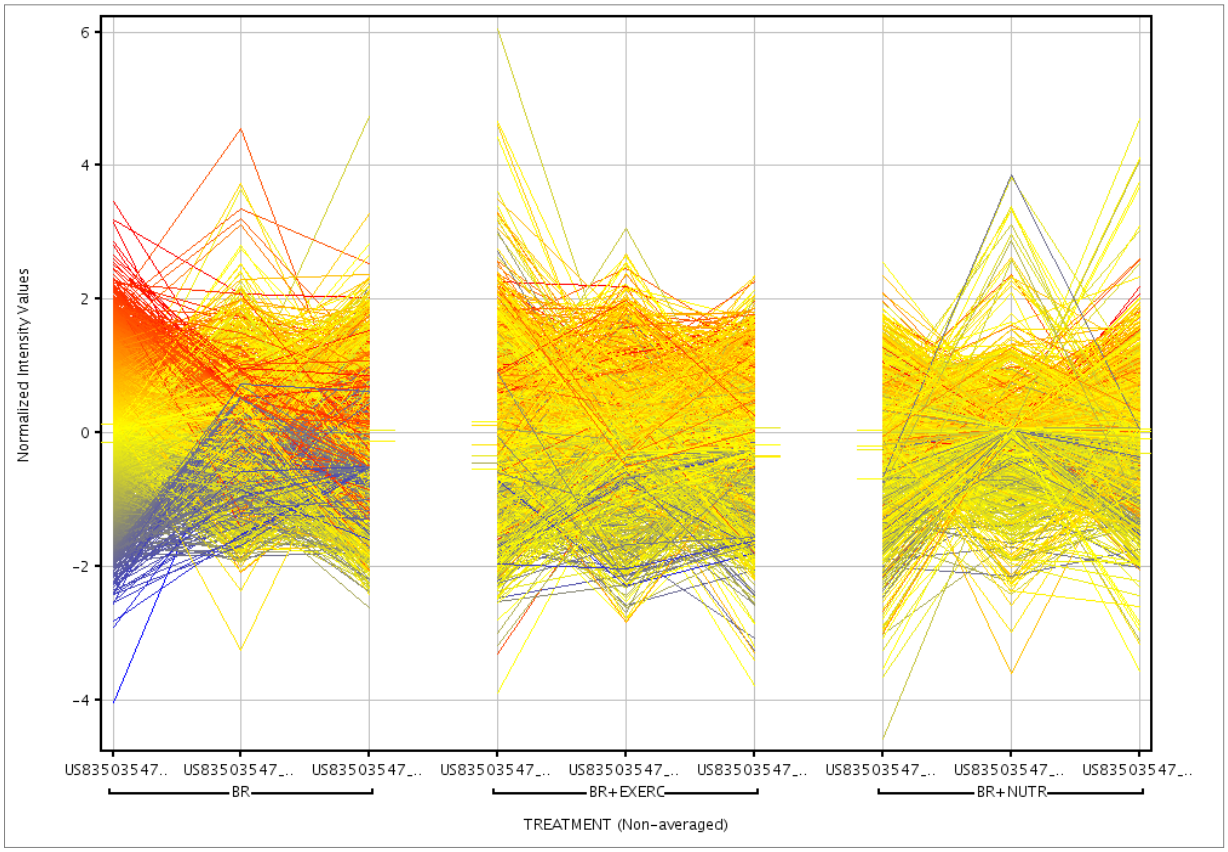

Figure 22: Gene expression variability during bed rest (BR), bed rest and exercise (BR+EXERC), bed rest and nutrition (BR+NUTR), graphical design provided by Dr. De Boever

As figure 21 gives an overview of all known genes at the present time, and I like to consider only genes affected regarding the coagulation system, we scanned all

genes of this profile associated with the endothelium. We ended up with over hundred genes related to endothelial function. For interest raw data of probes related to endothelial function can be submitted.

