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Association of Acquired Coagulation Changes and Genetic Polymorphisms on Microvascular Free Flap Thrombosis in Reconstructive Surgery

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Abstract

Microvascular free flap surgery possesses an ability to cover a broad range of tissue defects for reconstructive purpose fostering functional and aesthetic recovery. The restoration of blood flow is essential to ensure the viability of the transplanted tissue, as adequate vascular anastomosis facilitates the delivery of oxygen and nutrients, thereby preventing tissue ischaemia and necrosis.

Despite remarkable advances in microsurgical techniques, instrumentation, and perioperative management protocols, thrombosis remains a significant complication that can hinder surgical success, resulting in dissatisfaction among patients and surgeons, as well as increased hospital stays and costs. The incidence of thrombosis ranges from 2 % to 9 %.

The pathogenesis of thrombotic events in microvascular surgery appears multifactorial. Variants in genes regulating coagulation pathways, platelet function, and fibrinolysis might contribute to a predisposition for thrombotic events. Understanding these genetic influences might improve risk stratification and would personalise treatment strategies in microvascular surgery.

The aim of the study was to evaluate the association between specific single nucleotide variant (SNV) and acquired coagulation changes with thrombotic complications in microvascular free flap surgery with the goal of developing genetic risk stratification parameters for individualised perioperative care.

A prospective cross-sectional study was conducted, enrolling 155 adult patients scheduled for microvascular free flap surgery at the Centre of Plastic and Reconstructive Microsurgery of Latvia between December 2016 and July 2019. The study protocol was approved by the Latvian Central Medical Ethics Committee (No 1/28-11-16).

Patient assessment included standardised interviews to collect data on thrombotic history, medication use, family history, and inherited thrombophilia. SNV genotyping was performed for five variants: rs6025 (in the Factor V gene, known as Factor V Leiden), rs1799963 (in the prothrombin gene, known as G20210A), rs2066865 (in the fibrinogen gamma chain gene), rs2227589 (in the SERPINC1 gene), and rs1801133 (in the MTHFR gene, known as the C677T variant). Preoperative coagulation parameters were assessed using standardised laboratory protocols, including fibrinogen concentration, activated protein C resistance, prothrombin time, antithrombin activity, and homocysteine levels.

The study population consisted of 118 males (76.1 %) and 37 females (23.9 %), with a mean age of 45.07 ± 14.94 years. Flap thrombosis was observed in 14 patients (9.0 %), resulting in complete flap loss in 8 cases (5.16 %) and partial necrosis in 5 cases (3.22 %), yielding an overall flap success rate of 94.84 %. A statistically significant association was

identified between the rs2066865 variant in the FGG gene and plasma fibrinogen concentrations. Homozygous A/A genotype carriers exhibited significantly elevated fibrinogen levels (5.57 ± 1.81 g/L, $p=0.004$) compared to G/G genotype carriers (4.08 ± 1.32 g/L) and G/A genotype carriers (4.64 ± 1.74 g/L, $p=0.04$). One patient with a heterozygous Factor V Leiden variant experienced recurrent thrombotic complications in multiple surgical procedures. However, no statistically significant association was found between any of the analysed single nucleotide variants and the incidence of microvascular free flap thrombosis. Acquired thrombophilia factors and standard coagulation parameters were insufficient as standalone predictors of flap thrombosis. A combined analysis of genetic and acquired factors showed only modest, non-significant trends toward an increased rate of thrombosis, and the resulting predictive models demonstrated poor discriminatory power (AUC: 0.61-0.69).

While yielding negative results regarding the association of specific SNVs with thrombosis risk, this study provides valuable scientific information that redirects future research toward more comprehensive, multifactorial approaches to risk assessment. Fibrinogen polymorphisms were found to modulate plasma fibrinogen levels but lacked predictive utility for thrombosis. The current combination of genetic and acquired factors is inadequate for reliable preoperative risk stratification in microvascular free flap surgery. Future research should focus on large-scale, multicentre validation studies and the development of predictive models that integrate polygenic risk scores for personalised thromboprophylaxis in microvascular reconstruction.

Keywords: microvascular free flap surgery, microvascular free flap thrombosis, single nucleotide variant, thrombophilia, Factor V Leiden, Fibrinogen Gamma chain gene, fibrinogen, antithrombin deficiency, hyperhomocysteinemia

Anotācija

Iegūto koagulācijas izmaiņu un ģenētisko polimorfismu saistība ar mikrovaskulārā brīvā lēvera trombozi rekonstruktīvajā ķirurģijā

Mikrovaskulārā brīvā lēvera ķirurģija nodrošina plašu audu defektu rekonstrukcijas iespējas, kas nodrošina gan funkcionālu bojāto audu atjaunošanu, gan estētisku uzlabojumu.

Neraugoties uz mikroķirurģijas tehnikas un perioperatīvās aprūpes attīstību, tromboze attīstās līdz 2-9 % un ir galvenais lēvera ķirurģijas neveiksmes cēlonis, kas izraisa pacienta un ķirurgu neapmierinātību, pagarina hospitalizācijas laiku un palielina kopējās izmaksas.

Trombotisko notikumu patoģenēze mikrovaskulārajā ķirurģijā ir multifaktoriāla, un ģenētiskajiem faktoriem, kas ietekmē hemostāzi, iespējams ir būtiska loma indivīdu predispozīcijā trombotiskajiem sarežģījumiem.

Pētījuma mērķis bija izvērtēt specifisko viena nukleotīda variantu (VNV), kas regulē hemostatisko funkciju un iegūto koagulācijas izmaiņu saistību ar trombotiskajiem sarežģījumiem mikrovaskulārajā brīvā lēvera ķirurģijā, ar mērķi izveidot ģenētiskās riska stratifikācijas parametrus individualizētai perioperatīvai aprūpei.

Prospektīvā šķērsgriezuma pētījumā tika iekļauti 155 pacienti, kuriem tika veikta mikrovaskulāro brīvā lēvera ķirurģija Latvijas Plastiskās un rekonstruktīvās mikroķirurģijas centrā laika posmā no 2016. gada decembra līdz 2019. gada jūlijam. Pētījuma protokols tika apstiprināts Latvijas Centrālajā Medicīnas ētikas komitejā (Nr. 1/28-11-16).

Pacientu novērtējums ietvēra standartizētas intervijas, apkopojot datus par trombožu anamnēzi, medikamentu lietošanu, ģimenes anamnēzi un iedzimtu trombofiliju. Tika veikta VNV genotipēšana pieciem variantiem: rs6025 (V faktora gēnā, pazīstams kā Leidena V faktora mutācija), rs1799963 (protrombīna gēnā, pazīstams kā G20210A), rs2066865 (fibrinogēna gamma ķēdes gēnā), rs2227589 (SERPINC1 gēnā) un rs1801133 (MTHFR gēnā, variants pazīstams kā C677T). Pirmsoperācijas koagulācijas parametri tika novērtēti, izmantojot standartizētus laboratorijas protokolus, ieskaitot fibrinogēna koncentrāciju, aktivētā proteīna C rezistenci, protrombīna laiku, antitrombīna aktivitāti un homocisteīna līmeni.

Pētījuma populāciju veidoja 118 vīrieši (76,1 %) un 37 sievietes (23,9 %) ar vidējo vecumu 45,07 ± 14,94 gadi. Lēvera tromboze tika konstatēta 14 pacientiem (9,0 %), rezultējoties pilnīgā lēvera zudumā 8 gadījumos (5,16 %) un daļējā nekrozē 5 gadījumos (3,22 %), nodrošinot kopējo lēvera veiksmes rādītāju 94,84 %. Statistiski nozīmīgas saistības tika identificētas starp rs2066865 variantu *FGG* gēnā un plazmas fibrinogēna koncentrācijām. Homozigotie A/A genotipa nesēji uzrādīja būtiski paaugstinātus fibrinogēna līmeni

5.57 ± 1.81 g/L, p = 0.004 salīdzinājumā ar genotipa G/G nesējiem 4.08 ± 1.32 g/L un G/A genotipa nesējiem 4.64 ± 1.74 g/L, p = 0.04. Vienam pacientam ar V faktora Leidena variants heterozigotā stāvoklī piedzīvoja atkārtotus trombotiskus sarežģījumus vairākās ķirurģiskās procedūrās.

Netika konstatēta statistiski nozīmīga saistība starp kādu no analizētajiem viena nukleotīda variantiem un mikrovaskulāro brīvo lēveru trombozes biežumu. Iegūtie trombofilijas faktori un standarta koagulācijas parametri izrādījās nepietiekami kā atsevišķi lēvera trombozes prognozētāji. Kombinētā ģenētisko un iegūto faktoru analīze uzrādīja tikai nelielas, statistiski nenozīmīgas tendences uz paaugstinātu trombozes risku, un izveidotie prognozēšanas modeļi demonstrēja vāju diskriminācijas spēju (AUC: 0,61–0,69).

Pētījums, lai arī ar negatīvu rezultātu attiecībā uz specifisko viena nukleotīda varianta saistību ar trombozes risku, sniedz vērtīgas zināšanas novirzot turpmākos pētījumus uz visaptverošu, daudzfaktoru pieeju riska novērtēšanai. Pašreizējā ģenētisko un iegūto faktoru kombinācija nav pietiekama uzticamai pirmsoperācijas riska stratifikācijai mikrovaskulāro brīvo lēveru ķirurģijā. Nākotnes pētījumiem jākoncentrējas uz liela mēroga daudzcentru validācijas pētījumiem un prognozējošu modeļu izstrādi.

Atslēgvārdi: mikrovaskulāra brīvā lēvera ķirurģija, mikrovaskulāra brīvā lēvera tromboze, viena nukleotīda variants, trombofīlija, V faktora Leidena mutācija, fibrinogēna gamma ķēdes gēns, fibrinogēns, antitrombīna deficīts, hiperhomocisteinēmija.

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Abbreviations used in the Thesis

ADP	adenosine diphosphate
AH	arterial hypertension
ALT	Anterolateral thigh flap
APC	activated protein C
APTT	Activated Partial Thromboplastin Time
ASA	American Society of Anesthesiologists
AT	Antithrombin
ATP	Adenosine triphosphate
AUC	Area under the curve
CVP	Central venous pressure
DIEP	Deep inferior epigastric perforator flap
DVT	Deep vein thrombosis
EPCR	Endothelial cell protein C receptor
FF	Fibula flap
FGG	Fibrinogen Gamma chain gene
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FII	Coagulation factor II, prothrombin
FV	Coagulation factor V
FVai	Inactivated factor Va
FV Leiden	Coagulation factor V Leiden
FVIIIai	Inactivated factor VIIIa
FIXa	Activated Factor IX
FXa	Activated factor X
FXI	Coagulation factor XI
GP	Glycoprotein
HGVS	Human Genetic Variation society
HMWK	High-molecular weight kininogen
IBP	Invasive blood pressure
LAF	Lateral arm flap
LMWH	Low-molecular-weight heparin
LDL	Low density lipoprotein
MI	Myocardial infarction
NIRS	Near-infrared spectroscopy
NPWT	Negative pressure wound therapy
ns	Nonsignificant
PAI-1	Plasminogen activator inhibitor-1
PA	Plasminogen activator
PS	Protein S
PT	Prothrombin
RFF	Radial forearm flap

ROC	Receiver operating characteristic
SERPINC1	Serine protease inhibitor 1 gene
SIEA	Superficial Inferior Epigastric Artery
SNVP	Single nucleotide polymorphism variant
STSG	Split thickness skin graft
TAFI	Thrombin-activable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNF-α	Tumor necrosis factor alfa
u-PA	Urokinase-type plasminogen activator
t-PA	Tissue-type plasminogen activator
vWF	von Willebrand factor
Δt	Temperature difference

Introduction

Microvascular free flap surgery has emerged as a cornerstone technique in reconstructive surgery, representing a significant advancement in the field of plastic and reconstructive medicine. The fundamental principle underlying successful microvascular reconstruction lies in the meticulous restoration of blood flow through small-vessel anastomoses, typically involving vessels with diameters ranging from 1–3 millimetre, which is crucial for tissue oxygen delivery and nutrient supply to the transplanted tissue. Despite remarkable advances in microsurgical techniques, instrumentation, and perioperative management protocols, thrombotic complications continue to represent the primary cause of flap failure, occurring in 2–9 % of cases (Bowman, 2011; Friedman, 2010).

Successful microvascular anastomosis requires not only technical precision but also a comprehensive understanding of the various factors that may influence thrombosis formation. The development of thrombotic complications in microvascular surgery represents a complex pathophysiological process governed by the classical elements of Virchow’s triad: hypercoagulability, endothelial dysfunction, and haemodynamic alterations (Kumar, 2010). While mechanical and technical aspects of surgery have been extensively studied, the role of genetic factors in thrombotic complications has received relatively less attention in the context of microvascular surgery (Khansa, 2011).

Recent advances in molecular genetics have highlighted the potential influence of inherited factors on thrombotic events (Friedman, 2010). Single nucleotide variants (SNVs) in genes regulating coagulation pathways, platelet function, and/or fibrinolysis may contribute significantly to individual variation in thrombotic risk. Understanding these genetic influences could provide valuable insights for risk stratification and personalised treatment approaches in microvascular surgery.

Several genetic variants have been implicated in thrombotic disorders:

Factor V gene Leiden variant rs6025, legacy name R506G represents the most prevalent inherited thrombophilia in Caucasian populations, with frequencies ranging from 2–15 % and conferring 3–8-fold increased venous thrombosis risk in heterozygotes (Rees, 1995; Rosendaal, 1995). This gain-of-function mutation results in Factor Va resistance to inactivation by activated protein C, prolonging procoagulant activity approximately 10-fold compared to normal Factor Va (Segers, 2007).

Prothrombin gene variant rs1799963 legacy name G20210A represents the second most common inherited thrombophilia in Caucasian populations, with carrier frequencies of 1–3 % conferring approximately 2–3-fold increased venous thrombosis risk (Simone, 2013). This gain-of-function variant in the 3’ untranslated region of the prothrombin gene results in

elevated plasma prothrombin levels, enhancing thrombin generation potential and subsequent thrombotic risk (Poort, 1996).

Fibrinogen Gamma Chain variant rs2066865 is a risk factor for venous thrombosis, characterised by alterations in fibrinogen γ′ chain expression that affect clot structure and stability (de Willige, 2005; Grünbacher, 2007). Fibrinogen, as an acute-phase reactant, demonstrates elevated levels during inflammatory states, trauma, and malignancy, potentially amplifying thrombotic risk in surgical settings (van Hylckama Vlieg, 2003).

***SERPINC1* variant, rs2227589** affects antithrombin, the principal physiological inhibitor of thrombin and Factor Xa. Antithrombin deficiency, first described by Egeberg in 1965, represents one of the earliest recognised inherited thrombophilias, conferring 10–20-fold increased thrombosis risk (Zöller, 1999). The critical importance of antithrombin is underscored by the embryonic lethality observed in homozygous deficiency states.

***MTHFR* variant rs1801133, legacy name C677T** influences homocysteine metabolism through altered methylenetetrahydrofolate reductase activity (Goyette, 1998). The resulting hyperhomocysteinemia has been associated with both arterial and venous thrombotic events, though the mechanistic relationship remains subject to ongoing investigation (Liew, 2015).

While extensive epidemiological data support associations between these genetic variants and thrombotic risk in general populations, their specific relevance to microvascular surgical outcomes remains inadequately described. The unique haemodynamic environment of microvascular anastomoses, combined with the heightened prothrombotic state induced by surgical trauma, may amplify the clinical significance of genetic thrombophilic variants beyond their effects in spontaneous thrombotic events and thus the complex interplay between genetic factors and surgical outcomes presents an important area.

Previous investigations have demonstrated that Factor V Leiden variant carriers exhibit 3–7-fold increased venous thrombosis risk (Kujovich, 2011), while prothrombin G20210A variant carriers show 2–3-fold elevated risk (Simone, 2013). However, limited studies have focused specifically on genetic contributions to thrombosis in microvascular free flap surgery, representing a significant limitation in the field. Identification of genetic markers associated with increased thrombotic risk could potentially revolutionise preoperative risk assessment and guide personalised anticoagulation strategies in microvascular surgery. This approach aligns with the growing emphasis on personalised medicine and may contribute to improved surgical outcomes.

The Thesis aims to investigate the association of acquired coagulation changes, specific genetic variants and thrombotic complications in microvascular free flap surgery.

The findings of this research may have significant implications for:

1) preoperative risk assessment;
2) patient-specific anticoagulation protocols;
3) postoperative monitoring strategies;
4) overall surgical success rates.

The main goal is to bridge the gap between genetic predisposition and clinical outcomes in microvascular surgery, potentially leading to more personalised and effective treatment approaches for patients undergoing free flap procedures.

Aim of the Thesis

The aim of the Thesis was to determine whether specific genetic polymorphisms associate with increased thrombotic complications in microvascular free flap surgery with goal of developing genetic risk stratification parameters for individualised perioperative care.

Objectives of the Thesis

1. To identify patients with single nucleotide variants: rs6025 in *FV* gene; rs1799963 in prothrombin gene; rs2066865 in *FGG*; rs2227589 in *SERPINC1* and rs1801133 in *MTHFR*.
2. To examine and evaluate the association between determined single nucleotide variants and microvascular free flap thrombosis incidence.
3. To evaluate the impact of acquired thrombophilia factors on microvascular free flap thrombosis incidence.
4. To estimate the thrombosis risk in patients carrying combined factors (i. e. single nucleotide variants and acquired thrombophilia factors).

Hypothesis of the Thesis

Patients carrying variants in genes affecting haemostatic function (rs6025 in FV Leiden; Prothrombin rs1799963; rs2066865 in *FGG*; rs2227589 in *SERPINC1*; and/or rs1801133 in *MTHFR*) would demonstrate increased incidence of flap thrombosis.

Novelty of the Thesis

This study presents a novel approach to understanding thrombotic complications in microvascular free flap surgery by establishing assessment model that combines genetic polymorphism analysis with standardised perioperative protocols. The main aspect lies in the systematic evaluation of both inherited thrombophilic traits and acquired coagulation changes, providing a foundation for personalised risk assessment and targeted prophylactic strategies in reconstructive microsurgery. An integrated approach offers insight into

the multifactorial nature of flap thrombosis and presents potential pathways for improving surgical outcomes through genetically-informed clinical decision-making.

Organisation

The study was performed in the Anaesthesiology Clinic of Riga East Clinical University Hospital and in the Centre of Plastic and Reconstructive Microsurgery of Latvia. Microvascular flap transfers were performed by highly trained specialists in microsurgery.

Personal Contribution

The author took part in each stage of the study: patient selection, preoperative and postoperative patient evaluation, intraoperative patient management, and collection and analysis of data. The author also did review of the literature, performed statistical analysis, and interpreted the results

Ethical Concerns

The study protocol and patient informed consent form, including the request to donate genetic material were approved by Latvian Central Ethics Committee (Nr. 1/28-11-16). All patients provided written, informed consent.

1 Literature review

1.1 Haemostasis

The blood coagulation system is a complex physiological process that prevents bleeding following vascular injury through coordinated interactions between the vascular endothelium, platelets, and coagulation factors while at the same time impeding the systemic activation of clotting system. The equilibrium between vascular occlusion and haemostasis is determined by the complex interaction between procoagulant and anticoagulant components in the blood, as well as factors associated with the vasculature and subvascular structures. Procoagulant and fibrinolytic processes occur concurrently, with numerous positive and negative feedback mechanisms regulating the haemostasis process. Under normal physiological conditions, procoagulant and anticoagulant mechanisms are delicately balanced (Davie, 1991; Svensson, 1994). Upon completion of vascular repair, the clot undergoes fibrinolysis ensuring the restoration of normal blood flow. Any dysregulation in this process could contribute to thrombosis or aberrant clot formation (Lejniece, 2002).

Back in 1905, Paul Morawitz was the first who proposed the classical theory of coagulation. A more elaborate description of the coagulation system has characterised it as a cascade. Macfarlane, along with Davie and Ratnoff, proposed that the intrinsic pathway, which involves solely plasma, initiates blood coagulation through a series of sequential reactions leading to fibrin clot formation (Davie, 1991).

The coagulation cascade encompasses two distinct but convergent pathways: the tissue factor (extrinsic) pathway and the contact activation (intrinsic) pathway (Figure 1.1). The tissue factor pathway serves as the principal initiator of coagulation under physiological conditions, while the contact activation pathway, although traditionally described as an alternate route, is not associated with bleeding disorders when compromised, suggesting its auxiliary role in haemostasis (Brummel-Ziedins, 2013).

Both pathways comprise sequential enzymatic reactions involving multiple components:

- zymogens (inactive precursor proteins),
- activated coagulation factors (serine proteases),
- cofactors,
- regulatory proteins and inhibitors,
- cell surface-assembled enzyme complexes.

These pathways converge at the activation of factor X, leading to thrombin generation and subsequent fibrin formation, ultimately resulting in a stable blood clot. The process is tightly regulated by various inhibitory mechanisms to prevent pathological thrombosis while maintaining vascular integrity.

