Thesis for doctoral degree (Ph.D.)
2020

Coagulation in Liver Failure. The Role of Thromboelastometry and Fibrinogen

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Stockholm 2020

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB

ISBN 978-91-7831-985-5

COAGULATION IN LIVER FAILURE.
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” […] according to the principles of quantum physics, which is an accurate description of nature, a particle has neither a definite position nor a definite velocity unless and until those quantities are measured by an observer. It is therefore not correct to say that a measurement gives a certain result because the quantity being measured had that value at the time of the measurement. In fact, in some cases individual objects don’t even have an independent existence but rather exists only as part of many.”

Stephen Hawking & Leonard Mlodinow, *The Great Design*

ABSTRACT

Liver failure is undeniably accompanied by distortions of the coagulation system. The modern theory of coagulation in patients with compensated liver disease (acute or chronic) states that the coagulation system is in a rebalanced state, also displaying prothrombotic tendencies particularly towards the end stages. Conventional coagulation tests are proved inappropriate in the evaluation of bleeding or thrombotic risks in these patients. Viscoelastic tests, such as thromboelastometry, are used clinically as point-of-care testing to determine the balance of coagulation and have proven particularly effective in the treatment of critical situations with bleeding difficult to control. Also, there have been reports that viscoelastic testing might be used for prognosis purposes in liver cirrhosis. A thromboelastometric characterization of an unselected population with an indication for liver transplantation was assumed to be useful. Additionally, a clarification of blood coagulation abnormalities in conjunction with the liver dysfunction following massive liver resections was needed.

The aim of studies 1 and 2 was to characterize coagulation status in patients with liver conditions using thromboelastometry. In study 1 we included patients with stable chronic liver disease with an indication for liver transplantation. Aside from a viscoelastic description, the primary aim of study 1 was to assess thromboelastometry usefulness in assessing the prognosis in these patients and eventually be used to improve the existing scoring systems. In general, the patients were normo- and hypocoagulable with no significant signs of hypercoagulability expressed through thromboelastometry. No relation could be found between thromboelastometry and the Child-Pugh and MELD scores. The conclusion of study 1 was that we could not find any use for thromboelastometry in evaluating the gravity of the stable chronic liver disease with an indication for liver transplantation.

In study 2 we included patients who underwent hemihepatectomies and extended hemihepatectomies. Using thromboelastometry we found that postoperatively the coagulation system was rebalanced apparently in the same manner as in chronic liver disease and that the bleeding risk signalled by PT-INR was groundless. We also found that the postoperative trends of plasma fibrinogen concentration were related to the size of resection.

The findings in study 2 made us hypothesize that a deficiency in synthesis due to the loss of liver mass may be the factor which affects the plasma fibrinogen concentrations postoperatively. To explore this hypothesis, we needed to quantitatively assess the de novo synthesis of fibrinogen (and albumin) in these patients. To this end, in study 3, we adjusted the isotope tracer flooding dose technique to enable repetitive measurements in longitudinal studies.

The technique described in study 3 was then used in study 4 to determine the postoperative synthesis rates of fibrinogen and albumin in patients undergoing hemihepatectomies and pancreatectomies (as controls). An extraordinary capacity of the liver to increase fibrinogen synthesis on postoperative day one was discovered. In this study, we found that the loss of liver mass through elective hemihepatectomies did not incapacitate a sharp increase in the synthesis rates of fibrinogen compared to pancreatectomies. Plasma fibrinogen concentrations may therefore reflect the dynamic changes in synthesis related to fibrinogen utilization. The postoperative synthesis course maintained effective fibrinogen levels for balanced coagulation. Albumin synthesis rates confirmed good posthepatectomy synthetic function in the remnant liver tissue and eventually in the regenerated liver tissue.

LIST OF SCIENTIFIC PAPERS

I. **Thromboelastometry: Relation to the severity of liver cirrhosis in patients considered for liver transplantation**.
Dumitrescu G, Januszkiewicz A, Agren A, Magnusson M, Wahlin S, Wernerman J, *Medicine (Baltimore)* 2017, **96**(23):e7101

II. **The temporal pattern of postoperative coagulation status in patients undergoing major liver surgery**.
Dumitrescu G, Januszkiewicz A, Agren A, Magnusson M, Isaksson B, Wernerman J, *Thromb Res* 2015, **136**(2):402-407

III. **Repeated quantitative measurements of De Novo synthesis of albumin and fibrinogen**.
Dumitrescu G, Komaromi A, Rooyackers O, Klaude M, Hebert C, Wernerman J, Norberg A: *PLoS One* 2017, 12(3): e0174611

IV. **The temporal pattern of fibrinogen and albumin synthesis rates perioperatively in major abdominal surgery**.
Dumitrescu G, Januszkiewicz A, Agren A, Magnusson M, Sparrelid E, Rooyackers O, Wernerman J,
Manuscript submitted for publication

CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ALI	acute liver insufficiency
ANOVA	one-way analysis of variance
AT	antithrombin
aPTT	activated partial thromboplastin time
APC	activated protein C
APE	atom percent (%) excess
ASR	absolute synthesis rate
BV	blood volume
cAMP	cyclic AMP
CFT	clot formation time
CLD	chronic liver disease
CPS	Child-Pugh score
CT	coagulation time
DOACs	direct oral anticoagulants
DVT	deep vein thrombosis
ECMO	extracorporeal membrane oxygenation
ecto-- ADPase	ecto-adenosine diphosphatase
Ep	tracer steady state enrichment
EPCR	endothelial protein C receptor
ESLD	end-stage liver disease
ETP	endogenous thrombin potential
fVIIa, etc	activated coagulation factors
FDPs	fibrin degradation products
FDT	flooding dose technique
fpB	fibrinopeptide B
FSR	fractional synthesis rate
GC	gas chromatography
GC-MS	gas chromatography- mass spectrometry
GP	glycoprotein
HCC	hepatocellular cancer
HES	hydroxyethyl starch
HMK	high molecular weight kininogen
IL	interleukin
INR	International Normalized Ratio

IPMN	intraductal papillary mucinous neoplasm
ISI	international Sensitivity Index
ISI_{liver}	ISI calculated for patients with liver cirrhosis
ISTH	International Society on Thrombosis and Hemostasis
M	atomic mass
MCF	maximum clot firmness
MELD	Model for End-stage Liver Disease
ML	maximum lysis
MPE	mole percent (%) excess
MS	mass spectrometry
HASH	nonalcoholic steatotic hepatitis
PAI-1	plasminogen activator inhibitor 1
PAP	complexes plasmin-antiplasmin
PARs	G-protein–linked protease-activated receptors
PBC	primary biliary cirrhosis
PE	pulmonary embolism
PHLF	posthepatectomy liver failure
PK	pre-kallikrein
POD	postoperative day
POC	point-of-care
PSC	primary sclerosing cholangitis
PT	prothrombin time
PTT	partial thromboplastin time
PV	plasma volume
PVE	portal vein embolization/ligation
ROC	receiver operating characteristic
ROTEM	thromboelastometry
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complexes
TEG	thromboelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGT	thrombin generation test
TM	thrombomodulin
tPA	tissue plasminogen activator
t-RNA	transfer-RNA

TTR	tracer to tracee ratio
uPA	urokinase-type plasminogen activator
VETs	viscoelastic tests
VKA	vitamin K antagonists
VTE	venous thromboembolism
vWD	von Willebrand disease
vWF	von Willebrand factor
WHO	The World Health Organization

1 INTRODUCTION

1.1 NORMAL HAEMOSTASIS

1.1.1 Primary haemostasis

The term primary haemostasis refers to the formation of the platelet thrombus. A normal primary haemostasis means also an impediment of cloth formation when it is not necessary. The normal endothelium has a protective role against thrombo-genesis. Three biochemical mechanisms controlled by the endothelium are responsible for the prevention of platelet activation: nitric oxide, prostacyclin and the endothelial ecto-adenosine diphosphatase (ecto-ADPase/CD 39) pathways [1].

In case of endothelium damage, locally generated vasoactive agents determine vasoconstriction which restrict the bleeding [2] and primary haemostasis begin with the platelet adhesion to the sub-endothelium and further, through the platelet's aggregation in overlapping layers, to the formation of the platelet plug.

Platelets are essential for primary haemostasis, but this complex process also involves adhesive substrates, receptors on the platelets surface (integrins and non-integrins) and binding proteins like fibrinogen.

1.1.1.1 Platelets adhesion and activation

When the endothelium is injured, molecules such as collagen, von Willebrandt factor (vWF), laminin, fibronectin, and trombospondin are exposed to the bloodstream [3]. These are ligands for the platelet's membrane receptors and mediate platelets adhesion to the sub-endothelium. Their relationship with the platelets depends on the blood flow dynamics [3]. In rapid flow conditions (arteries) platelets adhesion is mediated by the vWF [4]. At low flows (veins) direct binding to collagen, laminin, fibronectin prevails [3].

vWF is synthetized by endothelium and megakaryocytes and exists immobilised at sub-endothelial level as well as in soluble form in plasma [4]. vWF can also be expressed on the platelet surface [5]. Normal vWF has a high affinity to specific platelets receptors and to collagen [3].

The platelet **adhesion** is initiated by the interaction between the platelet membrane receptor glycoprotein Ib (GP Ib) (a non-integrin which coexist in a complex with GP V and IX) and the sub-endotehelial vWF [4]. GPIb-vWF bindings will decelerate platelets carried by the blood flow [4, 6]. Thus, the platelets are seized in a "rolling adhesion" which eventually allows establishment of more stable bonds between platelet receptor GP IIb/IIIa (integrin $\alpha IIb\beta 3$) and the sub-endothelial vWF [4, 6]. P-selectin expressed by endothelial cells, as well released by activated platelets, facilitate the adhesion process [1, 4].

A stable adhesion is assured by a direct firm fastening to collagen completed through the platelet surface receptors GP Ia/IIa (integrin $\alpha 2\beta 1$) and GP VI (a non-integrin) [3].

The binding of vWF on GP Ib , as well as other interaction between different platelet receptors and their agonists, will determine the platelet **activation** [3, 7, 8] which will generate platelets degranulation, cytoskeletal modifications with subsequent shape changes of the platelets and finally the clot retraction [9, 10].

During platelet degranulation, several different mediators are released, e.g. arachidonic acid which attracts other circulating platelets to the site of injury, or important agonists such as

adenosine diphosphate (ADP), thrombin, epinephrine, and thromboxane A2 (TXA_2). They play a critical role in amplifying and sustaining the subsequent aggregation by interacting with receptors expressed on the platelets surface.

1.1.1.2 Platelets aggregation. The fibrinogen role in stabilizing the platelet thrombus.

The platelet aggregation is dependent on the interaction between platelet receptors GP Ib, GP IIb/IIIa and vWF and fibrinogen in a complex manner depending on blood flow share rates [4, 5, 8]. Although GPIb-vWF binding is required for both low and high flows [8], GP IIb/IIIa is the main receptor for platelets aggregation at high velocities [3].

While platelet activation takes place, GP IIb/IIIa is expressed, and undergoes a conformational change that increases its affinity for fibrinogen and the vWF [10, 11]. *GP IIb/IIIa binds enduringly the soluble fibrinogen* which molecular dimeric structure allows adjacent activated platelets to cross-link to underlying ones [10]. vWF competes with fibrinogen on GP IIb/IIIa, especially at elevated levels of platelets layers, allowing the initial contact between platelets [5, 10]. On mice vWF alone is not sufficient to ensure the stability of the platelet plug and fibrinogen is absolutely necessary in maintaining a stable platelet thrombus [10, 12].

Thrombin, which is rapidity generated around activated platelets, is a potent activator of the platelets and promotor of platelet aggregation [10].

1.1.1.3 Connection with the secondary haemostasis

The processes described by the cell-based model of haemostasis occur on the surface of the activated platelets.

The platelets contain pre-mRNA for tissue factor and in conjunction with platelet activation the synthesis of the bioactive tissue-factor is triggered. Once synthetized, the tissue factor will be expressed on the surface and will initiate the secondary haemostasis [13]. Activated platelets also release factor V, which is partially activated at the same time [14].

In the amplification stage of the coagulation process, the receptor GP Ib/IX on the surface of activated platelet binds thrombin which activate fVIII and fXI (**Figure 1**) [15, 16]. Platelets express as well high-affinity binding sites for flXa, fXa, and fXI [16].

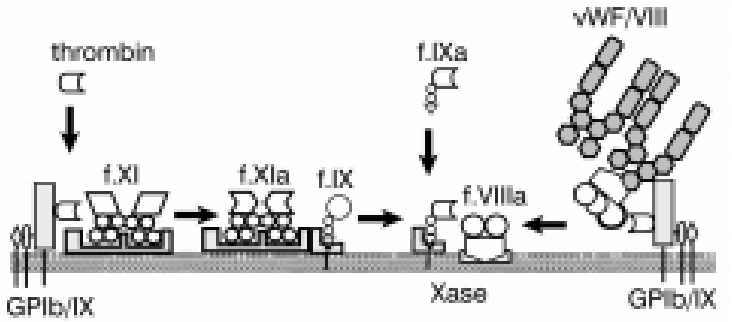

Figure 1. *Activation of factors VIII and XI by thrombin on the platelet surface (Reproduced from [16] with permission of the publisher).*

Negatively charged phospholipids are exposed by the platelets as well and play an important catalytic and stabilizing role in the fibrin formation [9].

1.1.2 Secondary haemostasis

Secondary haemostasis refers to the process of fibrin formation. Over time secondary coagulation theory has gone through several modifications according to the evolution of the understanding of the coagulation mechanisms.

In the 1930s the model of coagulation was very simple and regarded exclusively the activation of prothrombin under the action of thromboplastin and calcium and thus converting itself in thrombin which determined the fibrin formation [17]. The design of the coagulation test known as the prothrombin test (PT) is based on this simple model [17].

The model called “coagulation cascade” was presented in 1964 [18, 19]. The cascade model proposes a sequential proteolytic activation of proenzymes into enzymes (i.e. coagulation factors as serine proteases), with the two pathways intrinsic and extrinsic, which ultimately results in the generation of thrombin that causes fibrinogen to be converted to insoluble fibrin [18, 19].

A significant drawback of the cascade model was that the coagulation tests PT (which reflects the extrinsic pathway) and aPTT (which explores the intrinsic pathway) do not correlate with the risk of bleeding in all clinical situations. Different punctual deficiencies of various coagulation factors involved in the extrinsic pathway do not have the same risk of bleeding. Thus haemophilia (deficiencies in factor VIII (fVIII) or IX (fIX) have an increased risk of bleeding, while fXII or XI deficiencies have a significantly lower risk or not at all [17]. Furthermore, the cascade model cannot explain how haemophilia cannot be compensated by an extrinsic pathway that functions normally [16]. The in vivo activation of the intrinsic pathway was also questionable because deficiency of fXII, high molecular weight kininogen (HMK) or pre-kallikrein (PK) does not result in bleeding diathesis [16].

Other major deficiencies of this model were that the natural anticoagulation pathways were not included, and the platelets remained only a part of the primary haemostasis without making a connection with the secondary haemostasis.

1.1.2.1 A Cell-Based Model of haemostasis

The new model was published in 2011 and named "A cell-based Model of Haemostasis". It was the result of preliminary studies that brought new knowledge about how platelets and clotting factors interact [16].

It was demonstrated for example that activated platelets external surface is a place for activation of fXI [15], accordingly fXII, HMK and PK might not be mandatory for haemostasis [16].

Important findings such as that the complex tissue factor (TF) - fVIIa can activate factor IX [20] was key information in understanding the interconnexion between the extrinsic and intrinsic pathways.

The cell-based model explains that haemostasis occurs on the cell surface and involves three successive steps (or stages) (**Figure 2** on p.4**)**) [16]:

Stage 1: **Initiation**
Stage 2: **Amplification**
Stage 3: **Propagation**

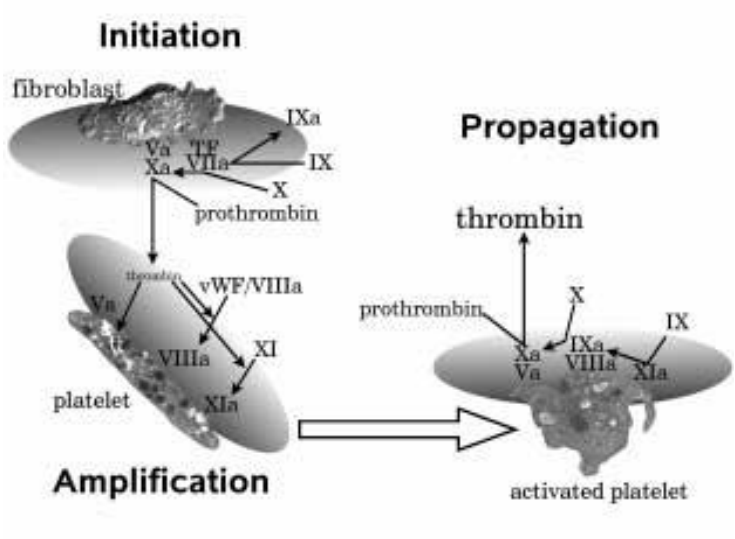

Figure 2. *A cell-based model of coagulation (Reproduced from [16] with permission of the publisher)*

Initiation *of coagulation on TF-bearing cells*

It has been demonstrated that TF is highly expressed by sub-endothelial pericytes in small vessels, and by adventitial cells around larger vessels under normal conditions. As long it remains sub-endothelial these TF-bearing cells do not trigger coagulation [21, 22].

An endothelial defect will allow TF-bearing to contact plasma and the TF will bind and activate fVII (fVIIa) [23]. The complex TF- fVIIa will then activate factor X (fXa) [23].

Activated platelets release partially activated factor V which will form with fXa complexes called prothrombinase complexes which will convert small amounts of prothrombin into thrombin [16].

The small amounts of thrombin are insufficient to promote fibrinogen polymerization at a large scale. It will act instead as a pro-coagulant signal for the next stage - amplification [16].

The next two stages take place on the surface and near the platelets trapped in the platelet plug.

Amplification

The small amounts of thrombin will determine maximal platelet activation [16]. Consecutively larger quantities of partially activated factor V will be released which will be completely activated by thrombin and fXa [16, 24].

Thrombin activates fVIII after releasing it from complexes von Willlebrand factor-fVIII which are already fasten to the activated platelets on specific receptors [16].

Thrombin will directly activate fXI on the platelet surface [15, 16, 25].

Propagation

In this phase fXIa will activate factor IX into fIXa which binds to fVIIIa on the platelet surface resulting complexes (previously called "intrinsic tenase") [16].

In the presence of the activated fIXa-fVIIIa complexes, factor X is activated to fXa which will associate fVa and form complexes (previously called "prothrombinase") that will determine an explosive thrombin generation [16].

Notes

Activation of fXI on the surface of the platelets in the amplification step explains why fXII is not required for normal haemostasis in vivo [17].

It is highlighted by the authors of the cell-based model that even if the haemostatic process is described occurring in distinct and successive steps, these stages are ongoing overlapping processes [16, 17].

1.1.2.2 Fibrin network formation

Thrombin will determine fibrinogen cleavage into soluble fibrin monomers which will polymerize into soluble fibrin complexes. The process is described in the section fibrin formation (see section 1.2.3 on p.8)

1.1.2.3 The natural anticoagulation

The human organism is equipped with anticoagulation mechanisms that localize the formation of clots to the appropriate sites and surfaces [16]. Natural occurring inhibitors of coagulation regulate thrombin through direct inhibition or by limitation of thrombin generation.

Protease Inhibitors

Antithrombin (AT) is synthetized by the liver and is a protease inhibitor of thrombin, fXa and other activated coagulation factors (p 355 in [26]).

Tissue factor pathway inhibitor (TFPI) is a protease inhibitor produced by the endothelium and other cells such as megakaryocytes which restrain the coagulation process by inhibition of fXa through TFPI-fXa complexes which bind and inactivate TF-fVIIa [27].

AT and TFPI will instantly inhibit the fXa if it leaves the cell surface on which it is formed and thus suppress the propagation of coagulation [16]. On the cells surface fXa seems to be relatively protected from the AT and TFPI [17, 28].

Activated Protein C- Protein S

Proteins C and S are synthesized in the liver and are vitamin K–dependent factors.

Protein C from plasma is attached by the endothelial protein C receptor (EPCR) and as a consequence its activity increase [29].

Thrombomodulin (TM), which is a cell surface receptor for thrombin, will bind thrombin which is produced at the site of injury. While thrombin attached to TM, it loses the effect on fibrinogen [16]. The complex TM-thrombin has the function to activate the protein C which is attached via EPRC on the endothelium. In the next step, protein S form a complex with the activated protein C (APC) increasing the activity of the already activated protein C [30, 31].