3. Coagulation genes

Using Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 220217) we extracted 191 genes that were annotated to the ontology term coagulation in the Haematological System Development and Function tree.

These 191 genes are then mapped back to Agilent probes (one gene can have multiple probes (signals) on the array). A total of 294 Agilent probes (related to ontology term coagulation) were retrieved. The fold change (ratio of post-bed rest versus pre-bed rest) for these probes are given in Figure 22. The message is that the fold changes are limited in range and that there can be significance between individual variability. Most of coagulation related genes did not change their expression during the experiment of long-term bed rest (representing yellow band in Fig.22). Only a few genes show a remarkable shift (see red line in Fig. 22). What we can also see is that there is no constant up regulation for a coagulation gene, which would be displayed as a flat red line. In contrary there is an individual variability in up- or down regulation of gene expression. For instance is the expression of gene x (red line) in subject 1 at a fold change of approximately 1.8, which means an 80% higher gene expression at the end of bed rest compared to gene expression before bed rest. Subject 2 only showed a fold change of approximately 1.2, which means an expression of twenty percent plus at the end of bed rest campaign. Whereas subject 3 shows a fold change less than 1.

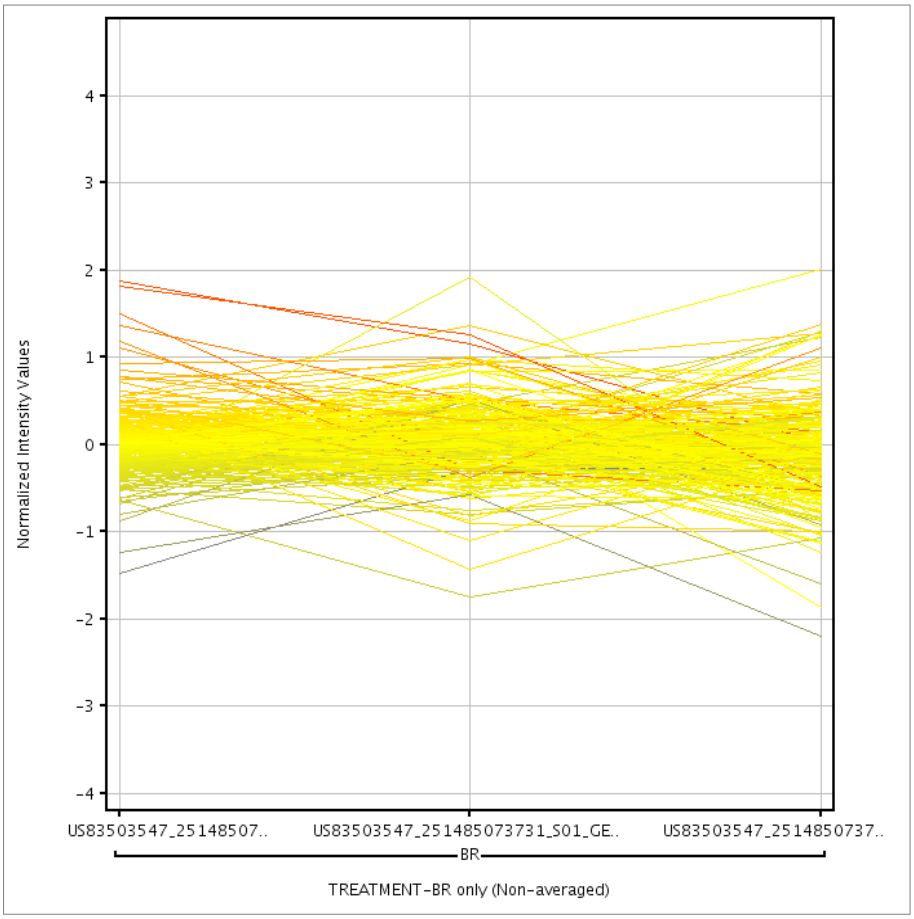

Figure 23: Expression of coagulation genes signals of three study subjects doing bed rest only (BR). The y-axis represents the level of fold change. Positive numbers represent an increase, negative numbers a decrease of gene expression. The x-axis corresponds to study subjects. Each line (red, orange and yellow) corresponds to a coagulation gene.