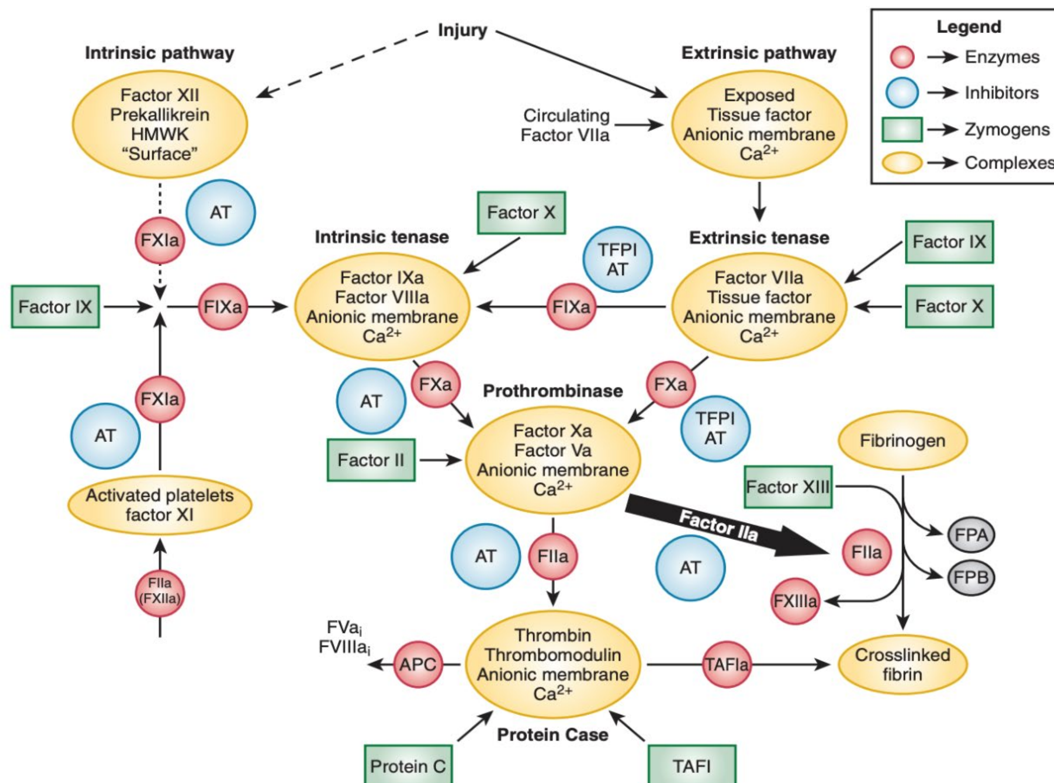


Figure 1.1. **Overview of Haemostasis** (Brummel-Ziedins K, 2013)

(The multistep process of haemostasis described in text.) APC, Activated protein C; AT, antithrombin; FIXa, factor IXa; FPA, fibrinopeptide A; FPB, fibrinopeptide B; FVai, factor Vai; FVIIIai, factor VIIIai; FXa, factor Xa; HMWK, high-molecular-weight kininogen; TAFI, thrombin-activatable fibrinolysis inhibitor; TFPI, tissue factor pathway inhibitor.

1.1.1 The vitamin K dependent proteins

Vitamin K-dependent protein complexes play a pivotal role in maintaining haemostatic balance. Synthesised in the liver, they are essential for regulating blood coagulation via both procoagulant and anticoagulant mechanisms (Oldenburg, 2008).

These complexes consist of a serine protease enzyme, a cofactor that acts as a receptor or enhancer for the enzyme, Ca^{2+}, and a negatively charged membrane surface provided by activated or damaged cells (such as endothelial cells, monocytes, and platelets).

Four major vitamin K-dependent complexes exist, including the extrinsic tenase complex: zymogens of procoagulant factors VII, IX, X, and prothrombin, as well as anticoagulants such as protein C, protein S, and protein Z (Nelsestuen, 2000).

After activation through proteolytic cleavage, the vitamin K-dependent proteins, except for protein S and protein Z, function as serine proteases, related to the trypsin and chymotrypsin superfamily. The cleavage at specific peptide bonds converts these zymogens into their active serine protease forms (Brenner, 2009).

Vitamin K is essential for the synthesis of clotting factors by facilitating the cyclic oxidation and modification, enabling the interaction of vitamin K-dependent proteins with calcium ions (Ca^{2+}) and membrane surfaces (Chatrou, 2011).

When a serine protease binds to its corresponding cofactor on an appropriate membrane surface in the presence of Ca^{2+}, the rate of the reaction is accelerated by a factor of 10^4–10^9 compared to the reaction of the protease with its substrate alone. To compare, blood clotting in a healthy individual typically occurs within 4 minutes, in the absence of the membrane and cofactor, this process would take approximately 3.8 years (Suttie, 1987). This illustrates the critical role of these complexes in haemostatic plug formation.

Cofactor proteins

There are two types of cofactor proteins: the cell bound cofactors – tissue factor and thrombomodulin and the soluble plasma-derived procoagulant cofactors – factor V and factor VIII with von Willebrand factor as circulating carrier (Nelsestuen, 2000).

Cell bound cofactors

Tissue factor

Tissue factor is a transmembrane protein that acts as a cofactor for factor VIIa in the extrinsic tenase complex. Under normal conditions, tissue factor is not expressed on cell surfaces in contact with circulating blood (Butenas, 2007). The exposure of tissue factor to the circulation is the primary trigger for the activation of the procoagulant pathway (Edington, 1991). There are no documented mutations or deficiencies of tissue factor in humans, and its deletion in mice results in embryonic lethality, indicating its critical importance for survival (Toomey, 1996).

The exposed subendothelium during vascular injury acts as a source of activation of tissue factor as well as monocytes activated by cytokines. There is ongoing debate regarding the source and presentation of active tissue factor, particularly whether functional tissue factor circulates in the bloodstream under both normal and pathological condition (Butenas, 2005).

Thrombomodulin

Thrombomodulin is a type 1 transmembrane protein on the surface of vascular endothelial cells. It is a high-affinity receptor for all forms of thrombin and serves as a cofactor in the thrombin-mediated activation of protein C. The endothelial cell protein C receptor provides cell-specific binding sites for both protein C and its activated form, activated protein C (APC) (Esmon, 2006). Upon binding to thrombomodulin, procoagulant properties of

thrombin – such as fibrin generation and the activation of factors V, VIII, XI, and platelets – are inhibited and its inactivation by antithrombin is enhanced (Rezaie, 2010).

The formation of activated protein C by the thrombin-thrombomodulin complex leads to the inactivation of procoagulant cofactors factor Va and factor VIIIa, thus inhibiting further thrombin generation. Moreover, this complex, known as the "protein Case" complex, also has antifibrinolytic properties through the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (Heylen, 2011).

Thrombomodulin activity on endothelial cells is reduced by inflammatory cytokines (Conway, 2012) leading to hypercoagulable conditions during inflammatory states (Brummel-Ziedins, 2013).

Soluble Plasma Pro-cofactors

Factor V

Factor V is a large single-chain glycoprotein that circulates in human plasma and is stored in the α-granules of platelets, accounting for approximately 18–25 % of the total factor V present in platelets. As a procofactor, factor V is cleaved by α-thrombin to produce the active cofactor, factor Va (Monkovich, 1990). Factor Va acts both as a receptor for factor Xa and enhances the catalytic activity of factor Xa within the prothrombinase complex. Factor Va is inactivated through proteolytic cleavage by activated protein C.

This regulatory mechanism importance is highlighted by the "APC resistance" syndrome, which is often linked to the factor V Leiden variant (Dahlbäck, 1993). Factor Va Leiden retains normal cofactor activity within the prothrombinase complex but is inactivated by APC at a slower rate than normal factor Va. This variant impairs the initial cleavage by APC, allowing cleaved factor Va Leiden to retain some cofactor activity, thereby continuing to promote thrombin generation thus contributing prothrombotic state (Castoldi, 2010).

Factor VIII

The soluble procofactor factor VIII circulates in plasma in complex with the large multimeric protein von Willebrand factor (vWF) (Leyte, 1989). vWF regulates the plasma concentration of factor VIII. Factor VIII is activated through thrombin-mediated cleavage resulting in the formation of the heterotrimeric cofactor VIII (Butenas, 1997). The vWF binding site is removed from factor VIII by thrombin cleavage allowing vWF-free factor VIIIa to form a complex with the serine protease factor IXa, Ca^{2+}, and a platelet-derived membrane, thus forming the intrinsic tenase complex (Brummel-Ziedins, 2013).

Factor VIIIa activity is downregulated by the relatively rapid dissociation of the non-covalently associated A2 subunit, which is produced during activation. Factor VIII

shares approximately 40 % identity with factor V. A deficiency of factor VIII, known as haemophilia A, is a well-established X-linked bleeding disorder, predominantly affecting males, with a prevalence of 1 in 5000 to 1 in 10 000 (Mannucci, 2001).

Von Willebrand Factor

Von Willebrand factor (vWF) serves essential functions in the coagulation process. It is synthesised in endothelial cells and stored in the α-granules of platelets (Brummel-Ziedins, 2013). vWF is a large adhesive glycoprotein that circulates in plasma as a heterogeneous mixture of disulphide-linked multimers, with sizes ranging from dimers to ultra large multimers exceeding 20 million kDa. vWF contains binding sites for factor VIII, heparin, collagen, platelet glycoprotein (GP) Ib, and GP IIb–IIIa (Goto, 1995). It acts as a bridge between platelets, promoting platelet aggregation. The primary receptor for vWF on platelets is the GPIb–IX–V complex, which is active on unstimulated platelets, allowing them to adhere and aggregate to vWF without prior activation (Furlan, 2002).

vWF also functions as a structural protein within the subendothelial matrix. Endothelial cells release vWF multimers that are larger than those found in plasma. These large multimers bind to and aggregate platelets under conditions of high shear stress. The degradation of these multimers is mediated by the metalloprotease ADAMTS-13 (Chung, 2002).

AB0 blood type significantly influences vWF levels, with individuals of blood types A, B, or AB exhibiting higher vWF levels compared to those with type 0 blood (Gill, 1987). Additionally, vWF acts as an acute-phase reactant, with levels increasing in response to stress, pregnancy, or surgical trauma (Pottinger, 1989).

1.1.2 The Intrinsic (Accessory) Pathway Proteins

The precise physiological role of the intrinsic pathway is not fully understood, though it is believed to contribute to thrombus stability and to play a role in pathological conditions such as disseminated intravascular coagulation (Woodruff, 2011). Accessory pathway also plays a role in milieu with high shear stress i.e. cardiopulmonary bypass system, where blood interacts with synthetic surfaces. Moreover, factor XI is highlighted as a key intersection between the intrinsic and extrinsic pathways, with factor XI deficiency presenting a variable bleeding phenotype. In the coagulation cascade, the activation of factor XI to factor XIa is proposed to be catalysed by thrombin, acting as a part of a positive-feedback mechanism that amplifies thrombin generation (Lawson, 1994).

Protein deficiencies related to the intrinsic pathway, including factor XII, prekallikrein, and high-molecular-weight kininogen (HMWK), are generally not associated with excessive bleeding, even during surgical procedures (Schmaier, 2008).

Proteinase Inhibitors

Proteases are enzymes responsible for maintaining homeostasis between activation and inhibition in clot formation and fibrinolysis systems via hydrolysation of peptide bonds (Oldenburg, 2008; Grover, 2022).

In the bloodstream, protease inhibitors represent a significant portion of circulating proteins. Key inhibitors involved in regulating clot formation include antithrombin, tissue factor pathway inhibitor, heparin cofactor II, and protein C inhibitor. Both specific and broad-spectrum inhibitors act to localise, constrain, and control haemostasis. The proteases that initiate coagulation and fibrinolysis possess highly specific substrate affinities, ensuring precise regulation of the coagulation cascade (Brummel-Zieidns, 2013).

Antithrombin

Antithrombin (AT), a member of the serpin (serine protease inhibitor) encoded by the *SERPINC1* gene family, circulates in the blood as a single-chain glycoprotein with a molecular weight of approximately 137 kDa (Perry, 1996). AT is present in plasma at a concentration of approximately 120 µg/ml with a circulating half-life of roughly 3 days (Bjork, 1997). Antithrombin exerts broad-spectrum inhibitory activity, primarily targeting serine proteases involved in the coagulation cascade. Its main targets include thrombin, factor Xa, factor IXa, factor VIIa in complex with tissue factor, factor XIa, factor XIIa, kallikrein, and high-molecular-weight kininogen (HMWK). The inhibitory action of antithrombin is significantly enhanced by heparin and heparan sulphate (Pike, 2005). When bound to heparin, antithrombin's rate of inhibition of several coagulation proteases is increased by up to 10 000-fold (Zheng, 2014).

The mechanism of inhibition involves the interaction of the active site of the protease with the reactive centre loop of antithrombin, resulting in the formation of a stable, equimolar (1:1) complex. Inactivation occurs through covalent bond formation between antithrombin and the protease, followed by conformational rearrangements in both proteins (Harper, 1991). Beyond its role in coagulation, antithrombin also exhibits antiproliferative and anti-inflammatory properties, predominantly due to its inhibition of thrombin. Moreover, cleaved or latent forms of antithrombin possess antiangiogenic activity (O'Reilly, 1999).

Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is a multivalent Kunitz-type protease inhibitor found in plasma. It circulates as a heterogeneous mixture of partially proteolysed forms, with up to 90 % of circulating TFPI associated with lipoproteins, particularly low-density

lipoprotein. TFPI is primarily cleared from circulation by the liver and has short half-life, compared to other protease inhibitors (Broze, 1994).

TFPI inhibits the factor VIIa–tissue factor complex in a factor Xa-dependent manner, inhibition of the extrinsic factor tenase complex occurs only after generation of factor IXa and factor Xa. TFPI exerts its inhibitory effect by forming a stable quaternary complex consisting of tissue factor, factor VIIa, TFPI, and factor Xa, or by directly forming a factor Xa–TFPI complex. As the primary inhibitor of the extrinsic factor tenase complex, TFPI is essential in maintaining haemostatic balance through its regulation of the factor VIIa–tissue factor pathway (Crawley, 2008).

The critical role of TFPI in blood coagulation is demonstrated in transgenic mice completely lacking TFPI, which exhibit embryonic lethality. However, this lethality can be rescued by either heterozygous or homozygous factor VII deficiency, suggesting that lowering factor VII levels reduces the necessity for TFPI-mediated inhibition of the factor VIIa–tissue factor pathway during embryonic development. Similarly, low-normal levels of TFPI combined with factor V Leiden mutation result in lethality. Furthermore, mice with heterozygous TFPI deficiency and homozygous apolipoprotein E deficiency develop a more extensive atherosclerotic burden, indicating that TFPI may play a protective role against atherosclerosis in addition to its function as a regulator of thrombosis (Westrick, 2001).

Heparin Cofactor II

Heparin cofactor II, a member of the serpin family, is present in plasma at concentrations ranging from 0.5–1.4 µmol/L, with a plasma half-life of approximately 2.5 days (Grover, 2022). Similar to antithrombin, heparin cofactor II inhibits thrombin, a process that is enhanced over 1000-fold in the presence of heparin but does not affect other coagulation enzymes (Liaw, 1999). The rate at which heparin cofactor II inhibits thrombin, in both the absence and presence of heparin or heparin-like molecules, is slower than that of antithrombin under equivalent conditions (Liu, 1995). Given that the plasma concentration of heparin cofactor II is 25–50 % of that of antithrombin, and reduced levels of heparin cofactor II are not strongly associated with thrombosis, the effectiveness of heparin cofactor II as a systemic thrombin inhibitor has been called into question (Giri, 2005).

In vitro, thrombin inhibition by heparin cofactor II is enhanced by dermatan sulphate proteoglycans produced by fibroblasts and vascular smooth muscle cells. This suggests that heparin cofactor II may be specifically adapted to regulate extravascular thrombin in areas of vascular injury, where dermatan sulphate from the subendothelium stimulates its activity. Furthermore, heparin cofactor II may contribute to the regulation of acute inflammation and

wound healing, as it contains a chemotactic peptide for neutrophils and monocytes that is released through leukocyte proteolysis (Church, 1991). Although low levels of thrombin–heparin cofactor II complexes are typically present in normal plasma, elevated levels have been observed in patients with disseminated intravascular coagulation. While inherited deficiencies of heparin cofactor II have been linked to thrombosis, this association is not consistently observed (Weisdorf, 1991).

Protein C Inhibitor

Protein C inhibitor is a 46 kDa serine protease encoded by the *SERPINA5* gene. It circulates in the bloodstream at a concentration of roughly 5 µg/mL and has a plasma half-life of one day (Meijers, 2011). When in complex with activated protein C, the clearance rate from circulation is significantly accelerated, with a half-life of around 20 minutes (Rezaie, 1995).

Protein C inhibitor is considered a nonspecific inhibitor, as it targets a wide array of enzymes, including procoagulant serine proteases, anticoagulant factors, fibrinolytic enzymes, plasma and tissue kallikreins, etc. Despite its broad range of targets, its primary substrate is activated protein C. Furthermore, Protein C inhibitor has been implicated in the regulation of thrombin-activatable fibrinolysis inhibitor activation through its inhibition of the thrombin–thrombomodulin complex, suggesting a potential role as a dual regulator of both coagulation and fibrinolysis (Mosnier, 2001). Additional targets of protein C inhibitor include human plasma kallikrein, factor XIa, factor Xa, and thrombin. No documented cases of protein C inhibitor deficiency exist, and its precise physiological role in vivo has yet to be fully clarified (Rezaie, 1995).

α2-Macroglobulin

α2-Macroglobulin is a broad-spectrum protease inhibitor, present in human plasma at concentrations between 2–4 µmol/L and can be found at higher levels in extravascular fluids. It is synthesised by various cell types, including hepatocytes, fibroblasts, and macrophages, and circulates in plasma as a tetramer (Lagrange, 2022).

The inhibitory mechanism of α2-macroglobulin underlies its broad substrate specificity. The initial step involves the proteolysis which triggers conformational changes that entrap the protease within the α2-macroglobulin molecule. This mechanism allows α2-macroglobulin to inhibit a wide variety of proteases, including members of all four major classes: serine, cysteine, aspartic, and metalloproteinases (Ignjatovich, 2007). In plasma, α2-macroglobulin functions as a secondary inhibitor of serine proteases, including thrombin, kallikrein, and plasmin. It may also play a role in preventing thromboembolic events, particularly in conditions

involving congenital antithrombin deficiency or acquired deficiencies, such as in sepsis (Abbink, 1991).

In addition, α2-macroglobulin also binds and inhibits several growth factors and cytokines, including transforming growth factor-α (TGF-α), interleukin (IL)-1β, IL-6, (acidic and basic fibroblast growth factors, tumor necrosis factor-α (TNF-α), and IL-2 (Vandooren, 2021). Reduced plasma levels of α2-macroglobulin have been observed in patients with chronic obstructive pulmonary disease and metastatic cancer. A complete deficiency of α2-macroglobulin has not been reported, suggesting that its absence is incompatible with life (Brummel-Ziedins, 2018).

1.1.3 Endothelium

Blood cells and vasculature are essential components in maintaining normal haemostasis. The endothelium plays a key role as the interface between organs, tissues, and the bloodstream (Versteeg, 2013). The structure and function of the endothelium vary across different regions of the vasculature. Therefore, dysfunction in the endothelium can have significant physiological implications. Structural abnormalities in the endothelial cell layer or its supporting matrix can result in excessive bleeding. Similarly, impaired expression or secretion of plasminogen activator inhibitor-1 (PAI-1) by endothelial cells can lead to increased fibrinolytic activity and associated bleeding (Neubauer, 2022).

The role of cellular membranes in supporting the formation and activity of procoagulant complexes is critical, although their nature remains incompletely understood. Mechanically damaged cells expose the anionic phospholipids from the inner leaflet of their membrane bilayers, which can support the formation of procoagulant complexes (Figure 1.2). However, specific cellular activation events also generate binding sites on intact cells, such as those for the factor IXa–factor VIIIa and factor Xa–factor Va complexes on activated platelet membranes (Ahmad, 2003).

1.1.4 Platelets

Platelets are essential procoagulant mediators and contributors to the fibrinolytic process. Small, irregularly shaped cell fragments are derived from megakaryocytes, with an average lifespan of approximately 5–9 days. Platelets are central to maintaining the balance between haemostasis and thrombosis (Scridon, 2022).

In their quiescent state, platelets present a non-thrombogenic surface, similar the endothelium. Critical components of platelet function include surface adhesion protein complexes and secretory granules, specifically α-granules, lysosomes, and dense granules. The α-granules contain a variety of procoagulant and adhesive proteins, such as fibrinogen,

fibronectin, thrombospondin, von Willebrand factor P-selectin, high-molecular-weight kininogen, platelet factor 4, osteonectin, factor V, and factor XI. Furthermore, α-granules also store proteins involved in anticoagulant processes, including α1-antitrypsin, protein S, tissue factor pathway inhibitor, and a platelet-specific inhibitor of factor XI (Twomey, 2019).

1.1.5 Fibrinolysis Proteins

Clot formation is linked with the process of clot breakdown, known as fibrinolysis. Fibrinolysis occurs in two forms: primary fibrinolysis, a normal physiological process, and secondary fibrinolysis, which results from external factors such as pharmacological agents, medical conditions, or other triggers. The biochemical mechanisms underlying fibrinolysis is the fibrin-specific activation of plasminogen to plasmin, the enzyme responsible for fibrin degradation. Plasminogen is the zymogen of plasmin, the key enzyme responsible for the proteolysis of fibrin. The principal proteins involved in this process include plasminogen, tissue plasminogen activator, urokinase plasminogen activator, and inhibitors such as plasminogen activator inhibitor-1, α2-antiplasmin, and thrombin-activatable fibrinolysis inhibitor (Risman, 2023).

