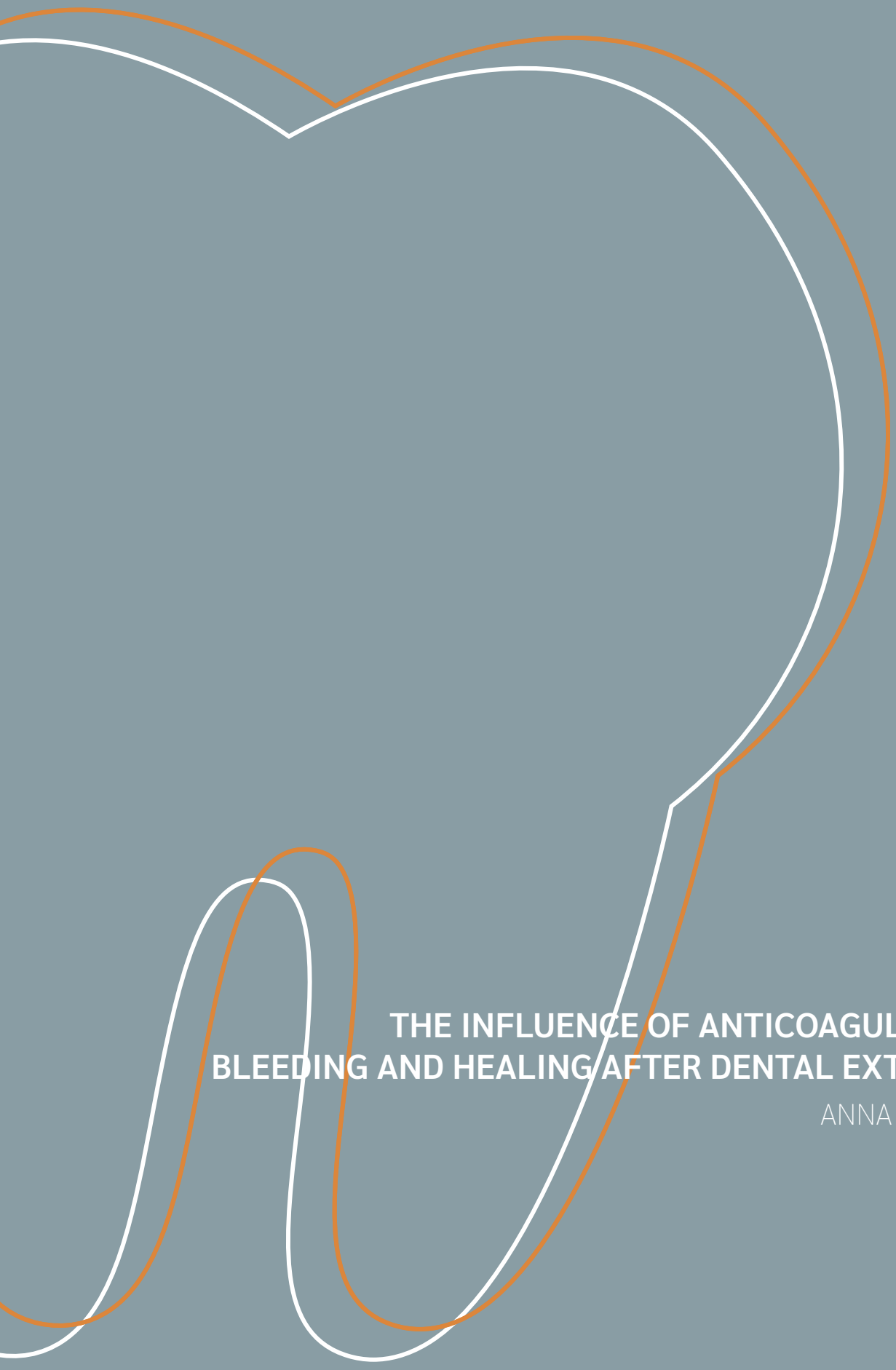

THE INFLUENCE OF ANTICOAGULANTS ON BLEEDING AND HEALING AFTER DENTAL EXTRACTION

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2021

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KU LEUVEN

DOCTORAL SCHOOL
BIOMEDICAL SCIENCES

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

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September 2021

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KU LEUVEN

DOCTORAL SCHOOL
BIOMEDICAL SCIENCES

DE INVLOED VAN ANTICOAGULANTIA OP BLOEDING EN HELING NA TANDEXTRACTIES

Anna OCKERMAN

Proefschrift
voorgedragen tot het
behalen van de graad
van Doctor in de
Biomedische
Wetenschappen

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September 2021

PREFACE

This doctoral thesis consists of 3 research chapters (Chapters 2–4), proceeded by a general introduction (Chapter 1) and concluded by a general discussion (Chapter 5). The research chapters follow the standard scientific IMRAD structure (Introduction, Methods, Results and Discussion). The thesis was based on the following peer-reviewed publications:

Chapter 1: General introduction, aims and hypotheses.

Ockerman A, Miclotte I, Vanhaverbeke M, Verhamme P, Poortmans L, Vanassche T, Politis C, Jacobs R. Local haemostatic measures after tooth removal in patients on antithrombotic therapy: a systematic review. Clin Oral Invest. 2019;23(4):1695–708.

Ockerman A, Vanassche T, Garip M, Vandenbriele C, Martens J, Jacobs R, Politis C, Verhamme P. Tranexamic acid for the prevention and treatment of bleeding in surgery, trauma and bleeding disorders: a narrative review. Thromb J. 2021;19(1):1–16.

Chapter 2: Original Research

Ockerman A, Vanhaverbeke M, Miclotte I, Belmans A, Vanassche T, Politis C, Jacobs R, Verhamme P. Tranexamic acid to reduce bleeding after dental extraction in patients treated with non-vitamin k oral anticoagulants: design and rationale of the EXTRACT-NOAC trial. Br J Oral Maxillofac Surg. 2019;57(10):1107–12.

Ockerman A, Miclotte I, Vanhaverbeke M, Vanassche T, Belmans A, Vanhove J, Meyns J, Nadjmi N, Van Hemelen G, Winderickx P, Jacobs R, Politis C, Verhamme P. Tranexamic acid and bleeding after dental extraction in patients treated with non-vitamin k oral anticoagulants: the EXTRACT-NOAC randomized clinical trial. Plos Med. 2021; 18(5):e1003601.

Chapter 3: Original Research

Ockerman A, Braem A, EzEldeen M, Castro A, Coucke B, Politis C, Verhamme P, Jacobs R, Quirynen M. Mechanical and structural properties of leukocyte- and platelet-rich fibrin membranes: an in vitro study on the impact of anticoagulant therapy. J Per Res. 2020; 55(5):686–93.

Chapter 4: Original Research

Ockerman A, Hendrickx A, Willekens W, Fehervary H, Vastmans J, Coucke W, Verhamme P, Constantinus P, Vanassche T, Braem A, Quirynen M, Jacobs R. Mechanical properties and cellular content of leukocyte- and platelet-rich fibrin membranes of patients on antithrombotic drugs. 2021. *Under review.*

Chapter 5: General discussion, conclusions and future perspectives

PERSONAL ACKNOWLEDGEMENTS

De voorbije vier jaren heb ik geleerd om betrouwbaar onderzoek op te zetten en uit te voeren, en om mijn werk te evalueren, verbeteren en te waarderen. Ik wil alvast volgende personen bedanken om dat proces mee te ondersteunen.

Mijn promotors **Prof. dr. Reinhilde Jacobs, Prof. dr. Constantinus Politis** en **Prof. dr. Peter Verhamme** ben ik zeer dankbaar om de kans die ze me gaven om dit veelzijdige onderzoek binnen Mond-, Kaak- en Aangezichtschirurgie uit te voeren. Ik kreeg veel vrijheid en vertrouwen van jullie om de studies tot een goed einde te brengen, en dat maakt me fier. Bedankt dat ik steeds bij jullie terecht kon voor raad en daad.

Dank aan mijn interne juryleden **Prof. dr. Marc Quirynen** en **Prof. dr. Pierre Delaere** voor de waardevolle feedback doorheen deze jaren en de tijd die jullie namen om mijn werk te evalueren. Het deed me deugd toen Prof. dr. Marc Quirynen zich liet ontvallen me 'een beetje geadopteerd te hebben in zijn team'. Dank u aan prof. Delaere om de rol van secretaris op te nemen bij mijn verdediging.

Bedankt **dr. Bruno Collaert** voor uw interesse in mijn master- en doctoraatsthesis en om voor een tweede keer te willen zetelen in mijn externe jury. Onze ontmoetingen zijn steeds plezierig. I also thank **Prof. dr. Benjamin Salmon** as external jury member, who is a great supporter of our Research Team. Thank you both for your valuable time spent reading and revising my manuscript.

Dank aan **Prof. dr. Tania Roskam** om mijn verdediging te willen voorzitten en aan **Prof. dr. Ghislain Opdenakker** voor de ondersteuning tijdens de finale fase van mijn doctoraat.

Bijzonder veel dank aan enkele geweldige collega-vrienden. **Myrthel, Catalina, Irem** en **Pierre**, zonder jullie wijze raad, fijne koffiepauzes en aanmoediging had ik dit doctoraat niet tot een (goed) einde gebracht. De babbels met jullie gaven me perspectief en deden me doorbijten. Graag bedank ik ook **Gabriela**, op wie we steeds konden rekenen.

Big thanks to **Karla, Bennaree** and **Tamara**, who were always cheerful and in for a chat and a bite. I would like to extent my appreciation to the entire OMFS-IMPATH group. It was very enriching to have met people from all over the world.

Onderzoek is teamwerk en de voorbije jaren mocht ik vele studenten onder mijn hoede nemen. **Liesbeth, Laura-Lien** en **Olivier** wil ik bedanken voor de vrolijke noot tijdens hun thesisjaar. **Amber** en **Laurence**, jullie waren ongelooflijk zelfstandig. Heel erg bedankt voor het harde werk. Veel dank aan nog veel andere studenten die meewerkten aan de onderzoeksprojecten: **Jeroen, Birgit, Evelyne, Chiara, Glynnis, Ana M, Zoë, Laure, Laura, Justin, Wim, Tom, Céline, Jonas, Frederik, Aaron, Lena, Elise, Melisa** en **Charlotte**.

Ook een woord van dank aan mijn collega's van de Journal Club. **Matthias, Ruben** en **Béatrice**, onze bijeenkomsten blijven me bij als aangenaam en leerrijk. Dank aan **Prof. dr. Thomas Vanassche** voor de organisatie en ook een terechte dankuwel voor uw inbreng in mijn onderzoek.

Voor de realisatie van de studies, ben ik erkentelijk voor de hulp van een bonte groep van onderzoekers en medewerkers. **Barbara, Valérie, Maarten** en **Isabel** hielpen mij goed op weg en bij hen kon ik steeds terecht met vragen. **Ann**, **Ipek** en **Wim** tackelden samen met mij de statistische analyses. Ann en Wim mag ik gerust mijn statistische mentoren noemen, waarvoor ik hen heel dankbaar ben. Ik leerde enorm veel bij. **Peter Van Rompaey, Els Ampe, Liese Vander Bruggen** en **Yannicke Gijbels** stonden klaar voor logistieke ondersteuning. Een speciaal woord van dank aan **dr. Jan Vanhove** om in mij en *mijn* onderzoek te geloven, aan **dr. Sarah Van Slycke** voor de vlotte samenwerking te Genk en aan **Elke Van de Casteele** voor het warme welkom in Antwerpen.

Volgende Mond-, Kaak- en Aangezichtsarts-specialisten in opleiding wil ik bedanken voor hun medewerking: **Koenraad, Jan, Pieter-Jan, Fréderic, Dries, Julie, Lisa, Philippe, Laury, Jannick, Jasper, Bert, Robin, Neil, Jeroen, Benjamin, Michaël, Liesbeth, Thomas A, Thomas D** en **Brecht**.

Bedankt aan de dienst Parodontologie, **Prof. dr. Christel Dekeyser, Prof. dr. Andy Temmerman, Prof. dr. Wim Teughels, Martine Pauwels, Ana C, Simón P, Simon C, Mihai** en **Rutger** om hun kennis met mij te delen en me met open armen te ontvangen. Ook **Mostafa** wil ik bedanken om me wegwijs te maken in het labo.

Ik bedank verder graag **Prof. dr. ir. Annabel Braem** en **prof. dr. ir. Nele Famaey** voor de samenwerking waarvan ik veel heb opgestoken, alsook **Julie Vastmans, Wouter Willekens**, **Kimberly Crevits** en **Heleen Fehervary** voor hun ondersteuning en aandeel in het experimenteel werk en de dataverwerking.

Bij uitbreiding bedankt aan alle chirurgen, assistenten, verplegend en administratief personeel en de deelnemende patiënten in UZ Leuven, Heilig Hart Leuven, Ziekenhuis Oost-Limburg, UZ Antwerpen, AZ Monica Antwerpen en AZ Monica Deurne. Zij maakten het allen mee mogelijk om dit onderzoek uit te voeren.

Verder ben ik heel gelukkig dat ik volgende personen tot mijn beste vrienden kan rekenen. **Elke,** *merci* voor de uitstapjes en de heerlijke babbels. **Annelotte, Barbara, Astrid, Laure, Anke, Eveline, Margo, Joëlle en Anne-Laure**, jullie zijn als een *Famille* voor mij, en zorgden de voorbije jaren voor veel geweldige momenten en welkome ontspanning.

Papa, mama, Lucas, Emilia, Victor en **Arthur**, jullie zijn de constante in mijn leven, en dat koester ik meer en meer. Heel erg bedankt voor het warme nest en de onvoorwaardelijke steun. Een extra dankjewel aan Emilia voor het coverontwerp van mijn doctoraatsboekje.

Tot slot, een dikke knuffel en een dankjewel aan **Michiel**. Je leerde me niet alleen de programmeertaal R, wat zijn vruchten afwierp voor de figuren en statistische analyses van dit doctoraat, maar had ook het geduld om samen grafieken te ontwerpen en wel honderd keer aan te passen. Dank je wel om altijd voor me klaar te staan. Bij jou vind ik rust, troost en plezier. Mijn thuis is waar jij bent.

Table of contents

List of abbreviations

CI	Confidence interval
DOAC	Direct oral anticoagulant
EDTA	Ethylenediaminetetraacetic acid
EHRA	European Hearth Rhythm Association
DMSO	Dimethyl sulfoxide
L-PRF	Leukocyte- and platelet-rich fibrin
NIHDI	National Institute for Health and Disability Insurance
NOAC	Non-vitamin K oral anticoagulant
PPP	Platelet poor plasma
RBC	Red blood cells
RCF	Relative centrifugation force
RIZIV	Rijksinstituut voor Ziekte- en Invaliditeitsverzekering
RPM	Rotations per minute
SD	Standard deviation
TF	Tissue factor
tPA	Tissue plasminogen activator
TXA	Tranexamic acid
uPA	Urokinase plasminogen activator
VAS	Visual analogue scale
VKA	Vitamin K antagonist

GENERAL INTRODUCTION

CHAPTER 1
General introduction, aims and hypotheses

Ockerman A[1]

[1]KU Leuven, Department of Imaging and Pathology, OMFS-IMPATH research group; and University Hospitals Leuven, Department of Oral & Maxillofacial Surgery, Leuven, Belgium.

This chapter was partly based on two reviews:

1. **Ockerman A**, Miclotte I, Vanhaverbeke M, Verhamme P, Poortmans LL, Vanassche T, Politis C, Jacobs R. Local haemostatic measures after tooth removal in patients on antithrombotic therapy: a systematic review. Clin Oral Investig. 2019;23(4):1695–708.

2. **Ockerman A**, Vanassche T, Garip M, Vandenbriele M, Martens J, Jacobs R, Politis C, Verhamme P. Tranexamic acid for the prevention and treatment of bleeding in surgery, trauma and bleeding disorders: a narrative review. Thromb J. 2021;19(1):1–16.

1. Bleeding and hemostatic management of patients treated with antithrombotic drugs in oral surgery

In the oral and maxillofacial practice, there is no consensus about the best management of patients treated with antithrombotic medication to obtain good hemostasis and to avoid bleeding complications after surgery. This concern has grown in importance in light of recent drug and treatment developments.

Two issues have arisen.

> **First**, a new type of oral anticoagulants, the non-vitamin K or direct oral anticoagulants (NOACs or DOACs), has entered the market and it is uncertain how to manage patients treated with these drugs and having teeth extracted.

Oral bleeding after dental extraction in these patients is a frequent problem and occurs in 20–25% of the cases.[1,2] Post-extraction bleeding often prompts unplanned medical contact and surgical re-interventions to stop the bleeding.[3] Bleeding events might also lead to unscheduled interruption of the NOAC treatment, so they become an important risk factor for subsequent and potentially life-threatening thromboembolic events.[3] To reduce the risk of post-extraction bleeding and thrombosis a well-considered anticoagulant and hemostatic management is key. The first part of this PhD will describe a randomized clinical trial that evaluates the effectiveness of a hemostatic mouthwash, additional to planning the dental extraction at trough level of the NOAC, in reducing bleeding in patients taking non-vitamin K oral anticoagulants.

> **Second**, variability of the morphology of leukocyte- and platelet-rich fibrin (L-PRF) membranes between patients was noted, which could have clinical implications. The reason of this variation is not yet fully understood.

L-PRF membranes are a bioactive surgical additive, composed of fibrin networks entrapping platelets and leukocytes, and are proven to boost wound healing and regeneration of both soft and hard tissues in oral surgery.[4,5] L-PRF can be used as socket filling after tooth extraction to limit the resorption of bone and to enhance socket healing[6], as well as hemostatic material to reduce bleeding.[7] To generate L-PRF, a few tubes of a patient's own blood are collected and immediately centrifuged. The contact

between the blood and the blood collection tubes activates the blood coagulation pathway.[8–11] The coagulation together with the centrifugation process, results in the formation of L-PRF clots. L-PRF clots are removed from the tubes and gently compressed to squeeze out the blood plasma, resulting in L-PRF membranes. Antithrombotic drugs that inhibit blood clotting processes, may as such affect the formation of L-PRF and, with that, the properties of L-PRF membranes. The second part of this PhD project describes two experimental studies evaluating some basic characteristics of L-PRF membranes between anticoagulant, antiplatelet and control groups. A first study evaluates the possible influence of the anticoagulant drug enoxaparin on L-PRF's properties *in vitro*. A second study compares the L-PRF membranes of patients on antiplatelets or anticoagulants to patients not taking these types of drugs.

2. The coagulation cascade and anticoagulant medication

Blood coagulation is the process of blood clotting and relies on the intrinsic, extrinsic and common pathways, resulting in the maturation of fibrin. Each step of the coagulation cascade involves a coagulation factor (abbreviated with F), a substrate and a cofactor, which are key players in a balanced system with several feedback loops.[12,13]

Intrinsic pathway

The intrinsic pathway is also known as the contact activation pathway. All factors involved in the intrinsic pathway are present in the blood. This pathway is activated in vivo by trauma inside the vascular system, through contact of the blood with negatively charged molecules as found on activated platelets, exposed endothelium, chemicals, or on collagen. Collagen is a structural protein of the blood vessel wall and it is exposed in case of blood vessel injury. This pathway can also be activated ex vivo, through contact of the blood with certain negatively charged surfaces, such as glass blood-collecting tubes or silica-coated plastic tubes. The trigger of the negatively charged molecules or surfaces activates FXII, leading to the sequential activation of FXI and FIX (Figure 1.1). FIXa ('a' stands for activated) together with its cofactor FVIIIa then activates FX to FXa.[12,13] Factor Xa is the first factor of the common pathway.

Extrinsic pathway

The extrinsic pathway is also called the tissue factor (TF) pathway, as this factor is the initiator of the cascade (Figure 1.1). TF is not present in the blood without trauma, but is secreted by damaged cells after vascular damage and is then exposed to the circulating coagulation factor VII. Together these factors form the TF-FVIIa complex that further activates factors IX and X to Xia and Xa.[12,13]

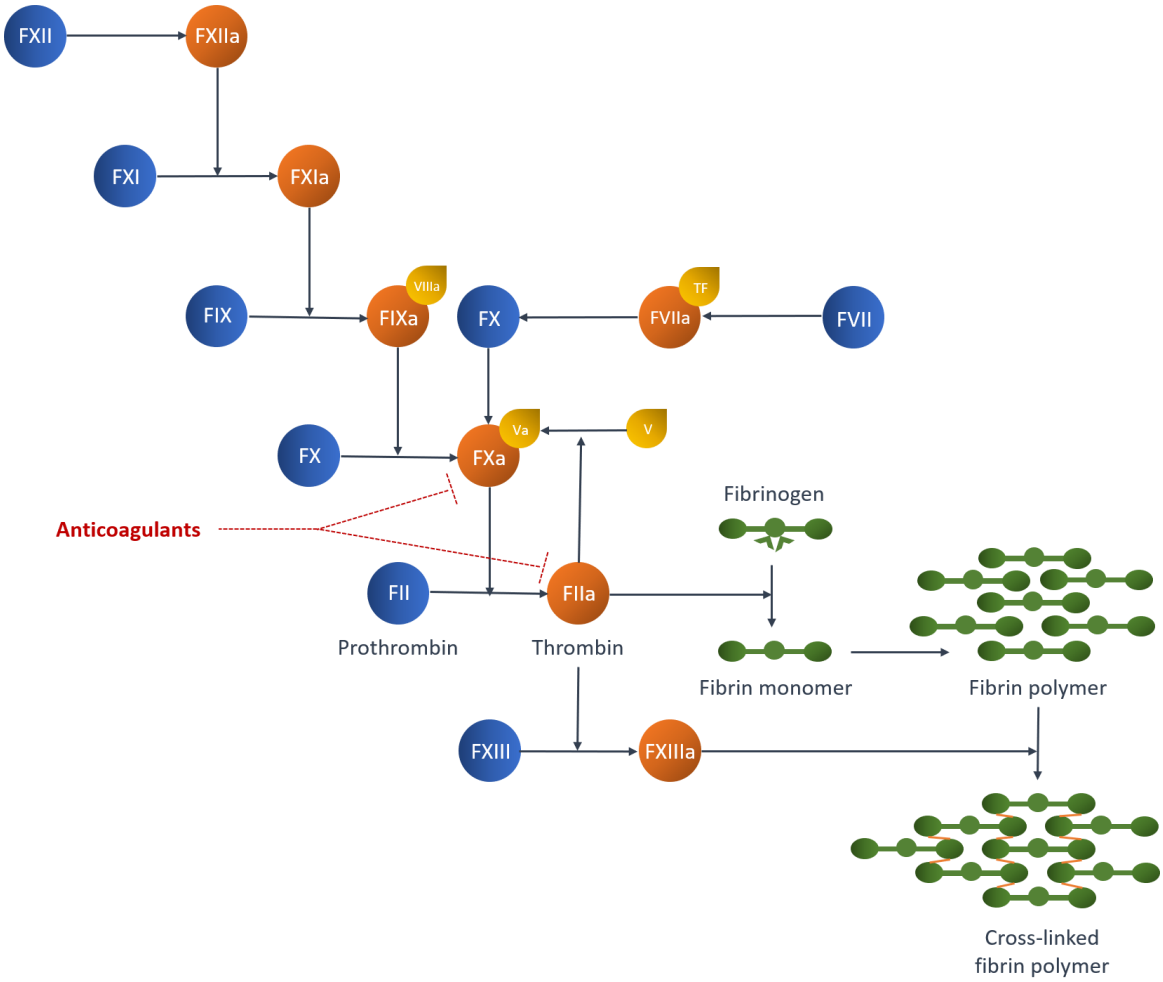

Figure 1.1. The coagulation pathways resulting in fibrin clot formation. The blood coagulation is initiated through contact of the blood with negatively charged molecules or surfaces (intrinsic pathway) or by the release of tissue factor (extrinsic pathway). Inactive factors are presented in blue, active ones in orange, and cofactors in yellow. Anticoagulants inhibit either FXa (apixaban, edoxaban, rivaroxaban), or FIIa (dabigatran), or both (heparins, such enoxaparin and fraxiparin).

Common pathway

FXa, at its turn, forms a complex with its cofactor FVa (called the prothrombinase complex) that catalyzes the cleavage of prothrombin (FII), bound to glycoprotein IIa/IIIa on the surface of activated platelets, to thrombin (FIIa) (Figure 1.1). Thrombin then

converts fibrinogen to fibrin monomers that spontaneously polymerize to fibrin polymers. Thrombin also activates FV, that further stimulates the conversion of FX to FXa, and FXIII to FXIIIa, which forms covalent bonds that crosslink the fibrin polymers.[12,13]

Anticoagulant medication

Anticoagulants (or blood thinners) inhibit the coagulation of the blood and exist in injectable and oral formulations.

The most widely used injectable anticoagulant is heparin and exist in three forms: unfractionated heparin (UFH), low molecular weight heparin (LMWH) and ultra-low-molecular weight heparin (ULMWH). It can be injected intravenously or subcutaneously. Heparin binds to and stimulates antithrombin, an inhibitor enzyme that inactivates thrombin, FXa and other proteases.[14] Heparin must be given frequently or as a continuous infusion because of its short half-life, and is usually only used to treat acute coronary syndromes, to prevent thrombotic complications after surgery, or in hospitalized patients.

The two types of oral anticoagulants are vitamin K antagonists (VKAs) and non-vitamin K or direct oral anticoagulants (NOACs or DOACs). Both are being prescribed for prevention of stroke in patients with atrial fibrillation, treatment and prevention of pulmonary embolism and deep venous thrombosis, and prevention of mechanical heart valve thrombosis. VKAs, standard of care for many years, inhibit hepatic synthesis of clotting factors II, VII, IX and X. NOACs, on the other hand, directly inhibit FXa or FIIa (Table 1.1).

Table 1.1. Characteristics of the non-vitamin K oral anticoagulants.