T-Test

By per probe t-test we determined if there is a differential gene expression. We performed 294 independent t-tests and obtained 294 p-values. Then, one can use different statistical thresholds (p-value) and/or biological thresholds (FC=fold change) to determine differentially expressed probes. Commonly a p-value of <0.05 as a statistical threshold and as a biological threshold a fold change of >1.5. By doing so we received two probes which we consider as significant. These two probes are the genes for Coagulation Factor II (F2), also named Thrombin, and Plasminogenactivator (PLAT). To avoid false positives we did no correction for multiplicity otherwise no probes would be significant.

However, we have a very limited number of samples and thus low power. In this case, statistical corrections are not very useful. This is more a pilot experiment with some exploratory analysis. A total of 294 Agilent probes (related to ontology term coagulation) were retrieved.

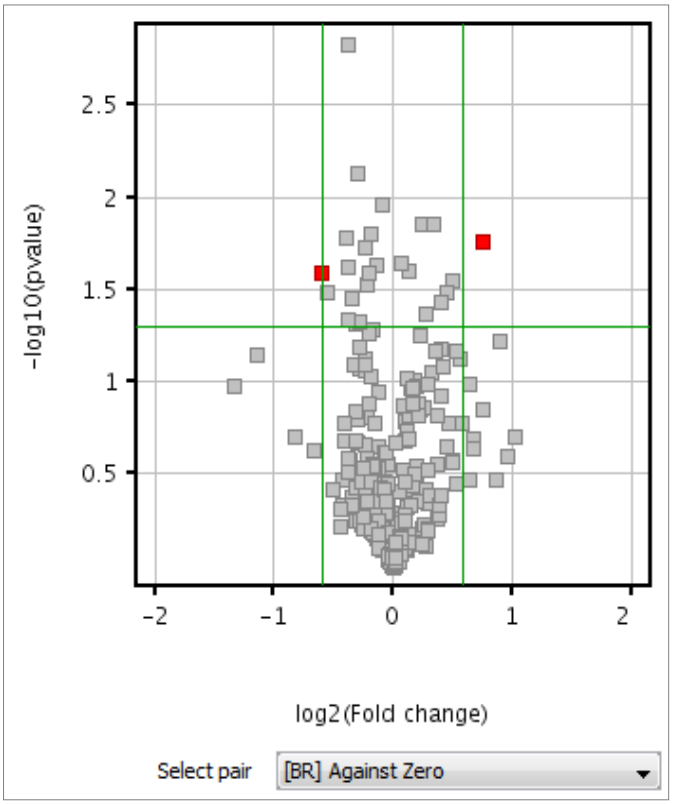

Figure 24: t-test results showing significant genes (2 red squares) with a fold change of 1.5, no correction of p-value

The next table shows genes which altered in expression (absolute fold change of >1.5 without p-value) when comparing pre bed rest and the post bed rest condition. This table shows data of only three study subjects. Therefore, data should be seen as supportive for other data or exploratory.

Table 6: Gene expression with absolute fold change >1.5

Gene	Fold Change	Expression polarity	Description
CSF3	1.68	up	Colony stimulating factor 3 (granulocyte) [NM_000759]
F2*	-1.53	down	Coagulation factor II (thrombin) [NM_000506]
PLAT*	1.68	up	Plasminogen activator [NM_000930]
CAV1	1.93	up	Caveolin 1 [NM_001753]
SERPINC1	2.03	up	Serpin peptidase inhibitor, clade C (antithrombin), member 1 [NM_000488]
PLG	1.72	up	Plasminogen (PLG) [NM_000301]
CXCL12	-2.22	down	mRNA for FLJ00404 protein [AK090482]
F7	1.59	up	Coagulation factor VII (serum prothrombin conversion accelerator) [NM_000131]
CCL11	-1.59	down	Chemokine (C-C motif) ligand 11 [NM_002986]
KIAA1715	1.56	up	mRNA for KIAA1715 [NM_030650]
F7	-1.78	down	Coagulation factor VII (serum prothrombin conversion accelerator) [NM_000131]
F2RL3	-2.53	down	mRNA for protease-activated receptor 4 [AF055917]
FZD6	1.81	up	Frizzled family receptor 6 [NM_003506]
C9	1.56	up	Complement component 9 [NM_001737]