Plasminogen activation occurs via three distinct pathways: 1) the intrinsic activator system, analogous to the contact system in coagulation, 2) extrinsic activators such as t-PA and u-PA, and 3) exogenous activators including fibrinolytic drugs (Larsen, 2020).

The extrinsic pathway, mediated by t-PA and u-PA, is the predominant mechanism for plasminogen activation in vivo, although the intrinsic and exogenous pathways also play important roles in certain disease states (Risman, 2023).

t-PA and u-PA are secreted by endothelial cells and once activated, plasmin enzymatically degrades fibrin, producing a series of fibrin degradation products, including fragment X, fragment Y, and the core fragments D and E. The degradation of fibrin begins with the cleavage of the α chains, exposing the coiled-coil regions that are subsequently cleaved to release fragments of various sizes. Fibrinogen has a trinodular structure composed of D–E–D domains, and during fibrin polymerisation, cross-links form between alternating D domains (D=D). Plasmin degrades these cross-links, generating fragments, the smallest of which is the D-dimer (Mr = 180 000 kDa). These degradation products are ultimately cleared by proteolytic enzymes or via renal and hepatic pathways (Brummel-Ziedins, 2013).

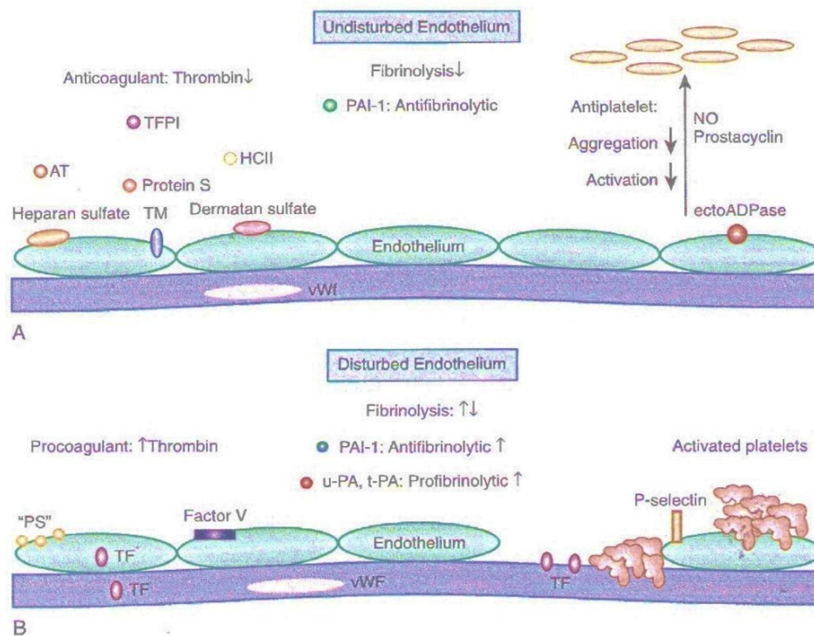


Figure 1.2. **Schematic overview of changes in endothelium after injury**
(Brummel-Ziedins K, 2013)

AT, Antithrombin; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; TFPI, tissue factor pathway inhibitor; TF, tissue factor; TM, thrombomodulin; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; vWF, von Willebrand factor.

1.1.6 Haemostasis stages

Initiation

Following vascular disruption, the early stages of haemostasis are primarily mediated by the extrinsic tenase complex (tissue factor–factor VIIa). The complex comprises tissue factor exposed by vascular injury or cytokine stimulation, calcium ions (Ca^{2+}), and the serine protease factor VIIa, which circulates in its active form at approximately 1 %–2 % of total factor VII zymogen. Before binding to tissue factor, factor VIIa remains inert and resistant to plasma protease inhibitors. Factor VII competes with factor VIIa for binding to tissue factor, serving as a negative regulator of the coagulation process. Additionally, factor VII-activating protease can activate factor VII independently of tissue factor, though its physiological role remains unclear, potentially involving inflammatory responses (Smith, 2015).

The extrinsic tenase complex facilitates the activation of small amounts of factor X and factor IX, converting them into their active serine protease forms, factor Xa and factor IXa, respectively. This process is subject to tight regulatory control to ensure proper coagulation responses. The extrinsic tenase complex initiates the conversion of factor X and factor IX into their active forms, factor Xa and factor IXa, respectively (Walker, 2002). Factor Xa is the more efficient and plays a critical role in generating the limited amounts of thrombin necessary to begin clot formation. The activity of the extrinsic tenase complex is tightly regulated by tissue

factor pathway inhibitor, which can bind both the factor VIIa–tissue factor complex and the product, factor Xa, to inhibit further activation (Autin, 2015).

If the initial procoagulant stimulus exceed the inhibitory capacity of TFPI and antithrombin, a threshold is reached, allowing for the formation of downstream coagulation complexes. The small amount of factor Xa that escapes inhibition binds to membrane surfaces and catalyses the activation of prothrombin into thrombin. This phase, referred to as the initiation phase of blood coagulation, generates very small amounts of thrombin, which is crucial for the activation of platelets and the coagulation cofactors factor V and factor VIII. Thrombin also activates factor XI to factor XIa, initiating the intrinsic or accessory pathway, which further enhances factor IX activation and amplifies the coagulation response (Miyazawa, 2023).

During tissue factor–induced coagulation in whole blood, thrombin generation is primarily confined to the early stages, prior to significant fibrin clot formation. The small quantities of thrombin produced during the initiation phase are sufficient to activate platelets, factor XIII, factor V, factor VIII, and release fibrinopeptides A and B from fibrinogen to initiate fibrin polymerisation. Although only a small fraction of thrombin (less than 2 %) is generated in this phase, its role in activating the necessary cofactors and platelets is essential for the subsequent propagation phase, during which most of the thrombin (~95 %) is produced (Wolberg, 2007).

Propagation

The accumulation of activated platelets facilitates the enhanced formation of intrinsic tenase and prothrombinase complexes on their surfaces via platelet receptors. As local inhibitor concentrations are oppressed, these platelet-bound catalysts drive the propagation phase of coagulation, during which the main amount (~95 %) of thrombin are produced. This phase of thrombin generation continues independently of the initial tissue factor, if there is a continuous supply of blood to deliver procoagulant factors, platelets, and fibrinogen to the site of vascular injury (Smith, 2015).

Factor Xa is essential for the formation of the prothrombinase complex. Factor Xa is unique in that it is generated by both the intrinsic and extrinsic tenase complexes. Under normal physiological conditions, factor Xa concentration is the rate-limiting component of the prothrombinase complex, while platelets and factor Va are rapidly activated in excess, in ready state for activity. However, the coagulation mechanism can become sensitive to factor V or platelet availability in the presence of congenital deficiencies, thrombocytopenia, platelet disorders, or pharmacological interventions (Mann, 2003).

Initial factor Xa generation occurs via the tissue factor–factor VIIa complex during the initiation phase, with further factor Xa being produced by the intrinsic tenase complex (factor IXa–factor VIIIa–membrane–Ca^{2+}). The concentration of the extrinsic tenase complex (tissue factor–factor VIIa) is higher than that of the intrinsic tenase complex (factor VIIIa–factor IXa), which requires activation and assembly. However, as the coagulation process progresses, the contribution of the intrinsic tenase complex to factor Xa production surpasses that of the extrinsic pathway. The intrinsic tenase complex is significantly more efficient, catalysing factor X activation at a rate 50–100 times higher than the extrinsic tenase complex (Kamikubo, 2017).

The rise in factor Xa production exceeds factor Xa inhibitors, such as tissue factor pathway inhibitor, leading to maximal prothrombinase activity and amplification of the procoagulant response. Approximately 95 % of thrombin is produced during the propagation phase, following the formation of the fibrin clot. Without the formation of the intrinsic tenase complex, as seen in haemophilia A or B, insufficient levels of factor Xa are generated, which impairs the propagation phase of thrombin production (Hackeng, 2011).

Termination

Once blood flow is halted by the formation of a fibrin–platelet barrier the high concentration of circulating inhibitors, including tissue factor pathway inhibitor, antithrombin, heparin cofactor II, α2-macroglobulin, α1-antitrypsin, and protein C inhibitor, act to inhibit the dissociated procoagulant factors. In the intact vasculature surrounding the growing thrombus, procoagulant enzymes and cofactors that escape the site of injury are rapidly neutralised under normal conditions by the inhibitory systems in the blood, in cooperation with elements of the vascular endothelium (Esmon, 2006).

Free serine proteases, such as thrombin, factor IXa, and factor Xa, are promptly inactivated by the excess of antithrombin molecules, a process accelerated by the interaction between antithrombin and heparan sulphate proteoglycans constitutively expressed on the surface of endothelial cells (Miyazawa, 2023).

Any thrombin that shades the wound site may bind to thrombomodulin. Once bound, thrombin is converted from a procoagulant enzyme to an anticoagulant enzyme. The thrombin–thrombomodulin complex activates protein C, which subsequently downregulates the intrinsic tenase and prothrombinase complexes by cleaving the activated forms of factor VIIIa and factor Va, respectively. The inactivation of factors Va and VIIIa by activated protein C is enhanced by protein S. Additionally, thrombin–thrombomodulin also

activates thrombin-activatable fibrinolysis inhibitor, which delays fibrin clot degradation (Autin, 2015).

Through the cleavage of factor Va and the inhibition of thrombin generation, APC also reduces TAFI activation, ensuring a balance between procoagulant activity and preventing systemic activation of the coagulation system. This mechanism effectively maintains localised haemostasis and prevents excessive coagulation. However, when vascular damage occurs internally, this balance can be disrupted, leading to pathological coagulation (Heckeng, 2011).

Fibrinolysis

The haemostatic system maintains physiological equilibrium through concurrent thrombotic and fibrinolytic processes. Procoagulant activation simultaneously initiates fibrinolysis (elimination) phase and initiates tissue repair processes through dissolution of fibrin-platelet thrombi. This process requires plasmin activity for both fibrin degradation and activation of matrix metalloproteinases, facilitating damaged tissue removal and cellular migration into injured areas (Rijken, 2009).

The plasminogen activation system operates through two distinct mechanisms: tissue plasminogen activator (t-PA)-mediated plasmin generation at fibrin surfaces, regulating fibrin homeostasis (Medcalf, 2007) and urokinase plasminogen activator (u-PA)-mediated pericellular plasmin generation via cellular u-PA receptor (u-PAR) binding, facilitating tissue remodeling and cellular migration.

Vascular endothelial cells synthesise and secrete both t-PA and u-PA, with regulation mediated by cellular cytokines and coagulation cascade components, notably thrombin (Collen, 1991). t-PA demonstrates minimal enzymatic efficiency in the absence of fibrin; however, concurrent binding of t-PA and plasminogen to fibrin surfaces results in an approximately 100-fold enhancement in plasminogen activation efficiency, thereby localising fibrinolytic activity to sites of fibrin deposition (Kim, 2011).

Fibrinolytic regulation is achieved through multiple inhibitory proteins: plasminogen activator inhibitor-1 (PAI-1) and -2 (PAI-2) (Declerck, 2013); α2-antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI) (Collen, 1999).

The antagonistic relationship between plasminogen activator inhibitors and their respective activators establishes an activation threshold for fibrinolysis, analogous to the procoagulant-anticoagulant equilibrium in coagulation. α2-antiplasmin functions as the primary physiological plasmin inhibitor, with reduced inhibitory activity against fibrin-bound plasmin due to competitive binding site interactions (Sakata, 1982).

1.2 Single nucleotide variants (SNVs)

1.2.1 rs6025 in FV gene

Factor V Leiden variant is the most widespread genetic variant causing inherited thrombosis in the Caucasian population (Rees,1995, Ridker, 1997; Rosendaal, 1995). The carrier frequency does not differ between sexes. Approximately 3–7 percent of patients of Northern European, Scandinavian (Caucasian) origin are heterozygous carriers. The incidence is considerably lower incidence among Spanish, African, Asian, and Native American populations (Ridker, 1997; Slavik, 2009). Thrombosis risk increases 3–8 times for heterozygous carriers and up to 80 times for homozygous forms (Rosendaal, 1995). It is the most common genetic condition in patients with thrombosis (Rees,1995), as it can be detected in 20 percent of patients with venous thrombosis and in more than half of selected families with thrombophilia (Rosendaal, 1995). The prevalence of the homozygous form is 1.5 percent of the general population (Rees, 1995). From population database gnomAD v1.1.0 data homozygotes in European (non-Finnish) population is 0.06 % (370 from 589584 genotyped individuals). The emergence of this variant is estimated to have occurred more than 21 000 years ago (Zivelin, 2006) and with high probability, all descendants originated from a single individual. The high prevalence of FV Leiden may indicate a greater survival advantage for descendants when encountering injuries (Lindqvist, 2001).

Factor V Leiden is a missense variant in which the 506th amino acid, arginine, is replaced by glutamine (Figure 1.3), resulting in cessation of inactivation of factor Va by APC (Zoller, 1994) approximately 10 times more slowly than normal (Friedman, 2010).

APC resistance (APCR) is attributable to the FV Leiden variant in up to 95 percent of cases (Segers, 2007). The literature lacks convincing data regarding increased risk for arterial thrombosis development (myocardial or cerebral ischaemia) in this population (Segers, 2007; Rahemtullah, 2007). The thrombotic risk is altered by other genetic and environmental risk factors, for example, hyperhomocysteinemia, ageing (Ridker, 1997), pregnancy (Dizon- Towson D, 1997).

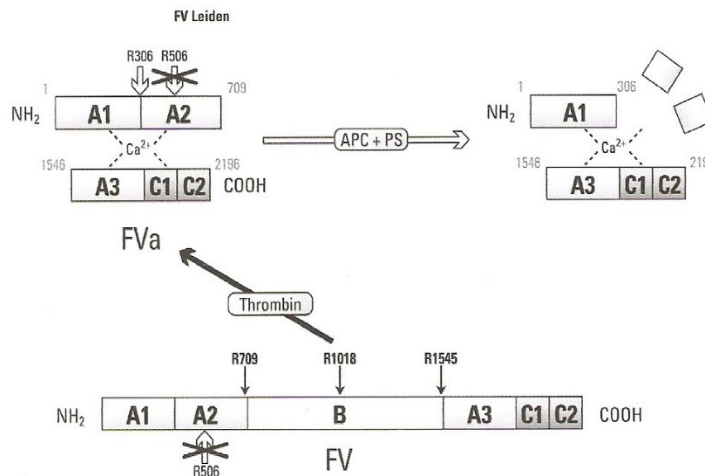


Figure 1.3. **Structure of procoagulant FV Leiden**
(Segers, 2007)

Native FV has little procoagulant activity until it is activated through limited proteolysis by thrombin or FXa at Arg709, Arg1018 and Arg1545. Following activation, the activated FV molecule (FVa) is a heterodimer that consists ofa105 kDa heavy chain (A1-A2 domains) and a 71/74 kDa light chain (A3-C1-C2 domains) that are non-covalently associated inacalcium-dependent manner.

Factor V is synthesised in the liver and then enters circulation in plasma. In cases where an individual has FV Leiden mutation, this causes APC resistance. However, if such an individual receives a liver transplant from a donor without FV Leiden variant, normal APC will subsequently circulate in the recipient's plasma, but DNA tests will still show FV Leiden variant, as DNA testing is performed using leukocytes. However, if an individual with existing FV Leiden receives a bone marrow transplant from a donor without FV Leiden variant, the recipient will show negative DNA FV Leiden variant but will have APC resistance (Van Cott, 2010).

Anticoagulant use does not affect FV Leiden DNA results, but the use of direct thrombin inhibitors (dabigatran, argatroban, bivalirudin) as well as Factor Xa inhibitor (rivaroxaban) may cause falsely normal APC resistance tests (Van Cot, 2016; Shaikh, 2009).

1.2.2 rs1799963 variant in the Prothrombin gene

The second most common variant that causes a predisposition to thrombosis formation is rs1799963 variant in the Prothrombin gene with legacy name G20210A (Bertina, 1998). Prothrombin is encoded by a 21-kb gene located on chromosome 11 (11p11 - q12). It consists of 14 exons separated by 13 introns with two untranslated regions at the 5' and 3' ends (Poort, 1996). In the prothrombin gene, within the 3' untranslated region at position 20210, an adenine-to-guanine substitution occurs. This variant is located outside the coding region; therefore, the molecular structure of prothrombin is not affected, and its procoagulant function following activation to thrombin remains unchanged.

The G20210A variant causes increased mRNA and protein synthesis of prothrombin, resulting in elevated prothrombin levels in plasma – 133 percent above the upper limit of normal (Jadaon, 2011). Increased prothrombin levels may promote elevation of thrombin-activatable fibrinolysis inhibitor (TAFI), which represents an additional thrombogenic mechanism. The prevalence of the prothrombin G20210A variant among Caucasian Europeans with deep vein thrombosis ranges from 3–17 %, while the mutation frequency among healthy individuals is 1–8 %. In the gnomAD database, the variant frequency among non-Finnish Europeans is 1.29 % (homozygotes 0.017 % or 92 out of 535 449 individuals). Prothrombin is a serine protease precursor of thrombin and represents a key component in ensuring haemostasis. It possesses procoagulant, anticoagulant, and fibrinolytic activities (Poort, 1996).

1.2.3 rs2066865 in the Fibrinogen Gamma Chain gene (*FGG* gene)

Thrombin induced conversion of fibrinogen to fibrin is essential part of haemostasis. An increased levels of fibrinogen have been associated with a change of blood viscosity and platelet aggregation (Eber, 1993; Lowe, 1986).

Fibrinogen is a plasma glycoprotein with a molecular weight of 340 kDa and is synthesised by hepatocytes. Fibrinogen molecules are elongated structures 45 nm long comprised of two sets of three polypeptide chains – A alpha; B beta; and gamma (Henschen, 1983). The three chains are encoded by three separate genes: fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*), and fibrinogen gamma (*FGG*) (Mosesson, 2005). Variant rs2066865 is a haplotype-tagging SNV characterised by a C to T substitution at nucleotide 10034 of the *FGG* gene (HGVS nomenclature NG_008834.1: g.13627C>T) and has been proposed as a risk factor for deep vein thrombosis in the Leiden Thrombophilia Study (de Willige, 2005), as well as in the later studies (Grünbacher, 2007, El-Galaly, 2013).

The γ chain is also reported to protect against inactivation by antithrombin, which is thought to facilitate heparin's resistance to clot-bound thrombin and further contribute to thrombin activity on the clot surface, thus increasing the time for thrombus formation (Farrell, 2012).

Fibrinogen is viewed also as an acute phase reactant during inflammation and its rise in relation to ageing (van Hylckama Vlieg, 2003), pregnancy and in case of pathophysiological changes such as trauma (Schlimp, 2016) and malignancy (Tang, 2010).

1.2.4 rs2227589 in SERPINC1 gene

Antithrombin (AT) deficiency was the first identified genetic risk factor for venous thrombosis. In 1965, shortly after the first assay for AT in plasma from clinical patients was presented, Egeberg reported presence of antithrombin deficiency in a family suffering from

thrombosis. However, the first proposal of antithrombin as a factor liable for the inhibition of thrombin activity was already mentioned in 1905 by Morawitz.

Inherited AT deficiency is a rare autosomal dominant disorder. Prevalence in general population is 1:2000 to 1:5000 and is associated with a 10-fold to 20-fold increased risk of thrombosis, mainly in venous origin. Male and female are affected equally and there is no racial or ethnic proclivity (Zöller, 1999).

Homozygous antithrombin deficiency is incompatible with life, almost always fatal in utero. Heterozygous type I antithrombin deficiency (quantitative) is characterised by reduced levels of antigen and reduced functional activity. Prevalence in general population is about one- eighth of all antithrombin deficiencies; however, it is found in 60 % of patients with symptomatic thrombosis. Type II (qualitative) – functional defects composing major part of antithrombin deficiency (80 %) is subdivided in three types by the site the defect arises: IIa-thrombin-binding domain mutants (reactive site domain) (Figure 1.4); IIb-heparin-binding site mutants (HBS) – is thought to be with a less severe thrombotic tendency compere to the other groups; IIc-mutants with pleiotropic effects (PE) – the group characterised by complex flaws affecting the heparin binding site, the reactive site and the plasma concentration of AT antigen, caused by reduced synthesis, secretion or as a result of increased catabolism (Kumar, 2015).

The occurrence of thrombosis during pregnancy and *puerperium* (post-partum) is not rare, but unlike to patients with antithrombin deficiency, usually develop during the last trimester of pregnancy or within two weeks following delivery (Hart, 2022). AT is associated with the highest risk of VTE of all known inherited thrombophilias. And mainly affects deep veins in lower extremities, however unusual sites are also reported such as cerebral veins and sinuses, mesenteric, portal, hepatic, renal and retinal veins. Most of VTE episodes in AT deficiency are unprovoked, and only 40 % are triggered by transient risk factors. First thrombotic events usually occur by the age of 30 years and by the age of 50 years, 50 % of patients having AT deficiency will have had thrombosis episode, generally DVT (Patnaik, 2008).