	Rivaroxaban (Xarelto®)	Apixaban (Eliquis®)	Edoxaban (Lixiana®)	Dabigatran (Pradaxa®)
Working mechanism	FXa inhibitor	FXa inhibitor	FXa inhibitor	FIIa inhibitor
Standard dose*	20 mg qd	5 mg bd	60 mg qd	150 mg bd
Reduced dose	15 mg qd	2.5 mg bd	30 mg qd	110 mg bd
Half-life (h)	5–9	12	10–14	12–17
Renal elimination (%)	35	27	35	80

*Standard dose in atrial fibrillation, with qd = once daily, bd = twice daily.

NOACs currently on the market are rivaroxaban (Xarelto®), apixaban (Eliquis®), edoxaban (Lixiana®) and dabigatran (Pradaxa®).[15–18] These were proven to be either equivalent to or more effective than VKAs in stroke prevention, with an acceptable risk of bleeding and a superior overall safety profile compared to VKAs.[19,20] Other advantages of NOACs over

VKAs include a more rapid onset and offset of action, fewer drug interactions, no influence by dietary intake of vitamin K and no requirement of routine coagulation monitoring.[20] Because of these advantages, NOACs have become the recommended therapy for most patients with atrial fibrillation and venous thromboembolism.

The prevalence of atrial fibrillation, the main indication for which NOACs are prescribed, has doubled in the last decade and increases with age.[21] It ranges from 4% in patients aged 60–70 years up to 15% in patients aged 80 years and older.[21] In Europe, it is estimated that the number of new cases of atrial fibrillation will be between 120,000 and 215,000 per year, which equals to 14–17 million people with atrial fibrillation by 2030.[21]

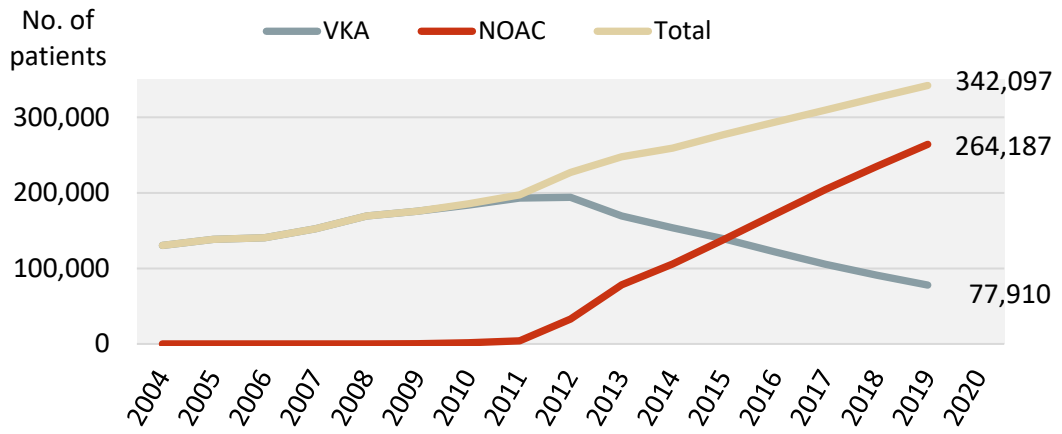

Figure 1.2. RIZIV numbers of Belgian patients treated with oral anticoagulants from 2004 to 2019. Since 2015, there are more patients treated with non-vitamin K oral anticoagulants (NOACs) than with vitamin K antagonists (VKAs). In 2019, about 342,000 patients were treated with oral anticoagulants of who ±77,900 were treated with VKAs and more than 264,000 were taking NOACs. The number of patients treated with NOACs is still increasing.

Because the increasing incidence of atrial fibrillation, and as the aging population is growing bigger, the number of patients treated with NOACs is increasing as well (Figure 1.2). Numbers from the National Institute for Health and Disability Insurance (NIHDI or Rijksinstituut voor ziekte- en invaliditeitsverzekering, RIZIV) show that from 2015 onwards, there were more patients treated with NOACs than with VKAs in Belgium, and that in 2019 the number exceeded 264,000 patients.[(A)] Since many of these elderly patients need dental care, often repeatedly, the results of the study presented in this PhD manuscript are applicable to a very large patient population.

(A) Numbers were requested from NIHDI/RIZIV in October 2020.

3. Oral bleeding after dental extraction in patients on non-vitamin K oral anticoagulants is a frequent problem

On a yearly basis, about 18,000 patients treated with NOACs have to undergo a dental extraction and these patients are at increased risk of bleeding after this dental procedure compared to patients who do not take anticoagulants.[1,2] A meta-analysis on the risk of post-extraction bleeding pointed out that patients on NOACs have a three times higher risk (risk difference of 3.04, 95% confidence interval (CI) 1.31–7.04) than patients not taking NOACs (22). The incidence of post-extraction bleeding in these patients is 15–20%, corresponding to 3,500 to 4,000 patients per year in Belgium.[1,2,23] Moreover, bleeding often result in patients re-consulting a dentist or a surgeon, even some of these patients require a surgical intervention to stop the bleeding, which is a burden for both the patients and healthcare services. Considering the high number of patients treated with NOACs and the high need for dental interventions, the immediate cost associated with medical care for bleeding events is thus high.

Importantly, bleeding events also lead to an increased risk of possibly life-threatening thrombotic complications, as bleeding may prompt the interruption of anticoagulant therapy. Hence, a safer periprocedural management will also help to reduce the risk of thrombotic events. Although less frequent, the morbidity and costs of the thromboembolic events are extremely high.

4. There is no consensus about the management of patients on non-vitamin K oral anticoagulants in the dental practice

A much-debated question is whether oral anticoagulants should be interrupted before oral surgery. On the one hand, continuing the medication increases the risk of bleeding during and after surgery, while on the other, interrupting the medication exposes the patient to an increased risk of thrombosis.[24]

The **perioperative management of VKAs** is not simple because VKAs have a long half-life, interact with several other drugs and are influenced by dietary vitamin K intake. Temporary interruption of VKA treatment confers both a thrombotic and a bleeding risk, due to the slow offset and onset of VKAs.[3] For minor oral surgery such as dental

extractions, previous research has pointed out that patients can continue their VKAs, provided the international normalized ratio is within the therapeutic range, as this confers no increased risk of bleeding compared to discontinuing or altering the dose of VKAs.[25]

The **perioperative management of NOACs** is easier than VKAs, because of the shorter half-life, rapid onset of action, and less drug-drug or drug-food interactions. However, it is known that the risk of mucosal bleeding is higher in patients taking NOACs compared to VKAs.[26] As the oral mucosa is inevitably damaged during dental extraction, post-extraction bleeding is a particular concern in NOAC-treated patients. Nevertheless, there is currently no consensus about how to minimize post-extraction bleeding risk in this patient population.

Patient's profile		Day before extraction		Day of extraction		Day after extraction	
NOAC regimen	Timing dental extraction	morning	evening	morning	evening	morning	evening
1x/d morning (Xarelto®/Lixiana®)	Before or afternoon	✓		✗		✓	
1x/d evening (Xarelto®/Lixiana®)	Before noon		✗		✓*		✓
	Afternoon		✓		✓*		✓
2x/d (Pradaxa®/Eliquis®)	Before noon	✓	✗	✗	✓*	✓	✓
	Afternoon	✓	✓	✗	✓*	✓	✓

Figure 1.3. NOAC management for planning minor oral procedures.[27] A green tick mark indicates that the patient can take the NOAC dose, whereas a red cross indicated that it is not advised to take the NOAC dose. A tooth extraction can take place 18 to 24 hours after the last NOAC dose, and the first NOAC after the procedure should be taken at least 6 hours after surgery (*), provided good hemostasis is achieved.

The European Hearth Rhythm Association (EHRA) formulated advice on the management of NOACs in patients having a surgical intervention.[27] The EHRA guideline intends to provide a unified approach, that is as simplified as possible, to allow its broad implementation.[27] For dental extractions or periodontal surgery, they advise to plan the intervention at NOAC trough level, or 18 to 24 hours after the last NOAC intake (Figure 1.3).[27] This recommendation can be brought into practice by withholding one NOAC dose. Considering the rapid onset of action of NOACs, the first NOAC should be taken at least 6

hours after surgery.[27] Taking a NOAC dose earlier would expose the patient to a high anti-coagulant activity promptly to the procedure, increasing the bleeding risk. Of course, the patient's risk profile for bleeding and thrombotic complications should always be considered while planning surgical interventions.

5. Tranexamic acid may be a good hemostatic agent to reduce bleeding

Beside the anticoagulant management, the bulk of the literature emphasizes the importance of adequate local hemostatic measures after minor oral surgery. Effective hemostatic reduce both post-extraction bleeding complications and inappropriate anticoagulant discontinuation following bleeding. We conducted a systematic review of 15 articles to investigate which local hemostatic measures are effective for preventing post-operative bleeding in patients continuing oral antithrombotics.[28] Following hemostatic methods were studied: sutures, gauze (pressure), gelatin or collagen sponges, fibrin glues or adhesives, mouthwashes, and wax or bone grafting materials.[28] Most studies failed to show significant differences between the various hemostatics, but overall the reported bleeding events were low.[28] In patients treated with vitamin K antagonists, tranexamic acid mouthwash significantly reduced bleeding compared to placebo.[28] Because all studies included in our review compared different hemostatic measures, it was difficult to point out which hemostatic treatment is optimal.

A great deal of the research (8 out of the 15 studies) on hemostatic agents focused on tranexamic acid (TXA)[28], an antifibrinolytic agent that reversibly inhibits plasminogen, preventing plasmin from degrading fibrin TXA (note 1.1 and Figure 1.4).

> **Note 1.1. The working mechanism of tranexamic acid.** The conversion of plasminogen to plasmin is mediated by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Plasminogen contains a kringle domain and a serine protease-binding domain. The kringle domain consists of five loops or kringles of triple disulfide-linked peptide regions with lysine-binding sites.[29] Via these lysine-binding sites, plasminogen binds to lysine residues on fibrin. The binding of plasminogen to fibrin is stabilized by tPA, that binds to both plasminogen and fibrin. This is called the ternary complex and makes plasminogen activation very efficient. uPA on the other hand, does not stabilize the binding of plasminogen to fibrin. Therefore, uPA cannot activate plasminogen as

efficiently as tPA.[29–31] The protease-binding domain of plasminogen contains a peptide bond. Both tPA and uPA cleave this peptide bond and stimulate the conversion of plasminogen to plasmin. Active plasmin then cuts the cross-linked fibrin strands into fibrin degradation products, resulting in the breakdown of blood clots.[29,30] TXA is a lysine analogue that impedes the binding of plasminogen to fibrin by blocking (the kringles of) plasminogen. The ternary complex cannot be formed anymore. This blockage leads to a powerful inactivation of tPA-driven plasminogen activation. In case of uPA, this mechanism is less efficient, simply because uPA does not bind to fibrin. The inhibition of the conversion of plasminogen to plasmin impairs the process of fibrinolysis and stabilizes the blood clot.

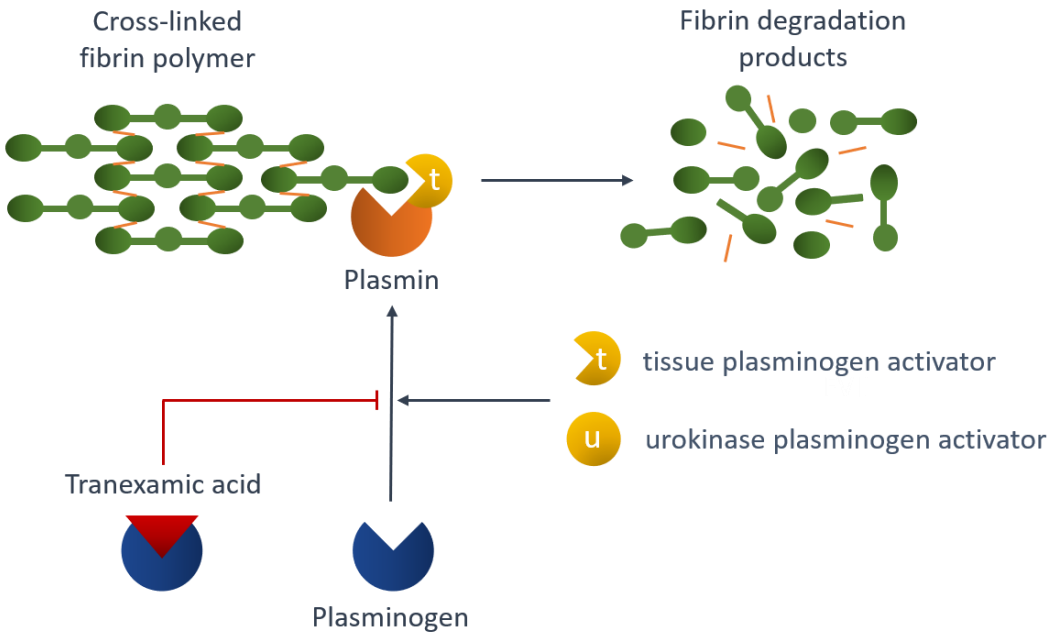

Figure 1.4. Fibrinolysis and its inhibition by tranexamic acid. Plasminogen is cleaved to plasmin by both the tissue plasminogen activator (tPA or t) and the urokinase plasminogen activator (uPA or u). Tranexamic acid binds to plasminogen, inhibiting its conversion to plasmin, so that plasmin cannot break down the fibrin clot.

TXA is widely used for the prevention and treatment of hemorrhage in trauma and several types of elective surgery (Figure 1.5).[32] TXA is also used in patients with underlying bleeding disorders and in patients who take antithrombotic drugs. Because of the importance of TXA in the management of bleeding, the World Health Organization has added TXA to the list of essential medicines.[33]

The FDA approved the oral administration of TXA to prevent bleeding in patients with hemophilia undergoing dental extraction. However, there is an increased interest in topical administration of TXA as systemically administered TXA (such as oral tablets) may results in prothrombogenic effects in an unselected patient population or accumulation of TXA in case of renal insufficiency. After all, systemic administration of TXA leads to high plasma levels (7µg/mL) without detectable levels in the saliva, while TXA mouthwash leads to sustained therapeutic TXA levels in the saliva, with minimal systemic exposure (plasma levels below 2µg/mL).[34] Therefore, the use of TXA mouthwash is considered safe without evidence for prothrombotic effects and is an attractive hemostatic agent after dental extraction.[35] Moreover, two studies demonstrated that TXA mouthwash significantly reduced bleeding in patients on vitamin K antagonists compared to placebo.[36,37] Its use in NOAC-treated patients, however, has never been studied.

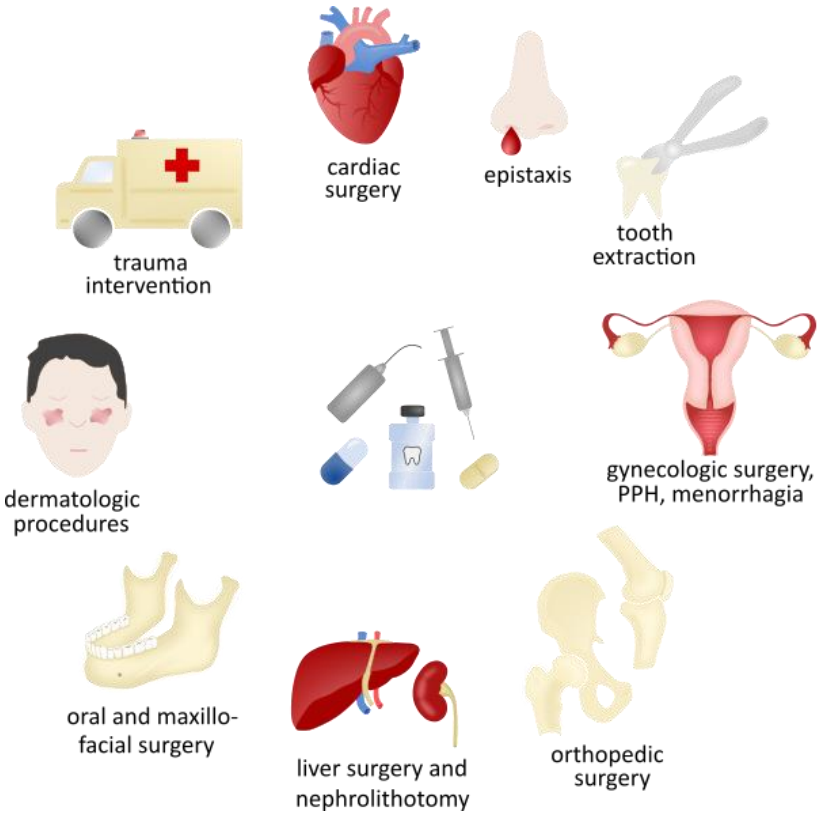

Figure 1.5. Tranexamic acid is used in multiple formulations to treat or prevent bleeding for various indications. TXA can be administered as a mouthwash, orally, topically or intravenously. PPH is postpartum hemorrhage.

The existing literature on NOAC-treated patients having oral surgery is limited and many of the published studies are case-series or retrospective case-control studies, often lacking information on NOAC discontinuation and on the use of local hemostatic measures.[38] The question thus remains how to reduce the bleeding risk after dental extraction in patients on NOACs. We have therefore set-up a randomized clinical trial to evaluate if the use of TXA mouthwash after dental extraction reduces bleeding in patients treated with NOACs. The intervention is pragmatic, low-cost, and readily available, with no important known side-effects. Once proven beneficially and applied in oral practice, this strategy is likely to result in a very favorable cost-benefit profile and therefore very appealing.

6. Natural guided healing techniques on the rise

The development of bioactive surgical additives, based on blood components, such as fibrin and platelets, releasing a wide range of signaling molecules and growth factors, is a major area of interest within the field of surgery. In oral surgery, leukocyte- and platelet-rich fibrin, a second-generation platelet concentrate, has received considerable attention as it was proven to be beneficial for enhancing healing and regeneration of soft and hard tissue, stimulating immune processes and minimizing bleeding.[11,39,40]

A short history of platelet concentrates

A first product described as biological adhesive is fibrin glue, also known as fibrin sealant or fibrin tissue adhesive. It is formed by polymerizing fibrinogen with the aid of thrombin and calcium.[5,8] Fibrin glues can be obtained commercially by industrial fractionation of pools of donated plasma, carrying a small risk of disease transmission, or autologously from the patient's blood.[5,8] However, the quality of autologous fibrin glue is rather low because of the low concentrations of fibrinogen in the plasma.[5,8] Further, the use of fibrin glue remains limited due to the complexity and the cost of its production protocol.

Later, fibrin glue optimization led to the development of platelet-rich plasma products. Platelets contain high concentrations of growth factors that play an important role in cell proliferation, matrix remodeling, and angiogenesis. Because of high commercial interests

in platelet-rich plasma products, a wide range of preparation protocols, kits and centrifuges have emerged. All protocols for generating platelet-rich plasma are based on a common technique, aimed at concentrating the platelets by two centrifugation cycles and triggering the activation of the platelets, so that they release growth factors, and at fibrin polymerization by adding thrombin and calcium chloride.[5,8]

Leukocyte- and platelet-rich fibrin membranes

After some time, because of legal restrictions on biomechanical blood handling, another type of platelet concentrate was introduced: leukocyte- and platelet-rich fibrin. L-PRF is derived from the patient's own blood and is composed of a fibrin network entrapping platelets and leukocytes.[5,8,9,41]

Contrary to other platelet concentrates, there is no need for biochemical modification of the blood for generating L-PRF, this means, no anticoagulants, thrombin or calcium chloride are required. Consequently, L-PRF is 100% autologous and its biosafety is therefore assured. Other advantages of L-PRF over preceding platelet concentrates are its availability and its simple and cost-effective preparation.[9,41] L-PRF was proven capable of favoring healing and boosting the immune system.[11,39,42] L-PRF is currently being used in oral surgery as socket filling after tooth extraction to limit the resorption of bone, to enhance socket healing[6], to reduce pain and swelling afterwards, especially after third molar extraction[43], and/or as hemostatic material to prevent bleeding.[7,40,44] L-PRF can also be applied during sinus lifting and for improving the stability of implants.[11,45,46]

For preparing L-PRF, a few patient blood samples are taken and immediately centrifuged. The contact of the blood with the negatively charged surface of the glass or silica-coated plastic blood tubes activates the intrinsic (or contact) coagulation pathway (Figure 1.1). The centrifugation process, at its turn, leads to the separation of platelet-poor plasma at the top of the tube, fibrin, leukocytes and platelets in the middle, and a layer of red blood cells at the bottom of the tube (Figure 1.5).[5,8,9,41] The fibrin network polymerizes naturally and slowly, and entraps the leukocytes and platelets.[5] This way, the coagulation and centrifugation processes together lead to the formation of an L-PRF clot in the middle of the tube. The L-PRF clot can be easily collected with tweezers and gently compressed to generate an L-PRF membrane.

7. General characteristics of leukocyte- and platelet-rich fibrin membranes

L-PRF membranes can be divided in two areas: the body and the face (Figure 1.5). The body is the largest, whitish part of the membrane mainly containing platelets, and the face is the red area which is full of leukocytes (and some platelets as well), and is bordered with red blood cells, as this was the part of the L-PRF clot neighboring the red blood cells in the blood tube.[47,48]

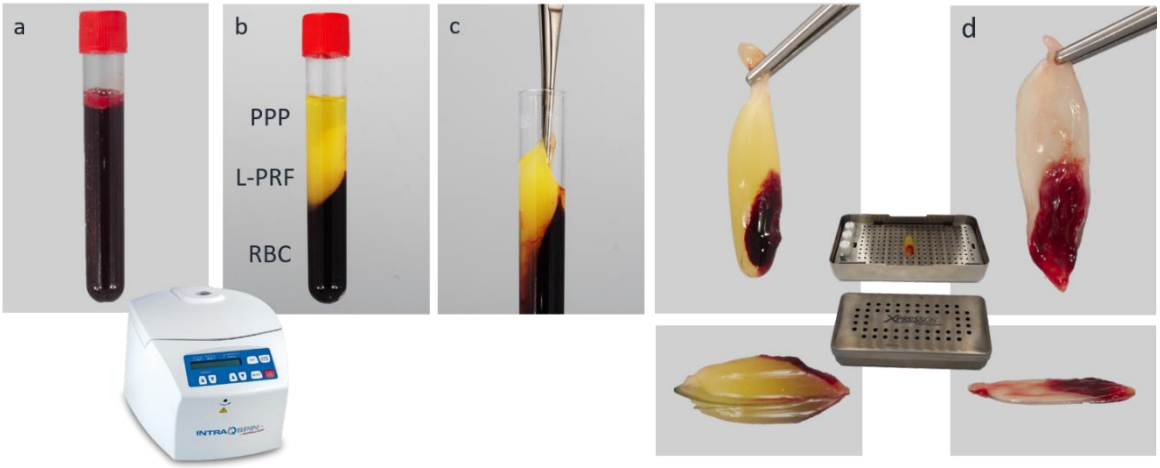

Figure 1.5: L-PRF generation process. (a) After blood collection, the blood tubes are immediately centrifuged. (b) After centrifugation, three layers can be distinguished: platelet poor plasma (PPP) at the top, a leukocyte- and platelet-rich fibrin (L-PRF) clot in the middle, and red blood cells (RBC) in the bottom of the tube. (c) The L-PRF clot is taken out with tweezers and placed in an L-PRF box to gently compress by aid of a lit. In the box, blood plasma is squeezed out of the clot and an L-PRF membrane is formed. (d) The red part of the membrane is called 'the face' and the whitish part is called 'the body'.

Some studies examined the cell content of L-PRF and showed that the membranes contained up to 80% platelets and 50–70% leukocytes compared to the initial blood of patients.[48,49] This is a high percentage and it is important because the platelets and leukocytes release a great number of growth factors and cytokines, which play an important role in wound healing.[49,50]

L-PRF is further composed of a polymerized fibrin matrix that acts as a scaffold entrapping the platelets, leukocytes and cytokines. The fibrin matrix results from a slow and physiological fibrin polymerization during L-PRF generation.[4,51,52] The reason of this slow

polymerization is that only the autologous thrombin (there is no bovine thrombin addition) acts on the fibrinogen present in the blood sample.[4,51,51] The resulting fibrin network consists of equilateral junctions, allowing platelet and leukocyte enmeshment and migration, and gives great elasticity to the fibrin matrix.[4,51,52] On the contrary, adding bovine thrombin would result in fast polymerization and the formation of bilateral junctions, impeding cell enmeshment.[4,51,52]

Some studies on the mechanical properties of L-PRF membranes pointed out that L-PRF membranes are capable of deforming significantly as they only rupture after being stretched to more than two times their initial length.[42,43,48] L-PRF membranes are also quite strong: it is estimated that an average membrane can withstand forces up to 1.5 Newton, which is the equivalent of applying a load of ±150 grams.[53,55,57,58] Last, L-PRF membranes showed elastic moduli between 0.01 MPa and 0.5 MPa[53,54,57,58], which is a large range and comparable to other human soft tissues, such as the skin (0.1 to 0.6 MPa) and tendons (±0.03 MPa).[59,60] However, all the studies had different protocols for blood centrifugation and tensile testing, making any comparison difficult.

Studies on the mechanical characterization of L-PRF membranes are rather scarce, yet important because they teach us how elastic or stiff and how weak or strong these membranes are. Indeed, it is difficult to evaluate the fibrin network through imaging (alone). Having knowledge about the mechanical properties thus makes it possible to compare membranes in a quantified way, and not only visually, of various patient populations or generated by various protocols or centrifuges.

8. Some factors can influence the generation and properties of leukocyte- and platelet-rich fibrin membranes

There are various protocols and centrifuges for generating L-PRF.[61] These protocols and the centrifugation characteristics determine the composition of L-PRF membranes.[62] Recent evidence suggests that each centrifuge is characterized by its own vibration profile depending on the rotational speed, and together with the timing of centrifugation, it directly impacts the architecture of the fibrin network, the cell content and the growth factor release of L-PRF clots.[62,63]

Each centrifugation protocol is characterized by three parameters: speed of spinning (rotations per minute or RPM), g-force of the centrifuge (relative centrifugation force or RCF), and time of spinning (in minutes). For each centrifuge, the following formula applies:

$$RCF = 11.18 * r * (\frac{RPM}{1000})^2$$

with r equals to the radius from the center of the centrifugation rotor to the sample. It is shown that various centrifuges produce heavier and longer membranes with a strongly polymerized fibrin matrix or lighter and shorter membranes with a lightly polymerized fibrin matrix.[62] One study showed that reducing the speed and time increases the number of platelets in the L-PRF membranes, but decreases the density of the fibrin network.[63] Following the original L-PRF preparation protocol, blood samples should be centrifuged at an RCF of 408g for 12 minutes.[64] Because of their impact, the use of the specific centrifuge and centrifugation protocol should be clearly described in study protocols and clinical guidelines to avoid confusion and inaccurate results.