Pathway Connection

Using Ingenuity Pathway Analysis (http://www.ingenuity.com, application build 220217) we have extracted 191 genes that were annotated to the ontology term coagulation in the Hematological System Development and Function tree.

The following graph shows the coagulation pathway with interactions between genes. Red means up regulation in expression at the end of the bed rest period, whereas green means deregulation. The bar chart next to the gene (corresponding to up- and down regulation in red or green) indicates the fold changes for the blood samples of the three individuals.

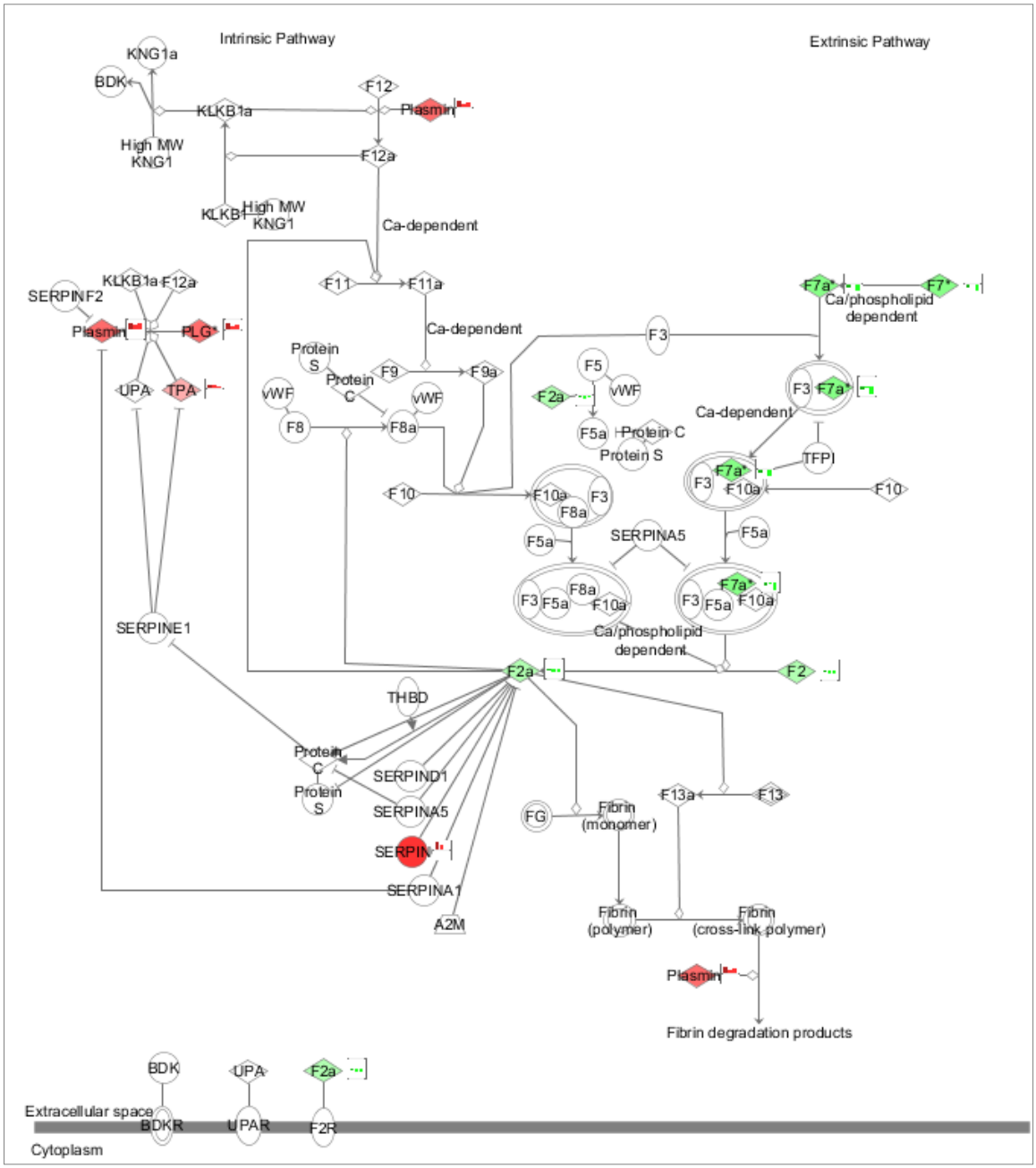

Figure 25: Molecular Interaction Network

V. DISCUSSION

With this study “Immobilization & Coagulation: Molecular mechanisms” we wanted to show the consequences of twenty-one days strict bed rest on gene expression particular on coagulation and fibrinolysis. This study showed that long-term bed rest itself has no significant impact on the expression of most of the coagulation genes. This findings correlate with a study carried out simultaneously in Toulouse in which alteration of pro- and anticoagulant factors during long-term bed rest were investigated.

In literature different findings concerning immobilization and changes in the coagulation system have been discussed. There is high evidence that long-term bed rest influences the coagulation system in a negative way. In previous literature long-term immobilization itself was linked with a high risk to develop thrombosis (55). But on the other hand Rosenfield et al. could state no critical increase of hemostasis during bed rest as estimated but pointed out a change of the fibrinolysis activity which might be a protective physiological reaction (56). It was believed that bed rest is a main cause of an increase of procoagulatory factors and therefore a high risk factor of developing thrombosis with its further complications. Although there was no study concerning bed rest without a previous event such as a major fracture or previous undergoing of a major surgery, it was highly believed that thrombosis is primarily developed by immobility.