Plasma antithrombin levels tend to be higher in younger age groups and decrease with age. An oestrogen-containing hormone therapy may also decrease plasma and serum antithrombin levels. Interestingly, a low antithrombin concentration does not inevitably ensue thrombosis. In his report on families with inherited antithrombin deficiency, van der Meer mentioned a few cases of pedigrees with low antithrombin concentration and no convincing history of thrombosis (van der Meer, (1973).

Antithrombin is a member of serpin (serine proteinase inhibitor) family, posing the ability to regulate several enzymes of the coagulation pathway including thrombin and factor Xa and accounting for 70 % of the total anticoagulation activity. It is circulated as a single- chain glycoprotein (M_r -58.2 kDa) composed of 432 amino acids and is produced in the liver with a half-life of 2–3 (2.4) days. AT in circulation exists as two isoforms, α (90–95 %) and β (5–10 %). The β isoform possess higher affinity to heparin (Zheng, 2014).

Under normal conditions, antithrombin exhibits weak anticoagulant activity; however, after binding to heparin or other heparin-like glycosaminoglycans, as endogenous heparan sulphate exposed on endothelial surface, effect increases thousand-fold. Conformational changes of antithrombin after interaction with heparin-like substances lead to inactivation of coagulation factor Xa. To inhibit thrombin (factor II), a ternary complex is created. Antithrombin first interacts with heparin and subsequently thrombin binds to a remote domain of heparin, thus converting appropriately for inhibition. This thrombin-antithrombin (TAT) complex is then promptly cleared from circulation (Olson,1994, Zöller, 1999, Zheng, 2014).

Antithrombin gene comprises 7 exons and 6 introns, and the protein contains 3 disulphide bonds and 4 glycosylation sites: a heparin-binding domain at the N-terminus and the reaction site at the C-terminus (Figure 1.4). Over 200 variants are mentioned in the antithrombin gene.

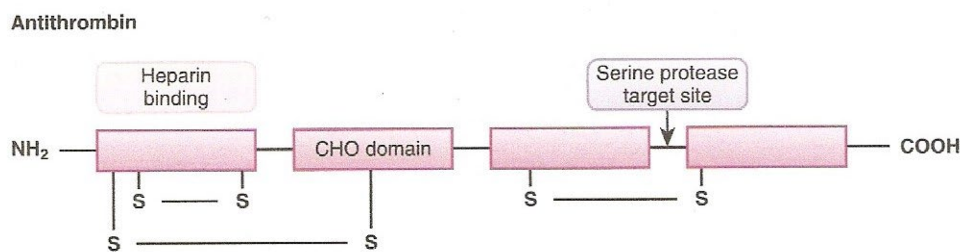


Figure1.4. **Antithrombin schematic illustration**
(Brummel-Ziedins, 2013)

Antithrombin (AT) contains two intrachain disulphide bonds (-S-S-) in its NH2-terminus and one in its COOH-terminus with a carbohydrate-rich domain (CHO) in between. The region of interaction between the active sites of target proteases and AT is illustrated (reactive centre loop). Heparin binding occurs in the NH2-terminus and enhances the rate of inhibition of serine proteases.

The variant rs2227589 (HGVS nomenclature NM_000488.4: c.41+141G>A) is one of frequent variants tested in the *SERPINC1* gene showing that it might be risk factor of VTE, but more evidences are demanded (Jiang, 2017).

1.2.5 rs1801133 in the MTHFR gene

The prevalence of variant varies depending on ethnicity and region. It is more frequently encountered among Italians and Spaniards, and less common among African Americans and sub-Saharan populations (Botto, 2000). Among Europeans, the homozygous allele is most prevalent in Italians and least common in Germans (Adams, 1996). In the United Kingdom, the homozygous prevalence in the population is approximately 13 percent, while outside Europe, the prevalence among Caucasians (Canada, America, Brazil, Australia) ranges from 10–14 % (Wilcken, 1996). Zero percent prevalence is observed in sub-Saharan African populations (Pepe, 1998; Schneider, 1998). In population database gnomAD the variant frequency in Europeans (non-Finnish) 0.3371 and homozygote frequency is 0.1144 (67492 from 589929 individuals). Lower *MTHFR* gene variant prevalence is noted among older individuals (> 80 years) at 7 percent, compared to younger groups: (55–79 years) 14 percent and (14–55 years) 19 percent (Matsushita, 1997).

Homocysteine is a sulphur-containing intermediate in methionine metabolism. Under normal circumstances the 5,10-Methylenetetrahydrafolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which produces methyl donor for the conversion of homocysteine to methionine. The process is catalysed by methionine synthase that is found in all mammalian tissues where thiamine is used as a cofactor (Goyette, 1998).

The C677T variant is nucleotide substitution of cysteine for thymine causing the substitution of alanine for valine in 677 aminoacid in the MTHFR enzyme. This results in the enzyme being unable to convert 5,10-methylenetetrahydrafolate to 5-methyltetrahydrofolate, which leads to increased plasma homocysteine levels in the homozygous mutated subjects (Liew, 2015).

MTHFR C677T variant and an increase in plasma homocysteine levels have been reported to be associated with an increased risk of myocardial infarction in young / middle-aged Caucasians (Liew, 2015). Elevated plasma homocysteine levels have been described as a risk factor for the development of atherosclerosis and coronary artery disease (Stanger, 2004). Forty percent of patients with coronary artery disease, cerebral or peripheral arterial diseases have elevated levels. The precise mechanism of vascular damage remains unclear; it is possible that homocysteine induces atherogenesis and thrombogenesis, which potentiates fibrosis and smooth muscle cell hyperplasia (Welsch, 1997). However, individuals with the *MTHFR* 677TT variant and a low folate status had a significantly higher risk of coronary heart disease (Klerk, 2002). Individual publications have emerged regarding elevated homocysteine levels and their association with the risk of developing cerebral infarction, as

well as myocardial infarction risk in younger / middle-aged individuals, particularly among the Caucasian race (Yu, 2011; Xuan, 2011).

Contrasting viewpoints regarding elevated homocysteine levels and their impact have also appeared. In 2012, Clarke and colleagues argued that mildly elevated homocysteine levels do not cause coronary heart disease or have a minimal impact on it, attributing the previously stated contrary opinion to methodological inaccuracies in earlier studies (Clarke, 2012). In addition, studies conducted with the aim of reducing plasma homocysteine levels did not reduce cardiovascular risk-associated complications and mortality (Bonaa, 2006), and the *MTHFR* C677T gene variant is only weakly associated with the risk of developing venous thromboembolism (Ray, 2002).

Furthermore, research originating from the same Latvian Centre for Reconstructive and Microsurgery provides critical context on nonhereditary risk factors. The work by Vanags et al. (Vanags, 2020), further expanded in the doctoral thesis by Stepanovs (2025), investigated a cohort of 103 trauma patients undergoing microvascular reconstruction. Their findings underscored the paramount importance of acquired and surgical variables. Specifically, they identified prolonged surgical duration (>240 minutes) as the primary risk factor in early post-traumatic reconstructions and rotational thromboelastometry (RTE)-detected hypercoagulability as the key predictor of thrombosis in late reconstructions. This body of work highlights that in this specific clinical setting, the prothrombotic state is powerfully influenced by the timing of surgery, patient comorbidities, and surgical trauma itself (Stepanovs, 2025).

1.3 Microvascular free flap surgery

Microvascular free flap surgery or free tissue transfer. A flap represents a composite tissue unit with autonomous blood circulation that is transferred from one anatomical location (donor site) to another (recipient site). In microvascular flap procedures, vascular anastomoses are performed (typically involving one artery and two veins), thereby ensuring oxygen and nutrient supply to the flap tissues at the recipient site. This procedure can also be classified as autotransplantation within the confines of a single body (Fu-Chan, 2017).

Indications:

- Tissue defects resulting from amputation and trauma
- Tissue defects due to infection
- Tissue defects following benign and malignant tumor extirpation
- Treatment of congenital pathologies

Classification of Flaps:

- Fasciocutaneous (comprising skin, subcutaneous tissue, and superficial fascia)

- Fasciomyocutaneous (comprising skin, subcutaneous tissue, fascia, and muscular tissue)
- Muscular flaps
- Osteocutaneous (comprising skin, subcutaneous tissue, fascia, and bone)
- Any tissue that can be isolated on a vascular pedicle may be classified as a flap

Commonly Encountered Flaps:

1. Radial Forearm Flap: Based on the radial artery angiosome. Predominantly utilised in head and neck oncological reconstruction. Classified as a fasciocutaneous flap.
2. Lateral Arm Flap: Based on the posterior radial collateral artery. Primarily employed in head and neck oncological reconstruction for moderate tissue defects. Classified as a fasciocutaneous flap.
3. Anterolateral Thigh Flap: Based on the descending branch of the lateral circumflex femoral artery. Utilised in larger tissue defect reconstruction.
4. Fibula Flap: Based on the peroneal artery angiosome. May be harvested as either an osseous or fasciocutaneous flap. Utilised in various osseous defect reconstructions.
5. Latissimus Dorsi Muscle Flap: Based on the thoracodorsal artery territory. Employed in scalp reconstruction and extensive tissue defects. Comprises purely muscular tissue.
6. Scapular and Parascapular Flap: Based on the circumflex scapular artery territory. Utilised in extensive tissue defects. Comprises skin, subcutaneous tissue, and fascia. Can be harvested as a composite flap including a portion of the scapular bone.
7. LADO (Latissimus Dorsi) Flap: Comprises muscle and skin, based on the thoracodorsal artery territory. Utilised in extensive defect reconstruction. Commonly employed in breast reconstruction (rarely as a microvascular flap, more frequently as a pedicled flap).
8. TRAM (Transverse Rectus Abdominis Myocutaneous) Flap: Comprises skin, subcutaneous tissue, fascia, and a segment of rectus abdominis muscle. Based on the superior epigastric artery territory. Utilised in post-mastectomy breast reconstruction (Figure 1.5).
9. DIEP (Deep Inferior Epigastric Perforator) Flap: Comprises skin, subcutaneous tissue, and fascia. Based on the superior epigastric artery territory. Utilised in breast reconstruction.

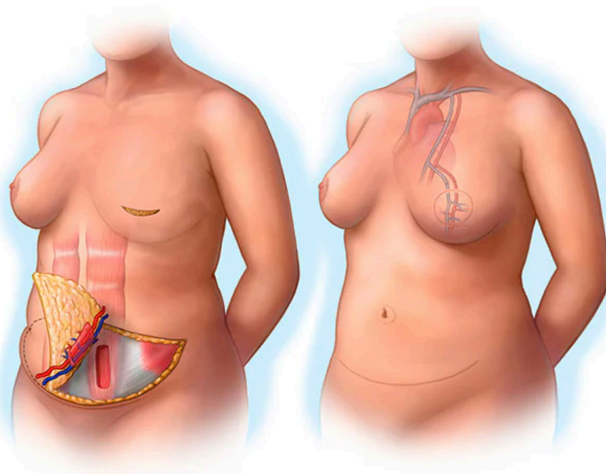


Figure 1.5. **Breast reconstruction with TRAM (transverse rectus abdominis myocutaneous) flap following mastectomy**

(Picture taken from mayoclinic.org Accessed 20.02.2025)

1.4 Anaesthesia management in microvascular free flap surgery

1.4.1 Physiology

Anaesthetic management impacts flap survival. Adequate flap perfusion and oxygen delivery are a core elements of free flap survival (Hagau, 2008). The provision of appropriate anaesthesia and perioperative care from an anaesthesiologist play a crucial role in the successful survival of a free flap. When providing anaesthesia for microvascular free flap surgery, it is essential to understand the physiology of tissues that have lost circulation and innervation. Following transplantation, despite losing innervation after transfer, the microvascular free flap remains capable of responding to chemical, physical, and humoral stimuli. Blood flow in the flap decreases by around half in the early postoperative period, returning to previous levels within several days to one week. The free flap lacks lymphatic drainage, which accelerates the potential for oedema formation, thereby compromising blood flow within the flap (Wu, 2022).

Principles of perfusion

At physiological shear rates blood plasma demonstrates Newtonian fluid behaviour and the Hagen-Poiseuille equitation is applicable (Figure 1.6). This principle explains the relationship between blood pressure, blood flow, and vascular resistance. The blood volume flowing through a vessel is directly proportional to the pressure drop along with the distance of the vessel (P_1–P_2) and the fourth power of the radius of the vessel and is inversely proportional to the length of the vessel (L) and the viscosity of the blood (η). Choice of anaesthesia can alter the vascular tone by acting on smooth muscle and thus altering radius (r). Maintenance of blood pressure (ΔP = difference between systemic arterial and venous pressure). And blood viscosity

(η) to facilitate higher possible flow. The rate or velocity of blood flow varies inversely with the total cross-sectional area of the blood vessels (Koeppen, 2017).

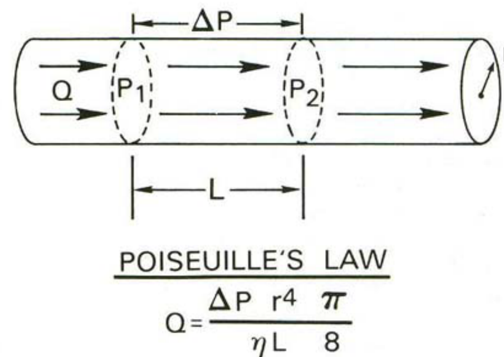


Figure 1.6. **Hagen–Poiseuille equitation** (Koeppen, 2017)

Q – flow, ΔP = difference between pressure, P – pressure,
L – length, η – viscosity, r – radius

Laminar flow represents optimal fluid movement efficiency; however, its presence in arterial circulation is limited. This flow pattern predominantly manifests in vessels with diameters below 5 mm. Consequently, microvasculature involved in free tissue transfer primarily exhibits laminar flow characteristics. Turbulent flow patterns arise under specific conditions including excessive flow velocity, irregular vessel surface characteristics, and altered vessel geometry (MacDonald, 1985).

The transition between laminar and turbulent flow is quantified by the Reynolds number (Re), expressed as: $Re = (v \times d \times p) / \eta$, where v represents flow velocity, d denotes vessel diameter, p indicates fluid density, and η represents dynamic viscosity. Critical Reynolds number thresholds demonstrate that turbulence occurs at vessel branching points when Re exceeds 200, and in straight vessel segments when Re exceeds 2000. Conversely, capillary vessels maintain laminar flow due to their small Reynolds numbers.

The application of Bernoulli's principle to vascular haemodynamics reveals that vessel diameter reduction results in elevated flow velocity with concomitant reduction in lateral wall pressure (Figure 1.7). This phenomenon leads to diminished perfusion pressure gradients across tissue beds, potentially compromising microcirculatory perfusion. Under conditions of maintained volumetric flow rate, blood velocity demonstrates an inverse relationship with vascular cross-sectional area. According to the continuity equation, as blood traverses progressively narrower vascular lumens, flow velocity increases proportionally to maintain constant volume flow (Adams, 2003).

Clinical meaning extends to areas of disturbed flow at bifurcations or regions of altered vessel geometry, where changes in flow velocity and direction correlate with increased thrombogenic risk. Venous stasis, often resulting from extended surgical procedures or

postoperative immobilisation, may trigger anticoagulation cascades, potentially compromising flap viability (Doh, 2021).

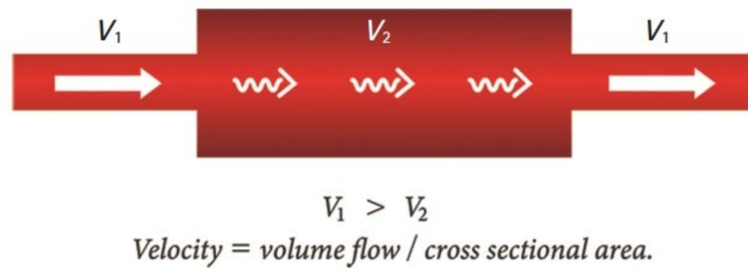


Figure 1.7. **Bernoulli's principle**

Haemodynamic changes in stenotic vessels. Bernoulli's principle illustrates the inverse relationship between vessel diameter and flow velocity. (Doh, 2021)

According to Laplace's law (Figure 1.8), the vascular anastomosis site tends to maintain a constant homeostatic pressure between arteries. Where there is a constant pressure, as the radius of the post-anastomotic artery diminished, the tension within the artery's wall also simultaneously decreased (Hagau, 2008).

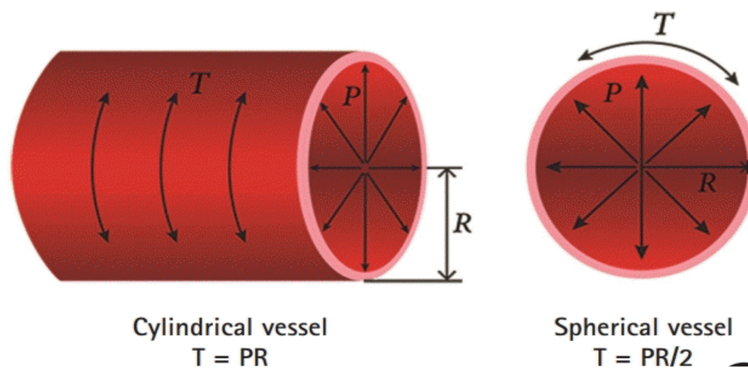


Figure 1.8. **Laplace's law**

T – wall tension, P – pressure, R – radius (Doh, 2021)

Oxygen Transport and Metabolism

Cellular metabolism in human physiology requires continuous oxygen (O_2) supply for optimal function. Both oxygen deficiency (hypoxia) and excess (hyperoxia) can result in significant physiological changes.

Oxygen transport in blood primarily occurs through binding with haemoglobin (Hb). Following diffusion across the alveolar membrane into pulmonary capillaries, oxygen molecules form oxyhaemoglobin. Each haemoglobin molecule possesses the capacity to bind four oxygen molecules. At physiological haemoglobin concentrations (15 g/dl), the maximum oxygen-carrying capacity is 20.85 ml O_2/100 ml, corresponding to approximately 1.39 ml O_2/g Hb (Hüfner's constant) (Dunn, 2016).

Total arterial oxygen content comprises both haemoglobin-bound and plasma-dissolved oxygen. The quantitative expression for arterial oxygen content per 100 ml of blood is defined by:

$$CaO_2 = (1.31 \times Hb \times SaO_2 \times 0.01) + (0.0225 \times PaO_2)$$

Where:

- 1.31 represents Hüfner's constant
- Hb denotes haemoglobin concentration (g/dl)
- SaO_2 represents arterial haemoglobin saturation (percentage)
- 0.0225 indicates oxygen solubility coefficient at physiological temperature
- PaO_2 represents arterial oxygen partial pressure (kPa)

Haemoglobin–Oxygen Dissociation Curve: Clinical Implications in Microvascular Surgery and Anaesthesia

Oxygen transport occurs through two primary mechanisms: convection, an energy-dependent active process and diffusion – a passive process driven by partial pressure gradients. Oxygen transport and delivery are critical components in microvascular surgery outcomes. The haemoglobin–oxygen dissociation curve (Figure 1.9) provides essential insights into oxygen transport dynamics during surgical procedures and anaesthesia management.

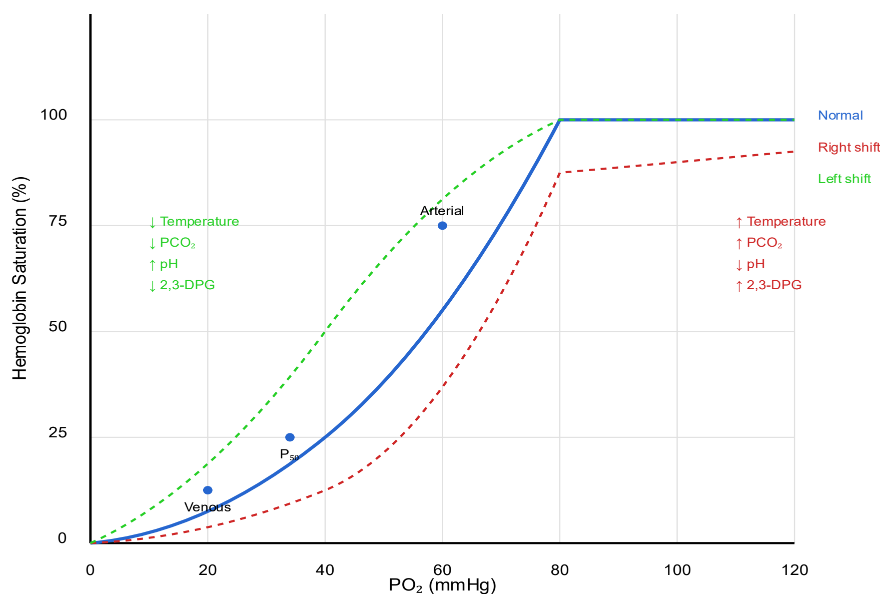


Figure 1.9. **Haemoglobin-oxygen dissociation curve** (Koeppen, 2013)

The haemoglobin-oxygen dissociation curve illustrates the relationship between partial pressure of oxygen (PO_2) and haemoglobin saturation. The sigmoid-shaped curve demonstrates three physiological states: normal (blue, solid line), right shift (green, dashed line), and left shift (red, dashed line).