Further, the time between blood draw and centrifugation also plays an important role. The longer the time interval, the more the fibrin will polymerize in a diffuse way[5] and the shorter the L-PRF membrane will be.[47,67] The recommended interval time to obtain standard-sized L-PRF membranes is 60–90 seconds.[47]

Besides protocol-related factors that can affect L-PRF, there are important patient-related factors that should be considered.

A few studies on the influence of age on L-PRF characteristics showed a decrease in density, a more irregular arrangement of the fibrin network, and a reduced number of platelets and leukocytes in L-PRF membranes with increasing age.[63,66] Another study showed that females and elderly patients produced larger membranes, although the latter finding was not significant.[47] The larger membranes might be explained by the lower levels of hematocrit (ratio of the volume of red blood cells to the total volume of blood): the less red blood cells in the blood collection tube, the more of other blood products there are present, of which L-PRF can be formed.[47]

Surprisingly, the effects of antithrombotic medication on the formation and properties of L-PRF has not yet been studied. As L-PRF is dependent on the coagulation process of blood, anticoagulants drugs that interfere with the coagulation pathway or antiplatelets that inhibit platelet aggregation may have an important impact on this biomaterial.

As wound healing largely depends on the fibrin structure and the cell concentration[67], it may be that diverse properties of L-PRF membranes have important implications for their use in clinic. Ergo, it is important to determine if and which factors affect the cell content, fibrin network and mechanical characteristics of L-PRF membranes. In this PhD manuscript, I will focus on the potential influence of antithrombotic medication. Therefore, we have set-up two experiments to evaluate the mechanical properties and cellular content of L-PRF membranes and the possible influence of antithrombotics on these properties. In the future, we hope that our results will contribute to the management of L-PRF in patients taking anticoagulants. Understanding the impact of protocol- and patient-related factors on L-PRF will also lead to advanced L-PRF techniques.

Aims and hypotheses

Bleeding is a common complication after dental extraction in patients treated with oral anticoagulants and there is no consensus about the best strategy to obtain good hemostasis and to prevent bleeding. More specifically, **this PhD project focused on two concerns**.

First, oral bleeding after dental extraction is frequent in patients treated with non-vitamin K oral anticoagulants. Post-extraction bleeding often prompts unplanned medical contact and surgical re-interventions to stop the bleeding. Bleeding may also lead to unscheduled interruption of the NOAC treatment, which is an important risk factor for subsequent and potentially life-threatening thromboembolic events.

We therefore have set up a multicenter, randomized, double blind, placebo-controlled, clinical trial to evaluate if the use of tranexamic acid mouthwash after dental extraction, additional to planning the intervention at NOAC trough level, reduces bleeding in patients taking non-vitamin K oral anticoagulants.

> The **hypothesis** was that the use of a tranexamic acid mouthwash after dental extraction in patients treated with non-vitamin K oral anticoagulants would reduce post-operative bleeding.
>
> The **aim** of this trial was to implement a safer peri- and post-operative strategy for reducing bleeding for patients treated with NOACs and undergoing dental extraction, and to implement this strategy in current practice and clinical guidelines.

Second, leukocyte- and platelet-rich fibrin membranes are used to enhance healing and as hemostatic material with oral surgery. As the formation of a fibrin clot is crucial for the generation of L-PRF, it may be that antithrombotic drugs affect the properties of L-PRF membranes, possibly having clinical implications. Antithrombotic drugs both interfere with coagulation (anticoagulants) and platelet aggregation (antiplatelets) processes, interfering with the formation of fibrin, so that both the fibrin network plus its mechanical

properties and the cells that the network entraps may be affected. Therefore, we have set up two experimental studies to examine the mechanical characteristics and cell content of L-PRF membranes between anticoagulated and non-anticoagulated blood. In a first experiment, we supplemented blood samples with the anticoagulant drug heparin. In a second experiment, we compared blood samples from patients treated with anticoagulants or antiplatelets and patients not taking these drugs.

The **hypothesis** was that the mechanical properties and cellular content of leukocyte- and platelet-rich fibrin membranes from blood supplemented with or from patients taking antithrombotic drugs would be different from those of controls.

The **aim** of these experiments was to improve our knowledge about the properties of L-PRF membranes and the possible influence of antithrombotics on these properties, which has never been studied before. We believe that this will lead to the optimization of both L-PRF's generation and its use in clinic.

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TRANEXAMIC ACID TO REDUCE BLEEDING

CHAPTER 2

Tranexamic acid to reduce bleeding in patients treated with non-vitamin k oral anticoagulants undergoing dental extraction

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This research chapter was adapted from:

1. **Ockerman A**, Vanhaverbeke M, Miclotte I, Belmans A, Vanassche T, Politis C, Jacobs R, Verhamme P. Tranexamic acid to reduce bleeding after dental extraction in patients treated with non-vitamin K oral anticoagulants: **design and rationale** of the EXTRACT-NOAC trial. Br J Oral Maxillofac Surg. 2019;57(10):1107–12.

2. **Ockerman A**, Miclotte I, Vanhaverbeke M, Vanassche T, Belmans A, Vanhove J, Meyns J, Nadjmi N, Van Hemelen G, Winderickx P, Jacobs R, Politis C, Verhamme P. Tranexamic acid and bleeding after dental extraction in patients treated with non-vitamin k oral anticoagulants: the EXTRACT-NOAC **randomized clinical trial**. Plos Med. 2021;18(5):e1003601.

Abstract

Background: Oral bleeding after dental extraction in patients on non-vitamin K oral anticoagulants (NOACs) is a frequent problem. We investigated whether 10% tranexamic acid (TXA) mouthwash decreases post-extraction bleeding in patients treated with NOACs.

Methods: The EXTRACT-NOAC study is a randomized, double blind, placebo-controlled, multicenter, clinical trial. Patients were randomly assigned to 10% TXA or placebo mouthwash, and were instructed to use the mouthwash prior to dental extraction, and three times a day for three days thereafter. The primary outcome was the number of patients with post-extraction oral bleeding up to day 7. Secondary outcomes included peri-procedural bleeding (0 to 10 VAS scale) and early and delayed bleeding.

Results: Of 222 randomized patients, 218 patients were included in the full analysis set of which 106 patients were assigned to TXA (74.8 (±8.8) years; 81 men) and 112 to placebo (72.7 (±10.7) years; 64 men). Post-extraction bleeding occurred in 28 (26.4%) patients in the TXA group and in 32 (28.6%) patients in the placebo group (relative risk, 0.92; 95% confidence interval [CI], 0.60 to 1.42; p=0.72). There were 46 bleeds in the TXA group and 85 bleeds in the placebo group (rate ratio, 0.57; 95% CI, 0.31 to 1.05; p=0.07). TXA did not reduce the rate of peri-procedural bleeding (bleeding score 4±1.78 versus 4±1.82, p=0.80) and early bleeding (rate ratio 0.76; 95% CI, 0.42 to 1.37). Delayed bleeding (rate ratio, 0.32; 95% CI, 0.12 to 0.89) and bleeding after multiple extractions (rate ratio, 0.40, 95% CI, 0.20 to 0.78) were lower in the TXA group. The main study limitation was the premature interruption of the trial following a futility analysis.

Conclusion: In patients on NOACs undergoing dental extraction, tranexamic acid does not seem to reduce the rate of peri-procedural or early post-operative oral bleeding compared to placebo. Tranexamic acid appears to reduce delayed bleeds and to reduce post-operative oral bleeding if multiple teeth are extracted.

Introduction

Oral bleeding is a frequent complication after dental extraction in anticoagulated patients and may occur in up to 25% of these patients.[1,2] A meta-analysis on the risk of post-extraction bleeding pointed out that these patients have a three times higher risk of bleeding than patients not taking anticoagulant drugs.[3] Bleeding often results in patients re-consulting a dentist or oral and maxillofacial surgeon and may require a reintervention. These unplanned visits to the dental practice or hospital increase healthcare costs. In addition, if the anticoagulant therapy is interrupted, bleeding may turn into a risk factor for thrombotic events.

Although the number of patients treated with non-vitamin K oral anticoagulants (NOACs) is increasing, data on how to prevent and manage bleeding in patients undergoing dental extractions is limited.[4] Moreover, evidence from clinical trials shows a potential increased risk of mucosal bleeding in certain NOAC regimens.[5] Guidelines advise performing dental extraction at trough level of a NOAC, which can be implemented by skipping a NOAC dose on the morning of the day of the dental extraction.[6] This strategy appeared to be safe in a recent prospective pilot study, although there was a signal towards excess delayed bleeding compared to non-anticoagulated patients.[1] Based on these preliminary findings, the current clinical trial was designed.

Tranexamic acid (TXA) is an attractive hemostatic agent for oral surgery as it can be applied locally, resulting in low systemic absorption.[7,8] Previous research has shown that the use of TXA mouthwash is effective in decreasing bleeding after dental extraction in patients treated with vitamin K antagonists, but its use in NOAC-treated patients has not yet been studied.[9,10]

The current interventional EXTRACT-NOAC study was designed to assess whether a 10% TXA mouthwash reduces bleeding after dental extraction in patients on NOACs.

Methods

The EXTRACT-NOAC study is a prospective, randomized, double blind, placebo-controlled, multicenter, investigator-initiated, clinical trial. The study obtained written consent by the

Ethics Committee Research of the University Hospitals of Leuven in July 2017 (S60131). The trial is registered at ClinicalTrials.gov (Identifier: NCT03413891).

Patients

Patients older than 18 years were eligible if they were treated with a NOAC (rivaroxaban, apixaban, edoxaban or dabigatran), were scheduled for a dental extraction, and provided informed consent. Patients who were pregnant or lactating and patients who had a known allergy for tranexamic acid were excluded.[11] Patients visiting the oral and maxillofacial departments of the following Belgian hospitals were screened for eligibility: University Hospitals Leuven, Regional Hospital Heilig Hart Leuven, General Hospital St-Jan Genk and AZ Monica Antwerp.

Randomization and study procedures

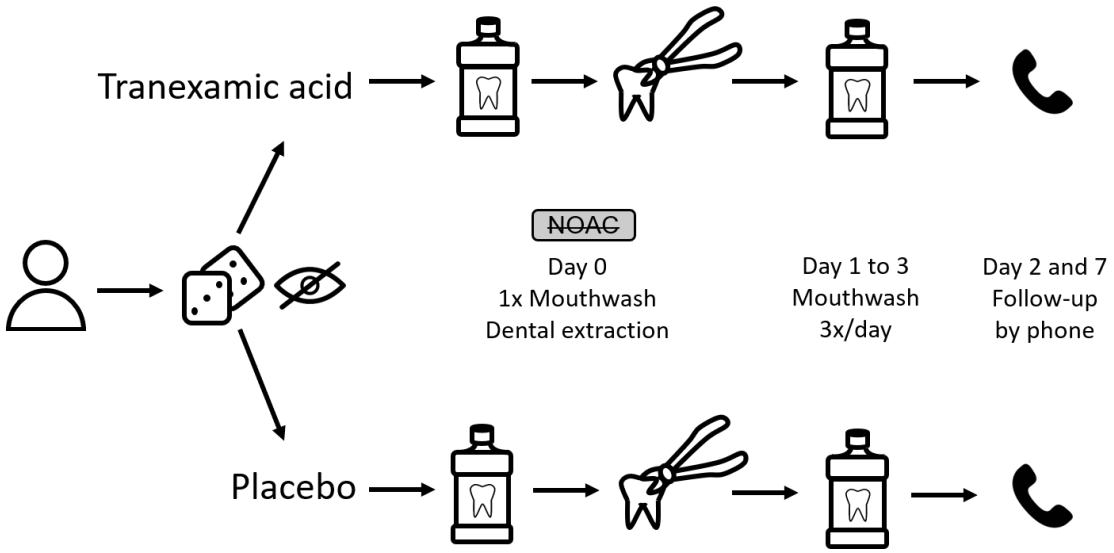

Figure 2.1. Study design of the EXTRACT-NOAC study. After informed consent, patients were randomized to TXA mouthwash or to placebo mouthwash. Patients were instructed to use the mouthwash immediately prior to the dental extraction (day 0), and thereafter three times a day for three days (day 1 to 3). Patients were contacted on day 2 and day 7 after the dental extraction to assess all outcomes and their compliance with the study protocol.

All patients were instructed to skip the morning dose of their NOAC on the day of the dental extraction, in agreement with the European Hearth Rhythm Association (EHRA) guidelines and as previously validated by our group.[1,6] No other precoagulation screening was performed, in agreement with the EHRA guidelines.[6] If the procedure was planned

early in the day, it was also allowed to skip the anticoagulant dose the evening before, aiming for an 18 to 24 hours window before the last dose.

Patients were randomized through an interactive web recognition system to a 1g/10mL (10%) TXA mouthwash or a taste and color-matching placebo (water with 2.5mL of 45% cherry aroma), both manufactured by the Leuven Centre for Clinical Pharmacology, University Hospitals Leuven, Belgium. The mouthwash was packed into oral syringes of ten milliliter. A computer-generated block-randomization list was generated by an independent person (Leuven Centre for Clinical Pharmacology, University Hospitals Leuven, Belgium) for treatment allocation. The randomization was not stratified. Patients were enrolled in the study and assigned to the study interventions by clinicians. Patients, clinicians and treating surgeons were blinded to the allocated intervention.

Patients were instructed to use the mouthwash immediately before the dental extraction and subsequently three times a day for three days starting the day after the extraction (Figure 2.1). In order not to mechanically dislodge the immature coagulum, we advised no immediate post-extraction mouthwash on the day of the dental extraction. They were instructed to use the mouthwash for 1 minute and to spit it out afterwards. Extraction-wound management was left at the discretion of the physician. Patients received instructions on how to stop minor oral bleeding and are asked to record any bleedings. They were instructed to resume the NOAC the day after dental extraction unless there were hemostasis issues. Investigators blinded to the allocated treatment contacted the patients by phone on day two and day seven after dental extraction to assess compliance with the study protocol, the occurrence of any bleeding and other secondary and safety outcomes.[11]

Outcomes

The primary outcome was the number of patients with any oral bleeding, defined as overt bleeding within the oral cavity. Secondary outcomes were the number of bleeding events in the different bleeding categories, peri-procedural bleeding (defined as a bleeding score measured on a visual analogue scale ranging from 0 to 10), unplanned medical contacts, reinterventions following oral bleeding and unplanned NOAC interruptions.

Oral bleeding events were categorized as minor, clinically relevant or major, as previously published[1], and as early (on the day of the extraction or the day after) or delayed (on day two or later) (Table 2.1).[11] Surgical reinterventions were defined as any procedure in the oral cavity for the treatment of bleeding, except for rinsing the extraction socket with saline, performed by a health care professional. Facial hematomas were recorded as well. A blinded adjudicator adjudicated all suspected bleeding events and reinterventions.

Table 2.1. Definitions of oral bleeding events

Severity of the bleeding event	
Major	Oral bleeding events requiring blood transfusion, hospitalization or resulting in death.
Clinically relevant non-major	Oral non-major bleeding events requiring unplanned medical contact or additional hemostatic measures (except for gauzes), with or without surgical reintervention.
Minor	Oral bleeding events such as bleeding requiring the use of additional gauzes, blood on the pillow, clear red bleeding when spitting out the mouthwash, etc.
Timing of the bleeding event	
Early	Oral bleeding events occurring after the extraction up to and including day 1 after dental extraction.
Delayed	Oral bleeding events occurring between day 2 and day 7 after dental extraction.

Safety outcomes were thrombotic events, including myocardial infarction, stroke, systemic embolism and venous thromboembolism, any allergic reactions to the mouthwash and non-oral bleeding events (such as epistaxis, eye bleeding or bruises), up to the end of the study follow-up.[11]

Statistical analysis

The study was designed to test the hypothesis that tranexamic acid mouthwash would be superior to placebo with respect to the number of patients with any oral bleeding. Based on a pilot study the sample size was set at 236 patients, which would allow to detect a treatment difference of 15% between the TXA and placebo group using a chi-square test with an expected proportion of patients with any bleeding of 30% in the control group.[1] The statistical power was set at 80% and the two-sided significance level at 5%. All analyses were performed using SAS version 9.4 and SAS/STAT version 15.1 for Windows.

The primary analysis was conducted in the full analysis set, which included all randomized patients, but excluded three patients for whom no follow-up data could be obtained and one control patient who used off-trial TXA.[12] A sensitivity analysis was performed whereby the primary endpoint for these patients was imputed according to a worst-case-scenario (i.e. occurrence of oral bleeding), but yielded similar results. A further sensitivity analysis was performed on the per-protocol set, additionally excluding patients who had not complied with the assigned treatment for at least 80%.

The primary outcome (i.e. number of patients with any oral bleeding) was analyzed using a chi-square test. The effect of treatment was estimated by the risk ratio and presented along with its 95% confidence interval.

The secondary outcomes were analyzed and a pre-specified subgroup analysis was performed, by means of a logistic regression (for the number of patients with bleeds) and negative-binomial regression model (for the number of bleeds). The confidence intervals from the negative-binomial regression were calculated using the normal approximation. The treatment effect was estimated as a rate ratio (the number of bleeds per patient during the first seven days after dental extraction). The subgroup analysis included a risk analysis for bleeding events accounting for patient demographics and procedural characteristics, stratified per treatment group. Additionally, a post-hoc exploratory analysis was performed, analyzing all oral bleeds plus facial hematomas by means of a logistic regression (for the primary endpoint) and negative-binomial regression model (for the number of bleeds).

Importantly, an independent Data Safety Monitoring Board performed an unplanned interim analysis on the number of patients with oral bleeding in May 2020 with the aim of assessing futility or reassessing the sample size. At that time, the trial was suspended because of the outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and 222 of the 236 planned patients had been randomized. The Data Safety Monitoring Board recommended not to re-initiate the study based on the number of patients with bleeding. The study team remained blinded to all study results. The full details of the methodology of the interim analysis are included in the online supplement.

Results

Recruitment, baseline characteristics and follow-up

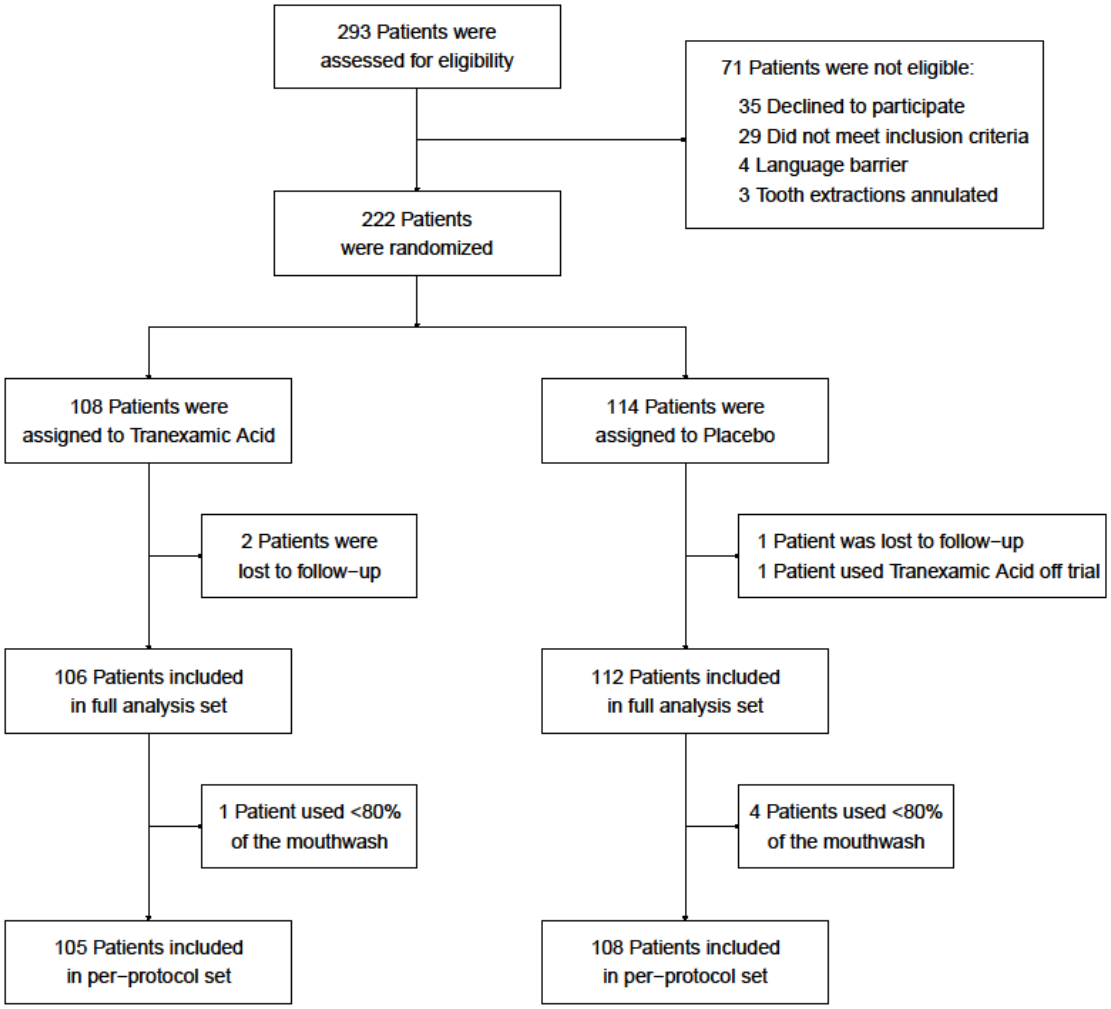

Figure 2.2. Screening, randomization, follow-up and analysis of patients.

The study flow-chart is shown in Figure 2.2. The first patient was enrolled in February 2018 and the trial was closed in May 2020. Of 293 patients referred for dental extraction, 222 eligible patients were randomized: 108 patients were assigned to TXA mouthwash and 114 to placebo. Three patients were lost to follow-up and one patient took TXA off-trial, so that 106 TXA-treated patients and 112 placebo-treated patients were included in the full analysis set.

The baseline characteristics of the patients are shown in Table 2.2. The two groups were well-balanced, except for a difference in sex. The mean age was 74.8 (±8.8) years in the TXA group and 72.7 (±10.7) years in the placebo group. Details of the dental extractions are presented in Table 2.5.

Table 2.2. Baseline characteristics of the patients.

Characteristics	Tranexamic acid (N=106)	Placebo (N=112)
Age – yr	74.8±8.8	72.7±10.7
Male sex – no. (%)	81 (76.4)	64 (57.1)
Smoking status – no. (%)	-	-
Active smokers	10 (9.4)	15 (13.4)
History of smoking	53 (50.0)	47 (42.0)
Never smoked	43 (40.6)	50 (44.6)
Alcohol consumption – no. (%)	-	-
≤2 units daily	99 (93.4)	105 (93.8)
>2 units daily	7 (6.6)	7 (6.3)
Medical background – no. (%)[1]	-	-
Chronic heart failure	29 (27.4)	24 (21.4)
Hypertension	76 (71.7)	76 (67.9)
Diabetes	21 (19.8)	15 (13.4)
Stroke	21 (19.8)	29 (25.9)
Coronary artery disease	35 (33.0)	36 (32.1)
Peripheral artery disease	11 (10.4)	9 (8.0)
CHADS-VASc score	3.7±1.7	3.7±1.6
NOAC type – no. (%)	-	-
Rivaroxaban	38 (35.8)	40 (35.7)
Apixaban	29 (27.4)	33 (29.5)
Edoxaban	21 (19.8)	19 (17.0)
Dabigatran	18 (17.0)	20 (17.9)
Indication for NOAC – no. (%)	-	-
Atrial fibrillation	88 (83.0)	88 (78.6)
Venous thromboembolism	11 (10.4)	14 (12.5)
Other or unknown	7 (6.6)	10 (8.9)

Percentages may not total 100 because of rounding. Plus-minus values are means ±SD.
[1]These data are not mutually exclusive.

Primary and secondary bleeding outcomes

The primary and secondary bleeding outcomes are summarized in Table 2.2. There was no difference in the number of patients with any oral post-extraction bleeding in the TXA group (28 patients, 26.4%) versus the placebo group (32 patients, 28.6%) (relative risk, 0.92; 95% CI, 0.60 to 1.42; p=0.72). When assessing all oral bleeding events, there were 46 oral bleeds in the TXA group versus 85 in the placebo group (rate ratio, 0.57; 95% CI,

0.31 to 1.05, p=0.07). The procedural bleeding score was similar between both groups: 4±1.78 on a VAS scale (0–10) for TXA-treated patients and 4±1.82 for placebo-treated patients (p=0.80).

Patients in the TXA group had **less delayed oral bleeds** than patients in the placebo group (11 versus 36 delayed bleeds, rate ratio, 0.32; 95% CI, 0.12 to 0.89). TXA-treated patients had fewer bleeds related to the dental extraction, which were oral bleeds and facial hematomas, compared to patients assigned to placebo (52 versus 99 bleeds, rate ratio 0.56; 95% CI, 0.32 to 0.96).

All oral bleeds in this trial were managed with local hemostatic measures, except for one major oral bleeding in a patient in the placebo arm who needed a blood transfusion and who was hospitalized for two days.

Of the 60 patients who reported oral bleeding, 28 experienced more than one bleeding (Figure 2.3). No patient had four or more oral bleeds in the TXA group, whereas ten patients in the placebo group had. Figure 2.4 shows the number of oral bleeds per day after dental extraction. TXA had no influence on early post-extraction oral bleeding (day 0 and day 1) (see also Table 2.3). In contrast, the number of oral bleeds was lower on day 2, 3 and 4 after extraction for patients in the TXA group compared to the placebo group (see also Table 2.3).

