Universitat Autònoma de Barcelona

BLEEDING ASSOCIATED COAGULOPATHY: NEW TREATMENT STRATEGIES BASED ON COAGULATION FACTOR CONCENTRATES

Tobias Bernhard Koller

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Doctoral Thesis

Tobias Bernhard Koller

Barcelona, 2022

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“The greatest enemy of knowledge is not ignorance
it is the illusion of knowledge."

— Stephen Hawking—

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I dedicate this work to my beloved family,

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LIST OF THE ARTICLES MAKING UP THE THESIS

Thesis in the form of a compendium of articles.

The thesis consists of 2 main objectives which are addressed by the following 2 publications:

1. Koller T, Parera Ruiz A, Diaz-Ricart M, Gómez Caro AM: Role of fibrinogen concentrates for treatment of critical perioperative hemorrhage. Drugs of Today 2021; 57:219–39

2. Koller T, Kinast N, Caste llanos AG, Garcia SP, Iglesias PP, Vintro XL, Arranz JM, Seto NV, García MVM, Moreno-Castaño AB, Aznar-Salatti J, Albaladejo GE, Diaz-Ricart M: Normalization of blood clotting characteristics using prothrombin complex concentrate, fibrinogen and FXIII in an albumin based fluid: experimental studies in thromboelastometry. Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine 2021; 29:57

ABBREVIATIONS

aF	activated Coagulation Factor
A5	Amplitude at 5 minutes
A10	Amplitude at 10 min
CFC	Coagulation Factor Concentrate
CRF	Coagulation Resuscitation Fluid
Cryo	Cryoprecipitates
FC	Fibrinogen Concentrate
FFP	Fresh Frozen Plasma
FGN	Fibrinogen
FXIIIC	FXIII Concentrate
MCF	Maximum Clot Firmness
PCC	Prothrombin Complex Concentrate
POC	Point of Care
TIC	Trauma Induced Coagulopathy
TEM	Thromboelastometry
TRALI	Transfusion-Related Lung Injury
VHA	Viscoelastic Haemostatic Assays
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
t-PA	tissue-type Plasminogen Activator
TRALI	Transfusion-Related Lung Injury
VHA	Viscoelastic Haemostatic Assays

DISCLOSURE

The presented in vitro study was supported and funded by a grant from CSL Behring

SUMMARY

Massive bleeding in clinical settings like trauma, obstetrics, cardiac, and other high-risk surgery is a severe and life-threatening complication. It is associated with an elevated mortality rate and causes a heavy economic burden for public health care systems (1)(2)(3). Bleeding is caused by a surgical lesion of blood vessels, by disorders of the blood coagulation system, or both. The underlying severe blood loss leads to severe haemorrhagic shock and a lethal outcome unless adequate treatment is offered immediately to the patient. Resuscitation fluids are infused into the bleeding patient´s venous system to correct the intravascular volume deficit and to restore an adequate macro- and microcirculation to prevent the deleterious consequences of prolonged and uncontrolled shock (4). For this purpose, physicians have different kinds of fluids available. Regardless of their nature, be it crystalloids, artificial-, or natural colloids, all currently available fluids have a negative impact on the complex mechanisms of the coagulation system whose functionality and integrity are essential for the effective control of ongoing bleeding. To avoid a vicious circle of fluid administration leading to coagulopathy leading to enhanced bleeding, which in turn necessitates more fluid administration, it is indispensable to guarantee the rapid and consistent treatment of emerging coagulation disorders as diagnosed by standard laboratory testing or point-of-care (POC) monitoring like thromboelastometry (TEM). For the treatment of coagulopathies with deficiency of coagulation factors, the transfusion of plasma products is still a clinical standard in most hospitals and forms an integral part of their institutional protocols for massive blood transfusion. However, there is a certain paucity of high-quality evidence supporting this clinical praxis and plasma transfusion is not free from serious adverse events. The concept of plasma transfusion aiming at substitution of coagulation factors in severe bleeding is currently challenged by new alternative treatment concepts based on the replenishment of coagulation proteins in form of coagulation factor concentrates (CFC). Fibrinogen Concentrate (FC), Prothrombin Complex Concentrate (PCC) and Factor XIII Concentrate (FXIIIC) have been increasingly used in recent years for the treatment of bleeding-associated coagulopathies, with growing scientific evidence for a better outcome in several clinical endpoints when compared to plasma transfusion (5)(6)(7). However, plasma possesses unique

qualities as a resuscitation fluid, providing reliable intravascular volume effects combined with a close to physiological coagulation factor composition, which might partially explain the high acceptance of this product among physicians in massive bleeding scenarios. Currently, no alternative products are available that share these therapeutical components with human plasma. The objective of this doctoral thesis is, 1st, to perform a thorough review of the current evidence on the role of FC in perioperative bleeding, 2nd, to analyse the capacity of commercial coagulation factors dissolved in a plasma-free albumin solution to form a stable fibrin clot and, 3d, to generate an albumin-based colloid solution enriched with coagulation factors showing a blood clot generation with a normal TEM pattern. Such fluids with a coagulation capacity comparable to whole blood when tested in presence of platelets could represent a new class of colloids which will be referred to as Coagulation Resuscitation Fluid (CRF) in the further course of this work. CRFs could result in alternative transfusion strategies equally combining reliable intravascular volume effects with the capacity to maintain adequate haemostatic properties for bleeding control. Follow-up studies could investigate if plasma-free transfusion strategies based on CRF would provide acceptable hemodynamic and haemostatic properties with a non-inferior efficacy and safety profile when compared to plasma.

INTRODUCTION

1. INTRODUCTION

1.1. The Haemostatic System

The formation of a stable blood clot at the site of vessel injury is a highly regulated physiological process, which is determined by the complex interplay between endothelial cells, platelets, and coagulation factors. Platelet aggregation and the activation of coagulation factors are two closely interwoven mechanisms that mutually regulate and influence each other. This process is currently best described by models highlighting the mechanisms of coagulation factor activation in close spatial proximity to membrane surfaces of activated platelets (cell-based model) within three overlapping phases:

1. Initiation phase in which low amounts of activated coagulation factors are generated,
2. Amplification phase which prompts an important increase in the concentration of activated coagulation factors, and
3. Propagation phase with formation of fibrin next to highly procoagulant membranes of activated platelets (8).

Cell-based models of haemostasis challenge the classical, purely enzymatic concept of two separated coagulation cascades (intrinsic and extrinsic coagulation cascade) and give more precise answers to timely and spatial interactions between the vessel wall, platelets and coagulation factors. The haemostatic effects provided by a platelet plug embedded and stabilized in a fibrin mesh are indispensable for bleeding control but must be limited to the site of the endothelial lesion. Inhibitory coagulation factors and the fibrinolytic system provide potent control mechanisms to avoid excessive activation of the platelet-coagulation interplay and to minimize the thromboembolic risk at downstream sections of the involved blood vessel (9).

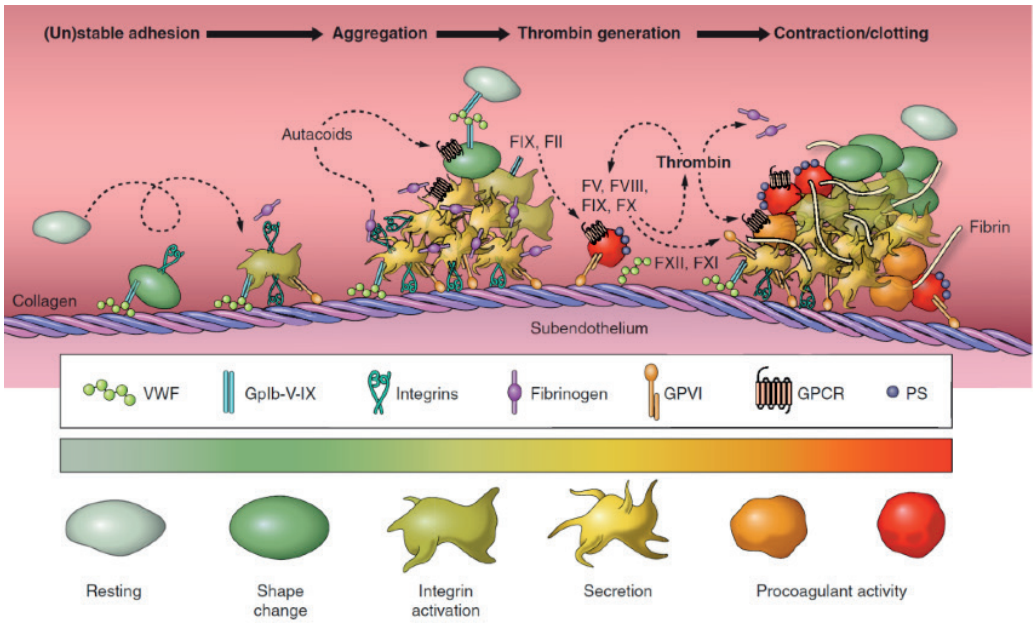
Platelet Aggregation

Resting platelets, characterized by a discoid shape are circulating in high abundance (up to 400 billion cells in one litre of blood) through the human blood vessels screening the endothelial wall for its integrity (10). A large number of different receptors and adhesion molecules from the integrin family and G-protein (GP)-coupled receptors are available at the platelet membrane surface regulating the cellular mechanisms of platelet adhesion and aggregation. The most abundant platelet membrane receptor GP-IIbIIIa (αIIbβ3-integrin) experiences a conformational change after platelet activation exposing binding sites for its natural ligands fibrinogen (FGN), fibronectin and von Willebrand factor and exhibits a crucial role in granule secretion, irreversible platelet aggregation and clot retraction (11) (12) (13).

Under high shear rates (1000 -4000 s^{-1}), as found under flow conditions in arterial vessels, platelet aggregation depends on the intermediary role of soluble von Willebrand factor (14). Soluble von Willebrand factor is captured by exposed collagen molecules from the subendothelial connective tissue and experiences a configurational change that leads to binding to the platelet GP-1bα receptor. These first platelet-collagen interactions mediated by soluble von Willebrand factor are initially transient under high shear, leading to slowing down and rolling of platelets along the injured vessel wall. This gives raise to further interactions between platelet membrane receptors and ligands in the extracellular matrix thus generating a first layer of adherent stationary and activated platelets. During rolling along the vessel wall the intracellular signalling induced by the GP-1bα interaction with the A1 domain of collagen-bound von Willebrand factor leads to activation of further membrane receptors and transmembrane adhesion molecules, especially of α2β1-integrins, which then enhance further GP-VI-mediated collagen-platelet binding. It is this α2β1-collagen interaction that converts the initially reversible nature of the platelet-collagen interaction into a stable bonding with high resistance to shear stress (15). α2β1-integrin and GP-VI synergistically stimulate degranulation of α- and δ-granules from the platelet cytosol, Ca++-signalling, changes in the platelet membrane shape with phosphatidylserine (PS) exposure and further platelet aggregation (16)(17)(18).

The forming microenvironment of negatively charged PS-based platelet membranes and degranulated and partially activated coagulation factors (FV, von Willebrand factor) set the prerequisite for the effective interplay between platelets and activated coagulation factors produced during secondary haemostasis (19). These early cell-bound mechanisms of primary haemostasis occur mostly in parallel and simultaneously to the activation of the coagulation processes of secondary haemostasis driven by tissue factor exposure. Both processes significantly interact and influence each other within a shared biochemical environment in close proximity to the surface of activated platelets.

Figure 1: Molecular mechansims of platelet aggregation



(20)

Cell-based model of coagulation

- Initiation Phase

During the initiation phase of haemostasis plasma comes into contact with exposed tissue factor-bearing cells of the subendothelial space leading to binding and complex formation between tissue factor and FVIIa (21). Tissue factor-FVIIa

complexes then induce several proteolytic factor activation steps that belong to the extrinsic coagulation cascade. It cleaves plasma coagulation FIX and FX to convert them into their activated forms FIXa and FXa. FXa activates FV and again FVII, which - in a positive feedback loop - reinforces the initiation phase of coagulation. Furthermore, FXa produces trace amounts of thrombin from prothrombin. Thrombin is the key enzyme to

1. activate factor V and FVIII
2. activate FXI which cleaves FIX to FIXa, and
3. activate platelets by protease-activated receptors 1 and 4 (PAR1, PAR 4).

The thrombin-driven activation of factors needed for assembling the tenase (FVIIIa/FXIa), and prothrombinase (prothrombinase, FVa/FXa) complex and further activation of adhering platelets are important mechanisms to perpetuate and intensify the haemostatic process during the amplification and propagation phase (20).

- Amplification Phase

The assembly of the stable procoagulant complexes tenase, consisting of activated FIX and FVIII, and prothrombinase, consisting of activated FX and FV, stands in the centre of the amplification phase.

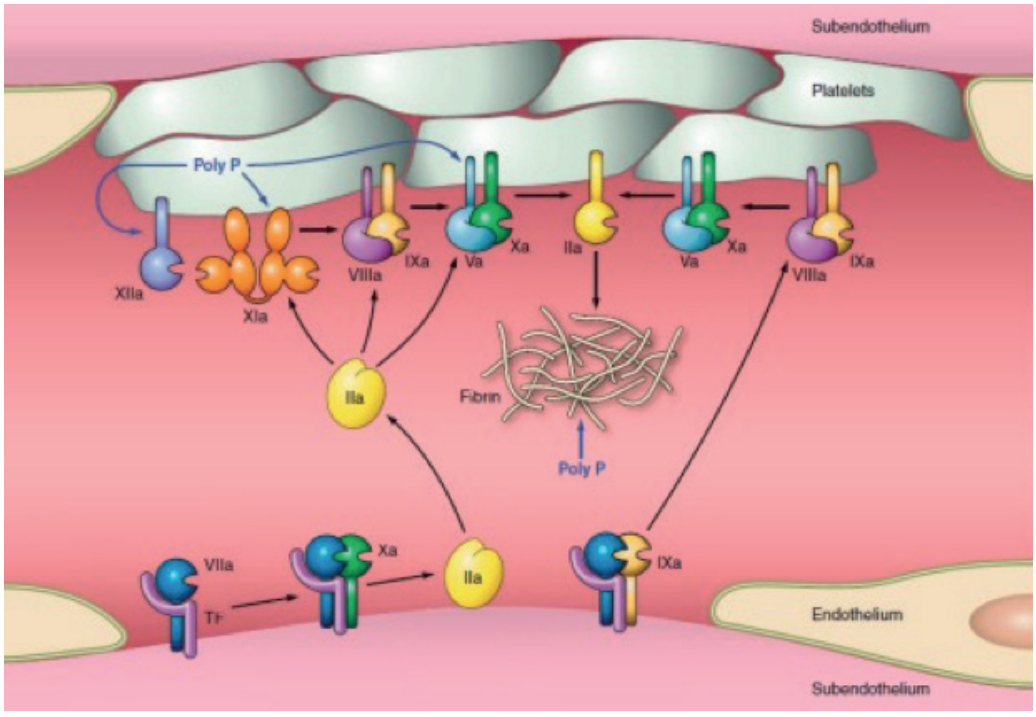
The trace amounts of thrombin generated during the initiation phase serve as a strong activator for platelets via activation of PAR-1 and PAR-4 receptors. Thrombin-activated platelets release partially activated FV from α-granules, which – after degranulation - become fully activated by contact with thrombin and FXa.

FXa from the initiation phase is partially deactivated by tissue factor pathway inhibitor and antithrombin to guarantee that no procoagulant stimulus is transmitted to the downstream healthy parts of the blood vessel (22). By comparison, FIXa, generated during the initiation phase by trace amounts of thrombin is not targeted by tissue factor pathway inhibitor/ antithrombin and can diffuse to membranes of previously activated platelets to further support the formation of thrombin.

Thrombin bound to the platelet surface activates FXI to FXIa, which cleaves FIX to FIXa and plays an important role in the activation of von Willebrand factor-bound FVIII. After sufficient cleavage of the von Willebrand factor/FVIII-complex the released and activated FVIII remains at the membrane surface of platelets (23).

The totality of these individual processes provides sufficient platelet-bound cofactors FVIIIa and FVa required for assembling with FIXa and FXa to form the stable procoagulant complexes tenase (FVIIIa/FXIa) and prothrombinase (FVa/FXa) and to induce the further enhancement of thrombin production during the propagation phase.

Figure 2: Coagulation factor activation steps during initiation-, amplification-, and propagation phase



(20)

- Propagation Phase

The assembly of the tenase (FVIIIa/FXIa)- and prothrombinase (FVa/FXa)-complex takes place within proximity to procoagulant, activated platelets. These platelets have previously passed activation steps mostly driven by collagen and thrombin. They are characterized by a balloon-like shape exposing phosphatidylserine (PS) on their membrane surface. The assembly of the tenase complex occurs on the "cap"-like regions of ballooned platelets where a high density of adhesive proteins can be identified (24). The local distribution of activated coagulation factors on the platelet surface seems to be relevant in supporting the haemostatic procedures during the propagation phase. Once the tenase-complex, formed by activated FIX/VIII, cleaves FX to FXa the prothrombinase-complex consistent of FXa and FVa can assemble. The presence of calcium (Ca++) at physiological concentrations is essential for the assembly of tenase- and prothrombinase-complexes to the anionic phospholipids exposed on the platelet membrane. The prothrombinase-complex finally leads to a high local concentration of thrombin (thrombin burst) that induces effective cleaveage of FGN and promotes polymerization of insoluble fibrin.

Fibrin Polymerization

The FGN molecule is a key element of haemostasis, integrating the fluid phase components of coagulation with cell-mediated mechanisms of primary haemostasis. Thrombin mediates the cleavage of an N-terminal peptide sequence in the α-chain, thus forming soluble fibrin monomers (Figure 3) (25). A second slower cleavage process on the β-chain increases the polymerisation capacity of the molecule. Double-stranded fibrils are generated by end-to-middle alignment of the D- and E-domains of several fibrin molecules and assemble with each other, either in parallel forming four-stranded fibrils (bilateral branch junctions), or in an end-to-side configuration (equilateral branch junctions) (26) (Figure 4). Altogether, the thrombin-induced cleavage process induces dramatic changes in solubility and the formation of an insoluble, complex fibre structure, the fibrin clot.

Figure 3: Molecular structure of fibrinogen

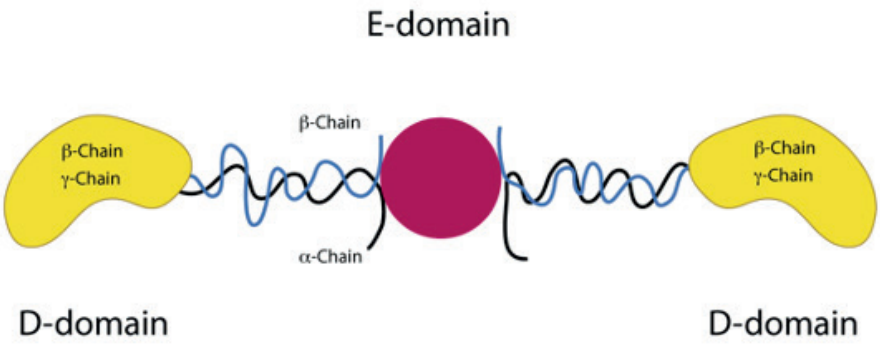
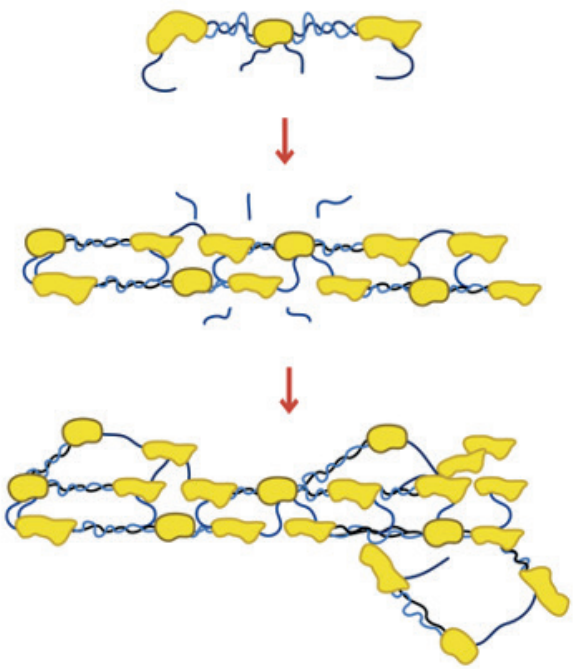


Figure 4: Fibrin polymerization



Fibrin molecules are crosslinked by FXIIIa by covalent bonds within the generated clot, thereby terminating the coagulation process. FXIII crosslinks lead to more resilient and elastic properties while protecting the clot from plasmin-mediated fibrinolytic activity (27)(28)(29). Both FGN and fibrin directly interact with platelets (30). FGN is a physiological ligand to the platelet membrane-glycoprotein GP-IIbIIIa (αIIbβ3-integrin) and its interaction with FGN promotes platelet aggregation and degranulation. The binding of fibrin within forming clots to GP-IIbIIIa enhances clot retraction, which further increases clot stability and resilience.

Inhibition of the Coagulation System and Fibrinolysis

The activation of the coagulation system and blood clot formation has to be spatially limited to the site of vessel injury to avoid harmful thrombosis or thromboembolism. This is provided by a complex inhibitory system consisting of protease inhibitors (e.g. antithrombin, tissue factor pathway inhibitor) and the endothelial-based Protein C/Protein S enzymatic activity.

- Antithrombin

Antithrombin is a plasma glycoprotein with regulatory effects on the proteolytic activity of the procoagulant proteases of both the extrinsic and intrinsic coagulation cascade (31)(32). It exerts inhibitory effects on all activated coagulation factors but its main targets seem to be thrombin, FXa and FIXa. Its inhibitory interaction with coagulation factors is markedly enhanced in the presence of heparins or glycosaminoglycans from endothelial HSPG (heparin sulfate proteoglycans)-receptors (33). Antithrombin is considered to be one of the most relevant inhibitors of thrombin. Its clinical relevance is supported by the observation of a 10-fold elevated risk for thromboembolic events under heterozygous antithrombin deficiency in humans (34). Complete antithrombin deficiency seems to be incompatible with life (35).

- Tissue factor pathway inhibitor

Tissue factor pathway inhibitor is a multivalent, serine protease inhibitor and a major physiological regulator of tissue factor-induced blood coagulation. Tissue factor pathway inhibitor is located in platelets and microvascular endothelium (36). Tissue factor pathway inhibitor inactivates the enzyme activity of several proteases like FVIIa and FXa (37). Tissue factor pathway inhibitor-mediated inhibition of the coagulation system affects both the tissue factor/FVIIa/Xa complex and isolated FXa molecules. The inactivation step of tissue factor/VIIa is dependent on the presence of FXa that is previously formed at the tissue factor/VIIa complex (38). Protein S is an important cofactor for isolated tissue factor pathway inhibitor induced FXa-, but not tissue factor/VIIa-inactivation (39)(40). This leads to inhibition of early forms of the prothrombinase-complex during the initiation of blood coagulation (41). FIXa on the other hand is not affected by tissue factor pathway inhibitor and can therefore diffuse more easily to platelet membranes to exercise their important role during the propagation phase.

- The protein C/protein S pathway

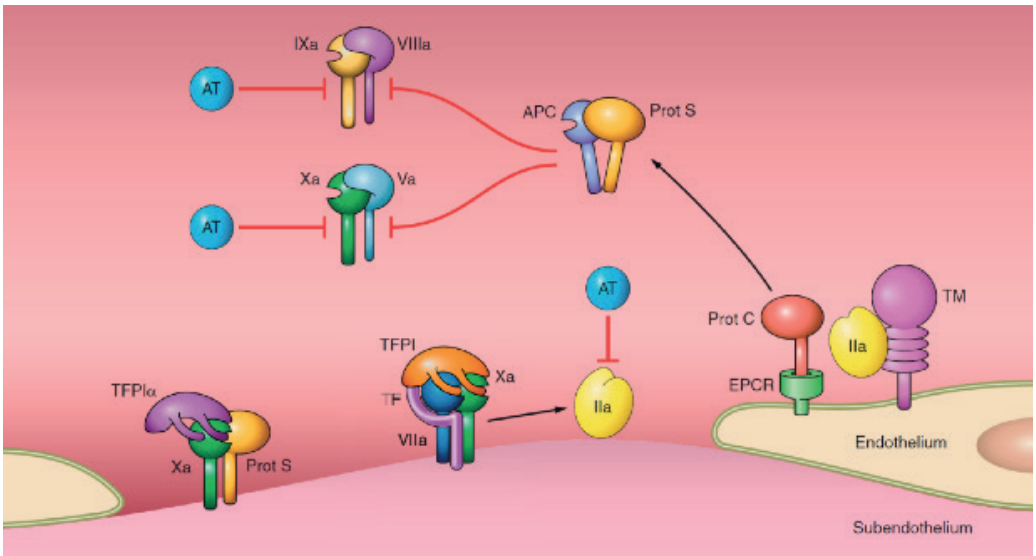
The protein C/protein S pathway is a further enzymatic control system to avoid overshooting coagulant activity, thrombosis and inflammatory processes (42). Protein C has high homology to vitamin K-dependent procoagulant proteins. Rising thrombin concentrations during coagulation bind to thrombomodulin expressed in endothelial cells (43). Thrombin/thrombomodulin then lead to cleavage and activation of protein C that is bound to endothelial protein C receptor (44). After cleavage, and still in close proximity to the endothelial membrane, activated protein C binds to its cofactor protein S (635 amino acids, vitamin K-dependent protein) as a prerequisite to attain full anticoagulant activity (45) leading to effective downregulation of prothrombinase activity (46). Being an endothelial-bound process the overall anticoagulant efficiency can be expected to be especially high in the capillary bed as a function of a favourable blood-volume/endothelial-surface-ratio. In bigger arterial vessels with a less favourable ratio, this seems to be compensated by a higher expression of endothelial protein C receptor (47). Activated protein C dependent FV-inactivation seems to be only

effective at the site of endothelial but not platelet membranes, suggesting that it exerts a rather thrombotic protection within healthy vessels than a switch-off signal on coagulation near activated platelets (48).