APC does not act in the fluid phase as usual anticoagulants do [17]. Its antithrombotic activity is limited to the endothelial surfaces [17].

The APC-Protein S complex inactivates fVa and fVIIIa on the endothelial surface disabling thrombin production [30].

AT, PC and PS deficiencies determine a pro-thrombotic tendency as a result of excessive thrombin generation [32-34]. Total TFPI deficiency does not exist in humans and partial deficiency is associated with a weak pro-thrombotic status [27].

1.1.3 Fibrinolysis

Fibrinolysis is initiated as soon as the first fibrin polymers are born and will carry on as long as these filaments exist.

The enzyme which is responsible for fibrinolysis is plasmin. Plasmin is the result of activation of plasminogen, its zymogen, under the action of tissue plasminogen activator (tPA) or, in special circumstances of urokinase-type plasminogen activator (uPA). Fibrin polymers attaches plasminogen and tPA on binding sites exposed after fibrinogen cleavage [17].

Under normal circumstances, fine-tuning mechanisms are triggered to maintain the balance of fibrinolysis. Plasminogen activator inhibitor 1 (PAI-1), a serine protease inhibitor which also fastens to forming fibrin network, is the main inhibitor of the fibrinolytic system [36]. Hence, tPA is blocked by PAI-1 by forming inactive tPA -PAI-1 complexes [37]. The rate of PAI-1 inhibition on tPA is related to its plasma concentration [35].

As well other proteins are involved in fibrinolysis inhibition by preventing the plasmin action. Thrombin activatable fibrinolysis inhibitor (TAFI) protects the clot from lysis by removing plasminogen binding sites from fibrin, α-antiplasmin (α AP) inactivates the plasmin by forming complexes plasmin-antiplasmin (PAP), α_2 macroglobulin (α_2 MG) inactivates plasmin as well.

The fibrinolysis factors are produced in the liver and endothelium in different proportions. Plasminogen is synthesized exclusively by hepatocytes. PAI-1 is produced by the liver but also by endothelium and other tissues as well [17]. TAFI, α AP and most of α_2 MG are synthesized in the liver.

tPA is synthesized entirely by the endothelium under stimuli such as thrombin, ischemia or hypoxia while *uPA* is produced in different tissues but not in the hepatocytes [17]. Liver intervenes on tPA by clearing it from plasma, with a half time for active tPA of 2-3 min and for tPA-PAI-1 complexes of 5-6 min [17].

Fibrinolysis generates fibrin degradation products (FDPs) and D-dimers which are markers for fibrinolysis. These markers have high sensitivity but low specificity for venous thrombo-embolism (VTE) [38-40], and thus have less reliable positive predictive value [41]. However, because of high sensitivity, a negative D-Dimer test can exclude thrombotic events [40].

Plasmin also has degrading action on fibrinogen resulting in fibrinogen degradation products which are similar but not identical to fibrin degradation products [42]. D-dimers are not markers of fibrinogenolysis (p.13 in [35]).

Fibrinogen degradation product fragment D have the potential to stimulate the fibrinogen synthesis by releasing the “hepatocyte stimulating factor” (i.e. IL-6) [43] in macrophage cell lines [44].

1.2 FIBRINOGEN

1.2.1 Fibrinogen structure

Fibrinogen is a glycoprotein with a molecular mass of 340 kDa. Fibrinogen structure consists of two identical chains of polypeptides. Each chain is made of three polypeptides namely Aα, Bβ and ϒ which are helically intertwined around each other and bind together by disulfide bonds [45, 46]. In the middle of the molecule, between the two symmetrical (Aα, Bβ, ϒ) chains, there is a smaller globular central region called **E region** which consists of fibrinopeptides A and B situated at the N-terminal of the peptide-chains Aα and Bβ respectively. Each end of the fibrinogen molecule presents a globular region called **D region** which contains the β and ϒ peptide nodules [46].

1.2.2 Fibrinogen metabolism (distribution, synthesis, catabolism)

1.2.2.1 Fibrinogen distribution in the human body

Fibrinogen's normal plasma concentration is 1.5-4.0 g/L (measured with the Clauss method) [42].

Plasma fibrinogen concentration is altered under pathological conditions. It rises as a result of an acute inflammatory process [47] or decline as in liver insufficiency [48, 49].

Although most of the fibrinogen (72%) is located in plasma [50, 51], other body compartments like the lymph nodes and the interstitial fluid can also contain fibrinogen [42]. The platelet alpha granules contain fibrinogen though it is not certain if it is structurally and functionally identical to the plasma fibrinogen [42].

In studies with radio-isotopically labelled fibrinogen transcapillary escape rate (TER) is estimated to be around 60 % of the plasma pool per day [50, 51].

1.2.2.2 Fibrinogen synthesis in humans

In humans, fibrinogen is mainly (at least 98%) synthesized by the liver, both under normal circumstances and during the acute phase reaction [52]. Extra-hepatic tissues such as epithelial cells synthesize fibrinogen [52, 53] but it is unlikely that the extra-hepatic synthesis contributes significantly to the plasma concentration [52].

Fibrinogen synthesis rates

The normal rate of synthesis for fibrinogen in adults is about 1.7-5.0 g fibrinogen per day, and the normal half time is 3-5 days [42, 51].

Fibrinogen synthesis is quantified, only in a few studies which used isotopic tracer techniques. In healthy young non-smokers subjects' studies using the flooding dose technique reported mean fractional synthesis rate (FSR) of 14%/day and absolute synthesis rate (ASR) of 22 mg/kg/day [54, 55]. The synthesis rates in healthy volunteers depends on factors such as age, feeding or smoking [54, 55]. In elderly fibrinogen synthesis is reported to be reduced [55], while smoking and feeding increase the fibrinogen production [54, 55].

Inflammation and fibrinogen synthesis

Fibrinogen is an acute-phase protein and systemic inflammation is associated with a significant increase in its plasma concentration [47]. Interleukin 6 (IL-6) is the major inducer

of the acute-phase response in humans [43, 56], as demonstrated on human hepatocyte cultures where IL-6 determine increases of fibrinogen synthesis [43, 56]. Interleukin 1β (IL-1β) inhibits the stimulating action of IL-6 on the synthesis of acute-phase proteins, including fibrinogen [43, 56]. TNF-α which is one of the first cytokines released after trauma has no stimulating effect on hepatocytes synthesis [43].

1.2.2.3 Fibrinogen catabolism

Some effort has been made in elucidating the fibrinogen catabolic pathways, still, these remain broadly unknown [42]. The calculated fractional catabolic rate, using iodine-labelled fibrinogen as a tracer, is around 24% of the plasma pool/day [50].

The catabolic pathways of fibrinogen are not completely understood. Contrary to what might be expected, fibrin formation-fibrinolysis seems not to be the main mechanism of fibrinogen catabolism under normal physiological circumstances [42, 50]. One study suggests that the thrombin pathway could be responsible for a maximum of 3% of fibrinogen catabolism under normal conditions [57]. However, in prothrombotic conditions or perioperatively the pathway of fibrin formation can gain significant proportion [57, 58].

Other proposed mechanisms such as degradation by plasmin have not been demonstrated to support substantially the fibrinogen catabolism [42, 44, 50].

1.2.3 Fibrin formation

During secondary haemostasis, fibrinogen plays a fundamental role in the construction of the clot. The process of fibrin clot formation comprises three phases:

1. *Conversion of fibrinogen to soluble fibrin monomers under the action of thrombin.* Thrombin binds to fibrinogen and removes fibrinopeptids A (at fast rate) and B (at slower rate) in the central E region which convert the fibrinogen molecule in a fibrin monomer [59]. Thus, polymerization sites are exposed, and the fibrin monomers will spontaneously form noncovalent bonds with each other.

2. *Spontaneous polymerization of the fibrin monomers* by central (E) regions to lateral (D) regions interaction will give birth to oligomers then to protofibrils and further to branched polymeric structures [59].

3. *Stabilization of the fibrin network by F XIII*. Thrombin activates F XIII which by covalent cross-linking adjacent fibrin strings will stabilize the fibrin structure [59].

The presence of soluble fibrin monomers and polymers in plasma is coupled with early phases of fibrin formation after cleavage of fibrinogen by thrombin. These markers have the potential to predict thrombosis even in the pre-thrombotic phases, but the cut-off values are not yet defined [39].

1.2.3.1 Fibrinogen and the quality of the fibrin network

The quality of the fibrin clot depends on various factors including fibrinogen quantity and quality, aside thrombin generation and platelets number and functionality [59].

Electron microscopy can visualize the structure of the fibrin network and features like fibre diameter and density, as well number and distances between the branch points and the size of the pores can be explored and eventually explain clinical implications of different abnormalities [59].

The porosity and the permeability of the fibrin network can be measured using biophysical techniques [59, 60]. These parameters may be influenced by oxidative modifications in the fibrinogen molecule which is seen in patients with liver cirrhosis [60].

1.2.4 Fibrin involvement in the repairing process of the injured liver tissue

Fibrinogen, FDPs, and plasmin modulate inflammation and promote repair processes as part of the complex interrelation inflation-coagulation following the acute phase response to injury [53]. It was suggested recently that intrahepatic fibrin deposition following liver surgery is a factor that stimulates liver regeneration and counteracts the postoperative liver failure [61].

1.3 COAGULATION IN LIVER FAILURE

Liver insufficiency is characterized by complex haemostasis abnormalities which, besides many similarities, have distinct characteristics for different clinical entities.

Many proteins with function that promote or inhibit the coagulation system are synthetized by the liver and a decrease of the liver synthetic capacity may affect their plasma concentration.

The liver synthetic function is affected in chronic or acute liver diseases or after extensive surgical liver resections. Liver insufficiency and overlapping pathophysiological mechanisms such as hypersplenism or endothelium activation will result in substantial changes in the haemostatic system.

1.3.1 Coagulation in chronic liver insufficiency

1.3.1.1 Primary haemostasis

Low platelet counts are frequently present in patients with liver cirrhosis due to multiple reasons, such as splenic and hepatic sequestration, low thrombopoietin levels and activity, or antiplatelet autoantibodies [9, 62, 63].

Platelets in cirrhotic patients may be dysfunctional, complex defects regarding platelets adhesion and aggregation as well concerning receptors and mediators have been reported [9, 64, 65]. However, many of the earlier studies are questioned due to methodological faults [66-68]. A newer report provides evidence for increased platelet activation in vivo conditions, still it is not enough to elucidate the enigma of normality or abnormality of platelet function in chronic liver disease [67-69].

A remarkable observation regarding the platelet function in liver cirrhosis is related to their adhesion function [65]. In these patients von Willebrand factor (vWF) have lower than normal binding capacity to platelets (via receptor glycoprotein Ib) and collagen [65]. However, the platelets (from patients with cirrhosis or normal controls) suspended in plasma from cirrhotic patients demonstrate hyper-adhesion to collagen under flow conditions and this is attributed to the high plasma concentration of vWF [65]. In cirrhotic plasma vWF antigen levels are higher than normal and correlates with the Child-Pugh score, presenting substantially increased levels in end-stage liver disease and this is suggested to overrun the deficit in its function [65].

Additionally, the vWF-cleaving protease ADAMTS-13 has a lower than normal activity [65] especially in end-stage liver disease [65]. Subsequently, this deficit contributes to the higher levels of vWF [62].

Even though platelet dysfunction in CLD is not entirely understood and a subject of controversy [67], most experts agree that thrombocytopenia may be compensated, at least partially, by higher levels of vWF in stable liver cirrhosis [17, 66].

1.3.1.2 Secondary haemostasis

In CLD the pro-coagulation factors which are synthetized by the liver are at lower than normal plasma levels; factor VIII (fVIII), apparently produced by the liver sinusoidal endothelial cells and not by the hepatocytes [70, 71], have higher than normal plasma concentrations [70]. High fVIII levels are in relation with increased levels of vWF that binds fVIII restraining thus its clearance [70, 72].

At the same time, naturally occurring anticoagulant proteins**,** specifically protein C, protein S and antithrombin (AT) have low plasma concentrations [73]. In addition, the function of AT and protein C may also be decreased in advanced stages of liver disease [74]. Tissue factor pathway inhibitor (TFPI), which is synthetized by the endothelium and not by the liver, has normal concentrations but decreased anticoagulant activity due to low levels of protein S (its cofactor) [75].

Because of these concomitant modifications in both pro and anticoagulant pathways the generation of thrombin is unaltered (or even enhanced) in stable liver cirrhosis [76-78].

1.3.1.3 Fibrinolysis

Many of the pro- and anti-fibrinolytic factors are synthesized and cleared by the liver and therefore affected by liver insufficiency [17]. Thus, the levels of fibrinolytic factors dependent of liver synthesis such as plasminogen, α-2 antiplasmin, thrombin-activatable fibrinolysis inhibitor (TAFI) have low plasma concentrations while others, synthesized by the endothelium, are increased (e.g. tPA) [79]. PAI-1, which is likewise removed by the liver, sometimes shows high plasma levels due to elevated endothelium production especially in cholestasis [80] and non-alcoholic steatohepatitis (NASH) [81].

These disturbances may and will in certain circumstances unbalance the fibrinolytic system. However, there is no obvious correlation between the degree of liver insufficiency and the degree of fibrinolysis malfunction [17].

1.3.1.4 The concept of re-balanced coagulation in liver cirrhosis and the global coagulation assays

Until the early 2000s the standard view on cirrhotic patients assumed that a prolonged prothrombin time (PT) and international normalized ratio (INR) combined with a low platelet count was associated with a high risk for bleeding, and bleeding prophylaxis with plasma and platelets prior surgery was common. On the other hand, per-operative bleeding in patients with liver cirrhosis during surgery, and especially during liver transplantation, was far less extensive than expected based on the above-mentioned assumptions [64, 73, 82].

2005 a remarkable finding demonstrated, that thrombin generation is normal in patients with liver cirrhosis if thrombomodulin is added to the samples [76]. When the thrombin generation test is measured in the presence of activated protein C, which is not happening if thrombomodulin is not added, conditions move closer to the in vivo settings and the results became surprisingly different [83].

Although most of the coagulation factors are low in liver disease, fVIII plasma concentrations are increased, which in combination with a low level of the natural anticoagulation factors AT, protein C and protein S, maintain a normal or even enhanced, thrombin generation [76, 78, 84].

In the context of a lower than expected blood loss during surgery, demonstration of a normal thrombin generation together with platelet hyper-adhesion properties grounded the new concept of rebalanced coagulation in CLD [85, 86].

Conventional coagulation tests such as PT-INR are no longer considered predictors for bleeding in patients with liver disease [85, 86], and a patient with stabile liver disease is considered being in haemostatic balance even if the so-called standard coagulation tests are out of the normal range [79, 87].

Because thrombin occupies a central role in the coagulation system, a normal thrombin generation gives the coagulation system the basis for re-balance [76, 85]. However, in order to have functional haemostasis, fibrinogen, the thrombin's substrate, must be sufficient both in terms of quantity and quality [62].

Thrombin generation tests (TGT) are the gold standard in the assessment of coagulation balance. A limitation is that they can only provide information about the coagulation process up to the point of fibrin formation, which does not include platelet function and fibrinolysis. [62]. Besides, they are laborious and expensive which limits their use in clinical practice.

Viscoelastic tests (VETs) on the other hand measure the whole blood clot formation and lysis and this can give an advantage in assessment of the coagulation balance.

It is controversial whether or not fibrinolysis is re-balanced in patients with stable liver cirrhosis status[88]. There are observations suggestive for hyperfibrinolysis in stable CLD using plasma clot lysis assays [89-91]. In contrast, lack of hyperfibrinolysis in a study searching the clot lysis suggests rebalanced fibrinolysis [92]. Fibrinolysis assessed by thromboelastometry with the addition of tPA shows absence of hyperfibrinolysis in cirrhosis [49].

An important issue in getting a consensus over the habitual fibrinolysis balance in non-surgical patients with liver cirrhosis is the difficulty to assess fibrinolysis globally and that the clot lysis analyses used are home-made, with limited external validity [74, 88].

1.3.1.5 The risk of losing coagulation balance in liver cirrhosis

There is a **vulnerability** of the coagulation system in liver failure because the rebalanced structure has a certain degree of fragility [62]. The low levels of pro- and anticoagulant factors narrow the margins to become deficient in these factors at additional liver decompensation. Sepsis or renal failure may as well induce imbalances and predispose to bleeding and/or thrombosis [62, 86, 93].

The more advanced liver insufficiency, the more vulnerable the coagulation system will become. Particularly in end-stage liver disease (ESLD) imbalances in both directions are possible, sometimes with coexisting bleeding and thrombotic events [62].

The bleeding risk in liver cirrhosis

Bleeding tendency and even spontaneous bleedings are common complications in advanced liver cirrhosis. However, variceal bleeding, the most frequent bleeding complication in liver

cirrhosis, is not caused by hypocoagulability but by exacerbations of portal hypertension [85]. The magnitude of the variceal bleeding as well depends mainly on the portal pressure [94].

As stated above, the clotting time PT-INR has no predictive value regarding the risk of bleeding in patients with CLD [62, 85]. This relates to the fact that PT - INR reflects only the levels of the procoagulant factors, and not the activity of anticoagulant proteins, platelets or fibrinolysis [17]. In this respect PT – INR does not evaluate the coagulation process as a whole and therefore cannot assess the balance of coagulation.

The tendency towards thrombosis and hypercoagulability in liver cirrhosis

Cirrhotic patients have approximately double the risk of deep vein thrombosis (DVT) compared to the general population [95]. Portal vein thrombosis diagnosed with imaging techniques has a wide prevalence in different studies of 1-25 % [96, 97], being more observed in advanced stages of liver disease [96, 97]. Notably, a prevalence of 2% is reported in patients awaiting liver transplantation and 4% in those undergoing liver transplantation [74, 97].

In particular ***cholestasis*** is associated with hypercoagulability [74, 98]. The autoimmune forms of liver disease i.e. primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), as well autoimmune hepatitis, are associated with antiphospholipid antibodies [99] and with thrombosis events perioperatively in liver transplantation [74]. An upregulation of certain pro-coagulant factors is described in subgroups of patients with cholestasis [100, 101].

Also, patients with ***non-alcoholic fatty liver disease*** may or may not present a pro-coagulant imbalance in different studies, likely due to analytical differences regarding the thrombin generation tests methodology [102, 103].

It is demonstrated that thrombin generation is higher than normal in end stages of liver disease [49, 77] possibly related to resistance to the effects of thrombomodulin [78] , predisposing to an imbalance towards hypercoagulability and a pro-thrombotic state [77, 78, 104].

An enhanced platelet count in the presence of high levels of VWF is associated with prothrombotic risk, therefore actions to increase platelet count in non-bleeding cirrhotic patients should be avoided [105].

Dysfibrinogenemia. Fibrinogen synthesized by a liver with advanced liver disease may be dysfunctional due to the hypersialylation and is then characterized by a delayed fibrin polymerization [106]. Recent studies suggest that in liver cirrhosis fibrinogen molecule, because of oxidative modifications, is more thrombogenic and produces a fibrin network characterized by reduced permeability which makes the cloth less susceptible to lysis [60].

1.3.1.6 Intrahepatic activation of coagulation

Animal and human studies demonstrate presence of intrahepatic activation of coagulation in liver disease, which could be a factor in the disease progression [62, 107, 108]. Intrahepatic coagulation activation with formation of microthrombi in the sinusoid may be a consequence of the tissue factor expression by injured hepatocytes, as suggested by studies in mouse with acute induced liver failure [109].

1.3.1.7 Endothelial dysfunction in liver disease

In liver failure endothelium suffers a dysfunction under the influence of nitric oxide dysregulation and inflammatory disturbances [110]. The levels of coagulation and fibrinolysis factors such as fVIII, vWF, PAI-1 and tPA are high and this is related to the endothelial

production [74]. Endothelial dysfunction due to endotoxemia and oxidative stress, is suggested to play a role in the pro-thrombotic abnormalities in the portal circulation [74].

1.3.1.8 Assessment of the coagulation balance using viscoelastic tests

Viscoelastic tests in stable CLD

Clinically, VETs are mainly used in patients with liver failure as point-of-care tests to guide the administration of pro-coagulant treatment during and after surgery with a high risk of massive bleeding. VETs are effective to reduce the administration of blood products in liver transplantation [111, 112] and also before invasive procedures in CLD [113]. However, VETs have not been proven to predict bleeding in liver failure [62].

Both thromboelastography (TEG®) and thromboelastometry (ROTEM®) have been used in studies to characterize coagulation in patients with liver insufficiency. In compensated patients with chronic or acute liver disease, TEG® demonstrates that the coagulability remains in normal ranges [114], while studies performed with ROTEM® show normo- or hypocoagulability [49, 115, 116].

PBC and PSC are associated with thromboelastographic signs of hyperactive coagulation [98, 117], while HCC in the absence of cirrhosis is not [118]. TEG® can detect hypercoagulability in liver cirrhosis [98, 117], but there are no such reports for ROTEM®.