In this study we could falsify the current opinion about immobilization and increased coagulation through an expected change of gene expression. In a parallel bed rest study in Berlin adaptation of the coagulation system during sixty days bed rest was investigated. Also in this Berlin bed rest study blood results showed no indication of increased coagulation and any kind of thrombus formation could not be detected via ultrasound (12).

Anti-thrombotic therapy is predominantly recommended as prevention in patients undergoing surgery presenting acute deep vein thrombosis as well as surgical patients with high, intermediate and low clinical suspicion of a thrombotic event (57). Thrombosis prophylaxis is also discussed and recommended in hospitalized

medical ill patients, patients with cancer and chronically immobilized persons (58-61). Decision of anti-thrombotic medication in nonsurgical patients shall be under consideration of unwanted side-effects such as bleeding (59) .

We are of the opinion that factors such as major surgery, smokers, taking medication, that influence the procoagulatory system such as oral contraceptives, genetic disorders of the coagulation system are mainly responsible for an increased response of blood clotting. And therefore these factors are the major reasons to develop thrombosis. The results of this study hence bring a new view on this chapter and might be from great interest regarding thrombosis prophylaxis.

In conclusion the present study as well as the parallel study showed clearly that immobilization itself has in healthy persons no negative effect on the coagulation system respectively on the molecular level (expression of coagulation genes).

VI. REFERENCES

(1) Laszlo Z, Rossler A, Hinghofer-Szalkay HG. Cardiovascular and hormonal changes with different angles of head-up tilt in men. Physiol Res 2001;50(1):71-82.