The activated protein C cofactor Protein S is mainly bound in plasma (> 60%) to the complement regulatory protein C4b-binding protein (C4BP). The anticoagulant activity of protein S is not strictly limited to its cofactor function for activated protein C. It also exerts anticoagulant effects by directly inhibiting FV, FXa, and by contributing to FXa/ tissue factor pathway inhibitor interactions (39)(49)(50).

Heterozygous deficiencies for both protein C and protein S are correlated to a higher risk for venous thrombosis whereas homozygous deficiencies are not compatible with life indicating a highly relevant physiological role for controlling haemostatic and thrombotic processes (51)(52).

Figure 5: Inhibition of the coagulation system



- *The fibrinolytic system*

After the generation of a functional haemostatic fibrin clot the fibrinolytic system, consisting of a cascade of serum proteases converting the zymogen plasminogen into its active form of plasmin, will finally promote effective fibrinolysis and thrombolysis. The efficiency of enzymatic fibrinolysis is determined by fibrinogen (FGN) polymorphism, mechanical characteristics of the fibrin molecule, thrombin generation rate, thrombus-associated cells (platelets), and the surrounding biochemical environment.

Fibrin itself enhances the conversion of plasminogen to plasmin by simultaneously binding of tissue-type plasminogen activator and plasminogen augmenting the catalytic potential of tissue-type plasminogen activator by two orders of magnitude (53). The number of plasminogen binding sites to the fibrin molecule (lysine residues) increases under the cleavage process resulting in high plasmin activity in direct neighbourhood to its target substrate fibrin.

To avoid premature clot degradation thrombin activatable fibrinolysis inhibitor bound by thrombomodulin provides a regulatory mechanism by directly removing COOOH-terminal lysine residues from the cleaved fibrin molecule and thus reducing the number of potential binding sites for tissue-type plasminogen activator (54).

Overshoot downstream activity of the potent protease plasmin is controlled and limited by naturally occurring enzymes like α2 antiplasmin, plasminogen activator inhibitor 1 and 2 (55).

Although not finally elucidated plasmin-independent mechanisms seem to play a role in fibrinolysis as both homozygous plasminogen deficiencies and reduced tissue-type plasminogen activator activities do not account for increased thrombotic events (56).

1.2. Coagulation Monitoring

Standard Laboratory Testing and Viscoelastic Haemostatic Assays.

Coagulation Monitoring is an indispensable component of clinical management in situations of uncontrolled bleeding. Haemostatic pharmacological and/or transfusional interventions in clinical practice should best be guided by well-established diagnostic criteria provided by adequate test results. In clinical practice two general principles for coagulation monitoring can be distinguished:

- Conventional Standard Laboratory Testing with routine monitoring generally including prothrombin time, activated partial thromboplastin time, mechanical or photo-optical determination of plasma FGN concentration (Clauss method) and platelet count. More specific testing like thrombin time, reptilase time, etc. can help to determine more specific coagulopathic disorders.
- POC-monitoring with Viscoelastic Haemostatic Assays (VHA) is based on the registration of physical blood clot characteristics usually applied as point-of-care monitoring.

In recent years POC monitoring has gained clinical relevance in the management of perioperative bleeding due to several advantages compared to standard laboratory testing. In the first place, the time to availability of test results guiding treatment decisions is significantly shorter with reported turnaround times for diagnosis-treatment cycles of up to 100 minutes for standard laboratory testing (57) compared to only 5 – 10 minutes for VHA (58). This difference is highly relevant, as rapid and correctly indicated therapeutic decisions in the treatment of massive bleeding events can be life-saving.

Next, VHA is performed on whole blood samples including information on the contribution of platelets and red blood cells on the clotting process whereas standard laboratory testing is performed on centrifuged plasma samples excluding this relevant information (59).

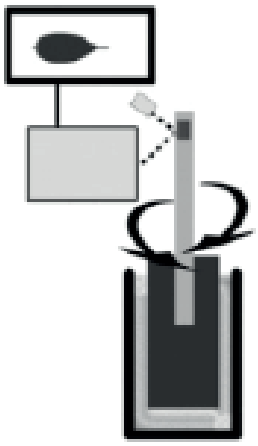
Finally, blood samples can be tested directly at the bedside of the bleeding patient. This facilitates the execution of the test directly by the treating team close to the patient. The analysed blood probes reflect real temperature conditions of the bleeding patient´s blood without previous correction to 37°C standard temperature as done for standard laboratory probes.

- Basic principles of VHA

Thromboelastometry (TEM) was first introduced in 1948 by Hartert et al. (60) but broader clinical acceptance and introduction as POC monitoring for perioperative and emergency settings was not achieved until 1996 when technical and methodological improvements led to significant shorter interpretation times and higher reliability of the obtained results. Today several commercial VHA systems are available in the market. The most commonly used and best studied analysers are rotational thromboelastometry (ROTEM®, Instrumentation Laboratory, Bedford, MA, USA) and thromboelastography (TEG®, TEG Haemonetics Corporation, Boston, MA, USA) . In the further course of this thesis unless explicitly stated otherwise, reference is made to the ROTEM system since the experimental part of this work was carried out on ROTEM equipment and the interpretation of the results obtained must be made with reference to Rotem standard parameters.

During TEM temporal and mechanical characteristics of viscoelastic clot properties of a forming blood clot are measured within a sample of whole blood under low shear conditions. A small rotating pin immersed in a whole blood sample registers changes of blood viscosity after coagulation activation and the onset of platelet-fibrin bonding that leads to increased clot firmness. The corresponding limitation of the free rotation of the rotating pin is transmitted via an optical detector system (Figure 6) generating a graphical display resulting in the typical thromboelastograph (Figure 7). The thromboelastograph plots the time course on the X axis and the increase in clot firmness on the Y axis. (Viscoelasticity is expressed as “mm” corresponding to the loss of motion of the optical signal on the detector as the rotational capability of the pin reduces under rising resistance of the clotting blood sample). The normal ranges for standard ROTEM parameters are presented in Table 1.

Figure 6: Rotational thromboelastometry (ROTEM®)



(60)

Figure 7: Typical thromboelastograph

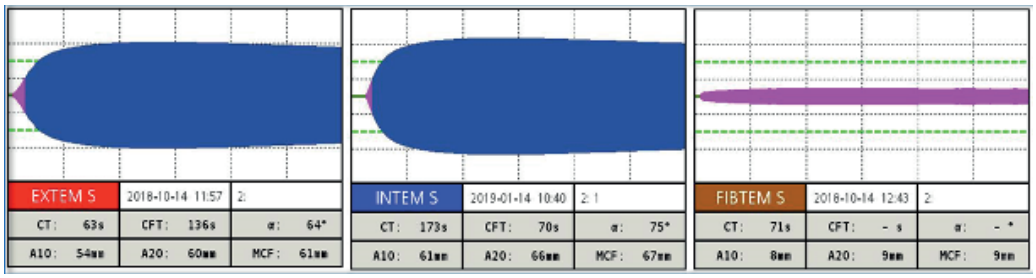


Table 1: Normal values for Rotem parameters

Sex related differences in the main parameters of ROTEM

	Age (years)	CT (s)	CFT (s)	Alpha angle (°)	MCF (mm)
INTEM					
females ($n = 79$)	41.2 (16.7)*	187.9 (26.8)	62.5 (16.4)*	77.6 (2.8)*	62.5 (4.8)*
males ($n = 76$)	49.8 (17.7)*	184.4 (25.9)	69.4 (16.4)*	76.2 (3.5)*	60.1 (4.8)*
EXTEM					
females ($n = 130$)	41.3 (15.8)*	55.2 (8.2)*	91.2 (27.1)	72.5 (5.3)	60.2 (5.2)
males ($n = 72$)	46.3 (15.6)*	58.7 (9.2)*	97.0 (25.9)	71.6 (4.8)	59.6 (5.4)
FIBTEM					
females ($n = 93$)	38.8 (15.1)	n.d.	n.d.	n.d.	16.7 (3.9)*
males ($n = 50$)	38.9 (13.4)	n.d.	n.d.	n.d.	15.1 (4.0)*

Shown are mean values and standard deviations, *P value < 0.05 (two tailed unpaired t-test) in the comparison of female and male subgroup.

(61)

- VHA-guided diagnosis

ROTEM® provides different coagulation tests which allow for specific diagnosis of a variety of coagulopathies. Tests differ in the way coagulation is activated with either tissue factor used for extrinsic activation (i.e. for EXTEM-, FIBTEM- and APTEM-subtests) or ellagic acid for intrinsic activation (i.e. for INTEM- and HEPTEM-subtests). Further reactants can additionally help to discriminate between different coagulopathic triggers that can result in identical ROTEM patterns. E.g., FIBTEM subtests containing cytochalasin D as a potent platelet inhibitor will effectively eliminate the contribution of platelets to the clot firmness and leave only information on clotting proteins, mainly FGN and FXIII (62).

Therapeutical decision-making is guided by a series of parameters from the viscoelastometric measurement:

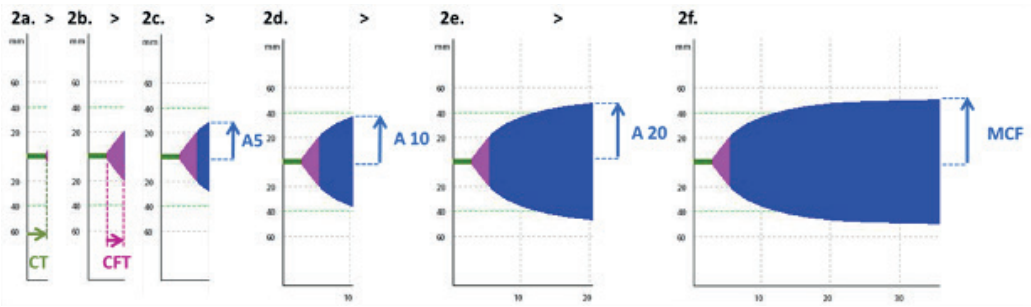
Clotting Time (CT) is defined as the duration from coagulation activation to the formation of the first fibrin molecules with a recordable increase in viscoelasticity amplitude to 2 mm. In function of the applied coagulation activator (intrinsic or extrinsic) CT gives information on the corresponding coagulation factors and their potential to generate thrombin for initiating FGN cleavage with consecutive fibrin polymerization.

Clot Formation Time (CFT) is the time needed to increase clot firmness from 2 to 20 mm reflecting the quality and dynamic of fibrin polymerization.

The physical clot strength measured during the coagulation process is expressed in ***Maximum Clot Firmness (MCF)***. FGN, FXIII and platelets, are the main determinants of this parameter. Platelet inhibition with Cytochalasin D (FIBTEM) allows for a quantitative estimate of FGN levels (and FXIII) contributing to MCF (59). Results on clot firmness obtained from EXTEM or FIBTEM are available within 5- 10 minutes after coagulation activation and provide an early guide for clinicians to indicate FGN treatment and calculate a corresponding treatment dose.

The effects of the fibrinolytic system on the stability of the formed blood clot are detected by the ***Maximum Lysis (ML)*** expressed as the percentage decrease calculated from the maximum clot firmness over 60 minutes according to the total duration of the TEM measurement.

Figure 8: Parameters from the thromboelastometrical measurement used for therapeutical decision making



Based on these standard TEG parameters several treatment algorithms are published for different clinical high-risk bleeding scenarios and clinically validated on evidence-based criteria (58). A general principle to follow according to these treatment algorithms is to substitute haemostatic blood components in accordance with goal-directed algorithms as indicated by ROTEM parameters that are outside the normal range and known to be associated with an increased risk of bleeding. Isolated or combined coagulation factor deficiencies are increasingly substituted by the administration of CFC as an alternative to indiscriminate plasma transfusion. There is evidence that the concept of VHA-guided, goal-directed coagulation management is cost-effective, reduces transfusion rates, and has beneficial effects on the overall outcome, especially in trauma and cardiac surgery as shown in cross-sectional metaanalysis (63)(64) .

Most clinical guidelines currently include monitoring with VHA as an alternative or even superior to standard laboratory testing in a variety of clinical situations (3)(65)(66)(67)(68).

1.3. Bleeding-associated coagulopathy

Background

Disruption of a well-balanced coagulation system can be both, cause and consequence of severe bleeding. Different clinical high-risk areas like trauma, obstetrics, or cardiac surgery are characterized by specific and unique patterns of disruptive mechanisms in the coagulation system. As a consequence treatment algorithms have to be adjusted to the specific underlying disorder, leading to significant differences in treatment algorithms between bleeding patients in various clinical settings (3)(69)(70). Nevertheless, independent of the specific underlying coagulation disorder, all bleeding patients share one common pathomechanism aggravating their original coagulation disorders, which is **dilutional coagulopathy** (71). Dilutional coagulopathy is caused by resuscitation fluids administered to avoid severe intravascular hypovolemia and to guarantee adequate cardiac output and tissue perfusion. Treatment of bleeding patients with resuscitation fluids must therefore be seen as a cornerstone of emergency care – despite their adverse effects on blood coagulation - to avoid haemorrhagic shock and its serious consequences. The attractiveness of plasma transfusion lies in its potential to simultaneously provide stabilising effects on the coagulation system in addition to its volume effects, properties that no other volume therapeutic agent can match. In other words, plasma is currently the only option to provide adequate volume therapy during massive bleeding while simultaneously avoiding the occurrence of severe dilutional coagulopathy.

Trauma-Induced Coagulopathy (TIC)

Trauma is one of the ten most common causes of death, with a worldwide annual total of over 5 million victims (2). One of the main causes of the high mortality rate among trauma patients is severe, uncontrolled blood loss leading to exsanguination (72). Patients with a severe injury pattern who reach the emergency department alive show signs of ongoing coagulopathy in around one third of cases (73)(74), which entails a nearly three to four-fold higher mortality risk (75)(76) compared to those without associated coagulation disorders.

Tissue damage and shock are the driving forces that contribute to the development of TIC. It is crucial to distinguish blunt trauma without shock from severe penetrating trauma with shock. In the absence of shock, the clinical picture is rather characterized by hypercoagulable conditions with increased thrombin generation potential. Shock and hypoperfusion in the context of penetrating trauma mechanisms, however, can result in severe hypocoagulability and hyperfibrinolysis with an enhanced bleeding tendency (77). Early treatment of shock is therefore of paramount importance to improve the overall survival rate (3). Various pathological downstream processes may occur in varying degrees of severity after the trauma-associated shock has been established.

The following section refers exclusively to hypocoagulopathic alterations in patients with trauma-associated shock as hypercoagulable situations stand out of context to this work.

The main mechanisms involved in the onset of TIC are reduced thrombin generation potential, hyperfibrinolysis, hypofibrinogenemia, and endothelial damage:

Hypoperfusion-triggered activation of the protein C pathway converting protein C into activated protein C together with an endothelial overexpression of thrombomodulin are the main factors that can induce an overall reduced thrombin generation potential (78). Activated protein C induces direct inhibition of aFVIIIa and aFVa contributing to the hypocoagulable state. Increased thrombomodulin and decreased protein C levels are associated with higher bleeding rates and mortality (78). It was also suggested that hypocoagulable conditions might have protective functions against microvascular thrombosis under shock with low blood flow conditions (79)(80).

Hyperfibrinolysis under severe trauma with shock is today seen as a consequence of massive endothelial overexpression of tissue-type plasminogen activator rather than activated protein C-driven plasminogen activator inhibitor 1 inhibition (81). Trauma patients with VHA patterns of hyperfibrinolysis (lysis at 30 minutes > 3%) are at the highest risk for trauma-associated mortality when compared to patients with physiological or even reduced fibrinolytic capacity (82).

TIC-associated hyperfibrinolysis and dilutional effects from prehospital fluid resuscitation cause a high incidence of FGN depletion below critical thresholds which strongly relates to higher mortality (83). A total of 73% of patients with Hb levels < 10 g/L at hospital admission have FGN levels below the critical threshold of 1.5 g/L (84).

Shock and hypoxemia also act as central triggers in emerging endothelial damage among trauma patients. As a consequence glycocalyx shedding of heparans and thrombomodulin from damaged endothelial cells amplify coagulopathic mechanisms in trauma (85). Loss of endothelial barrier function leads to elevated permeability for intravascular fluids and the onset of peripheral oedema. As a result a vicious circle of even more severe intravascular hypovolemia and again aggravated shock and hypoperfusion can be induced (77).

Coagulopathy in Peripartum Haemorrhage

Severe Peripartum Haemorrhage, defined as blood loss higher >500 ml/>1000ml during vaginal/cesarean delivery, is the most frequent cause of peripartum morbidity and is estimated to be responsible for 34% of maternal deaths worldwide (86)(87).

Physiological changes of the coagulation system during pregnancy lead to an overall prothrombotic state of the parturient at term, with a significant increase in procoagulant factors and reduced activity of anticoagulant and fibrinolytic factors. E.g., FGN is reported to increase up to twice the levels before pregnancy with plasma concentrations reaching up to 6 g/l (88)(89). Accordingly, thromboelastometric reference ranges among parturients differ from non-obstetric populations (90) with FIBTEM values for obstetric vs non-obstetric populations of 13–28 mm vs 6-22 mm for amplitude at 5 minutes (A5), 14 – 30 mm vs 7 – 23 mm for amplitude at 10 minutes (A 10), 16 – 34 vs 9 – 25 mm for maximum clot firmness (MCF). These findings confirm previously published data and clearly reflect the initially hypercoagulable state during pregnancy (91)(92).

Coagulopathies as the primary trigger for bleeding are rare. The main obstetrical bleeding causes are uterine atony, abnormal placentation and genitouretral trauma. Acquired bleeding associated with coagulopathy in these settings, caused by

dilution, local and/or disseminated consumption, and increased fibrinolysis can become a life-threatening complication. Especially hypofibrinogenemia < 2-2,5 g/l, is frequently responsible for bleeding propagation towards severe and massive blood loss. In case of severe bleeding, the raised plasma FGN concentration at term apparently confers certain resistance to dilutional effects (93) under fluid administration for shock therapy. (94). However, this reserve appears to exhaust when FGN levels drop to the critical level of 2 g/l.

In placental abruption and amniotic fluid embolus hypofibrinogenemia is seen in 100% of the affected patients (95) and eventually high FGN dosage is needed (up to 18g in extreme cases) to correct plasma FGN levels (96).

Given the heterogeneity of underlying conditions for peripartum haemorrhage it is questionable if a formulaic transfusion concept with fixed ratios of fresh frozen plasma (FFP) to red blood cells should be applied. Goal-directed correction of single- or multifactor deficiencies guided by POC testing as introduced in many obstetric centres seem to reduce overall transfusion rates, but no clear impact on morbidity or mortality could be shown so far (97).

Coagulopathy in Cardiac Surgery

Up to 15% of all cardiac surgical patients with cardiopulmonary bypass suffer from relevant postoperative haemorrhage (98)(99) causing an overall consumption of up to 20% of all transfused blood products worldwide (100). Besides the highly relevant economic burden, severe bleeding and transfusion of allogeneic blood products show a clear negative impact on patient morbidity and mortality (100) (101).

The onset of coagulopathy in cardiac surgery is a complex interplay of patient-, cardiopulmonary bypass-related-, and surgical factors. Coagulopathic bleeding in cardiac surgery can have several causes, including:

- Disruption of the patient´s homeostasis with hypothermia, acidosis, anaemia, and/or hypocalcaemia
- Disruption of primary haemostasis due to pre-existing antiplatelet treatment, mechanical disruption of platelet function caused by the extracorporeal

bypass pump or acquired deficit of von Willebrand factor in patients carrying intermediate and long-term cardiac assist devices (102)

- Disruption of the plasmatic coagulation capacity in the context of coagulation factor deficiency or inhibition. Systemic and local coagulation activation through direct blood exposure to air and thrombogenic surfaces in the cardiopulmonary bypass circuits, consumption, dilution, loss and rebound of heparin effects are the main contributers.
- The hyperfibrinolytic activity caused by intrinsic contact activation with artificial cardiopulmonary bypass surfaces and extrinsic activation by systemic endothelial tissue-type plasminogen activator release from the vascular wall (103)

The complexity of the underlying coagulopathies in cardiac-surgery-related bleeding led to a broad acceptance of TEM-based monitoring systems, especially in European centres. Increasing scientific evidence, as summarized in two recently published meta-analysis, is supporting its use (104)(105). Both investigators found consistent results on the efficiency of VHA monitoring to reduce transfusion rates. A reduction in mortality rates was only observed in the smaller sample published by Wikkelsø (104). One relevant study in this field was interrupted after an interim analysis (50 patients enrolled of 100) due to a highly significant reduction of red blood cell transfusion in the VHA arm (106).

Under VHA monitoring, hypofibrinogenemia as a single coagulation factor deficit is - next to heparin rebound in plasma - among the most frequently detected coagulopathies in cardiac surgery (107). Intraoperative and postoperative plasma FGN levels falling below thresholds of 1,5 – 2,2 g/l are associated with more severe blood loss (108)(109). Ranucci et al. reported a negative predictive value of 100% for plasma FGN levels of 2.87 g/l or 98% for FIBTEM-MCF values of 14 mm. Some controversy is still ongoing about the positive predictive value of FGN with a considerable risk for overtreatment if pre-emptive strategies were applied (110)(111). As suggested by current guidelines on bleeding management in cardiac surgery FC administration can be considered if FGN levels fall below 1.5 g/l and should be based on VHA monitoring if available (65)(112).

There is clear consensus in favour of reversal of oral vitamin-K-antagonist-associated multifactor deficiencies with four-factor prothrombin complex

concentrates (PCCs) as it is shown to be faster and safer than plasma-based reversal (113). However, the currently available evidence on the best treatment of multifactor deficiencies in the context of loss and consumption under long-lasting bleeding and/or cardiopulmonary bypass is limited. There is some evidence from clinical studies that PCC-based management might be more effective than plasma transfusion to reduce postoperative blood loss and associated transfusion rates (114)(115)(116)(117). This evidence however does not currently translate into clear guideline recommendations in favour of plasma-free management of multifactor deficiencies as some safety concerns associated to PCC administration like higher rates of thromboembolic events and acute kidney failure exist (115).

Dilutional Coagulopathy

Regardless of the underlying specific coagulopathies in the clinical high-risk settings like trauma, obstetrics and cardiac surgery, all severe bleeding situations share a common pathologic pathway that perpetuates pre-existing coagulopathies: ***dilutional coagulopathy***. Dilutional coagulopathy is caused by coagulation factor-free resuscitation fluids administered to compensate for blood loss and to maintain an adequate volume status of the patient. The overall detrimental effect of resuscitation fluids on coagulation mechanisms is the sum of the unspecific dilution of haemostatic components (coagulation factors and platelets) in the intravascular space and specific anticoagulant side-effects of resuscitation fluids from different pharmacological classes (71).

1.4. Resuscitation fluids

Several fluid types are available for the treatment of clinically relevant fluid deficits in the intra- and/or extravascular components of the human body. Crystalloid fluids (as balanced and unbalanced salt solutions), semisynthetic colloids like dextrans, starches or gelatines, and natural colloids like albumin solutions must be distinguished. Despite being one of the most frequently applied medical interventions it stays unclear for fluid administration which class of fluid is best for different clinical situations. Under severe bleeding, clinicians try to choose from fluid types with minimum impact on the haemostatic competence of the bleeding patient.

In general, crystalloids and albumin solutions seem to trigger less deterioration of coagulation mechanisms than semisynthetic colloids, such as dextrans or starches. *In vitro* and *in vivo* VHA assays show a significant reduction of FGN-dependent clot firmness under 30% dilution with colloids and only at 40 – 60% dilution with crystalloids (118)(119). A significantly lower intravascular volume effect with less dilution of coagulation components compared to colloid solutions could play a role in this context.

All colloids used as plasma substitutes exert specific anticoagulant side effects on both, the plasmatic coagulation and platelet function. Impaired fibrin polymerization with reduced clot stability, decrease in von Willebrand factor and FVIII plasma levels, enhanced fibrinolytic response and reduced platelet adhesion and aggregability are the underlying mechanisms for the specific anticoagulant effects of colloid plasma substitutes (Table 2).

Albumin

Albumin is considered to be the colloid solution with the least negative effects on the coagulation system, although it has also some theoretical anticoagulant effects. It can directly inhibit platelet aggregation through induction of nitric oxide, without showing direct platelet coating effects as seen from other colloid solutions. Moreover, the binding of antithrombin may result in an anti-Xa effect (120)(121).