It should be emphasized that TEG® and ROTEM® results are sometimes conflicting, which could de explained through differences in sensitivity [119, 120].

The viscoelastic coagulation profile in decompensated liver failure

Studies including viscoelastic coagulation description in decompensated chronic liver disease are rare. In a study on acute decompensated chronic liver disease TEG demonstrates imbalance of the coagulation with hypo-coagulability in most parameters compared to healthy controls [121].

A recent study, using ROTEM®, compared patients with acute-on-chronic liver failure (ACLF) with patients in acute decompensation of the liver disease (AD) [122]. Hypo-coagulability in three or more ROTEM® parameters, is observed to a greater extent in patients with ACLF (60%) than AD (30%), hypercoagulability is exceptional [122]. The ROTEM® parameters are not significantly changed at 72 hours after admission [122]. Patients with ACLF have a higher prevalence of bleeding but this is not correlated with hypo-coagulability on ROTEM® parameters in this study [122]. INR and aPTT are significantly higher in ACLF and correlated with mortality [122]. Platelets and fibrinogen concentrations are more affected in ACLF than AD [122].

In another study fibrinolysis in patients with ACLF and AD, explored with a global fibrinolysis test which could indicate the risk for thrombosis in case of hypofibrinolysis, show a large variability with patients in normo-, hypo- and hyperfibrinolysis state [123]. In this study hypofibrinolysis is associated to sepsis, organ failure and higher mortality [123].

1.3.1.9 Thromboelastometry (ROTEM®) and assessment of prognosis in chronic liver failure

Since the early 2000s, the reliability of PT-INR, as the only coagulation test included in the Child-Pugh score and Model for End-stage Liver Disease (MELD) score is questioned [73, 124]. Due to the high interlaboratory variability for INR, there are concerns over the impact

of an uncertain INR on the MELD score and further on the objectivity in the evaluation of priority to liver transplantation [86, 125, 126].

In one of the few published studies using viscoelastic tests in patients with stable CLD, it is suggested that thromboelastometry, particularly maximum clot firmness (MCF), may be useful in assessing prognosis in patients with stable CLD [115]. The reason is that parameter MCF correlated with Child-Pugh and MELD scores similarly to PT-INR [115]. Another study, published in 2016, show a good correlation between MCF and MELD but concludes that ROTEM® is inappropriate to assess global haemostasis in liver cirrhosis [116]. Both studies included patients with liver cirrhosis based mainly on viral and alcoholic aetiology with the greater part of the patients in earlier stages of liver disease according to Child-Pugh and MELD scores.

A recent study (published 2018) reports a significant association between the parameter CT (on INTEM and EXTEM) and increased mortality in patients with acute-on-chronic liver failure [122].

There are so far no reports on patients with an indication for liver transplantation.

1.3.2 Coagulation in acute liver insufficiency

Diagnosis of acute liver failure (ALF) require, by definition, a PT- INR ≥1.5 and the presence of encephalopathy [127].

Compared with liver cirrhosis, patients with acute liver insufficiency exhibit a higher reduction in pro-coagulant factors reflected by the substantially higher PT-INRs, and also a lower level of the anticoagulant proteins AT and protein C [127]. Conversely, high levels of fVIII and vWF are present, as well as a low ADAMTS13 concentration in plasma [127].

Thrombocytopenia is common in acute liver failure, however not as pronounced as in chronic liver failure [114, 128].

In contrast to liver cirrhosis, fibrinolysis is lower than normal in acute liver insufficiency, with impeded lysis activity on the overall fibrinolysis potential tests [129].

Coagulation in acute liver insufficiency is re-balanced almost in the same manner as in CLD with tendencies rather towards thrombosis than to bleeding [127]. Thrombin generation is normal [129] and TEG show most normal clot formation, and even hypercoagulability if sepsis is associated [130].

Patients with ALI rarely present significant spontaneous bleedings [127, 131], or massive bleedings during liver transplantation [131].

1.3.3 Coagulation changes following major liver surgery

The haemostasis abnormalities associated with major liver surgery are complex and not thoroughly elucidated. A better description of the coagulation processes in these patients is desirable.

Major hepatic resections are associated with postoperative liver dysfunction, which in most cases is spontaneously reversible in a relatively short time period (days) [132, 133]. During this time, however, coagulation abnormalities are present.

1.3.3.1 The risk of bleeding in connection with liver surgery

Most centres routinely follow conventional coagulation tests in the postoperative period after major liver surgery. A higher than normal PT-INR occurs immediately postoperatively in the majority of cases [132, 134]. This sudden increase of PT-INR is often interpreted as an increased bleeding risk and this sometimes results in prophylactic plasma administration. Furthermore, concerns over the risk for postoperative bleeding is the background to the controversy whether postoperative thrombosis prophylaxis can or cannot be administered to these patients [135, 136].

The perioperative bleeding incidence in retrospective studies is low, around 6% [137, 138], and postoperative transfusions are only given in 0.8 % of cases [139]. In this respect, it is questionable if PT-INR alone is good to assess the postoperative bleeding risk after major liver resections.

1.3.3.2 The risk of thromboembolism in connection with liver surgery

In contrast to the low risk for postoperative bleeding following hepatic surgery, there is an increased prothrombotic risk. The incidence of deep venous thrombosis (DVT) is between 0.8% and 3.6%, and of pulmonary embolism (PE) between 1.4% and 7.1% [135, 139, 140]. The highest probability to occur for venous thromboembolism (VTE) is between postoperative days 5 and 7 after major liver resections [140, 141]. On a multivariate analysis (n= 375,748) the patients undergoing hepatectomy has among highest odds ratios (2.55) of developing VTE, higher than nephrectomy (1.19), colorectal surgery (1.87), pancreatectomy (2.07) or even esophagectomy (2.47), relative to bariatric surgery which has the lowest risk for VTE [142].

There are evidences of postoperatively increased levels of ***coagulation activation markers*** such as prothrombin fragments 1 and 2, or thrombin-antithrombin complexes in living liver donators or liver resections for primary and secondary malignancies which are indicating a hypercoagulable state [134, 143]. In addition, a hypofibrinolytic state early postoperatively following liver resections is described recently, in the same extent as following pancreas resections [144].

It is also reported a decline not only in pro-coagulant factors, but also in anti-coagulant proteins such as AT and protein C during the postoperative days following major liver surgery [118, 134]. It has been unclear if this is a factor contributing to the high prothrombotic risk in these patients.

A systematic review reports that thrombosis prophylaxis in patients with liver resections decrease the incidence of VTE events from 4.6 % to 2.6 % [145]

1.3.3.3 Global coagulation testing in major liver surgery

Thrombin generation tests postoperatively after hemihepatectomy show contradictory results, with increased as well normal values in different studies [146, 147].

Viscoelastic testing. TEG® is studied postoperatively in patients with major liver resections. In living liver donors, TEG® indicates hypercoagulability [148]. In patients undergoing major liver surgery mainly for malignancy a hypercoagulable state on postoperative day one (defined as decreased *R-value* on TEG®*)* that returned to the preoperative values in the following postoperative days is reported [149]. This is not reported by another study following liver resections in patients with hepatocellular cancer TEG® which shows normocoagulability with no signs of hypercoagulability postoperatively [118]. Despite the signs of hypercoagulability on TEG, none of these reports' present associations with clinically relevant thrombotic events.

2 METHODS

2.1 STANDARD COAGULATION TESTS

A standard laboratory evaluation of the haemostasis consists of a battery of a few conventional tests which are usually performed in patients at risk or presenting bleeding of different grades that needs treatment.

2.1.1.1 The prothrombin time (PT)

The prothrombin time (PT) test was developed by Armand Quick who published the original method in 1935 [150].

His methodology conformed to contemporary theory on haemostasis that thrombin results from prothrombin in the presence of thromboplastin and calcium [151]. At that time the clotting time of the recalcified plasma was already described, Quick tested different thromboplastins (tissues extracts) to get more constant results [151].

The PT test measures thus the time required for a clot to begin to form, once the protein called tissue factor and calcium are added to citrated platelet-poor plasma. Adding in excess thromboplastin (which contain tissue factor, and phospholipids) to plasma, clotting time will be dependent only on the prothrombin level (in conditions in which calcium level is constant) [150, 151].

Noticeably, the methodology does not cover the part of the coagulation system that is affected by haemophilia and PT is normal in these patients. On the contrary, the test is prolonged in patients with "obstructive jaundice" which allowed Quick to conclude that prothrombin levels are decreased in these patients [150].

However, the PT test is sensitive to changes in coagulation factors II, V, VII, X, and fibrinogen [86].

Depending on the method used to run the test, thromboplastin may either be plain (extractive or recombinant), as used in the Quick method or combined with "additives", as used in the Owren method [152]. The Owren method use as additives factor V and fibrinogen together with the tissue factor, making the PT test specific only to factors II, VII and X [152]. This makes the PT more precise by eliminating two variables and lowering the inter-laboratory variability in patients with chronic liver disease [153].

PT results have a large coefficient of variation between different testing centres, due to the differences in the quality of the thromboplastin which is supplied by many different manufacturers. This makes it extremely difficult to monitor PT in patients treated with vitamin K antagonists (VKA). The inconsistencies in results led to the development of a method of standardization [154].

International normalized ratio (INR)

The standardization of PT occurs according to a system of calibration recommended by The World Health Organization (WHO) [155].

Each manufacturer of commercial PT systems must calibrate its regent (recombinant human thromboplastin or thromboplastin of bovine or rabbit origin) against one of the WHO international standards. PT is measured in normal subjects and in patients treated with vitamin K antagonists, using both the actual reagent and a WHO reference reagent. The results (pair values) are plotted on a log-scale and a regression line is drawn. The slope of the

line is calculated, and the result is the **correction factor** named International Sensitivity Index (**ISI**).

The ISI is used to convert the PT results obtained into the international scale called International Normalized Ratio (INR), as follows:

INR= ($PT_{patient}/PT_{normal}$)$^{\mathbf{ISI}}$ [155]

The INR scale is most accurate in the range of 1.5–4.5[86].

It is important to emphasize that ISI (implicitly INR) is *valid only for patients on vitamin K antagonists* [86].

Significant differences have been reported between the INR calculated with an ISI for the patients on vitamin K antagonists, and the INR based on ISI_{liver} , calculated for patients with liver cirrhosis [156].

The International Society on Thrombosis and Haemostasis (ISTH) is aware of these differences but a standardization of the INR in liver disease is regarded too laborious to be carried out currently [157].

2.1.1.2 Activated partial thromboplastin time (aPTT)

The test called partial thromboplastin time (PTT) emerged from the need to quantify the coagulation defect in haemophilia (as PT test was insensitive to haemophilia). Observations over the clotting time of plasma with *reduced levels of thromboplastin* showed that considerably longer clotting times could discriminate haemophilic plasma from normal [158]. A new clotting time test was designed based on this finding and the term used for this new test was partial thromboplastin time. An important drawback for PTT was the considerable variability. By adding *kaolin as the activator*, more stable clotting time was achieved, with a better reproducibility [159]. This test was named activated PTT (aPTT).

aPTT is sensitive to the deficiencies of the intrinsic pathway factors II, V, VIII, IX, X, XI and fibrinogen.

2.1.1.3 Platelet count

Clinically, the platelet count is in many cases the only investigation necessary to assess platelet contribution to haemostasis, except for situations involving antiplatelet therapy or acquired or hereditary platelet dysfunctions in which case platelet function testing is mandatory.

Platelet count is currently performed using impedance measurements by automated analysers [160]. The normal range for platelet count is between 150,000-400,000 /μL.

2.1.1.4 Fibrinogen plasma concentration

Automated assays are available to perform this test according to **the Clauss method**. With this method, fibrinogen concentrations are evaluated indirectly. *What is measure is the time to fibrin formation when thrombin in excess is added to diluted plasma samples.* Fibrinogen plasma concentrations are calculated then using calibration curves [161]. Although there are reports indicating variability of the assay between laboratories, Clauss fibrinogen correlates well with other coagulation assays both in healthy subjects and patients with dysfibrinogenemia [162]. It is reported that haemodilution with hydroxyethyl starch (HES) 130/0.4 affects the fibrinogen concentration estimates [163].

2.1.1.5 Quantitative D-dimer

D-dimer are quantified using automated immuno-turbidity assays. D-dimer is formed through fibrinolysis and is a very sensitive marker for the activation of coagulation [164]. D-dimer increases postoperatively [165] or in any conditions associated with elevated fibrin-formation such as massive bleeding, venous thromboembolism (VTE) or disseminated intravascular coagulation (DIC), but also in inflammation, malignancies or pregnancy [35] (p13). Being present in many pathological conditions not necessary associated with VTE, D-dimer have low specificity for pro-thrombotic states [164]. However, because of high sensitivity, D-Dimer plasma values < 500 μg/L have negative predicting value for VTE [38, 164]. The effectiveness of VTE diagnostic becomes higher if the cut-off value is age-adjusted [40].

2.1.1.6 Antithrombin, protein C, protein S

Functional assays measure the inhibitory activity of anticoagulant proteins on their substrates. For AT it is measured the activity against fXa, for protein C and S the activity against fVIIIa and fVa[26]. Quantitative antigenic assays measure their concentration in plasma [26].

2.1.1.7 Limitations of SCTs

The standard coagulation tests are well standardized and, despite signalled variability, they are adequate for analysing punctual aberrations in the coagulation system and are effective in cases with clear, unequivocal reasons for coagulation non-normality.

When complex coagulation disturbances occur, the assessment of the coagulopathy is more challenging. It is the case of massive bleeding associated with trauma or different surgical interventions, as well in liver insufficiency, which implies multiple concomitant coagulation abnormalities. In these situations, the fragmented information given by the SCTs is not enough for a global evaluation of the haemostasis.

2.2 VISCOELASTIC TESTING

2.2.1 Introduction

1948 Dr. Hellmut Hartert published the principles of thromboelastography (TEG), a technique able to measure the phases of blood coagulation during clot formation [166]. The attribute of measurement was the viscoelasticity of the blood and its changes over time, which was graphically depicted by an ingenious and simple oscillatory mechanism. The trombelastographic principle is currently used in the computerized version of the TEG® (Haemonetics, Braintree, USA).

In 1996 the thrombelastographic principle was adapted, and a new technique invented, namely rotational thromboelastometry (ROTEM®) [167].

The generic term of viscoelastic coagulation tests (VETs) is common when we refer to TEG® or ROTEM® or other similar techniques which investigate viscoelasticity during clot formation.

Viscoelastic tests investigate the coagulability of the whole blood, which contains all the protein, enzymatic and cellular components involved in coagulation and fibrinolysis. Hence, VETs give a global assessment of the coagulation function and, although several components are missing, reflects better the *in vivo* haemostasis [168].

2.2.1.1 The TEG® and ROTEM® techniques.

Although TEG® and ROTEM® are related and share similarities, there are significant differences between the two systems, in terms of both mechanical and software operation, as well as differences in activators and additives [168, 169].

TEG® detects the viscoelastic changes of the whole blood using a bowl which oscillates around a plunger connected to a torsion wire, while ROTEM® uses a pin axis which oscillates in a cuvette [170].

The ROTEM® modification in which the pin axis is stabilized with a ball bearing, which reportedly can avoid movement artefacts, gives greater robustness than TEG® [169].

For conversion of the movements in items of information TEG® is equipped with an electromagnetic transducer-amplifier while ROTEM® has an optic sensor [170].

The collected data is processed by a computer and the viscoelastic changes which occur during coagulation are visualized by a graphic representation of clot formation and lysis. A set of parameters is delivered, giving information about the times until different levels of coagulation are achieved and the mechanical strength of clot (viscoelasticity) which is illustrated as amplitudes of the graph [170].

2.2.2 The ROTEM® device. The thromboelastometric curve and its parameters. ROTEM® analysis

2.2.2.1 The ROTEM® delta device

ROTEM® delta is a semiautomatic device with 4 independent measurement channels that allow the parallel plotting of four different thromboelastographic curves.

During the analysis, the pin is inserted in the sample which is placed in a cuvette fixed in a metal cup, with a temperature that can be controlled. Usually, the temperature is set at 37 degrees Celsius. The pin axis oscillates 4.75 degrees 12 times per minute. The clot fibres formed after coagulation initiation, bind the pin to the cuvette wall impairing the pin rotation as the clot strength increases in intensity. This reduction in the movement of the pin is measured and presented graphically as amplitud [167].

The pin movement is detected by a LED light detector via a mirror mounted on the moving axis. The signal from the light detector is electronically processed by the embedded computer and thus the thromboelastometric curve is generated. The device is equipped with an electronic display showing the progressive thromboelastometric curves and thromboelastometric parameters (p.40 in [35]).

2.2.2.2 The thromboelastometric curve and its parameters on ROTEM® delta device

The thromboelastometric curve is plotted against x/y axis where x is the time (in seconds) and y is the amplitude (in millimetres). The amplitude reflects the clot strength, or more technically said the clot firmness (**Figure 3** on p.21).

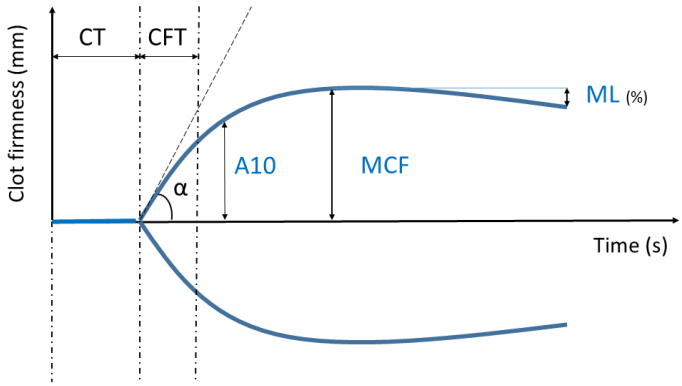

Figure 3. *Parameters of the thromboelastometric curve (Graphics created using PowerPoint)*

This graphic representation is depicted as a mirror image with the positive values extrapolated in the negative area of the graph so that the image is symmetrical and imitate the image generated by thromboelastography in its mechanical, non-computerized model.

The standard thromboelastometric parameters are (p 43 in [35]):

Coagulation time (CT) is the time in seconds from the start of measurement until the clot firmness reaches 2 mm in amplitude;

Clot formation time (CFT) is the time in seconds between clot firmness 2 and 20 mm;

The alpha (α) angle (degrees), is the angle formed by the tangent to the graphical trace at an amplitude of 2 mm;

A5, A10, A20 - the amplitude in millimetres reflecting the clot firmness after 5, 10 and 20 min;

Maximum clot firmness (MCF) - is the amplitude in millimetres reflecting the maximum clot firmness reached during the time the analysis is running;

Maximum lysis (ML) - presented as a percentage of MCF- represents the maximum decay in amplitude reported to MCF and reflect the maximum lysis detected during analysis (usually this value is reached at the end of the analysis);

Lysis index at 30 and 60 min (Li 30, Li 60) - presented as a percentage of MCF - represents the decay in amplitude reported to MCF after 30 and 60 min from the analysis start.

Depending on the phase of the coagulation which is investigated, the parameters can be classified as:

Coagulation activation and clot polymerization parameters: CT, CFT, The alpha (α) angle;

Clot firmness parameters: A5, A10, A 20, and MCF;

Clot lysis parameters: (which reflect the residual clot firmness) – Li 30, Li 60, ML.

2.2.2.3 ROTEM® analysis

The blood for ROTEM® analysis is collected on citrated samples.

Activation of coagulation. For analysis, the samples are recalcified and activation of coagulation is made in two ways, with *tissue factor* which investigate the extrinsic pathway (the thromboelastometric curve which results is called therefor **EXTEM**) or *ellagic acid and phospholipids* for the intrinsic pathway (which initiate the thromboelastometric curve called **INTEM)** [35].

Different *additives* can be used to investigate different coagulation aspects.

Fibrinogen polymerization is assessed by adding *Cytochalazin-D* to block the platelets activation in the sample, while coagulation is initiated through the extrinsic pathway (hereby the analysis is a modified EXTEM). The thromboelastometric curve which results is called **FIBTEM** [35].

To diagnose hyperfibrinolysis and discriminate between fibrinolysis and other reasons for hypo-coagulability, an inhibitor of fibrinolysis can be used. Commonly *Aprotinin* is added to inhibit fibrinolysis while coagulation is initiated through the extrinsic pathway (thus the analysis is a modified EXTEM). The thromboelastometric curve which results is called **APTEM**. More recently *tranexamic acid* was introduced instead of Aprotinin, and the analysis is called tAPTEM [35].

To reveal *heparinization or heparin-like effects*, *Heparinase* can be used in the sample while coagulation is initiated through the intrinsic pathway (thus the analysis is a modified INTEM). The thromboelastometric curve which results is called HEPTEM [35].