Other secondary outcomes

Fewer patients treated with TXA (6.6%) had an **unplanned medical contact** after dental extraction compared to patients using placebo (16.1%) (relative risk, 0.41; 95% CI, 0.18 to 0.94) (Table 2.4). The most frequent reasons for unplanned medical contact were bleeding and infection (Table 2.5). The number of patients requiring a reintervention for oral bleeding or that interrupted their NOAC was comparable between both groups (Table 2.4). The majority of unplanned NOAC interruptions was because of bleeding complications (Table 2.6).

Table 2.3. Primary and secondary bleeding outcomes.						
Outcome	**Patients with bleeding** - *no. (%)*			**Number of bleeds** – *no.*		
	Tranexamic acid (N=106)	**Placebo (N=112)**	**Relative Risk** *(95%CI)*	**Tranexamic acid (N=106)**	**Placebo (N=112)**	**Rate ratio** *(95%CI)*
Primary outcome	-	-	-	-	-	-
Any oral bleeding	**28 (26.4)**	**32 (28.6)**	**0.93 (0.60;1.42)**	**-**	**-**	**-**
Secondary outcomes	-	-	-	-	-	-
Oral bleeding[1]	-	-	-	46	85	0.57 (0.31;1.05)
Clinically relevant[2]	4 (3.8)	10 (8.9)	0.42 (0.14;1.31)	5	13	0.44 (0.14;1.42)
Minor	27 (25.5)	29 (25.9)	0.98 (0.63;1.55)	41	72	0.60 (0.32;1.12)
Delayed	7 (6.6)	17 (15.2)	0.44 (0.19;1.01)	11	36	0.32 (0.12;0.89)
Early	25 (23.6)	27 (24.1)	0.98 (0.61;1.57)	35	49	0.76 (0.42;1.37)
Facial hematomas[1]	6 (5.7)	14 (12.5)	0.45 (0.18;1.14)	6	14	0.45 (0.17;1.18)
Total bleeding	**31 (29.2)**	**40 (35.7)**	**0.82 (0.56;1.21)**	**52**	**99**	**0.56 (0.32;0.96)**

The relative risks are obtained by means of logistic regression. The rate ratios are obtained by means of a negative-binomial regression model.
[1]Patients may have had both clinically relevant and minor oral bleeds, may have had both delayed and early oral bleeds, and may have had oral bleeding plus a facial hematoma or only a facial hematoma.
[2]One patient in the placebo group had a major oral bleeding for which the patient needed a blood transfusion and was hospitalized for two days.

Table 2.4. Other secondary and safety outcomes.						
Outcome	**Number of patients** - *no. (%)*			**Number of events** – *no.*		
	Tranexamic acid (N=106)	**Placebo (N=112)**	**Relative Risk** *(95%CI)*	**Tranexamic acid (N=106)**	**Placebo (N=112)**	**Rate ratio** *(95%CI)*
Secondary outcomes	-	-	-	-	-	-
Unplanned medical contact	7 (6.6)	18 (16.1)	0.41 (0.18;0.94)	10	27	0.39 (0.16;0.98)
Reintervention after oral bleeding	4 (3.8)	10 (8.9)	0.42 (0.13;1.31)	7	13	0.57 (0.17;1.91)
Unplanned NOAC interruption	6 (5.7)	9 (8.0)	0.70 (0.26;1.91)	6	9	-
Safety outcomes	-	-	-	-	-	-
Thrombotic events[1]	0	1 (0.9)	-	0	1	-
Allergic reactions	2 (1.9)	2 (1.8)	1.06 (0.15;7.37)	2	2	-
Epistaxis	2 (1.9)	4 (3.6)	0.53 (0.10;2.83)	2	9	-
Eye bleeding	1 (0.9)	2 (1.8)	0.53 (0.05;5.74)	1	2	-
Bruises[2]	2 (1.9)	4 (3.6)	0.53 (0.10;2.83)	2	4	-
Hematuria or blood in faces	1 (0.9)	1 (0.9)	0.95 (0.06;15.3)	1	1	-

The relative risks are obtained by means of logistic regression. The rate ratios are obtained by means of a negative-binomial regression model.
[1]One patient suffered from a transient ischemic attack while interrupting the NOAC in preparation for the tooth extraction.
[2]Bruising caused by, for example, bumping a limb or body part into something, or after blood sampling.

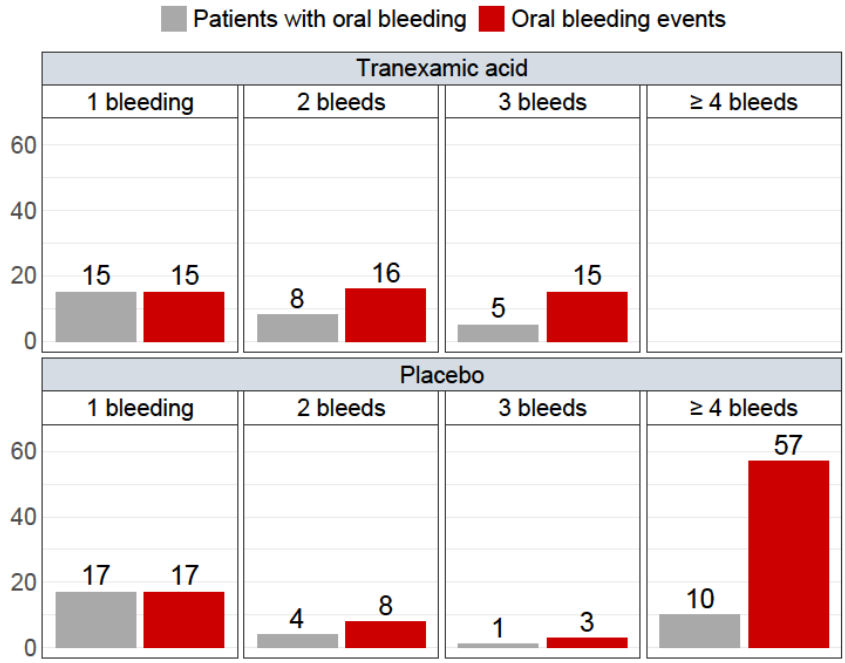

Figure 2.3. Post-extraction oral bleeds in patients who suffered from 1, 2, 3 or ≥ 4 bleeds. The number of patients reporting bleeding was similar (relative risk 0.93; 95% CI 0.60–1.42). There were 46 oral bleeds in the tranexamic acid group versus 85 in the placebo group (rate ratio 0.57; 95% CI 0.31–1.05).

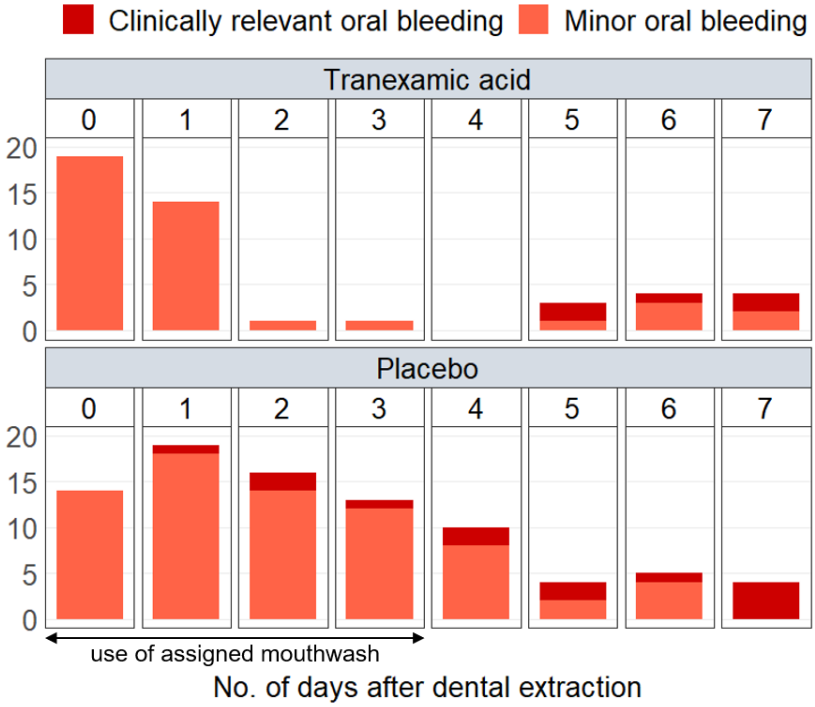

Figure 2.4. Clinically relevant and minor oral bleeds by day after dental extraction. The number of minor bleeding, clinically relevant bleeding, and early bleeding (on day 0 and 1) events was similar. Patients treated with tranexamic acid reported less delayed bleeding (later than day 1) compared to placebo (rate ratio 0.32; 95% CI 0.12–0.89).

Safety outcomes

Only one patient included in the placebo group suffered from a thrombotic event in preparation for dental extraction (Table 2.4). The participant had stopped NOAC treatment two days before dental extraction and had a transient ischemic attack. There were no thrombotic events in the TXA group. Allergic reactions were rare. Two patients in both treatment groups reported a tingling feeling in their mouth after using the mouthwash (Table 2.4).

Subgroup analysis

The exploratory subgroup analysis is shown in Figure 2.5. A significant treatment interaction (p=0.01) was identified in patients requiring extraction of 2 or more teeth. In this subgroup, there were 33 bleeds in 19/61 (31.2%) patients assigned to tranexamic acid and 77 bleeds in 24/57 (42.1%) of patients assigned to placebo (rate ratio, 0.40; 95% CI, 0.20 to 0.78). In patients who were 75 years or older, there were 22 bleeds in 13/59 (22.0%) patients assigned to tranexamic acid and 55 bleeds in 16/51 (31.4%) patients assigned to placebo (rate ratio, 0.35; 95% CI, 0.15 to 0.79), although this interaction did not reach statistical significance (p=0.07).

Subgroup	Patients with bleeding - no./No. (%) Tranexamic acid	Patients with bleeding - no./No. (%) Placebo	Number of events - no. Tranexamic acid	Number of events - no. Placebo	Tranexamic acid vs. Placebo Rate ratio (95%CI)	P value for interaction
Gender						**.67**
Male	20/81 (24.7)	19/64 (29.7)	31	45	0.54 (0.26;1.16)	
Female	8/25 (32.0)	13/48 (27.1)	15	40	0.72 (0.25;2.09)	
Age						**.07**
< 75 years	15/47 (32.0)	16/61 (26.2)	24	30	1.04 (0.44;2.46)	
≥ 75 years	13/59 (22.0)	16/51 (31.4)	22	55	0.35 (0.15;0.79)	
Number of extracted teeth						**.01**
1 tooth	9/45 (20.0)	8/55 (14.6)	13	8	1.99 (0.69; 5.71)	
≥ 2 teeth	19/61 (31.2)	24/57 (42.1)	33	77	0.40 (0.20;0.78)	
Type of extracted teeth						**.91**
Incisors/canines	1/16 (6.3)	1/12 (8.3)	1	1	0.75 (0.04;15.75)	
Molars	15/54 (27.8)	20/70 (28.6)	23	47	0.63 (0.29;1.38)	
Incisors/canines and molars	12/36 (33.3)	11/30 (36.7)	22	37	0.50 (0.19;1.31)	
Burring						**.33**
no	23/83 (27.7)	23/84 (27.4)	38	56	0.69 (0.34;1.37)	
yes	5/23 (21.7)	9/28 (32.1)	8	29	0.34 (0.09;1.20)	
Additional hemostatics						**.90**
no	17/58 (29.3)	21/73 (28.8)	29	63	0.58 (0.27;1.25)	
yes	11/48 (22.9)	11/39 (28.2)	17	22	0.63 (0.23;1.70)	
Chlorhexidine mouthwash prescribed						**.81**
no	18/58 (31.0)	12/46 (26.1)	31	43	0.57 (0.25;1.32)	
yes	10/48 (20.8)	20/66 (30.3)	15	42	0.49 (0.20;1.20)	
Leukocyte- and Platelet-Rich Fibrin						**.58**
no	26/97 (26.8)	26/95 (27.4)	42	77	0.53 (0.28;1.01)	
yes	2/9 (22.2)	6/17 (35.3)	4	8	0.94 (0.14;6.34)	
NOAC reintake after dental extraction						**.25**
< 24 hours	21/74 (28.4)	24/82 (29.3)	32	73	0.49 (0.24;0.98)	
≥ 24 hours	7/32 (21.9)	8/30 (26.7)	14	12	1.09 (0.33;3.57)	
Total population						
All bleeding events	28/106 (26.4)	32/112 (28.6)	46	85	0.57 (0.31;1.04)	

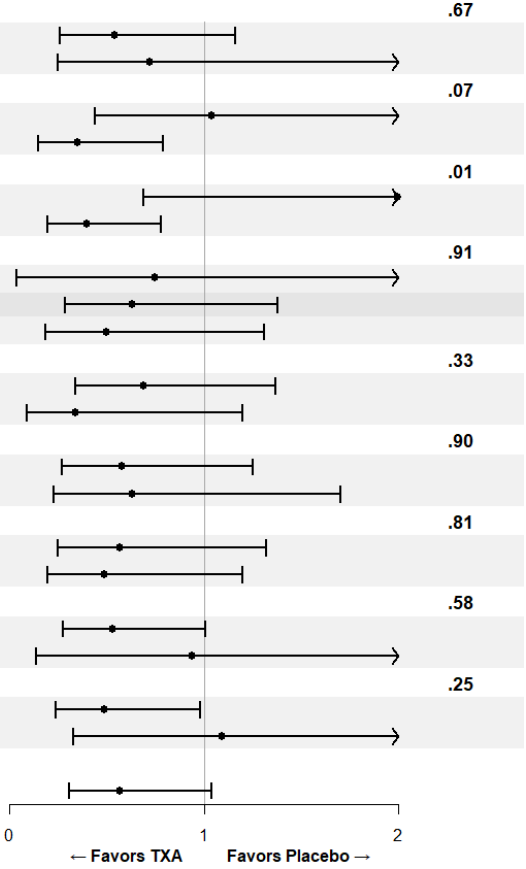

Figure 2.5. Estimated rate of oral bleeding according to patient and surgical characteristics. With no./No. = the number of patients with oral bleeds divided by the total number of patients in the subgroup; no. = number of oral bleeding events. The rate ratios are given for the number of oral bleeding events and are obtained by means of a negative-binomial regression that included factors for treatment, subgroup and their interaction. A rate ratio for which the associated 95% confidence interval does not include 1 indicates a significant treatment effect. The p-value for interaction was obtained from the aforementioned negative binomial model by an F-test and assessed whether the effect for treatment differs between the subgroups. A significant p-value for interaction indicates that the treatment with TXA works differently in the given subgroups. Additional hemostatics included gauze compression and oxidized cellulose materials.

Table 2.5. Details of dental extractions according to treatment group.		
Variable	**Tranexamic acid (N=106)**	**Placebo (N=112)**
Indication of dental extraction – no. (%)[1]	-	-
Abscess	23 (21.7)	20 (17.9)
Periodontal disease	37 (34.9)	33 (29.5)
Tooth decay	70 (66.0)	74 (66.1)
Other indications[2]	5 (4.7)	10 (8.9)
Surgical procedures – no. (%)[1]	-	-
Stitches	105 (99.1)	111 (99.1)
Burring	23 (21.7)	28 (25.0)
Additional hemostatics[3]	48 (45.3)	39 (34.8)
Antibiotics	22 (20.8)	27 (24.1)
Chlorhexidine mouthwash prescribed	48 (45.3)	66 (58.9)
Leukocyte- and Platelet-Rich Fibrin	9 (8.6)	17 (15.2)
Number of extracted teeth – no. (%)	-	-
1 tooth	45 (42.5)	55 (49.1)
2 teeth	14 (13.2)	18 (16.1)
3-4 teeth	22 (20.8)	21 (18.8)
≥ 5 teeth	25 (23.6)	18 (16.1)
Maxilla or mandible – no. (%)	-	-
Maxilla	40 (37.8)	41 (36.6)
Mandible	43 (40.6)	48 (42.9)
Maxilla and mandible	23 (21.7)	23 (20.5)
Incisors/canines or molars – no. (%)	-	-
Incisors/canines	16 (15.1)	12 (10.7)
Molars	54 (50.9)	70 (62.5)
Incisors or canines and molars	36 (34.0)	30 (26.8)

Number of patients are reported. Percentages may not total 100 because of rounding.
[1]These data are not mutually exclusive.
[2]Other most-common indications for tooth extraction were root resorption, tooth extraction prior to organ transplantation or to an oncological treatment or to the use of bisphosphonates.
[3]Additional hemostatic included gauze compression and resorbable oxidized cellulose materials.

Table 2.6. Details of unplanned medical contact and NOAC interruption.		
Outcome	**Tranexamic acid (N=106)**	**Placebo (N=112)**
Unplanned medical contact – no. (%)[1]	-	-
Bleeding	7 (6.6)	15 (13.4)
Infection	3 (2.8)	7 (6.3)
Other	0	5 (4.5)
NOAC interruption – no. (%)	-	-
Bleeding	3 (2.8)	6 (5.4)
Accidental	1 (0.9)	2 (1.8)
Doctor's advice	1 (0.9)	0
Patient's preference	1 (0.9)	1 (0.9)
In preparation for surgery	2 (1.9)	3 (2.7)

Number of patients are reported.
[1]These data are not mutually exclusive.

Discussion

This is the first randomized double blind clinical trial assessing the efficacy of TXA to reduce post-extraction bleeding in patients treated with NOACs. The results of our trial provide new clinical data in the debate of the optimal anticoagulant and hemostatic management of patients on NOACs undergoing dental extractions.[4] We observed no reduction in the number of patients with oral bleeding when comparing TXA mouthwash with placebo. Tranexamic acid did not reduce the rate of peri-procedural bleeding or early bleeding after extraction, but reduced delayed bleeding and bleeding after multiple dental extractions. Patients using TXA also had fewer unscheduled medical contacts after dental extraction (59% reduction), predominantly related to bleeding.

Our results confirm the high event rate of oral bleeds in patients treated with NOACs undergoing tooth extraction, with more than one out of four patients reporting any bleeding. Moreover, half of the patients with a bleeding reported multiple oral bleeding events. In the TXA group, no patients had four or more bleedings, compared to 10 patients in the placebo group. Fewer patients in the TXA group thus suffered recurrent bleeding.

Two patients in each group experienced allergic reactions after the use of the assigned mouthwash and no patients had thrombotic complications after the use of TXA, highlighting that TXA is well tolerated and safe to use. The intervention with TXA mouthwash is also affordable, readily available on the market and therefore easy to implement in clinical practice.

In our study, six patients in the TXA group and nine patients in the placebo group interrupted their NOAC (without it being planned in preparation for surgery). Unplanned interruption of anticoagulants is a concern in clinical practice, since it is associated with increased risk of cardiovascular events.[13] Patient adherence and compliance with the doctor's advice remains important.

An exploratory subgroup analysis showed that patients who underwent extraction of two or more teeth and elderly patients and patients who restarted their NOAC within 24 hours after the dental extraction might benefit more from treatment with TXA. However, the latter finding was not statistically significant. These findings support the hypothesis that the potential benefit of a hemostatic agent is likely to be the largest in patients with friable

mucosa and more extensive tissue damage. An immature coagulum combined with re-initiation of the anticoagulant may then result in bleeding. Future studies may focus on this high-risk population.

After enrolling 222 out of 236 planned patients, the trial was prematurely stopped, during the SARS-CoV-2 pandemic after a futility analysis by the Data Safety Monitoring Board based on the number of patients with oral bleeding. This was the main limitation of the study. Another limitation was that we relied on self-reported information gathered during a follow-up by phone to assess the patients' compliance with the study protocol. However, the studied intervention was a simple intervention and patients got both oral and written instructions on a schematic leaflet about the protocol. A last limitation is that there was no fixed time point on restarting the NOAC after dental extraction. Patients were instructed on when to restart their NOAC, but the exact timing of the NOAC re-intake was left at the patient's decision. As a result, there was a spread in the timing of the NOAC restart amongst the studied patients and the possible effect hereof on bleeding after dental extraction is unclear. The question on timing of NOAC restart needs further study.

Conclusion

The use of a 10% TXA mouthwash did not reduce the number of patients with any oral bleeding after dental extraction, nor the peri-procedural bleeding or early bleeding after extraction. Tranexamic acid appears to reduce delayed bleeding and bleeding after multiple dental extractions. Follow-up studies may explore potential benefit in high-risk patient populations.

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LEUKOCYTE- AND PLATELET-RICH FIBRIN

CHAPTER 3

Mechanical and structural properties of L-PRF membranes: study on the impact of anticoagulants

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This research chapter was adapted from:

Ockerman A, Braem A, EzEldeen M, Castro A, Coucke B, Politis C, Verhamme P, Jacobs R, Quirynen M. Mechanical and structural properties of leukocyte- and platelet-rich fibrin membranes: an in vitro study on the impact of anticoagulant therapy. J Periodontal Res. 2020;55(5):686–93.

Abstract

Background: Little is known about structural and mechanical properties of Leukocyte- and Platelet-Rich Fibrin (L-PRF) membranes and even less about the influence of antithrombotic drugs on L-PRF. The aim of this is therefore to investigate mechanical properties, fibrin structure and cell content of L-PRF membranes and the impact of anticoagulant therapy on L-PRF.

Methods: Blood samples were obtained from 12 volunteers and supplemented with either no, 1.25 IU, 2.5 IU, 5 IU or 10 IU of the anticoagulant drug enoxaparin. L-PRF membranes were characterized with tensile testing, scanning electron microscopy and measurement of platelets and leukocytes. Control and enoxaparin-supplemented L-PRF membranes were compared.

Results: At 10 IU enoxaparin no L-PRF membranes could be generated, whereas the low doses of 1.25 and 2.5 IU had no influence on L-PRF properties. The mechanical properties, fibrin networks and number of platelets and leukocytes of 5 IU supplemented membranes were unlike the control membranes, but were not found to be significantly different because of limited sampling and inter- and intra-variability.

Conclusion: Low doses of the anticoagulant enoxaparin do not affect mechanical properties, fibrin network, nor cellular content of L-PRF, whereas high doses impair L-PRF generation.

Introduction

Leukocyte- and platelet-rich fibrin, also known as L-PRF, is the most recent generation of platelet concentrates. L-PRF is a biomaterial derived from patient's own blood, composed of a fibrin network entrapping platelet concentrates and immune cells and characterized by a constant release of growth factors for up to 14 days.[1-4] Since L-PRF contains all the constituents of blood that favor healing and are capable of boosting the immune system, it is currently being used in various surgical procedures to promote bone and tissue healing and regeneration.[5-6] Major advantages of L-PRF include its biosafety because of its 100% autologous nature, availability and cost-effective preparation. L-PRF is generated via a very simple procedure. Some blood samples are collected from a patient. The moment the blood is in contact with the blood tubes, the contact coagulation pathway is activated, leading to the formation of fibrin. Meanwhile, the centrifugation process separates the blood into three distinguishable layers: platelet-poor plasma (PPP) at the top, followed by an L-PRF clot (the buffy coat) in the middle, and a layer of red blood cells at the bottom of the tube (Figure 3.1).[7-9] The L-PRF clot is then transformed into a L-PRF membrane through gentile compression, squeezing out the plasma.[1]

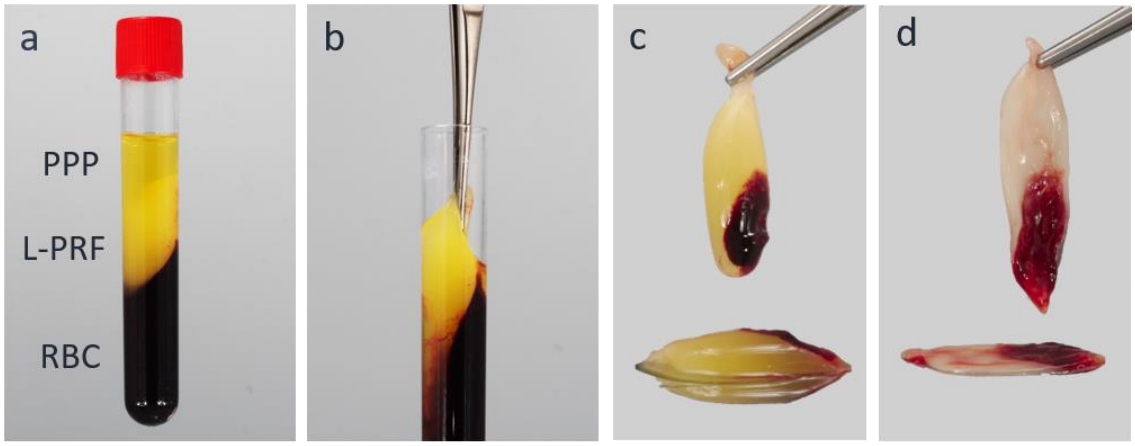

Figure 3.1. L-PRF generation process. (a) After blood collection, the tubes were centrifuged leading to three distinguishable layers: PPP = platelet-poor plasma, L-PRF = leukocyte- and platelet-rich fibrin and RBC = red blood cells. (b) The L-PRF clot was removes from the tube with tweezers and compressed to generate (d) an L-PRF membrane.

In recent years, L-PRF membranes are increasingly being used in oral surgery. L-PRF can be applied during tooth extraction to promote socket and bone healing and to reduce pain and swelling.[10,11]

The morphology of L-PRF membranes can vary. Aside from various centrifugation protocols, also patient-related factors including medication can explain this observed variability.[7] More specifically, as L-PRF is dependent on the coagulation process of blood, antithrombotic drugs that modify blood coagulation may influence its properties. The impact of antithrombotics on L-PRF membranes may as such have important implications on the composition of L-PRF, and with that its biological and mechanical properties.

Even though L-PRF's use in practice and its method of preparation have been examined extensively, studies on its structural and mechanical properties are scarce. The aim of this study is to record the mechanical properties, fibrin structure and cell content of L-PRF membranes of healthy subjects, and to analyze a possible impact of antithrombotic medication.