These albumin-specific properties can translate into the alteration of coagulation monitoring parameters, including ROTEM parameters (122). Significantly prolonged clot-formation time and reduced MCF were
demonstrated after albumin substitution under therapeutic plasma exchange.
A further study, comparing coagulation parameters of healthy volunteers with high, physiologic and low plasma albumin levels showed enhanced primary haemostasis, platelet aggregation, and clot formation in groups with low albumin levels, suggesting that a reduced anticoagulant effect under low albumin concentration could cause a prothrombotic condition (123).

There is some evidence from animal studies that albumin administration might be beneficial in terms of reducing blood loss and improving survival (124). Clinical studies comparing human albumin against hydroxyethyl starch (HES 130/0.4) showed consistently lower perioperative haemorrhage in the albumin arms (124) (125)(126)(127). In a randomized controlled trial comparing albumin solutions with lactated Ringer in major non-cardiac surgery, no difference was seen between study groups (128). However, another study comparing albumin with crystalloids indicates that albumin might increase the overall risk for perioperative blood loss (129) in the perioperative setting.

The use of albumin solutions and its safety- and benefit-profile in the ICU setting is also the subject of intensive research. In 1998 a highly regarded meta-analysis published by the Cochrane Injuries Group Albumin Reviewers suggested higher mortality rates in critically ill patients with hypovolaemia, burns, or hypoalbuminemia comparing administration of albumin or plasma protein fraction with no administration or with the administration of crystalloid solution (130). These alarming data on the use of albumin as resuscitation fluid could not be confirmed in huge follow-up studies. Especially, the large Saline versus Albumin Fluid Evaluation (SAFE) study, a blinded, randomized, controlled trial, to examine the safety of albumin in 6997 adults in the ICU could not confirm higher mortality or end-organ failure rates in the albumin study group (131). A subgroup analysis suggested a potential survival benefit in patients with severe sepsis.

Semisynthetic colloids

Starches belonged to the most frequently used colloids, often used as first-line therapy for goal-directed volume replacement in the perioperative setting. Accumulating evidence, however, demonstrates a significant 21 - 35% increase of risk for renal replacement therapies in critical care patients and an increase in risk for death (relative risk, 1.17; 95% CI, 1.01 to 1.30; P = 0.03) (132)(133)(134). Their detrimental effect on coagulation parameters is also well documented, especially among high molecular weight preparations (>200kD). These slowly degradable starches exert coating of the platelet surface with colloidal macromolecules (135) resulting in an inhibition in the conformational changes and/or interaction of glycoproteins IIb–IIIa and Ib with their ligands. Simultaneously, a detrimental effect on fibrin polymerization with a clear impact *in vitro* and *in vivo* on the MCF in TEM has been shown (136)(137). Altogether this translates into a significant increase in perioperative blood loss in most non-cardiac surgery settings when comparing starch solutions against crystalloids. During cardiovascular surgery metaanalysis data did not demonstrate a significant difference in haemorrhage comparing HES and crystalloid groups (129)(138) - (148).

Crystalloids

Crystalloids are considered to be the safest resuscitation fluids in terms of negative side effects on the haemostatic system in patients with ongoing bleeding. Sodium chloride or normal saline (0.9%) has to be distinguished from “balanced” or “physiological” crystalloid solutions. The term “normal saline” was introduced, based on red-cell lysis studies performed by the Dutch physiologist Hartog Hamburger in 1882/1883. Although the “normal” saline content of extracellular fluids was later shown to be 0,6% and not 0.9% the term “normal saline” was maintained (149). Administration of larger volumes of normal saline can be associated with the onset of hyperchloremic metabolic acidosis and clinical adverse events like immune and renal dysfunction have been related to this problem (150)(151).

As a consequence balanced crystalloid solutions with a more physiologic composition like Ringer Lactate ®, Hartmann solution® or Plasmalyte® have gained increasing relevance as resuscitation fluids. Theoretical safety concerns are linked

to the chemical composition and buffer molecules (lactate, acetate, gluconate or malate) including elevated risk for cerebral oedema (relative hypotonicity), hyperlactatemia (lactate containing solutions like Ringer or Hartmann solutions), hyperkalemia or cardiotoxicity (acetate containing solutions like Plasmalyte). A relevant number of randomized clinical studies however give evidence that balanced crystalloids compared to normal saline are not associated with worse organ function or higher mortality (152)(153). On the contrary, lactated Ringer Solution seems to be associated with reduced amounts of perioperative blood loss and reduced transfusion of red blood cells compared to normal saline as shown in a metaanalysis including 28 clinical surgical and non-surgical studies (154).

Dilution of Coagulation Factors caused by Resuscitation Fluids

Dilutional effects of the intravenous administration of resuscitation fluids are proportionate to their intravascular volume effect. Decreased pro- and anticoagulant coagulation factors concentration in plasma and a decreased platelet count without affecting platelet function are the direct consequences of dilution leading to nonspecific dilutional coagulopathy (155)(156). Although all resuscitation fluids exert unspecific dilutional effects on coagulation factors it seems that *in vivo* dilution is context-sensitive and the detrimental effects of crystalloids are clinically less relevant than those of colloids. This effect is most likely related to the reduced volume effect in the intravascular space induced by equal volumes of crystalloids or colloids infused(157)(158). The integrity of the endothelial glycocalyx layer and the associated capacity of the vascular wall to retain plasma fluid in the intravascular space (159) play a possibly relevant role in this context.

Overall minimal changes are to be expected under dilution rates below 20% for most resuscitation fluids. In viscoelastic tests, a hypercoagulable state can be detected under dilution grades of < 20% (40%) being unclear the clinical relevance of these findings (160). Methodological artefacts or a relevant reduction of antithrombin activity are discussed in this context.

With rising dilutional grades hypofibrinogenemia seems to be the first relevant coagulopathic mechanism to emerge in form of a single-factor deficiency. The overall impression that FGN is the first coagulation factor dropping to critically

low plasma levels with detrimental effects on coagulation is widely supported by mathematical, animal models and *in vivo* studies (161)(162)(163).

In more severe dilutional models within the range of 60% dilution degree and higher, a significant impact beyond clot strength impairment caused by hypofibrinogenemia can be observed including deterioration of thrombin generation potential and relevant decrease of platelet count. This manifests in form of prolonged CT in VHA, and prothrombin time and activated partial thromboplastin time in standard laboratory testing and further enhanced reduction of blood clot stability (reduced A5, A10 MCF) (164). These changes reflect a coagulopathic propagation from single-factor- towards multifactor-deficiencies under increasing dilution rates.

Table 2: Summary of coagulopathic mechanisms caused by different fluid types

Albumin:
• Dilutional, nonspecific decrease of plasma coagulation factor activity and reduced platelet count
• Specific effects:
  • Inhibition of platelet aggregation
  • Binding and inactivation of antithrombin

Crystalloids: • Nonspecific decrease of plasma coagulation factor activity and platelet count • Hypercoagulability at a low degree of dilution. Artefact vs reduced activity of antithrombin.

Synthetical Colloids:
• Dilutional, nonspecific decrease of plasma coagulation factor activity and platelet count
• Specific effects:
  • decrease in FVIII activity
  • Detrimental effects on fibrin formation and crosslinking
  • Inhibition of platelet dysfunction
  • Activation of fibrinolytic mechanisms

1.5. Haemostatic therapy of coagulation factor deficiencies in massive bleeding

Perioperative bleeding associated coagulopathies caused by coagulation factor deficiencies is a dynamic process and progress from hypofibrinogenemia as a single-factor deficiency in the initial phase of the bleeding event to a multifactor deficiency owing to ongoing loss, consumption, and dilution. Clinicians have to thoroughly substitute those coagulation factors that drop below critical plasma levels that are proven to be related to maintained coagulopathic bleeding. Several treatment options with products containing coagulation factors are available to achieve this goal: allogeneic plasma products, cryoprecipitates (Cryo) and CFCs like FC, PCC and FXIII concentrate (FXIIIC).

Transfusion of allogeneic plasma products

During the second half of the last century, transfusion of separated blood components like plasma, platelets and red blood cells largely replaced the previous standard of care of allogeneic whole blood transfusion. The potential advantage of this change of procedure was seen primarily in the improved preservability and storability of the separated blood components, as well as in enabling laboratory-guided transfusion strategies based on real clinical necessity, which should limit the overall amount of blood transfusion and thus also the rate of associated side effects. Since the introduction of blood components, a huge body of scientific work on identifying clinical indications for safe and beneficial plasma transfusion has accumulated but clear evidence is still lacking for most clinical scenarios (165). In the clinical scenario of massive bleeding plasma is transfused to avoid, or correct, clinically relevant coagulation factor deficiencies as detected by standard laboratory testing or VHA. Interestingly, most guidelines and massive transfusion protocols have returned to suggest high ratios of plasma: red blood cells of up to 1:1 which equals the transfusion of reconstituted whole blood (166).

- Efficacy of plasma transfusion for haemostatic purposes

Once separated from red blood cells after whole blood donation, or by direct apheresis, plasma can be maintained in a liquid state but is mostly frozen for better preservation. Plasma products that can be found in clinical use today include liquid quarantined plasma, frozen plasma, lyophilised plasma, and cryoprecipitate-reduced plasma. (166). To improve the safety of plasma products, several methods like leucocyte filtration and pathogen inactivation with methylene blue (single donor and virus-inactivated), or solvent/detergent-treatment (pooled and virus-inactivated) have been introduced to reduce leukocyte-triggered immune responses and viral transmission. Most of these pre-transfusion treatments are associated with a decrease in clotting factor activity and thus with an overall decrease in the haemostatic quality of the plasma product (167). The amount of citrate added as an anticoagulant also has a significant effect on the activity of several coagulation factors (FVIII, FV, FIX)(168)(169). Other factors that influence the plasma quality are time from donation to plasma separation, storage time and leucocyte filtration (170). An experimental study reconstituting whole blood from previously separated components showed a significantly reduced clotting activity when compared to fresh whole blood with dilutional effects not only on coagulation factor activity but also on haematocrit and platelet count (171) . Especially FGN levels were just within the limits for recommended substitution thresholds (1.5 – 2.0 g/l) as proposed by most treatment guidelines (3)(172). The critically low FGN level of most plasma preparations makes plasma appear to be an inappropriate source for correction of clinically relevant hypofibrinogenemia. This assessment is supported also in mathematical models predicting an exponentially increase of transfused plasma volume to correct FGN concentrations at, or slightly below, the substitution thresholds (173). The low activity of most coagulation factors found in pathogen-inactivated preparations like solvent/detergent or methylene blue plasma are in good agreement with the clinical experience that very large plasma volumes of up to 30 ml/kg are needed to correct coagulation disorders with prolonged prothrombin time as an expression of reduced thrombin generation potential (174). In a recently published Cochrane meta-analysis, major uncertainty was expressed by the authors concerning safety and effectiveness of plasma for the treatment of coagulopathies under major bleeding. This is in line with the results of a major review previously published

by Kozek et al. on the topic (175). A recent retrospective study reported increased mortality risk or worse clinical outcomes in different surgical populations (176).

The lack of evidence in favour of plasma transfusion for severe bleeding stands in a certain contrast to treatment recommendations in guidelines and massive transfusion protocols and still wide acceptance among physicians. Actually, a significant rise in the use of plasma products is registered in the past decades (177). This is mainly driven by evidence from trauma studies showing a mortality benefit of high plasma: RBC ratios of 1:1 to 1:2 versus low ratios or versus coagulation factor-free fluids. These results from damage-control resuscitation in trauma have been included as a treatment recommendation in most guidelines not only for trauma- but also for non-trauma-related bleeding.

- Adverse effects

Plasma-related adverse effects can be divided into three major groups: immunological (TRALI, anaphylactic events), physicochemical transfusion-associated circulatory overload (TACO), intolerance towards chemical additives like citrate or latex), and infectious reactions like transmission of bacterial-/viral- or prion-related infectious diseases.

TRALI is defined as acute respiratory distress related to the transfusion of components of allogenic blood, with the highest risk for plasma-containing components like platelets or FFP (178). It is considered to be the second most important cause of transfusion-related morbidity or mortality after blood group incompatibilities with an overall frequency of 0.08 – 15.1% per patient or 0.01 – 1.12% per product (179), with a relevant chance of underreporting. A higher vulnerability is found among critical patients with the previous activation of neutrophils due to shock, sepsis, or previous surgery (two-hit hypothesis). Transfused donor antibodies against recipient leucocytes (e.g. anti HLA II antibodies) seem to be responsible for a strong immune reaction leading to the production of proinflammatory mediators and onset of increased vascular permeability with acute pulmonary oedema within 6 to 72 hours after transfusion. Fluid overload in the context of massive transfusion presents with a similar clinical picture but is associated with high hydrostatic pressure with low plasmatic protein levels in contrast to the usually low central venous pressure

found in TRALI patients (179). Preventive strategies for TRALI like antibody screening (anti-HNA and anti-HLA antibodies) and deferral of all female donors (use of male-only plasma donors) led to a significant reduction of TRALI events in recent years (180).

The incidence of allergic/anaphylactic transfusion reactions is reported to be between 1:591 and 1:2,184 units FFP transfused with a clinically mostly mild course consisting of urticaria, pruritus and or flushing although full-blown anaphylactic reactions with shock symptoms are also reported (181)(182).

The risk for transmission of infectious disease has been considerably reduced through medical screening and testing for infectious diseases of donors and the introduction of pathogen inactivated/reduced plasma preparations like solvent/detergent or methylene blue-treated plasma. Currently, the transmission rate for acquiring HIV, HCV, and HBV through transfusion is 1:1.467000, 1:1.149000, and 1:280000 donations, respectively (183)(184).

Fibrinogen Concentrates

FCs are obtained from human plasma pooled from up to 60.000 donors and treated with virus inactivation/removal processes. Several products are commercially available: Haemocomplettan®, or RiaSTAP® (CSL Behring, Marburg, Germany) in the US and some other countries, Clottafact® (LFB, Les Ulis, France) and Fibryga® (Octapharma, Langenfeld, Germany) are the best documented products and licensed in several countries (185). Other more regional products are Fibrinogen HT® (Benesis, Osaka, Japan) and FibroRAAS® (Shanghai RAAS, Shanghai, China). Riastap is distributed worldwide but is currently not licensed for acquired hypofibrinogenemia. By contrast, Haemocomplettan and Fybriga include this indication for some clinical situations such as obstetric bleeding. The original indications of FCs are congenital hypofibrinogenemia, afibrinogenemia and dysfibrinogenemia.

Pharmacokinetic data for Haemocompletttan® have been published for patients with congenital hypo- and afibrinogenemia (186). The median increase in plasma FGN lies in the range of 1.5 – 1.7 mg/dl per substituted mg of FGN per kg body weight, resulting in an *in vivo* recovery of 60%. Distribution volume is reported

to lie between 80 – 140 ml/kg with a median half-life (t1/2) of 77.1 h for FGN activity. Differences in composition and pharmacokinetic characteristics between different FCs are described and briefly reviewed in Table 3.

Table 3: Pharmacokinetic data of commercially available fibrinogen concentrates

	Pharmacological preparation	FGN Antigen Level	Factor XIII IU/ml	Clerance (ml/h/kg)	Distribution Volume (ml/kg)	Fibronectin (g/l)
Haemocomplettan/RiaSTAP (CSL Behring, Germany)	1g/50ml	23.4	7.2	0,82 – 2.55	89	3.3
Clottafact (LFB, Les Ulis, France)	1,5g/100ml	na	na	0,53	50,7	na
Fibrinogen HT (Benesis, Osaka, Japan)	1g/50 ml	na	na	na	na	na
FibroRAAS (Shanghai RAAS, China)	0,5 g/25 ml	na	na	na	na	na
Fibryga (Octapharma, Lachen, Switzerland)	1g/50 ml	29.5	10,1	0,66	70.2	0.19

(185)(187)(188)

The dose needed to correct hypofibrinogenemia to a predefined goal is best calculated and guided by FIBTEM-MCF values. Dose calculations with Clauss-derived FGN concentrations are based on the published pharmacokinetic data. The time delay between sample extraction and report of results together with bleeding dynamics has to be taken into consideration when FGN dose is calculated with Clauss values:

Figure 9: Equations for dose calculation based on FIBTEM or Clauss values (validated for Haemocomplettan®)

- **Equation for FIBTEM-guided FGN replacement:**[1]

$$FGN\ dose\ (g) = (Target\ FIBTEM\ MCF\ -\ Actual\ FIBTEM\ MCF) \times 0.5 \frac{g}{mm} \times \frac{body\ weight}{70}$$

- **Equation for Clauss-guided FGN replacement:**[2]

$$FGN\ dose\ (g) = \frac{(Target\ plasma\ con. - Actual\ plasma\ con.)\ g/l}{1.7\ mg/dl} \times 0.1 \times body\ weight\ (kg)$$

[1]: Validated and published (20), [2]: From the author´s institutional treatment algorithm

FC seems to have a good safety profile. A pharmacovigilance report analysing data from a 27-year period informed of only 1 adverse event per 24600 g FC administered. The risk for bias due to underreporting of adverse effects has to be taken into consideration, but consistent data were found in an analysis of published clinical studies on FC in bleeding scenarios (189). The infusion rate of FC should not exceed 5 ml/min, although safe administration of 1g FC in 20 seconds or up to 14 g in less than 5 minutes under emergency conditions has been reported (190).

Table 4: Comparison of different fibrinogen sources

	FC	Cryo	Plasma
FGN Concentration	Standard Preparation	variable: 15 – 17 g/l	variable 2 – (3) g/l
Unit/Volume/Content	Vial/50 ml/0.9- 1.3 g	pooled bag with 5 units/100-150ml/1,5-2g	bag/200-280 ml/0,5g
Volumen needed to administer 4 g FC	200ml	250 - 300 ml	2000ml
Coagulation Factors	FGN/FXIII/ Fibronectin	FGN, FXIII, VWF, FVIII, Fibronectin	All coagulation factors
Shelf life (conditions)	60 months (25°C)	12 months (-20°C)	12 months (-18°C)
Risk for transmission of infectious agents	Extremely low	low	low
Need for blood group compatibility check	No	Required if large volumes transfused	Yes
Need for cold chain	No	Yes	Yes
Preparation time	5 min	10 min	30 min
Adverse events	Possibly low/minimal risk for thromboembolic complications	(same as plasma)	TRALI, TACO, TRIM, TTI, infection, allergic reactions, blood group incompatibility
Virus removal/inactivation	Nanofiltration, Pasteurization cryoprecipitation	(same as plasma)	Psoralen Solvent-detergent (SD) Methylene blue Quarantine

Cryoprecipitate:

Cryoprecipitate (Cryo) is produced by controlled thawing of FFP (191). Precipitated high molecular weight proteins are separated from plasma supernatant (exhausted plasma) by centrifugation, resuspended in a small volume of plasma (15 – 20 ml) and deep frozen for final storage at -20°C. After thawing Cryos should be infused immediately, although a storage time of 4-6h at 20 – 24°C appears to be acceptable (192). Cryo is not only a rich source of FGN and von Willebrand factor, but also contains coagulation FVIII and FXIII, fibronectin, α2-antiplasmin and minimal amounts of immunoglobulins. A substantial amount of contaminating platelet membrane micro-particles and phospholipids, generated during the freezing and thawing process, possible influences the therapeutical coagulation effects of Cryo. FGN concentration in Cryo can vary between different units but usually lies between 15 – 17 g/l (95). Five units of Cryo are usually pooled in a single bag. Standard doses of 4 g FGN in bleeding conditions would correspond to a treatment dose of two pooled bags. Most of the conducted clinical studies comparing FC and Cryos for treatment of hypofibrinogenemia under severe bleeding showed no clear difference in efficacy and safety profiles (193). Thawing time, the risk for viral transmission and heterogeneous factor composition affecting coagulation beyond the role of FGN are some of the drawbacks that makes Cryos appear as a less suitable product, compared to FC to control perioperative hypofibrinogenemia.

Prothrombin Complex Concentrate

Prothrombin Complex Concentrates (PCCs) are pathogen-reduced, lyophilized concentrates of the vitamin K-dependent coagulation factors FII, VII, IX, X. Their protein content is standardized according to their corresponding FIX concentration (Tabla 5). They are produced by ion-exchange chromatography from the Cryo supernatant of large plasma pools after the removal of antithrombin and FXI (194). 3-factor PCCs with sub-therapeutic or without FVII must be distinguished from 4-factor PCCs that include therapeutic levels FVII and are known to reverse more effectively the anticoagulant effects of oral vitamin K antagonists (195). Heparin is added to the product to avoid factor coagulation. Some preparations also contain other anticoagulants like protein C, protein S, and/or substituted antithrombin. PCCs were originally developed as a source of

FIX to treat patients with haemophilia B but since the introduction of recombinant factor preparations, it is no longer used for this indication (196). In recent years, the field of application for PCC has been shifted towards several other clinical indications like emergency reversal before surgical interventions of vitamin K antagonists and direct FX inhibitors and the treatment of acquired coagulopathies in bleeding scenarios with coagulation factor deficiencies causing impaired thrombin generation potential (7)(197)(198). PCCs offer several advantages over FFP in these new indications. Most importantly, after reconstitution, the activity of the corresponding coagulation factors is 20 – 30 times higher than in plasma products which allows for the highly effective replacement of these factors without the need for high volume administration. Second, there is no time delay due to ABO compatibility testing or thawing. And finally, PCCs are leucocyte-free and antibodies are removed during the manufacturing process which substantially reduces the risk for immunological transfusion reactions and TRALI (199)(200)(201). All this renders PCCs to be an attractive alternative to plasma for managing factor deficiencies in a clinical emergency situation with substantial bleeding risk. Experimental *in vitro* studies and animal models could show that PCC has good potential to improve and correct reduced thrombin generation potential induced by previous dilution (202)(203). In the last ten years, several clinical studies on the use of PCCs in acquired bleeding-associated coagulopathies have been performed to examine their potential to improve the outcome in bleeding patients. In a recently published metaanalysis, including 3060 patients investigated in a total of 17 clinical studies, the use of PCCs, mostly in addition to plasma, was associated with mortality reduction for bleeding trauma patients (204). A reduction in blood loss in cardiac surgery and a decrease in red blood cell transfusion was seen in a wide range of other surgical bleeding patients when compared to standard of care. The dosage of PCC used was based on clinical judgment in most groups and ranged from 10 to 50 IU/ml and the coagulation effect was mostly monitored by viscoelastic testing (CT EXTEM) (7).

Clinical side effects after PCC administration remain a major concern among clinicians.

The risk for transmission of infectious diseases or TRALI is low due to the applied viral inactivation steps and/or nanofiltration for most preparations. The overall risk of thrombotic or thromboembolic adverse events, being the major safety

concern for PCC treatment is low as shown from pharmacovigilance reports and reviews on the topic (205). An elevated risk for thromboembolism under PCC treatment could arise from an accumulation of prothrombin with repetitive PCC administration because the half-life of prothrombin (and FX) is significantly longer than that of other coagulation factors, and therefore accumulation of these factors is easily possible (206). Some studies also reported an elevated risk for perioperative acute renal failure which might be explained by the comparatively reduced volume administration with a compensatory use of vasoconstrictors when plasma is replaced or complemented by PCC (115).

Table 5: Composition of PCC in the World Federation of Haemophilia register of clotting factor concentrates

		International units relative to factor IX					
Brand name	**Manufacturer**	**Factor II**	**Factor VII**	**Factor IX**	**Factor X**	**Viral inactivation**	**Additional information**
Bebulin VH	Baxter BioScience, Austria	120	(13)	100	100	Vapour heat, 60°C for 10 hours at 190 mbar, then 80°C for 1 hour at 375 mbar	Heparin added
Beriplex P/N	CSL Behring, Germany	128	68	100	152	Pasteurisation at 60°C for 10 hours, and nanofiltration	Protein C; antithrombin, heparin and albumin added
Cofact	Sanquin, the Netherlands	56-140	28-80	100	56-140	Solvent/detergent and 15 nm nanofiltration	Antithrombin added
KASKADIL	LFB, France	148	40	100	160	Solvent/detergent	Heparin added
Octaplex	Octapharma, Austria and France	44-152	36-96	100	50	Solvent/detergent and nanofiltration	Heparin added; low activated factor VII content
Profilnine SD	Grifols, USA	148	(11)	100	64	Solvent/detergent	–
Prothrombinex VF	CSL Bioplasma, Australia	100	(–)	100	100	Dry heat, 80°C for 72 hours and nanofiltration	–
Prothromplex T	Baxter BioScience, Austria	100	85	100	100	Vapour heat, 60°C for 10 hours at 190 mbar, then 80°C for 1 hour at 375 mbar	Antithrombin and heparin added
UMAN Complex D.I.	Kedrion, Italy	100	(–)	100	80	Solvent/detergent and dry heat, 100°C for 30 minutes	Antithrombin and heparin added

Composition of prothrombin complex concentrates (PCCs) listed in the World Federation of Hemophilia register of clotting factor concentrates [54], excluding concentrates for national markets only or for which the concentrations of factors relative to factor IX were not available from the relevant product information sheets. Factor VII presented in parentheses for three-factor PCCs.

(206)

Factor XIII Concentrates

FXIIIC is a plasma-derived, highly purified and pasteurized product which is licensed for the treatment of congenital FXIII deficiencies. Recombinant FXIII is also available for the treatment of FXIII deficiency but only plasma-derived FXIII is referred to in this work.

Congenital FXIII deficiencies are extremely rare conditions. By comparison, acquired FXIII is often reported in the context of major trauma, cardiac surgery, and burns and probably remains underdiagnosed (207).