2.2.2.4 The ROTEM® reference ranges

ROTEM® reference values were obtained from a large multicentre study in healthy volunteers [171].

Reference ranges reported in several studies are for orientation only because they can vary marginally from country to country (p.42 in [35]). It is recommended that each centre should validate the reference ranges [168].

2.2.3 Interpretation of ROTEM® analysis

2.2.3.1 Coagulation activation and clot polymerization parameters

The parameter **CT** in all ROTEM® analysis is the time between when the coagulation is activated until the clot network is of enough strength to restrain the movement of the pin. This parameter is influenced by the plasma level of the coagulation factors and their activity and reflects partially the thrombin generation process. It is thus influenced by anticoagulants and tissue factor expression on circulating cells (p.44, 52-54 in [35]).

CT INTEM mainly depend on coagulation factors of intrinsic pathway (p.42 in [35]). A prolonged CT INTEM is interpreted as a lack of coagulation factors involved in the intrinsic pathway. A shortened CT in **HEPTEM** compared to INTEM of the same analysis represent a heparinization effect.

CT EXTEM depend on coagulation factors of extrinsic pathway II, V, VII, X, as well on fibrinogen (p.41 in [35]). A prolonged CT EXTEM is interpreted as a lack of extrinsic pathway coagulation factors and can be used to guide the administration of coagulation

factors concentrate or plasma. In non-cirrhotic patients treated with warfarin CT EXTEM linearly correlates with PT-INR [172].

CFT and the **alpha (α) angle** in all ROTEM® curves reflects the kinetics of the clot formation and their velocity depends to a great extent on thrombin generation, but the contribution of fibrinogen-platelets functionality in these parameters is important (p.43 in [35]). In INTEM/EXTEM these parameters alone cannot discriminate deficits of fibrinogen from platelets [173]. Discrimination can be made by comparing clot firmness parameters from EXTEM and FIBTEM (p.54 in [35]).

2.2.3.2 Clot firmness parameters

Both in EXTEM and INTEM clot firmness parameters **A5, A10, A20,** and **MCF** reflect fibrinogens and platelet's contribution to the formation and stability of the clot. Lower than normal values of these parameters are interpreted as lack of fibrinogen and/or platelets. MCF have been used in the decision-making algorithms regarding treatment of severe bleeding; A5 and A10 are reported to correlate very well with MCF and thus allow a much earlier decision for treatment [174, 175], and have recently been included in algorithms [176, 177].

In FIBTEM the clot firmness only results from the fibrinogen polymerization because the platelets activation is blocked. Thus, the difference between clot firmness in EXTEM and FIBTEM can give an estimation of the platelet's contribution to the clot strength (p.54 in [35]).

Higher than normal values of clot firmness parameters, are interpreted as hypercoagulability [178, 179].

2.2.3.3 Clot lysis parameters

These parameters which are calculated for all ROTEM® analysis (INTEM, EXTEM, FIBTEM, etc.) give information regarding the activity of the fibrinolytic system. They indicate the residual clot firmness reported to MCF at different subsequent time points, usually at 30 and 60 min during the test run. The parameter ML is usually achieved at the end of the test and corresponds to Li 60 if the total test time is 60 min.

Clot lysis parameters are used in detecting hyperfibrinolysis. A higher than normal value could indicate hyperfibrinolysis, which is undoubtedly if the value is high.

It is reported that ROTEM® have a low sensitivity for the disclosure of a clinically relevant level of hyperfibrinolysis in trauma patients [180].

FIBTEM is more sensitive to hyperfibrinolysis then EXTEM or INTEM [181, 182]. In a study EXTEM is more sensitive to hyperfibrinolysis than INTEM [181]

As fXIIIa stabilizes the clot and increase the resistance to fibrinolysis, higher than normal clot lysis parameters on ROTEM® could indicate a deficiency of this coagulation factor (p.45 in [35]). However, this could not be demonstrated in patients with liver cirrhosis [183].

2.2.4 Limitations of viscoelastic testing

Limitations are related to the fact that viscoelastic tests are not sensitive to vWF activity and that protein C is not activated during the analysis [62].

A recent trial reports that ROTEM® is not sensitive to von Willebrand disease (vWD) while TEG® can discriminate vWD patients from healthy controls [184].

VETs are insensitive to antiplatelet therapy effects (p. 45 in [35]).

Limitations regarding extrapolation to in vivo reality are related as well to the fact that the endothelial factor is excluded and that the analysis is not performed under flow conditions [79].

Another limitation is low sensitivity to fibrinolysis [180, 185].

2.2.5 Clinical applications of viscoelastic testing

2.2.5.1 Viscoelastic testing in clinical situations involving bleeding

Cardiovascular surgery, liver transplantation, trauma, and obstetric haemorrhage have been the main indications of viscoelastic tests as point-of-care (POC) tests for haemostatic management in the bleeding patient.

Cut-off values for the management of coagulation are established in studies conducted with both TEG® and ROTEM®, and several transfusion algorithms are published and used per se or adapted to the practices of each centre [176, 186, 187].

In liver transplantation, VET based algorithms reduce red cell and fresh frozen plasma transfusion [111]. ROTEM® improves the use of coagulation factors, especially fibrinogen [188], and could also identify patients at risk for postoperative bleeding [189]. In cirrhotic patients, TEG® -guided transfusion before invasive procedures decreased the use of blood products transfusion compared to INR and platelet count guided protocol [113].

Viscoelastic testing has been investigated in smaller trials in other clinical areas such as severe sepsis, inherited bleeding disorders, anticoagulation with direct oral anticoagulants (DOACs), hypercoagulable states, liver surgery, neonatology and paediatrics, extracorporeal membrane oxygenation (ECMO), etc.

2.2.5.2 Viscoelastic testing and hypercoagulable states

Viscoelastic tests are used in studies to detect hypercoagulability. TEG® is able to detect hypercoagulability after major abdominal surgery and to predict thrombotic complications after surgical procedures [190, 191].

Furthermore, there are a few reports which show that ROTEM® has potential to diagnose hypercoagulability and even predict thromboembolism [35, 179, 192, 193]. Expanded clot firmness parameters are most commonly reported to diagnose hypercoagulability [178, 179], but shortened CT and CFT could also be appropriate indicators [192].

2.3 TRACER METHODOLOGY OF DE NOVO PROTEIN SYNTHESIS MEASUREMENT

The challenge in metabolic research is to investigate the dynamics of the metabolic mechanisms and the rates of changes in these processes. Among methods to investigate metabolism are isotopic labelling techniques, which allow dynamic measurements and remains highly reliable, despite their longevity [194, 195].

Almost any type of molecule can be labelled with one or more isotopes and become a tracer [195].

2.3.1 Isotopic tracers in metabolic research

2.3.1.1 The term of tracer and tracee

The term **tracee** is used to indicate a molecule (**the substrate**) that is involved in metabolism and is itself, or directly related to, the compound of interest for the research.

The biological behaviour of the tracee can be studied by administrating a traceable molecule, called **tracer**, which mimic the tracee course. Thus, the metabolism of a tracee can be estimated by studying the tracer dynamics in vivo if the tracer is metabolically identical with the tracee.

An **isotopic tracer** is the substrate (the tracee) which is labelled with an isotope. The tracer is thus chemically identical to the tracee but also different which make it distinguishable from the tracee.

An essential assumption to get reliable results using isotopic techniques is that the labelling does not influence the metabolism and the function of the substrate [194]. The tracer differs from the tracee only by physical properties which allows separation, quantification and ratios calculation.

2.3.1.2 Isotopes used for labelling of tracers

The isotopes of a chemical element have the same number of protons but differ by the number of neutrons they contain, which make their atomic masses different. The isotope with *the lowest atomic mass* can be written as *M+0* (M from atomic mass) [194]. As the atomic mass of a neutron is 1 Da, all the other isotopes can be written as M+i (i=1,2, 3 etc) where i = the number of extra neutrons in the isotope as compared with M+0.

Either radioactive isotopes or stabile isotopes have been used in metabolic research to label the tracers [195].

When radioactive isotopes are used, the tracer differentiation from the tracee is made by using scintillation counters [195].

The tracers labelled with stable isotopes can be distinct from the tracee by the differences in atomic/molecular mass. Mass spectrometers are used for this purpose.

2.3.1.3 Stable isotope tracers in metabolic research

When used in humans, an important advantage of the stable isotopes over the radioactive ones is the lack of disintegration and subsequent ionizing radiations. Hence, stable isotopes imply no risks or side effects when they are administrated in **recommended doses (sometimes** called **"accepted amounts")**, allowing even repeated measurements, which in most cases is not possible with radioisotopes [195].

2.3.1.4 The concept of natural abundance

The stable isotopes are naturally occurring. For the chemical elements most used as tracers (hydrogen, carbon and nitrogen) *the most commonly isotopes in nature are those with the lowest atomic mass*, which are **^{1}H, ^{12}C and ^{14}N** respectively [194]. The isotopes **^{2}H, ^{13}C, ^{15}N** are rare (0.02-1.1 %) [194]. Tracers are labelled with isotopes which are *not the most abundant natural occurring* (named as well "much-less-common" isotopes by some authors) [195].

2.3.2 Tracer mathematical models (pools, isotopic enrichment)

Many mathematical models are used to explore the metabolism by using isotope tracers. These analytical models aim to represent as accurate as possible the physiology of a metabolic process.

2.3.2.1 Body pools/compartments.

A pool is a theoretical concept defined as a compartment in the body related to a metabolic function [196]. The pools are abstractions which must be thought of apart from concrete anatomical structures.

Regarding metabolism and turnover of proteins, the term of pool is usually referring to the compartments containing free (unbound) amino acids as well protein(s) that bound the amino acids.

In the human body there are many extracellular as well subcellular compartments, containing free amino acids and/or proteins that bound amino acids. The free amino acids pools do not overlap the amino acid bound proteins pools and it is therefore appropriate to distinguish between them [196].

2.3.2.2 Assumptions in mathematical models

Considering the complexity of the physiological processes and the difficulties to get samples at cellular and especially subcellular levels, several assumptions are necessary in different models to complete the analysis.

For-instance *in non-compartment models, all the compartments*, or a group of them, could be seen as a unique pool and it is then assumed that they *are homogenous* [196]. This is a simplification and the term homogenous could be improper as homogeneity in this case involves reaching several balances between the compartments.

2.3.2.3 The concept of steady-state. Isotopic enrichment

When an isotopic tracer is administrated its concentration will rise in the different compartments of the body. Sooner or later depending on the rate of infusion, a *steady state* of the tracer concentration (enrichment respectively) will be reached in *the pool of the free tracer* [194].

The *isotopic enrichment* is defined as the *ratio of abundance* of *tracer to tracee* in the pool of interest at a certain time point [194]. Isotopic enrichment parameters express the *percentage of enrichment of the tracer (%enrichment*).

The primary parameter is the *tracer to tracee ratio (TTR) which* mathematically is expressed by the formula:

TTR = Tracer/tracee [194]

There are other ways such as *atom percent (%) excess (APE)* or *mole percent (%) excess (MPE)* which are used to express the isotopic enrichment.

APE= [TTR/ (1+TTR)] x 100 [194] or

APE (MPE) = [tracer/ (tracer + tracee)] x100 [194]

Thus, APE (MPE) are derivatives of TTR.

The term used for the *tracer steady state enrichment* is *"plateau enrichment"* (Ep) [196].

2.3.3 The tracer incorporation model

The incorporation model is used to assess the in vivo synthesis of a molecule. This model can be applied in studies of *de novo synthesis for individual proteins* [194].

The term *de novo* synthesis refers to a complete creation of a molecule and is opposed to the process of synthesis by recycling. For protein synthesis it refers to the ribosomal translation of RNA to a protein [196].

After its administration, usually intravenously, the tracer enters the amino acid pool for protein synthesis and is incorporated in the protein which is the subject of research. In this process the amino acid is called a *precursor* whereas de novo synthetized protein is *the product* [194].

The incorporation model contains an important *assumption* that the size of the total pool and the incorporation rate after the administration of the tracer does not change during the time of the study [194].

2.3.3.1 Administrating the tracer

The tracer can be given either orally or intravenously. Mostly the intravenous administration is used to determine protein synthesis. One or more tracers can be infused during a certain time interval.

There are a few ways to intravenously administrate the tracer which settle the names to specific methods.

1. The constant infusion method. The tracer is then administered at a constant rate. An inconvenience is that the steady state will often not be achieved until after a long time period (most often several hours of infusion). A large number of samples are necessary just to assure that the steady state has been achieved, and the patient should then be maintained in a metabolic steady state during this extended period [195, 197]. Another disadvantage with this method is the so called "precursor problem" which is presented below.

2. Primed continuous infusion method. Here a bolus of tracer is given to quickly reach the steady state, followed by a continuous infusion to maintain it [194].

3. A large unique bolus (The flooding dose technique): The tracer is administrated intravenously in a pulse dose which compulsorily equilibrate to steady state the enrichment of the tracer in all compartments [197]. An important advantage is that the measurements are performed under a shorter time which is necessary in clinical situations with rapid changes in metabolism. A potential disadvantage is that the flood in itself may affect what is supposed to be measured.

2.3.3.2 Sampling

Samples should ideally be taken from the pools in which the tracer show the greatest amplitude of dilution [195].

Repeated samples are taken. The interval of sampling depends on the flow and transit time of the tracee and its tracer trough the organ which synthetize the protein, the time needed to achieve steady state and the presumed rate of incorporation of the tracer into the protein [195].

Plasma and tissue sampling are commonly used for free amino acids enrichments. To explore the synthetic rates of proteins which remain intracellularly tissue sampling is needed to determine the enrichment of tracer into protein [195].

For proteins which are exported in plasma such as albumin or fibrinogen plasma samples can be used to determine enrichments both for the precursor and the product [198].

2.3.3.3 Calculations

For calculation of the protein synthesis rate two sets of variables are necessary:

1) *The precursor enrichment*-The isotopic *enrichment of the free amino acid* in in the precursor pool

2) *The product enrichment*-The *enrichment of the amino acid incorporated into the protein*.

By the methods of *constant infusion* and *primed continuous infusion* the protein synthesis rate can be mathematically calculated by dividing the protein enrichment to the area under the curve for the free amino acid pool (Ep) vs. time (a steady state is required).

Mathematically:

$$Protein\ synthesis\ rate\ (\%\ per\ hour) = \Delta\ \mathrm{Eprotein}/\int \mathrm{Ep}\ \mathrm{x}\ 1/\mathrm{t}$$

[194, 195]

Where

$\Delta\ E_{protein}$= change in protein enrichment

$\int E_p$ = area under the curve for precursor enrichment

t= time

2.3.3.4 The precursor problem

A core problem in metabolic research using isotope labelling for assessment of protein synthesis is the adequately sampling of the pool of interest. It is important to accurately indicate the precursor enrichment in the appropriate pool in order to get realistic estimations of the protein synthesis [195].

The "precursor problem" emerge from in the fact that *the real precursor pool for protein synthesis is the aminoacyl-transfer-RNA (tRNA) pool* [199, 200] which is extremely difficult to reach and get sample for measurements [200]. In intracellular pools, near the ribosomes we don't have access for sampling and the dilution of the tracer is unknown [195].

De facto, most studies do not take samples from the aminoacyl-tRNA pool but use samples taken at "distance" from the real precursor pool and estimate the processes ongoing in the real precursor pool. The *assumption* is that the intracellular amino acid pool is homogenous in all compartments including the site of protein synthesis. This enable to extrapolate the isotopic enrichment from tissue (and even plasma) samples to the aminoacyl-tRNA pool [194]. However, it is known that the amino acid pool is not homogenous in the subcellular compartments [196].

The precursor problem consists in the fact that, *in the constant infusion technique the plasma level of precursor enrichment differs significantly from the intracellular levels* [196, 197].

The precursor problem concern as well the source of the tracer at the site of protein synthesis in long-lasting continuous infusions. If enough time have passed after the start of tracer administration, *breakdown* of the molecules that already have incorporated the precursor *release precursors which re-enter the amino acid pool* [197].

With regard to these problems concerning the precursor pool enrichments, questions over the accuracy of the results obtained with the continuous infusion method have been raised and the flooding dose technique emerged as a solution [197].

2.3.4 The flooding dose technique (FDT)

The flooding dose technique (FDT) was developed to discard the inconveniencies with the constant infusion method, in particular the precursor problem [197].

In the flooding method the precursor is administrated intravenously *as a single large bolus of a mixture of tracee and tracer*. This pulse dose eliminates the differences between the tracer enrichments in the free precursor pool and the real precursor pool [201, 202] and give the advantage for a more accurate estimation of the real precursor pool [197].

If a large amount of a known mixture of labelled and unlabelled amino acid (much larger than the endogenous amino acid free pool) is administrated, this will flood the endogenous amino acid free pool and provide a uniform enrichment [202, 203].

The method was used for the first time on mice to study the protein turnover in different tissues using radio-isotopes, initially ^{14}C-leucine [204],^{3}H-Valine, then L-[4-^{3}H] phenylalanine [205]. Following 10 years of refinement on different animal species, the method was applied in humans using stable isotopes [197]. As precursor was used ^{13}C-Leucine [206, 207], L-[1-^{13}C] phenylalanine [207] and finally $^{2}H_5$-phenylalanine [201, 208].

The flooding dose induce a high peak in plasma precursor enrichment. The reached levels of precursor enrichment are far above the steady-state. An enrichment equilibrium in all the body pools (plasma/tissue/t-RNA) will accomplice in a very short time interval [209].

Not being dependent on a steady state this method is appropriate to study unstable clinical states like in perioperative or critically illness situations [197, 202].

In humans the precursor enrichment falls after the initial peak at rate of about 25% per hour [201]. This is not a problem as the precursor free pool after the bolus injection is much larger (several fold) than what normally is [197]. Despite rapid falling, the initial very high level of precursor enrichment will allow enough time for sampling.

An important *assumption* in the FDT is that the protein synthesis is not altered by the administration of amino acids in a pulse dose. Because the calculated fractional synthesis rates were larger using flooding dose compared with the constant infusion rates of tracer, aroused the supposition that the high amounts of amino acid could stimulate the protein synthesis [210]. Studies on skeletal muscle protein synthesis infirmed this hypothesis [197].

FDT have been used to assess protein synthesis in different tissues (such as liver or muscles) both in normal and pathological conditions [197, 202].

2.3.4.1 Precursors used in FDT

The precursor is preferably an essential amino acid. Non-essential amino acids are problematic when used as tracers in FTD for protein synthesis assessments as they are synthetized de novo in the organism and will give less accurate results.

At the outset of the FDT method ^{13}C-labelled *leucine* was mainly used because of its free pool of small size which allow a rapid achievement of steady state in continuous infusion techniques and because leucine assure a very good agreement between enrichments in plasma and liver tissue [210]. But leucine has been reported to stimulate protein synthesis in muscles [211] and affect nitrogen balance during starvation [212]. Moreover, the precursor enrichment was possible to determine by using GC-MS but the enrichment in protein necessitate more sensitive instruments such as isotope-ratio mass spectrometry (IRMS) [202, 208].

To overcome these disadvantages, leucine was replaced by multiple ^{2}H-labelled *phenylalanine* in most studies. Notably, with multiple ^{2}H-labelled *phenylalanine* all the measurements are possible with standard GC-MS [201, 208].

Studies using large amounts of phenylalanine in flooding dose technique do not show a stimulation of protein synthesis by the pulse dose [205, 213].

Studies have shown that for phenylalanine the curves of enrichments in plasma and muscle do not differ significantly [201]. There is a very good agreement between the enrichments in the free amino acid pool and the aminoacyl -RNA pool in muscle and liver with FDT using phenylalanine [209].

2.3.4.2 The pulse doses

In humans the pulse doses of phenylalanine are between 30-50 mg/kg, [197, 201, 214]. Commonly 45 mg/kg (approximately 3g /70 kg) are infused intravenously during 10 min interval in from of a 2% phenylalanine solution which contain labelled phenylalanine mixed with unlabelled phenylalanine at the desired enrichment [201].

2.3.4.3 Sampling

During and after administration of the tracer, repeated blood samples are taken. The number of blood samples are determined by the number of points necessary to draw the enrichment curves. Usually blood samples are taken every 5-10 minutes.

The time for sampling is 90 min [202, 214], in some studies 120 min [215, 216].

2.3.5 Gas chromatography-mass spectrometry

The samples contain a huge number of molecules which need to be separated to analyse compound(s) of interest.

Chromatography is doing the separation of the compounds.

Both gas chromatography (GC) and liquid chromatography are used for isotopically labelled compounds.

Gas chromatography is the elected method for separation of compounds labelled with ^{2}H as in liquid chromatography a lot of ^{2}H will be lost by ion exchanges. To facilitate the volatility of compounds of interest from the sample a chemical *derivatization* is performed before processing gas chromatography.