(2) Norsk P, Stadeager C, Johansen LB, Warberg J, Bie P, Foldager N, et al. Volume-homeostatic mechanisms in humans during a 12-h posture change. J Appl Physiol 1993 Jul;75(1):349-356.

(3) Hitosugi M, Niwa M, Takatsu A. Rheologic changes in venous blood during prolonged sitting. Thromb Res 2000 Dec 1;100(5):409-412.

(4) Gauer OH, Thron HL. Postural changes in the circulation. Handbook of Physiology 1965;Circ III:2409-2439.

(5) Kenichi TA, Nigel KS, Jerrold LH. Blood Coagulation: Hemostasis and Thrombin Regulation. Anesth Analg 2009;108(5):1433-1446.

(6) Thaler J, Ay C, Pabinger I. Clinical significance of circulating microparticles for venous thrombembolism in cancer patients. Hamostaseologie 2011;32:127-131.

(7) Jen CJ, McIntire LV. Characteristics of shear-induced aggregation in whole blood. J Lab Clin Med 1984 Jan;103(1):115-124.

(8) Böcker D, Heitz. Störung der Blutstillung und Blutgerinnung. In: W.Böcker, H.Denk, Ph.U.Heitz, H.Moch, editor. Repetitorium Pathologie. 2nd ed.: Urban & Fischer; 2008. p. 91-97.

(9) Edelsberg J, Hagiwara M, Taneja C, Oster G. Risk of venous thromboembolism among hospitalized medically ill patients. Am J Health Syst Pharm 2006 Oct 15;63(20 Suppl 6):S16-22.

(10) Creditor MC. Hazards of hospitalization of the elderly. Ann Intern Med 1993 Feb 1;118(3):219-223.

(11) Pabinger I, Ay C. Risk of venous thromboembolism and primary prophylaxis in cancer. Should all patients receive thromboprophylaxis? Hamostaseologie 2012;32(2):132-137.

(12) Haider T, Gunga HC, Matteucci-Gothe R, Sottara E, Griesmacher A, Belavy DL, et al. Effects of long-term head-down-tilt bed rest and different training regimes on the coagulation system of healthy men. Physiol Rep 2013 Nov;1(6):e00135.

(13) Geerts WH, Pineo GF, Heit JA, Bergqvist D, Lassen MR, Colwell CW, et al. Prevention of venous thromboembolism: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2004 Sep;126(3 Suppl):338S-400S.

(14) Definition Immobilization. Available at: http://han.medunigraz.at/han/pubmed/www.ncbi.nlm.nih.gov/mesh/?term=immobilization. Accessed September, 2013.

(15) Arinell K, Christensen K, Blanc S, Larsson A, Frobert O. Effect of prolonged standardized bed rest on cystatin C and other markers of cardiovascular risk. BMC Physiol 2011 Dec 9;11:17-6793-11-17.

(16) Asher RA. The dangers of going to bed. Crit Care Update 1983 May;10(5):40-1, 51.

(17) Poulsen JB. Impaired physical function, loss of muscle mass and assessment of biomechanical properties in critical ill patients. Dan Med J 2012 Nov;59(11):B4544.

(18) Charansonney OL. Physical activity and aging: a life-long story. Discov Med 2011 Sep;12(64):177-185.

(19) Pavy-Le Traon A, Heer M, Narici MV, Rittweger J, Vernikos J. From space to Earth: advances in human physiology from 20 years of bed rest studies (1986-2006). Eur J Appl Physiol 2007 Sep;101(2):143-194.

(20) Fitts RH, Trappe SW, Costill DL, Gallagher PM, Creer AC, Colloton PA, et al. Prolonged space flight-induced alterations in the structure and function of human skeletal muscle fibres. J Physiol 2010 Sep 15;588(Pt 18):3567-3592.

(21) LeBlanc A, Lin C, Shackelford L, Sinitsyn V, Evans H, Belichenko O, et al. Muscle volume, MRI relaxation times (T2), and body composition after spaceflight. J Appl Physiol 2000 Dec;89(6):2158-2164.