Both forms of FXIII deficiency, congenital and acquired, can be pharmacologically treated with FXIIIC. Currently, several plasma-derived products are available with Fibrogammin®, Corifact®, Cluvot® which are all distributed by CSL Behring. Other possible sources for FXIII substitution are direct plasma or Cryo transfusions. The factor concentration in plasma is considerably lower with only around 300 UI per transfused plasma unit (factor activity of 1.0 to 1.2 IU/ml). Very large transfusion volumes would therefore be required to effectively compensate for a relevant FXIII deficit (208).

FXIII is a transglutaminase which plays an important role at the end of the coagulation process through the covalent cross-linking and stabilization of the fibrin network (209). It also exerts an important role in many other physiological processes like angiogenesis, wound-healing and immunological defence against bacterial infections. FXIII is composed of two A and two B subunits and circulates in its inactive form in plasma (210). It can be converted into its active form by a thrombin-induced enzymatic cleavage process which mediates a conformational change, which then allows forming covalent crosslinks between soluble fibrin polymers resulting in a stable blood clot. The FXIII-mediated incorporation of α2-antiplasmin, plasminogen activator inhibitor 2 and thrombin activatable fibrinolysis inhibitor (TAFI) provides good protection of the forming fibrin clot against proteolytic degradation by plasmin (211).

FXIII can contribute to the overall mechanical firmness of the fibrin clot. *In vitro* studies could demonstrate that FXIII supplements added to blood probes from ICU patients significantly improved the stability of blood clots and the resistance

to hyperfibrinolysis measured by VHA (212). It is also shown from *in vitro* studies and animal models for trauma bleeding that high-dosed FXIII supplements improve clot adhesion and haemostasis (213)(214). A significant reduction of FXIII levels in trauma patients and postoperative ICU patients is reported with factor activities dropping to 60% or below (212)(215). These levels of FXIII activity can impose a relevant risk of bleeding in certain surgical areas (216). Two major clinical studies following CFC-based algorithms including a goal-directed substitution of FXIII showed reduced transfusion rates and improved outcomes on several clinical endpoints, although the individual impact of each coagulation factor was not definitely attributable (217)(218).

Treatment safety of FXIII deficiencies with plasma-derived FXIIIC has been investigated in several studies. FXIIIC is considered to be safe for both congenital and acquired deficiencies (219)(220). Especially the risk for thromboembolic events or viral transmission seems to be extremely low.

1.6. Treatment Strategies for coagulation factor deficiencies in bleeding-associated coagulopathies

Acquired Hypofibrinogenemia

Consumption in extensive wound fields in peripartum haemorrhage, trauma or by cardiopulmonary bypass circuits and decreased synthesis (liver transplant) are among the principal underlying causes of FGN depletion. The degree of FGN shortage in these clinical situations can thereby show great variability. As no storage and no overexpression can compensate for consumption and loss, the FGN replacement necessity can be significantly beyond that calculated by a simple dilutional phenomenon (221).

By contrast, dilutional effects under aggressive resuscitation fluid therapy are constant and well-studied. Blood dilution with all fluids induces detrimental effects on FGN levels reflected by standard laboratory and VHA parameters (119)(164)(222). As previously mentioned severity of the dilutional effects depends both on the fluid type and the dilutional grade.

FGN replacement has a very strong potential to improve and correct dilutional effects on viscoelastic clot characteristics confirmed *in vitro* (223) and in bleeding models in swine (221). The overall efficacy of FGN seems to depend on the underlying fluid applied for dilution. Hydroxyethyl starches, unlike crystalloid- and albumin solutions, impair the complete normalization of clot properties by FGN supplements (224).

A generally accepted consensus exists to maintain a minimum plasma concentration of FGN for effective blood clot formation (3)(66)(228). The optimal treatment threshold to guide FGN supplementation in different clinical situations is still controversial, although a general tendency toward more generous plasma FGN levels has been observed over the last 2 decades (3)(229).

Clinicians can choose from three different FGN sources: FC, Cryos, and plasma. The potential of plasma to correct a low FGN level compared to FC and Cryo

is significantly reduced due to the underlying low FGN concentration in most plasma preparations.

Acquired multifactor deficiencies: Coagulation factor concentrates as an alternative to fresh frozen plasma.

FGN deficiency seems to be the first mechanism to emerge under dilution, as clearly supported by mathematical and *in vivo* approaches (162)(226). However, in more severe dilutional models of 60% and higher, a significant impact beyond clot strength impairment can be observed including deterioration of the thrombin generation potential, with prolonged CT in VHA, and prothrombine time and activated partial thromboplastine time in standard laboratory testing (165). Overall, these changes during severe dilutional conditions reflect propagation from single-factor- towards multifactor-deficiencies under increasing dilution rates.

The efficacy of FGN substitution in severe bleeding-associated multifactor-coagulopathies strongly depends on a maintained thrombin generation potential (227), since effective fibrin polymerisation starts with thrombin-mediated cleavage of the FGN molecule. Other CFCs, especially PCCs, seem to guarantee an adequate thrombin activity in this context.

The high efficacy of CFC supplements to reverse coagulation factor deficiencies increasingly challenges the concept of plasma transfusion for this purpose.

Most massive transfusion protocols suggest high ratio plasma transfusion for severe bleeding, based on a large body of evidence clearly demonstrating superiority of plasma transfusion against volume resuscitation with ***coagulation-factor-free*** crystalloid or colloid fluids. This aims to avoidance of volume therapy-induced dilutional coagulopathy. In trauma patients, a timely transfusion at a 1:1:1 ratio with FFP, platelet, and red blood cell concentrates as primary resuscitation fluids are considered to be the currently safest strategy (225) (226). Nevertheless, despite timely management of massive bleeding with high ratio plasma transfusion, the onset of bleeding-associated coagulopathies is not completely avoidable. Although plasma contains all coagulation factors it exerts by itself dilutional effects on FGN, erythrocytes, and platelets (73)(174). The

limitation of plasma to efficiently maintain haemostasis has to be compensated by additional monitoring, ideally performed by point-of-care systems, to deliver goal-directed top-up replacement of CFCs. FGN deficiency and deteriorated thrombin generation are efficiently and safely optimized by CFC (FC, FXIII, and PCC) supplements to plasma transfusion. The necessity of additional monitoring and treatment strategies to compensate for the haemostatic limitation of fixed ratio transfusion of allogeneic blood components is reflected in practically all published guidelines on the management of massive bleeding (3)(69)(70).

It is of great clinical interest in how far plasma transfusion can be completely substituted by administration of CFC. Recently published data from *in vitro* studies by Schöchl et al. suggest it might be possible to maintain the basic haemostatic mechanisms under CFC administration even under complete avoidance of plasma. They reported higher efficacy of FC on clot firmness and PCC on thrombin generation compared to plasma if analysed under blood reconstitution conditions in presence of red blood cells and platelets (227).

HYPOTHESIS

2. HYPOTHESIS

Bleeding-associated coagulopathies often involve the deficiency of coagulation factors and are increasingly monitored by VHA. A dynamic evolution from a single factor deficiency in the form of hypofibrinogenemia with reduced viscoelastic clot properties towards multifactor deficiencies with additionally restricted thrombin generation potential expressed as prolonged clotting time in VHA can frequently be observed over the course of massive bleeding. Coagulation Factor Concentrates (CFCs) have shown *in vitro*, in animal, and in human studies to have good potential to restore VHA parameters in diluted blood samples and have become a clear treatment alternative to plasma transfusion to correct factor deficiencies. However, there is still a lack of evidence if their administration alone or in combination with plasma transfusion is superior to plasma transfusion alone.

Despite a paucity of high-quality evidence, plasma-based treatment strategies are supported by most guidelines and massive transfusion protocols. The high acceptance of plasma transfusion strategies among many physicians might be explained, not only by its stabilizing effect on the coagulation system by providing a close-to-physiological factor composition but also by its resuscitation fluid quality with good intravascular volume effects in patients with haemorrhagic shock. Currently, no alternative products are available that share these two characteristics with human plasma. CRF in form of colloid fluids combining adequate intravascular volume effects combined with maintained haemostatic properties could be an interesting future treatment component in massive transfusion. CRF would be an ideal comparator to plasma transfusion for the treatment of multifactor deficiencies in future clinical trials trying to analyse the non-inferiority of CRF-based versus-plasma-based algorithms. Together with FC for hypofibrinogenemia and top up corrections with CFCs for specific coagulopathies in different clinical scenarios, CRF would open up a theoretical option for plasma-free management of factor-deficiency-based coagulopathies and hypovolemia in massive bleeding.

We hypothesized that a theoretical basis could be established for a CFC-based, plasma-free treatment approach for acquired coagulation factor deficiencies as reflected by VHA during the time course of massive bleeding.

OBJECTIVES

3. OBJECTIVES

In order to confirm the previous hypothesis, the specific objectives of this doctoral thesis were the following:

1. To confirm that the evidence from current literature would justify a plasma-free treatment approach based on FC for the treatment of hypofibrinogenemia as a single-factor coagulopathy during active bleeding.

2. To investigate that the appropriate combination of FC as clotting substrate associated with other coagulation proteins facilitating thrombin generation (e.g. PCC) in a plasma-free albumin-based colloid solution would restore the basic mechanisms of coagulation and lead to the formation of a stable fibrin clot.

3. To demonstrate that a well-defined combination containing adequate concentrations of FC, FXIII and PCC, reconstituted in an albumin carrier solution and in presence of platelets would lead to normalized TEM parameters when compared to whole blood after extrinsic activation of coagulation.

RESULTS

4. RESULTS

Role of fibrinogen concentrates for treatment of critical perioperative haemorrhage

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SUMMARY:

A literature review was performed to analyse the role of FC in the management of critical haemorrhage in clinical high-risk scenarios like trauma, cardiac surgery, and peripartum haemorrhage.

Acquired hypofibrinogenemia is a frequent cause of maintained bleeding in perioperative high-risk settings. Loss, consumption and dilution under resuscitation fluid therapy are the principal causes of FGN depletion. Severe hypofibrinogenemia is frequently associated with an early bleeding complication that cannot be reliably avoided with high-ratio plasma transfusion strategies. Replenishment of FGN in uncontrolled bleeding events is currently recommended by most published guidelines, suggesting treatment thresholds to maintain a minimum of 1.5 g/L plasma FGN concentration for non-obstetrical haemorrhage, and 2 g/L for active, obstetric bleeding. Institutional cut-off values for viscoelastic testing of functional FGN should be established. FIBTEM-MCF values of 8 mm would most likely predict the critical plasma FGN concentration of 1.5 g/L. FIBTEM-A5 values of 12 mm indicate critical levels for peripartum haemorrhage. FC and cryos are currently the recommended sources of fibrinogen for the correction of clinically relevant hypofibrinogenaemia. It is recognised among experts that these products should be preferred to plasma transfusion, mainly because of their higher fibrinogen concentration

The currently available data from clinical studies provide good evidence to recommend against preemptive FC administration for most clinical situations. There is still a significant shortage of large, multicentre, high-quality randomizaed controlled trials that can clearly demonstrate FC-associated reduction in transfusion rates or even mortality. Inconsistent results between clinical trials are partially explained by inclusion criteria leading to a patient selection with bleeding events that were not necessarily related to critical FGN plasma levels. The main potential of FCs, however, is to be expected in bleeding situations associated with FGN levels below established, critical cut-off values. A huge body of scientific data from retrospective and observational studies clearly suggests that hypofibrinogenaemic conditions in actively bleeding patients significantly increase the risk for massive transfusion and higher mortality, and should therefore be adequately treated. Therefore, most experts agree on the concept of threshold-

guided treatment necessity. Some evidence from randomized controlled trials and observational studies suggests that plasma transfusion, especially within the framework of fixed component ratios, can be safely substituted by CFCs, guided by POC monitoring with a beneficial impact on transfusion rates and mortality.

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Clarivate Analytics

Review Article

Role of fibrinogen concentrates for treatment of critical perioperative hemorrhage

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Contents

Summary

Acquired hypofibrinogenemia is a frequent cause of maintained bleeding in perioperative high-risk settings. Loss, consumption and dilution under resuscitation fluid therapy are the principal causes for fibrinogen depletion. Severe hypofibrinogenemia is frequently associated with an early bleeding complication that cannot be reliably avoided with high-ratio plasma transfusion strategies. Real-time monitoring with viscoelastic hemostatic assays is a useful tool for timely diagnosis and treatment of detected coagulopathies. Replenishment of fibrinogen in uncontrolled bleeding events is currently recommended by most published guidelines, suggesting treatment thresholds to maintain a minimum of 1.5 g/L plasma fibrinogen concentration for nonobstetrical hemorrhage. Fibrinogen concentrates, originally licensed for treatment of bleeding episodes in patients with congenital

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hypo-, dys- or afibrinogenemia disorders, are used in many clinical situations as supplementary therapy for the treatment of acquired hypofibrinogenemia. This review seeks to provide an overview of the most relevant topics associated to fibrinogen replacement therapy for critical perioperative hemorrhage highlighting currently available evidence on the risk/benefit profile of purified Fibrinogen concentrates for this extended clinical indication.

Key words: Fibrinogen – Hypofibrinogenemia – Fibrinogen replacement therapy – Plasma fibrinogen concentration – Blood factors – Bleeding disorders – Hemostatics

Background

Critical hemorrhage in the perioperative setting is a considerable risk factor for elevated morbidity and mortality and a major economic burden for public healthcare systems. Early detection and treatment of bleeding-associated coagulopathies is becoming a central component of most transfusion protocols and guidelines on bleeding management (1). Hemostatic drugs such as Fibrinogen concentrates (FCs) are in the focus of clinical research looking for new therapeutical approaches to reduce the overall transfusion rates and associated adverse outcomes.

Fibrinogen (FGN)—the principle substrate for clot formation—is the first coagulation factor dropping to critical levels under severe bleeding with detrimental effects on hemostatic mechanisms (2). A generally accepted consensus exists to maintain a minimum plasma concentration of FGN for effective blood clot formation (1, 3, 4). The optimal treatment threshold to guide FGN supplementation in different clinical situations is still controversial, although a general tendency towards more generous plasma FGN levels has been observed over the last 2 decades (1, 5). If supplementation is indicated, clinicians can choose from FC or cryoprecipitate (Cryo) as FGN sources. The low average FGN concentration of 2 g/L in frozen plasma (FFP) makes this product unsuitable as an effective replacement therapy (6). Data from several clinical studies suggest that correctly indicated FC treatment has the potential to reduce overall transfusion rates in certain clinical high-risk conditions and that point-of-care (POC)-guided, goal-directed replacement can be cost-effective (7-9). However, the overall clinical information available is not consistent and large multicenter randomized clinical trials (RCTs) have failed to prove clear benefit on mortality or transfusion rates in some high-risk areas.

The aim of this review is to provide the reader with updated and comprehensive aspects of FC treatment in the management of severe bleeding and to explore the available evidence from clinical studies in different high-risk conditions.

Pharmacodynamics

Human FGN is the most abundant clotting factor in human plasma with a reference range from 2.4 to 4 g/L and a daily synthesis of approximately 3-5 g which is limited to hepatocytes. Pregnancy and inflammatory processes can lead to significant upregulation of the FGN expression (10).

Intracellular FGN synthesis leads to formation of a hexamer of two sets of disulfide-bridged α-, β- and γ-chains building up a long-stretched 45-nm structure with two outer D-domains connected to a central E-domain (11) (Fig. 1). Released from hepatocytes into the plasma compartment, FGN has a half-life of 3-5 days and serves as substrate for three different enzymes: plasmin, coagulation factor XIII (FXIII) and thrombin (12).

Thrombin mediates the cleavage of an N-terminal peptide sequence in the α-chain, thus forming soluble fibrin monomers (fibrin I) (13). A second slower cleavage process on the β-chain increases the polymerization capacity of the molecule. Double-stranded fibrils are generated by end-to-middle alignment of the D- and E-domains of several fibrin molecules and assemble with each other, either in parallel forming four-stranded fibrils (bilateral branch junctions), or in an end-to-side configuration (equilateral branch junctions) (14) (Fig. 2). Altogether, the thrombin-induced cleavage process induces the formation of a complex fibre structure, the fibrin clot.

Fibrin molecules are crosslinked by activated coagulation factor XIII (FXIIIa) by covalent bonds within the generated clot, thereby terminating the coagulation process. FXIII crosslinks lead to more resilient and elastic properties while protecting the clot from plasmin-mediated fibrinolytic activity (15).

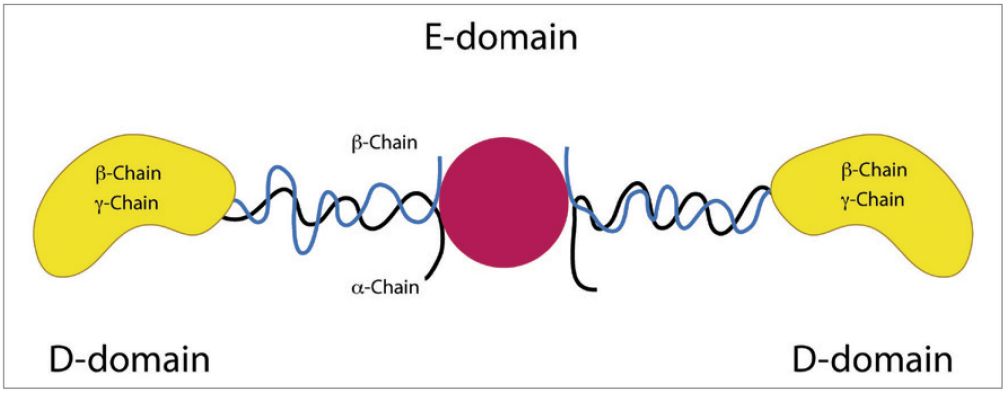

Figure 1. Molecular structure of fibrinogen.

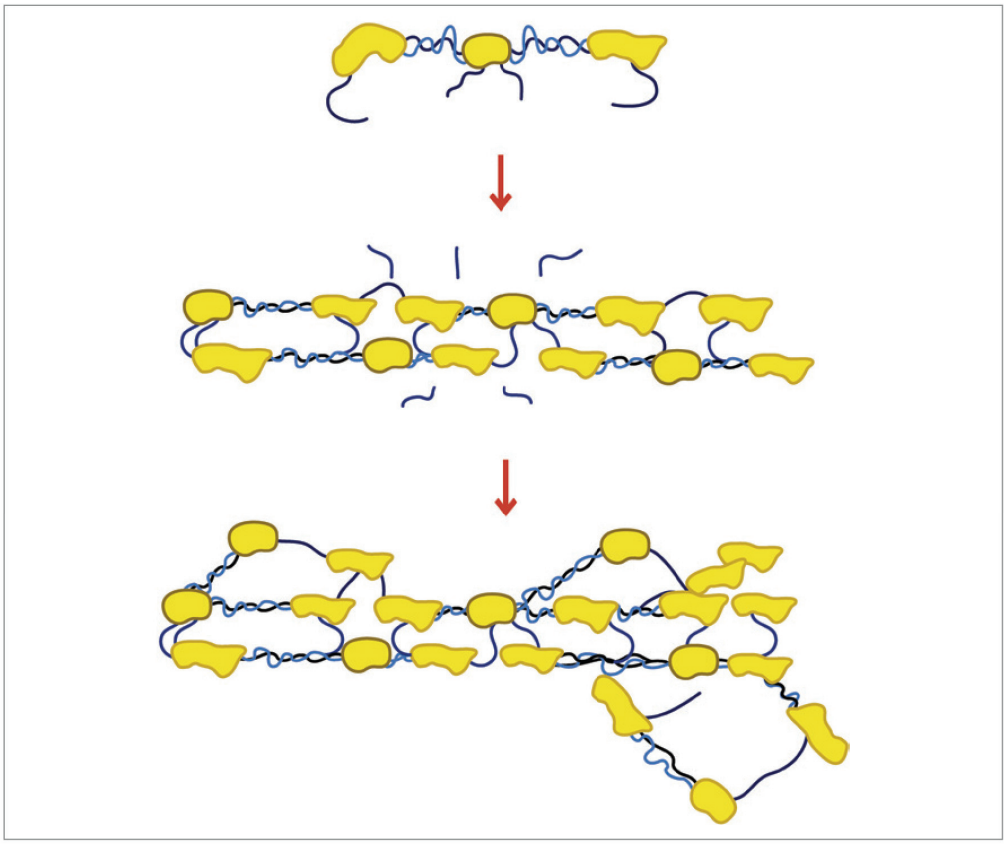

Figure 2. Fibrin polymerization.

Both FGN and fibrin directly interact with platelets (16). FGN is a physiological ligand to the platelet membrane-glycoprotein GPIIb-IIIa (also known as integrin $\alpha_{IIb}\beta_3$). The GPIIb-IIIa interaction with FGN promotes ADP-stimulated platelet aggregation. Binding of fibrin within forming clots to GPIIa-IIIb enhances clot retraction, which further increases clot stability and resilience (17).

In summary, the FGN molecule is a key element of hemostasis, integrating the fluid phase components of coagulation with cell-mediated mechanisms of primary hemostasis. Deficiency of this molecule inevitably leads to deterioration of hemostatic mechanisms.

Fibrinogen Monitoring

FGN monitoring for detection of hypofibrinogenemic conditions is the key element for goal-directed factor replacement under massive bleeding. In the last decades, POC monitoring systems have been introduced in many centers as alternatives to standard laboratory testing (SLT).

Standard laboratory testing

The Clauss method, considered as diagnostic gold standard, measures coagulation time of platelet poor plasma samples under thrombin excess and provides FGN concentration interpolated from a standard calibrated curve. Coagulation time is usually measured by photo-optical methods and, less frequently, by mechanical systems. Clinicians should know about the routine method used in their central laboratories as interference of colloid fluids with photo-optical systems, unlike mechanical systems, is shown to provide falsely high FGN values (18). Turnaround times for treatment decisions based on the Clauss method lie between 50 and 100 minutes (19). This delay makes real-time goal-directed actuation in high dynamic bleeding situations impractical. Further limitations of the Clauss method are related to the impact of high heparin concentrations, and the presence of direct thrombin inhibitors such as dabigatran, argatroban and bivalirudin (20-23). Previous interferences can result in misinterpretation of the obtained test results.

Point-of-care methods

Most guidelines recommend FGN monitoring with either the Clauss method or viscoelastic hemostatic assays (VHA) for bleeding management (1, 3, 24). VHA give comprehensive information on physical and timeline characteristics of the developing blood clot within whole-blood samples. The concept of VHA-guided, goal-directed, coagulation factor concentrate (CFC)-based management is cost-effective, and it seems to reduce transfusion rates while providing apparent beneficial effects on overall outcomes, including multi-organ failure and mortality in some, but not all, high-risk conditions (25, 26). The recently published iTACTIC study could confirm these VHA benefits compared with SLT only for trauma patients with traumatic brain injury, but not for the total of analyzed trauma patients (27). In the referred study, hemostatic treatment was partially predefined per protocol with empiric delivery of blood components at a 1:1:1 ratio (transfusion ratio of 1 unit red blood cells [RBCs], 1 unit fresh FFP and 1 unit platelet concentrate). Thus, it is possible that the benefits of POC testing will remain partially unrecognized if hemostatic treatment is predefined and not guided by diagnostic results which are more specifically for this purpose. Additionally, around 75% of the included trauma patients were not coagulopathic, with coagulopathic bleeding patients being the subgroup of patients that most likely would benefit from POC testing.

Several commercial VHA systems have been commercialized in the market. The most commonly used and best studied analyzers are rotational thromboelastometry (ROTEM) and thromboelastography (TEG). In essence, these systems are based on detection of mechanical resistance of a rotating pin within a blood sample containing cup. The physical clot strength measured during the activation of the coagulation system is expressed in maximum clot firmness (MCF) or maximum amplitude (MA) for ROTEM and TEG, respectively. FGN, FXIII and platelets are the main determinants of these parameters. Platelet inhibition with cytochalasin D (FIBTEM) or abciximab (FF-TEG) allows to estimate the FGN (and FXIII) contribution to MCF/MA (28). Results obtained from FIBTEM and FF-TEG are available within 5-10 minutes and provide an excellent guide for clinicians to indicate FGN treatment and to calculate the corresponding dose (29) (Fig. 3).

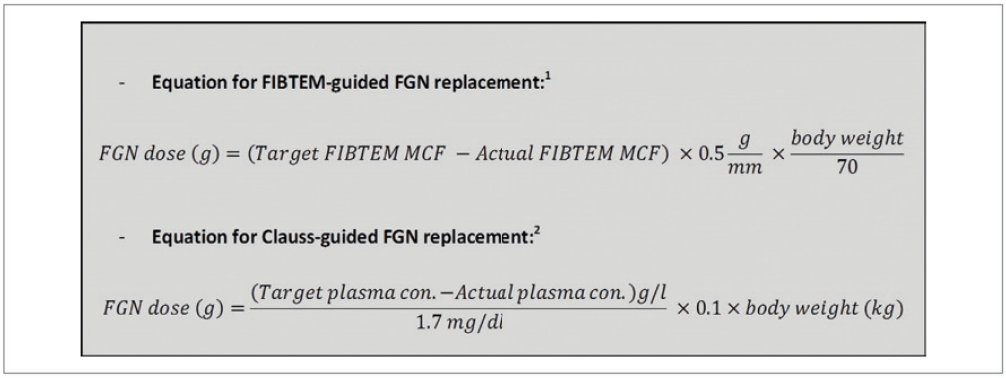

Figure 3. Equations for dose calculation of fibrinogen (FGN) replacement based on FIBTEM or Clauss values (validated for Haemocomplettan). MCF, maximum clot firmness. [1]Validated and published (30). [2]From the author's institutional treatment algorithm.