Mass spectrometry (MS): After passing the GC the chemical compounds are vacuum-aspirated in the mass spectrometer. MS measures the *abundance* of molecules for selected ranges of mass-to-charge ratios and display the results in form of a *mass-to-charge ratio (m/z) spectra.*

Known mass-to-charge ratios spectra are used to identify the molecules in the analysed sample.

2.3.5.1 Subtraction of the natural background

The natural background of the isotopes used in tracers must be taken into account for every determination and be subtracted from the enrichment of the samples taken after the administration of tracer [194]. If the tracer is the m+1 isotope, the subtracted is the value of the natural occurrence of m+1 when m+0 is normalized to 100% [194].

2.3.5.2 Determination of low levels of enrichment

GC-MS detect relatively high levels of enrichment as the precision of measurements is around 0.5 APE. Higher levels of enrichments of the free amino acid pool (0.5-20 APE) can be thus detected without problem by GC-MS. However, the *enrichments of protein bound-isotopic labelled amino acid* are much lower (0,002-0.1 APE), and due to that, much difficult to be detected and measured by CG-MS [194, 208].

A modified GC-MS technique has been used successfully to detect low enrichments of $^{2}H_{5}$-phenylalanine (0.002-0.09 APE) [201, 208].

The modification implies enzymatic conversion by *decarboxylation* of $^{2}H_{5}$-phenylalanine to $^{2}H_{5}$-phenylethylamine before the derivatization and analysing with GC-MS [208]. The measurements done on $^{2}H_{5}$-phenylethylamine are significantly augmented in precision by reducing the background noise in GC results almost to zero [208].

This technique implies the use of calibration curves of phenylethylamine and curves of known phenylalanine enrichments [208].

2.3.6 Calculation of the protein synthesis rates with FDT

The flooding technique have been used to determine synthesis of hepatic cellular proteins [217-219] as well as of export proteins like albumin [214, 220] and fibrinogen [54, 55, 198, 215].

CG-MS determinations deliver the two variables which are needed for calculations:

1) The isotopic *enrichment of the precursor* in the precursor pool

2) The *enrichment of the precursor incorporated into the protein*.

Two synthesis rates are calculated: *the fractional synthesis rates (FSR) and the absolute synthesis rate (ASR)*

1) The fractional synthesis rate (FSR) is de novo synthesis of the studied protein, expressed as the fraction of the intravascular protein pool synthetized per unit of time.

The background behind the formula for FSR calculation is the derivative equation for incorporation of the labelled precursor into the protein with respect to time [197]:

$$\mathrm{d}mB/\mathrm{d}t = \mathrm{v} \cdot \mathrm{eA} \quad (1)$$

Where
mB= mass of labelled precursor in protein (tracer)
t = time
v = absolute rate of incorporation of the amino acid into protein (mass per unit time)
eA= isotopic enrichment of the precursor

Dividing all over by the total mass of precursor (the protein pool size), derivative formula will be**:**

$$(\mathrm{d}m\mathrm{B}/\mathrm{d}t) \,/\, (\mathrm{MB} + \mathrm{mB}) = \mathrm{k} \cdot \mathrm{eA} \quad (2)$$

Where

MB = mass of unlabelled amino acid
mB = mass of labelled precursor in protein
k= the fractional rate of incorporation of the amino acid into protein
eA= isotopic enrichment of the precursor

The *assumption* is that *protein pool size is constant*. The other *assumption* is that *the pulse dose of precursor does not influence the rate of protein synthesis*. Thus, the size of the precursor pool can be ignored and the equation for protein synthesis will include only its isotopic enrichment [197], equation (2) may be integrated and the equation is:

$$\int \mathrm{eB} = \mathrm{k} \cdot \int \mathrm{eA}\mathrm{d}t \quad \text{or}$$

$$\mathrm{eB}(t) - \mathrm{eB}(\mathrm{o}) = \mathrm{k} \cdot \int \mathrm{eA}\mathrm{d}t \ (3)$$

Thus, the formula for calculation of the fractional synthesis rate is [197]**:**

$$\mathrm{k} = [\mathrm{eB}(t) - \mathrm{eB}(\mathrm{o})] \,/\, \int \mathrm{eA}\mathrm{d}t \quad (4)$$

Where

k= the fractional synthesis rate
$e_{B(o)}$ = enrichment of the precursor incorporated in the protein at time 0
$e_{B(t)}$ = enrichment of the precursor incorporated in the protein at time *t*
$\int e_A dt$ = area under the curve for precursor enrichment versus time

Usually FSR is given as percentage of synthetized protein per day, and equation (4) can be written as:

$$\mathrm{FSR} = \mathrm{Ep}/\mathrm{A}\,\mathrm{x}100 \qquad [202, 214]$$

Where:

Ep= the increase in enrichment of the precursor into protein
A= area under the curve for precursor enrichment versus time (expressed in days).

Thus, the equation for FSR calculation in FTD is similar to that used in the continuous infusion method.

It was questioned if it is correct to use this formula as FDT do not produce a steady state for the free amino acid pool [221] but instead could imply a massive inflow of precursor in the synthesis pool from the expanded amino acid pool. However, this assertion neglected the assumption that the flooding dose does not influence the rate of protein synthesis, which was demonstrated to be true after all [197]. It is *assumed* as well that *proteolysis is minimal* under the sampling time which is short and can be ignored [197]. Hence, equation (4) is correct.

2) The absolute synthesis rate (ASR) per day can be calculated by multiplying FSR with the total protein mass in the intravascular pool. Usually ASR is given as mg/kg/day [214].

2.3.7 The issue of multiple determinations using FDT

To investigate protein synthesis in longitudinal studies, sequential measurements are required. Assessment of the time that must pass before proceeding a new determination could be problematic and, if sufficient time have not passed, the results may be affected by the isotopic precursor baseline caused by the first measurement [213]. Repetitive sampling using FDT with short time intervals between determinations exploring synthesis of albumin and total protein in the liver has shown that this is a real problem and the results are difficult to interpret [222].

This issue was not settled, and further studies were therefore needed to determine how repeated measurements using the flooding dose technique should be made without compromising the acuity of the results.

3 AIMS

Objectives of the first part of the thesis:

In patients with liver failure, standard coagulation tests indicate a risk of bleeding but clinically in most cases, there is no obvious tendency to bleed related to coagulation. On the contrary, there is evidence of increased thrombotic risk. The objective of the first part of the thesis was to search in descriptive studies if thromboelastometry can add value to clinical assessment in liver failure in patients with chronic liver disease and after major liver resections with a loss of liver mass.

The specific aims of the first part were:

1. To characterize the coagulation in patients scheduled for liver transplantation. In this group of patients, we also investigated the potential of thromboelastometry to be used as a prognostic tool in chronic liver disease.

2. To characterize the coagulation in patients undergoing major liver surgery, in particular if there is a role for thromboelastometry in how to understand and handle potential hypo- and hypercoagulation.

Objectives of the second part of the thesis:

Work nr.2 of the first part of our project revealed that following liver resections fibrinogen plasma concentrations had different trends postoperatively depending on the resection size. The objective of the second part of the thesis was to investigate the fibrinogen synthesis following major liver resections.

The specific aims of the second part were:

1. To establish and validate a methodology of repetitive determinations of in vivo fibrinogen and albumin synthesis.

2. To study the perioperative de novo synthesis of fibrinogen and albumin in patients undergoing major liver resections or pancreas resections.

4 METHODOLOGICAL CONSIDERATIONS

4.1 STUDIES DESIGN (ALL STUDIES)

	Study 1	Study 2
Primary aim	To assess the ROTEM® potentiality to be a prognostic tool	To characterize the perioperative coagulation using routine coagulation tests and ROTEM®
Study population	Patients with stable chronic liver disease with indication for liver transplantation	Patients with indication for hemihepatectomi(1) and extended hemihepatectomi (2)
No. of subjects	N=40	N=16 n1= 8 n2= 8

	Study 3	Study 4
Primary aim	To establish tracer metodologi in longitudinal studies	To assess the perioperative synthesis rates of fibrinogen and albumin
Study population	Healthy volunteers in two protocols: A(1) and B(2)	Patients with indication for liver(1) and pancreas(2) resections
No. of subjects	N=16 n1= 10 n2= 6	N=15 n1= 9 n2= 6

Figure 4: *Synoptic view on aims, study population and number of subjects (N=total number of subjects in the study; n=number of subjects in subgroups; 1 and 2 indicate the subgroup, which in study 2 were 1= hemihepatectomy, 2= extended hemihepatectomy, in study 3 - 1=Protocol A, 2=protocol B and in study 4 - 1=hepatectomy, 2=pancreatectomy)*

Protocols

Study 1 was a prospective cross-sectional observational study. The patients were studied at the time of the final assessment for liver transplantation. The patients were consecutive in an effort to obtain a representative sample. Post hoc the patients were dichotomized in two subgroups according to the Child-Pugh and MELD scores to make comparisons.

Study 2 and 4. Both studies were prospective longitudinal observational studies. The participants were studied preoperatively, in study 2 just before the surgery and in study 4 at least 2 days before the operation. Postoperatively the participants in both studies were studied on postoperative day 1, and thereafter in study 2 every third postoperative day during the hospitalization, while in study 4 on just one more occasion between postoperative days 3 to 5.

Study 3 was a prospective longitudinal study with two protocols (**Figure 5**). Protocol A with determinations at two time points with 48 hours' time interval. Simple randomization was performed with 5 subjects in each subgroup with cross-over administration of ^{2}H5-phenylalanine (D5-phenylalanine) or ^{2}H8-phenylalanine (D8-phenylalanine) at the two occasions. Protocol B with determinations at three-time points i.e. at 5 hours and 7 days after the first determination. D5-phenylalanine was used at occasion 1 and 3, and D8-phenylalanine at occasion 2.

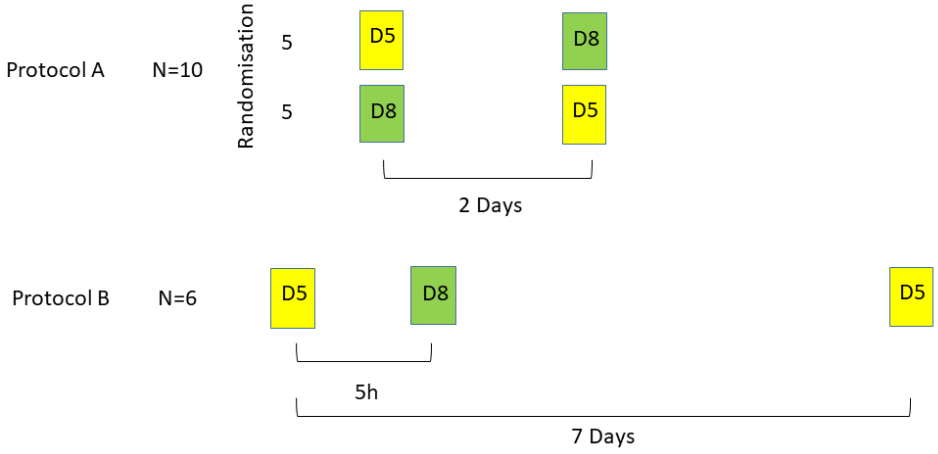

Figure 5. *The study 3 protocols (in rectangles is given which isotope was used for determinations)*

Settings

All participants in all studies were studied at Karolinska University Hospital, Stockholm, Sweden. Coagulation and biochemical analyses were performed at the Department of Clinical Chemistry while thromboelastometry was performed at the Department of Clinical Immunology and Transfusion Medicine at Karolinska Huddinge University Hospital. At the analytical laboratory of the ICU metabolism and nutrition research group at Karolinska Institutet were performed analyses for protein synthesis including GC-MS and ELISA analyses for biomarkers for coagulation activation.

4.2 COAGULATION AND BIOCHEMICAL TESTS (STUDIES 1, 2 AND 4)

Samples for coagulation tests were collected into citrated vacutainer tubes.

Standard coagulation and biochemical tests were analysed on automatic devices. Details about the reagents and devices which was used for each test are provided in the manuscripts of studies 1, 2 and 4.

The Owren method was used to determine the prothrombin time in all studies.

The Clauss method was used to determine plasma fibrinogen concentration in all studies.

Antithrombin, protein C and protein S levels were determined using functional assays.

ROTEM® analyses were executed on a ROTEM® delta device (Pentapharm GmbH, Munich, Germany) following the standard procedure according to the manufacturer's instructions, by specialized stuff trained to perform this analysis.

In study 4 the coagulation and biochemical tests were collected just before the procedure for protein synthesis through the vascular line dedicated for sampling.

4.3 THE FLOODING DOSE TECHNIQUE (STUDIES 3 AND 4)

Vascular access for analysis. For protein synthesis determinations, all subjects had two vascular lines. One line was used for the administration of the tracer, the other for sampling.

Tracer administration: Unlabelled phenylalanine together with $^{2}H_{5}$-phenylalanine (or $^{2}H_{8}$-phenylalanine) 99 molar percent excess (MPE) (Apotek Production & Laboratorier AB, Kungens Kurva, Sweden) was mixed to a concentration of 20 mg/ml (2g/100 ml saline) at different levels of enrichments according to the study protocol. The 2% phenylalanine solution was administrated intravenously in a dose of 45 mg/kg as an infusion for 10 min.

In **study 3** we used $^{2}H_{5}$-phenylalanine (alternatively $^{2}H_{8}$-phenylalanine) 10 MPE for the doses with 5 h and 48 h interval, and 20 APE (after seven days). In **study 4** we used an incremental dose of $^{2}H_{5}$-phenylalanine i.e. 5 MPE preoperatively, 10 MPE on POD1 and 30 MPE on POD 3-5.

Sampling: Blood samples were taken before the start of the tracer infusion and at 5, 10, 15, 30, 40, 50, 60, 70, and 90 minutes after.

The samples were collected into EDTA vacutainer tubes, kept on ice until centrifugation which was performed at 2500 x G for 10 min at $4^{O}C$, then plasma was transferred in small plastic tubes and stored at - $80^{O}C$.

Measurements were performed by biochemists with long experience in the domain. Chemical separations are described in study 3. For enrichment measurements was used gas chromatography- mass spectrometry.

Calculation of synthesis rates

To determine the synthesis rate for the liver export proteins the flooding method has been adapted and pays attention to distinguish between the time of the administration of the precursor and the time point when presumably begin the secretion of the protein into plasma called the *secretion time* (t_s) [214].

Accordingly, for the calculation of FSR in study 3 and 4 the following formula was used:

$$FSR(\%/day) = (P2 - P1)/A\ x\ 1440\ x\ 100 \quad [214]$$

Where

P_2-P_1= the increase of enrichment of the precursor incorporated in the protein secreted during time points t_1 and t_2 (which determined the regression line, after enrichment becomes lineal).

A= area under the curve for precursor enrichment between t_1-t_S and t_2-t_S (t_S is subtracted from the secretion times to select the part of the area under the curve according to the time points presumed to agree with the synthesis times).

The factors 1440 and 100 were used to give FSR as %/24 hours.

Absolute synthesis rate (ASR) was calculated by multiplying FSR with the quantity of the protein in plasma:

$$ASR\ (mg/kg/day) = FSR/100 \cdot PC \cdot PV/W$$

Where

PC = protein concentration
PV = plasma volume
W = weight

For estimation of the plasma volume in studies 3 and 4 we used *the anthropometric method, specifically* the *height cubed + body mass formula (Nadler formulas)*. These formulas give an excellent prediction of the blood volume determined by the isotopic method [223].

*The formula for **blood volume** (BV) calculation according to Nadler* [223] is:

For men: $BV = 0.6041 + 0.3669\ H^3 + 0.03219\ W$

For women: $BV = 0.1833 + 0.3561\ H^3 + 0.03308\ W$

Where
W= weight in kilograms
H= Height in meters

A correction factor of 0.91 representing the difference between peripheral body haematocrit and average body haematocrit was applied [223].

***Plasma volume** (PV)* was calculated with the formula:

$$PV = (1 - hematocrit)\ x\ BV\ x\ 0.91$$

4.4 STATISTICS (ALL STUDIES)

The statistical methods used in different papers are given in **Table 1**.

Table 1. *The statistical methods used in different studies are marked by grey shading.*

	Study 1	Study 2	Study 3	Study 4
Mann-Whitney U test				
Wilcoxon's matched pairs test				
One-way ANOVA				
Mixed effects model analysis				
Friedman's ANOVA				
Two-way ANOVA				
Spearman's correlation				
Pearson's correlation				
Fisher's exact test				
Dahlberg's formula				
ROC curves				

Nonparametric analyses were used if the assumption of normality was not confirmed. Normality was tested with Shapiro-Wilk test, D'Agostino & Pearson test or Kolmogorov-Smirnov test.

For nonparametric data, Mann-Whitney U test was used to compare two independent samples while Wilcoxon's matched pairs test was used to compare two related samples in repeated measurements.

One-way analysis of variance (ANOVA) was used to analyse the differences between means in repeated measures within groups. The lack of sphericity was corrected with Geisser-Greenhouse correction. Friedman's ANOVA with Dunn's post-hoc test was used for non-parametric data. Two-way ANOVA was used to analyse differences in changes of repeated measures between groups. In study 4, a mixed-effects model analysis (in Prism® 8) was performed to compare repeated measures data within and between groups which allowed maintaining in the analysis of patients with missing values due to random causes. In study 4 post hoc multiple comparisons were performed using Tukey's test.

Receiver operating characteristic (ROC) curves were used in study 1 to assess the ability of the test to distinguish between the subgroups with different mortality risks. For correlations, we used the Pearson's method for normally-distributed data. For non-normally-distributed variables, the Spearman's method was used. In study 3, Dahlberg's formula was used to calculate, the so-called pool coefficient of variance based on two observations (the imprecision of repeated measurements) under conditions assuming no systematic changes between the two occasions.

The accepted significance level (p) was under 0.05.

Statistical analyses were performed with Prism® 5, 6 or 8 (GraphPad Software Inc. La Jolla,CA) and Statistica 13.2 (Dell Inc. US)

4.5 ETHICS (ALL STUDIES)

All study protocols were following Helsinki Declaration and were approved by the Regional Ethical Committee in Stockholm, Sweden. All the subjects included in our studies received verbal and written information about the study protocols and provided written informed consent when we were sure that all information was understood.

5 RESULTS AND DISCUSSIONS

5.1 STUDIES 1 AND 2 (AND PARTLY 4)

In our studies we intended to characterize the patients with hepatic insufficiency also from the perspective of a viscoelastic test, which, we hypothesized, could have add value to the clinical assessment. We chose ROTEM® because this was the standard viscoelastic test in our institution. ROTEM® had been used during liver transplantations as a point of care testing of coagulation, with good results, especially reducing transfusion of blood and blood products. Nevertheless, a full thromboelastometric characterization of coagulation in the population accepted for liver transplantation or following major liver resections was not available at the time when we began our project.

5.1.1 The ROTEM®'s role in assessment of prognosis in chronic liver disease (study 1)

There were only a few publications regarding the viscoelastic test assessing of coagulability in patients with liver cirrhosis [49, 115, 224] and just one which regarded the mortality risk assessment by thromboelastometry [115]. Based on a good correlation of severity scoring systems Child-Pugh and MELD with some ROTEM® parameters, Tripodi et al. suggested that thromboelastometry could be applied not only to assess coagulopathy and the risk for bleeding but also for investigating the severity of liver cirrhosis. The most interesting parameter to be considered for prognosis proposes was MCF and to a lesser extent CFT [115].

Tripodi's report was promising considering that PT-INR, as the only coagulation parameter included in the scoring systems Child-Pugh and MELD, was questioned as potentially misleading the liver cirrhosis severity assessment and thus the priority for liver transplantation [73, 124, 126].

However, the study was not sufficient to conclude this issue mainly because of the low number of patients in end-stage liver disease (only 12% of the included patients were in Child-Pugh C stage), and because of narrow etiological spectrum consisting in hepatitis liver cirrhosis (68 %) and alcoholic liver cirrhosis (17%) [115].

In **study 1** we tested the hypothesis that ROTEM® can discriminate the degree of severity in stable chronic liver disease. A secondary aim was to assess ROTEM®'s ability to predict bleeding or thrombosis.

For this purpose, we enrolled consecutive patients undergoing an evaluation for liver transplantation. We dichotomized the patients into two groups to compare them. One group consisted of severe liver cirrhosis patients in Child-Pugh class C. Lesser sick patients in Child-Pugh A and B stages formed in the other group. We dichotomize as well according to MELD score at a cut off 17. These two groups, according to Child-Pugh and MELD score, had almost the same size and were reasonably sized to give a statistical power of 0.8.