Materials and methods

The Ethics Committee Research of the University Hospitals of Leuven granted a positive advice for the study in May 2018 (S61315). All volunteers signed an informed consent prior to the blood collection.

Blood collection and L-PRF generation

Blood samples were taken from 12 healthy volunteers that were non-smokers, had no systemic diseases, were not pregnant or lactating, and did not take or had no history of taking medication affecting blood coagulation (five men, seven women, mean age 25±4 years). Seven blood samples per volunteer were collected in 9 mL silica-coated plastic tubes without anticoagulant (red cap, BVBCTP-2, Intra-Lock®, Boca). Four were intended for tensile testing, three for cell counting and scanning electron microscopy. Blood samples were either supplemented with 0.5 mL of normal saline solution as a control, 1.25 IU, 2.5 IU, 5 IU or 10 IU enoxaparin in 0.5 mL normal saline using a syringe (Figure 3.2).[13]

Additionally, one blood sample in a 5 mL EDTA tube (purple cap, K2E (EDTA), Becton Dickinson Vacutainer®) was collected from each volunteer for cell counting. Only in the first phase of the current study, high doses of 10 IU enoxaparin were tested.

L-PRF membranes were prepared as described by Choukroun *et al*.[14] The red cap blood samples were centrifuged at 408 g RCF (2700 rpm) for 12 minutes (IntraSpin™, Intra-Lock®, Boca). Thereafter, L-PRF clots were removed from the tubes with sterile tweezers, separated from the red blood cell phase and placed in a L-PRF compression box (Xpression™ kit, Intra-Lock®, Boca) for gentle compression during 5 minutes.

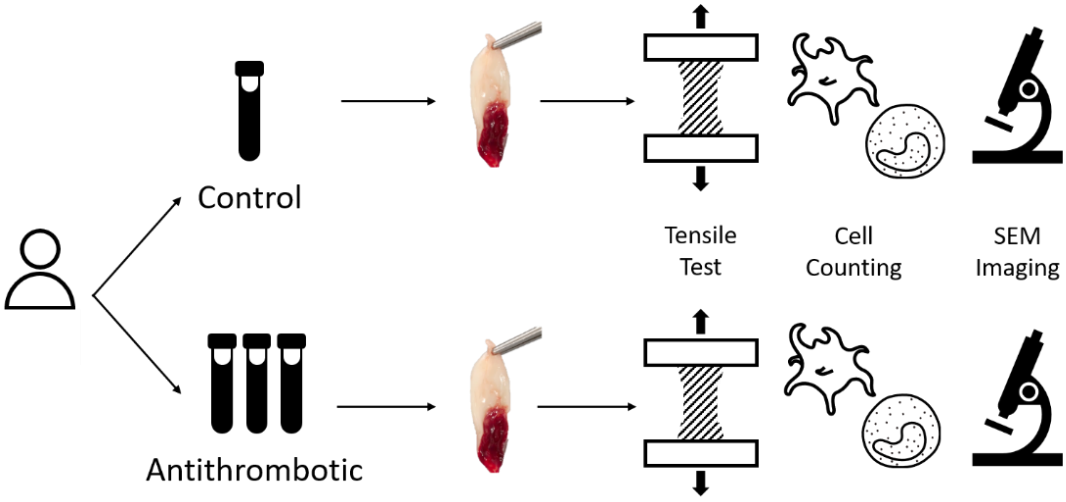

Figure 3.2. Study design. The blood samples were supplemented with 0.5 mL of normal saline solution as a control, 1.25 IU, 2.5 IU, 5 IU, or 10 IU of the anticoagulant drug enoxaparin. However, when adding 10 IU enoxaparin, the generation of L-PRF failed in the majority of the blood samples. L-PRF membranes were generated and submitted to tensile testing to evaluate the mechanical characteristics, cellular counting and scanning electron microscopy (SEM) to have a look at the fibrin network.

Tensile Testing

A press mall was used to shape L-PRF membranes into rectangles of 5 mm x 22 mm. The L-PRF membranes were mounted on a 10 N testing bench of a universal testing machine (Instron® 5943, Norwood) without any tension on the membrane. The membranes were stretched at a crosshead speed of 1 mm/min until the membranes ruptured. Applied force [N] and tensile extension [mm] were recorded via an Instron Bluehill 3 software. E-modulus [MPa], ultimate tensile strength [MPa] and stretch at rupture [-] were calculated from the stress-strain curves. The E-modulus, representing the stiffness of the membrane,

was determined as the slope of the stress-strain curve within the apparent strain range of 50–150 %. Stretch at rupture is a measure of how much the membrane could been stretched before it ruptured.

Cell counting

After the L-PRF clots were removed from the tubes, the remnant in the tubes was diluted with normal saline up to a volume of 9 mL. The L-PRF exudate, obtained after compression of the L-PRF clots, was collected and diluted with normal saline up to a volume of 9 mL. The tubes were carefully shaken to become a homogeneous mix. From the tubes with remnant and exudate and from the EDTA tube, 900 µL was taken out and transferred to three Eppendorfs. To avoid formation of crystals inside the cells a 100 µl of 10% dimethylsulfoxide was added to each Eppendorf and the content was carefully mixed with a pipette. The cell counting of the remnant, exudate and whole blood samples was performed with a hematology analyzer (Abbott Cell-Dyn 3700 Hematology Analyzer). By subtracting the cells in the remnant and exudate of the cells in the whole blood in the EDTA tube, the cell content of the L-PRF membranes was assessed. Platelets and white blood cells were measured. Cell counts were performed for control and 5 IU supplemented samples.

Scanning Electron Microscopy

L-PRF membranes were fixed in 2.5% glutaraldehyde diluted in a phosphate-buffered saline solution for 4 hours, dehydrated in increasing concentrations of ethanol (50%, 75%, 90% and 100%) for 30 minutes each, and treated with 100% hexamethyldisilane for 10 minutes. The membranes were placed on absorbent paper overnight inside a laminar flow cabinet. After the drying process, the L-PRF membranes were coated with platinum and the middle part of each membrane was scanned using a scanning electron microscope (Philips XL30 ESEM FEG). Thereafter, we measured and compared the thickness of the fibrin bundles and the density of the fibrin network between control and 5 IU enoxaparin-supplemented membranes. ImageJ software (Fiji, Version 1.51) was used for these measurements. Two control and two 5 IU L-PRF samples were analyzed. Each membrane

was scanned at 15000x, 20000x and 25000x magnifications. Five images per magnification were used for measuring the fibrin thickness and fibrin density.

Statistics

To evaluate for differences in mechanical properties, a linear mixed model was fit for the obtained data with volunteer as random factor and the enoxaparin-supplementation as fixed factor. A normal quantile plot of the residual values and a residual dot plot were made in order to test the assumptions underlying the model. E-modulus, ultimate tensile strength and stretch at rupture were compared between four L-PRF membrane groups: control, 1.25 IU, 2.5 IU and 5 IU enoxaparin. (No L-PRF membranes supplemented with 10 IU enoxaparin could be tested.) The comparison was also performed per gender. The statistical significance level was set at $p<0.05$. Comparisons were corrected for simultaneous hypothesis testing according to Tukey.

Results

In the present study sample, no gender or age- (the L-PRF samples originated of volunteers with more or less the same age) effects were noted.

Tensile testing

When adding 10 IU enoxaparin, the generation of L-PRF failed in the majority of the blood samples, or resulted in L-PRF membranes that were friable and remarkably smaller than control L-PRF membranes, making them inadequate for further experiments. Therefore, during the continuation of the study, blood samples were supplemented with 1.25 to 5 IU of enoxaparin.

For each membrane, a stress-strain curve was generated (Figure 3.3a) and the E-modulus, ultimate tensile strength and stretch at rupture were compared between control (enoxaparin-free) and enoxaparin-supplemented L-PRF membranes (Table 3.1). Six samples were excluded from the analyses as they were supplemented with a wrong concentration of enoxaparin and one sample got lost during preparation.

The **E-modulus** (Figure 3.3b) was on average 0.07 MPa for the control L-PRF membranes and 0.06 MPa for the 1.25 IU, 2.5 IU and 5 IU supplemented membranes, and did not significantly differ between the groups (control vs 1.25 IU, p=0.27; control vs 2.5 IU, p=0.83; control vs 5 IU, p=0.42; 1.25 IU vs 2.5 IU, p=0.76; 1.25 IU vs 5 IU, p=0.10 and 2.5 IU vs 5 IU, p=0.89). The E-modulus of the 5 IU enoxaparin-supplemented membranes showed the highest variability.

Table 3.1 The mechanical characteristics of the L-PRF membranes.

L-PRF groups	n	E-modulus [MPa]	Strength [MPa]	Stretch [-]
Control	12	0.07 ± 0.01	0.29 ± 0.08	2.78 ± 0.35
1.25 IU enoxaparin	10	0.06 ± 0.01	0.25 ± 0.10	2.46 ± 0.47
2.5 IU enoxaparin	10	0.06 ± 0.01	0.31 ± 0.08	2.66 ± 0.33
5 IU enoxaparin	9	0.06 ± 0.02	0.20 ± 0.12	2.34 ± 0.64

Plus-minus values are means ± standard deviation. With n = number of L-PRF membranes, strength = ultimate tensile strength and stretch = stretch at rupture.

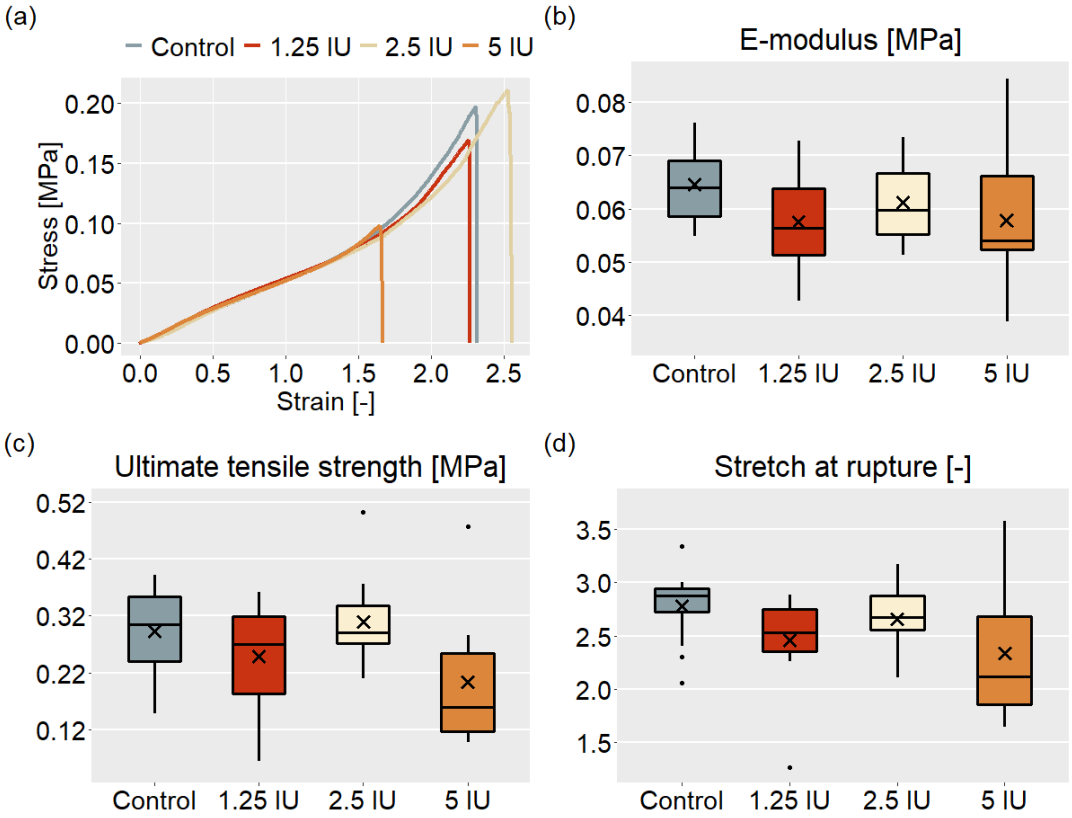

Figure 3.3. The mechanical characteristics of L-PRF membranes. (a) A representative stress-strain curve, (b) the E-modulus, (c) the ultimate tensile strength, and (d) the stretch at rupture are shown. The mean (X) is indicated. The control (without enoxaparin), 1.25 IU, 2.5 IU, and 5 IU enoxaparin-supplemented L-PRF membranes were compared.

The **ultimate tensile strengths** of control, 1.25 IU and 2.5 IU membranes were comparable: 0.29 MPa, 0.25 MPa and 0.31 MPa, respectively and did not significantly differ (Figure 3.3c). The 5 IU membranes were, on average, the least strong with an ultimate tensile strength of 0.20 MPa, and significantly differed from the 2.5 IU membranes (p=0.03), but not from the controls (p=0.09) or the 1.25 IU membranes (p=0.61).

Control membranes could be **stretched** up to 2.8 times their initial length until they ruptured, 1.25 IU membranes up to 2.5 times, 2.5 IU membranes up to 2.7 times and 5 IU membranes up to 2.3 times (Figure 3.3d). Note that the stretch at rupture of the 5 IU membranes was on average the shortest and showed a remarkably high variability, but no statistically significant differences were noted (5 IU vs control, p=0.10; 5 IU vs 1.25 IU, p=0.92 and 5 IU vs 2.5 IU, p=0.34).

Cell counting

Two control and two enoxaparin-supplemented samples got lost during preparation for cell counting and could not be tested. Approximately 72% **platelets** and 51% **leukocytes** of the whole blood sample were present in control L-PRF membranes and 47% platelets and 68% leukocytes in 5 IU enoxaparin-supplemented (Table 3.2). There was some variation in the concentration of lymphocytes, neutrophils, eosinophils, basophiles and monocytes between 5 IU and control membranes, but there were no significant differences.

Table 3.2. Cellular concentrations of the L-PRF membranes.

	Control		5 IU enoxaparin	
	Mean (10^3/µL)	**%**	**Mean (10^3/µL)**	**%**
Platelets	200.98 ± 77.20	72.2±18	129.72 ± 133.45	46.6±28
Leukocytes	2.68 ± 0.64	50.5±10	3.58 ± 2.13	67.5±12
Neutrophils	0.67 ± 0.85	80.2±18	0.71 ± 0.83	84.2±20
Lymphocytes	0.66 ± 0.78	29.1±17	1.10 ± 1.43	48.5±26
Monocytes	0.07 ± 0.40	11.1±30	0.41 ± 0.48	69.9±26
Eosinophils	1.02 ± 1.29	77.2±15	1.12 ± 1.11	84.3±16
Basophils	0.16 ± 0.18	56.7±13	0.24 ± 0.35	85.6±7

Plus-minus values are means ± standard deviation. The percentage of cells in the L-PRF membranes are the relative proportions of the cells to the initial whole blood.

Scanning Electron Microscopy

Fibrin bundles of the control membranes seemed to have a more regular fibrin **microstructure** compared to 5 IU enoxaparin-supplemented ones that showed an irregular structure (Figure 3.3). However, there were no differences between the fibrin bundle thickness and the fibrin network density between both L-PRF groups (Table 3.3).

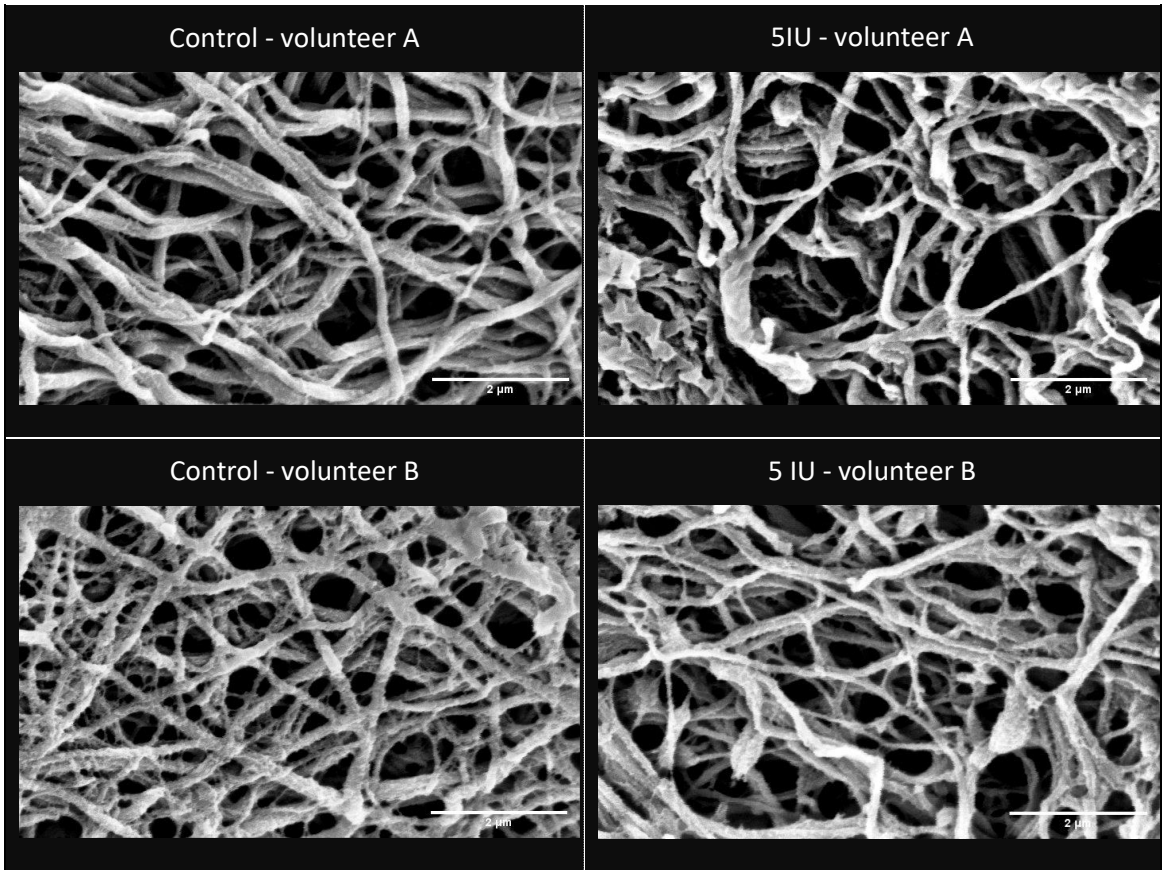

Figure 3.3. Scanning electron microscopy images of L-PRF membranes. The fibrin network of control and 5 IU enoxaparin-supplemented L-PRF membranes are alike. The presented images are magnified with a factor 15000×.

Table 3.3. The fibrin bundle thickness and network density of L-PRF membranes.		
	Control	**5 IU enoxaparin**
Fibrin bundle thickness [nm]		
15000×	135 ± 14	133 ± 17
20000×	135 ± 13	131 ± 13
Fibrin network density (%)		
15000×	71.5 ± 2.5	70.9 ± 1.8
20000×	72.0 ± 1.3	72.2 ± 2.3
25000×	73.7 ± 3.9	73.5 ± 3.7

Plus-minus values are means ± standard deviation. The fibrin bundle thickness was evaluated on images magnified with a factor 15000× and a factor 20000×, and the and fibrin network density on images of 15000×, 20000× and 25000× images.

Discussion

Leukocyte- and platelet-rich fibrin membranes serve as a scaffold for immune and blood cells and play a key role in the regeneration of soft and hard tissue after surgery. Diverse mechanical and structural properties of L-PRF membranes might have important implications for their use in clinic. We performed this study because there is a lack of knowledge regarding the mechanical properties, fibrin network microstructure and cell content of L-PRF membranes, and no studies have previously investigated the influence of antithrombotic drugs on these membranes.

Our study showed a mean E-modulus of 0.07 MPa for the control membranes (Table 3.4). This results is comparable to one study found in literature.[15] However, two other studies showed higher E-moduli[16-17], and one study lower results.[18] The ultimate tensile strength of the control membranes was 0.29 MPa, which is higher than reports of the strength of L-PRF in multiple other studies.[15,17-19] The stretch at rupture of the control membranes in the current study was about 2.8 times their initial length, which is higher, comparable and lower than three other studies that recorded a stretch of 1.45, 2.17 and ±3.50, respectively.[15-16,19] In short, the current results of tensile testing are quite similar or differ slightly from results obtained in other studies (Table 3.4). All previously mentioned studies had different protocols for blood centrifugation and tensile testing though[15-19], which makes any comparison difficult.

The time of blood centrifugation, the g-force of the centrifuge, the shape of the L-PRF membrane (rectangular or dogbone shaped) with tensile testing and the crosshead speed of the tensile test have to be taken into consideration (Table 3.4). It has been shown that centrifugation characteristics directly influence the L-PRF's architecture[20] and, possibly, indirectly the mechanical characteristics. Additionally, it might be that the shape of the tested L-PRF membranes and the crosshead speed of the tensile test influence the results, although this should be investigated. Therefore, it is difficult to clarify similarities and discrepancies of the mechanical properties of L-PRF membranes reported in various studies and comparisons of the results must be interpreted with caution.

Table 3.4. Comparison of multiple studies on the mechanical properties of L-PRF.						
	Studies[1]					
	No.1	**No.2**	**No.3**	**No.4**	**No.5**	**No.6**
Centrifugation						
Time [min]	12	10	12	10	12	5
G-force [g]	408	400	400	400	408	1700
Speed [mm/min]	1	2	10	2	?	10
Tensile test protocol						
Number of membranes	12	3	6	10	10	4
Shape membrane[2]	R	R	D	D	D	R
Tensile test results						
E-modulus [MPa]	0.07	0.08	0.47	0.13	0.01	NA
Strength [MPa]	0.29	0.11	NA	0.20	0.15	0.14
Stretch at rupture [-]	2.78	1.45	2.17	NA	NA	2.95

[1]Various studies on the mechanical properties of L-PRF membranes are listed. No.1 is de present study, No.2 Aggour et al. 2018[15], No.3. Madurantakam et al. 2015[16], No.4 Khorshidi et al. 2016[17], No.5 Khorshidi et al. 2018[18] and No.6 Kardos et al. 2018.[19]
With NA = a parameter that was not assessed and '?' = unknown information.
[2]For the tensile tests, the membranes were (R) rectangular or (D) dogbone shaped.

The current study was also designed to investigate if antithrombotic medication influences the properties L-PRF. With low doses of enoxaparin (1.25 IU and 2.5 IU supplementation), corresponding to enoxaparin at trough levels of therapeutic anticoagulation or administered in prophylactic dosing, the mechanical characteristics of L-PRF membranes were similar to membranes not supplemented with anticoagulant. With an intermediate dose of 0.5 IU/ml (5 IU supplementation), corresponding to a clinically relevant anticoagulant activity, L-PRF's generation was not impaired, but its mechanical properties became more variable. Since L-PRF could no longer be generated in the presence of therapeutic anticoagulant activity at peak level (10 IU supplementation), our findings highlight the importance of planning oral surgery at through anticoagulant levels. Importantly, the presence of low levels of enoxaparin, which may still be present at surgery, did not interfere with L-PRF generation and its properties.

Differences in the fibrin network structure of control and enoxaparin-supplemented L-PRF membranes may explain the varying mechanical properties. Scanning electron microscopy analysis of the membranes revealed that the fibrin network architecture rather than the fibrin fibers of these networks seems to be different between control and 5 IU membranes.

Various factors may influence the fibrin network of L-PRF membranes. First, the centrifugation process, with characteristics as speed and g-force, directly impacts the architecture of L-PRF.[20] Second, the age of the patient of whom blood is taken for L-PRF influences the fibrin network of L-PRF.[21] These two factors must be kept in mind for *in vivo* applications of L-PRF, but could not have been influencing factors in this study as all L-PRF membranes were generated according to the same centrifugation protocol and were taken of volunteers with more or less the same age. A third factor is the time from blood collection to centrifugation.[22] After blood collection, platelets are activated the moment they are in contact with the wall of the blood collection tube, which triggers the coagulation cascade and stimulates the conversion of fibrinogen into fibrin by the circulating thrombin. The fibrinogen is initially concentrated in the high part of the tube and by centrifugation the fibrinogen gets concentrated in the middle part of the tube.[22] Therefore, if the blood collecting and launching centrifugation takes too much time, the fibrinogen does not get concentrated and fibrin will polymerize in a diffuse way.[22] In the present study, we ensured that the blood was transferred to the centrifuge within one minute of blood collection to avoid diffuse fibrin polymerization. Last, the type of junctions formed during polymerization of fibrin strands influence the three-dimensional fibrin network. Bilateral junctions between fibrin strands will lead to thickening of fibrin polymers and a rigid network, while equilateral junctions will lead to a flexible and strong network, supporting cytokines enmeshment and cellular migration.[23-24] The presence of physiological thrombin allows the formation of equilateral junctions by slow polymerization.[23,25] As enoxaparin decreases the concentration of thrombin, enoxaparin may interfere with the polymerization process of fibrin. This assumption is supported by findings of an *in vitro* study showing that low molecular weight heparins indeed modulate fibrin polymerization and fibrin membrane structure.[26] Accordingly, enoxaparin (a low molecular weight heparin) may have caused the observed changes in the three-dimensional network of enoxaparin-supplemented L-PRF membranes in the current study as well.

We did not only observe a slight impact of enoxaparin on the mechanical properties and the fibrin network of L-PRF, but also on the cellular content. Because the cellular content is important for the healing process, this difference may be relevant. Considering these

observations, it would be interesting to investigate if and how strength, elasticity, fibrin network and cellular content of L-PRF membranes contribute to successful surgery and to examine the properties of L-PRF of patients on chronic anticoagulant treatment. Indeed, a limitation of the study is the *ex vivo* supplementation of enoxaparin to L-PRF.

The current study had further limitations. First, it is difficult to extrapolate from in vivo doses of anticoagulant to in vitro concentrations, including additional effects that these drugs can have in vivo. Simulating the consequences of long-term in vivo exposures to antithrombotic medication in vitro or ex vivo is impossible. Therefore, it would be of interest to investigate L-PRF membranes of patients taking antithrombotics for a while. Second, only enoxaparin was used to spike the blood samples, and no other antithrombotic agents were tested. Third, there is a risk at bias from blood variety. To minimize this risk, we selected volunteers without systemic diseases and with a small age range. Nevertheless, we cannot simply extrapolate the results of this study to a broader population. Fourth, the indirect subtraction method of the cell counting might not be very precise. This method does not consider the possibility of a significant number of platelets present in the clotted red blood cell fraction, which then were not measured by this method, and the possible loss of platelets during processing for cell counting.[27] Last, we only examined four L-PRF membranes with scanning electron microscopy.