Substantial variability of VHA testing in intra- and inter-center evaluations, and between different analyzer types, has been demonstrated (30). Although the Clauss method also shows certain limitations in the reproducibility of its results, triggering thresholds for substitutive FGN treatment are published in most guidelines for Clauss values only, and no recommendations are usually given for VHA (31). A recently published meta-analysis on data correlating Clauss and FIBTEM/FF-TEG results determines the best predictor values for a Clauss level of 1.5 g/L at 8 mm for FIBTEM-MCF and 12 mm for FF-TEG (32). The correlation between Clauss and viscoelastic FGN testing may worsen during bleeding management, probably due to variable contribution of FGN, platelets or FXIII on the overall clot firmness (33). This circumstance has led to corresponding test pairs with Clauss levels as high as > 2 g/L and FIBTEM-MCF values as low as 8 mm (32, 34). Clinicians should be aware of these confounders when indicating and calculating FGN treatment (35).

Newer systems for assessing clot formation parameters, like TEG-6S and the Quantra QPlus, have emerged in recent years. TEG-6S applies multifrequency harmonic oscillation to a blood drop, whose oscillation response is measured by an optical sensor. The optical signal is converted into a TEG-similar graph. FGN assessment seems to deliver acceptable results, although some limitations in its correlation with standard TEG and ROTEM parameters were reported (36, 37). Due to the absence of heparinase in FF-TEG, the test results under systemic heparinization have to be critically evaluated.

The Quantra QPlus system (HemoSonics, LLC, Charlottesville, VA) applies a novel ultrasound technique to the clotting blood sample, measuring resonant frequency changes. Good correlation to ROTEM parameters, especially to FIBTEM values in cardiac surgery, could be demonstrated (38, 39).

Acquired Hypofibrinogenemia and Effects of Fibrinogen supplementation. Preclinical Data

The onset of hypofibrinogenemia has a multifactorial origin. Dilutional effects, loss, consumption, fibrinolysis, reduced synthesis (hypothermia, liver failure) and increased breakdown (acidosis) are the principle underlying causes (40-42). The magnitude of FGN deficiency in severe bleeding can therefore be significantly beyond that caused by dilutional effects under treatment with resuscitation fluids (43).

Dilutional effects under aggressive resuscitation fluid therapy are constant and well studied. Blood dilution with different fluids such as Ringer Lactate, albumin and gelatin solutions, dextrans and hydroxyethyl starches induces detrimental effects on FGN levels reflected by SLT and VHA parameters (44-46). Severity of the dilutional effects depends

both on the fluid type and the dilutional grade. Crystalloids and albumin solutions seem to trigger less deterioration on coagulation mechanisms than synthetical colloids, such as dextrans or starches. In vitro and in vivo VHA assays show a significant reduction of FGN-dependent clot firmness under 30% dilution with colloids and under 40-60% dilution with crystalloids (45, 47).

FGN replacement has a very strong potential to improve and correct dilutional effects on viscoelastic clot characteristics confirmed in vitro (48) and in bleeding models in swine (42). The overall efficacy of FGN seems to depend on the underlying fluid applied for dilution. Hydroxyethyl starches, unlike crystalloid and albumin solutions, impair complete normalization of clot properties by FGN supplements (49).

FGN deficiency seems to be the first mechanism to emerge under dilution, as clearly supported by mathematical and in vivo approaches (2, 50). In more severe dilutional models of 60% and higher, a significant impact beyond clot strength impairment can be observed including deterioration of the thrombin generation potential, with prolonged clotting time (CT) in VHA, and prothrombin time (PT) and activated partial thromboplastin time (aPTT) in SLT (44). Overall, these changes during severe dilutional conditions reflect propagation from single-factor towards multifactor deficiencies under increasing dilution rates.

The efficacy of FGN substitution in severe bleeding-associated multifactor coagulopathies depends on a maintained thrombin generation potential (51), since effective fibrin polymerization starts with thrombin-mediated cleavage of the FGN molecule. Other CFC, especially 3- and 4-factor prothrombin complex concentrates (PCCs), seem to guarantee an adequate thrombin activity in this context (52). PCCs, alone or in combination with FC, are increasingly studied for treatment of multifactor coagulopathies with deteriorated thrombin generation under massive bleeding. This strategy challenges traditional indications for “plasma” transfusion. So far, a limited number of RCTs have compared CFC- against plasma-based treatment, suggesting a potential benefit for CFC-treated bleeding patients (53, 54).

Fibrinogen Sources

Three different FGN sources are available for the treatment of hypofibrinogenemic conditions under severe bleeding: FC, Cryo and FFP. FC and Cryo have substantially higher FGN concentrations than FFP (Table I), and most experts agree on the limited potential of FFP to correct hypofibrinogenemia. FCs can be administered immediately after reconstitution, and the time from indication to infusion is reported to lie between 20 and 30 minutes (43). By contrast, Cryo and FFP have to be thawed and time to transfusion can be significantly delayed with a reported median time of 60 minutes (55). Additionally, the less effective viral inactivation and a risk for blood group incompatibility raise safety concerns. Currently, no clear recommendation based on good quality evidence can be given with respect to preference of FC or Cryo for treatment of acquired hypofibrinogenemia. While Anglo-American and British guidelines recommend Cryo, most continental European Societies prefer FC (1, 3, 4), but administration of the alternative product is foreseen as acceptable if the first choice product is unavailable.

Fibrinogen concentrates

FCs are obtained from human plasma pooled from up to 60,000 donors and treated with virus inactivation/removal processes. Several products are commercially available: Haemocomplettan, marketed under the trademark RiaSTAP (CSL Behring, Marburg, Germany) in the U.S. and some other countries; Clottafact (LFB, Les Ulis, France), marketed with the trademark FibCLOT in some European countries; and Fibryga (Octapharma, Langenfeld, Germany), marketed with the trademark Fibryna in the U.S., are the best documented products (56). Other more regional products are Fibrinogen HT (Benesis, Osaka, Japan) and FibroRAAS (Shanghai RAAS, Shanghai, China). RiaSTAP is distributed worldwide, but is currently not licensed for acquired hypofibrinogenemia, which also applies to FibCLOT and Fibryna. By contrast, Haemocomplettan, Clotttafact and Fybriga include this indication for some clinical situations such as obstetric bleeding. The original indications of FCs are congenital hypofibrinogenemia, afibrinogenemia and dysfibrinogenemia.

Table I. *Comparison of different fibrinogen (FGN) sources.*

	FC*	Cryo	Plasma
FGN concentration	Standard preparation	Variable: 15-17 g/L	Variable 2-(3) g/L
Unit/volume/content	Vial/50 mL/0.9-1.3 g	Pooled bag with 5 units/ 100-150 mL/1.5-2 g	Bag/200-280 mL/0.5 g
Volume needed to administer 4 g FC	200 mL	250-300 mL	2000 mL
Coagulation factors	FGN/FXIII/fibronectin	FGN, FXIII, vWF, FVIII, fibronectin	All coagulation factors
Shelf life (conditions)	60 min (25 °C)	12 min (−20 °C)	12 min (−18 °C)
Risk for transmission of infectious agents	Extremely low	Low	Low
Need for blood group compatibility check	No	Required if large volumes transfused	Yes
Need for cold chain	No	Yes	Yes
Preparation time	5 min	10 min	30 min
Adverse events	Possibly low/minimal risk for thromboembolic complications	(Same as plasma)	TRALI, TACO, TRIM, TTI, infection, allergic reactions, blood group incompatibility
Virus removal/ inactivation	Pasteurization	(Same as plasma)	Psoralen, SD, methylene blue, quarantine

Cryo, cryoprecipitate; FC, fibrinogen concentrate; SD, solvent/detergent; TACO, transfusion-related circulatory overload; TRALI, transfusion-related acute lung injury; TRIM, transfusion-related immune modulation; TTI, transfusion-transmissible infections; vWF, von Willebrand factor.
*Data representative for Haemocomplettan.

Pharmacokinetic data for Haemocompletttan have been published for patients with congenital hypo- and afibrinogenemia (57). The median increase in plasma FGN lies in the range of 1.5-1.7 mg/dL per substituted mg of FGN per kg body weight, resulting in an in vivo recovery of 60%. Distribution volume is reported to lie between 80 and 140 mL/kg with a median half-life ($t_{½}$) of 77.1 h for FGN concentration. Differences in composition and pharmacokinetic characteristics between different FCs are described and briefly reviewed in Table II.

The dose needed to correct hypofibrinogenemia to a predefined goal is best calculated and guided by FIBTEM-MCF values. The good correlation between amplitude at 5 minutes (A5) or 10 minutes (A10) with the final MCF values allows clinicians to integrate these parameters into dose calculation resulting in shorter turnaround times (29). Dose calculations with Clauss-derived FGN concentrations are based on the published pharmacokinetic data. The time delay between sample extraction and report of results together with bleeding dynamics has to be taken into consideration when FGN dose is calculated with Clauss values.

FC seems to have a good safety profile. A pharmacovigilance report analyzing data from a 27-year period informed of only 1 adverse event per 24,600 g FC administered. Risk of bias due to the underreporting of adverse effects has to be taken into consideration, but consistent data were found in an analysis of published clinical studies on FC in bleeding scenarios (58). The infusion rate of FC should not exceed 5 mL/min, although safe administration of 1g FC in 20 seconds or up to 14 g in less than 5 minutes under emergency conditions has been reported (43).

Cryoprecipitates

Cryos are produced by controlled thawing of FFP (59). Precipitated high-molecular-weight proteins

Table II. *Pharmacokinetic data and composition of commercially available fibrinogen (FGN) concentrates (56, 57, 107-109).*

	Pharmacological preparation	Clearance (mL/h/kg)	V_{ss} (mL/kg)	IVR mg/dL per mg/kg BW	FGN AL (g/L)	Factor XIII (IU/mL)	Fibronectin (g/L)
Haemocomplettan/ RiaSTAP (CSL Behring, Germany)	1g/50 mL	0.8	77.7	1.7	23.4	7.2	3.3
Clottafact/FibCLOT (LFB, Les Ulis, France)	1.5 g/100 mL	0.6	53.5	2.2	NA	NA	NA
Fibryga/Fibryna (Octapharma, Lachen, Switzerland)	1 g/50 mL	0.6	61.0	1.8	29.5	10.1	0.19
FibroRAAS (Shanghai RAAS, China)	0.5 g/25 mL	NA	NA	NA	NA	NA	NA
Fibrinogen HT (Benesis, Osaka, Japan)	1 g/50 mL	NA	NA	NA	NA	NA	NA

AL, antigen level; BW, body weight; IVR, in vivo recovery (mg/dL per administered mg/kg BW); NA, not available; V_{ss} distribution volume at steady state.

are separated from plasma supernatant (exhausted plasma) by centrifugation, resuspended in a small volume of plasma (15-20 mL), and deep frozen for final storage at −20 °C. After thawing, Cryos should be infused immediately, although a storage time of 4-6 hours at 20-24 °C appears to be acceptable (60). Cryo is not only a rich source of FGN and von Willebrand factor, but also contains coagulation factors VIII and XIII, fibronectin, α2-antiplasmin and minimal amounts of immunoglobulins. A substantial amount of contaminating platelet membrane micro-particles and phospholipids, generated during the freezing and thawing process, possibly influences the hemostatic effects of Cryo on the coagulation mechanisms. FGN concentration in Cryo can vary between different units but usually lies between 15 and 17 g/L (61). Five units of Cryo are usually pooled in a single bag. Standard doses of 4 g FGN in bleeding conditions would correspond to a treatment dose of two pooled bags. Most of the conducted clinical studies comparing FC and Cryos for treatment of hypofibrinogenemia under severe bleeding showed no clear difference in efficacy and safety profiles (62). Thawing time, risk for viral transmission, and heterogeneous factor composition affecting coagulation beyond the role of FGN are some of the drawbacks that makes Cryos appear as a less suitable product compared with FC to control perioperative hypofibrinogenemia.

Plasma

FFP contains variable amounts of coagulation factors depending on processing, virus inactivation and donors' blood group (63). The effective FGN concentration in FFP varies from 1.0 to 3.0 g/L. Methylene blue treatment may reduce FGN levels below 2 g/L. Collins et al. (6) demonstrated that the amount of plasma rises exponentially the closer the treatment objective lies to the actual plasma FGN concentration. For instance, if the target FGN level is set at 2 g/L (in accordance to European guidelines), and the underlying plasma product is considered to contain 2 g/L, it is not feasible to effectively treat a hypofibrinogenemic condition with FFP. A further drawback for using FFP as FGN source is the timely delay caused by thawing time, which is of special importance in the emergency room for the treatment of severe trauma patients. Safety concerns and a variety of severe adverse effects, such as anaphylactic reactions, volume overload (transfusion-related circulatory overload), transfusion-related acute lung injury, etc., are further arguments against uncritical use of plasma transfusion (64).

Clinical Data from Various Critical Bleeding Conditions

Peripartum hemorrhage

Background

Preexisting coagulopathies as a primary trigger for peripartum hemorrhage (PPH) are rare. However, acquired bleeding-associated coagulopathy, especially hypofibrinogenemia < 2-2.5 g/L, is frequently responsible for bleeding propagation towards severe and massive blood loss. In placental abruption and amniotic fluid embolus, hypofibrinogenemia is seen in 100% of the affected patients (65) and eventually high FGN doses are needed (up to 18 g in extreme cases) to correct plasma FGN levels (66).

Coagulation and pregnancy

Physiological changes of the coagulation system during pregnancy lead to an overall prothrombotic state of the parturient at term. FGN is reported to increase to twice the baseline levels during pregnancy with plasma concentrations reaching up to 6 g/L (67). Accordingly, thromboelastometrical reference ranges among parturients differ from nonobstetric populations. The raised plasma FGN concentration at term seems to provide a hemostatic reserve capacity for the parturient. Among 244 patients with a mean infusion of 1329 mL (interquartile range [IQR], 900-2000 mL) of crystalloids, only a very small percentage (2%) suffered hypofibrinogenemia levels below 2g/L (68) (Table III). This hemostatic reserve appears to exhaust when FGN levels drop to the critical level of 2 g/L, leading to a significant risk for advanced hemostatic interventional procedures like embolization or hysterectomy in PPH (69). Charbit et al. (70) found a positive predictive value of 100% for severe PPH (4 RBCs transfused) for FGN levels ≤ 2.0 g/L. Consistently, FIBTEM has been shown to be an independent predictor for progression towards massive bleed > 2500 mL, with FIBTEM-A5 values < 10 mm being associated to more prolonged bleeds and longer intensive care unit (ICU) stays (71). Collins et al. suggested in their RCT OBS2 that FGN replacement is not required if the FIBTEM-A5 is > 12 mm or Clauss FGN > 2 g/L. Beneficial effects below these levels were not excluded (72).

Clinical data on fibrinogen treatment of PPH

Pre-emptive FGN administration has been analyzed in different trials. In a double-blinded, multicenter RCT on 249 parturients, no beneficial effect of pre-emptive FC (26 mg/kg) could be demonstrated (68). This study, however, has been criticized as FGN supplements were indicated by an estimated blood loss of 1500 mL, and not by critical low FGN levels.

A multicenter, randomized, double-blind, placebo-controlled trial conducted by Collins et al. also failed to prove FC benefit on blood loss and transfusion rates in 55 randomized subjects with ongoing PPH and FIBTEM-A5 values ≤ 15 mm. In this study the number of treated women with Clauss plasma FGN levels < 2 g/L was also very low (a total of 7 patients), leading to some controversy over the applied inclusion criteria (72).

An observational, nonrandomized, open-label study in parturients with ongoing PPH (defined as blood loss > 1500 mL) demonstrated a significant reduction of transfusion requirements in coagulopathic parturients (defined as FIBTEM-A5 < 7 mm or FIBTEM-A5 7-12 mm in ongoing or high risk of hemorrhage). The study compared FC treatment (3 g) to management with formulaic allogeneic blood transfusion in the form of shock packs (66) (Table III). The overall incidence of PPH was reported with 2.7% of all deliveries over a 4-year observation period. Among these parturients with severe PPH, hypofibrinogenemia (FIBTEM-A5 ≤ 12 mm) was diagnosed in about 25%.

Conclusion

The currently existing RCTs in the field of PPH include only a very small number of patients suffering from severe PPH with simultaneously diagnosed hypofibrinogenemia. Consequently, little information is provided on the effects of FC treatment in clinical circumstances where the highest benefit could be expected. On the basis of the available RCTs, current guidelines on PPH recommend against pre-emptive FC treatment and in favor of goal-directed, POC (e.g., FIBTEM-A5 < 12 mm) treatment (24) (Table IV). FGN replacement can equally be based on Cryo or FC (24). FFP transfusion, although still controversially discussed, should not be performed for hypofibrinogenemia, and should be reserved for multifactorial

Table III. *Clinical studies of fibrinogen (FGN) in peripartum hemorrhage (PPH) and trauma.*

Study	Study type	Sample size/ intervention	Interventional trigger	FGN dose	Comparator	Outcome on transfusion rates	TEE/adverse events	Comment
PPH								
Wikkelsø 2015 (68)	Pre-emptive RCT	244 obstetric patients	Pre-emptive	2 g	Placebo	No difference	No	Severe and rapid PPH not included
Mallaiah 2015 (110)	Interventional prosp CT	93, presenting PPH	FIBTEM-A5 ≤ 12 mm	IQR 0-12 g	Shock packs	Significant reduction	No	Two-phase study, historic comparator
Collins 2017 (72)	Interventional RCT	55, presenting PPH	FIBTEM-A5 < 15 mm + continued bleeding	3-4 g	Placebo	No difference	1/1 TG/CG	Possible benefit below A5 < 12 mm
McNamara 2019 (66)	Interventional prosp CT	110 PPH with FC ttm	FIBTEM-A5 ≤ 12 mm	IQR 0-18 g	Shock packs	Significant reduction	No data	Extension study to Mallaiah 2015
Trauma								
Innerhofer 2013 (111)	Prosp cohort study	144, trauma	FIBTEM-MCF < 7 mm FGN < 150-200 mg/dL	4 g FC	FFP	Significant reduction	No data	4-factor PCC allowed in TG and CG
Nascimento 2016 (43)	Interventional RCT	50, trauma	Hypotension and RBC transfusion	6 g FC	Placebo	No difference	No difference TG vs. CG	Early infusion of FC is feasible
Innerhofer 2017 (53)	Interventional RCT	100, trauma	FIBTEM-A10 < 9 mm	8 g	FFP	Significant reduction	No difference TG vs. CG	4-factor PCC allowed in TG
Akbari 2018 (82)	Interventional RCT	90, trauma	FGN level < 2 g/L	2 g	1. FFP 2. Control	Significant reduction	No	Risk of bias
Curry 2018 (80)	Interventional RCT	39, trauma	Activation of MTP	6 g	Placebo	No difference	No difference TG vs. CG	Early FC administration within 45 min not feasible
Ziegler 2020 (81)	Interventional RCT	67, trauma	Major bleeding, need for volume replacement	1.5 g per 30 kg BW	Placebo	No difference	No difference	Preclinical FC administration feasible

BW, body weigh; CG, control group; FC, fibrinogen concentrate; FFP, frozen plasma; IQR, interquartile range; MTP, massive transfusion protocol; PCC, prothrombin complex concentrate; prosp, prospective; RBC, red blood cell; RCT, randomized clinical trial; TEE, thromboembolic events; TG, treatment group; ttm, treatment.

Table IV. *Guideline recommendations on fibrinogen (FGN) treatment in perioperative bleeding.*

	FC in AHF*: LoR	Pre-emptive FC	Ttm threshold	VHA monitoring: LoR	First-line FGN source	Cost effectiveness
PPH	- Strong[1,4] - Recom[3]	Strongly against[1]	- 2 g/h[4,5] - FIBTEM-MCF 12 mm[4,5,9]	- Moderate[1] - Strong[4] - Recom[3,5]	- FC[4] - Cryo[3,4]	NA
Trauma	- Strong[6] - Recom[3]	NA	- 1.5-2 g/L[1,6] - 1.5 g/L[3]	- Strong[6] - Recom[3]	- FC[6] - Cryo[3]	Yes[1]
Cardiac surgery	- May be considered[2] - Strong[1] - Recom[3]	Not recom[2]	1.5 g/L[1,2,3]	- Strong[1] - Recom[3]	- FC[1] - Cryo[3]	Yes[1]
Liver transpl	- Recom[1]	NA	1.5 g/L[1]	- Recom[1]	- FC[1]	NA

AHF, acquired hypofibrinogenemia; Cryo, cryoprecipitate; FC, fibrinogen concentrate; LoR, level of recommendation; NA, not applicable; recom, recommended; transpl, transplantation; Ttm, treatment; VHA, viscoelastic hemostatic assay.
*If bleeding is present.
[1]Kozeck-Langenecker et al. 2017 (3); [2]Pagano et al. 2018 (4); [3]Khanna and Bhatt 2018 (112); [4]Muñoz et al. 2019 (24); [5]Collins et al. 2016 (113); [6]Spahn et al. 2019 (1).

coagulopathies, as expressed by prolonged aPTT, PT in SLT or CT in VHA (24).

Published results of one ongoing trial are still pending at the moment of publication of this review (73).

Severe bleeding in trauma patients

Background

Trauma is the leading cause of death in people aged 18-44 years, and patients with comparable injury patterns suffer higher rates of fatal outcome if coagulopathy develops (74). Shock and hypoperfusion are the principal triggers of the complex mechanisms of trauma-induced coagulopathy (TIC), with activation of the protein C pathway and endothelial tissue plasminogen activator (tPA) release resulting in a hypocoagulable and hyperfibrinolytic state with a high risk of bleeding (40). TIC-associated hyperfibrinolysis and dilutional effects from prehospital fluid resuscitation cause a high incidence of FGN depletion below critical thresholds which relates to higher mortality (75). A total of 73% of patients with Hb levels < 10 g/L at hospital admission have FGN levels below the critical threshold of 1.5 g/L (76).

Monitoring

Reduced clot strength in FIBTEM subtests has shown to be a strong predictor for massive transfusion (FIBTEM-A10/MCF 4 mm/7 mm) and mortality (FIBTEM-MCF 7 mm) in the initial phase of trauma (77). There is preliminary evidence that VHA monitoring combined with goal-directed therapy might reduce transfusion rates and overall exposure to allogeneic blood products in trauma bleeding (78). As mentioned previously, the recent international RCT iTACTIC could show VHA benefit only for a subgroup population with traumatic brain injury (27).

Evidence from clinical studies

FGN replacement efficiently corrects alterations of FGN plasma levels and deteriorated clot strength in functional VHA assays (79).

Feasibility studies analyzing the fast availability of FC/Cryo (E-FIT 1/Cryostat 1) showed a shorter median time from hospital admission to treatment for FC (39 minutes) than for Cryo (60 minutes) (55, 80). Both FC and Cryo seem to reliably raise plasma FGN levels (43, 81). This has been also confirmed by the more recently published FlinTIC study, which has shown preclinical FC administration to be safe and feasible and to protect against early FGN depletion (81).

Benefit from FGN replacement approaches on survival and transfusion rates was demonstrated in several military, civilian, prehospital and intrahospital

settings (53, 78, 82, 83). In a retrospective study, civilian patients treated with FC as a first-line treatment showed significantly reduced mortality compared with score prediction (mortality of 24.4% vs. calculated mortality by TRISS of 33.7%, $P = 0.032$) (83). The same group reported reduced overall transfusion rates under VHA-guided FC/PCC treatment (78), with 29% of patients free from any transfusion in the CFC arm versus only 3% in the FFP study arm.

The highly considered RETIC study published by Innerhofer et al. compared CFC versus FFP in managing bleeding trauma patients. Bleeding signs of severely injured trauma patients with plasma coagulopathy assessed by ROTEM were the primary eligibility criteria. After study inclusion, patients were assigned to treatment with 15 mL/kg FFP or 50 mg/kg FC as first-line CFC treatment. The study was disrupted prematurely owing to safety concerns after 100 patients were included due to a high rate of rescue therapy necessary in the FFP group (52% vs. 4%, $P < 0.0001$), increased massive transfusion rates (30% vs. 12%; $P = 0.042$), and a tendency toward higher multiple organ failure (66% vs. 50%, $P = 0.15$) and venous thrombosis rates (18% vs. 8%; $P = 0.22$) (53).

Two recently published meta-analyses raised concerns about the efficacy and safety profile of FGN replacement in trauma patients by reporting a 2-fold risk of sustained thromboembolic complications compared with patients who did not receive FC (84, 85). Of note, the results of the RETIC study were not included in the final analysis as the direct, FC-associated thromboembolic event risk could not be evaluated. The overall quality of the evidence of the included studies was low and a high risk of bias was highlighted by the authors. This relevant risk of bias when interpreting current available data in the field of trauma is also well reflected in another meta-analysis on this topic (86). Several clinical trials are currently ongoing which will provide further insight into the efficacy and safety profile of FGN replacement in trauma patients (ClinicalTrials.gov Identifier NCT02864875, NCT02745041, Cryostat 2). The unpublished FEISTY study (NCT02745041), which reports a completed recruitment status, could provide new insights into the comparison between FC and Cryo in bleeding trauma patients.