Statistical comparisons showed no differences in ROTEM® parameters between these groups of patients in earlier stages of liver disease compared with late stages (**Figure 6** on p.43**)**. ROC curves were produced to assess the performance of ROTEM® parameters to predict Child-Pugh or MELD scores. For all ROTEM® parameters, the area under the curves (AUC) of corresponding ROC curves indicated that this test could not discern if patients were on a lower level of gravity or in end-stage liver disease (for MCF see **Figure 7** on p.44) (see also **published material study 1**).

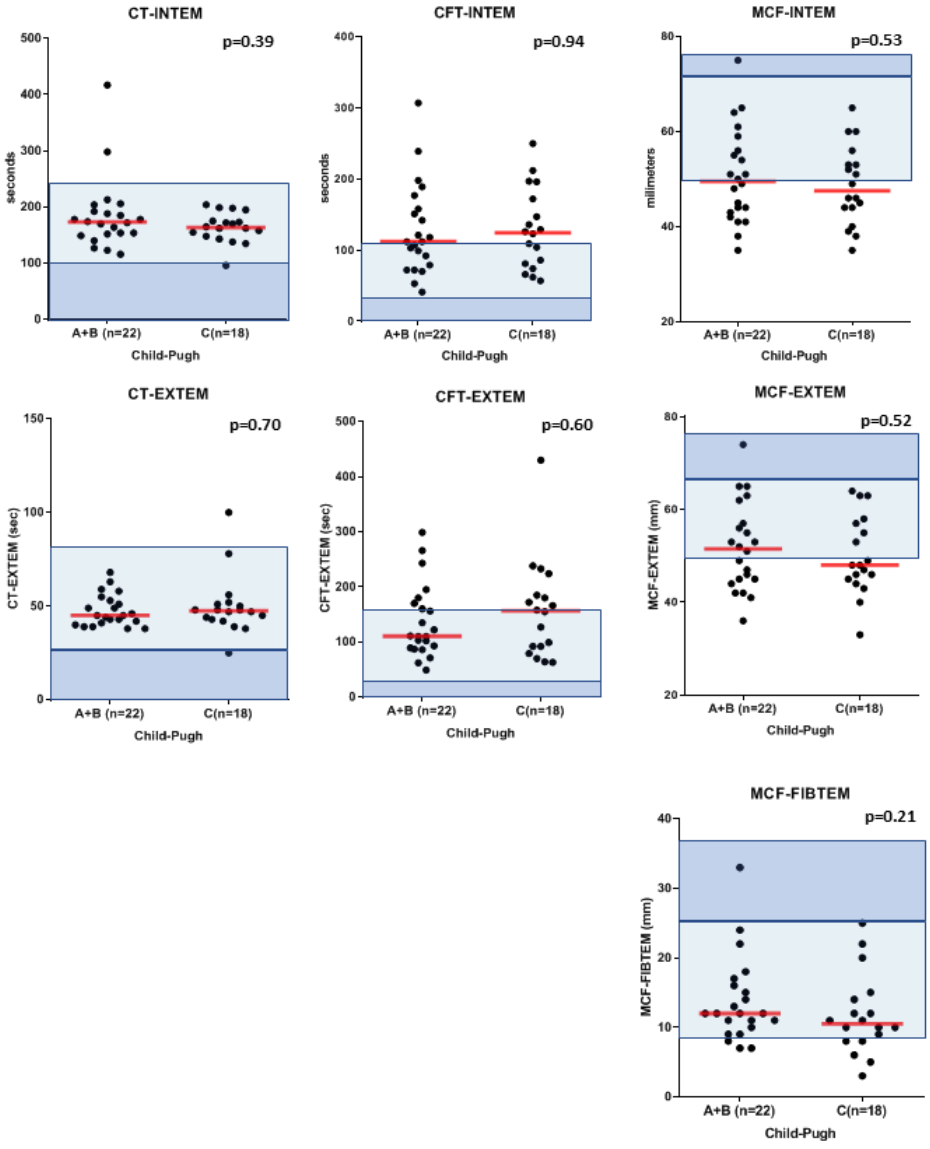

Figure 6. *The ROTEM® parameters in patients (n=40) included in study 1. The horizontal line represents the median value. Comparations were made by Students t-test or Mann-Whitney U test as appropriate and the p value is given in the upper right corner.(The blank area=hypocaoagulability,the intense shaded area= hypercoagulability)*

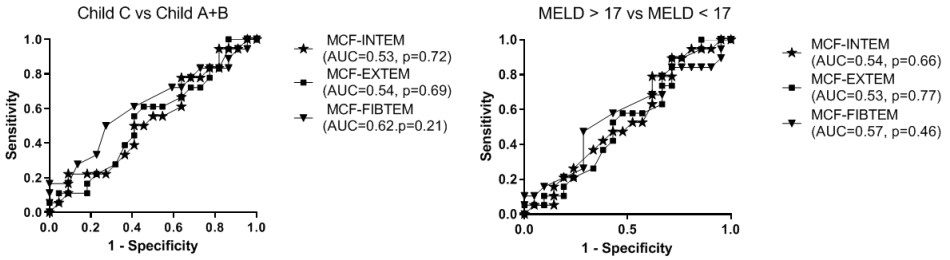

Figure 7. *Receiver operating characteristic (ROC) curves of MCF concerning the subgroups of patients according to Child-Pugh and MELD scores in study 1.*

We performed a post hoc regression analysis for MCF against the MELD score, which revealed extremely low coefficients of determination r squared (r^2) (**Figure 8, panels A-C**). MCF (INTEM, EXTEM or FIBTEM) explained only 5-8% of MELD variance.

Interestingly, advanced stages of liver disease had other relationships with ROTEM® parameters compared to early stages. Hence, this post hoc analysis disclosed that in the subgroup of the less sick patients (MELD ≤ 16) there was a good correlation of the scoring systems with the parameters CFT and MCF, explaining between 31-43% of MELD variance, while in the subgroups of sicker patients the correlation was almost 0 given an r^2 coefficient of .01(**see Figure 8, panels D-F)**. The same pattern was observed in Child-Pugh scoring (**Figure 9** on p.45). Thus, when the patients in the advanced stages are added to the group, the correlation decrease and became insignificant for the entire cohort for most of the ROTEM® parameters. Hence, what Tripodi et al. alleged might be correct for patients within less advanced stages of liver disease but should not be a rule *for the entire spectrum of severity, especially if a significant number of patients with advanced liver disease are included.*

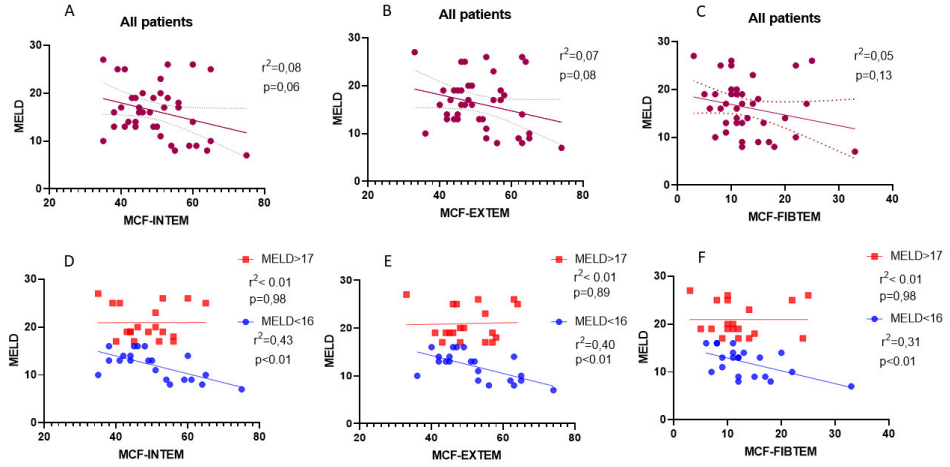

Figure 8. *Regression analysis MCF vs. MELD score in patients with chronic liver disease included in study 1, for all patients (n=40) (panels A-C) and for subgroups MELD ≤16 (n=21) and MELD ≥17 (n=19)*

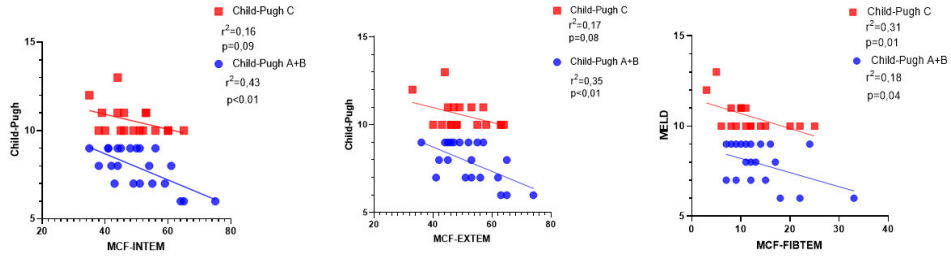

Figure 9. *Regression analysis MCF vs. Child-Pugh score A+B (n=22) and C (n=18) in patients with chronic liver disease included in study 1.*

MCF-FIBTEM correlation with Child-Pugh score (and to some extent and a lower significance with MELD score) seeks an explanation which we don't have now. This correlation is lacking in other cohorts with proportionally fewer patients in Child-Pugh C, although the prevalence of lower values of MCF-FIBTEM is significantly higher in patients with advanced chronic liver disease [48]. A possible explanation could be an increasingly dysfunctional fibrinogen molecule [60, 225] in end-stage liver disease that might have an impact on MCF FIBTEM. In support of this hypothesis is that in a previous study MCF FIBTEM could not differentiate healthy subjects from a cohort of stabile cirrhotics predominantly in stage Child-Pugh A and B [115].

Our findings in study 1 let us to conclude that we found no use for ROTEM® parameters for prognosis purposes in an unselected group of patients with stable chronic liver disease, similar to our sample.

Our finding disagrees with the suggestion that, as a rule, thromboelastometry, in particular, MCF, could be used to assess disease severity [115, 116].

Our conclusion that thromboelastometry has a limited role in predicting outcome in stable liver cirrhosis was an original finding. Although the external validity was limited by the case-mix for liver transplantation at our centre, the characteristics of the cohort investigated was similar to other European liver transplantation centres [226]. Another more recently study investigating the same issues with thromboelastography came out with a similar conclusion [227]. The results of a very recent study using thromboelastometry on patients with stable liver cirrhosis confirmed our conclusion [228]

A different situation could be in decompensated chronic liver failure. A recent study reported the parameter CT EXTEM > 80 s, at admission in the ICU, as a predictor of 28-days mortality in patients with acute-on-chronic liver failure [122].

5.1.2 Coagulation after major liver surgery (study 2)

We explored in our **study 2** the hypothesis that viscoelastic tests can add value to the evaluation of coagulation status and risk assessment regarding bleeding and thrombosis following major liver resections.

We, as others before us [118, 134], showed in **study 2** that during the first postoperative days, there is a decrease not only in pro-coagulant factors, which is reflected by PT-INR and fibrinogen plasma levels but also in anti-coagulant proteins such as AT and protein C.

To characterize the coagulation process, we analysed our samples with thromboelastometry, which showed the patients in coagulation re-balance throughout the entire observed postoperative period. In some individuals' signs of hypercoagulability was observed on postoperative days 4 and 7 (**Figure 13** on p.51).

5.1.3 The ROTEM®'s role in assessment of coagulations balance in patients with live failure (studies 1, 2 and 4)

The status of coagulation (the coagulation balance in particular), when assessed by viscoelastic tests, requires an interpretation of all parameters together. Individual ROTEM® parameters could be used to assess the balance within the segment of haemostasis that is reflected by each of them.

The CT parameter reflects the coagulation factors and the anticoagulants, while the MCF gives information mainly on the fibrinogen-platelets functionality. CFT is in between CT and MCF and related to thrombin generation as well.

Overall the results of **study 1** showed normo- and hypocoagulability on ROTEM® for the entire cohort (**Figure 6** on p.43).

CT was exceedingly normal, which indicated a rebalanced secondary haemostasis in any stage of liver failure, even in the end stages.

Regarding CFT and MCF in INTEM and EXTEM, they showed balanced coagulation only in 50 % of the patients with liver cirrhosis and hypocoagulability for the rest of the group (**Figure 6** on p.43).

Other studies report similar results in cirrhotic patients although with different proportions in balanced coagulation according to different ROTEM® parameters which could be due to differences in the etiological and severity case-mix of the samples [49, 115, 116].

Studies using TEG® show the same pattern although more balanced than ROTEM® based studies [114] while in patients during liver transplantation TEG® provides signs such as high G values (a clot firmness parameter derived from the maximum amplitude MA which correspond to MCF in ROTEM®) and short R times (which correspond to CT in ROTEM®), suggesting hypercoagulability in some but not all patients with cholestasis pathologies like PBS and PSC [117]. However, we could not see hypercoagulability in our **study 1** cohort also including patients with these conditions.

In patients with liver dysfunction after major liver resections (study **2**), we could demonstrate a different kind of rebalanced coagulation compared to chronic liver failure, with a majority of patients remaining inside the normal ranges in all ROTEM® parameters during the entire postoperative period (**Figure 10** on p.47).

The results of **study 2** showed that despite significant coagulation abnormalities in pro- and anticoagulant pathways as reflected by standard coagulation tests, ROTEM® indicated *balanced coagulation with a stable trend postoperatively*. Hypocoagulability on ROTEM® was exceptional and associated with complications such as grave liver failure or subcapsular hematoma of the liver. In some patients, hypercoagulability on postoperative day 4 and 7 was indicated by CT EXTEM, CFT/MCF EXTEM, and MCF FIBTEM (**Figure 13** on p.51).

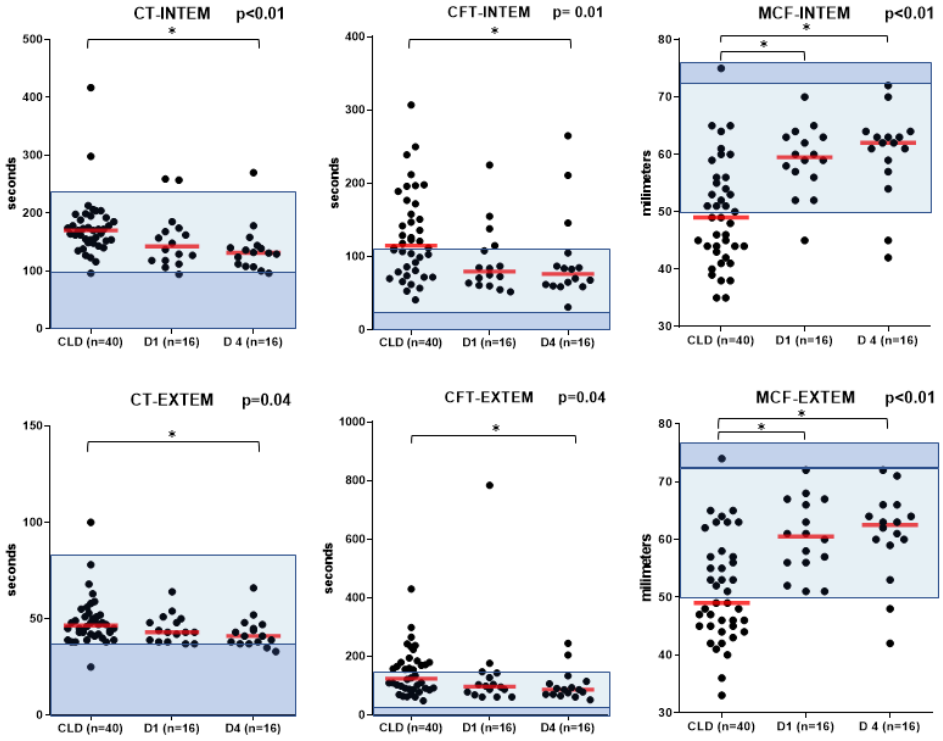

Figure 10. *The ROTEM® parameters from studies 1 and 2 in INTEM and EXTEM. The horizontal line represents the median value. The blank area=hypocaoagulability, the intense shaded area= hypercoagulability. Comparisons were made by Kruskal-Wallis test with and the p values are given in the upper right corner. * denotes Dunn's post-hoc test statistical significance p < 0.05. (**CDL**= chronic liver disease in **study 1**; **D1, D4**= postoperative day 1 and 4 in **study 2**).*

The coagulation re-balance following major liver resections, reflected by a very stable CT, may come by concomitant decrease in procoagulant factors (mirrored by the PT-INR) and the natural anticoagulant factors AT and protein C. At the same time, a firm clot is assured by the fibrinogen-platelets functionality. This approach in thinking grounded the concept of rebalanced coagulation in liver cirrhosis [85], and we showed in **study 2** using ROTEM® that a similar situation is present during the liver dysfunction following major liver resections. *We noted as well that a tendency towards hypercoagulability existed on postoperative day 4 and onwards, more expressed after hemihepatectomy.*

The main conclusion in **study 2**, that coagulation might be re-balanced following major liver resection similarly to chronic liver disease is original. We also showed that because of rebalancing, the risks for bleeding in these patients are small, even though standard coagulation tests could indicate such a risk.

Our results were in agreement with the results published by De Pietri et al., who used TEG® [118] with the exception that they didn't report signs of hypercoagulability. To be remarked that at that time, TEG® was not equipped with functional fibrinogen analysis.

A while after **study 2**, a study was published using ROTEM® on patients undergoing major liver resections, which have similar results and conclusions [147].

In **study 4** we noted no perioperative differences in ROTEM® parameters CT, CFT and MCF between patients undergoing major liver surgery and pancreatectomies (see additional file 2 in study 4). This confirmed the findings in study 2 and showed that after the loss of a considerable liver mass by surgical resection the re-balancing of coagulation occurred in comparable ranges with the pancreatectomy control group with intact liver.

In **studies 1, 2 and 4** the alpha (α) angle had a good correlation with CFT and similar results in the statistical analyses as CFT (for this reason the alpha (α) angle data was not included in this paper). The ROTEM® clot lysis parameter ML didn't have any denoting relation with the studied outcomes in studies 1, 2 and 4 and due to this was not included in this paper.

5.1.4 The fibrinogen (and platelets) function reflected by ROTEM® in liver failure (studies 1, 2 and 4)

The relationships between fibrinogen plasma levels and ROTEM® parameters are described in **study 1**, **2 and 4**. Essential, the action of fibrinogen on ROTEM® parameters regarding the clot firmness, should always be combined with the effect of the platelets on these parameters. Considering subtracting platelets contribution, MCF FIBTEM reveals the fibrinogen action only.

Fibrinogen has a vital role in balancing the coagulation disorders [62, 229].

Both in **study 1** and **studies 2 and 4** the patients had balanced coagulation according to MCF-FIBTEM.

80% of the patients included in **study 1** had an MCF FIBTEM inside the normal ranges, while only 57 % of the patients had normal plasma fibrinogen (**Figure 11**). Thus, many patients with plasma fibrinogen under normal range may still have a normal MCF FIBTEM which, in itself, can be considered a measure of balanced coagulation in the fibrinogen domain. This indicates the fact that fibrinogen plasma levels alone cannot give full information about how balanced fibrin-specific clot formation is, and a viscoelastic test should confirm this.

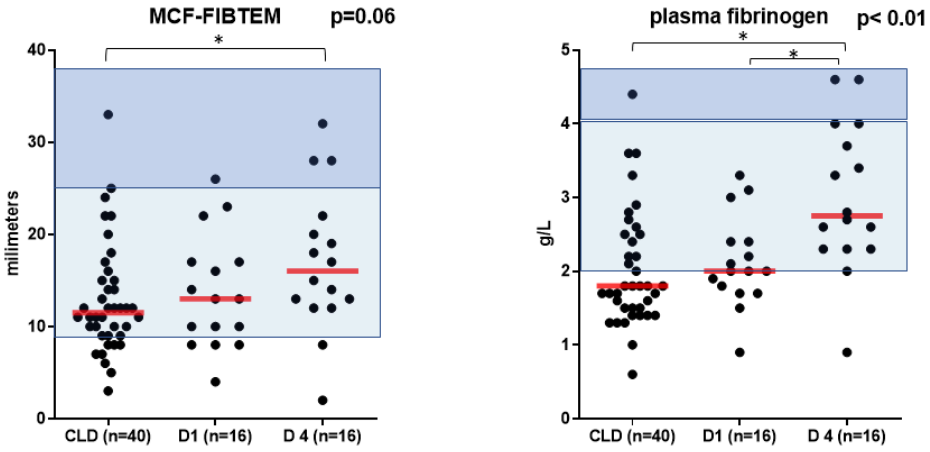

Figure 11. *MCF FIBTEM and plasma fibrinogen from studies 1 and 2. The horizontal line represents the median value. The blank area=hypocaoagulability, the intense shaded area= hypercoagulability. Comparisons were made by Kruskal-Wallis test with and the p values are given in the upper right corner. * denotes Dunn's post-hoc test statistical significance p < 0.05. (**CDL**= chronic liver disease in **study 1**; **D1, D4**= postoperative day 1 and 4 in **study 2**).*

The absence of correlation of MCF (INTEM and EXTEM) with scoring systems in **study 1** most likely relies on fibrinogen and platelets influence on this parameter. Patients in end-stage liver disease had low as well as high plasma levels of fibrinogen to the same extent as patients in earlier stages of liver disease. This was a piece of essential information that helped to clarify our findings regarding the relation between ROTEM® and scoring systems in the studied sample of the population.