Conclusion

The current study provides better insight in mechanical and structural properties of L-PRF. Our findings suggest that low therapeutic concentrations of enoxaparin do not influence L-PRF's mechanical properties, fibrin network or cellular content significantly, and that higher concentrations may do. These findings will need to be verified in controlled clinical trials.

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CHAPTER 4

Mechanical properties and cellular content of L-PRF membranes of patients on antithrombotic drugs

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This research chapter was adapted from:

Ockerman A, Hendrickx A, Willekens W, Fehervary H, Vastmans J, Coucke W, Verhamme P, Constantinus P, Vanassche T, Braem A, Quirynen M, Jacobs R. Mechanical properties and cellular content of leukocyte- and platelet-rich fibrin membranes of patients on antithrombotic drugs. 2021. *Under review.*

Abstract

Background: Anticoagulants and antiplatelets interfere with blood coagulation and may therefore influence the generation of leukocyte- and platelet-rich fibrin (L-PRF) membranes. The aim of the current study was to examine a potential influence of antithrombotic drugs on properties of L-PRF membranes.

Methods: Tensile tests and cell counts were performed to compare mechanical properties and cellular content of L-PRF membranes originating from patients on anticoagulants and antiplatelets versus patients not taking antithrombotics (controls).

Results: For the tensile tests, 35 patients donated blood: 13 control, 12 on anticoagulants and 10 on antiplatelets. Compared to controls, membranes from anticoagulated donors were weaker (ultimate tensile strength 0.57±0.24 MPa versus 0.80±0.27 MPa, p=0.03) and could not be stretched as far (1.8±0.3 versus 2.0±0.3 times the initial length, p=0.01). For the cell counting, 55 patients donated blood: 23 control, 16 on anticoagulants and 16 on antiplatelets. The percentage of platelets was approximately 50% in the three groups. The percentage of leukocytes in the L-PRF membranes was lower in the anticoagulant group compared with membranes from controls (69±10% versus 78±8%, p=0.04). There was no difference between membranes from the control group and the group on antiplatelets.

Conclusion: Our results indicate that L-PRF membranes originating from patients on an anticoagulant therapy, but not on antiplatelets, are weaker, stretch less far, and contain less leukocytes than L-PRF membranes of patients not taking these drugs.

Introduction

Leukocyte- and platelet-rich fibrin (L-PRF) is a promising platelet concentrate in natural-guided healing, as it boosts wound healing and regeneration of soft and hard tissues.[1,2] In clinical practice, the morphology of L-PRF membranes is variable, which may be important as the fibrin network together with the entrapped cells determine wound healing.[3] The reason for this variation is not yet fully understood, and the potential impact of antithrombotic therapy has not yet been studied.

L-PRF is generated from a patient's own blood: blood is collected and immediately centrifuged.[4] The moment the blood gets in contact with the negatively-charged surface of the glass or silico-coated collection tubes, the coagulation pathway is activated (Figure 4.1).[5] Activation of coagulation factor (F) XII leads to the sequential activation of FXI, FIX and FX, and results in the cleavage of prothrombin (FII) to thrombin (FIIa). Thrombin then converts fibrinogen to fibrin monomers that spontaneously polymerize to fibrin polymers. Thrombin also activates FXIII, a factor that forms covalent bonds crosslinking the fibrin polymers.[5–7] The slowly polymerizing fibrin entraps platelets and leukocytes. Activation of the coagulation cascade works in parallel with platelet activation and aggregation. Platelet activation enhances the coagulation response, as the activation of FX occurs mainly at the level of the membranes of activated platelets and leads to platelet aggregation, a process that causes the release of certain coagulation factors and cofactors.[6,7] On the other hand, coagulation also increases platelet activation. Thrombin is a potent platelet activator by interacting with its receptors on the platelet membrane.[6,7] Platelet aggregation and coagulation are thus strongly linked. Meanwhile, the centrifugation process separates blood into red blood cells at the bottom of the tube, plasma at the top, and a fibrin clot in the middle. This way, the combination of coagulation activation and centrifugation leads to the formation of a leukocyte- and platelet-rich fibrin clots.[8–10]

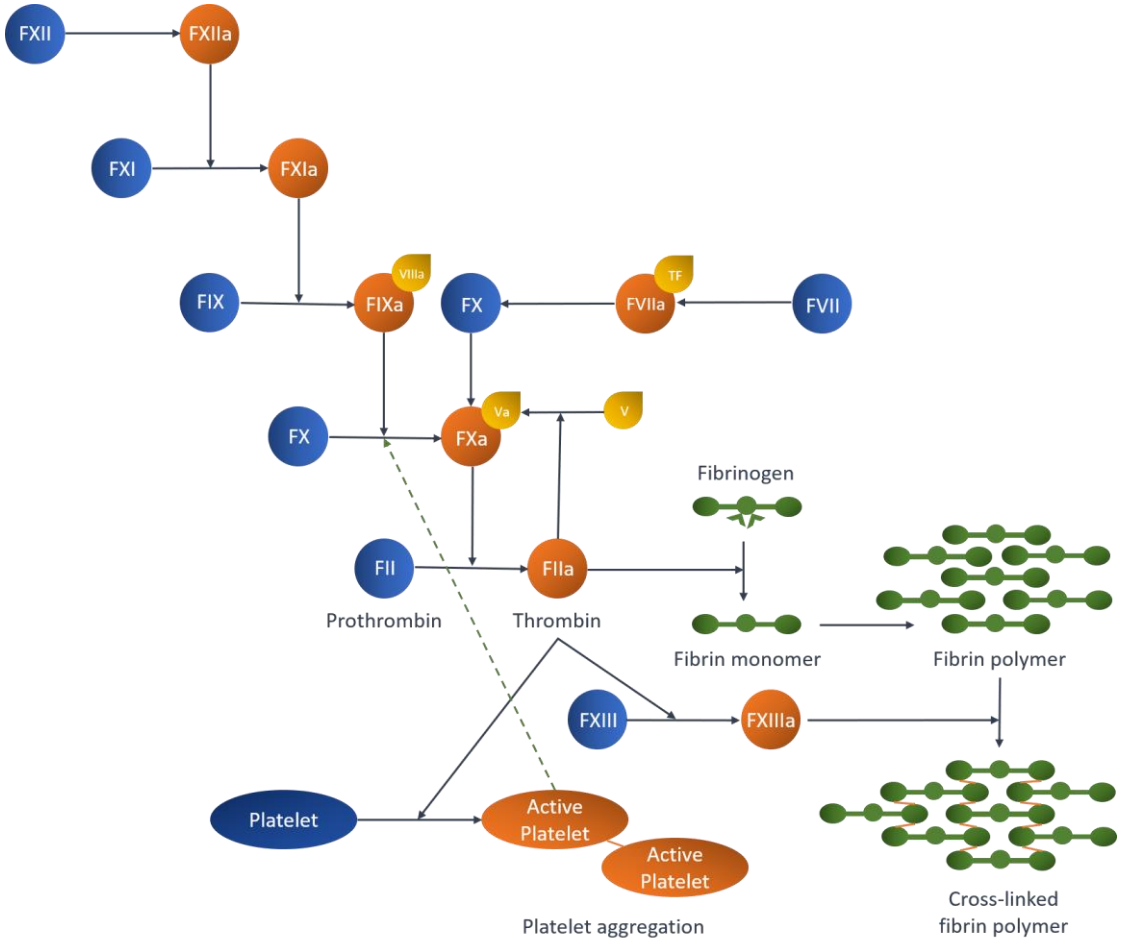

Figure 4.1. The coagulation pathway and platelet aggregation. Ex vivo, blood coagulation is initiated through contact of the blood with negatively charged surfaces, such as glass blood-collecting tubes or silica-coated plastic tubes. The coagulation cascade leads to thrombin formation and thrombin stimulates platelet activation and aggregation and fibrin clot formation. There is a close interaction between platelet activation and the blood coagulation cascade (indicated with the green dotted arrow). Inactive factors are presented in blue, active ones in orange and cofactors in yellow.

It is hypothesized that antithrombotic drugs, that inhibit the coagulation pathway (anticoagulants) or platelet function (antiplatelets), influence the generation and with that the properties of L-PRF membranes. Both the fibrin network structure and cellular content of L-PRF membranes may differ between patients taking antithrombotic drugs and those who do not. One way to compare the structural properties of L-PRF is through tensile testing, as the mechanical properties are most likely determined by the fibrin network, which is the only load bearing constituent present in the membrane. Additionally, it was proven that the properties of fibrin fibers underlie the mechanical properties of fibrin clots.[11] Indeed, it is difficult to evaluate the fibrin network through

imaging (alone). Furthermore, the fibrin network allows the enmeshment of platelets and leukocytes and their migration.[1,12–15] If anticoagulant drugs affect the network, it may be that the network cannot entrap cells as well anymore. Platelets and leukocytes release growth factors and cytokines, regulating healing and tissue regeneration as well as inflammation.[1,16–18] For this reason, membranes containing less cells may be less effective in supporting these processes. Moreover, the release of bioactive factors from platelets is linked to platelet activation , and can thus be altered by antiplatelet drugs.

We previously performed experiments for which we supplemented blood samples of volunteers with the anticoagulant heparin after sampling. We generated L-PRF membranes and evaluated mechanical properties and cellular content of the membranes.[19] The study indicated that low therapeutic concentrations of heparin probably do not affect L-PRF membranes. However, it was suggested that high concentrations impede the generation of the membranes, indicating a potential influence of anticoagulants on L-PRF.[19] The current study, for which blood samples were collected of patients on antithrombotic drugs, further explores this hypothesis.

The aim of the present comparative study was to evaluate the dimensions, tensile properties and cellular content of L-PRF membranes originating from patients treated with anticoagulants drugs, patients treated with antiplatelets and controls.

Materials and methods

The Medical Ethics Committee UZ/KU Leuven approved the study in May 2018 (S61315) and all patients signed an informed consent prior to blood collection.

Patients and blood collection for L-PRF generation

Blood samples were taken from patients visiting the Oral and Maxillofacial Department, University Hospitals of Leuven (Belgium), who were 18 years or older and who were not pregnant or lactating. Patients that had systemic or chronic diseases such as diabetes or active cancer were excluded. In agreement with the European Heart Rhythm Association (EHRA) guidelines, patients on anticoagulants skipped their non-vitamin K oral

anticoagulant (NOAC) if the intake was within 18 hours before the oral intervention, and patients on antiplatelets continued their antithrombotic medication.[20] Blood samples for preparing L-PRF membranes were collected in 9 mL silica-coated plastic tubes without anticoagulant (red cap, BVBCTP-2, Intra-Lock®). Within 1 minute from blood collection, the samples were centrifuged at 408 g RCF (2700 rpm) for 12 minutes (IntraSpin™, Intra-Lock®). L-PRF clots were separated from the red blood cell phase and placed in an L-PRF compression box (Xpression™ kit, Intra-Lock®) for gentle compression during 5 minutes. One additional whole blood sample per patient was collected in a 5 mL EDTA tube (purple cap, K2E (EDTA), Becton Dickinson Vacutainer®) for cell counting.

Tensile Testing

Uniaxial tensile tests were performed at FIBEr KU Leuven Core Facility for Biomechanical Experimentation. L-PRF membranes were placed on a cutting mat with centimeter tick marks and a picture was taken perpendicular to each membrane. The picture was loaded into Inkscape (GPL, version 0.92.4), the Inkscape ruler was calibrated with the centimeter tick marks, and the length of the membranes was measured. The L-PRF membranes were cut into dogbone shapes and marked with two black spots of graphite powder, used to track the stretching behavior during the tensile test (Figure 4.2). By aid of a micro laser scanner, the geometry of each sample was accurately measured. This resulted in a greyscale intensity image and a point cloud of each sample. The point cloud represented the outer surface of the sample with a spatial resolution of 0.05 mm in the x and y direction and a resolution of 0.03 mm in the z-direction. The thickness and width of the membranes were derived from these scans.

The membranes were mounted on 2 clamps of a tensile bench (MessPhysik, Zwick, Austria) without any tension (Figure 4.2). The bench was submerged in a saline solution (0.9% NaCl) bath at 37°C to approximate physiological conditions and to avoid dehydration of the L-PRF membranes. During the test, the forces and actuator displacements at the 2 sides of the sample were acquired with a 200 N class 1 load cell with a 1 µm spatial resolution, respectively, both at a rate of 20 Hz. In addition, images of the membranes were recorded with a Manta G-917B camera (Allied Vision, Germany) to track the deformation of the sample during the test, also at a sample rate of 20 Hz.

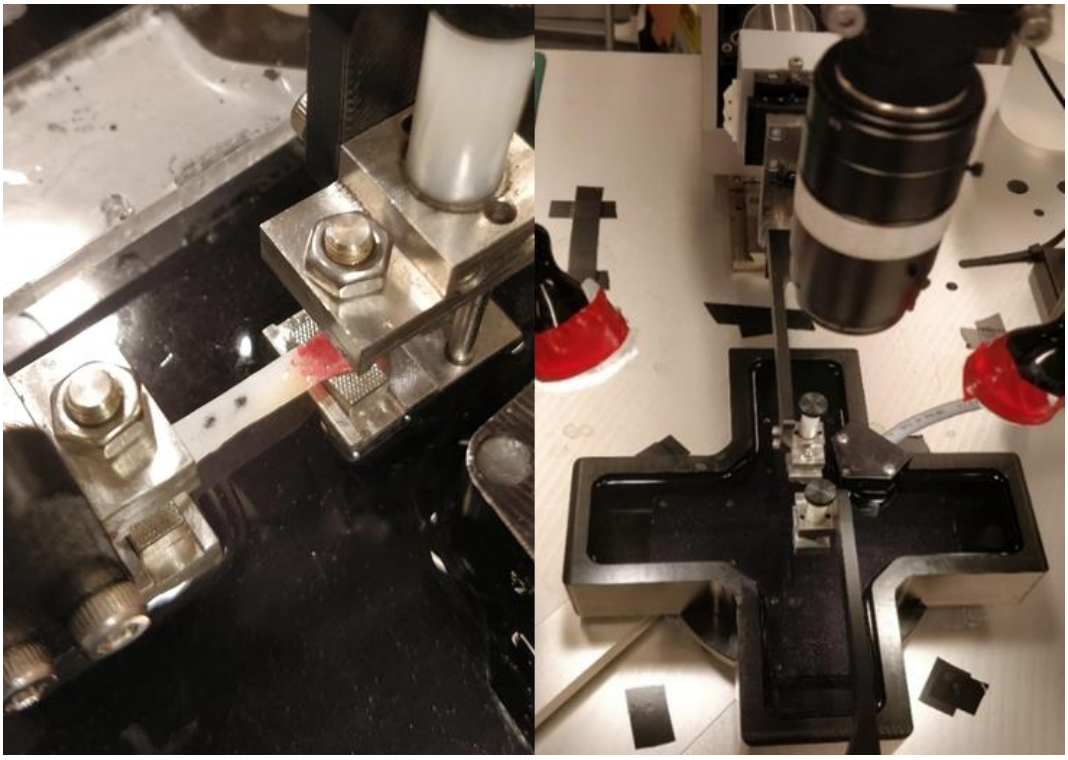

Figure 4.2. Set-up of the tensile test. (Left) An L-PRF membrane was dog-bone shaped, marked with two black spots of graphite powder, and mounted without tension on two clamps of a tensile bench (MessPhysik, Zwick, Austria). (Right) The membrane was submerged in a saline solution (0.9% NaCl) bath at 37°C (the cross-shaped black box) during the test. Two adjustable lights (in red) and a Manta G-917B camera (Allied Vision, Germany) were installed perpendicular to the membrane to track the black markers on the membrane.

The membranes were loaded with a preload of 0.03 N to avoid sagging. A loading of 5% and 10% strain was applied to facilitate synchronization between force measurements and camera images. Thereafter, the membranes were stretched until rupture with a loading speed of 1% nominal strain per second. The force measurements at both sides of the membrane were averaged and the Cauchy or True stress was calculated by dividing the averaged forces by the undeformed area and multiplying with the current stretch of the membrane (Table 4.1). The undeformed area was calculated by multiplying the undeformed (dogbone) neck width with the undeformed thickness of the membrane. The elongation of the membrane was measured by tracking black markers (black spots of graphite powder) on the images using in house developed software and the stretch was calculated by dividing the current distance between the markers by the initial distance (i.e. the distance at the preload level). True stress-True strain curves were plotted and the

E-modulus or Young's modulus [MPa], ultimate tensile strength (or maximum true stress) [MPa] and stretch at rupture [-] were calculated. The True strain was calculated as the natural logarithm of the obtained stretch. The E-modulus, the slope of the stress-strain curve, was calculated within multiple strain-ranges.

Table 4.1. Formulas of the tensile test parameters.

Formula	Parameter	
f	Average force applied at both sides of a membrane	
$\lambda = \frac{l}{l_0}$	Stretch of a membrane measured by tracking the black markers on the images	l = current length l_0 = initial length
$A = W * T$	Initial surface area of a membrane where force is applied on	W = initial width T = initial thickness
$\varepsilon = \ln(\lambda)$	True strain or logarithmic strain	λ = stretch of the sample
$\sigma = \frac{f}{A} \times \lambda = \frac{f}{a}$	True stress or Cauchy stress	f = average force A = initial surface area a = current surface area

Cell counting

Cell counting was performed by means of a hematology analyzer (Abbott Cell-Dyn 3700 Hematology Analyzer). After removal of the L-PRF clots from the collection tubes, the remaining blood in the tubes was preserved. After compression of the L-PRF clots, the exudate was gathered in tubes as well. The concentrations of the cells (platelets, leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils) were measured in both the remaining blood and the exudate, as well as in the initial whole blood sample in an EDTA tube from each patient. The concentration of cells in the L-PRF membranes were then calculated by subtracting the cells measured in the blood remnant and the L-PRF clot exudate from the cells measured in the initial whole blood sample (EDTA tube).

Statistics

The study was designed to test the null hypothesis that there are no differences between patients on anticoagulants or antiplatelets and patients not taking these drugs (controls). The power calculation was based on the variability and differences of mechanical properties between L-PRF samples detected in a previous study performed by our group (21). The final sample size was set at 10 patients per group, which would allow detecting an effect size of 0.6 using an Anova test. To account for dropouts, more patients per group were included. The statistical power was set at 80% and the two-sided significance level at 5%.

The statistical analysis was performed using R Software, version 4.0.0. The dimensions, mechanical properties and cellular concentrations were compared between control, antiplatelet and anticoagulant L-PRF membranes by fitting an Anova model with group as fixed factor. A residual analysis by means of a residual dot plot and a normal quantile plot showed that residuals had the same variance across treatment groups, but were not always normally distributed, for which a logarithmic transformation (data of the true stress and force) or arcsine transformation (data of the neutrophils, monocytes, eosinophils and basophils) were applied. Further, one outlier was removed for the analysis of the data of the thickness and the force, as these data points affected the underlying assumption of normality. Comparisons between treatment groups for the Anova models were corrected for simultaneous hypothesis testing according to Tukey. Additionally, the relation between thickness and force was assessed. As both variables are subject to measurement error, an orthogonal regression model was fit after exclusion of an outlier and a leverage point. Prior to orthogonal regression, an Ancova model showed that there was no evidence of a different relation between thickness and force for the three groups. For this reason, the orthogonal regression was performed without distinguishing between the groups.

Results

For the tensile tests, 35 patients donated blood, of whom 13 patients were not taking any type of antithrombotic drug (controls), 12 patients took anticoagulants (NOACs) and 10 were treated with antiplatelets (acetylsalicylic acid). For the cell counting, 55 patients were included, of whom 23 were controls, 16 were treated with anticoagulants and 16 with antiplatelets. The baseline characteristics of the patients are shown in Table 4.2.

Table 4.2. Baseline characteristics of the patients.

Tensile test	**Control (N=13)**	**Anticoagulant (N=12)**	**Antiplatelet (N=10)**
Age – yr	64±8	76±9	73±10
Male gender	3	5	7
Smoking status			
Active smokers	1	0	0
History of smoking	3	4	5
Never smoked	9	8	5
Alcohol ≤2 units daily	10	11	8
Cell counting	**Control (N=23)**	**Anticoagulant (N=16)**	**Antiplatelet (N=16)**
Age – yr	63±8	75±8	72±8
Male gender	10	5	11
Smoking status			
Active smokers	2	0	0
History of smoking	5	5	7
Never smoked	16	11	9
Alcohol ≤2 units daily	19	14	13

Plus-minus values are means ± standard deviation.

Membrane dimensions

The dimensions, as well as the mechanical properties and cellular content of the control, anticoagulant and antiplatelet membranes are shown in Table 4.3. The overall **length** of the membranes was similar between the groups, but the thickness tended to differ. On average, the anticoagulant membranes were **thinner** than the control membranes, but the difference of 0.1 mm was not significant (95% CI [-0.01;0.41], p=0.08), possibly due to high variability.

Table 4.3. Dimensions, tensile test and cellular content of the L-PRF membranes.			
	Control	**Anticoagulant**	**Antiplatelet**
Membrane dimensions			
Length [mm]	34.5±5.6	35.9±3.3	38.1±5.4
Thickness [mm]	0.50±0.11	0.41±0.09	0.48±0.09
Tensile test			
Maximum Force [N]	0.77±0.30	0.49±0.18	0.63±0.14
Maximum True Stress [MPa]	0.80±0.27	0.57±0.24	0.87±0.34
Stretch at rupture [-]	2.07±0.26	1.79±0.27	2.08±0.28
Cellular count			
Platelets (%)	52±19	50±16	54±24
Leukocytes (%)	78±8	69±10	71±15
Neutrophils (%)	74±25	63±20	73±22
Lymphocytes (%)	77±9	66±11	75±11
Monocytes (%)	84±19	79±24	88±11
Eosinophils (%)	90±7	81±22	81±18
Basophils (%)	68±24	72±17	70±26

Plus-minus values are means ± standard deviation. The percentage of cells in the L-PRF membranes are the relative proportions of the cells to the initial whole blood.

Tensile testing

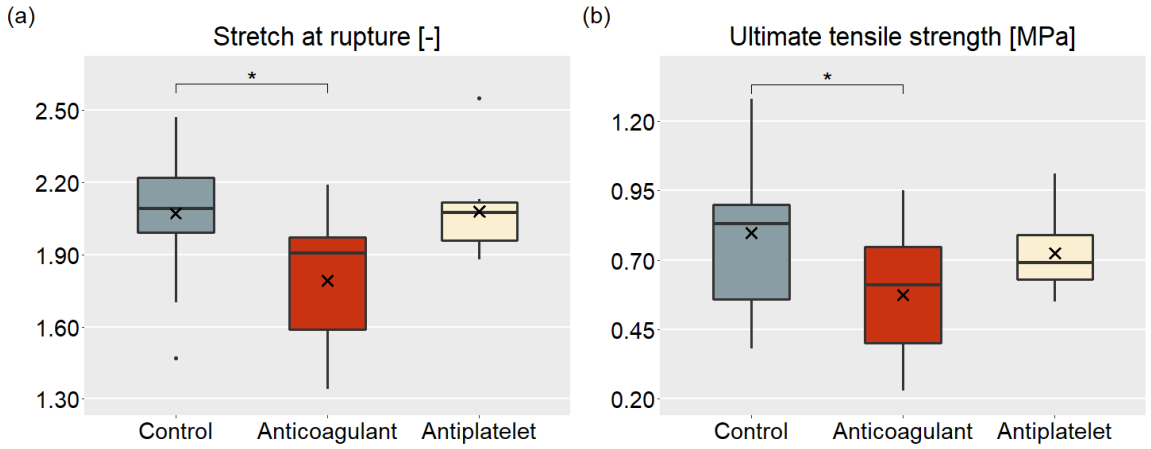

Figure 4.3. The stretch at rupture and ultimate tensile strength of the L-PRF membranes. The mean (x) and significant differences (*) are indicated. (a) The anticoagulant membranes could not be stretched as far as control membranes (p=0.01) and (b) were weaker than controls (p=0.03), regardless of the high variability. Antiplatelet membranes showed comparable results as the control membranes.

Figure 4.3a shows the maximum **stretch at rupture**. On average, the anticoagulant membranes failed earlier during the tensile test, whereas control membranes could be stretched further (1.8±0.3 times the initial membranes' length versus 2.1±0.3 with a difference of 0.2, 95% CI [0.05;0.41], p=0.01). Antiplatelet membranes showed a similar stretch as controls. Figure 4.3b shows the **ultimate tensile strength** (or maximum True stress) and it was shown that anticoagulant membranes were weaker (0.57±0.24 MPa) than control membranes (0.80±0.27 MPa) with a difference of 0.33 MPa (95% CI [0.02;0.63], p=0.03). The antiplatelet membranes, on the other hand, did not significantly differ from controls.

A correlation test showed that the thicker a membrane, the higher the maximum force it could bear before it ruptured (r=0.6, p<0.001).