High-ratio plasma transfusion versus algorithm-based concentrate transfusion approaches

Most protocols for massive transfusion in trauma and other high-risk situations suggest hemostatic resuscitation by high-ratio transfusion (1:1 to 1:2) of FFP:RBC to counteract hemorrhagic shock, and to conserve the hemostatic potential by stabilizing the plasma coagulation factor activity (87). However, the potential to fully prevent coagulopathy of damage control resuscitation based on blood component therapy is limited. Algorithm-based, goal-directed hemostatic resuscitation has emerged as an alternative to aggressive FFP transfusion concepts with compelling results in studies comparing these treatment concepts to ratio-driven FFP transfusion (53, 78).

Conclusion

Current guidelines on the management of bleeding trauma patients recommend hemostatic resuscitation based on VHA-guided and goal-directed FC or Cryo treatment (Table IV). The iTACTIC results, currently providing the best-quality evidence on this topic, will have to be integrated into future guideline versions. FGN replacement is indicated as per pathological viscoelastic FGN assays or corresponding Clauss levels below thresholds of 1.5-2 g/L. These treatment thresholds for trauma, suggested by the European guidelines on management of major bleeding following trauma, are currently based on expert consensus (1, 3). Well-defined thresholds from large prospective RCTs as provided for PPH or cardiac surgery are still lacking for the clinical setting of trauma-associated bleeding (72).

Excessive bleeding during cardiac surgery

Background

Onset of coagulopathy in cardiac surgery is a complex interplay of patient- and cardiopulmonary bypass (CPB)-related factors. Intraoperative and postoperative plasma FGN levels falling below thresholds of 1.5-2.2 g/L are associated with more severe blood loss (88, 89). Ranucci et al. reported a negative predictive value of 100% and 98% for plasma FGN levels of 2.87 g/L and FIBTEM-MCF of 14 mm, respectively. Some controversy is still ongoing about the positive predictive value of FGN, with

a considerable risk of overtreatment if pre-emptive strategies were applied (90).

Monitoring

A relevant number of clinical trials on the efficiency of VHA monitoring in surgical procedures are summarized in a recently published meta-analysis, including data of more than 8,000 patients; 16 of 21 RCTs included in this meta-analysis have been performed in a cardiac surgery setting (26). The investigators found a significant reduction in mortality, which was also confirmed for a subgroup analysis for coagulopathic patients after cardiopulmonary bypass.

Evidence from clinical studies

Pre-emptive effects of FGN supplements, as in other clinical entities, could not show a significant reduction in transfusion rates as demonstrated by several RCTs (91-93). As in studies from other high-risk areas (68), these trials included a substantial number of individuals whose FGN levels lay significantly above the established thresholds related to bleeding (> 2 g/L). Eligibility criteria increasing the number of patients at "real" risk for hypofibrinogenemia-induced bleeding applying VHA-guided treatment algorithms could possibly result in more significant effects of FGN supplements.

A large number of trials support the hypothesis that interventional FGN replacement significantly reduces the transfusion rates of allogeneic blood components (54, 94-96) (Table V).

However, the recently conducted large-scale trials, designed to provide final conclusions concerning the efficiency of FGN replacement, could not confirm a significant reduction of exposure to allogeneic products (97, 98). The REPLACE study reported surprising results with higher transfusion rates in the FGN treatment arm. Again, the applied inclusion criteria led to recruitment of patients missing strict criteria for hypofibrinogenemia and the interventional trigger was not POC-guided. Instead, FGN supplements were indicated by 5-minute bleeding mass, which does not necessarily reflect an indication for FGN supplements. Low adherence to the study protocol was also commented by the authors.

Conclusion

Current European guidelines on bleeding management in cardiac surgery recommend against FGN administration as a prophylactic hemostatic agent (3, 4) (Table IV). Assessment of bleeding risk by analysis of preoperative FGN concentrations may be considered. If FGN levels fall below 1.5 g/L FC administration can be considered. Indication for FGN treatment should be based on VHA monitoring if available.

Other high-risk bleeding surgeries

Further evidence is provided by clinical studies from other high-risk surgery. In liver transplantation, FC supplements are indicated if hypofibrinogenemia with a plasma concentrations of < 1.5 g/L (corresponding to FIBTEM-MCF levels < 8 mm) are diagnosed, with a weak recommendation given by the European guidelines on bleeding management (3). The combination of POC monitoring and goal-directed intervention with CFCs has been shown to significantly reduce transfusion rates, with a significant increase in FC administration (99). Pre-emptive strategies, however, as in many other high-risk settings, could not improve transfusion rates (100). These findings have been also confirmed in a study performed in patients submitted to transurethral prostatectomy showing no additional benefit of pre-emptive supplements of 2 g FC (101). In cystectomy patients with dilutional coagulopathy, expressed by deteriorated clot firmness under VAH monitoring, FC treatment significantly improves the measured clot firmness and leads to reduction of transfusion requirement (102).

Clinical evidence from other high-risk surgeries confirms the results obtained from previous trials in different clinical entities demonstrating a negligible impact of pre-emptive strategies, in contrast to effective interventions under VAH monitoring and guided treatment of established hypofibrinogenemic conditions.

Cost Effectiveness

FC prices in most countries are substantially higher than those for Cryo (price FC:Cryo = 4:1); however, the overall difference after including costs for blood

Table V. *Clinical studies of fibrinogen (FGN) in cardiac surgery and other high-risk surgery.*

Study	Study type	Sample size/ intervention	Interventional trigger	FGN dose	Comparator	Outcome on transfusion rates	TEE/adverse events	Comment
Cardiac surgery								
Rahe-Meyer 2009 (94)	Interventional RCT	15, AVR 42, retrosp.	5-min bleeding mass ≥ 60 g	5.7 g	Transfusion algorithm	Significant reduction	No	Investigators aimed at FIBTEM 22 mm
Karlsson 2009 (93)	Pre-emptive RCT	20, CABG	Pre-emptive	2 g	No infusion	No difference	2 cases TG	CG maintained FGN level > 2 g/L
Rahe-Meyer 2013 (95)	Interventional RCT	61, aortic surgery	5-min bleeding mass > 60 g	8 g	Placebo	Significant reduction	No	Investigators aimed at FIBTEM 22 mm
Sadeghi 2014 (92)	Pre-emptive RCT	60, CABG	Pre-emptive	1 g	Placebo	No difference	No	FGN > 2 g/L in both arms
Tanaka 2014 (96)	Interventional RCT	20, valve replacement	Bleeding scale	4 g	Apheresis platelets 1 U	Significantly fewer platelets	No	FGN > 1.65 g/L in CG (FIBTEM-MCF > 9 mm)
Ranucci 2015 (54)	Interventional RCT	116, mixed CS with CPB	FIBTEM-MCF < 22 mm	4 g	Placebo	Significant reduction	No	PCC allowed if clotting time prolonged > 80 s
Jeppsson 2016 (91)	Pre-emptive RCT	48, CABG patients	Pre-emptive	2 g	Placebo	No difference	No	CG maintained FGN level > 2g/L
Bilecen 2017 (97)	Interventional RCT	120, elective high-risk CS	5-min bleeding volume > 60 mL	3.1 g	Placebo	No significant difference	7/3 TG/CG	Primary outcome: intraoperative blood loss
Rahe-Meyer 2016 (98)	Interventional RCT	152, aortic surgery	5-min bleeding mass > 60 g	6.9 g	Placebo	Significant increase	No difference TG vs. CG	High FGN levels in pretreatment analysis
Morrison 2019 (114)	Interventional RCT	20, aortic aneurysm	FIBTEM-A10 < 8 mm	8.5 g	FFP	Significant reduction	1/1 TG/CG	Included abdominal aortic surgery
Kwapisz 2020 (115)	Pre-emptive RCT	62, elective high-risk CS	Pre-emptive	3.2 g	Placebo	No difference	No difference TG vs. CG	FGN 1.83 g/L in CG at end of surgery
High-risk surgery								
Fenger-Eriksen 2009 (102)	Interventional RCT	20, radical cystectomy	30% dilution	45 mg/kg	Placebo	Significant reduction	No data	FC improves MCF
Lancé 2012 (116)	Interventional RCT	43, mixed CVS, AS, OS	Bleeding scale	2 g	FFP	No differences	No	TG received also FFP
Najafi 2014 (117)	Pre-emptive RCT	30, hip surgery	Pre-emptive	30 mg/kg	Placebo	No differences	No	-

(Continued)

Table V. *Clinical studies of fibrinogen (FGN) in cardiac surgery and other high-risk surgery. (Cont.)*

Study	Study type	Sample size/ intervention	Interventional trigger	FGN dose	Comparator	Outcome on transfusion rates	TEE/adverse events	Comment
Sabaté 2016 (100)	Pre-emptive RCT	99, LT	Pre-emptive	3.54 g	Placebo	No differences	1/5 TG/CG	FC ttm in both groups if plasma concentration <1 g/L
Soleimani 2017 (101)	Pre-emptive RCT	60 TUR-P	Pre-emptive	2 g	Placebo	No differences	No	Preoperative FGN significantly lower in TG

AS, abdominal surgery; AVR, aortic valve replacement; CABG, coronary aortic bypass graft; CG, control group; CPB, cardiopulmonary bypass; CS, cardiac surgery; CVS, cardiovascular surgery; FC, fibrinogen concentrate; FFP, frozen plasma; LT, liver transplantation; MCF, maximum clot firmness; OS, orthopedic surgery; PCC, prothrombin complex concentrate; retrosp, retrospective; RCT, randomized clinical trial; TEE, thromboembolic events; TG, treatment group; ttm, treatment; TUR-P, transurethral resection of the prostate.

group compatibility testing, thawing and administration is less significant (price FC:Cryo = 1.5:1) (103). Similar data were presented from an economical simulation model: applying all costs, including Cryo wastage and technicians, the calculated price was USD 414/g FGN for Cryo and USD 740/g FGN for FC (104).

Several studies analyzed the cost effectiveness of VHA-guided algorithms integrating goal-directed, pharmacological interventions with FC and other CFCs (PCCs, FXIII). A meta-analysis concluded cost effectiveness of VHA-based strategies for cardiac surgery and trauma patients (7). In the majority of the reviewed studies, implementation of VHA monitoring led to a significant decrease in allogeneic blood component transfusion accompanied by a substantial increase in consumption of FC, with an overall cost-effective balance. The evidence on this topic has been reinforced by further publications following this meta-analysis (8, 9).

In a retrospective study on visceral surgery and liver transplantation, FFP and platelet transfusion decreased by 89% and 58%, respectively, with an FC consumption increasing from 68 to 745 g per year. The overall cost savings on allogeneic blood products including costs on consumed CFCs plus ROTEM machines and analysis was calculated to be in excess of 250,000 Euros in a German tertiary university hospital (total cost reduction of 36%). Similar data were reported by Spalding et al. on cardiac surgery patients. ROTEM introduction in their hospital resulted in a 2-fold increase of FC consumption, with significant reduction of RBC and platelet transfusion rates (105). The calculated average monthly savings lay at 50,000 euros in a German cardiac surgery center.

Conclusions

Most guidelines on the management of massive bleeding recommend FGN administration triggered by the established treatment thresholds of 1.5 g/L for active, nonobstetric bleeding and 2 g/L for active, obstetric bleeding. Institutional cut-off values for viscoelastic testing of functional FGN should be established and integrated into center- and setting-specific treatment algorithms (29). FIBTEM-MCF values of 8 mm and FF-TEG values of 12 mm would most likely predict the critical plasma FGN concentration of 1.5 g/L. FIBTEM-A5 values of 12 mm indicate critical levels for PPH.

The currently available data from clinical studies provide good evidence to recommend against pre-emptive FC administration for most clinical situations. There is still a significant shortage of large, multicenter, high-quality RCTs that can clearly demonstrate FC-associated reduction in transfusion rates or even mortality (Tables III/V). Inconsistent results between clinical trials are partially explained by inclusion criteria leading to a patient selection with bleeding events that were not necessarily related to critical FGN plasma levels. The main potential of FCs, however, is to be expected in bleeding situations associated to FGN levels below established, critical cut-off values. A huge body of scientific data from retrospective and observational studies clearly suggests that hypofibrinogenemic conditions in actively bleeding patients significantly increase the risk for massive transfusion and higher mortality, and should therefore be adequately treated. Therefore, most experts agree on the concept of threshold-guided treatment necessity. Some evidence from RCTs and observational studies suggests that plasma transfusion, especially within the framework of fixed component ratios, can be safely substituted by CFCs, guided by POC monitoring with a beneficial impact on transfusion rates and mortality (53, 54, 66). In the last 2 decades, FCs have been incorporated into institutional protocols for massive bleeding, leading to a significant rise in the overall FC consumption for these indications (106). Future studies with a more rigorous focus on patients with massive bleeding and simultaneous hypofibrinogenemia, established by timely VHA testing, and aiming to reduce plasma transfusion might reinforce the evidence derived from RCTs on the therapeutic benefits resulting from FGN replacement.

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Conclusions

- Acquired hypofibrinogenaemia under perioperative bleeding is associated with a significant increase in the risk for massive transfusion and higher mortality
- Fibrinogen substitution should be indicated by thresholds related to higher bleeding risk.
- Monitoring of fibrinogen concentration with viscoelastic POC systems is cost-effective, reduces transfusion rates, and has beneficial effects on the overall outcome.
- Fibrinogen Concentrates should be preferred to plasma transfusion for single-factor coagulopathies
- Plasma transfusion for multifactor deficiencies can be safely substituted by CFCs, guided by POC monitoring with a possibly beneficial impact on transfusion rates and mortality.

Normalization of blood clotting characteristics using prothrombin complex concentrate, fibrinogen and FXIII in an albumin based fluid: experimental studies in thromboelastometry

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SUMMARY:

Colloid fluids supplemented with adequate combinations of CFCs with the capability to restore coagulation could be a desirable future treatment component in massive transfusion. Such fluids with the potential to preserve basic coagulation mechanisms are referred to as CRF.

Starting from a coagulation factor and blood cell-free albumin solution we added PCC, FC and FXIIIC in different combinations and concentrations to analyse their properties to restore TEM parameters without the use of plasma. Further analysis under the presence of platelets was performed for comparability to whole blood conditions.

We could demonstrate that plasma-free albumin solutions enriched with PCC and FC show restoring coagulation potential. In a first step we could demonstrate that PCC showed sufficient thrombin formation for inducing FGN polymerization after extrinsic activation of coagulation. The combination of PCC and FC in a plasma-free albumin carrier solution led to the formation of a stable *in vitro* fibrin clot.

In a second step we analysed the effects of different combinations and concentrations of three different coagulation factor concentrates (PCC, FC, FXIII). In a final experiment the effects of different platelet concentations was tested. These tests were equally performed in a plasma-free albumin-based carrier solution.

We found that FGN and FXIII have an excellent capacity to improve fibrin clot firmness expressed as Amplitude at 10 min and Maximal Clot Firmness. FGN alone, or in combination with FXIII, was able to restore normal Amplitude at 10 min and Maximal Clot Firmness values. In the presence of platelets, the TEM surrogate parameter for thrombin generation (Clotting Time) improves and normalizes when compared to whole blood. Under optimized concentrations of PCC; FC, FXIII and platelets, as determined in different dilutional series, all clinically relevant ROTEM parameters were within the normal range for human whole blood.

We conclude from our findings that combinations of CFCs suspended in albumin solutions can restore basic coagulation mechanisms as expressed by TEM parameters in the absence of plasma. This kind of artificial colloid fluids with coagulation-restoring characteristics might offer new treatment alternatives for hypovolemic patients with massive bleeding and ongoing dilutional coagulopathy.

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ORIGINAL RESEARCH Open Access

Normalization of blood clotting characteristics using prothrombin complex concentrate, fibrinogen and FXIII in an albumin based fluid: experimental studies in thromboelastometry

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Abstract

Background: Colloid fluids supplemented with adequate combinations of coagulation factor concentrates with the capability to restore coagulation could be a desirable future treatment component in massive transfusion.

Methods: Starting from a coagulation factor and blood cell-free albumin solution we added Prothrombin Complex Concentrate, Fibrinogen Concentrate and Factor XIII in different combinations and concentrations to analyze their properties to restore thromboelastometry parameters without the use of plasma. Further analysis under the presence of platelets was performed for comparability to whole blood conditions.

Results: Albumin solutions enriched with Fibrinogen Concentrate, Factor XIII and Prothrombin Complex Concentrate at optimized concentrations show restoring coagulation potential. Prothrombin Complex Concentrate showed sufficient thrombin formation for inducing fibrinogen polymerization. The combination of Prothrombin Complex Concentrate and Fibrinogen Concentrate led to the formation of a stable in vitro fibrin clot. Fibrinogen and Factor XIII showed excellent capacity to improve fibrin clot firmness expressed as Amplitude at 10 min and Maximal Clot Firmness. Fibrinogen alone, or in combination with Factor XIII, was able to restore normal Amplitude at 10 min and Maximal Clot Firmness values. In the presence of platelets, the thromboelastometry surrogate parameter for thrombin generation (Clotting Time) improves and normalizes when compared to whole blood.

Conclusions: Combinations of coagulation factor concentrates suspended in albumin solutions can restore thromboelastometry parameters in the absence of plasma. This kind of artificial colloid fluids with coagulation-restoring characteristics might offer new treatment alternatives for massive transfusion.

(Continued on next page)

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Trial registration: Study registered at the institutional ethic committee "Institut de Recerca, Hospital Santa Creu i Sant Pau, with protocol number IIBSP-CFC-2013-165.

Keywords: Massive bleeding, Blood transfusion, Fibrinogen, Factor XIII, Prothrombin complex concentrate, Fluid therapy, Hemostatic resuscitation, Plasma substitutes

Background

Massive bleeding can cause severe intravascular hypovolemia with significant hypoperfusion of peripheral tissues leading to life-threatening hemorrhagic shock. The correction of the underlying hypovolemic state with conventional resuscitation fluids like crystalloid or colloid solutions contributes to bleeding associated coagulopathies by dilution of plasma coagulation factors [1]. Deterioration of physical clot strength and, in a later stage of prolonged bleeding, reduced thrombin generation potential are the principal coagulopathic patterns found when monitored by viscoelastic testing [2]. Viscoelastic testing, like rotational thromboelastometry (TEM), is frequently used as a point-of-care tool in severe bleeding, providing comprehensive information about the viscoelastic and temporal characteristics of blood clot formation [3]. Thereby identified coagulopathies caused by single- or combined coagulation factor deficits are increasingly treated with coagulation factor concentrates (CFC), like Fibrinogen Concentrate (FC), Prothrombin Complex Concentrate (PCC) and Factor XIII Concentrate (FXIIIC) [4–6]. The principle goal of CFC-based treatment strategies is to reduce transfusion rates of allogeneic blood products and adverse events associated with plasma transfusion [7]. There is growing scientific evidence that coagulation factor deficiencies in bleeding patients can be effectively and safely treated with CFCs with some studies showing better outcomes for CFCs when compared to plasma transfusion [8–10]. By contrast there is a certain paucity of high-quality evidence in favor of plasma, although plasma transfusion is still a widely accepted standard of trauma and non-trauma massive transfusion protocols [11]. The high acceptance of plasma transfusion among many physicians might be explained, not only by its stabilizing effect on the coagulation system providing a close-to-physiological factor composition but also by its resuscitation fluid quality with good intravascular volume effects in patients with hemorrhagic shock. Currently, no alternative products are available which share these two characteristics with human plasma. Therefore, colloid fluids providing adequate intravascular volume effects combined with maintained hemostatic properties could be an interesting future treatment component in massive transfusion and damage control resuscitation, helping to reduce plasma transfusion and its associated side effects under maintenance of the stabilizing effects on coagulation and hemodynamics.

We hypothesized, that an optimized and well-balanced combination of different coagulation factors, reconstituted in an albumin-based carrier solution, would provide basic clotting characteristics with TEM responses compatible with whole blood if tested under the presence of platelets.

In this study, we used a thromboelastometric approach to analyze modifications of viscoelastic parameters in a plasma-, and blood cell-free environment. It is technically feasible to perform thromboelastometric analysis in such conditions, although this approach has been limited to its use in laboratory investigations [12, 13].

Methods

Experimental design

This study (protocol number IIBSP-CFC-2013-165) was designed to explore in vitro the capability of CFCs to restore coagulation properties. A series of experimental studies were performed to define the optimal factor concentrations of such coagulation resuscitating fluid (CRF). Starting from a coagulation factor and blood cell-free solution of 5% human albumin we added PCC, FC and FXIIIC in different combinations and concentrations to analyze their properties to restore thromboelastometric parameters without the use of plasma. The optimal CFC composition was further analyzed under the presence of platelets to improve comparability to whole blood conditions.

All coagulation factors – fibrinogen (FGN), factor XIII (FXIII) and prothrombin complex (PC) factors II, VII, IX and X - were derived from commercial CFCs (FC, FXIIIC and PCC). The optimal concentration of FGN and FXIII in CRF was determined by direct comparison to clot firmness parameters of plasma from an internal control group. The optimal concentration of PC coagulation factors was determined by the shortest obtained clotting time (CT) value. CRF was considered as having a coagulation restoring potential to allow consideration as plasma substitute, if thromboelastometric responses of the final CRF composition lay within the normal range for whole blood when tested in presence of platelets [14].

Plasma reference values for thromboelastometry parameters

TEM parameters were determined from plasma from healthy volunteers to define the reference range for

clotting time (CT), the amplitude at 10 min (A10) and maximum clot firmness (MCF) under blood cell-free conditions. For this purpose, we collected blood samples from 33 healthy volunteers who had not taken medication affecting coagulation- or platelet-function in the last 10 days. A sample of 4.5 ml of blood was drawn from each donor in citrated tubes (0.12^9 M) and centrifuged at 3200 rpm for 25 min. Plasma supernatant was used for FIBTEM analysis. FIBTEM was used to minimize viscoelastic signals associated with residual platelets after centrifugation. The 95% confidence interval of the obtained values was defined as the reference range for plasma. The established reference ranges were later used for comparison with TEM responses of CRF samples to determine the optimized CRF factor concentration.

Study samples

Composition of study samples

Study samples were composed of an artificial fluid solution (AFS) based on 5% human albumin together with different CFC combinations and concentrations. 20% human Albumin (Grifols®, Spain) was diluted with an isotonic, balanced, crystalloid solution (Viaflo Plasmalyte® 148, Baxter, Spain) to a final albumin concentration of 5% correspondent to high physiologic plasma concentrations for albumin. Calcium gluconate was added to achieve a physiological free ionized calcium concentration of 1.0–1.2 mmol/l. The solution was buffered with TRIS buffer (1 M) to a physiological pH range between 7.36–7.45. Electrolyte concentrations and pH were measured on the blood gas analyzer Radiometer®ABL 90 Flex to confirm the physiological composition of our stem solution.

All factor concentrates were provided by CSL Behring. The different coagulation factor concentrates (CFC) were reconstituted in stock solutions. PCC (Beriplex, CSL Behring GmbH, Germany), FC (Riastap, CSL Behring GmbH, Germany) and FXIIIC (Fibrogammin/Cluvot, CSL Behring GmbH, Germany) were used as CFCs for this in vitro analysis. The lyophilized proteins were reconstituted with the minimum amount of the accompanied provider's solution necessary for protein dissolving, resulting in final concentrations of 0.025 IU/µl for factor IX (FIX) as reference protein in PCC, 0.1 mg/µl for FGN, and 0.05 IU/µl for FXIII. High final protein concentrations in the stock solutions were necessary to avoid dilutional effects during the preparation of the final study samples. The stock solution was directly used or stored at − 70 °C for later use. The final composition of AFS free of proteins and blood cells was tested as a negative control with EXTEM and FIBTEM subtests as described in the thromboelastometry section.

Preparation of study samples

Aliquots of the stock solution containing coagulation factors were added to the AFS within citrated tubes to reach the defined final factor concentration. The study samples were warmed to 37 °C in the provided warming chamber of the ROTEM® machine before testing.

Variable CFC concentrations and platelet count

The effect of various combinations of CFC-derived coagulation factors suspended in AFS on their functional contribution to clot formation was evaluated by TEM. The study samples were distributed in different test series. Within the same test series the protein concentration of only one component (FGN, FXIII or PC), or platelet count, was gradually modified while the concentration of the other components was left unchanged. The protein concentration of PC used in our in vitro-experiments is provided as IU/ml referring to the underlying factor IX activity, being the PC reference protein. Three main series of tests were performed combining different CFC-derived proteins added to the AFS:

a) Increasing PC concentrations (0.05, 0.1, 0.25, 0.5, 1, 2 and 4 IU/ml) over a fixed FGN concentration of 4 g/l; b) Increasing FGN concentrations (0.5, 1, 2, 4, 8 and 12 g/l) over a fixed PC concentration of 1 IU/ml; c) Increasing FXIII concentrations (0.1, 0.5, 1, 2, 4 and 8 IU/ml) over a fixed PC concentration of 1 IU/ml and a fixed FGN concentration of 4 g/l. Each concentration step of the changing factor component defined one study sample that was tested by TEM. The study samples were analyzed for viscoelastic properties with the FIBTEM-S subtest. A TEM response within the defined reference values (Table 1) for A10 and MCF derived from internal controls determined the optimal FGN and FXIII composition of the CRF. Combined FGN/FXIII preparations with TEM responses within the normal range for A10/MCF were given priority to single factor preparations (FGN alone) for defining the final CRF composition. The shortest CT value obtained in a series with increasing PC concentrations defined the final PC concentration of CRF.