In **study 1** and **study 2,** as expected, there were correlations of fibrinogen concentration in plasma with CFT and MCF, but not with CT.

For **studies 1, 2** and **4** the fibrinogen plasma concentrations had similar correlation coefficients with MCF EXTEM/INTEM as with MCF-FIBTEM (**Table 2** on p 54). According to Fisher r-to-z transformation there was no significant difference between the correlation coefficients either within or between the groups.

Table2. *Fibrinogen vs. MCF, Spearman correlation coefficients (r_s) with corresponding p values (CDL= chronic liver disease, D1/D4/D3-5= postoperative day 1/day4/day3-5)*

Group/subgroup	**MCF INTEM**	**MCF EXTEM**	**MCF FIBTEM**
CLD	r_s**= 0.73** p <0.01	r_s**=0.71** p <0.01	r_s**=0.66** p <0.01
D1 (study2)	r_s**=0.60** p =0.01	r_s**=0.63** p <0.01	r_s**=0.50** p =0.01
D4 (study2)	r_s**=0.62** p =0.01	r_s**=0.70** p <0.01	r_s**=0.55** p =0.01
D1 (study4)	r_s**=0.71** p =0.03	r_s**=0.77** p =0.01	r_s**=0.82** p <0.01
D3-5 (study4)	r_s**=0.86** p <0.01	r_s**=0.89** p <0.01	r_s**=0.82** p <0.01

An interesting finding in a post hoc analysis was that in chronic liver disease, platelets count correlates with MCF –FIBTEM **(study 1)**, which is not the case for non-cirrhotic patients after major liver resections **(study2) (Figure 12** on page 50**)**. The questions which emerge from this observation is either if cirrhotic platelets are resistant to Cytochalazin-D or if cirrhotic plasma could affect the results giving a better coagulability and cloth firmness in FIBTEM samples inhibited with Cytochalazin-D. If this finding is confirmed by other studies, it is a reason to rethink how MCF FIBTEM should be interpreted in patients with liver cirrhosis and eventually adjust the method.

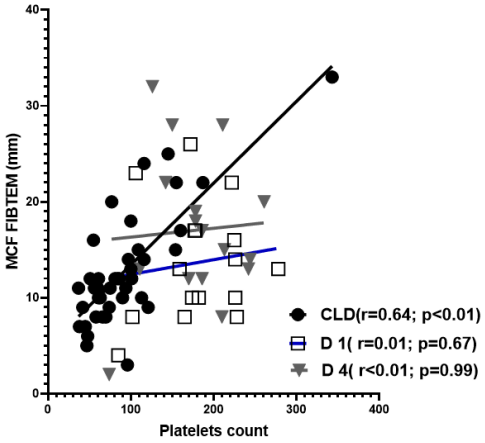

Figure 12. *Spearman correlation coefficients (r) of platelets count with MCF-FIBTEM for study 1(n=40) and study 2 (n=16) cohorts (CDL= chronic liver disease, D1/D4= postoperative day 1/day4) (r and p values are given in the parenthesis for each analysis)*

5.1.5 The ROTEM®'s role in predicting bleeding and thrombosis in liver failure (studies 1and 2)

5.1.5.1 The bleeding risk assessed by ROTEM® in liver failure

There is no evidence that viscoelastic tests (VETs) can be used to predict bleeding in patients with liver cirrhosis [230], and our **study 1** did not clarify the matter. The material was insufficient in size to be conclusive, and it was therefore hypothesis-generating only. Here ROTEM® analyses were made at a time distance from the liver transplantation and had no predictive value for bleeding. The possible predictive value for VETs on peroperative bleeding, when taken immediately before the procedure, remains to be elucidated [230].

The main factor for bleedings in liver cirrhosis is hypertension in the portal system and not coagulation disorders as the most stabile patients are in a rebalanced haemostasis [62]. Our **study 1** demonstrated that 92 % of patients with stable liver disease had a balanced coagulation status according to CT, but only 50% of them according to MCF. However, we found no association between indirect signs of portal hypertension and bleedings on the waiting list period either.

In **study 2**, the patients were generally balanced according to ROTEM®, and there were no postoperative bleedings of clinical importance. Hence, we could not draw any conclusions regarding the predictability of postoperative bleeding.

5.1.5.2 Predictability of thrombosis based on ROTEM®

Lately there is an increasing focus upon the tendency to venous thrombosis [95] and hypercoagulability, especially in the end stages of liver disease [74, 78, 231]. It is reported that the thrombin generation tests in the presence of thrombomodulin increase with liver disease progression to the end stages [49]. It is actually suggested that, rather than insufficient haemostasis, an imbalance towards hypercoagulability is more likely to occur in end-stage liver disease [78, 79].

In our **study 1**, no single patient presented signs of hypercoagulability on ROTEM®, defined as being out of normal range in more than 1 ROTEM® parameter, either in the early or in the end stages of liver disease in our cohort of patients. It could be speculated that in advanced stages of liver disease, exacerbated thrombin generation could be drawn back by a defected, slower polymerization due to dysfunctional fibrinogen [60, 225]. Thus, the thrombogenesis will not deviate to hypercoagulability. Another possible explanation is that ROTEM® cannot reveal hypercoagulability in liver cirrhosis because the normal ranges are not adapted to this rebalanced haemostasis. Other ROTEM® based studies failed to show hypercoagulability in patients with chronic liver failure as well [49, 116].

In **study 1,** the patients with portal vein thrombosis at the time of sampling had no signs of hypercoagulability on ROTEM®. However, the mechanism of thrombosis is often multifactorial, and Virchow's triad also point out portal flow disturbances and the endothelium as pathogenic factors for portal thrombosis.

In **study 2**, a tendency towards hypercoagulability could be noted on postoperative day 4 and postoperative day 7 on specific ROTEM® parameters, especially on CT EXTEM and MCF FIBTEM (**Figure 13**).

The rapid recovery of procoagulant factors reflected by PT-INR and fibrinogen plasma levels compared to anticoagulation proteins AT and protein C, which remained at lower levels a much longer period, could explain the signs of hypercoagulability which are more expressed in patients with hemihepatectomy then after enlarged hemihepatectomy.

The two patients included in the study who developed VTE, both had ROTEM® parameters that indicated the tendency to hypercoagulability and one of them undoubtedly had hypercoagulability signs on CT and CFT EXTEM and MCF FIBTEM days before the lung emboli occurred (**Figure 13**). The MCF FIBTEM trend in both patients showed a rapid recovery on postoperative day 4 with a sharper angle than the median. These cases suggest that ROTEM® could indicate the risk for thrombosis after major liver resections but are far from sufficient to set limits of risks.

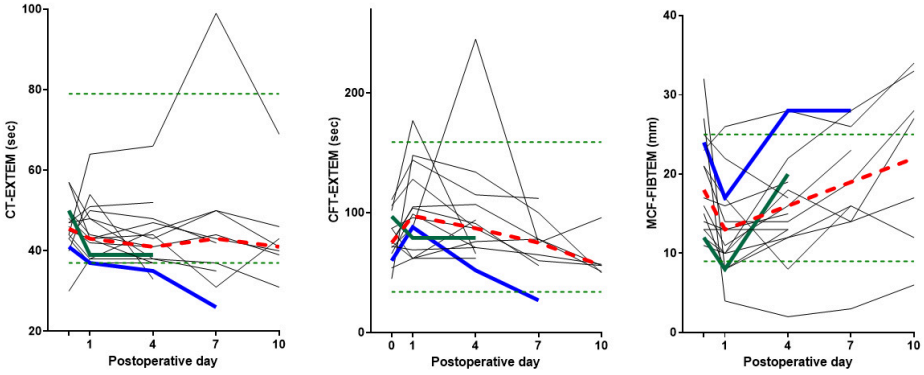

Figure 13. *ROTEM® parameters in patients (n=16) included in study 2. The normal range is indicated by dotted green lines. Median value is presented as a dotted red line for all patients. The two patients with pulmonary embolism are shown in bold blue and green line which are interrupted the day before they were diagnosed with lung emboli (postoperative day 5 and 8 respectively).*

5.1.5.3 The need for defining ROTEM® risk ranges for patients with chronic liver disease

Preoperative ROTEM® parameters A10 and CFT are suggested as predictors of postoperative thrombotic complications after major non-cardiac surgery [179]. May this be extrapolated to major liver surgery or liver cirrhosis?

Studies with ROTEM®, including our **study 1** failed to show hypercoagulability in cirrhotic patients. Why? Was there a case-mix problem or were the "normal ranges "for liver failure inappropriate? Or simply, there was no hypercoagulability? ROTEM® analysis could raise this supposition although it contradicts the broad consensus that patients with stabile liver cirrhosis are normo- or hypercoagulable [67].

Half of our cirrhotic patients included in **study 1** had a normal CT, but CFT and MCF were in the hypocoagulation area. How should this be interpreted in terms of predictions?

It seems that with ROTEM®, we are stuck in the same dilemma that we had with PT-INR which is more or less useless in predicting bleeding in liver cirrhosis. ROTEM® was not shown to be of much help for either bleeding or thrombosis predictions in chronic liver failure in our hands and in our patient case-mix. So, the utility of viscoelastic tests may be limited to the status of point-of-care tests during surgery.

Reference ranges specific for patients with liver insufficiency may surpass this limitation. However, defining what the valid group of reference subjects is may be difficult due to variability between patients considering the aetiology and stage of the liver disease.

The use of ROTEM® has been very helpful in surgical procedures associated with major bleedings, like liver transplantation, trauma, and cardiac surgery. When transfusion algorithms were directed by conventional coagulations tests like PT-INR, there was a tendency towards over-transfusion. Currently there are algorithms for treatment based on ROTEM® with cut-off values which are quite similar for liver transplantation (p.265 in [35]) and severe bleeding in trauma, and cardiac surgery [35, 177, 187]. Considering modifications in coagulation pathways, which subsequently rebalance the coagulation system on lower levels of pro- and anticoagulant factors, it is unlikely that liver cirrhosis patients are similar to the non-cirrhotic population in terms of risks assessed by ROTEM® parameters. Recently published algorithms' set lower thresholds on A5 for administrating of pro-coagulant therapy for bleeding under liver transplantation compared to other types of operations, however there are minimal differences in the CT thresholds compared to non-cirrhotic bleedings [176].

5.2 STUDIES 3 AND 4 (AND PARTLY 2)

5.2.1 Methodological clarifications concerning the FDT methodology for multiple determinations of fibrinogen and albumin synthesis in humans (study 3)

In **study 2** we found that the pattern of fibrinogen concentration in plasma postoperatively was related to the size of liver resection. Therefore, we considered a study where this pattern would be explored combined with measurements of de novo fibrinogen synthesis rates. The working group was experienced in quantitative measurements of another export protein from the liver, albumin. Therefore, the same methodology was considered also for synthesis rates of fibrinogen. Before launching a patient study a few methodological issues were to be solved, and also our laboratory needed a run-in study for the new analysis of fibrinogen synthesis rate. The main methodologic question was, how frequent multiple assessments of

fibrinogen synthesis rate were possible with maintained accuracy. This may be solved by an increase in the tracer to tracee ratio in a second measurement, or by introducing a different tracer in a second measurement. To sort this out we launched a study in healthy volunteers. In the protocol of **study 3** the flooding dose technique for determination of de novo fractional and absolute synthesis rates (FSR and ASR) of fibrinogen and albumin with repeated measurements taken at different time intervals using two isotopic traces. We analysed the differences in variance between determinations. In this study, we used the stable isotope $^{2}H_{5}$-phenylalanine (D5-phenylalanine), which is standard in this method [198]. We also investigated the possibility of introducing a second isotopic label, $^{2}H_{6}$-phenylalanine (D8-phenylalanine), trying to shorten the interval between measurements (**Figure 5** on p. 37). This was an original idea and part of the hypothesis in this study.

The complete dataset and the statistical comparisons are provided in the published study 3.

The study showed that an interval of at least 48 hours between measurements was appropriate when the flooding dose technique was used in longitudinal studies. In addition, the results showed that 5 hours' time interval was to short and gave less accurate results even when two different isotopic labels for phenylalanine are used. Hence, the results from **study 3** gave guidance regarding the time intervals between measurements in longitudinal studies of liver export proteins. Additionally, the study demonstrated the non-inferiority of the tracer D8-phenylalanine compared to D5-phenylalanine in the flooding dose technique. However, introducing a second isotopic label did not allow shortening the time intervals to 5 hours between measurements.

5.2.1.1 Fibrinogen and albumin synthesis rates

A few studies in the literature report synthesis rates of fibrinogen in healthy persons. Due to the low number of participants in the studies, it is improper to define a normal range for fibrinogen or albumin synthesis rates. However, comparisons between the reported synthesis rates values can be made if the determinations are performed with the same technique [54, 55, 216, 232].

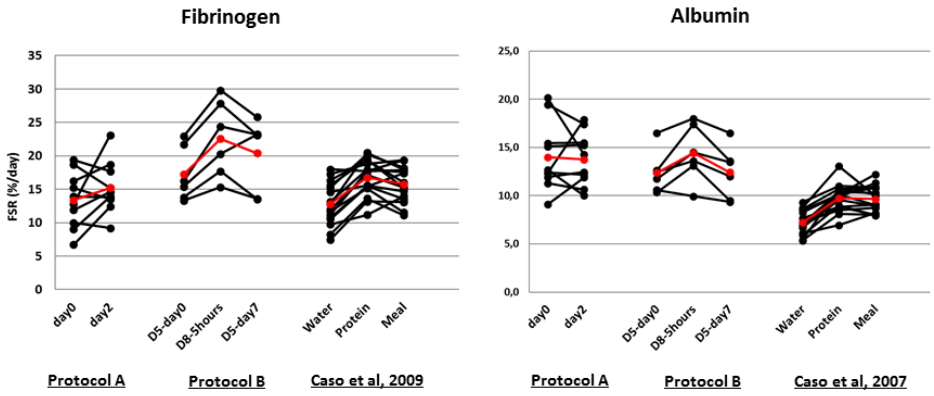

Figure 14. *Fractional synthesis rates of fibrinogen and albumin in healthy persons included in study 3. For comparison, results are given from two previous studies published by Caso et al. [55, 233]. (D5, D8= D5-, D8-phenylalanine. In red are depicted the median values).*

In **study 3**, FSRs for fibrinogen in protocol A were comparable with what is reported previously, a bit higher in the protocol B [55], while for albumin FSRs were higher than what is reported in a previous study [233] (see **Figure 14**). These differences may be related to the administration of a standardized meal including proteins in our study protocol before the

measurements, as the synthesis rates of fibrinogen and albumin increase following feeding, especially with meals with high protein content [55, 233, 234]. In contrast, the protocols of Caso et al. were performed in the post-absorptive state [55, 233].

Study 3 emphasized a well-known fact, that standardization during sampling is very important to reduce variability. The difference between the coefficients of variation regarding determinations with 7 days' time interval was higher for fibrinogen synthesis than for albumin synthesis which may be related to physiologic intercurrent inflammatory factors rather than to protocol or analytical causes.

5.2.2 The pattern of fibrinogen synthesis and fibrinogen plasma concentration postoperatively in major liver surgery (study 4)

In study 4 we determined the de novo synthesis rates for fibrinogen and albumin following hepatectomies and pancreatectomies (as controls). The driving force behind this research was the results of study 2 showing that the size of the remnant liver may be a factor influencing the postoperative alterations in plasma fibrinogen concentrations [235].

The study 4 results indicated that after hemihepatectomies, the liver synthetic function concerning fibrinogen was remarkable good. Fibrinogen synthesis on postoperative day 1 was roughly 2.5 to 3.5 times higher than preoperatively. As compared to the pancreatectomy group, with no loss of liver tissue, the variability of the increase in fibrinogen synthesis rate in hepatectomies was much greater. On postoperative days 3-5 a reversal was observed in both types of surgery and the synthesis returned towards lower levels, still higher than the preoperative ones, particularly following liver surgery (**Figure 15**).

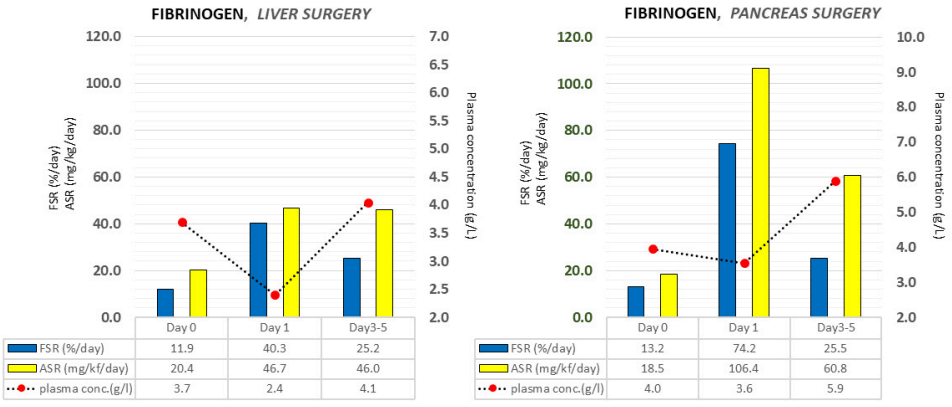

Figure 15. *Fibrinogen synthesis rates and plasma concentrations given as medians, in patients undergoing pancreas and liver surgery (study 4), preoperatively (Day 0) and postoperatively (Day 1, and Day3-5). Statistical analyses are provided in the study 4 manuscript.*

5.2.3 Plasma fibrinogen concentration, a reflection of the dynamic balance synthesis-catabolism-extravasation (study 4)

Plasma concentration of a protein does not reflect only the synthesis but also usage, degradation and trans-capillary migration of the protein [214]. Hence, the levels of plasma fibrinogen can only be explained by all these factors combined.

Despite the notable increase in fibrinogen synthesis on postoperative day 1, the plasma concentrations did not respond accordingly, remaining unchanged after pancreatectomy and decreasing marginally after hepatectomies, thus suggesting that important amounts of de novo synthetized fibrinogen was used within a relatively short time interval.

Following liver surgery, evidences indicate that the highest level of coagulation activation and thus fibrinogen utilization is at the end of surgery [143]. According to the levels of coagulation markers TAT and fpB, fibrin formation was not massively increased on postoperative day 1. However, following liver surgery, a higher level of ongoing fibrin formation even on postoperative day 1 was indicated by the soluble fibrin concentrations. Presumably the rise of plasma fibrinogen concentration, which most likely commenced early following pancreas and liver surgery [118, 147] was slowed by ongoing utilization especially following liver surgery.

It is possible that fibrinogen plasma concentration was not solely dependent on production and utilization in fibrin formation. Alternative catabolic pathways for fibrinogen may also be involved in fibrinogen disappearance from plasma to a large extent [50, 236].

It is not known if fibrinogen transcapillary transit occurs in inflammatory conditions. Extrapolating the behaviour of albumin to fibrinogen, the passage may accelerate, which would then have consequences on plasma fibrinogen concentrations. However, size of fibrinogen molecule is very different from albumin, more than 5 times larger.

In study 4 we found no correlation between fibrinogen plasma concentrations (or changes in concentrations) and the synthesis rate of fibrinogen postoperatively following major abdominal surgery, with or without loss of liver tissue. This lack of correlation may result from the plasma concentration being controlled not only by de novo synthesis, but also by its usage in the coagulation process, degradation by alternative catabolic pathways and possible increased extravasation.

An issue concerning liver cirrhosis is worth mentioning here. Fibrinogen catabolism in liver cirrhosis, measured with techniques using fibrinogen labelled with radioactive iodine, is reported to be increased by 30% compared to healthy subjects [237]. However, coagulation markers do not indicate an exacerbation of coagulation, and thrombin generation is reported to be normal in stabile cirrhosis [76, 238]. Although there are evidences of slightly increased fibrinogenolysis in liver cirrhosis [239], this cannot explain such a large increase in fibrinogen utilization.

5.2.4 The regulation of the fibrinogen synthesis (study 4)

The fibrinogen plasma concentration, reflecting the balance synthesis-utilization-extravasation, is as it seems regulated and maintained inside a range which allows for a normal coagulation. The mechanism which regulates the plasma fibrinogen levels is not elucidated. *It is not known if fibrinogen concentration itself has a role in this mechanism by modulating its own synthesis.*

5.2.4.1 The fibrinogen synthesis stimulation

As indicated by rat and human cell lines studies, IL 6 is the main inducer of fibrinogen synthesis [43, 56, 240].