Haemoglobin–oxygen dissociation curve shifts demonstrate significant clinical implications in perioperative management. The rightward curve displacement, characterised by decreased oxygen affinity, enhances tissue oxygen delivery. This phenomenon occurs in

response to several perioperative conditions: metabolic acidosis from significant blood loss (Base deficit > 5 mEq/L), which elevates P50 by 1–2 mmHg per 0.1 unit pH reduction, respiratory acidosis, inducing CO_2 retention and enhanced Bohr effect temperature elevation, causing 2–3 % increase in P50 per degree Celsius and prolonged surgical duration affecting 2,3-DPG dynamics.

Conversely, leftward curve displacement indicates increased haemoglobin–oxygen affinity, potentially compromising tissue oxygen delivery. Alkalotic states, both respiratory (induced by mechanical hyperventilation) and metabolic (from excessive buffer therapy), enhance haemoglobin–oxygen binding, consequently reducing tissue oxygen availability (Koeppen, 2017).

The relationship between haematocrit, blood viscosity, and oxygen delivery represents a critical balance in microvascular surgery. Blood viscosity demonstrates an exponential relationship with haematocrit levels, significantly affecting microvascular flow dynamics and oxygen delivery capacity (Figure 1.10).

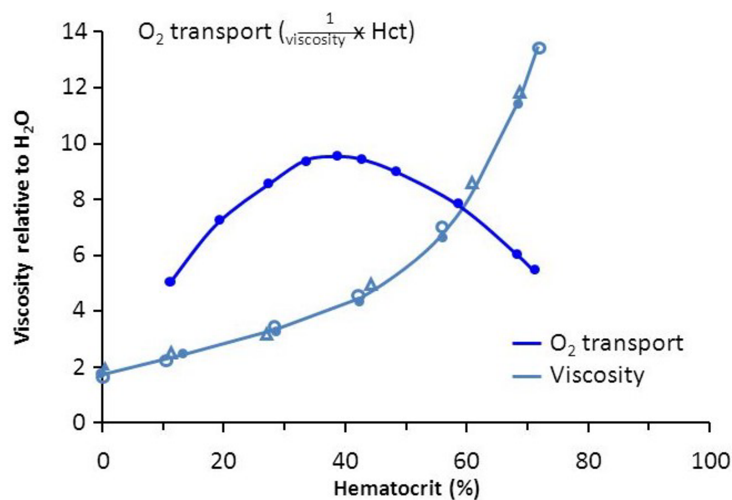


Figure 1.10. **Relationship between haematocrit, blood viscosity and oxygen delivery** (Wayne, 1993)

Hct – haematocrit, the oxygen transport increases with increase in haematocrit, until the point where viscosity reaches impact on shear rates, Hct of 30% is described as optimal for microvascular surgery, as best compromise between share rate/ viscosity and oxygen delivery.

The oxygen transport effectiveness increases with increased shear rate and higher haematocrit is warrant, however when shear rate is reduced lower haematocrit levels could be appropriate for effectiveness of optimal oxygen delivery (Piety, 2017). A haematocrit level of 30 % has been identified as optimal for microvascular surgery, representing the best compromise between oxygen-carrying capacity and blood viscosity (Wayne, 1993). This level maintains adequate oxygen delivery while minimising the adverse effects of increased viscosity on microvascular flow (Menu, 2000; Kim, 2018).

1.4.2 Perioperative Management

Pre-operative patient assessment

Preoperative patient assessment is a crucial prerequisite for minimising patient and surgical risk factors. The development of an anticipated anaesthetic plan is aimed at ensuring optimal blood flow in the microvascular free flap (Khouri, 1998).

Patient age *per se* is not a risk factor in microvascular surgery (Serletti, 2000). However, comorbidities that affect circulation are the primary risk factors that may cause difficulties in providing the necessary measures for successful free flap survival (Vincent, 2019).

Patients with documented ischaemic heart disease and/or left ventricular dysfunction find it difficult to tolerate administered fluid loads, and their monitoring requires special attention (ST depression, decreased urine output, increased serum lactate, core-to-peripheral temperature gradient changes). Therefore, in patients with documented preoperative left ventricular insufficiency, free flap compromise is predictable, and the planned surgical scope should be modified for such patients (Sou, 2022). Patients taking angiotensin-converting enzyme inhibitors should discontinue them 24 hours before the planned surgery due to the high probability of promoting hypotension (Roshanov, 2017). Preoperative anaemia is linked to increased flap failure and thrombosis when haemoglobin falls below 10 g/dL; preoperative transfusion is warranted if levels drop below 8 g/dL, especially in ischaemic heart disease cases (Hill, 2012).

Chronic obstructive pulmonary disease and smoking, as one of the leading aetiological factors, cause hypercapnia, hypoxia, and reduced FEV1, which are associated with a higher risk of developing postoperative ventilation disorders (Nakagava, 2001). Hypoventilation with subsequent respiratory acidosis reduces erythrocyte deformability and increases endogenous catecholamine release, which directly affects flow in newly created vascular anastomoses. Smoking cessation should be recommended for at least 6–8 weeks before the planned surgery (Kuri, 2005).

Negative effects are also observed in cases of hyperoxia and hyperventilation with respiratory alkalosis – cardiac output decreases (MacDonald, 1985) and elevated pO_2 causes vasoconstriction with subsequent reduction in tissue perfusion (Tsai, 2003).

Diabetes mellitus is the most commonly encountered endocrine pathology associated with increased morbidity in surgical patients. Acute and chronically persistent hyperglycaemia are associated with increased vascular wall permeability, resulting in an increased likelihood of interstitial oedema formation, which compromises microvascular flap blood flow (Hagau, 2008). In diabetic patients with poorly controlled glucose levels suboptimal blood vessels

integrity and immunosuppression increased risk of surgical site infection. Haemoglobin A1C level below 8 % and blood glucose levels within 4–12 mmol/l (Mak, 2020).

Adequate perioperative nutrition has also been consistently demonstrated to improve outcomes (Dort, 2017; Leung, 2017). The Enhanced Recovery After Surgery (ERAS) Society strongly recommends comprehensive preoperative nutritional assessment, with particular attention to dysphagia severity and refeeding syndrome risk. (Kinzinger, 2017). Preoperative nutritional optimisation in malnourished patients has been shown to reduce postoperative adverse events, including impaired wound healing and surgical site infections (Mortensen, 2014).

Preoperative patient education is an essential yet frequently underemphasised component of microvascular free tissue transfer preoperative management, with patient comprehension of surgical information representing the greatest challenge (Turkdogan, 2022). The preoperative period provides opportunities to address behavioural risk factors such as tobacco and alcohol use, discuss cessation strategies, and establish realistic postoperative expectations to improve patient understanding and psychosocial well-being (Crawley, 2019). Innovative educational approaches, including multimedia platforms and animated surgical videos, have demonstrated promise in enhancing patient education and satisfaction while overcoming health literacy barriers (Turkdogan, 2022). Enhanced preoperative understanding reduces patient anxiety before admission and sustains decreased clinical anxiety and depression for up to 6 months postoperatively (Boyu, 2024). Structured preoperative education programmes have shown to be associated with shorter hospital stays, reduced complications and decreased costs (Schmid, 2022).

Intraoperative Management

Goal-Directed Fluid Therapy and Haemodynamic Optimisation

Goal-directed fluid therapy has emerged as a superior approach compared to traditional fluid management protocols in microsurgical procedures. To ensure adequate perfusion in transplanted tissues, it is essential to maintain hyperdynamic circulation, high cardiac output, and peripheral vasodilation through increased cardiac filling pressure. The target central venous pressure (CVP) should be maintained at 10–15 cm H_2O or 3–5 cm H_2O above baseline levels. However, since CVP does not accurately reflect peripheral circulatory status, it should be evaluated in conjunction with other circulation-related parameters, including urine output, peripheral-central temperature differential, and intra-arterial pressure curve changes (Chalmers, 2012).

László et al. conducted a randomised clinical trial comparing crystalloid versus colloid fluid therapy effects on microcirculation during free flap surgery, demonstrating that individualised fluid administration based on haemodynamic parameters significantly improved

microcirculatory function (László, 2019). Al Saied et al. further compared the impact of vasopressors versus goal-directed fluid therapy on free flap reconstruction management, emphasising the importance of balanced haemodynamic approaches in intensive care unit monitoring (Al-Saied, 2020). These findings support the implementation of dynamic fluid monitoring using parameters such as stroke volume variation and cardiac output optimisation rather than fixed-volume protocols, while carefully balancing vasopressor use to maintain adequate perfusion pressure without compromising flap microcirculation (Goh, 2019; Chen, 2010).

Fluid Selection and Haematocrit Management

For fluid infusion selection in microvascular surgery, preference should be given to solutions that do not freely cross damaged epithelium, remaining within the intravascular bed, and possess sufficiently long half-lives to protect against hypovolemia development during the postoperative period. Combinations of crystalloids and colloids are most commonly used. Third-generation hydroxyethyl starch preparations with low molecular weight (130 kD) remain longer in the intravascular bed, reducing the risk of interstitial oedema development and improving rheology (Menu, 2000).

A linear relationship between blood viscosity and haematocrit does not exist; when haematocrit exceeds 40 %, blood viscosity increases dramatically. Haemodilution, by reducing blood viscosity, increases blood flow. Most patients can tolerate haematocrit reduction to 20 %; however, oxygen transport capacity decreases rapidly. An optimal haematocrit of 30 % is considered to provide balance between blood viscosity and oxygen content for tissue transport (Hagau, 2008).

Monitoring and Risk Factor Management

A comprehensive analysis by Pattani et al. identified critical intraoperative factors contributing to free flap failure including modifiable risk factors as haemodynamic instability, inadequate tissue perfusion, and suboptimal surgical conditions. Continuous monitoring of core and peripheral temperatures, alongside with tissue oximetry and haemodynamic variables (Karamanos, 2022) with intermittent measurements of blood gases, electrolytes, and haemoglobin concentration serves as critical guides for therapeutic interventions (Pattani, 2010).

Optimal tissue perfusion requires careful attention to acid-base homeostasis and oxygenation parameters during microsurgical procedures. Blood gas monitoring should guide ventilation strategies, with buffer therapy indicated when pH falls below 7.25 and ventilation adjustment required when pH exceeds 7.45. Maintaining PaO_2 above 80 mmHg ensures adequate oxygen delivery to transplanted tissues, while monitoring gradient changes in blood

gas parameters provides early indicators of metabolic derangement or inadequate perfusion (Adams, 2003).

Temperature Control

Temperature control plays a significant role both intraoperatively and postoperatively. Temperature reduction causes peripheral vasoconstriction and increased haematocrit with subsequent viscosity increase, potentially affecting blood flow. Maintaining optimal temperature can be challenging in cases with prolonged exposure of extensive surgical fields. For temperature maintenance, warm air blankets, heated tables, and warmed infusion fluids should be used, with operating room temperature maintained at 24–25°C. Central-peripheral temperature differential monitoring provides indirect indication of patient volume status and cardiac output (Tc: Tp < 1°) (Adams, 2003; Hagau, 2008).

Active warming measures should be initiated when core temperature falls below 36 °C, as hypothermia impairs microcirculation and increases the risk of thrombotic complications (Moellhoff, 2021). Conversely, cooling measures are indicated when temperature exceeds 37.5 °C to prevent hyperthermia-induced complications.

Anaesthetic Management and Preconditioning

General anaesthesia, regional anaesthesia, or a combination of both may be used for anaesthetic management. Regional anaesthesia provides excellent pain control both intraoperatively and postoperatively, reduces deep vein thrombosis incidence, and promotes faster recovery. Inhalation anaesthesia offers advantages for maintening general anaesthesia; sevoflurane reduces capillary filtration coefficient, thereby decreasing plasma extravasation and reducing the potential for oedema formation (Bruegger, 2002). Sevoflurane also possesses protective effects, safeguarding endothelial cells from ischaemia-reperfusion injury (Hagau, 2008). Among opioids, remifentanil is preferred as it provides rapid arterial pressure control, systemic vasodilation and maintenance of hyperdynamic circulation. Anaesthetic preconditioning is a novel approach that enhances tissue tolerance during the ischaemia-reperfusion events that are inherent in microsurgical procedures.

Claroni et al. demonstrated in a randomised controlled trial that sevoflurane preconditioning provided significant protective effects against ischaemia-reperfusion injury in patients undergoing microsurgical flap reconstruction (Claroni, 2016). The study showed reduced markers of cellular damage and improved postoperative outcomes in the sevoflurane preconditioning group compared to controls.

Anticoagulation

Intraoperative heparin use shows no statistically significant effect on microvascular thrombosis incidence. A single intraoperative heparin dose does not increase haematoma development risk but also does not prevent microvascular thrombosis formation (Chen, 2008).

Post-operative management and flap assessment

Monitoring Requirements and Clinical Assessment

Postoperative care principles are fundamentally based on measures that ensure optimal conditions for the survival of free microvascular flaps, i. e. strategies to prevent secondary ischaemia and thrombosis. Clinical flap checks are usually done every 2–4 hours for the first 2–3 days after surgery, as 95 % of failed flaps occur within 72 hours of surgery (Hosein, 2016). As early tissue reperfusion determines flap survival, assessing the flap in the operating theatre may be a future alternative (Schraven, 2023). Clinical monitoring may consist of a combination of assessments, including flap skin colour, surface temperature, rate of bleeding with pinprick or scratch, capillary refill time, and acoustic cutaneous Doppler to detect intravascular arterial and venous blood flow (Kohlert, 2019). Timely re-exploration can increase the rate of flap salvage. Successful salvage rates range between 28 % and 87.5 %, with return-to-theatre rates between 13 % and 20 % (Torabi, 2025).

Circulatory and Haemodynamic Management

To maintain optimal circulation, hypovolemia, hypotension, development of increased peripheral vascular resistance, and haematocrit reduction below 30 % must be avoided. Keeping the patient well hydrated and preventing anaemia are important steps to prevent microvascular thrombosis. Haematocrit should be kept between 25 % and 30 % (30 % for patients with a history of heart disease) (Lighthall, 2013). Diuresis control should be ensured (approximately 1 ml/kg/h).

Pain Management and Complications Prevention

Adequate analgesia and anxiety reduction are necessary to protect against the release of endogenous catecholamines. NSAIDs should not be avoided (both COX1 and COX2 inhibitors) as they do not increase the risk of bleeding or haematoma formation (Joshi, 2025; Bonde, 2017). The development of shivering increases oxygen consumption, causes peripheral vasospasm and reduces blood flow in the free flap (Moellhoff, 2021). Therefore, it should be prevented immediately by using warm blankets, adjusting the ambient temperature or administering clonidine.

Near-infrared spectroscopy (NIRS) provides superior flap salvage and survival rates compared to clinical examination (Bian, 2022). The survival of flaps with vascular compromise is inversely proportional to the time taken to detect the defect and the time taken to re-explore the area (Hosein, 2016).

Thromboprophylaxis Protocols

Currently, no consensus exists regarding postoperative thromboprophylaxis or the use of antiplatelet agents versus anticoagulants (Khansa, 2013). Most microvascular surgeons use some form of antithrombotic prophylaxis, such as heparin, aspirin, dextran, or other antithrombotic agent. However, no clinical reviews have so far conclusively shown any regimen to optimise free flap success (Pan, 2014).

2 Materials and methods

2.1 Study design and patient enrolment

The study was designed as an observational prospective case series study with patient enrolment from December 2016 to July 2019. A total of 155 adult patients who were scheduled to undergo microvascular free flap surgery at the Centre of Plastic and Reconstructive Microsurgery of Latvia were included. The selection process was governed by a stringent set of inclusion and exclusion criteria.

Prior to any procedural steps, a detailed informed consent form was presented to each participant and outlined the study's scope, including but not limited to the surgical procedures, potential risks, benefits, and the specific request for the donation of genetic material for research purposes was rigorously upheld through the approval of research protocol and informed consent form by the Latvian Central Ethics Committee, under the reference number No 1/28-11-16. (see Annex 1). This approval underscored the study's adherence to international ethical guidelines and the protection of patient rights, ensuring that all research activities were conducted with the highest ethical considerations.

Patient inclusion criteria

- All adult patients with microvascular free flap surgery
- A signed informed consent form

Patient exclusion criteria

The exclusion criteria were carefully established to mitigate any risk to participants and enhance the reliability of the study findings.

- *Pregnancy and Peripartum Period.* Due to the confounding effects of gestational and postpartum physiological alterations, this group of patients were excluded from the study.
- *Recent Transfusions.* Patients who received transfusions of allogeneic blood components and/or coagulation factors within 72 hours prior to the surgery were excluded. This measure was taken to prevent confounding variables related to blood product transfusions that could affect outcome.
- *Cardiac Conditions.* Individuals with proven left ventricular failure were not included in the study to avoid complications related to their condition, which could interfere with the surgery's success and their recovery process.
- *Bone Marrow and Liver Transplantations.* Patients who had undergone allogenic bone marrow transplantation or liver transplantation were excluded due to

the complex interplay of factors affecting their recovery and the potential impact on the outcome.

- *End-stage Kidney Diseases.* Patients manifesting end-stage kidney disease were excluded from the analysis due to confounding systemic alterations.
- *Recent Medication Use.* The study protocol required the cessation of direct oral anticoagulants 72 hours before sample collection to eliminate inaccuracies in testing. Similarly, for those on vitamin K antagonists, medication was halted based on the patient's international normalised ratio (INR) level 4–5 days before sample collection. Patients with a high thrombosis risk were switched to low-molecular-weight heparin (LMWH) and discontinued use 12 h prior to the surgery.

An interview was conducted to collect patient history, with particular interest in any previous thrombotic events, the use of antithrombotic medications, regular medications (including oral contraceptives), family history of thrombotic events, and any previously diagnosed inherited thrombophilias. All factors contributing to tissue injury were recorded, with a recent trauma period defined as occurring within the last 30 days in cases of trauma or polytrauma aetiology.

Regarding demographics, medical history, and family history, interviews were performed by the same clinician. A positive history of thrombosis was classified as any thrombotic event of either arterial or venous origin.

2.2 Genotyping

Blood samples were collected preoperatively, prior to anaesthetic induction and crystalloid administration, and transported to the Latvian Biomedical Research and Study Centre for subsequent analysis.

Genomic DNA was extracted from peripheral blood leukocytes utilising a standardised phenol-chloroform extraction protocol. The extracted DNA underwent quality assessment via spectrophotometric analysis (260/280 ratio) and concentration determination prior to subsequent analysis. DNA integrity was verified through gel electrophoresis.

Single nucleotide variant (SNV) genotyping was performed using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) on a ViiA 7 Real-Time PCR system (Applied Biosystems). The following specific assays were employed: C_11975250_10 for Factor V Leiden (rs6025), C_1799963_10 for prothrombin gene, C_11503414_10 for Fibrinogen Gamma (rs2066865), C_16180170_10 for SERPINC1 (rs2227589), and C_1202883_20 for MTHFR (rs1801133).

The PCR reaction mixture comprised 12.5 μL TaqMan Universal PCR Master Mix, 0.625 μL SNP Genotyping Assay (40×), 2 μL DNA template (10 ng/μL), and 9.875 μL nuclease-free water, yielding a total reaction volume of 25 μL. Amplification was performed under the following thermal cycling conditions: initial AmpErase UNG activation at 50 °C for 2 minutes, followed by polymerase activation at 95 °C for 10 minutes, succeeded by 40 cycles of denaturation (95 °C, 15 seconds) and annealing/extension (60 °C, 1 minute). The protocol concluded with a final extension at 72 °C for 1 minute.

Quality control measures included concurrent analysis of known homozygous wild-type, heterozygous, and homozygous variant controls, alongside no-template controls. Genotype determination was accomplished through allelic discrimination plots and cluster analysis. Validation criteria encompassed a minimum call rate of 95 %, Hardy-Weinberg equilibrium assessment, and control sample concordance verification. Internal quality assurance was maintained through duplicate testing of 10 % of samples and cross-platform verification where applicable.

All procedures were executed according to the manufacturer's recommendations, with comprehensive documentation of reaction conditions, reagent lot numbers, and equipment calibration status. Data analysis was performed using the manufacture's software (Applied Biosystems), with adherence to standardised analytical parameters.

2.3 Clinical laboratory investigation

Blood samples were drawn on the day of surgery prior to the induction of anaesthesia and any crystalloid infusion. All tests were processed within an hour.

Total plasma fibrinogen concentration was measured by the Clauss method (Mackie, 2003) (reference range 2–4 g/L) in citrated plasma using the STA-R COMPACT (Diagnostika Stago, Asnières-sur-Seine, France). Activated protein C resistance testing methodology: Modified APC-R Test – Patient plasma diluted 1:4 with FV-deficient plasma. Parallel testing with/without APC / APTT-based clotting time measurement (reference ranges: normal > 2.1, borderline 1.8–2.1, abnormal < 1.8, Heterozygous FVL typical: 1.5–1.8, Homozygous FVL typical: < 1.5). For prothrombin time, the reagent containing thromboplastin and calcium chloride is mixed with the patient's plasma, and the time taken for clotting to occur is measured photo-optically. The reference range is 70–130 %. Homocysteine concentration was investigated with Chemiluminescent Immunoassays (CLIA) with reference range 5–12 mkmol/l. Antithrombin analysis was performed using a chromogenic assay with a reference range of 75–125 % (All laboratory investigations were carried out at the "SIA NMS Laboratorija" facility).