In a fourth test series the effect of platelets on samples containing the final CRF composition of PC (1 IU/ml), FGN (4 g/l) and FXIII (1 IU/ml) was evaluated adding an increasing number of washed platelets (12.5, 25, 50, 100, 200 and 400 platelets 10^3/µl) obtained from healthy, non-medicated donors. The samples were analyzed with the FIBTEM-S and EXTEM-S subtests.

Preparation of platelet suspensions

Blood was collected into citrate/phosphate/dextrose (final concentration of citrate of 19 mM) and centrifuged (120 x g for 15 min) to obtain platelet-rich plasma. Washed platelets were obtained by mixing PRP with

Table 1 Reference ranges for standard TEM parameters CT, A10, MCF. Reference ranges are presented for plasma and whole blood. Plasma ranges are derived from an internal control group. Values from an external control group from previous studies are also highlighted for comparison. Whole blood reference ranges are shown for comparison with TEM results of CRF under presence of platelets. The recommended treatment thresholds are presented to provide a clinical context of the obtained TEM results

Reference ranges	Plasma (Internal control)[a]	Plasma (External control)[b]	Whole blood[c]	Treatment threshold[d]
CT (*sec*)	47–54	53–71	42–74	> 80
A10 (*mm*)	17–24	n.a.	43–65	< 7
MCF (*mm*)	18–26	17–35	49–71	< 14

[a] Values derived from fresh plasma from 33 healthy, non-medicated volunteers. Reference range corresponds to 95% confidence interval
[b] Values derived from fresh plasma as published by Schörgenhofer et al. [13]
[c] Values derived from whole blood as published by Lang et al. [14]
[d] Treatment thresholds for whole blood EXTEM-CT and whole blood FIBTEM-A10/MCF as published by Schöchl and Schlimp [15]. and Ranucci et al. [16]

equal volumes of citrate/acid citric/dextrose (93 mM sodium citrate, 7 mM acid citric, and 140 mM dextrose), pH 6.5 containing 5 mM adenosine and 3 mM theophylline (CCD−AT) [17]. The final pellet was resuspended in a Hanks' balanced salt solution (136.8 mM NaCl, 5.3 mM KCl, 0.6 mM Na2HPO4, 0.4 mM KH2PO4, 0.2 mM NaH2PO4-2H2O) supplemented with dextrose (2.7 mM) and NaHCO3 (4.1 mM), pH 7.2, and maintained for 50 min at 37 °C before experiments were performed. Concentrated washed platelets were added to the study samples to reach the established platelet counts.

Thromboelastometry

TEM analysis was performed on the ROTEM® delta machine (Rotation Thromboelastometry, TEM International, Munich, Germany). Plastic cups were filled with 300 µl of pre-warmed (37 °C) solutions. FIBTEM-S/EXTEM-S subtests were used providing extrinsic coagulation activation with/without cytochalasin-based deactivation of platelets. FIBTEM is essentially dependent on the FGN function. This test inhibits the platelet contribution to clot formation, leaving only the clotting proteins. Thus, one can observe the contribution of functional FGN to clot formation. A minimum of three independent measurements on different, freshly prepared samples were performed for each concentration step. Thromboelastometry measurements were performed immediately after combining the different protein and/or cellular components of the study samples. Standard TEM parameters were obtained for statistical analysis: CT, A 10, and MCF.

Statistical analysis

The 95% confidential interval defined the normal range for the obtained TEM parameters of the internal control group: CT, A10, and MCF. Statistical analysis was performed on SAS® 9.3 Statistical Software. The correlation analysis of the collected data was performed on basis of a dispersion graph for illustrating values of TEM parameters in function of the corresponding dose. Linear correlation was analyzed on basis of the underlying dispersion graph. In the case of linear correlation the Pearson coefficient for linear correlation was applied. For interpreting our data $p < 0.050.05$ were considered significant.

Results

Negative control of coagulation factor free AFS samples

No response was detected in FIBTEM or EXTEM subtests when performed on AFS that were not enriched with coagulation factors. The tests resulted in an infinite CT value and no clot formation could be detected.

Normal range of plasma tested by TEM

The 95% confidence interval of the TEM parameters determined in 33 plasma samples of healthy volunteers was defined as the "normal range" for plasma. The results are summarized in Table 1 as "Internal Control". The upper limit of normal for CT in our internal control group was 53 s for EXTEM subtests and 54 s for FIBTEM subtests. The obtained plasma reference range for A 10 and MCF was 17–24 mm and 18–26 mm, respectively. Previously published reference ranges for standard TEM parameters for plasma and whole blood and usually accepted treatment thresholds are shortly summarized in Table 1 for comparison [13–16].

Combination of PC and FGN leads to fibrin clot formation in a plasma and platelet free environment

The combination of PC and FGN in an artificial colloid solution free from other blood components leads to the formation of a thromboelastometrically measurable fibrin clot. The formed fibrin clots were stable as no significant deterioration of its viscoelastic integrity was observed during the 60 min TEM response. Figure 1 shows a typical TEM graph using a FGN concentration of 4 g/l and PC at 1 IU/ml (using the concentration of FIX as a reference). The viscoelastic properties of the tested fluids at this coagulation factor combination (CT 117 ± 20 s, A10 10.7 ± 0.6 mm, MCF 12 ± 1.7 mm) were located outside the aspired, predefined normal values (Table 1), especially CT was considerably prolonged.

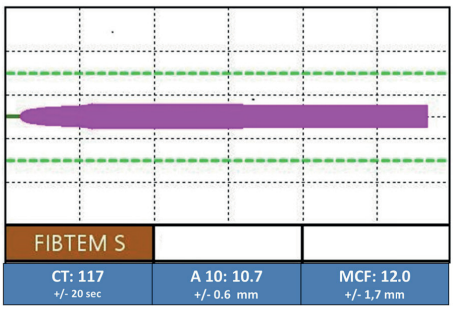

Fig. 1 Combination of PCC and FGN leads to fibrin clot formation in a plasma and platelet free environment. Figure is representative for fibrin clot formation under a combination of PCC at 1 IU/ml with FGN at 4 g/l.

Fibrin formation was dependent on the presence of PC as negative controls without PC proteins ruled out spontaneous fibrin formation during TEM analysis.

Increasing concentrations of PC caused progressive improvements in TEM parameters at a fixed FGN concentration of 4 g/l

As described in Table 2, a progressive shortening of CT was observed with increasing concentrations of PC. An inverse association between PC concentration and CT was observed within the 0.05–1 IU/ml concentration range with the shortest CT detected at a concentration of 1 IU/ml and an average CT of 117 s. This value was significantly prolonged when compared to the CT reference range of our internal control group, to external controls, and published treatment thresholds (Table 1). Higher PC concentrations (> 1 IU/ml) did not further shorten CT values. Even in very high concentrations like 4 IU/ml, CT did not further improve.

Effects of increasing concentrations of PC on fibrin clot strength measured by A 10 and MCF were less evident than those observed on CT. As shown in Table 2, a positive linear correlation between rising PC concentrations and thromboelastometry clot strength parameters was seen (Pearson correlation coefficient of rho = 0.65, 0.57 and p-values < 0.0013, 0.0063 for A10 and MCF respectively).

According to these data, a concentration of 1 IU/ml PC for the final CRF composition was determined.

Rising FGN concentrations improved clot strength related parameters at a fixed PC concentration of 1 IU/ml

Based on the previous results, a fixed PC concentration of 1 IU/ml was chosen for this test series as it provided the shortest CT values in our model. As summarized in Table 3, a negative non-linear correlation was seen between increasing FGN concentrations and measured CT values. This effect was more pronounced in the lower FGN range between 0.5 and 2.0 g/l and became less significant with higher FGN concentrations above 2 g/l.

Moreover, FGN concentrations rising from 0.5 to 12 g/l improved the TEM parameters that characterize the clot strength with a strong positive correlation between FGN concentrations and the corresponding TEM parameters (Pearson correlation coefficient of rho = 0.98 and p-values of < 0.0001 for both A10 and MCF respectively, Table 3). At a physiological FGN concentration of 4 g/l the measured TEM responses (11/12 mm for A 10/MCF) were slightly below the desired TEM range of 17 mm. The final FGN concentration for CRF composition of 4 g/l was determined in synopsis with the results achieved in combination with FXIII.

Increasing concentrations of FXIII (0.1, 0.5, 1, 2, 4, 8 IU/ml) enhanced clot strength at fixed concentrations of PC (1 IU/ml) and FGN (4 g/l)

Fixed concentrations of FGN (4 g/l) and PC (1 IU/ml) were used in this experimental setting. FXIII effects on fibrin clot strength were evaluated by A 10 and MCF. As shown in Table 4, a moderate to high positive

Table 2 Analysis of viscoelastic parameters in albumin-based colloid solutions enriched with prothrombin complex concentrate (PCC) and fibrinogen (FGN). Different PCC concentrations (0.05, 0.1, 0.25, 0.5, 1, 2, 4 IU/ml) were combined with fixed fibrinogen (FGN) concentrations of 4 g/l. The albumin concentration was maintained stable at 5%. Negative controls on PCC-free solutions did not show any measurable ROTEM response (infinite CT). A minimum of three repeats for each concentration step was performed. The value in bold letters is prolonged (>80s) but still reflects the optimal PCC response for CRF composition

Increasing PCC concentrations over FGN 4 g/l							
PCC conc.[a]	0,05	0,1	0,25	0,5	1	2	4
CT[b,*]	613 ± 251	337 ± 46	216 ± 50	168 ± 22	**117 ± 20**	95 ± 40	115 ± 18
A10[c,**]	5.7 ± 1.5	6.3 ± 1.5	6.7 ± 0.6	8.3 ± 1.5	10.7 ± 0.6	14 ± 4.3	11.7 ± 2.1
MCF[c,***]	6.3 ± 1.5	7.3 ± 2.1	7 ± 1	9.3 ± 2.3	12 ± 1.7	14.3 ± 4.9	12 ± 2.6

*p < 0,0001, Pearson rho 0,9 for1/CT, **p = 0,0013, Pearson rho 0,65, ***p = 0,0063, Pearson rho 0,57
[a]PCC concentrations in IU/ml refer to final factor IX concentrations as reference protein in this product
[b]CT in seconds. Mean values ± SD
[c]A10 and MCF in mm. Mean values ± SD

Table 3 Analysis of viscoelastic parameters in albumin-based colloid solutions enriched with fibrinogen (FGN) and prothrombin complex concentrate (PCC). Different FGN concentrations (0.5, 1, 2, 4, 8, 12 g/l) were combined with fixed PCC concentrations of 1 IU/ml. The albumin concentration was maintained stable at 5%. Negative controls on fibrinogen free solutions did not show any measurable ROTEM response (infinite CT). A minimum of three repeats for each concentration step was performed. The values in bold letters reflect the optimal FGN response for CRF composition

Increasing FGN concentrations over PCC 1 IU/ml						
FGN conc. [a]	0,5	1	2	**4**	8	12
CT [b,*]	4032 ± 1949	620 ± 620	221 ± 90	117 ± 20	154 ± 27	198 ± 1
A10[c,**]	n.a.	2.7 ± 0.6	3.6 ± 0.6	**10.7 ± 0.6**	25.7 ± 4.2	47 ± 1.4
MCF[c,***]	n.a.	3.7 ± 0.6	3.3 ± 0.6	**12 ± 1.7**	26.3 ± 3.1	49.5 ± 3.5

*$p < 0{,}0001$, Pearson rho 0,84 for1/FGN, **$p < 0{,}0001$, Pearson rho 0,98, ***$p < 0{,}0001$, Pearson rho 0,98
[a]FGN concentrations in g/l
[b]CT in seconds. Mean values ± SD
[c]A10 and MCF in mm. Mean values ± SD

correlation was observed for the Pearson correlation coefficient between rising FXIII concentrations and A10 and MCF (p-values of 0.004 and 0.002, respectively). The previously observed TEM response at a FGN concentration of 4 g/l (MCF 12 mm) in a FXIII-free environment (MCF 12 mm), significantly improved to MCF of 24 mm by adding 1 IU/ml of FXIII, thus reaching the upper limit of our predefined range for normality. No additional effect or statistical correlations were observed for other tested TEM parameters (CT). According to the combined data on FGN and FXIII, final concentrations of 4 g/l for FGN and 1 IU/ml for FXIII were determined for further analysis of CRF under the presence of platelets.

Platelets completely restore whole blood TEM parameters, including CT

The impact of increasing platelet counts (12.5, 25, 50, 100, 200, 400×10^3/µl) on various TEM parameters was investigated under the previously established conditions using fixed CRF concentrations of PC (1 IU/ml), FXIII (1 IU/ml) and FGN (4 g/l) as this combination provided optimal values for CT and clot strength in TEM studies.

The presence of platelets (in the optimally designed CRF) significantly improved fibrin clot strength parameters assessed by A10 and MCF (Fig. 2a and b). A moderate to high positive correlation was observed between rising platelet concentrations and A10 and MCF measurements (Pearson correlation coefficient of rho = 0.89/0.86, respectively and p-values of 0.0001). Clot strength values reached the normal range for EXTEM whole blood under a minimum platelet concentration around 100×10^3/µl, as calculated from a regression analysis. No effect on clot strength was observed for platelets in the FIBTEM tests performed in parallel.

As shown in Fig. 2c, the presence of platelets (in the optimal CRF) shortened CT. A moderate to high negative correlation was observed with rising platelet concentrations and measured CT (Pearson correlation coefficient of rho = – 0.80, $p < 0.0001$). TEM values reached normal whole blood CT values for EXTEM subtests under a minimum platelet concentration of around 100×10^3/µl.

As shown in Table 5, CRF at PC 1 IU/ml, FGN 4 g/l, FXIII 1 IU/ml reached TEM parameters comparable to whole blood EXTEM reference ranges when tested under the presence of 100×10^3/µl platelets.

Discussion

Data from our in vitro study demonstrate that it is possible to restore coagulation properties by combining defined concentrations of coagulation factor concentrates in an albumin-based colloid solution. The viscoelastic clot formation parameters A 10 and MCF observed in CRF were comparable not only to human plasma, but also to whole blood under the presence of platelets. The surrogate parameter for thrombin generation, CT, was prolonged when compared to our internal control group,

Table 4 Analysis of viscoelastic parameters in albumin-based colloid solutions enriched with factor XIII (FXIII), prothrombin complex concentrate (PCC) and fibrinogen (FGN). Different FXIII concentrations (0.1, 0.5, 1, 2, 4, 8 IU/ml) were combined with fixed PCC and FGN concentrations of 1 IU/ml and 4 g/l, respectively. The albumin concentration was maintained stable at 5%. Negative controls for FXIII- free solutions are represented by the correspond ng compositions shown in Tables 2 and 3. A minimum of three repeats for each concentration step was performed. The values in bold letters reflect the optimal FXIII response for CRF composition

Increasing FXIII concentrations over PCC 1 IU/ml and FGN 4 g/l						
FXIII conc. [a]	0.1	0.5	**1**	2	4	8
CT[b,*]	123 ± 12.7	128 ± 17.3	135.3 ± 12.7	122.3 ± 7.5	133.5 ± 29	133.3 ± 22.2
A10[c,**]	12.5 ± 0.7	16.3 ± 1.5	**21.3 ± 2.1**	24.3 ± 2.3	30.3 ± 11.4	30.7 ± 9.9
MCF[c,***]	13.5 ± 0.7	18.7 ± 2.1	**23.7 ± 3.1**	26.7 ± 4.6	32.3 ± 10.4	35.7 ± 12.4

*no correlation, **$p = 0{,}004$, Pearson rho 0,65, ***$p = 0{,}002$, Pearson rho 0,69
[a]FXIII concentrations in IU/ml
[b]CT in seconds. Mean values ± SD
[c]A10 and MCF in mm. Mean values ± SD

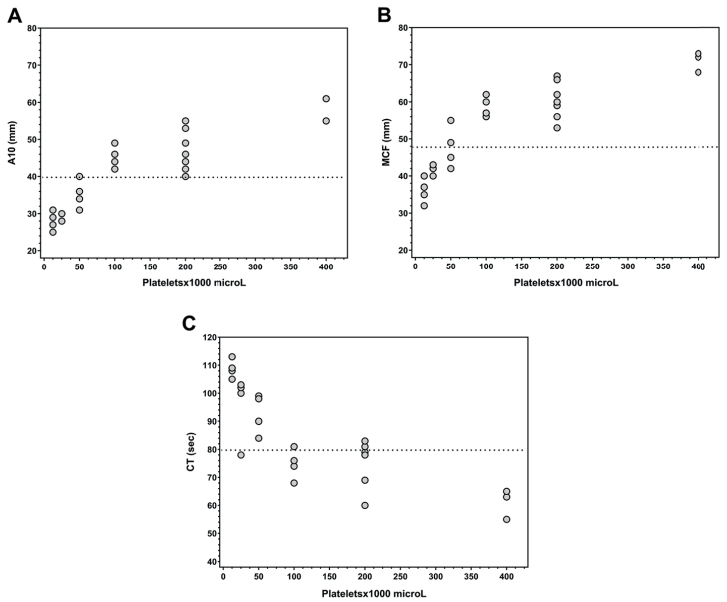

Fig. 2 Analysis of viscoelastic parameters of CRF, defined as albumin-based colloid solution containing PCC [1 IU/ml], FXIII [1 IU/ml] and FGN [4 g/l]). Different platelet concentrations (12.5, 25, 50, 100, 200, 400 × 10^3/μl) were combined with CRF. A minimum of three repeats for each concentration step was performed. The continuous line in the graph shows the lower limit of normal range for whole blood TEM parameters. **a** Effect of different platelet concentrations on A10. **b** Effect of different platelet concentrations on MCF. **c** Effect of different platelet concentrations on CT

but reached normal levels for whole blood when analyzed in presence of platelets at 100 × 10^3/μl [14]. Fluid substitutes like CRF could be an interesting treatment option, with clinical indications comparable to those of fresh frozen plasma.

Massive bleeding, independent of its etiology (trauma, obstetrical or surgical), usually includes high ratio FFP transfusion guided by institutional massive transfusion protocols, based on a large body of evidence favoring plasma against coagulation factor-free resuscitation fluids [11, 18]. This hemostatic resuscitation concept, however, is insufficient to avoid massive transfusion-associated multifactor coagulopathies [19]. Factor containing fluids for volume therapy, like CRF, that are characterized by optimized viscoelastic properties could be an appealing new treatment component. Additional point-of-care monitoring would still allow for goal-directed top-up corrections, but monitoring intensity could be reduced. Easy storage of the CRF components at 4 °C, immediate availability without thawing time, and the universal applicability, independent of blood group compatibility, could provide both logistic and clinical advantages of this new product compared to frozen plasma. The clinical indications for CRF administration could largely be analog to those of plasma, focusing on uncontrolled bleeding events related to multifactor deficiencies in hypovolemic patients, in which ongoing factor-free fluid therapy could cause further deterioration of the basic coagulation mechanisms. In these urgent scenarios CRF could provide shorter decision-to-treatment times than those known for plasma transfusion. CRFs could easily be held available, independent of blood bank facilities, in all areas exposed to massive bleeding scenarios, like operating theatres, ICUs, delivery rooms, emergency departments and even in prehospital emergency- or military settings.

Future clinical studies in massive transfusion scenarios will have to show if substituting plasma by CRF is feasible and if equal efficacy in terms of hemodynamic and

Table 5 Comparison of whole blood reference ranges with TEM results for CRF under presence of 100 × 10^3/μl platelets

	WHOLE BLOOD[a]	CRF + PLATELETS
CT (s)	42–74	74 +/− 5
A 10 (mm)	43–65	45 +/− 3
MCF (mm)	49–71	59 +/− 2

[a] Whole blood reference ranges as published by Lang et al. [14]

coagulation stability will be provided. Furthermore clinical trials will have to show if plasma-specific adverse events like "transfusion-related acute lung injury (TRALI)" or "transfusion-related immune modulation (TRIM)" would be less frequent under treatment of purified plasma-derived components like CFCs or albumin. It is not finally established from previous clinical studies, if the overall complication rate, especially when compared to solvent and detergent treated pooled plasma (S/D plasma), really results in a better safety profile for factor concentrates. Next to these still to be answered clinical issues, current prices for the final CRF components would imply a major economic obstacle for the implementation of this product into clinical routine, even if outcome superiority compared to plasma-based standard of care could be demonstrated in future clinical studies.

Human albumin 5% was chosen as carrier solution for the coagulation factor compound of our CRF for different reasons. First, colloids were favored against crystalloids, because accurately defined coagulation factor concentrations within a predefined volume of a resuscitation fluid would only make sense, if the underlying carrier showed adequate and sustained volume effects in the intravascular space. Second, the colloid should not interfere in a significant manner with the coagulation system. Although all colloid solutions show dilutional effects, albumin solutions, together with gelatins, seem to cause less colloid-specific, detrimental effects on platelet function or fibrin polymerization than other colloids, like dextrans, or starches [20, 21]. We preferred albumin against gelatins to optimize comparability to FFP in future trials. Experimental studies show that albumin-based colloid solutions provide stabilizing effects on the endothelial barrier and show intravascular plasma expander effects of nearly 100% [22, 23]. By contrast, no such effects on the endothelial barrier could be demonstrated for CFCs [24]. The administration of well-balanced coagulation factors in carrier solutions with constant intravascular volume effects might be a safe way to treat bleeding associated coagulopathies, as "overshot" peak plasma concentrations caused by the infusion of highly concentrated factor formulas (as under non-diluted CFC administration) would be avoided. The intravascular volume effect of colloidal resuscitation fluids seems to be context-sensitive and correlated to the integrity of the endothelial glycocalyx layer. There is growing evidence that in special clinical conditions like sepsis and trauma, characterized by elevated glycocalyx shedding rates, the volume expander rate of isooncotic colloids would be less than predicted. The underlying glycocalyx disruption seems to be partially driven by a "low-protein environment" caused by aggressive crystalloid or synthetical colloid fluid treatment. By contrast, protein containing resuscitation fluids like plasma or albumin-based colloids seem to provide protective effects against glycocalyx shedding. This albumin-mediated protection of the glycocalyx layer is currently demonstrated in mostly preclinical, in-vitro studies, and it remains a matter to future studies if this translates into a clinically detectable advantage of albumin containing resuscitation fluids [25, 26] .

Hemostasis is a result of coordinated interactions between platelets and coagulation mechanisms [27]. Coagulation mechanisms necessary for consolidation of platelet mediated primary hemostasis require a cascade of enzymatic reactions leading to the formation of fibrin [15, 28, 29]. Results of the present study indicate that coagulation mechanisms can be reproduced using a restricted number of coagulation factors suspended in a neutral fluid. To our knowledge, this is the first experimental study that has been able to demonstrate that the combination of commercially available CFCs in an initial coagulation factor- and blood-cell-free solution leads to the formation of a stable in vitro fibrin clot. The initiation of the coagulation in this fluid requires the use of EXTEM or FIBTEM reagents whose components (calcium, phospholipids and tissue factor) would trigger the activation of the prothrombin complex coagulation factors VII, IX, X and II contained in commercial PCCs [6]. These coagulation factors lead to sufficient thrombin generation and warrant the basic activating mechanism of the coagulation system to sustain in vitro fibrin polymerization. FGN provides the structural clotting substrate supporting secondary hemostasis [30]. The necessary FGN concentration in CRF to reach normal TEM values (when combined with FXIII) was found in the range of physiological plasma concentrations, around 4 g/l for FGN and 0,5–1 IU/ml for FXIII. FXIII cross-links fibrin, completing blood coagulation and protecting the hemostatic plug from the fibrinolytic activity at the clot formation site. In vitro studies demonstrated that supplementation with FXIIIC increases clot firmness assessed by TEM in perioperative patients with elevated FGN and reduced FXIII levels [31]. However, in another in vitro model of massive transfusion in trauma, combination therapies with FC and fresh frozen plasma, but not FXIIIC, improved both coagulation kinetics and fibrin-based clot strength [32, 33]. Our present study indicates that increasing concentrations of FXIII enhance clot strength at fixed concentrations of PC (1 IU/ml) and FGN (4 g/l). Consistently, there is further evidence that FXIII deficiency will impair FGN function and fibrin formation, suggesting an inverse link between low FXIII levels and enhanced thrombin generation, modifying the structure-function relationship of fibrin to support hemostasis [34]. Data derived from clinical studies propose maintenance of 50–60% of FXIII activity to avoid bleeding tendency in the perioperative setting [35].

CRF compositions without FXIII, yielding comparable clot strength in TEM when compared to our final composition, are possible from a theoretical point of view. We decided to add a purified source of FXIII to our final CRF composition despite the high potential of concentrate-derived FGN on viscoelastic clot strength to maintain a close-to-physiological factor composition.

The safe upper limit of FC treatment has not been precisely defined. It is currently suggested that plasma levels of FGN should reach 1.5 to 2 g/l in bleeding patients [36]. There is a clear tendency, as reported in different guidelines, to recommend elevating plasma FGN in some clinical situations [8, 37, 38]. Taking into consideration the results of our TEM studies it may be difficult to maintain a well-balanced coagulation factor composition during a long-lasting, high-dynamic bleeding event if supplements are only point-of-care driven and punctual. In this context, a fixed ratio of clotting factors in CRFs administered under volume therapy could provide more balanced stability within the complex multifactor system of blood coagulation than single factor substitutes as proposed in current algorithms.