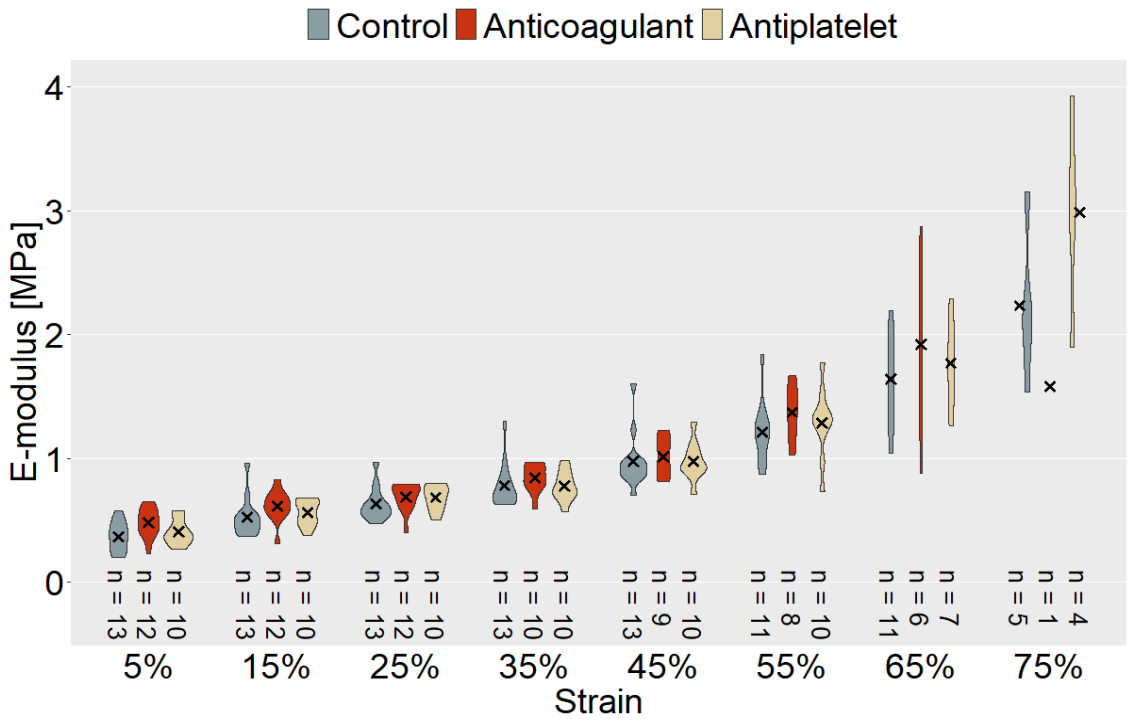

Figure 4.4: E-moduli data of the L-PRF membranes. The violin plot shows the full distribution of the data. The means of the E-moduli (x) and the number of membranes (n) are indicated. The E-moduli were calculated at different levels of strain. At each level, the E-moduli were similar over the three groups. In the three groups, the E-moduli increased with increasing strain, indicating stiffening behavior. More membranes in the anticoagulant group failed early during the tensile test, whereas control membranes could be stretched more far. Note that the outliers in the control group detected at 35%, 45% and 55% strain all originate from one patient.

Figure 4.4 outlines the **E-moduli**, the slopes of all True stress-True strain curves, at different levels of strain and shows increasing E-moduli with increasing strain in all groups. This indicates stiffening behavior. The E-moduli between the groups were similar at all levels of strain and the statistical analysis showed no significant differences. The number of membranes reaching a certain percentage of strain is indicated on the x-axis. More membranes in the anticoagulant group failed early during the tensile test (failing membranes from 35% strain onwards) compared to control and antiplatelet membranes that generally failed later (failing membranes from 55% and 65% strain onwards).

Cell counting

The ratio of **platelets** was very similar and close to 50% of the initial whole blood in the three groups (Table 4.3). The ratio of **leukocytes**, on the other hand, was the highest in the control membranes (78±8%), followed by the antiplatelet membranes (71±15%), and anticoagulant membranes (69±10%). The anticoagulant membranes contained significantly less leukocytes compared to the control group, with a difference of ±9% (95%CI [0.3%;17.6%], p=0.04). This difference was mainly driven by a lower percentage of **lymphocytes** (difference of ±12% compared to controls, 95%CI [3.3%; 19.8%], p=0.004). There were no significant differences between the control and antiplatelet groups.

Discussion

Antithrombotic drugs interfere with blood coagulation and platelet aggregation and may therefore influence the fibrin network formation and consequently the properties of leukocyte- and platelet-rich fibrin membranes. As the porous matrix of L-PRF membranes serves as a scaffold for cellular infiltration and migration, important for healing and regeneration, altered L-PRF membranes in patients on antithrombotics may have clinical implications.[3] We compared properties of L-PRF membranes of patients on anticoagulant or antiplatelet drugs with membranes of patients not treated with antithrombotic drugs.

Our results indicate that L-PRF membranes of patients on anticoagulants are weaker, stretch less far and contain approximately 9% less leukocytes than membranes of patients

not taking antithrombotic drugs. In contrast, antiplatelet agents did not affect these properties.

The question rises what may be at the base of the differences between anticoagulant and control membranes in our study. A plausible explanation lies in the three-dimensional network of the membranes. A high (but natural) thrombin or fibrinogen concentration (controls) leads to great fibrin branch point density and thinner fibers stimulating membrane rigidity, whereas a low concentration (anticoagulant membranes) results in less branching and thicker fibers.[14,21,22] It is also shown that fibrin fibers underlie the mechanical properties of the fibrin networks.[11] Possibly, membranes with a lower branching density are weaker and rupture faster when stretched.

In addition, the thinner a membrane, the lower the force it could withstand and the anticoagulant membranes were on average thinner than the controls. However, the difference was not significant. Moreover, the thickness of a membrane most likely depends mainly on its generation process by aid of an Xpression™ box (Intra-Lock®). The gap between the perforated tray and weighted cover of the box, between which an L-PRF clot is placed to compress, measures 0.5 mm (with tolerance that may go up to 0.9 mm). The thickness may also depend on how long the membranes are pressed.

In literature, there are other studies describing the mechanical characteristics of L-PRF membranes.[23–27] The average E-modulus of membranes in our study was 0.4–3.0 MPa, depending on the level of strain, while in the other studies the E-modulus varied between 0.01 and 0.5 MPa.[23–27] However, none of the studies found in literature reported at which strain the E-modulus was calculated.[23–27] In addition, often the calculation of the E-modulus was lacking. The E-modulus is theoretically calculated as the slope of true stress-true strain curve (as done in this paper), but in practice different formulations of stress and strain are used, which results in a kind of stiffness modulus, but not the E-modulus. The average strength in the current study was 0.6–0.9 MPa, which is higher than reported in other studies.[23–27] Nevertheless, because of various protocols any comparison should be made with caution.

Amongst factors that varied between the tensile protocols were the shape of the membrane (rectangular or dogbone shaped), crosshead speed of the test, and tensile protocol itself. Preconditional loading for tensile tests is good-practice for biological tissues and is important to allow meaningful comparison of the results within the study and with other studies. Nevertheless, to our knowledge, none of the studies found in literature applied preconditional loading.[23–27]

Next, the L-PRF centrifugation protocol is important to consider as it can influence the dimensions and structure of L-PRF membranes. First, the longer the time from blood collection to centrifugation, the shorter the L-PRF membrane.[28] This time interval should be as short as possible, ideally between 60–90 seconds.[28] Second, it is proven that some centrifuges produce lighter and shorter membranes than other centrifuges.[29] For our experiments, we used the IntraSpin™ centrifuge, producing heavy and long clots according to the aforementioned study.[29] Third, reducing the centrifugation speed and time results in a lower fibrin network density.[30]

Further, anticoagulant membranes in our study contained approximately 9% less leukocytes than control membranes. There were ±12% less lymphocytes in the anticoagulant group compared to the controls, but no difference in neutrophils, monocytes, eosinophils and basophils, meaning that the difference in leukocytes was mainly attributed to the difference in lymphocytes. A possible explanation might be the size of the cells. (Inactive) lymphocytes are amongst the smallest leukocytes with a diameter of 5–7 µm and may escape a more porous network with less branching of anticoagulant membranes, whereas bigger cells may not.[21,22,31] Active lymphocytes, involved in immune processes, can have a diameter of 14–20 µm though.[31] At first glance, this hypothesis is not supported by the finding that the proportion of platelets, cells with a diameter of 2–4 µm and smaller than lymphocytes, was similar amongst the groups, but platelets occur in activated form in L-PRF and aggregated platelets may not escape the porous network.[24,31,31] Nevertheless, the three groups showed a high variability, and the inter-patient variation should be considered when interpreting the differences between the groups.

Indeed, patient-related factors are important as they can influence L-PRF membranes.[30,33] In our study, control patients were on average 10 years younger than patients taking antithrombotic drugs were. A cytology study reported a more irregular arrangement of the fibrin network and a reduced number of platelets and leukocytes in L-PRF membranes with increasing age.[33] However, the study compared patients between the age of 20–39 years, 40–59 years and ≥60 years[33], while the average age of the patients included in the current study was above 60 years. Another patient-related factor to consider is the concentration of antithrombotic drugs in the blood related to the time between the last intake of the antithrombotic and the oral intervention. In our study, patients on NOACs skipped a dose if this dose was within 18 hours before the oral intervention with L-PRF. This practice could lead to relatively low NOAC levels. However, we believe our findings are relevant either way, because patients on NOACs who come for interventions with L-PRF would also be treated this way.

The clinical relevance of our findings is still unknown. Possibly, affected L-PRF membranes of patients on anticoagulants are less beneficial for wound healing than L-PRF membranes of patients not taking these drugs. In this case, it would be important to rule out the influence of anticoagulants. One way of doing so may be interrupting the anticoagulant therapy before a surgical intervention with L-PRF. The risk of thromboembolic complications that goes together with such interruption must be weighted though. For now, we advise to plan an oral intervention with L-PRF at trough level of an anticoagulant.

The current study has some strengths and limitations. To our knowledge, this is the first study to compare mechanical properties and cellular content of L-PRF membranes originating from patients on an anticoagulant or antiplatelet therapy to patients not taking antithrombotic drugs. A second strength is the temperature- and humidity-controlled condition under which tensile tests were performed, mimicking physiological conditions and ruling out the potential influence of dehydration on the tensile results. Additionally, the experimental protocols should always be considered when comparing results of various studies, for which a detailed description of the methods is crucial. We clearly described the centrifugation and experimental protocols in this manuscript, and hope that all researchers will do so in the future.

The current study has the following limitations. First, the intra- and inter-patient variability inherent to patient-related factors such as blood variety, and to the L-PRF generation process should be considered. Next, some types of antithrombotic medication were underrepresented. Although NOACs are now the most used type of oral anticoagulants, it is unclear whether our results would also translate to patients treated with vitamin K antagonists, which affect the coagulation system in a different way. To minimize the risk of bias, we selected patients without chronic and systemic diseases. Nevertheless, patients taking antithrombotic drugs are generally elderly patients with comorbidities and treated with co-medication. Ideally, we should conduct a prospective study aiming for patients in whom an antithrombotic is prescribed for the first time or after an interval without the intake of any antithrombotic, so that we could compare L-PRF samples of a same patient before and during the intake of an antithrombotic. This would exclude any confounding factors. Furthermore, it would be interesting to take L-PRF samples at different time points after the intake of an antithrombotic, and to measure the amount of antithrombotic in the blood of the patients, to better quantify the effect of the antithrombotic agents on the properties of L-PRF. Last, the method to assess the cellular content of the L-PRF membranes did not take into account the possible loss of cells during the sample processing and might therefore be imprecise.[34]

Conclusion

In conclusion, our results indicate that L-PRF membranes of patients on anticoagulants are weaker, stretch less far and contain less leukocytes than L-PRF membranes of patients not taking antithrombotics. In further research, an increased sample size and altered study design may eliminate confounding factors between patient groups. The clinical relevance of our findings should be investigated.

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GENERAL DISCUSSION

CHAPTER 5
General discussion, conclusions and future perspectives

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The increased risk of **bleeding and impaired healing after oral interventions in patients treated with antithrombotic medication** is a problem and worries patients and oral surgeons. The best management to obtain good hemostasis and avoid bleeding in these patients is under debate. In this light, the current **PhD project focused on two concerns**.

1. An increasing number of patients is treated with non-vitamin K oral anticoagulants and these patients have a higher risk of bleeding after dental extraction than patients not taking these drugs, while it is not sure how to reduce the risk of bleeding.

2. Antithrombotic drugs may interfere with the generation of leukocyte- and platelet-rich fibrin membranes and consequently alter the membranes' properties. As L-PRF membranes are used to guide natural healing and as hemostatic material after oral interventions, variations in their properties may have clinical implications. It is therefore of interest to study if L-PRF membranes of patients on chronic antithrombotic drugs differ from patients not taking these drugs.

In this chapter, the results of the PhD project are critically discussed, important considerations are put forward, and future perspectives are defined.

General discussion Chapter 2

Chapter 2 focused on oral bleeding after dental extraction in patients treated with NOACs, which is a frequent complication.[1,2] Tranexamic acid mouthwash is a potent hemostatic agent to reduce bleeding after dental extraction in patients on vitamin K antagonists[3], but its use in patients treated with NOACs has never been studied. We conducted the placebo-controlled, double blind, randomized, clinical **EXTRACT-NOAC trial** to evaluate if tranexamic acid mouthwash also reduces bleeding after dental extraction in this latter patient population.

The EXTRACT-NOAC study is the first clinical trial assessing if TXA reduces post-extraction bleeding in patients on NOACs. The results provide new data in the debate of the optimal anticoagulant and hemostatic management of these patients having dental extractions.[4] We aim at implementing a safe(r) peri- and postprocedural management for reducing bleeding after dental extractions in patients treated with NOACs.

The results indicate that **TXA** does not reduce peri-procedural or early post-operative oral bleeding compared to placebo, but may **reduce delayed bleeding and post-extraction bleeding if multiple teeth are extracted**. Possibly, elderly patients and patients who restart their NOAC within 24 hours after a dental extraction may benefit more from treatment with TXA compared to younger patients and patients having their first NOAC after 24 hours from the dental extraction. Follow-up studies involving these high-risk patient populations should be performed to further evaluate these findings.

Four other patient **subgroups** were analyzed, but the analysis did not show that TXA was better in one of these particular subgroups. A first subgroup was osteotomy versus a simple extraction. Second, it did not matter if patients were treated with other hemostatic agents (such as spongostan), additionally to the use of TXA, during the dental procedure or not. Next, some patients also used a chlorhexidine mouthwash (with antibacterial properties) besides TXA mouthwash. Again, there was no difference in bleeding between patients that did and did not use chlorhexidine. Last, the rate of bleeding in patients having incisors or canines versus molars removed was similar.

Another important factor to consider is **infection**. Infection by certain types of bacteria, such as Staphylococcus aureus and Streptococcus pyogenes, found in subgingival plaque,

or Prophyromonas gingivalis, strongly associated with periodontitis, can enhance the bleeding tendency of the gums due to the release of proteinases from these bacteria interfering with the clotting potential by degrading fibrinogen or fibrin.[5,6] It is, in other words, plausible that patients with infection have a higher risk of bleeding after dental extractions than patients without infection. It may be that the antifibrinolytic effect of TXA overcomes the fibrinolytic effect of the bacteria, so that TXA is beneficial for patients presenting with an oral infection. In addition, we may wonder if antibiotics can decrease the higher bleeding tendency, or if an additional treatment with TXA is designated. Unfortunately, our study was not designed to answer these questions. Identifying and studying patients presenting with caries or periodontal disease was hard because of two reasons. First, a patient could have had a dental extraction because of multiple indications instead of caries or periodontal disease alone. Second, a patient may have presented with one of these underlying conditions, but had a tooth extraction because of another indication.

The main **strength** of the EXTRACT-NOAC trial is its design: a multicenter, double blind, placebo-controlled, randomized clinical trial, delivering the **highest level of evidence** in evidence-based medicine. Another strength was the broad inclusion criteria, reaching a large patient population and ensuring maximal external validity. All patients older than 18 years treated with a NOAC and scheduled for dental extraction were eligible for inclusion. We included all four currently available NOAC types, which are edoxaban, rivaroxaban, apixaban and dabigatran. According to numbers of the RIZIV, at least 18,000 patients on NOACs a year have dental extractions in Belgium. Third, the intervention we studied is safe, low-cost and easily implementable in practice. TXA mouthwash is considered safe without evidence for prothrombotic effects, because of the maximal local effect and minimal systemic exposure, and as our study safety outcomes showed as well.[7] We designed the EXTRACT-NOAC study with the locally available concentration and dosing of TXA (10% TXA in 10 uni-doses).

The main **limitation** of the EXTRACT-NOAC study was its **premature discontinuation** after a futility analysis following the outbreak of the SARS-CoV-2 pandemic. At that time, 222 of the 236 planned patients were randomized. The Data Safety Monitoring Board advised to halt patient recruitment based on the number of patients with oral bleeding. However,

if they had considered the number of bleeding events as well, they might have suggested otherwise.

There are other study restrictions, aside from the limitations discussed in Chapter 2. We did not consider the local application of TXA on gauzes, while this method may reduce bleeding as well. The reason we focused on TXA mouthwash is that its efficacy for reducing bleeding after dental extraction in patients on vitamin K oral antagonists was proven in a previous clinical trial.[8] Additionally, we conducted a systematic review on the use of hemostatic agents after tooth extraction in patients on antithrombotic medication.[3] Eight of the 15 included studies focused on the use of TXA. Studies comparing TXA-soaked gauzes to the use of other hemostatic methods (such as sponges or gauze pressure) failed to show a difference.[3] A possible concern with the use of TXA on gauzes is that patients, by applying gauzes on the extraction site with their hands, bring bacteria into their mouth that may promote infection. In future work, it may be interesting to compare TXA mouthwash to TXA-soaked gauzes. Last, the increased bleeding tendency in patients with oral infection and the role of tranexamic acid in these patients needs further study.

Regardless of these limitations, our study showed an overall benefit of TXA for reducing delayed bleeding and bleeding after multiple dental extractions in patients on all four types of NOACs. Moreover, the use of **TXA** has more **advantages**.

First, our results indicated that TXA may reduce the number of patients with unplanned medical contacts, and with this the associated costs. In addition, as TXA reduces delayed bleeding and bleeding often leads to NOAC interruption, its use may avoid such unplanned interruptions. This is important since the interruption of an anticoagulant therapy is a risk factor for subsequent and potentially life-threatening thromboembolic events.[9] In our study, one patient had a thrombotic complication related to a two-day NOAC interruption prior to the dental extraction. The patient was hospitalized for 2 days after the event. Although less frequent, the morbidity and costs of thromboembolic events are extremely high. Last, as TXA is available on the Belgium market at low cost (€13 per patient), its routine use can be easily and directly implemented in daily clinical practice.

General conclusions Chapter 2

In the EXTRACT-NOAC study, we focused on a **frequent and still-increasing problem** with important associated risks and costs. The potential to improve patient care and prevent troublesome and potentially costly outcomes such as bleeding and thrombotic complications with a low-cost and easy intervention is of clear benefit to all involved parties.

The results of our clinical trial indicate that **tranexamic acid may reduce** delayed **bleeding** and bleeding after the extraction of multiple teeth in patients treated with non-vitamin K oral anticoagulants. The use of TXA mouthwash after dental extraction may also reduce the number of patients reconsulting a dentist or oral surgeon. Moreover, the intervention with TXA mouthwash is **cheap, safe, available on the market and easy to implement** in clinical practice.

General discussion Chapters 3 and 4

In **Chapter 3 and 4**, we studied the hypothesis that **antithrombotic drugs** may impede the generation of leukocyte- and platelet-rich fibrin membranes and, with that, influence the **properties of L-PRF membranes**. The variability of this biomaterial used to guide natural healing and tissue regeneration may have clinical implications.

The study described in **Chapter 3** evaluated blood samples of healthy volunteers supplemented with the anticoagulant enoxaparin. The results showed that **high concentrations hindered** the generation of L-PRF membranes, and that low therapeutic concentrations had no (great) impact on the generation, mechanical properties and cellular content of the membranes compared to controls. A second major finding was the high variability between L-PRF membranes. In a subsequent study (**Chapter 4**), L-PRF membranes between **patients** on anticoagulants or antiplatelets and patients not taking antithrombotic drugs were compared. The results showed that L-PRF **membranes from patients taking anticoagulants were weaker, stretched less far, and contained less leukocytes** compared to controls. We detected no differences between L-PRF membranes of patients on antiplatelets and controls. The clinical significance of our findings needs further study.

The finding of the **tensile tests** indicating that anticoagulant membranes are weaker and stretch less far may be attributed to the three-dimensional fibrin network. After all, anticoagulant drugs interfere with thrombin, resulting in lower levels of fibrin and leading to bilateral fibrin junctions and a lower branching density of the fibrin network.[10,11] Possibly, the affected network structure results in a weaker membrane.

What is more, the network with bilateral junctions impedes cell enmeshment, whereas equilateral junctions support cell enmeshment.[10] The impaired network of the anticoagulant membranes could therefore also be the cause of the lower rate of leukocytes that we observed with **cell counting**. This reduction can lower the efficacy of L-PRF in stimulating healing as these cells play a role in wound repair and mediate inflammation.[12] Nevertheless, the difference between the rate of leukocytes in anticoagulant and control membranes was only ±10%, which may be clinically irrelevant.

Importantly, in our study, mechanical properties and cellular concentrations showed a high variety, emphasizing the **inter-patient variability**. Gender, age, and comorbidities might be confounding factors. It is shown that females and elderly patients produce larger membranes (although the latter finding was not significant).[13] The influence of length on the mechanical properties or cellular content is unknown, though. Another (cytology) study showed a less dense and more irregular fibrin network and reduced number of platelets and leukocytes with increasing age.[14]

Besides patient-related factors, the **L-PRF generation and experimental protocols** are important to consider, as indeed these may influence L-PRF as well.[13,15,16] Studies about the mechanical properties of L-PRF found in literature often describe only very briefly their study protocols.[17–21] In addition, the protocols differ between studies. This makes comparison of tensile results difficult, if not impossible, or at least not useful. In future studies it is therefore crucial that the methods are well-described.

The main **strength** of our project studying the influence of antithrombotics on the properties of L-PRF membranes is its **novelty**. To our knowledge, no studies have previously investigated this. Another strength is that the research was conducted in an interdisciplinary scientific environment combining knowledge from dental, cardiovascular, and bioengineering disciplines. The research team of Periodontology and Oral Microbiology of KU Leuven, experienced in research on L-PRF for years, helped designing the current studies. The cell counting was performed at the Center for Molecular and Vascular Biology at KU Leuven. The team of Cardiovascular Sciences also advised us about the (working mechanisms of) blood thinning medication. Further, we could use the high-tech equipment of FIBEr, of the Biomechanics Section of KU Leuven, for the tensile testing described in Chapter 4. FIBEr is a core facility of KU Leuven for biomechanical experimentation specialized in human biomaterials. We could rely on the expertise of researchers working there, who thought us good-practice protocols for tensile tests and guided us in practice.

We have to consider some **limitations** as well. As little research has been performed on the mechanical characterization of L-PRF and because protocols of these tests are often not described in detail in studies found in literature, it took some time to figure out the best way to evaluate the mechanical properties. We first had to set up a network between

various departments and research groups and then had to find the best protocol for the tensile test. This was a matter of trial and error and involved 13 volunteers that all together gave 191 blood samples. In the end, the experiments required a lot of careful work at different departments, which was both an advantage and a challenge.

Another restriction of the current research is that we did not investigate biological properties such as the release of cytokines, coagulation factors and growth factors, or the activation of platelets in the membranes. These factors are important for the clinical efficacy of the membranes. Neither was the fibrin membrane structure thoroughly investigated through imaging such as scanning electron microscopy or micro computed tomography. Unfortunately, we were only able to perform scanning electron microscopy with a low number of membranes (Chapter 3) because of time and resource restrictions. We decided to reasonably focus on the mechanical tests and cellular counts. As mentioned before, we devoted a lot of time and attention to these experiments. The comparison of the biological properties and visual inspection of fibrin networks of L-PRF membranes between patients on antithrombotics and control patients would be an interesting topic for future research.

The current project **enhances our knowledge** about some basic properties of L-PRF. The tensile tests and cellular counts gave a better understanding of the mechanical behavior, strength, stretch and cellular composition of L-PRF membranes, as well as the potential influence of antithrombotic drugs on these properties. This information is useful for other medical disciplines aside from oral surgery, as L-PRF membranes are more frequently used in other specialisms as well. L-PRF is, for instance, applied to treat chronic wounds as leg ulcers and diabetic feet, or to close the dura mater with neurosurgery.[22–24] Further, we hope to **optimize the use and efficacy of this biomaterial** in clinical practice. We may think of a way to rule out the influence of anticoagulants, such as interrupting the anticoagulant therapy before the surgical intervention with L-PRF. The risk of thromboembolic complications that goes together with such interruption must be weighted though. Moreover, the clinical relevance of the influence of anticoagulants should first be explored. For now, we advise to plan an oral intervention with L-PRF at trough level of an anticoagulant.

General conclusions Chapters 3 and 4

We studied the possible influence of antithrombotic drugs on the properties of leukocyte- and platelet-rich fibrin membranes as this may cause variation amongst L-PRF membranes and may have clinical implications.

The results showed that **patients on anticoagulants**, but not on antiplatelets, may produce **weaker L-PRF membranes that stretch less far and contain less leukocytes** than patients not taking these drugs. The reason and clinical relevance of the difference have not yet been clarified.

Future perspectives

To date, two concerns have arisen about the management of antithrombotic drugs in patients undergoing dental extraction to avoid bleeding complications and achieve good tissue healing.

First, there is no consensus on the management of patients on non-vitamin K oral anticoagulants to reduce bleeding after dental extractions. Bleeding occurs with up to 25% of these patients and frequently calls for unplanned medical consults and re-intervention to stop the bleeding.[1,9,25] Moreover, the NOAC therapy is often interrupted because of bleeding, which is a risk factor for thrombotic complications.[9] To reduce the risk of bleeding, a straightforward and evidence-based anticoagulant and hemostatic guideline should be implemented in clinical practice. **We advise patients on NOACs to skip one NOAC dose before their dental extraction and to use tranexamic acid mouthwash afterwards three times a day for three days**, as our randomized clinical trial showed that this protocol may reduce delayed bleeding and bleeding after multiple dental extractions.

Future clinical trials can further evaluate the efficacy of TXA mouthwash compared to TXA-soaked gauzes and their use in high-risk populations such as elderly patients and patients taking their first NOAC within 24 hours after dental extraction.