2.4 Anaesthesia and intraoperative patient monitoring

Three types of anaesthesia were conducted according to the patients' needs: standardised general anaesthesia, regional anaesthesia, or a combination of regional and general anaesthesia.

Standardised general anaesthesia

Protocol included:

- Preoperative Phase: Pre-medication with Midazolam 0.02–0.04 mg/kg IV, maximum 2 mg. Prophylactic antibiotics per institutional guidelines; Antiemetic prophylaxis: Dexamethasone 8 mg IV + Ondansetron 4 mg IV
- Induction Phase: Pre-oxygenation – 100 % O_2 for 3 minutes via face mask; Induction Medications – Fentanyl 1–2 mcg/kg IV; Propofol 1.5–2.5 mg/kg IV; Cisatracurium 0.15–0.2 mg/kg IV
- Maintenance Phase: Inhalational Agent – Sevoflurane: MAC 0.8–1.2 in mixture of O_2/Air (FiO_2 0.4–0.5); Fentanyl 0.5–1 mcg/kg/h continuous infusion or Remifentanil 0.05–0.2 mcg/kg/min continuous infusion. For muscle relaxation – Cisatracurium 0.1–0.2 mg/kg/h is needed to maintain TOF 1–2 twitches.

Standardised regional anaesthesia

Protocol included:

- For upper and lower extremities long-acting agents – Ropivacaine 0.2–0.5 %, Bupivacaine 0.25–0.5 % volume 20–30 ml, with maximum dosage calculation. Adjuvants: Dexamethasone 4–8 mg. The neural blockade was performed utilising dual guidance methodology: real-time ultrasonographic visualisation and peripheral nerve stimulation (Stimuplex, B. Braun, Melsungen, Germany) to ensure precise anatomical localisation.

Fluid Management: Maintenance – Balanced crystalloid solution 4–6 mL/kg/h and additional boluses based on clinical assessment.

Central catheter placement and indwelling urinary catheter placement in surgery exceeding 2 hours.

Target Parameters:

- Haemodynamic Goals – Mean Arterial Pressure: 70–80 mmHg, Heart Rate: 60–80 beats/minute
- Central Venous Pressure (CVP) Monitoring: Optimal range for flap perfusion: 4–6 mmHg – not exceeding CVP > 10 mmHg due to risk of venous congestion
- Urinary Output Monitoring: Optimal range: 0.5–1.0 mL/kg/h

- Haematocrit: 30–35 %
- Temperature Management – Core temperature (nasopharyngeal): 36.0–37.0 °C – Δt (core-peripheral) ≤ 1 °C – Utilising fluid warmer and forced-air warming device

Multimodal Analgesia – Paracetamol 1g IV or Metamizole 1g IV and Dexketoprofen 50 mg IV (if not contraindicated).

Recovery Room Monitoring: Standard ASA monitoring, flap perfusion assessment, temperature monitoring, pain management.

2.5 Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) V.23 (IBM Korea, Seoul, Korea). The Kolmogorov-Smirnov test was used to check whether the variables followed a normal distribution. Normally distributed, continuous variables were presented as mean ± standard deviation (M ± SD) and categorical variables as percentages (%). In case values did not follow a normal distribution, the medians and interquartile ranges (IQRs) were presented. Odds ratios and 95 % confidence intervals were calculated to evaluate factor impacts between groups. Comparisons between genotype groups were performed with Kruskal-Wallis H tests for nonparametric variables and with ANOVA for parametric variables. Pearson's $x2$ correlation coefficient and p-values were calculated, and Spearman's rank correlation coefficient was used where applicable. Statistical significance was assumed as two-tailed $p < 0.05$.

For thrombosis risk assessment a primary analysis with logistic regression models for each SNP was performed with subsequent calculation of Odds Ratios (OR), 95 % Confidence Intervals and adjusted p-values. Allele distribution analysis using Chi-square test for genotype frequencies, Fisher's exact test (when n < 5) and Odds ratios for allele frequencies. Combined genetic risk analysis with multiple logistic regression and genetic risk score calculation with ROC curve analysis with AUC was used. Multivariable logistic regression was used for risk prediction models, with three categories of predictors defined:

- Genetic variants: *FV* rs6025, *FII* rs1799963, *FGG* rs2066865, *SERPINC1* rs2227589, *MTHFR* rs1801133;
- Acquired Coagulation Changes: Fibrinogen, Homocysteine, APCR, Prothrombin, Antithrombin;
- Confounding Factors: Age, Gender, Smoking;
- For statistical power and sample size power calculations for genetic association $\alpha = 0.05$ and $\beta = 0.20$ were assumed.

3 Results

3.1 Study population characteristics

Following the application of inclusion and exclusion criteria, a total of 162 consecutive patients scheduled for microvascular flap surgery were initially enrolled in the study. Seven patients were subsequently excluded from analysis due to incomplete data sets, resulting in a final study population of 155 subjects. The study cohort comprised 118 males (76.1 %) and 37 females (23.9 %), mean age of 45.07 years (SD ± 14.94). Table 3.1 summarises the demographic and clinical characteristics of the study population.

Table 3.1

Studied group characteristics (*n*, 155)

Demographic data	Number of patients, (%)
Gender, male	118 (70.8)
Age, years (Mean (±SD))	45.1 (±14.94)
History of thrombosis	10 (6.5)
Family history of thrombosis	21 (13.5)
Smoking	64 (41.3)
Alcohol abuse	18 (11.6)
Metabolic disturbance*	15 (9.7)
Recent trauma (< 30 days)	51 (32.9)
Defect aetiology: trauma	69 (44.5)
Polytrauma	12 (7.7)
Chronic inflammation	31 (20)
Malignancy	28 (18)
Combustion	6 (3.9)
Defect localization: Lower extremity	85 (54.8)
Upper extremity	36 (23.2)
Head/orofacial	29 (18.7)
Other (trunk, abdomen)	5 (3.2)

* *Diabetus mellitus, Adipositas (BMI > 25); n – number of patients*

Among patients included in the study, 14 patients (9 %) had flap thrombosis. Despite salvage attempts, 8 patients (5.16 %) experienced complete flap loss due to failed re-anastomosis, while 5 patients (3.22 %) had partial flap loss. Overall, the success rate for microvascular flap survival was 94.84 %.

The analysis of the 14 cases requiring surgical re-exploration is given in Table 3.2. The majority of complications occurred in lower extremity reconstructions (85.7 %, n, 12), with trauma and osteomyelitis representing the predominant underlying aetiologies. Venous thrombosis was more frequent than arterial thrombosis, occurring in 71.4 % of cases either alone or in combination with arterial complications.

Table 3.2

Microvascular Free flap complications requiring surgical re-exploration (*n*, 14)

Flap	Aetiology	Defect localisation	Thrombosis venue	Re-anastomosis	Flap necrosis	Salvage measure
Scapular/ Parascapular	osteomyelitis	lower extremity	venous	yes	no	additional vein anastomosis
Medial plantar artery flap	osteomyelitis	lower extremity	arterial	yes	no	additional vein anastomosis
LAF	trauma	lower extremity	venous	yes	no	additional vein anastomosis
Scapular/ Parascapular	osteomyelitis	lower extremity	venous	yes	no	NA
Scapular/ Parascapular	polytrauma	lower extremity	venous	yes	yes	additional vein anastomosis
Sartorius free flap	trauma	lower extremity	arterial and venous	no	yes	NPWT; STSG
Osteocutaneous FF	trauma	lower extremity	arterial and venous	yes	yes	local muscle flap and STSG
Osteocutaneous FF	osteomyelitis	lower extremity	arterial and venous	yes	yes	local flap
Serratus anterior muscle flap	osteomyelitis	lower extremity	arterial and venous	yes	yes	NPWT; STSG
RFF	malignancy	orofacial	arterial	yes	yes	local flap
LAF	trauma	upper extremity	venous	yes	yes	ALT flap
Scapular/ Parascapular	trauma	lower extremity	venous	no	yes	local flap; STSG
ALT	trauma	lower extremity	venous	no	yes	NPWT
SIEP, DIEP flap	trauma	lower extremity	venous	no	no	Rotation flap

NPWT – negative pressure wound therapy; STSG – Split thickness skin graft; LAF – Lateral arm flap; FF – Fibula flap; RFF – Radial forearm flap; ALT – Anterolateral thigh flap; SIEA – Superficial inferior epigastric artery; DIEP – Deep inferior epigastric artery perforators.

Despite aggressive surgical intervention, partial or complete flap necrosis occurred in 64.3 % of patients (n, 9), necessitating alternative reconstructive strategies including local flaps, split-thickness skin grafts (STSG), and negative pressure wound therapy (NPWT).

The heterogeneity of salvage measures reflects the individualised approach required for each case, with outcomes dependent on the extent of thrombosis, timing of recognition, and availability of alternative reconstructive options. Notably, cases involving both arterial and venous thrombosis demonstrated higher rates of flap necrosis, emphasising the critical importance of dual circulation compromise in determining final outcomes.

The case series (Table 3.3) examines the specific genetic variants and corresponding laboratory parameters in all 14 patients who experienced microvascular thrombosis. This analysis enables examination of whether certain combinations of genetic variants and phenotypic expressions may predispose to thrombotic events, despite the absence of statistically significant associations in the overall cohort analysis.

Table 3.3

Case series of patients with microvascular free flap thrombosis, determined SNVs and laboratory parameters (n, 14)

Flap	**Cause**	**Thrombosis venue**	**SNV and genotype**	**Laboratory parameters**
Scapular/ Parascapular	osteomyelitis	venous	rs6025 in *FV* C/T rs2066865 in *FGG* G/A	APCR 1.21 Fibrinogen 4.24 g/L
Medial plantar artery flap	osteomyelitis	arterial	rs2227589 in *SERPINC1* C/T rs1801133 in *MTHFR* G/A	AT 76 % Hmc 13.3 mkmol/l
LAF	trauma	venous	rs2066865 in *FGG* G/A	Fibrinogen 4.4 g/L
Scapular/ Parascapular	osteomyelitis	venous	rs1801133 in *MTHFR* G/A	Hmc 12.8 g/L
Scapular/ Parascapular	polytrauma	venous	rs2066865 in *FGG* G/A	Fibrinogen 4.7 g/L
Sartorius free flap	trauma	arterial and venous	rs2066865 in *FGG* G/A rs2227589 in *SERPINC1* C/T rs1801133 in *MTHFR* G/A	Fibrinogen 5.1.g/L AT 76 % Hmc 13.1mkmol/l
Osteocutaneous FF	trauma	arterial and venous	rs2227589 in *SERPINC1* C/T rs1801133 in *MTHFR* G/A	AT 81 % Hmc 14.1mkmol/l
Osteocutaneous FF	osteomyelitis	arterial and venous	rs2066865 in *FGG* G/A rs1801133 in *MTHFR* G/A	Fibrinogen 5.1 g/l Hmc 13.2 mkmol/l
Serratus anterior muscle flap	osteomyelitis	arterial and venous	rs2227589 in *SERPINC1* C/T	AT 111.8 %
RFF	malignancy	arterial	rs1801133 in *MTHFR* G/A	Hmc12.2. mkmol/l
LAF	trauma	venous	rs2066865 in *FGG* A/A rs1801133 in *MTHFR* G/A	Fibrinogen 4.5 g/L Hmc 14.4 mkmol/l
Scapular/ Parascapular	trauma	arterial and venous	rs1799963 in *FII* G/A rs2066865 in *FGG* G/A rs1801133 in *MTHFR G/A*	PT 81 % Fibrinogen 7.7 g/l Hmc 8.08 mkmol/l
ALT	trauma	venous	rs1801133 in MTHFR G/A	Fibrinogen 4.7 g/L APCR 1.68 AT 96.7 % Hmc 13.08 mkmol/l

Table 3.3 continued

Flap	Cause	Thrombosis venue	SNV and genotype	Laboratory parameters
SIEA; DIEP	trauma	venous	rs2227589 in *SERPINC1 C/T* rs1801133 in *MTHFR A/A*	AT 93.7 % Hmc 9.25 mkmol/l

SNV – single nucleotide variant; *FV* – Factor V gene; *FGG* – Fibrinogen Gamma Chain gene; *MTHFR* – methylene tetrahydrofolate reductase; F2 – Prothrombin gene; AT – antithrombin; APCR – activated protein C resistance; Hmc – Homocysteine; PT – prothrombin time; SIEA – Superficial inferior epigastric artery; DIEP – Deep inferior epigastric perforators; ALT – Anterolateral thigh flap; LAF – Lateral arm flap; FF – Fibula flap; RFF – Radial forearm flap; ns – nonsignificant.

The individual case analysis reveals several notable patterns among patients with thrombotic complications. All cases carried at least one genetic variant, with *MTHFR* rs1801133 being the most prevalent (10/14, 71.4 %), followed by *FGG* rs2066865 (7/14, 50 %) and *SERPINC1* rs2227589 (5/14, 35.7 %). Notably, only one patient with thrombotic complications carried the Factor V Leiden variant, and this patient also harboured the *FGG* variant with correspondingly elevated fibrinogen levels and reduced APCR values.

Laboratory abnormalities were consistently present in patients carrying corresponding genetic variants, with elevated fibrinogen levels observed in all *FGG* variant carriers and elevated homocysteine levels in most *MTHFR* variant carriers.

3.2 Genetic variant frequencies

The most prevalent genetic variants were *MTHFR,* rs1801133 (26.5 %), while Prothrombin G20210A (Factor II rs1799963) showed the lowest frequency (1.3 %), consistent with population studies (Table 3.4).

Table 3.4

Comparison of Genetic variant frequencies between study cohort and reference population

Gene, variant (legacy name)	Variant Allele Frequency in the large cohort* (n = 605)	Variant Allele Frequency in the study group, % (n = 155)	Frequency Fold change
Factor V, rs6025 (Factor V Leiden)	18/1210, 1.5 %	7/310, 2.1 %	1.33
Factor II, rs1799963 (Prothrombin G20210A)	15/1210, 1.2 %	4/310, 1.3 %	1.01
FGG, rs2066865	301/1210, 24.9 %	68/310, 22 %	0.93
SERPINC1, rs2227589	118/1210, 9.8 %	30/310, 9.7 %	1.01
MTHFR, rs1801133	356/1210, 29.4 %	82/310, 26.5 %	0.91

* Large cohort – Development of Latvian Population Genome Reference. European Recovery Fund funded project No 4.1.1.r.0/3/22/I/VM/001.

Comparison analysis showed very similar allele frequencies, with no statistically significant differences. This confirms the populations are comparable genetically, supporting the representativeness and validity of our study. Rare variants like Factor V Leiden and

G20210A had low frequencies, limiting power for rare variant analysis. Overall, the populations are genetically consistent, validating the broader applicability of our findings.

The frequency distribution of five thrombophilia-associated genetic variants was analysed according to thrombosis status in the study cohort (Table 3.5).

Table 3.5

Frequency of Genetic variants by Thrombosis status

Gene, variant (legacy name)	Variant allele frequency in thrombotic group, alleles/ % (n, 14)	Variant allele frequency in no-thrombosis group, alleles/ % (n, 141)	OR (CI, 95%)	*p*
Factor V, rs6025 (Factor V Leiden)	1/28, 3.6 %	6/282, 2.1 %	0.84 (0.05–15.60)	1
Factor II, rs1799963 (Prothrombin G20210A)	1/28, 3.6 %	3/282, 1.1 %	2.88 (0.26–32.30)	0.245
FGG, rs2066865	8/28, 28.6 %	60/282, 21.3 %	1.36 (0.67–2.76)	0.375
SERPINC1, rs2227589	5/28, 17.9 %	25/282, 8.9 %	1.46 (0.59–3.62)	0.448
MTHFR, rs1801133	11/28, 39.3 %	71/282, 25.2 %	1.28 (0.67–2.45)	0.5

FVL – Factor V Leiden; FII – prothrombin gene; FGG – Fibrinogen Gamma Chain gene; MTHFR – methylene tetrahydrofolate reductase. Odds ratios (OR) with 95% confidence intervals (CI) for five genetic variants. Statistical analysis was performed using Fisher's exact test, significance level $\alpha = 0.05$ (two tailed)

None of the examined genetic variants achieved statistical significance. The *p* ranged from 0.245 for rs1799963 to 1.000 for rs6025, indicating insufficient statistical power to detect associations with thrombosis outcome. The wide confidence intervals observed across all variants reflect the limited precision of effect estimates due to the relatively small sample size, particularly for rare variants such as Factor V Leiden and Prothrombin G20210A.

The genotype frequencies of examined variant were all in Hardy-Weinberg (HWE) equilibrium (Table 3.6) which describes a state where allele and genotype frequencies in a studied population remain constant.

Table 3.6

Genotype frequencies of analysed genetic variants in all study group

Gene, variant (legacy name)	Thrombosis Allele Frequency (p)	Thrombosis HWE (p)	No-thrombosis Allele Frequency (p)	No-thrombosis HWE (p)
FV, rs6025 (Factor V Leiden)	0.982	0.995	0.978	0.973
FII, rs1799963 (Prothrombin G20210A)	0.964	0.981	0.987	0.382

Table 3.6 continued

Gene, variant (legacy name)	Thrombosis Allele Frequency (p)	Thrombosis HWE (p)	No-thrombosis Allele Frequency (p)	No-thrombosis HWE (p)
FGG, rs2066865	0.732	0.808	0.788	0.993
SERPINC1, rs2227589	0.875	0.778	0.911	0.998
MTHFR, rs1801133	0.696	0.932	0.745	0.991

FVL – Factor V Leiden; FII – prothrombin gene; *FGG* – Fibrinogen Gamma Chain gene; *MTHFR* – methylene tetrahydrofolate reductase gene; HWE – Hardy-Weinberg equilibrium.

3.3 Association of genetic variants and coagulation parameters

The genotype-phenotype correlation analysis provides crucial insights into the biological significance of the identified genetic variants and their potential impact on haemostatic function (Table 3.7). The analysis encompassed all five targeted variants and their associated proteins: activated protein C resistance (APCR) for Factor V Leiden, prothrombin time (PT) for prothrombin G20210A, fibrinogen levels for *FGG* variant, antithrombin (AT) activity for *SERPINC1* variant, and homocysteine concentrations for *MTHFR* variant.

Table 3.7

Analysed SNVs and determined proteins in studied population (n, 155)

SNVs and genotype distribution in study group	Determined protein reference interval (min–max) M (±SD)	*p*
rs6025 *FV* (C>T) C/C (n= 148) C/T (n=7) T/T (n=0)	APCR (< 1.8) 2.02 (0.32) 1.19 (0.17) –	0.006
rs1799963 *F2* (G>A) G/G (n=152) G/A (n=2) A/A (n=1)	PT (70–130 %) 94.38 (20.34) 95.50 (20.51) 106.00	*ns*
rs2066865 *FGG* (G>A) G/G (n=92) G/A (n=54) A/A (n=9)	Fibrinogen (2–4 g/L) 4.08 (1.32)$^{\#}$ 4.64 (1.74)* 5.57 (1.81)$^{*\#}$	0.04* 0.004$^{\#}$
rs2227589 *SERPINC1* (C>T) C/C (n=126) C/T (n=28) T/T (n=1)	AT (75–125%) 89.57 (14.14) 97.89 (14.52) 92.10	*ns*
rs1801133 *MTHFR* (G>A) G/G (n=83) G/A (n=62) A/A (n=10)	Homocysteine (5–12 mkmol/L) 10.71 (4.17) 13.61 (5.44) 13.50 (5.8)	*ns*

Values are presented as mean, ±SD, sig. 2-tailed $p < 0.05$; SNV – single nucleotide variant; APCR – activated protein C resistance; FVL – Factor V Leiden; F2 – prothrombin gene; PT – prothrombin; *FGG* – Fibrinogen Gamma Chain gene; *MTHFR* – methylene tetrahydrofolate reductase gene; AT – antithrombin

The genotype-phenotype correlation analysis revealed significant associations between specific genetic variants and their corresponding protein functions. Most notably, Factor V Leiden carriers (C/T genotype) demonstrated significantly reduced APCR values (1.19 ± 0.17) compared to wild-type carriers (2.02 ± 0.32, p = 0.006), confirming the functional impact of this variant on protein C resistance as expected from established pathophysiology. The *FGG* rs2066865 variant showed a clear gene-dose effect, with fibrinogen levels progressively increasing from G/G carriers (4.08 ± 1.32 g/L) to G/A carriers (4.64 ± 1.74 g/L, p = 0.04) and A/A carriers (5.57 ± 1.81 g/L, p = 0.004 compared to G/G).

Conversely, the prothrombin G20210A, *SERPINC1*, and *MTHFR* variants did not demonstrate statistically significant associations with their respective protein products in this cohort, possibly due to small sample sizes for variant carriers or the influence of acute-phase reactions on protein levels.