CT in TEM is partially dependent on thrombin generation. Direct anticoagulants reducing thrombin generation definitively prolong CT [39]. Platelets contribute to enhance thrombin generation, accelerate CT, and increase MCF. Additionally, platelet phospholipids dramatically contribute to the amplification of coagulation mechanisms, thus potentiating thrombin generation and fibrin polymerization. Fibrin then interacts with activated platelets and plays a critical role in MCF. CT values of platelet-free CRF samples in our in-vitro experiments were significantly prolonged when compared to plasma CT levels of our internal control group. Several reasons may account for these findings:

First, our in vitro samples were completely free of any phospholipids or cell membrane fragments that could influence factor activation. Consequently, the addition of platelets to CRF containing 1 IU/ml PC, 4 g/l of FGN and 1 IU/ml FXIII leads to the normalization of CT and MCF. It could be assumed from our studies that, when combined with CRF, a platelet count around 100×10^3/µl should be required to fully reconstitute TEM parameters to levels observed in whole blood studies (see Fig. 2a-c). CT values above 80 s are considered to reflect pathological thrombin generation and are generally accepted as treatment threshold. CT values of CRF combined with platelets were significantly shorter than this generally recommended treatment thresholds [15] (Tables 1 and 5).

Second, the used PCC in our experiments contains heparin. Other study groups previously reported about CT sensitivity of extrinsically activated TEM tests [40]. It is questionable if this phenomenon has any clinical relevance. The currently scientific rationale rather suggests that PCCs might be associated to overshot thrombin generation with the potential to induce disseminated intravascular coagulation and that Antithrombin III supplements might mitigate this potentially dangerous adverse effect [41]. The complete absence of antithrombin in the final CRF composition is a major limitation of our experiments and the effects of PCC supplements in clinical situations with reduced antithrombin levels will have to be analyzed in future trials.

A further limitation of our experimental studies is the complete absence of red blood cells. Red blood cells seem to exert a more important role in primary hemostasis, whereas their modulating effect on secondary hemostasis seems to be negligible [13]. The fact is that, viscoelastic studies can be reliably performed in plasma samples [12, 13]. Surprisingly, an inverse relation between hematocrit and clot firmness was previously reported under experimental and clinical anemic conditions [42]. We cannot rule out that the presence of red blood cells in our in vitro model could lead to a measurable reduction of clot firmness parameters. However, following reports of Schoergenhofer et al. [13] no effects on other TEM parameters should be expected under whole blood conditions. Under massive transfusion using CRF as a plasma substitute, transfusion of red blood cells would be an integral part of the clinical management to uphold an adequate amount of oxygen carriers within the circulating blood volume.

Altogether, the transfer of our data into a clinical context must therefore be done very carefully. All factor components of CRFs have previously been safely administered in loose compositions for the management of bleeding associated coagulopathy [43]. PCCs show a reliable safety profile and are now the treatment of choice for the emergency reversal of Vitamin K antagonists [44, 45]. Nevertheless, a careful assessment of the thrombogenic potential of fixed factor combinations for the treatment of a multiple factor deficit under massive bleeding will have to be performed in future studies.

Conclusions

Coagulation factor concentrates suspended in albumin solutions have the potential to restore mechanisms of secondary hemostasis in the absence of any blood component, showing viscoelastic properties comparable to whole blood when tested in presence of platelets. Coagulation factor enriched albumin-based colloids could be a valuable tool to provide stable intravascular volume effects in hypovolemic conditions and to simultaneously maintain basic coagulation mechanisms. This could offer future alternatives to transfusion of fresh frozen plasma under resuscitation conditions.

Abbreviations
AFS: Artificial Fluid Solution; A10: Amplitude at 10 min; CFC: Coagulation Factor Concentrate; CRF: Coagulation Resuscitating Fluid; CT: Clotting Time; FC: Fibrinogen Concentrate; FGN: Fibrinogen; FXIII: Factor XIII; FXIIIC: Factor XIII Concentrate; MCF: Maximum Clot Firmness; PC: Prothrombin Complex; PCC: Prothrombin Complex Concentrate; TEM: Thromboelastometry

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Authors' contributions
TK designed the study. PP, JM, JA and VM reviewed the study protocol. TK, NK, AG, SP, NV and AM collected and assessed the experimental data. TK, GE and MD interpreted the data and drafted the manuscript. PP, JM, XL, AM and JA reviewed the manuscript and provided critical comments for the interpretation and discussion of the data. XL, TK and GE performed the statistical data interpretation. All authors read and approved the final manuscript.

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Availability of data and materials
The dataset used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee "Instituto de Recerca Hospital de la Santa Creu I Sant Pau" and registered under the protocol number IIBSP-CFC-2013-165.
Written informed consent was obtained from healthy volunteers before blood sample extraction.

Consent for publication
Not applicable.

Competing interests
Tobias Koller received a study grant from CSL Behring. Pilar Paniagua received lecture fees from CSL Behring. Jose Aznar-Salatti is working as CSL Behring Medical Affairs Manager at a Spanish Affiliate. The remaining authors have no conflict of interest to declare.

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Publisher's Note

Conclusions

- The combination of FC and PCC in colloid carrier solutions leads to the formation of a stable fibrin clot after extrinsic coagulation activation.
- An optimized combination of PCC, FC and FXIII has the capacity to restore coagulation without the additional use of plasma.
- PCC provides sufficient thrombin formation for inducing fibrin polymerization. FC and FXIII show a great potential to improve clot firmness.
- In the presence of platelets, an optimized combination of PCC, FC and FXIII reconstituted in human albumin carrier solutions provide normalized TEM parameters when compared to whole blood.
- CRFs could be a new treatment option providing hemostatic properties comparable to plasma but being based on products that might provide an improved risk/benefit profile when compared to plasma.

DISCUSSION

5. DISCUSSION

In the present doctoral thesis we hypothesized that a theoretical basis could be established for a plasma-free treatment approach for acquired single- and multi-coagulation factor deficiencies during the time course of massive bleeding. To verify the theoretical basis for this consideration a review of the currently available scientific evidence on the treatment of bleeding-associated coagulopathies with FC was performed as a first step. In a second step, a series of experimental trials should demonstrate that well-defined compositions of CFCs in plasma-free, albumin-based carrier solutions have the potential to conserve the basic coagulation mechanisms.

The performed literature review confirmed that FC, when used as threshold-guided treatment based on VHA monitoring, is an adequate treatment of acquired hypofibrinogenaemia and has a beneficial impact on transfusion rates and mortality. There is broad consensus among experts that FC supplements (or Cryo) should be given preference to plasma transfusion to correct critically low FGN levels as detected in standard laboratory testing or VHA-based monitoring.

From the results of our experimental *in vitro* investigations, we conclude that absent coagulation factors can be efficiently replaced with CFCs *in vitro*, restoring coagulation mechanisms with normal VHA parameters and without the use of plasma. This allows for the option to reconstitute CFCs in an optimal composition for creating a CRF based on plasma-free albumin solution as plasma-free carrier solution. CRFs could have the potential in clinical settings to eliminate the onset of dilutional coagulopathies which is yet to be proven in follow-up studies.

Altogether this work demonstrates that in the clinical setting of acquired hypofibrinogenaemia FCs are preferable to plasma and that an optimized composition of CFCs has the *in vitro* potential to restore VHA parameters starting from coagulation factor-free solutions without the requirement for using additional plasma.

Fibrinogen Concentrates in bleeding-associated coagulopathies

Coagulopathies in massive bleeding events are complex and multifactorial. In practically all clinical settings the onset of coagulation factor deficiencies seems to follow a two-step dynamic with plasma FGN being the first coagulation factor dropping to critically low levels resulting in a single-factor deficit at the early stage of bleeding (161). It has been demonstrated that dilution phenomena cause clinically relevant deficiencies of all coagulation factors, including FGN (77)(228). Other mechanisms like hyperfibrinolysis, loss, and consumption of clotting factors can explain that the magnitude of FGN deficiency in severe bleeding can be significantly beyond that caused by dilutional effects (190). This early single factor deficit of hypofibrinogenaemia is followed in a second step by the onset of multifactor deficiencies as the dilutional grade exceeds 60% or more (164) negatively affecting the thrombin generation potential. It is reasonable to perform monitoring of bleeding-related factor deficiencies applying VHA as its use is shown to reduce blood loss and transfusion rates of allogeneic blood products. VHA provides comprehensive information on the physical and timeline characteristics of the developing blood clot within whole-blood samples. The concept of VHA-guided, goal-directed, CFC-based management is cost-effective, and it seems to reduce transfusion rates while providing apparent beneficial effects on overall outcomes, including multi-organ failure and mortality in some, but not all, high-risk conditions (63)(229). The physical clot strength measured during the activation of the coagulation system is expressed in MCF. FGN, FXIII, and platelets are the main determinants of these parameters. To eliminate the impact of platelets on clot formation and to reduce the information of MCF to the contribution of FGN (and FXIII) to the firmness of the forming fibrin clot, platelet inhibition with cytochalasin D can be induced within FIBTEM subtests (59). Results obtained from FIBTEM are available within 5-10 minutes and provide an excellent guide for clinicians to indicate FGN treatment and to calculate the corresponding dose (58). It has been demonstrated that FGN replacement has a very strong potential to improve and correct dilutional effects on viscoelastic clot characteristics (223)(230). FC is therefore increasingly used in bleeding-associated coagulopathies with risk for acquired hypofibrinogenaemia (58). A huge body of scientific evidence has been published in recent years on the

efficacy of FC supplements in perioperative bleeding. In our Review on the topic, a total of 24 clinical studies were analysed comparing FC to placebo or other haemostatic agents (like plasma or platelets), in the clinical high-risk settings of trauma, peripartum haemorrhage, Cardiac Surgery, or other high risk surgery. The total of studies with an interventional design and applying an interventional trigger for FC supplements defined by previously thresholds proven to be related to increased bleeding risk have shown a significant reduction of transfusion rates when compared to placebo or other haemostatic agents as a comparator (5)(6)(96)(231)-(234). The best established thresholds for this purpose are currently 1.5 g/l for non-obstetrical bleeding or 2 g/l for obstetrical bleeding or corresponding FIBTEM values.

In contrast, ´the majority of studies that opted for a pre-emptive treatment strategy, or for an interventional trigger other than critical FGN thresholds, failed to show any benefit from FC supplements (94)(190)(235)-(245). These results could possibly be related to the fact that the highest FC efficacy can only be expected when bleeding is directly related to a clinically relevant hypofibrinogenaemia. Those studies that miss this inclusion criterion are at risk to include patients with bleeding causes other than hypofibrinogenaemia, who then would be insensitive to FC treatment.

Currently, no clear recommendation based on good quality evidence can be given concerning the preference of FC or Cryo for treatment of acquired hypofibrinogenemia. While Anglo- American and British guidelines recommend Cryo, most continental European Societies prefer FC (3)(65)(246), but administration of the alternative product is foreseen as acceptable if the first choice product is unavailable.

Taken together the current evidence definitely supports the use of FC (or Cryo) before plasma as a FGN source for replenishment of critically low FGN plasma levels in patients with ongoing bleeding and diagnostic criteria for acquired hypofibrinogenaemia established by VHA or standard laboratory testing.

The concept of Coagulation Resuscitation Fluids (CRF) as a potential plasma substitute

CRF with combined therapeutic effects on the circulating intravascular volume and on the maintenance of the basic coagulation mechanisms could be a desirable future treatment option. Currently, plasma is the only available product combining these two therapeutic principles which together form the mainstay of clinical management of coagulopathy-driven massive bleeding. In recent years, therapeutic concepts are increasingly applied to uncoupling volume- from haemostatic therapy by infusing crystalloids or colloids for haemodynamic stability and to correct coagulation deficiencies (mainly caused by the administered resuscitation fluids) with goal-directed administration of corresponding CFCs (247). This stands in clear contrast to formulaic strategies suggesting high-ratio transfusion of blood components of 1:1:1 for plasma:RBC: platelets (226). This strategy provides volume therapy with simultaneous administration of coagulation factors contained in the transfused plasma products. High transfusion rates of allogeneic blood products are the consequence, raising considerable concern among clinicians due to the overall limited evidence regarding the risk/benefit profile of plasma transfusion (175)(248). In this context, CRFs could be a new treatment option combining the same therapeutic properties as plasma but being based on products that might provide an improved risk/benefit profile when compared to plasma.

Data from our in vitro study demonstrated that it is possible to restore coagulation properties by combining defined concentrations of CFCs in an initially factor-free albumin-based colloid solution. The viscoelastic clot formation parameters A 10 and MCF observed in CRF were comparable not only to human plasma but also to whole blood under the presence of platelets. The surrogate parameter for thrombin generation, CT, was prolonged when compared to our internal control group, but reached normal levels for whole blood when analysed in presence of platelets at 100 × 103/µl (249).

Massive bleeding, independent of its aetiology (trauma, obstetrical or surgical), usually includes high ratio FFP transfusion guided by institutional massive

transfusion protocols, based on a large body of evidence favouring plasma against coagulation factor-free resuscitation fluids (250)(251). This haemostatic resuscitation concept, however, is insufficient to avoid massive transfusion-associated multifactor coagulopathies (252). Factor-containing fluids for volume therapy, like CRF, that are characterized by optimized viscoelastic properties could be an appealing new treatment component. Additional point-of-care monitoring would still allow for goal-directed top-up corrections to compensate for additional factor deficiencies (not dilution-related) but monitoring intensity could be reduced. Easy storage of the CRF components at 4°C, immediate availability without thawing time, and the universal applicability, independent of blood group compatibility, could provide both logistic and clinical advantages of this new product compared to frozen plasma. The clinical indications for CRF administration could largely be analog to those of plasma, focusing on uncontrolled bleeding events related to multifactor deficiencies in hypovolemic patients, in which ongoing factor-free fluid therapy could cause further deterioration of the basic coagulation mechanisms. In these urgent scenarios, CRF could provide shorter decision-to-treatment times than those known for plasma transfusion. CRFs could easily be held available, independent of blood bank facilities, in all areas exposed to massive bleeding scenarios, like operating theatres, ICUs, delivery rooms, emergency departments and even in a prehospital emergency- or military settings. Future clinical studies in massive transfusion scenarios will have to show if substituting plasma with CRF is feasible and if equal efficacy in terms of hemodynamic and coagulation stability will be provided. Furthermore, clinical trials will have to show if plasma-specific adverse events like “transfusion-related acute lung injury (TRALI)” or “transfusion-related immune modulation (TRIM)” would be less frequent under treatment of purified plasma-derived components like CFCs or albumin. It is not finally established from previous clinical studies, if the overall complication rate, especially when compared to solvent and detergent treated pooled plasma, really results in a better safety profile for factor concentrates. Next to these -still to be answered- clinical issues, current costs for the final CRF components would imply a major economic obstacle for the implementation of this product into clinical routine, even if outcome superiority compared to plasma-based standard of care could be demonstrated in future clinical studies.

The role of human albumin colloids as a carrier solution in CFR

Human albumin 5% was chosen as a carrier solution for the coagulation factor compound of our CRF for different reasons. First, colloids were favoured against crystalloids, because accurately defined coagulation factor concentrations within a predefined volume of a resuscitation fluid would only make sense if the underlying carrier showed adequate and sustained volume effects in the intravascular space. Second, the colloid should not interfere in a significant manner with the coagulation system. Although all colloid solutions show dilutional effects, albumin solutions, together with gelatins, seem to cause less colloid-specific, detrimental effects on platelet function or fibrin polymerization than other colloids, like dextrans, or starches (4)(253). There is some experimental *in vitro* evidence that albumin might exert anticoagulant and anti-platelet effects. These effects seem to be related to heparin-like mechanisms caused by direct binding to antithrombin and by an albumin-associated induction of nitric oxide affecting platelet aggregation (120) (121). These specific albumin actions on coagulation and platelet function do not appear to have a relevant clinical effect with increased bleeding tendency. In our experimental model different combinations and concentrations of coagulation factors and platelets were tested under physiological albumin concentrations. Therefeore, possible albumin effects on coagulation mechanisms should not affect the transferability of our results to *in vivo* conditions.

We preferred albumin against gelatins to optimize comparability to FFP in future trials. Experimental studies show that albumin-based colloid solutions provide stabilizing effects on the endothelial barrier and show intravascular plasma expander effects of nearly 100% (254)(255). By contrast, no such effects on the endothelial barrier could be demonstrated for CFCs (256). The administration of well-balanced coagulation factors in carrier solutions with constant intravascular volume effects might be a safe way to treat bleeding-associated coagulopathies, as "overshot" peak plasma concentrations caused by the infusion of highly concentrated factor formulas (as under non diluted CFC administration) would be avoided. The intravascular volume effect of colloidal resuscitation fluids seems to be context-sensitive and correlated to the integrity of the endothelial glycocalyx layer. There is growing evidence that in special clinical conditions like sepsis and trauma, characterized by elevated glycocalyx shedding rates, the volume expander rate of iso-oncotic colloids would be less than predicted.

The underlying glycocalyx disruption seems to be partially driven by a "low-protein environment" caused by aggressive crystalloid or synthetical colloid fluid treatment. By contrast, protein-containing resuscitation fluids like plasma or albumin-based colloids seem to provide protective effects against glycocalyx shedding. This albumin-mediated protection of the glycocalyx layer is currently demonstrated in mostly preclinical, in-vitro studies, and it remains a matter of future studies if this translates into a clinically detectable advantage of albumin-containing resuscitation fluids (257)(258).

Molecular Basis of CFC-driven coagulation processes

Haemostasis is a result of coordinated interactions between platelets and coagulation mechanisms (259). Coagulation mechanisms necessary for the consolidation of platelet-mediated primary haemostasis require a cascade of enzymatic reactions leading to the formation of fibrin (260)(261)(262). Results of our *in vitro* experiments indicate that coagulation mechanisms can be reproduced using a restricted number of coagulation factors suspended in a neutral fluid. To our knowledge, this is the first experimental study that has been able to demonstrate that the combination of commercially available CFCs in an initial coagulation factor- and blood-cell-free solution leads to the formation of a stable *in vitro* fibrin clot. The initiation of the coagulation in this fluid requires the use of EXTEM or FIBTEM reagents whose components (calcium, phospholipids, and tissue factor) would trigger the activation of the prothrombin complex coagulation factors VII, IX, X, and II contained in commercial PCCs (115). These coagulation factors lead to sufficient thrombin generation and warrant the basic activating mechanism of the coagulation system to sustain *in vitro* fibrin polymerization. FGN provides the structural clotting substrate supporting secondary haemostasis (263). The necessary FGN concentration in CRF to reach normal TEM values (when combined with FXIII) was found in the range of physiological plasma concentrations, around 4 g/l for FGN and 0,5–1 IU/ml for FXIII. FXIII crosslinks fibrin, completing blood coagulation and protecting the haemostatic plug from the fibrinolytic activity at the clot formation site. *In vitro* studies demonstrated that supplementation with FXIIIC increases clot firmness assessed by TEM in perioperative patients with elevated FGN and reduced FXIII levels. However, in another *in vitro* model of massive transfusion in trauma, combination therapies with FC and FFP, but not FXIIIC, improved both coagulation kinetics and fibrin-based clot strength

(264)(265). Our present study indicates that increasing concentrations of FXIII enhance clot strength at fixed concentrations of prothrombin complex (1 IU/ml) and FGN (4 g/l). Consistently, there is further evidence that FXIII deficiency will impair FGN function and fibrin formation, suggesting an inverse link between low FXIII levels and enhanced thrombin generation, modifying the structure-function relationship of fibrin to support haemostasis (266). Data derived from clinical studies propose maintenance of 50–60% of FXIII activity to avoid bleeding tendency in the perioperative setting (267). CRF compositions without FXIII, yielding comparable clot strength in TEM when compared to our final composition, are possible from a theoretical point of view. We decided to add a purified source of FXIII to our final CRF composition despite the high potential of concentrate-derived FGN on viscoelastic clot strength to maintain a close-to-physiological factor composition. The safe upper limit of FC treatment has not been precisely defined. It is currently suggested that plasma levels of FGN should reach 1.5 to 2 g/l in bleeding patients (268). There is a clear tendency, as reported in different guidelines, to recommend elevating plasma FGN in some clinical situations (5)(172)(269). Taking into consideration the results of our TEM studies it may be difficult to maintain a well-balanced coagulation factor composition during a long-lasting, high-dynamic bleeding event if supplements are only POC driven and punctual. In this context, a fixed ratio of clotting factors in CRFs administered under volume therapy could provide more balanced stability within the complex multifactor system of blood coagulation than single factor substitutes as proposed in current algorithms.

Impact of platelets on Clotting Time (CT) as a surrogate parameter for thrombin generation potential in CRF-based coagulation processes

CT in TEM is partially dependent on thrombin generation. Direct anticoagulants reducing thrombin generation definitively prolong CT (270). Platelets contribute to enhancing thrombin generation, accelerating CT, and increasing MCF. Additionally, platelet phospholipids dramatically contribute to the amplification of coagulation mechanisms, thus potentiating thrombin generation and fibrin polymerization. Fibrin then interacts with activated platelets and plays a critical role in MCF. CT values of platelet-free CRF samples in our in-vitro experiments were significantly prolonged when compared to plasma CT levels of our internal control group. Several reasons may account for these findings: First, our *in vitro*

samples were completely free of any phospholipids or cell membrane fragments that could influence factor activation. Consequently, the addition of platelets to CRF containing 1 IU/ml prothrombin complex, 4 g/l of FGN, and 1 IU/ml FXIII leads to the normalization of CT and MCF. It could be assumed from our studies that, when combined with CRF, a platelet count around 100 × 103/ µl should be required to fully reconstitute TEM parameters to levels observed in whole blood studies.

CT values above 80 s are considered to reflect pathological thrombin generation and are generally accepted as a treatment threshold. CT values of CRF combined with platelets were significantly shorter than the generally recommended treatment thresholds (260). Second, the used PCC in our experiments contains heparin. Other study groups previously reported on CT sensitivity of extrinsically activated TEM tests (271). It is questionable if this phenomenon has any clinical relevance. The current scientific rationale rather suggests that PCCs might be associated with overshot thrombin generation with the potential to induce disseminated intravascular coagulation and that AT supplements might mitigate this potentially dangerous adverse effect (272).

Study limitations

The complete absence of antithrombin in the final CRF composition is a major limitation of our experiments and the effects of PCC supplements in clinical situations with reduced antithrombin levels will have to be analysed in future trials. A further limitation of our experimental studies is the complete absence of red blood cells. Red blood cells seem to exert a more important role in primary haemostasis, whereas their modulating effect on secondary haemostasis seems to be negligible (273). The fact is that viscoelastic studies can be reliably performed in plasma samples (273)(274). Surprisingly, an inverse relation between hematocrit and clot firmness was previously reported under experimental and clinical anemic conditions (275). We cannot rule out that the presence of red blood cells in our *in vitro* model could lead to a measurable reduction of clot firmness parameters. However, following reports of Schoergenhofer et al. (273) no effects on other TEM parameters should be expected under whole blood conditions. Under massive transfusion using CRF as a plasma substitute, transfusion of red blood cells would be an integral part of the clinical management to uphold an adequate

amount of oxygen carriers within the circulating blood volume. Altogether, the transfer of our data into a clinical context must therefore be done very carefully. All factor components of CRFs have previously been safely administered in loose compositions for the management of bleeding-associated coagulopathy (276). PCCs show a reliable safety profile and are now the treatment of choice for the emergency reversal of Vitamin K antagonists (277)(278). Nevertheless, a careful assessment of the thrombogenic potential of fixed factor combinations for the treatment of a multifactor deficit under massive bleeding will have to be performed in future studies.

FINAL CONCLUSIONS

6. FINAL CONCLUSIONS

1. A huge body of scientific evidence from retrospective and observational studies supports the idea that hypofibrinogenaemic conditions in actively bleeding patients significantly increase the risk for massive transfusion and higher mortality, and should therefore be adequately treated.

2. Most experts agree on the concept of threshold-guided treatment necessity.

3. Evidence from randomized controlled trials and observational studies suggests that plasma transfusion, within the framework of fixed component ratios, can be safely substituted by CFCs, guided by POC monitoring with a beneficial impact on transfusion rates and mortality.

4. CFCs suspended in initially factor-free albumin solutions have the potential to restore mechanisms of secondary haemostasis in the absence of any blood component, showing viscoelastic properties comparable to whole blood when tested in presence of platelets.

5. Coagulation factor enriched albumin-based colloids could be a valuable tool to provide stable intravascular volume effects in hypovolemic conditions while simultaneously maintaining basic coagulation mechanisms.

6. This approach could offer future alternatives to transfusion of FFP under resuscitation conditions.

7. In the clinical scenario of hypofibrinogenaemia-related bleeding, there is current consensus favouring FC (or Cry) supplements before plasma transfusion to improve haemostatic potential and reduce the overall transfusion rates of allogeneic blood products.

8. CFC-based treatment of multi-coagulation factor deficiencies seems to be a feasible alternative to plasma-based treatment strategies in acquired bleeding-associated coagulopathies.

9. CFC reconstituted in albumin carrier solutions could have a future role as resuscitation fluids with haemostatic potential in severe bleeding.

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