(22) Rabin R, Gordon SL, Lymn RW, Todd PW, Frey MA, Sulzman FM. Effects of spaceflight on the musculoskeletal system: NIH and NASA future directions. FASEB J 1993 Mar;7(5):396-398.

(23) Vandenburgh H, Chromiak J, Shansky J, Del Tatto M, Lemaire J. Space travel directly induces skeletal muscle atrophy. FASEB J 1999 Jun;13(9):1031-1038.

(24) West JB. Physiology in microgravity. J Appl Physiol 2000 Jul;89(1):379-384.

(25) LeBlanc A, Schneider V, Shackelford L, West S, Oganov V, Bakulin A, et al. Bone mineral and lean tissue loss after long duration space flight. J Musculoskelet Neuronal Interact 2000 Dec;1(2):157-160.

(26) McCarthy EF. Perspective: skeletal complications of space flight. Skeletal Radiol 2011 Jun;40(6):661-663.

(27) Smith SM, McCoy T, Gazda D, Morgan JL, Heer M, Zwart SR. Space flight calcium: implications for astronaut health, spacecraft operations, and Earth. Nutrients 2012 Dec 18;4(12):2047-2068.

(28) Hughson RL, Shoemaker JK, Blaber AP, Arbeille P, Greaves DK, Pereira-Junior PP, et al. Cardiovascular regulation during long-duration spaceflights to the International Space Station. J Appl Physiol 2012 Mar;112(5):719-727.

(29) Hargens AR, Richardson S. Cardiovascular adaptations, fluid shifts, and countermeasures related to space flight. Respir Physiol Neurobiol 2009 Oct;169 Suppl 1:S30-3.

(30) Hughson RL. Recent findings in cardiovascular physiology with space travel. Respir Physiol Neurobiol 2009 Oct;169 Suppl 1:S38-41.

(31) Petersen LG, Damgaard M, Petersen JC, Norsk P. Mechanisms of increase in cardiac output during acute weightlessness in humans. J Appl Physiol 2011 Aug;111(2):407-411.

(32) Schlegel TT, Brown TE, Wood SJ, Benavides EW, Bondar RL, Stein F, et al. Orthostatic intolerance and motion sickness after parabolic flight. J Appl Physiol 2001 Jan;90(1):67-82.

(33) Schlegel TT, Benavides EW, Barker DC, Brown TE, Harm DL, DeSilva SJ, et al. Cardiovascular and Valsalva responses during parabolic flight. J Appl Physiol 1998 Nov;85(5):1957-1965.

(34) Epstein M. Renal effects of head-out water immersion in humans: a 15-year update. Physiol Rev 1992 Jul;72(3):563-621.

(35) Karmali F, Shelhamer M. The dynamics of parabolic flight: flight characteristics and passenger percepts. Acta Astronaut 2008 Sep;63(5-6):594-602.

(36) Nixon JV, Murray RG, Bryant C, Johnson RL,Jr, Mitchell JH, Holland OB, et al. Early cardiovascular adaptation to simulated zero gravity. J Appl Physiol Respir Environ Exerc Physiol 1979 Mar;46(3):541-548.

(37) Jost PD. Simulating human space physiology with bed rest. Hippokratia 2008 Aug;12 Suppl 1:37-40.

(38) Spronk HM, Govers-Riemslag JW, ten Cate H. The blood coagulation system as a molecular machine. Bioessays 2003 Dec;25(12):1220-1228.

(39) Bassenge E. Endothelial function in different organs. Prog Cardiovasc Dis 1996 Nov-Dec;39(3):209-228.

(40) Neumann H editor. Das Gerinnungssystem. Physiologie und Pathophysiologie - eine Einführung. 1st ed. Berlin: ABW Wissenschaftsverlag; 2007.