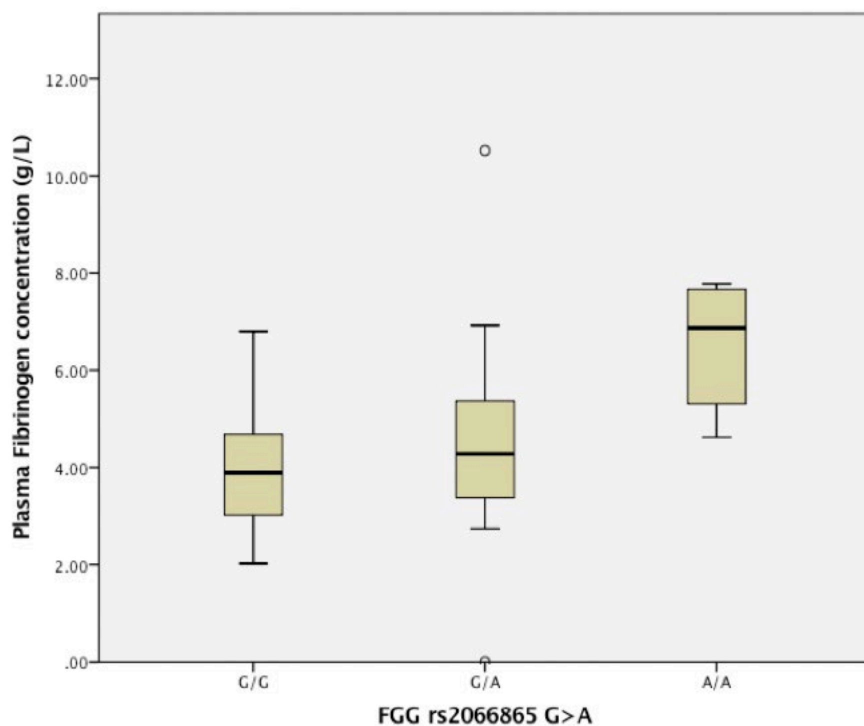


Figure 3.1. **Comparison of plasma fibrinogen concentration in patients with single nucleotide variant rs2066865 genotype in *FGG* gene**

Values are median [IQR range]

The single nucleotide variant rs2066865 in the FGG gene was significantly associated with plasma fibrinogen concentrations. Carriers of the A/A and G/A genotypes demonstrated elevated plasma fibrinogen levels compared to G/G carriers. Median plasma fibrinogen concentrations with interquartile ranges (IQR) were as follows: G/G genotype 3.91 g/L (IQR: 3.02–4.97), G/A genotype 4.51 g/L (IQR: 3.37–5.62), and A/A genotype 5.34 g/L (IQR: 4.60–7.07), as shown in Figure 3.1. Statistical analysis revealed significant differences

between A/A and G/A genotypes (p = 0.003) as well as between A/A and G/G genotypes (p = 0.001).

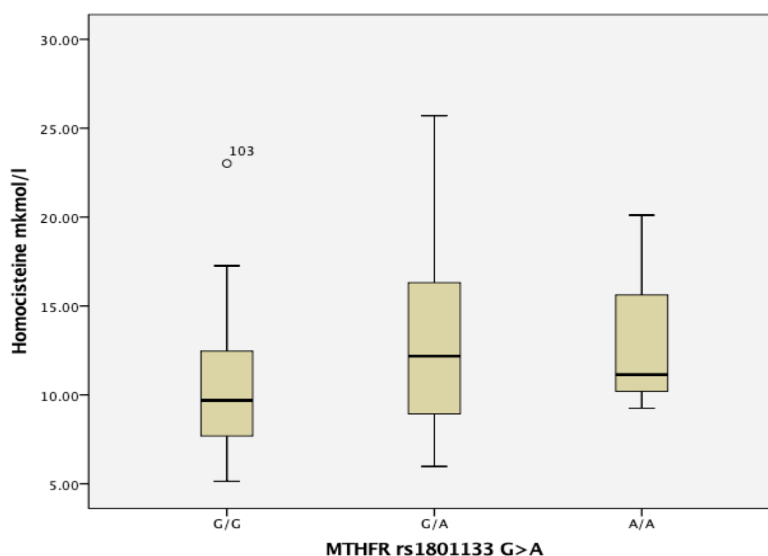


Figure 3.2. **Comparison of plasma homocysteine concentration in patients with single nucleotide variant rs1801133 genotypes in the *MTHFR* gene**

Values are median [IQR range]

The single nucleotide variant rs1801133 in the *MTHFR* gene was not significantly associated with plasma homocysteine concentrations when compared carriers of the G/G, A/A and G/A genotypes. In a total of 62 patients carriers of gene *MTHFR* G/A genotype were found, A/A genotype was found in ten patients. Median homocysteine concentration with interquartile ranges (IQR) were as follows: in patient carrying G/G genotype 9.69 mkmol/l (IQR: 7.11–12.28), G/A genotype 12.18 mkmol/l (IQR: 8.28–16.08), and A/A genotype 11.14 mkmol/l (IQR:10.76–15.37), as shown in in Figure 3.2. Statistical analysis revealed non-significant differences between G/G and G/A genotypes (p = 0.07) and did not show difference between G/A and A/A genotypes (p = 0.9).

In patients carrying genotype C/C and C/T for single nucleotide variant rs2227589 in *SERPINC1*, antithrombin activity in both groups did not differ, moreover in both groups antithrombin were in normal range. Median antithrombin activity with interquartile ranges (IQR) were as follows: in patient carrying C/C genotype 88.30 % (IQR: 77.95–98.65), C/T genotype 96.35 % (IQR: 82.15–110.55), as shown in Figure 3.3. Statistical analysis revealed non-significant differences between antithrombin activity in C/C and C/T genotype carriers (p = 0.09).

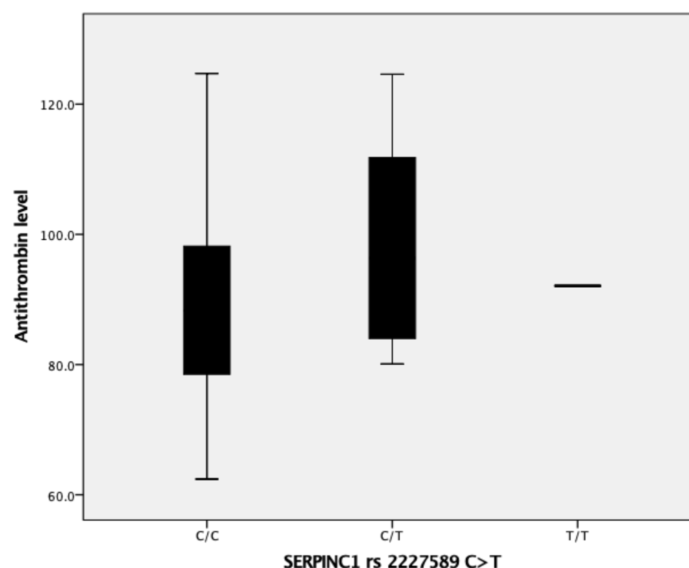


Figure 3.3. **Comparison of plasma antithrombin concentration in patients with single nucleotide variant rs2227589 genotypes in *SERPINC1***

Values are median [IQR range]

To detect differences in perioperative coagulation parameters, patients were stratified according to the presence or absence of flap thrombosis (Table 3.8).

Table 3.8

Baseline Coagulation Parameters According to Thrombosis Status

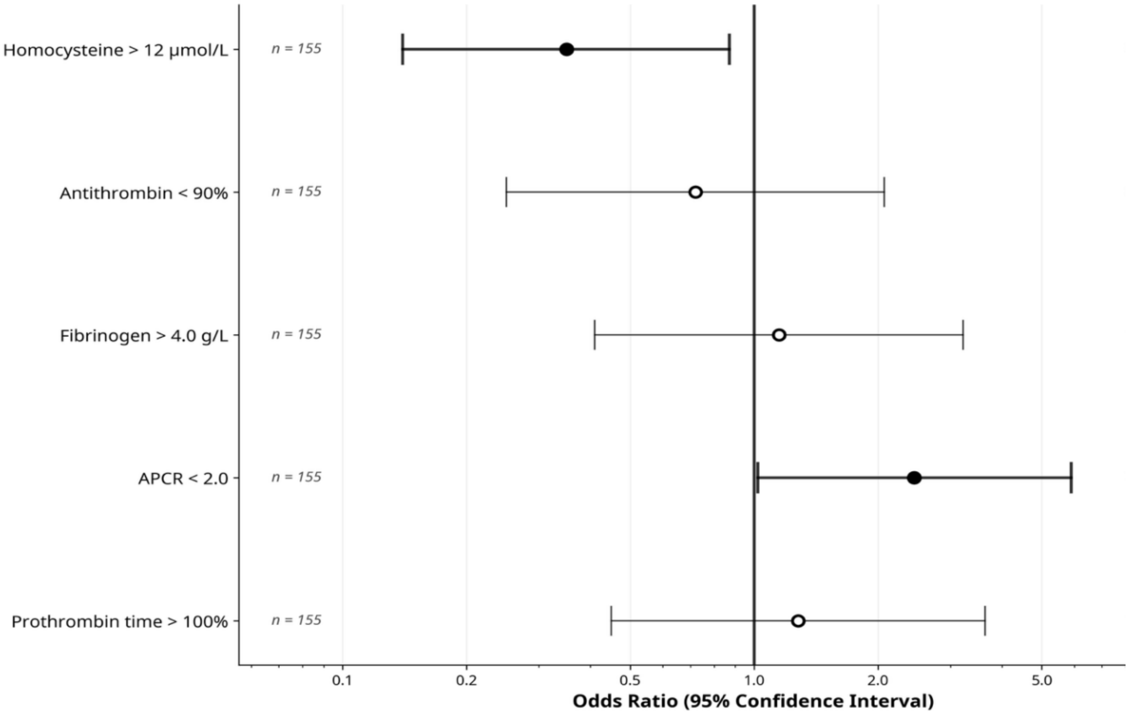
Variable	No-Thrombosis group n, 141	Thrombosis group n, 14	*p*
PT, %	97.0 (85.0–109.0)	102.5 (89.3–116.8)	0.26
APCR	2.01 (1.75–2.22)	1.83 (1.69–1.9)	0.043
Fibrinogen, g/L	4.08 (3.18–5.32)	4.28 (3.78–4.78)	0.60
AT	89.1 (79.9–97.4)	96.2 (85.4–111.8)	0.231
Hmc, mkmol/L	11.1 (8.4–14.6)	7.9 (7.1–8.4)	0.0029

Data are presented as median (interquartile range). P were calculated using the Mann-Whitney U test. $p < 0.05$ was considered statistically significant. PT – prothrombin time, APCR – activated protein C resistance, AT – antithrombin, Hmc – homocysteine

Among analysed coagulation parameters, only two coagulation parameters showed statistically significant differences between patients with and without thrombosis. Activated protein C resistance (APCR) was significantly lower in patients who developed thrombosis compared to those who did not (1.83 vs 2.01, $p = 0.043$).

Conversely, homocysteine levels were unexpectedly lower in patients with thrombosis (7.9 vs 11.1 μmol/L, $p = 0.029$), which contradicts the established association between hyperhomocysteinemia and thrombotic risk. However, this finding should be interpreted with caution due to the small sample size in the thrombosis group for homocysteine measurements. No significant differences were observed in prothrombin time, fibrinogen concentration, or antithrombin activity between the groups.

To evaluate the association between coagulation parameters and free flap thrombosis risk, analysis of baseline haemostatic markers between patients who developed thrombotic complications and those with successful outcomes was performed (Figure 3.4).



Parameter	OR (95% CI)	P-value	Sig.
Prothrombin time>100%	1.28 (0.45-3.64)	0.260	ns
APCR<2.0	**2.45 (1.02-5.89)**	**0.043**	*
Fibrinogen>4.0 g/L	1.15 (0.41-3.22)	0.600	ns
Antithrombin<90%	0.72 (0.25-2.07)	0.231	ns
Homocysteine>12 μmol/L	**0.35 (0.14-0.87)**	**0.029**	*

Figure 3.4. **Forest plot of coagulation parameters associated with free flap thrombosis**

Data points represent odds ratios with 95 % confidence intervals for coagulation parameters. Filled circles indicate statistically significant associations ($p < 0.05$), while hollow circles represent non-significant findings ($p \geq 0.05$). The vertical line at OR = 1.0 represents no effect. APCR < 2.0 was significantly associated with increased thrombosis risk (OR = 2.45, 95 % CI: 1.02–5.89, p = 0.043). Homocysteine > 12 μmol/L showed an unexpected protective association (OR = 0.35, 95 % CI: 0.14–0.87, p = 0.029). Sample size: n = 155 patients with 14 thrombotic events.

Five parameters were evaluated using clinically relevant cut-off values: prothrombin time > 100 %, activated protein C resistance (APCR) < 2.0, fibrinogen > 4.0 g/L, antithrombin < 90 %, and homocysteine > 12 μmol/L.

Forest plot analysis revealed distinct patterns in haemostatic biomarker associations with thrombotic outcomes. Among the analysed parameters, two demonstrated statistically significant associations with thrombosis risk, though with contrasting effects.

Activated Protein C Resistance (APCR) emerged as the most clinically relevant biomarker. Patients with APCR values below 2.0 demonstrated a significantly increased risk of

thrombosis compared to those with normal APCR levels (OR = 2.45, 95 % CI: 1.02–5.89, p = 0.043).

Homocysteine levels showed an unexpected significant association, with elevated levels (> 12 μmol/L) paradoxically associated with reduced thrombosis risk (OR = 0.35, 95 % CI: 0.14–0.87, p = 0.029). The small sample size in the thrombosis group and potential confounding factors may contribute to this paradoxical result.

Traditional coagulation parameters including prothrombin time > 100 % (OR = 1.28, 95 % CI: 0.45–3.64, p = 0.260), fibrinogen > 4.0 g/L (OR = 1.15, 95 % CI: 0.41–3.22, p = 0.600), and antithrombin < 90 % (OR = 0.72, 95 % CI: 0.25–2.07, p = 0.231) showed no significant associations with thrombosis risk, suggesting that routine coagulation screening may have limited predictive value in this clinical context.

3.4 Combined risk factor analysis

Three distinct logistic regression models were developed to assess the predictive power of different sets of factors. Logistic regression model for genetic predictors only for defined polymorphisms *FV* rs6025, *FII* rs1799963, *FGG* rs2066865, *SERPINC1* rs2227589, *MTHFR* rs1801133 showed the Pseudo R-squared of 0.02327 was very low, suggesting that genetic variants alone explain a very small proportion of the variance in flap thrombosis (Figure 3.5).

None of the individual genetic variants (*FV* rs6025, *FII* rs1799963, *FGG* rs2066865, *SERPINC1* rs2227589, *MTHFR* rs1801133) show statistically significant association with flap thrombosis (all p-values > 0.05). This suggests that these genetic factors alone are not strong predictors of flap thrombosis in the studied group.

Logistic regression model for coagulation predictors only showed pseudo-R-squared of 0.05983, while still modest, was higher than that of the genetic-only model, suggesting that these coagulation factors explain a slightly larger proportion of the variance in flap thrombosis (AUC = 0.65).

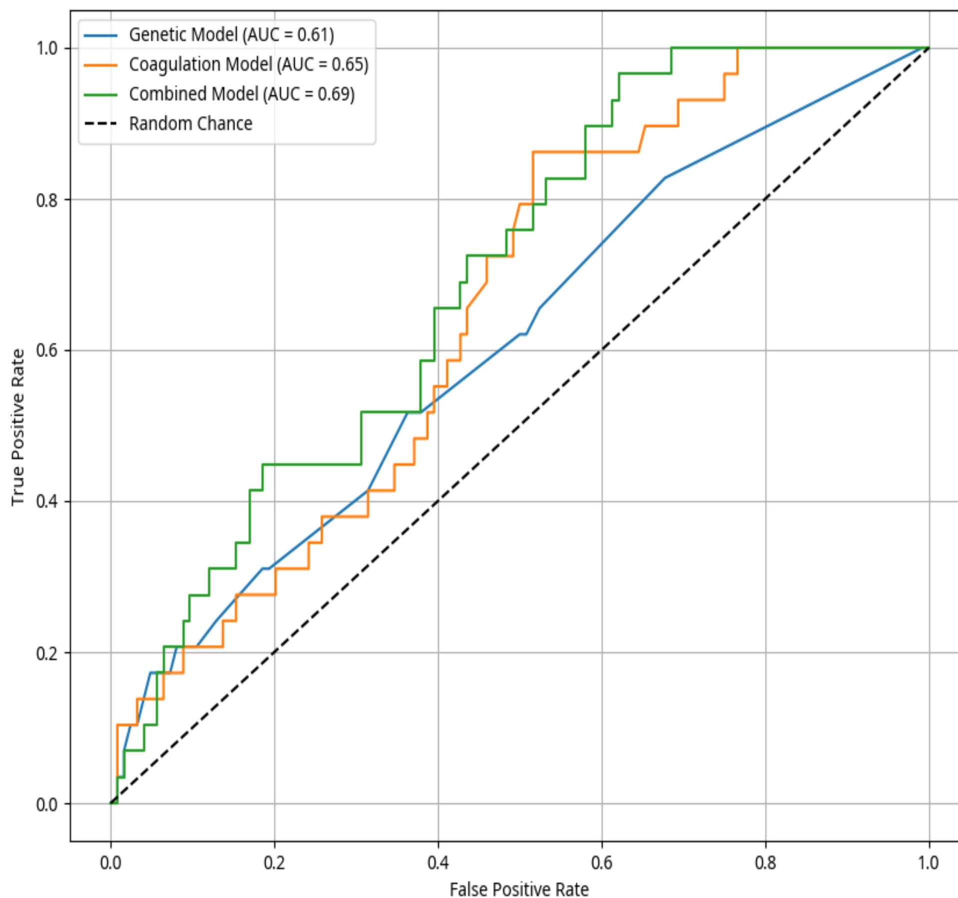


Figure 3.5. **ROC Curves for Logistic regression models for three categories of predictors**

Genetic Model. An AUC of 0.61 indicates that the genetic-only model has very poor discriminatory power, only slightly better than random chance (AUC = 0.50).

Coagulation Model. An AUC = 0.65. The coagulation-only model shows slightly better discriminatory power indicating a modest ability to distinguish between patients with and without flap thrombosis.

Combined Model. The combined model has the highest AUC at 0.69, suggesting it is the best performing model among the three, but its discriminatory power is still considered weak to moderate. This means that while it incorporates more information, its overall ability to predict flap thrombosis is limited.

When combined model with confounders the Pseudo R-squared of 0.08160 was the highest among the three models (AUC = 0.69), suggesting that including all genetic, coagulation, and confounding factors explains a slightly larger, but still limited, proportion of the variance in flap thrombosis. In this combined model, none of the genetic variants, coagulation factors, or confounding factors show a statistically significant association with flap thrombosis (all p-values > 0.05). The previously borderline significant Homocysteine ($p = 0.075$) and APCR ($p = 0.071$) were no longer below the 0.05 threshold, although they remained close. This suggests that when controlling for other factors, their individual predictive power diminishes below the conventional significance level.

The forest plot (Figure 3.6) presents the results of regression analysis examining the association between genetic polymorphisms and thrombotic complications in patients undergoing microvascular free flap surgery.

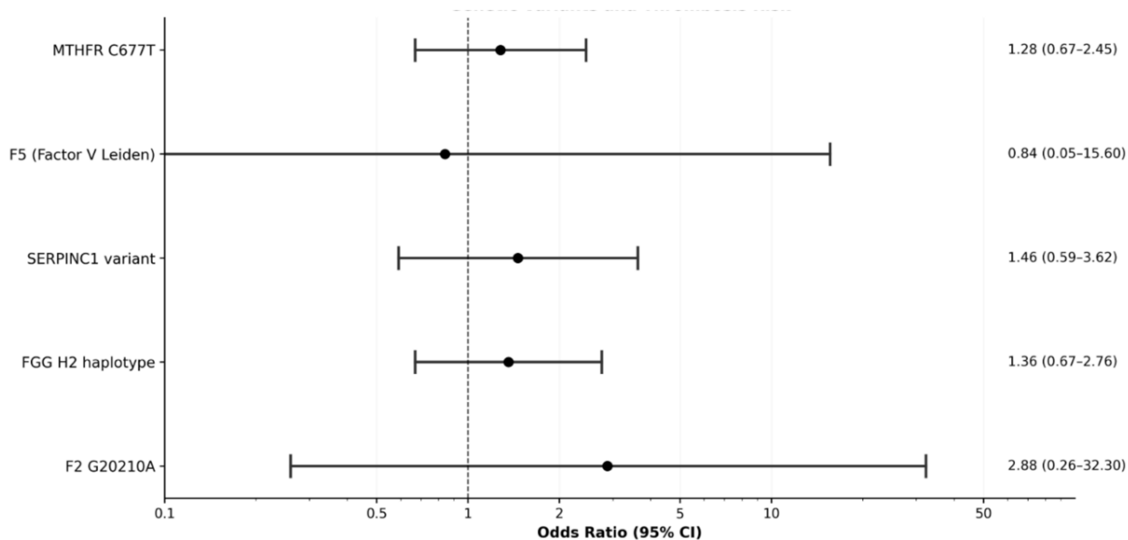


Figure 3.6. **Genetic variants associated with thrombosis risk: Forest Plot Analysis**

Odds ratios (OR) with 95% confidence intervals (CI) for five genetic variants. Point estimates (squares) and confidence intervals (horizontal lines) are shown. Vertical dashed line indicates OR = 1.0. No associations reached statistical significance (all p > 0.05). Statistical analysis was performed using Fisher's exact test for categorical variables. Odds ratios and 95 % confidence intervals were calculated using logistic regression.

F2 – prothrombin gene; *FGG* – Fibrinogen Gamma Chain gene; *MTHFR* – methylene tetrahydrofolate reductase gene; *SERPINC1* – serine protease inhibitor 1 (antithrombin) gene.