Following major abdominal surgery, the peak for plasma IL-6 occurs early (12-24 hours) postoperatively, and its concentration correlates with the magnitude and length of the operation, open liver resections and pancreatectomies having among the highest increases of IL6 compared to the preoperative values [241-243]. Thereafter the plasma levels of IL-6

decrease quickly towards normal levels at 72 hours postoperatively if no complications occur [242, 243]. ***This IL-6 plasma levels pattern follows exactly the time pattern of fibrinogen synthesis described in study 4.***

5.2.4.2 The connection to the coagulation balance

Thromboelastometry showed balanced coagulation postoperatively after both pancreas and liver surgery. Maintaining the coagulation in normal ranges is a complex process and was most likely acquired by different mechanisms following liver resections compared to pancreas surgery. However, fibrinogen is most likely a key point in this process.

We hypothesized in study 4 that after major abdominal surgery the ***fibrinogen synthesis was modulated to counterbalance the fibrinogen disappearance from the plasma pool*** and attain plasma concentrations with which the coagulation system was accustomed (these were the preoperative values), and it assured an essential substrate for coagulation. Also following hepatectomies this goal was achieved despite the loss of liver mass.

Our reasoning advanced thus the ***hypothesis that inputs coming from the coagulation system may influence the fibrinogen synthesis.*** *So hypothetically there may be a feedback mechanism to control the fibrinogen synthesis.* Too much fibrinogen synthesis may cause a hypercoagulability, an insufficient synthesis will put the patient at risk for bleeding.

***May this hypothesis be extended to the patients with chronic liver disease*?** The only study investigating the fibrinogen and albumin synthesis in liver failure is a case-series of patients with liver cirrhosis caused by hepatitis, published 1996 by Ballmer et al. [198].

In Ballmer's study, the liver function is measured by galactose elimination capacity and the aminopyrin breath test which doesn't correlate with fibrinogen synthesis, but it does correlate with albumin synthesis rates which are lower in advanced stages of liver disease. The lack of correlation may be caused by the fact that fibrinogen as an acute phase protein has a larger range of variance compared to albumin.

In Ballmer's study the patients in Child-Pugh score (CPS) A have a plasma fibrinogen median of 2.2 g/L and in CPS B 1.1 g/L while in CPS C of 1.5 g/L. In these patients' fibrinogens FSR/ASR is lowest in CPS B, while synthesis rates in CPS C are comparable with CPS A which, although lower, are in the proximity of the reported values in healthy population.

Even though the concept of rebalance coagulation didn't exist at that time, Balmer et al intuited this and stated that fibrinogen is "vital" for coagulation to work properly, and because of this the synthesis may be preserved even in advance stages of liver cirrhosis [198]. So, in advanced stages of chronic insufficiency the liver may have the capacity to modulate upwards the synthesis rates in order to achieve the plasmatic goal at which the coagulation system is rebalanced, which for the Ballmer's study advanced liver cirrhosis subgroup was around 1.5 g/L. However, Ballmer's study is limited by a low number of patients especially in CPS C stage.

5.2.5 Fibrinogen and albumin in the assessment of the intrinsic synthetic function of the remnant liver (study 4)

Our data from study 2 suggested that the critical liver mass which could not assure enough fibrinogen synthesis may be the remnant liver after extended right hemi-hepatectomy. It is very likely that a remnant liver of less than 20 % of the initial liver volume cannot avoid a severe hepatic insufficiency [244]. After performing study 4 and understanding the potentiality of fibrinogen synthesis in the liver, we can presume that an insufficiency of

fibrinogen synthesis can only occur in severe post-hepatectomy liver failure with an inadequate parenchymal volume and/or functionality. Conversely, *a slowness or inability in restoring fibrinogen plasma concentrations on postoperative days 3-5 at reasonable levels might be a marker of risk for severe post-hepatectomy liver insufficiency*.

In study 4 the albumin plasma concentrations decreased significantly on postoperative day 1 both after pancreatectomies and hepatectomies and remained low, which is the usual postoperative pattern following major abdominal surgery [245, 246] (**Figure 16**). It has already been shown that the postoperative drop of plasma albumin is mainly due to an increased albumin extravasation with redistribution to the extravascular space [247].

Following liver surgery, the albumin synthesis rates remained unchanged, while following pancreas surgery on postoperative day 1 they were de facto about 50% higher on postoperative day 1. Considering the important loss of liver tissue following hepatectomies, we may assume that the synthesis of albumin was increased per gram of tissue in the remnant liver too.

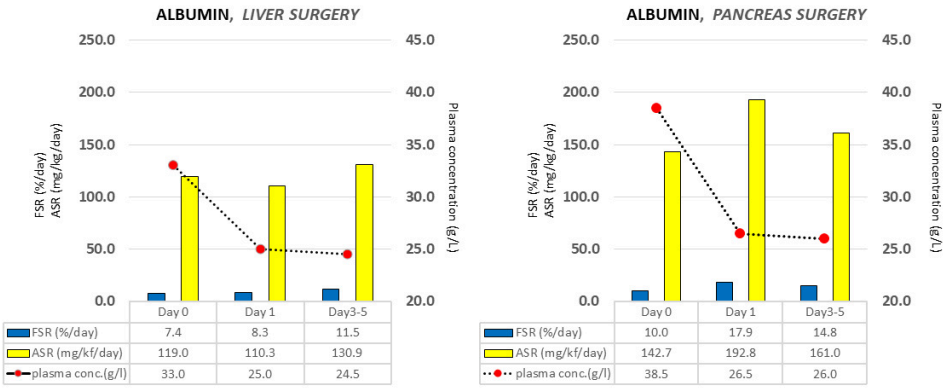

Figure 16. *Albumin synthesis rates and plasma concentrations given as medians, in patients undergoing pancreas and liver surgery (study 4), preoperatively (day 0) and postoperatively (day 1, and day3-5). Statistical analyses are provided in the study 4 manuscript.*

Completion of the concept Posthepatectomy Liver Failure (PHLF) regarding synthesis function of the liver mirrored by PT-INR

Hyperbilirubinemia and increased PT-INR during the first 5 postoperative days are signs of liver dysfunction [132, 248, 249]. However, these two signs are transitory in most patients and the so called "50-50 criteria" definition of post-hepatectomy liver failure (PHLF) does not include the first 5 postoperative days [132].

Aiming at a more precise statement, a consensus conference defined PHLF as inability of liver to "maintain its synthetic, excretory and detoxifying function" consistent with plasma INR and bilirubin [250].

In study 4 we found no correlation between PT-INR or bilirubin and the synthesis capacity in the liver for albumin and fibrinogen. Although PT-INR on postoperative day 1 increased above the normal values, the de novo synthesis of fibrinogen was substantially higher than preoperatively, and the albumin synthesis rates were not decreased.

Our findings indicate that the definition for PHLF may not be fulfilled considering that PT-INR cannot mirror the global synthetic capacity of the liver. PT-INR, although dependent on coagulation factors synthetized in the liver, is validated only to monitor the treatment with vitamin K antagonists and can only evaluate the functionality of the extrinsic pathway of haemostasis [86]. Yet, PT-INR is perceived as reflecting the synthetic function of the liver. The definition should therefore be reconsidered.

6 LIMITATIONS

In studies 1, 2 and 4 we did not perform sophisticated coagulation tests to explore thrombin generation and fibrinolysis.

It is debatable if viscoelastic tests can entirely estimate the balance of coagulation especially because these tests cannot measure the contribution of vWF and protein C. In studies 1, 2 and 4 we used a standard thromboelastometry, i.e. protein C was not activated during the test.

The relatively low number of patients in study 1 didn't give us the possibility to conclude over the risk of bleeding and thrombosis.

In studies 1, 2 and 4 we did not screen systematically with imagistic techniques for deep vein thrombosis and pulmonary embolism.

In study 2 we did not evaluate the effect of synthetic colloid administrated perioperatively on the ROTEM® parameters.

In studies 3 and 4 we used an anthropometric formula (Nadler formula) to estimate the plasma volume for calculation of the absolute synthesis rates.

In study 4 the relatively low number of subjects did not allow a description of synthesis rates trends in patients undergoing extended hemihepatectomy.

In study 4 we didn’t assess the disappearance rates of fibrinogen and albumin.

7 CONCLUSIONS

Our studies 1, 2, and 4 showed that thromboelastometry can provide important information to the clinician regarding the coagulation balance in liver insufficiency.

Study 1 demonstrated that in a heterogeneous cohort such as the patients scheduled for liver transplantation, the thromboelastographic parameters did not have a direct relationship to the stage of the liver disease. Our study 1 found no arguments for thromboelastometry to be used as a prognostic tool in chronic liver disease. However, our study 1 showed that thromboelastometry can assess the balance of coagulation in liver cirrhosis if the definition of unbalanced coagulation accepts at least one thromboelastometric parameter to be outside the normal range.

Study 2 contributed to a better understanding of the coagulation system postoperatively after major liver resections. We demonstrated, using thromboelastometry, that the hemostasis is rebalanced postoperatively in these patients and that standard coagulation tests could mislead the clinical judgment indicating a bleeding risk. Accordingly, decisions to treat coagulation aberrations should be taken in corroboration with a viscoelastic test. Study 2 gave support to the idea of routine thrombosis prophylaxis in patients undergoing liver resections and demonstrated that signs of hypercoagulability may appear on thromboelastometry which could signal the risk for venous thromboembolism in these patients.

Study 2 indicated also that the size of the remnant liver following, hemi- vs. extended hemihepatectmy, is a factor that can affect the postoperative plasma fibrinogen concentration trends.

Our studies 3 and 4 added knowledge in the domain of de novo synthesis of fibrinogen and albumin in healthy subjects and following major abdominal surgery,

Study 3, a methodological study, established the appropriate time intervals for repeated measurements in longitudinal studies exploring de novo synthesis of fibrinogen and albumin with maintained precision and accuracy.

Our study 4, guided by the methodology described in study 3, demonstrated that following hemihepatectomy the synthesis of fibrinogen and albumin were well preserved and had the capacity to increase over the preoperative values. Although of lower amplitude following hepatectomy, the synthesis rates of fibrinogen were not different from a comparable operation without loss of liver tissue (i.e. pancreatectomy). Hence it was discovered the extraordinary potentiality of the liver to increase the fibrinogen synthesis postoperatively. It was also revealed that the fibrinogen plasma concentrations postoperatively in major abdominal surgery, e.g. pancreatectomy or hemihepatectomy, were not solely dependent on fibrinogen synthesis but also on utilization.

Our study 4 demonstrated also that the synthesis of fibrinogen was sharply modulated which made possible prevention of substantial abnormalities of plasma fibrinogen concentration. This contributed to balanced postoperative coagulation, according to thromboelastometry, both following surgical interventions without liver implication or with loss of liver mass. It was also demonstrated that an overshot of fibrinogen concentration on postoperative days 3-5 was possible both after pancreatectomy or hemihepatectomy.

8 RESEARCH PERSPECTIVES

Directions for further research nave been opened by our studies as follows:

Study 1

To elucidate the correlation between MCF- FIBTEM and Child-Pugh score, a study can be designed to explore the relation between MCF-FIBTEM and fibrinogen in advanced stages of liver disease. The involvement of fibrin structure modifications might be searched in collaboration with centers with experience in dysfibrinogenemia research.

As a part of the completion of the validation for ROTEM®, studies to determine the sensitivity of ROTEM® to hypercoagulability in liver failure are required.

Establishing the ROTEM® reference range for the population with chronic liver disease is possible. This is done for TEG® at least in one transplantation centre [251].

A study to clarify the correlation between platelets count and MCF-FIBTEM in liver cirrhosis which was revealed in a post hoc analysis of data from study 1.

Study 2

We presume that after large liver resections the trend of the postoperative fibrinogen plasma concentration and MCF-FIBTEM, particularly the sharpness of the rebound, could be risk markers both for liver insufficiency and for hypercoagulability in these patients. Prospective studies to test this hypothesis can be designed. If it is proved to be true, it would add value to the prediction of serious risks.

Study 3

Research on the possibility to make protein synthesis determinations during the period between 5-48 hours after the first determination.

Develop methodologies to determine the synthesis of coagulation factors with much lower plasma concentrations than fibrinogen. Modern mass-spectrometry systems make it possible.

Study 4

A study focused on patients with extended liver resections to determine the critical remnant liver mass/volume for insufficient fibrinogen and albumin synthesis and the markers of risk. In parallel patients undergoing PVE with two-stage hemihepatectomy should be explored to determine the relation between the volume of the remnant liver and its synthetic function.

Studies focused on the postoperative rate of increase of plasma fibrinogen may give predictability of the postoperative levels.

Studies exploring the fibrinogen synthesis intraoperatively and early after the operation (major abdominal surgery involving liver and pancreas) may add valuable knowledge.

Eventually, studies regarding the synthesis of coagulation factors in major liver surgery may clarify the mechanisms underlying their plasma concentrations.

Study 4 reopen the issue of hypercoagulability following large hepatectomies. This issue is not settled jet.

The most exciting perspective opened by study 4 is, we believe, the incitement in deciphering the mechanisms of control and modulation of fibrinogen synthesis. Here is a complete lack of

knowledge. Studies can be made in animal models to see if induced variations in fibrinogen plasma concentrations alter the fibrinogen synthesis. Studies in humans are also possible to design.

Studies regarding fibrinogen and albumin synthesis in chronic liver disease, especially in end-stage liver disease, can solve the dilemma of how deficiencies in synthesis affect plasma concentrations of these two proteins.

Studies regarding fibrinogen extravasation could be difficult to design but not impossible. Such studies could help in understanding the mechanisms behind fibrinogen plasma concentration variations.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Vid leversvikt har levern nedsatt förmåga att syntetisera essentiella ämnen för en välfungerande metabolism samt att rensa bort metaboliska slutprodukter som inte längre behövs. Leversvikt finns i olika stadier, till följd av leversjukdomens utveckling. I allvarliga fall den är den enda behandlingen som kan rädda patienten med avancerad leversvikt en levertransplantation. Leverdysfunktion inträffar även postoperativt hos de patienter som genomgår stor leverkirurgi, där en stor del av levern tas bort. Koagulationsrubbningar ackompanjerar alla stadier av leversvikt, både tidigt och sent i förloppet. Orsaken till koagulationsrubbningarna vid leversvikt är komplexa och beror i stort sett på att de flesta koagulationsfaktorerna produceras i levern. Vi vet sedan tidigare att koagulationen vid stabil kronisk leversvikt blir re-balancerad genom mekanismer som uppstår för att kompensera den brist på koagulationsfaktorer och trombocyter som karakteriserar dessa patienter. Men vid svår dekompensation kan denna fragila balans upphöra. Patienter med leversvikt kan ha lätt för att blöda men samtidigt ha en större risk att bilda proppar.

För att bedöma koagulationsrubbningar tas vanliga koagulationsprover som kan avslöja punktuella förändringar i koagulationssystemet men, vid komplexa förändringar, dessa inte kan bedöma koagulationens funktionalitet som helhet. Tromboelastometri (ROTEM®) är en helblodsanalys där koagelbildning, koagelstyrka och koagelupplösning mäts i samma prov vilket ger en global syn över blodets levringsförmåga. På det sättet kan tromboelastometri ge upplysning om huruvida koagulationssystemet är balanserat eller inte. Det finns också rapporter om att tromboelastometri skulle kunna användas för att bedöma prognosen vid kronisk leversvikt.

Syftet med studierna 1 och 2 var att karakterisera patienter med leversvikt genom att använda tromboelastometri. I studie 1 karakteriserades koagulationen hos patienter med kronisk leverinsufficiens med indikation för levertransplantation. Vi använde ROTEM® och vanliga koagulationsprover. Ett huvudsyfte med den här studien var att undersöka huruvida tromboelastometri kan skilja på graden av leversvikt. Vi gjorde korrelationer mellan laboratoriedata och alvaret hos leversjukdomen enligt de två vanliga prognosverktyg Child Pugh och Model for End-stage Liver Disease (MELD). Slutsatsen var att vi inte kunde hitta någon användning av tromboelastometri för att värdera prognosen av den stabila kroniska leversjukdomen med indikation för levertransplantation.

Studie 2 var en deskriptiv pilotstudie med syftet att beskriva det temporala mönstret för koagulationsstatus hos patienter som genomgick större leverresektioner. Patienterna studerades perioperativt genom att använda en bredare koagulationstestning, inklusive ROTEM®. Parallellt registrerades blödning och trombotiska komplikationer. Studies huvudslutsats var att ROTEM® visade att patienterna hade en re-balanserad koagulationsstatus postoperativt trots avvikande svar på rutinmässiga koagulationsprover. Vi observerade även att de postoperativa fibrinogenivåerna i plasma skiljde sig åt beroende på resektionens storlek.

Ur resultaten i studie 2 framkom hypotesen att en bristande synthesförmåga på grund av förlust av levermassa kunde vara en faktor som påverkar fibrinogenets plasma koncentration. För att utforska denna hypotes behövde vi kvantitativt kunna mäta de novo syntesen av fibrinogen (och albumin) hos dessa patienter. För detta ändamål validerade vi i studie 3 den tracer metodiken med användnig av stabila isotoper, som vanligtvis används, för att möjligtgöra repetitiva mätningar i longitudinella studier.

I studie 4 användes metodiken som utvecklades i studie 3 för att undersöka fibrinogensyntesen i samband med leverkirurgi. Vi kunde då konstatera att fibrinogensyntesen ökade kraftigt den första postoperativa dagen. Resterande lever efter

leverresektion (hepatectomier) hade god förmåga att öka fibrinogensyntesen över de preoperativa nivåerna och det fanns ingen skillnad jämfört med en kontoll grupp av patienter som genomgick bukspottkörteloperation (pankreatektomier) utan förlust av levervävnad. Plasma fibrinogen koncentrationen speglar alltså inte bara syntesförmågan av fibrinogen, utan även förbrukningen av fibrinogen. Parallellt undersökte vi även syntesförmågan av albumin, som konfirmerade en god proteinsyntesfunktion i den resterande levern.

10 ACKNOWLEDGEMENTS

Jan Wernerman. The main supervisor of this paper. Thank you for all the advice and thought-provoking conversations over the years. Your guidance in this project, throughout my career, and your friendship over the past decade have been invaluable.

Anna Januszkiewicz, co-superviser. Thank you for the openness, encouragements and professional discussions, for the advices and all the support.

Anna Ågren, **Maria Magnusson**. My co-supervisors. Your attention to detail, feedback and explanations were fundamental in this process. I am forever grateful for all your advice and expert understanding of the field.

Olav Rooyackers, **Åke Norberg**, Thank you for the essential advices and help, discussions, challenging perspectives and great company.

Staffan Wahlin, **Ernesto Sparrelid** and **Bengt Isaksson**, my mentor. Co-authors and experts of their fields who contributed their time and knowledge. Thank you very much!

Kristina Kilsand, **Janelle Cederlund** and **Sara Ryden**. Our wonderful research nurses who supported us and made sure everything was done right. Thank you!

Maria Klaude, **Christina Hebert**, **Towe Jacobsson**, **Brigitte Tewlkmeyer**, **Eva Nejman** and **Nicolas Tardif**. The research lab team, whose knowledge, skilled expertise and precise analyses allowed us to undertake advanced and in-depth studies.

Suzanne Odeberg Wernerman. Former head of the Department of Anesthesiology at the Karolinska University Hospital Huddinge. Thank you for supporting me from the start of my research journey. I am grateful for your exceptional leadership.

Håkan Björne, **Lars Hållström** and **Lisbet Meurling**. Current and former heads of the Department of Perioperative Medicine and Intensive Care Karolinska University Hospital Huddinge. For fostering a great environment for work and clinical research.

Marie Stenbeck, Isabel Climent Johansson, **Nicoletta Raic** and **Agneta Wittlock**. Thank you for all the administrative guidance.

Gunilla Gryfelt, biomedical analyst. **Maura Krook** and **Ioana Apostolopoulou**, research nurses. Thank you for your kindness and all the support.

Co-workers of Perioperative Medicine and Intensive Care Karolinska University Hospital Huddinge. It is a pleasure working with you always, and I am proud to be your colleague.

Our patients and healthy volunteers for their invaluable contribution to the advancement of science.

At the most, I would like to thank my wife **Maria Dumitrescu**, and my daughter **Ioana Dumitrescu**. Thank you for being beside me, advising and helping me. Thank you for your love and endless patience.

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12 ERRATA TO PUBLISHED PAPERS

In study 1 on p.3: “Thrombelastometry and child pugh score” should have appeared as “Thrombelastometry and Child-Pugh score.”

In study 3, in the legends of Figure 1 and 2 (on p. 5): “Wilcoxon’s t-test for paired observations” should have appeared as: “Wilcoxon signed-rank test for paired observations”

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