(41) Hoffbrand A.V., Moss P.A.H. Platelets, blood coagulation and haemostasis. In: Hoffbrand A.V., Moss P.A.H., editors. Haematology. 6th ed.: Wiley-Blackwell; 2011. p. 314-329.

(42) Schmidt L, Heckmann. Blut und Immunabwehr. Physiologie des Menschen mit Pathophysiologie. 31st ed. Heidelberg: Springer; 2010. p. 492-497.

(43) McMichael M. New models of hemostasis. Top Companion Anim Med 2012 May;27(2):40-45.

(44) Rappaport SM, Smith MT. Epidemiology. Environment and disease risks. Science 2010 Oct 22;330(6003):460-461.

(45) De Boever P, Wens B, Forcheh AC, Reynders H, Nelen V, Kleinjans J, et al. Characterization of the peripheral blood transcriptome in a repeated measures design using a panel of healthy individuals. Genomics 2013 Dec 7.

(46) Clark DP, Pazedernik NJ. Chapter 3 DNA, RNA, and Protein. Molecular Biology. Second Edition ed.: Elsevier; 2013. p. 62-93.

(47) Watson JD, Baker T, Bell S, Gann A, Levine M, Losick R. Kapitel 2 Nucleinsäuren als Träger der genetischen Information. In: Watson JD, editor. Watson Molekularbiologie. 6. aktualisierte Auflage ed.: Person Studium; 2011. p. 25-49.

(48) Horn F editor. Biochemie des Menschen. 5. korrigierte Auflage ed. Germany: Thieme Georg; 2012.

(49) Koonin EV. Does the central dogma still stand? Biol Direct 2012 Aug 23;7:27-6150-7-27.

(50) Vlaanderen J, Moore LE, Smith MT, Lan Q, Zhang L, Skibola CF, et al. Application of OMICS technologies in occupational and environmental health research; current status and projections. Occup Environ Med 2010 Feb;67(2):136-143.

(51) Miller CS, Foley JD, Bailey AL, Campell CL, Humphries RL, Christodoulides N, et al. Current developments in salivary diagnostics. Biomark Med 2010 Feb;4(1):171-189.

(52) Liew CC, Ma J, Tang HC, Zheng R, Dempsey AA. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. J Lab Clin Med 2006 Mar;147(3):126-132.

(53) Van Hummelen P, Sasaki J. State-of-the-art genomics approaches in toxicology. Mutat Res 2010 Dec;705(3):165-171.

(54) Burczynski ME, Dorner AJ. Transcriptional profiling of peripheral blood cells in clinical pharmacogenomic studies. Pharmacogenomics 2006 Mar;7(2):187-202.

(55) Cushman M. Epidemiology and risk factors for venous thrombosis. Semin Hematol 2007 Apr;44(2):62-69.

(56) Rosenfeld BA, Faraday N, Campbell D, Sakima N, Bell W. The effects of bedrest on circadian changes in hemostasis. Thromb Haemost 1994 Aug;72(2):281-284.

(57) Kearon C, Akl EA, Comerota AJ, Prandoni P, Bounameaux H, Goldhaber SZ, et al. Antithrombotic therapy for VTE disease: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 2012 Feb;141(2 Suppl):e419S-94S.

(58) Amin A, Stemkowski S, Lin J, Yang G. Appropriate thromboprophylaxis in hospitalized cancer patients. Clin Adv Hematol Oncol 2008 Dec;6(12):910-920.

(59) Kahn SR, Lim W, Dunn AS, Cushman M, Dentali F, Akl EA, et al. Prevention of VTE in nonsurgical patients: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 2012 Feb;141(2 Suppl):e195S-226S.

(60) Streiff MB, Lau BD. Thromboprophylaxis in nonsurgical patients. Hematology Am Soc Hematol Educ Program 2012;2012:631-637.

(61) Violi F, Perri L, Loffredo L. Should all acutely ill medical patients be treated with antithrombotic drugs? A review of the interventional trials. Thromb Haemost 2013 Apr;109(4):589-595.