The translation of our research findings into practice **guidelines** is extremely important and will have societal implications. Our research group has been actively involved in the development and dissemination of the current guidelines on the management of antithrombotic drugs in the oral practice, which are also available on the following website: www.bloedverdunners-tandheelkunde.be.[26] Thanks to the publication of our randomized clinical trial in an international peer-reviewed journal, our findings will also have international impact.[27]

Second, the causing factors of variation amongst L-PRF membranes are not (yet all) unraveled, and antithrombotic drugs may play a role. Blood coagulation is crucial for generating L-PRF and as antithrombotic drugs impede this process, they may influence the properties of L-PRF. As L-PRF is used to stimulate wound healing and tissue regeneration with oral surgery, the potential influence of antithrombotics may have clinical implications. Nevertheless, the influence of these drugs on L-PRF has never been

studied. Our experiments **enhance the knowledge** about the dimensions, strength, stretching-ability and cellular content of L-PRF membranes. **Membranes from patients on anticoagulant drugs may be weaker, stretch less far, and contain less leukocytes** than membranes from patients not taking antithrombotics. Oral surgeons may have to take this into account in practice. However, the clinical implications of our findings should first be investigated.

The next step can be to conduct a **prospective study** evolving a higher number of patients in whom an antithrombotic is prescribed for the first time or who interrupt their antithrombotics for a period. This way, L-PRF membranes generated from patients' blood before and during the antithrombotic treatment can be compared, while excluding any confounding factors. Additionally, it would be of interest to measure the bioactive factors in L-PRF membranes and evaluate clinical outcomes of patients on antithrombotics and patients not taking antithrombotics treated with L-PRF.

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SUMMARY

The current **PhD project** is about patients on antithrombotic medication undergoing dental extractions and focusses on **two genuine concerns**.

1. Oral bleeding after dental extraction in patients on non-vitamin K oral anticoagulants (NOACs) is a frequent problem, and it is not sure how to reduce the risk of bleeding, whereas the number of patients treated with NOACs is increasing.

2. Variation of leukocyte- and platelet-rich fibrin (L-PRF) membranes was noticed and antithrombotic medication may be one of the causing factors as they interfere with blood coagulation, a process that is crucial for the generation of L-PRF. As L-PRF membranes are used to guide healing and enhance tissue regeneration and as hemostatic method, the variability amongst the membranes may have clinical implications.

In **Chapter 1**, these two **concerns are positioned**. The management of oral anticoagulants in patients undergoing dental extraction is described, as well as the potential of tranexamic acid to reduce the risk of bleeding. A short history and description of the properties of L-PRF membranes is given and factors potentially influencing the properties of this biomaterial are discussed.

Chapter 2 was about bleeding complications in patients on NOACs. We conducted the **EXTRACT-NOAC study**, a multicenter, placebo-controlled, double blind, randomized clinical trial to evaluate if the use of **tranexamic acid mouthwash**, an antifibrinolytic agent, after dental extraction reduces bleeding in patients on NOACs. Patients were instructed to skip their NOAC dose on the morning of the dental extraction and to use the

mouthwash once immediately prior to the dental extraction and three times a day for three days thereafter. Patients were followed up for seven days. In total, data of 218 patients was included in the full analysis set of who 106 were randomized to tranexamic acid and 112 to placebo mouthwash. The trial results indicated that tranexamic acid mouthwash does not reduce peri-operative bleeding or early bleeding (until one day) after dental extraction, but may reduce delayed bleeding (more than one day after) and bleeding in case multiple teeth are extracted. Moreover, TXA may be beneficial in patients ≥75 years and patients taking their NOAC within 24 hours after dental extraction.

Chapter 3 and 4 of the current work are about the properties of **L-PRF membranes** and the possible influence of antithrombotic drugs on these properties. In a first study (Chapter 3), blood of 12 volunteers without chronic or systemic diseases and not taking any drugs was supplemented with various concentrations of the anticoagulant drug enoxaparin. Tensile tests, cellular counts and scanning electron microscopy were performed to compare mechanical properties (stiffness, strength and stretch), cellular content (platelets and leukocytes) and fibrin networks of membranes with and without enoxaparin. The experiments showed that a low therapeutic concentration of enoxaparin probably does not influence the properties of L-PRF membranes, but also that a high concentration impedes the generation of L-PRF. Another important finding was the high variability amongst membranes.

In a second study (Chapter 4), blood samples of patients on anticoagulants, of patients on antiplatelets and of patients not taking antithrombotic drugs (controls) were taken. L-PRF membranes of 25 patients were used for tensile testing (13 controls, 12 anticoagulants and 10 antiplatelets) and 55 patients were included for cell counting (23 controls, 16 on anticoagulants and 16 on antiplatelets). It was shown that L-PRF membranes of patients on an anticoagulant therapy may be weaker, rupture faster and contain less leukocytes than membranes of patients not taking antithrombotic drugs. The difference is possibly explained by alterations in the fibrin network. On the contrary, antiplatelet and control membranes appeared to have more or less the same properties. Again, we saw a high inter-patient variability.

In **Chapter 5**, the studies are critically **discussed and future perspective** are outlined. Following the results of the randomized clinical EXTRACT-NOAC trial, we advise patients on NOACs to skip one NOAC dose before their dental extraction and to use tranexamic acid mouthwash afterwards three times a day for three days. We aim at publishing these findings in straightforward and evidence-based guidelines and implement them in clinical practice. It would be interesting if future studies focus on elderly patients and include the use of TXA on gauzes. Next, the experiments on L-PRF showed that the use of anticoagulants may result in weaker membranes that rupture faster and contain less leukocytes. Future studies should focus on confounding factors, the release of biologic factors from the membranes, and the clinical relevance of these findings.

SAMENVATTING

Dit **doctoraatsproject** gaat over tandextracties bij patiënten op chronische antitrombotische medicatie en focust op **twee bezorgdheden**.

1. Bloedingscomplicaties na tandextracties bij patiënten op nieuwe / niet-vitamine K of direct werkende orale anticoagulantia (NOACs) is veel voorkomend, maar het is niet zeker hoe men het risico op bloedingen kan verminderen, niettegenstaande het aantal patiënten behandeld met NOACs toeneemt.

2. In de kliniek ervaart men variabiliteit in 'L-PRF' bloedmembranen, maar het is niet zeker welke factoren die variabiliteit veroorzaken. Antitrombotische middelen zouden een rol kunnen spelen aangezien ze inwerken op de bloedstolling, een proces dat cruciaal is voor het vormen van deze membranen. Aangezien L-PRF-membranen gebruikt worden om de wondheling en weefselregeneratie te bevorderen, alsook als een hemostatisch middel, zou het kunnen dat de waargenomen variabiliteit klinische implicaties met zich meebrengt.

In **Hoofdstuk 1** worden deze twee bezorgdheden in het licht van de **huidige kennis** gepositioneerd. Het management van patiënten op orale anticoagulantia die tandextracties ondergaan wordt beschreven, alsook de potentie van tranexaminezuur om het risico op bloedingen te verminderen. Daarnaast wordt een kort historisch overzicht van L-PRF-membranen geschetst en worden de eigenschappen van deze membranen, tezamen met factoren die deze eigenschappen mogelijks beïnvloeden, besproken.

Hoofdstuk 2 beschrijft de **EXTRACT-NOAC-studie**, dewelke een multicentrische, placebo-gecontroleerde, dubbel-geblindeerde en gerandomiseerde klinische studie is. De studie werd opgesteld om te onderzoeken of het gebruik van een mondspoelmiddel op basis van **tranexaminezuur** (TXA), een antifibrinolyticum, bloedingscomplicaties na tandextracties bij patiënten behandeld met een NOAC kan verminderen. Patiënten die deelnamen aan deze studie werden opgedragen de NOAC-dosis de ochtend van hun tandextractie niet in te nemen en om na de tandextractie 3 keer per dag gedurende 3 dagen hun mond te spoelen met een toegewezen mondspoelmiddel (tranexaminezuur of placebo). Na de ingreep werden de patiënten 7 dagen opgevolgd. De data van 218 patiënten geanalyseerd waarvan er 106 patiënten tranexaminezuur en 112 placebo mondspoelmiddel kregen. De studieresultaten wijzen uit dat het gebruik van het TXA-mondspoelmiddel geen invloed heeft op bloeding tijdens en vroegtijdig na een tandextractie (tot één dag nadien), maar wel dat het laattijdige bloeding (meer dan één dag na de extractie) en bloeding in het geval dat meerdere tanden worden getrokken, zou kunnen verminderen. Bovendien zou TXA-mondspoelmiddel voordelig zijn voor patiënten ≥ 75 jaar en patiënten die hun NOAC-medicatie binnen de 24 uur na de ingreep innemen.

Hoofstukken 3 en 4 van deze thesis gaan over de eigenschappen van **L-PRF-membranen** en de mogelijke invloed van antitrombotische medicatie op deze eigenschappen. In een eerste studie (hoofdstuk 3), werden bloedstalen van 12 vrijwilligers zonder chronische of systemische aandoeningen en die geen medicatie innemen, gesupplementeerd met de verschillende concentraties van de anticoagulans enoxaparine. L-PRF-membranen gegenereerd van deze bloedstalen werden onderworpen aan mechanische trektesten, celtellingen en elektronenmicroscopie, om respectievelijk hun mechanische eigenschappen (stijfheid, sterkte en mate van uitrekking), celinhoud (bloedplaatjes en witte bloedcellen) en fibrinenetwerk te bestuderen. Deze experimenten toonden aan dat laag-therapeutische concentraties van enoxaparine de eigenschappen van de membranen niet beïnvloeden, en dat een hoog-therapeutische concentratie de aanmaak van L-PRF verstoort. Een tweede belangrijke bevinding was de hoge variabiliteit van de eigenschappen tussen verschillende membranen.

In een tweede studie (hoofdstuk 4), werd bloed genomen van patiënten op anticoagulantia, van patiënten op antiplaatjes, en van patiënten die geen van die types medicatie innamen (controle patiënten). Opnieuw werden de membranen onderworpen aan trektesten en celtellingen. Voor de trektesten gaven 25 patiënten bloedstalen (13 controle, 12 anticoagulans en 10 antiplaatjes), en voor de celtelling werden 55 patiënten geïncludeerd (23 controle, 16 anticoagulans en 16 antiplaatjes). De resultaten laten uitschijnen dat L-PRF-membranen van patiënten op anticoagulantia zwakker zijn, sneller scheuren en minder witte bloedcellen bevatten, wat mogelijks verklaard kan worden door veranderingen in het fibrinenetwerk van de membranen. De antiplaatjes medicatie, daarentegen, schenen deze eigenschappen niet te beïnvloeden. Opnieuw werd een hoge variabiliteit tussen de bloedmembranen van de patiënten gemeten.

In **hoofdstuk 5**, worden de **studies kritisch besproken en de toekomstperspectieven** geschetst. Naar aanleiding van de resultaten van de klinische EXTRACT-NOAC-studie, adviseren wij patiënten behandeld met NOACs om de ochtend van hun tandextractie een NOAC dosis over te slaan en na de tandextractie driemaal daags gedurende drie dagen tranexaminezuur-mondspoeling te gebruiken. Wij streven ernaar deze bevindingen te publiceren in en eenvoudige en evidence-based richtlijn en deze in de praktijk te implementeren. Toekomstige klinische studies zouden zich kunnen richten op oudere patiënten en de vergelijking van het gebruik van TXA op gaasjes en TXA als mondspoelmiddel. Verder bleek uit de experimenten met L-PRF-bloedmembranen dat het gebruik van anticoagulantia kan leiden tot zwakkere membranen die sneller scheuren en minder bloedcellen bevatten. Toekomstig onderzoek zou zich kunnen toespitsen op onderzoek naar de biologische effecten van de membranen en de klinische relevantie van onze bevindingen.

SCIENTIFIC ACKNOWLEDGEMENTS

This work could not have been established successfully without the help of some people, who I would like to acknowledge for their contribution.

Chapter 1

Anna Ockerman, KU Leuven: conceptualization of the reviews, search method, article screening and selection, data collection, drafting of the reviews, and revision and approval of the reviews

Reinhilde Jacobs, Constantinus Politis, Peter Verhamme, Isabel Miclotte, Maarten Vanhaverbeke, Christophe Vandenbriele, KU Leuven: revision and approval of (one or both) reviews

Laura-Lien Poortmans, Jeroen Martens, Melissa Garip, KU Leuven: article screening and selection, data collection, and approval of (one or both) reviews

Chapter 2

Anna Ockerman, KU Leuven: conceptualization of the trial, patient inclusion and follow-up, data collection, management and validation, statistical analysis, project administration, reporting to the competence authorities, drafting the article, and revision and approval of the article

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Constantinus Politis, Reinhilde Jacobs, Thomas Vanassche, KU Leuven: conceptualization of the trial, data validation, supervision, and revision and approval of the article

Isabel Miclotte, Maarten Vanhaverbeke, KU Leuven: conceptualization of the trial, project administration, and revision and approval of the article

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Nasser Nadjmi, Patrick Winderickx, Geert Van Hemelen, Bruno Vanassche, Frank Vanhove and Herman Vercruysse, ZMACK Association, University Hospital Antwerp and AZ Monica Antwerp/Deurne: patient inclusion and revision and approval of the article (NN, PW, GVH)

Laura-Lien Poortmans and Jeroen Martens, KU Leuven: patient inclusion, patient follow-up, and data collection

Ann Belmans and Ipek Guler Caamano Fajardo, KU Leuven: data validation, statistical analysis, and revision and approval of the article (AB)

Barbara Debaveye, University Hospitals Leuven: project administration and reporting to the competence authorities

Michiel Van der Haegen, KU Leuven: helped with figure design

Chapter 3

Anna Ockerman, KU Leuven: conceptualization of the study, sample collection and processing, scanning electron microscopy, tensile tests, cell counts, data collection and management, data validation, statistical analysis, project administration, drafting the article, and revision and approval of the article

Reinhilde Jacobs, Constantinus Politis, Peter Verhamme, KU Leuven: conceptualization of the study and revision and approval of the article

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Annabel Braem, KU Leuven: resources for tensile tests and revision and approval of the article

Tom Van der Donck and Mostafa EzEldeen, KU Leuven: scanning electron microscopy and revision and approval of the article (ME)

Laura-Lien Poortmans and Birgit Coucke, KU Leuven: sample collection and processing and data collection

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Chapter 4

Anna Ockerman, KU Leuven: conceptualization of the study, sample collection and processing, tensile tests, cell counts, data collection and management, data validation, statistical analysis, project administration, drafting the article, and revision and approval of the article

Reinhilde Jacobs, Constantinus Politis, Peter Verhamme, Marc Quirynen, KU Leuven: revision and approval of the article

Nele Famaey, Julie Vastmans, Heleen Fehervary, Wouter Willekens and Kimberly Crevits, KU Leuven: tensile tests (practical help and data processing) and revision and approval of the article (JV, HF, WW)

Amber Hendrickx, KU Leuven: sample collection and processing, tensile tests (practical help and data processing), cell counts, data collection, and approval of the article

Wim Coucke, Freelance Statistician: advice on statistical analysis and revision and approval of the article

Michiel Van der Haegen, KU Leuven: helped with figure design

PERSONAL CONTRIBUTION

Anna Ockerman is first author of all the research thesis chapters and corresponding manuscripts as she conceived the projects, collected, managed and analyzed the clinical and experimental data. Anna also wrote the peer-reviewed publications. This work was supported by her promotors Prof. dr. Reinhilde Jacobs, Prof. dr. Constantinus Politis and Prof. dr. Peter Verhamme, and all of her co-authors.

CONFLICT OF INTEREST

The author of this PhD Manuscript, Anna Ockerman, has no conflicts of interest to declare with respect to the publications of this work.

The EXTRACT-NOAC clinical trial (Chapter 2) was funded through the Applied Biomedical Research Grant with a Primary Social finality allocated by the Research Foundation Flanders (T002117N) and through a European Thrombosis Investigator Initiated Research Program (ERISTA) sponsored by Bristol Myers-Squibb and Pfizer. The leukocyte- and platelet-rich fibrin experiments of Chapters 3 and 4 were supported by an Internal KU Leuven Fund for Translational Biomedical Research (KOOR).

CURRICULUM VITAE

Anna Ockerman graduated in June 2017 as MSc in Biomedical Sciences (Faculty of Medicine, KU Leuven). Her Master's Thesis on the eruption potential of wisdom teeth was awarded with the Best Master's Thesis in Biomedical Sciences, third place. She was a PhD Researcher at the OMFS-IMPATH Research Group in cooperation with the Department of Cardiovascular Sciences from September 2017 to September 2021. Her promotors were Prof. dr. Reinhilde Jacobs, Prof. dr. Constantinus Politis (Department of Imaging and Pathology, KU Leuven) and Prof. dr. Peter Verhamme (Department of Cardiovascular Sciences, KU Leuven). Her PhD research focused on the management of postoperative bleeding in patients treated with antithrombotic drugs in the oral and maxillofacial surgery. She coordinated a placebo-controlled, double blind, randomized, clinical trial at the Departments of Oral and Maxillofacial Surgery in four Belgian hospitals, evaluating the use of a tranexamic acid mouthwash for reducing bleeding after dental extraction. She also managed an experimental study in cooperation with four Research Groups at three campuses of the KU Leuven concerning leukocyte- and platelet-rich fibrin membranes. Beside her PhD project, Anna was involved in multiple studies on oral radiology investigating third molar eruption (in cooperation with the Karolinska Institutet, Stockholm, Sweden), anatomical variations of the mandibular canal, and radiation doses with imaging, as well as a clinical trial to evaluate a possible therapy for the corona virus disease (COVID-19).

Contributions to (inter)national conferences and meetings

16/09/2021 CED-IADR/NOF Oral Health Research Congress
Brussels, Belgium

17/07/2021 International Society on Thrombosis and Haemostasis (ISTH) Congress
Philadelphia, USA

17/01/2021 International Academy for Oral and Facial Rehabilitation (IAOFR)
Online Meeting

08/09/2018 Enhanced Natural Healing in Dentistry (ENHD) Congress
Leuven, Belgium

14/06/2018 European Academy of Dentomaxillofacial Radiology (EADR) Congress
Lucern, Switserland

17/03/2018 Leuvense Universitaire Tandheelkundige Vereniging (LUTV)
Leuven, Belgium

Funds and Grants

2021 Fonds Wetenschappelijk Onderzoek, Grant study visit abroad (K202321N)

2018 Internal KU Leuven Fund for Translational Biomedical Research (KOOR)

2018 European Academy of Dentomaxillofacial Radiology (EADR), Travel Award

Supervision of undergraduate students

2017-2021 Mentor of 4 biomedical students during their masters' thesis year

Supervision of 25 (bio)medical and dental students:

- 7 lab rotation students
- 4 clinical interns
- 14 student researchers

List of publications

Publications related to the PhD manuscript

- **Ockerman A**, Hendrickx A., Vastmans J, Famaey N, Wouter W., Kimberly C., Verhamme P, Coucke W., Braem A., Verhamme P, Constantinus P, Quirynen M, Jacobs R. Mechanical properties and cellular content of leukocyte- and platelet-rich fibrin membranes of patients on antithrombotic drugs. *Under review.*

- **Ockerman A,** Miclotte I, Vanhaverbeke M, Vanassche T, Belmans A, Vanhove J, Meyns J, Nadjmi N, Van Hemelen G, Winderickx P, Jacobs R, Politis C, Verhamme P. Tranexamic acid and bleeding after dental extraction in patients treated with non-vitamin k oral anticoagulants: the EXTRACT-NOAC randomized clinical trial. Plos Med. 2021;18(5):e1003601.

- **Ockerman A**, Vandenbriele C, Vanassche T, Garip M, Martens J, Jacobs R, Politis C, Verhamme P. Tranexamic acid for the prevention and treatment of bleeding in surgery, trauma and bleeding disorders: a narrative review. Thromb J. 2021;19(1):1–16.

- **Ockerman A**, Braem A, EzEldeen M, Castro A, Coucke B, Politis C, Verhamme P, Jacobs R, Quirynen M. Mechanical and structural properties of leukocyte- and platelet-rich fibrin membranes: an in vitro study on the impact of anticoagulant therapy. J Per Res 2020;55(5):686–93.

- **Ockerman A**, Vanhaverbeke M, Miclotte I, Belmans A, Vanassche T, Politis C, Jacobs R, Verhamme P. Tranexamic acid to reduce bleeding after dental extraction in patients treated with non-vitamin k oral anticoagulants: design and rationale of the EXTRACT-NOAC Trial. Br J Oral Maxillofac Surg. 2019;57(10):1107–12.

- **Ockerman A**, Miclotte I, Vanhaverbeke M, Verhamme P, Poortmans L, Vanassche T, Politis C, Jacobs R. Local haemostatic measures after tooth removal in patients on antithrombotic therapy: a systematic review. Clin Oral Invest. 2019;23(4):1695–708.

Other publication in the field

- **Ockerman A**, Bornstein M, Leung Y, Li S, Politis C, Jacobs R. Incidence of bleeding after minor oral surgery in patients on dual antiplatelet therapy: a systematic review and meta-analysis. Int J Oral Maxillofac Surg. 2020;49(1):90–8.

Publications as collaborator of other research projects

- Saputri RI, De Tobel J, Vranckx M, **Ockerman A**, Van Vlierberghe M, Fieuws S, Thevissen P. Is third molar development affected by third molar impaction or impaction-related parameters? Clin Oral Investig. 2021. Epub ahead of print.

- Martens CP, Van Mol P, Wauters J, Wauters E, Gangnus T, Noppen B, Callewaert H, Feyen JHM, Liesenborghs L, Heylen E, Jansen S, Velásquez Pereira LC, Kraisin S, Guler I, Engelen MM, **Ockerman A**, Van Herck A, Vos R, Vandenbriele C, Meersseman P, Hermans G, Wilmer A, Martinod K, Burckhardt BB, Vanhove M, Verhamme P, Neyts J, Vanassche T. Dysregulation of the kallikrein-kinin pathway contributes to thromboinflammation in lungs of patients with COVID-19. 2021. *Under review.*

- Vanassche T, Engelen MM, Van Thillo Q, Wauters J, Gunst J, Wouters C, Vandenbriele C, Rex S, Liesenborghs L, Wilmer A, Meersseman P, Van den Berghe G, Dauwe D, Verbeke G, Thomeer M, Fivez T, Mesotten D, Ruttens D, Heytens L, Dapper I, Tuyls S, De Tavernier B, Verhamme P; **DAWn consortium members**. A randomized, open-label, adaptive, proof-of-concept clinical trial of modulation of host thromboinflammatory response in patients with COVID-19: the DAWn-Antico study. Trials. 2020;21(1):1005.

- Govaerts D, Piccart F, **Ockerman A**, Coropciuc R, Politis C, Jacobs R. Adjuvant therapies for MRONJ: a systematic review. Bone. 2020. Epub PMID: 33022455.

- Belmans N, Gilles L, Vermeesen R, Virag P, Hedesiu M, Salmon B, Baatout S, Lucas S, Lambrichts I, Jacobs R, Moreels M; **Dimitra Research Group**. Quantification of DNA double strand breaks and oxidation response in children and adults undergoing dental CBCT Scan. Sci Rep. 2020;10(1):2113.

- Oenning AC, Pauwels R, Stratis A, De Faria Vasconcelos K, Tijskens E, De Grauwe A, Jacobs R, Salmon B, **Dimitra research group**. Halve the dose while maintaining image quality in paediatric Cone Beam. CT Sci Rep. 2019;9(1):5521.

- Vranckx M, **Ockerman A**, Coucke W, Claerhout E, Grommen B, Miclotte A, Van Vlierberghe M, Politis C, Jacobs R. Radiographic prediction of mandibular third molar eruption and mandibular canal involvement based on angulation. Orthod Craniofac Res. 2019;22(2): 118–23.

- Moreno Rabie C, Vranckx M, Rusque Ignacia M, Deambrosi C, **Ockerman A**, Politis C, Jacobs R. The anatomic relation of third molars and retromolar canal appearance. Br J Oral Maxillofac Surg. 2019; Br J Oral Maxillofac Surg. 2019;57(8):765–70.

- Politis C, Schoenaers J, Hauben E, Lambrichts I, **Ockerman A**, Jacobs R. Goedaardige odontogene tumoren. Tandartspraktijk. 2017;38(10):4–12.

Anna Ockerman, the author of this PhD manuscript, graduated in 2017 as Msc in Biomedical Sciences (KU Leuven), and worked as a PhD researcher at the OMFS-IMPATH Research Group in cooperation with the Department of Cardiovascular Sciences from 2017 to 2021. Her promotors were Prof. dr. Reinhilde Jacobs (Department of Imaging and Pathology, KU Leuven), Prof. dr. Constantinus Politis (Department of Oral and Maxillofacial Surgery, UZ Leuven), and Prof. dr. Peter Verhamme (Department of Cardiovascular Sciences, KU Leuven). Her PhD research focused on the management of postoperative bleeding and healing after dental extraction in patients treated with antithrombotic drugs.

The current PhD project is about patients on antithrombotic medication undergoing dental extractions and focusses on two genuine concerns.

1. Oral bleeding after dental extraction in patients on non-vitamin K oral anticoagulants is a frequent problem, and it is not sure how to reduce the risk of bleeding. A placebo-controlled, double blind, randomized clinical trial was set up to evaluate if the use of tranexamic acid mouthwash, an antifibrinolytic agent, after dental extraction reduces bleeding in this patient population. The results indicated that tranexamic acid mouthwash does not reduce peri-operative bleeding or early bleeding (until one day) after dental extraction, but may reduce delayed bleeding (more than one day after) and bleeding in case multiple teeth are extracted.

2. Variation of leukocyte- and platelet-rich fibrin (L-PRF) membranes was noticed in the clinical practice and antithrombotic medication may be one of the causing factors as they interfere with blood coagulation, a process that is crucial for the generation of L-PRF. As L-PRF membranes are used to guide healing and enhance tissue regeneration and as hemostatic method, the variability amongst the membranes may have clinical implications. Therefore, experiments were set up to compare properties of L-PRF membranes between patients taking antithrombotics and controls. It was shown that L-PRF membranes of patients on an anticoagulant therapy, but not on antiplatelets, are weaker, rupture faster and contain less leukocytes than membranes of patients not taking antithrombotic drugs.