Factor V Leiden (G1691A) showed OR of 0.84 (95 % CI: 0.05–15.60, p = 0.762). Only one thrombotic event occurred among 7 carriers, though the wide confidence interval reflects the small sample size and lack of statistical power.

F2 G20210A (rs1799963) was present in 1/14 cases versus 2/141 controls (OR 2.88, 95 % CI 0.26–32.30, p=0.245) and showed the strongest association with flap thrombosis risk among all five variants represented here however as with the Factor V Leiden variant the wide confidence interval reflects the small sample size and lack of statistical power.

FGG gene rs2066865 emerged as the mild genetic predictor of thrombotic risk (OR = 1.36, 95 % CI: 0.67–2.76, p = 0.375). Despite not reaching statistical significance, the relatively narrow confidence interval compared to other genetic variants and the highest event rate suggests this may represent a clinically meaningful association worthy of further investigation.

SERPINC1 rs2227589 showed minimal association with thrombotic risk (OR = 1.46, 95 % CI: 0.59–3.62, p = 0.449).

The common *MTHFR* variant demonstrated a modest, non-significant increase in thrombotic risk (OR = 1.28, 95 % CI: 0.67–2.45, p = 0.500).

The results of both univariate and multivariate Cox proportional hazards regression analysis examining risk factors for thrombotic complications in microvascular free flap surgery is showed in Table 3.9. The analysis evaluating each variable independently (univariate

analysis) and then simultaneously adjusting for potential confounders in a multivariate model that included age, smoking status, and all genetic variants.

Table 3.9

Cox regression analysis: hazard ratios for thrombotic complications

Variable	Univariate HR (95% CI)	*p*	Multivariate HR (95% CI)	*p*
Factor V Leiden (C/T vs C/C)	0.74 (0.10–5.54)	0.743	0.68 (0.09–5.21)	0.697
Prothrombin G20210A (variant)	1.55 (0.21–11.45)	0.655	1.42 (0.19–10.67)	0.728
FGG rs2066865 (variant)	1.85 (0.65–5.28)	0.248	1.92 (0.66–5.59)	0.232
SERPINC1 rs2227589 (variant)	1.15 (0.38–3.45)	0.805	1.08 (0.35–3.29)	0.897
MTHFR rs1801133 (variant)	1.25 (0.45–3.47)	0.535	1.18 (0.42–3.31)	0.751
≥ 2 genetic variants	2.10 (0.75–5.89)	0.155	1.95 (0.68–5.56)	0.208
Age > 50 years	1.58 (0.56–4.45)	0.385	1.44 (0.50–4.13)	0.496
Smoking (current)	1.24 (0.45–3.42)	0.678	1.31 (0.47–3.66)	0.608

Univariate and multivariate Cox proportional hazards regression analysis for thrombotic complications. HR = hazard ratio; CI = confidence interval. Multivariate model adjusted for age, smoking status, and all genetic variants. No variables achieved statistical significance at α = 0.05 level.

Age greater than 50 years patients showed an elevated thrombotic risk (HR = 1.58, 95 % CI: 0.56–4.45, p = 0.386), with 8 events among 67 patients over 50 (11.9 % event rate). While not statistically significant, this represents the expected age-related increase in thrombotic risk.

Active smoking demonstrated a modest increase in thrombotic risk (HR = 1.24, 95 % CI: 0.45–3.42, p = 0.675), with 3 events among 29 current smokers (10.3 % event rate).

No variables achieved statistical significance at the conventional α = 0.05 level. This reflects several important limitations: the relatively low overall event rate (9.0 %), small sample sizes for individual genetic variants, and the inherent challenge of detecting modest genetic effects in clinical populations.

However, several clinically meaningful patterns emerge. The FGG variant and cumulative genetic burden (≥ 2 variants) showed the most consistent associations across both analytical approaches, with hazard ratios approaching 2.0 and *p* approaching statistical significance. These findings suggest potential clinical relevance that may become apparent with larger sample sizes or in higher-risk patient populations.

3.5 Survival analysis results: Timing and risk factors for flap thrombosis

To evaluate the timing and frequency of post-operative thrombotic complications assessment using time-to-event analysis were performed. Figure 3.7 illustrates the thrombosis-free survival probability over the first 10 days following surgery.

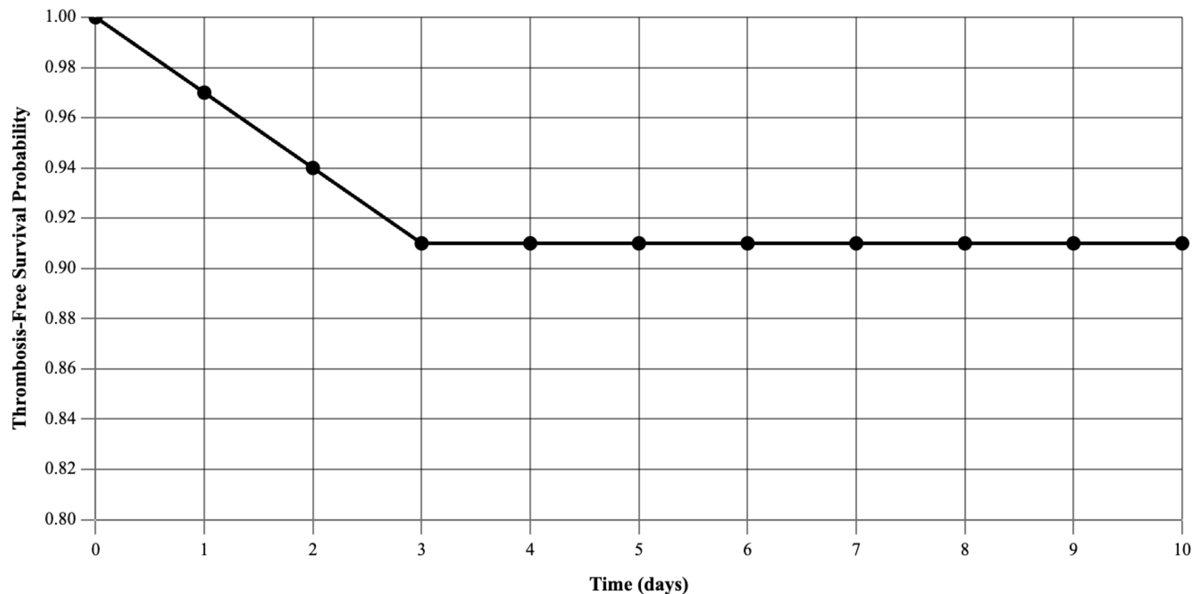


Figure 3.7. **Kaplan-Meier survival curve for thrombosis-free survival by time**

The curve demonstrates a 91% thrombosis-free survival rate with 14 thrombotic events (9.0%) occurring predominantly within the first 72 hours post-surgery. Median survival was not reached due to low event rate.

Thrombosis-free survival analysis revealed a characteristic biphasic pattern with distinct early and late phases. The survival curve demonstrated a steep decline during the initial 3-day post-operative period, with thrombosis-free survival probability decreasing from 100 % at baseline to approximately 97 % at day 1,94 % at day 2, and stabilising at 91 % by day 3. Following this critical early period, the survival curve plateaued, with no additional thrombotic events observed through day 10 of follow-up. This pattern indicates that the majority of thrombotic complications occurred within the immediate post-operative window, after which patients who remained event-free maintained stable thrombosis-free status. The final thrombosis-free survival probability of 91 % represents the proportion of patients who avoided thrombotic complications throughout the 10-day observation period.

To evaluate the impact of cumulative genetic variants on the risk of thrombosis, we analysed thrombosis-free survival over a 10-day period using Kaplan-Meier curves (Figure 3.8). The analysis revealed a stepwise decrease in survival probability with an increasing number of thrombophilic genetic variants.

Specifically, the group with zero variants maintained the highest thrombosis-free survival rate, which stabilised at approximately 87 % after day 3. In contrast, the group with

one variant showed a lower survival probability, plateauing at around 82 %. This trend continued with the groups having two and three or more variants, which exhibited progressively lower survival rates. Although there was a clear trend in the data suggesting that a higher genetic burden is associated with a lower likelihood of remaining thrombosis-free, the result of the log-rank test for trend did not reach statistical significance ($\chi^2 = 2.95$, $p = 0.086$).

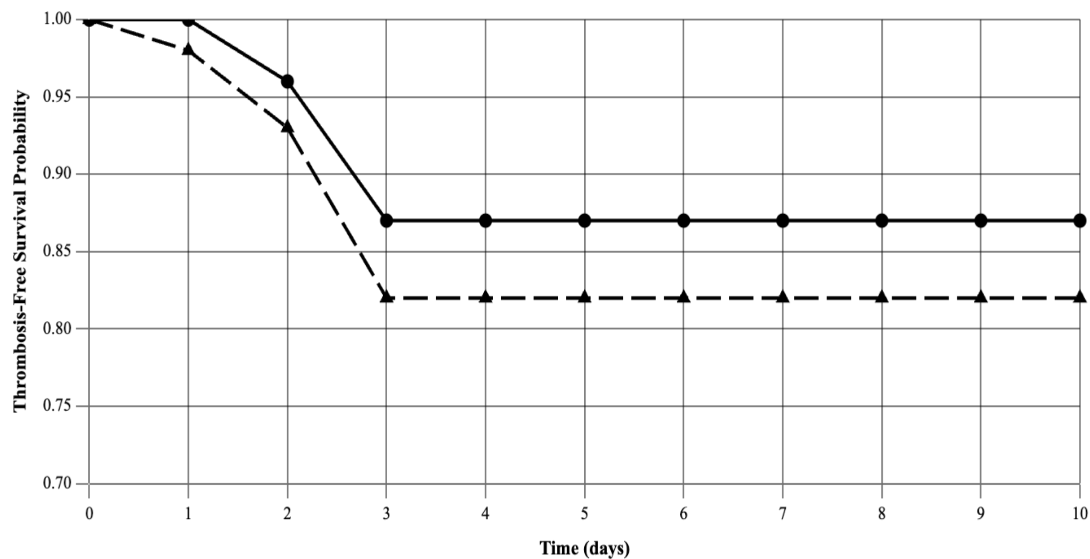


Figure 3.8. **Kaplan-Meier survival curve for thrombosis-free survival by time and cumulative genetic variant**

Kaplan-Meier survival curves examining the relationship between cumulative genetic variant burden and thrombosis-free survival in patients undergoing microvascular free flap surgery. Showing whether the presence of multiple thrombophilic variants creates an additive or synergistic effect on thrombotic risk, beyond what might be expected from individual variants alone. Survival curves stratified by number of thrombophilic genetic variants. 0 variants (n = 23, solid line), 1 variant (n = 45, long dashed). Progressive decrease in thrombosis-free survival observed with increasing genetic variant burden.

This near-significant result is particularly noteworthy given the study's sample size limitations and suggests biological effect that might reach statistical significance with larger patient cohorts. The similarity between the two-variant and ≥ 3-variant groups suggests that the genetic risk relationship may not be strictly linear, potentially indicating either: a ceiling effect where additional variants beyond two provide diminishing incremental risk, statistical power limitations preventing detection of further differences or biological interaction effects that modify simple additive genetic models.

The Kaplan-Meier curves for male and female patients showed some differences in the probability of flap thrombosis-free survival. However, the log-rank test (p-value = 0.1685) indicated that this difference was not statistically significant, suggesting that gender alone may not be a strong predictor of the timing of flap thrombosis in this cohort.

Patients with a history of thrombosis showed a trend towards lower thrombosis-free survival compared to those without a history. The log-rank test (p-value = 0.2151) did not reach

statistical significance, but the visual trend suggests that a history of thrombosis might be an important factor influencing the timing of future thrombotic events.

The curves for patients who received thromboprophylaxis versus those who did not also showed some visual differences, but the log-rank test did not indicate a statistically significant difference in survival times.

4 Discussion

Microvascular surgery, particularly in free tissue transfer, requires precise vascular anastomosis to re-establish circulation in small-calibre vessels. The viability of transferred tissue critically depends on adequate perfusion for oxygen delivery and metabolic requirements. While technical factors contribute significantly to surgical success, thrombotic complications remain a principal concern in microvascular procedures.

The present study represents comprehensive investigations examining the relationship between genetic polymorphisms affecting haemostatic function and thrombotic complications in microvascular free flap surgery. These findings provide important insights into the complex pathophysiology underlying flap failure while simultaneously challenging several established assumptions regarding genetic thrombophilia in surgical contexts.

The pathogenesis of thrombotic events in microvascular surgery appears to be multifactorial, with genetic factors potentially playing a substantial role. Polymorphisms in genes regulating haemostatic pathways including coagulation cascades, platelet function, and fibrinolytic systems may predispose certain individuals to thrombotic complications. Elucidating these genetic influences could enhance perioperative risk assessment and facilitate the development of individualised therapeutic approaches in microvascular surgery.

In the study, evaluation of the complex interplay between genetic predisposition, acquired thrombotic risk parameters, and thrombotic complications in microvascular free flap surgery were the main goal. The study population demonstrated demographic characteristics consistent with typical reconstructive surgery patients, with a predominance of male patients (76.1 %) and a mean age of 45.1 years.

4.1 Principal findings and clinical implications

The observed flap thrombosis rate of 9 % in our cohort aligns with previously published literature reporting complications ranging from 2–9 % (Bowman, 2011; Friedman, 2010), validating the representativeness of our study population. However, the overall flap success rate of 94.84 % demonstrates excellent clinical outcomes consistent with contemporary microsurgical practice standards. This success rate, while impressive, underscores the continued clinical significance of thrombotic complications, as even a small percentage of failures can result in devastating functional and aesthetic consequences for patients.

Contrary to our initial hypothesis, individual genetic polymorphisms demonstrated no statistically significant association with flap thrombosis risk. This finding challenges the direct extrapolation of genetic thrombophilia research from general populations to the specific context of microvascular surgery. The absence of significant associations between Factor V Leiden

(rs6025), Prothrombin G20210A (rs1799963), and other examined genetic variants suggests that the pathophysiological mechanisms underlying spontaneous venous thromboembolism may differ substantially from those governing microvascular anastomotic thrombosis. The multifactorial nature of thrombotic events in microsurgery necessitates a comprehensive analysis of both inherited and acquired risk factors.

4.2 Genetic variants

The prevalence of genetic variants in our study population generally corresponded to established population frequencies, with *MTHFR* rs1801133 showing the highest frequency (26.5 %) and Prothrombin G20210A the lowest (1.3 %). These distributions are consistent with European population studies (Rosendaal, 1995; Kujovich, 2011), confirming appropriate population sampling and validating our cohort as representative of the broader surgical population rather than a highly selected thrombophilic group. The variant allele frequencies demonstrated excellent concordance with the large Latvian population reference cohort (n = 605), with fold changes ranging from 0.91–1.33 across all examined loci, confirming the absence of significant selection bias in patient recruitment.

4.2.1 rs6025 in FV gene

Factor V Leiden remains the most prevalent inherited thrombophilia in Caucasian populations, with heterozygote frequencies of 3–7 % in Northern European populations and near absence in African and Asian populations (Rees, 1995; Ridker, 1997). The variant arose from a single founder mutation approximately 21 000–24 000 years ago, with its persistence suggesting potential evolutionary advantages, possibly related to improved haemostasis during childbirth or trauma (Lindqvist, 2001; Zivelin, 2006). The mechanistic basis of Factor V Leiden thrombophilia has been extensively characterised: the Arg506Gln substitution eliminates one of three activated protein C cleavage sites in factor Va, resulting in approximately 10-fold prolonged cofactor activity and sustained thrombin generation (Segers, 2007; Castoldi, 2010).

The literature demonstrates that aPCR is detectable in 20 % of venous thromboembolism (VTE) patients and in greater than 50 % of selected thrombophilic families (Rees, 1995; Rosendaal, 1995). The homozygous T/T genotype has been reported to occur in 1.5 % of the general population (Rees, 1995). This population frequency explains the absence of T/T genotype carriers of the *FV* gene at the analysed single nucleotide variant (SNV) locus in our study population. Despite extensive literature supporting 3–7-fold increased thrombotic risk in carriers (Kujovich, 2011), our study revealed no significant association with flap thrombosis (OR = 0.84, p = 0.762).

This finding may reflect several important considerations. First, the relatively small number of carriers (n = 7, 2.1 %) certainly limited our ability to detect modest effects, particularly given the low baseline thrombosis rate (9 %). Post-hoc power calculations indicate that detecting a 3-fold increased risk with 7 carriers and 9 % baseline thrombosis rate would require sample sizes exceeding 800 patients. Second, the haemodynamic environment of microvascular anastomoses, characterised by low flow states, small vessel calibres (1–3 mm), and significant surgical trauma, may create thrombotic conditions fundamentally different from spontaneous venous thromboembolism (Khouri, 1998).

Previous research has indicated that approximately 5 % of APCR cases arise through alternative mechanisms unrelated to Factor V Leiden (Segers, 2007), including elevated factor VIII levels, lupus anticoagulants, pregnancy, and oral contraceptive use (Castoldi, 2010). This phenomenon was exemplified in our cohort by a single patient exhibiting APCR (ratio 1.21) with Factor V Leiden heterozygosity who underwent two microvascular flap procedures within a two-year period, both resulting in thrombosis. This case illustrates the clinical relevance of functional APCR measurement beyond genetic testing, as the phenotypic expression of resistance influenced by both genetic and acquired factors may better predict thrombotic risk than genotype alone (Vos, 2006).

The absence of demonstrable Factor V Leiden association with microsurgical thrombosis aligns with limited prior literature in reconstructive surgery. While Factor V Leiden has been implicated in isolated case reports of flap failure, systematic investigations have failed to establish consistent associations (Khansa, 2011; Biban, 2019).

4.2.2 rs1799963 variant in the Prothrombin gene

The prothrombin G20210A variant represents the second most common inherited thrombophilia in European populations, occurring with frequencies of 1–3 % and conferring approximately 2–3-fold increased venous thrombosis risk (Simone, 2013; Bertina, 1998). Unlike Factor V Leiden, which affects protein function, the G20210A variant resides in the 3’ untranslated region of the prothrombin gene, resulting in elevated mRNA stability and increased protein synthesis without altering prothrombin’s enzymatic properties (Poort, 1996; Jadaon, 2011). Heterozygous carriers typically exhibit prothrombin levels 30 % above population means, though considerable overlap with normal ranges complicates phenotypic identification (Poort, 1996).

In our study cohort, one patient carrying variant in the prothrombin gene experienced free flap thrombosis, yielding a non-significant odds ratio of 2.88 (95 % CI: 0.26–32.30, p = 0.245).While this align with data conferring that patients carrying prothrombin gene variant

are with 2–3-fold increased risk for thrombosis in population studies (Simone, 2013) the extremely wide confidence interval reflects profound statistical uncertainty attributable to the variant's rarity. The variant frequency of 1.3 % in our population was consistent with typically reported (1–3 %), confirming appropriate population sampling with no selection bias.

Prothrombin plasma levels in patients carrying rs1799963 remained within normal reference intervals in our cohort. This observation merits discussion, as the variant's prothrombotic effect operates through subtle prothrombin elevation rather than frank deficiency or excess. Previous research established that prothrombin levels exceeding 95.19 % increase thrombotic risk by a factor of 2.34, while levels above 1.15 U/mL confer 2.1-fold increased VTE risk compared to levels below 0.95 U/mL (Poort, 1996). The threshold effect inherent in these observations suggests that G20210A carriers near the lower end of the elevated distribution may exhibit minimal phenotypic penetrance, potentially explaining the absence of clear associations in small studies.

The clinical significance of prothrombin G20210A in microsurgical populations remains controversial. The rarity of the variant in most populations, combined with modest effect sizes, renders adequately powered clinical trials impractical for this specific question.

4.2.3 rs2066865 in Fibrinogen Gamma chain gene

The association between FGG rs2066865 and elevated plasma fibrinogen concentrations represents the most significant genetic finding of this study. Carriers of the A/A genotype demonstrated markedly elevated fibrinogen levels (5.57 ± 1.81 g/L) compared to G/G carriers (4.08 ± 1.32 g/L), with statistically significant gene-dose effects ($p = 0.001$ for A/A vs. G/G; $p = 0.04$ for G/A vs. G/G). This strong genotype-phenotype correlation validates the functional relevance of rs2066865 as a quantitative trait locus modulating fibrinogen expression, consistent with previous reports from the Leiden Thrombophilia Study and subsequent investigations (de Willige, 2005; Grünbacher, 2007; El-Galaly, 2013).

Earlier investigations by Rosendaal established significant correlations between advancing age, elevated fibrinogen levels, and increased thrombotic events, particularly within the venous system (Rosendaal, 2009). Fibrinogen, as both an essential coagulation factor and acute-phase reactant, occupies a unique position in surgical haemostasis. Baseline genetic predisposition to elevated levels may be amplified during inflammatory states, trauma, and malignancy, creating a prothrombotic milieu that compounds surgical risk (van Hylckama Vlieg, 2003; Schlimp, 2016). Fibrinogen also contributes to blood viscosity a rheological property particularly relevant in low-flow microvascular systems where elevated viscosity may impair perfusion (Eber, 1993).

In this study population, despite subjects with homozygous A/A genotype exhibiting both higher mean age and elevated plasma fibrinogen concentrations, this did not translate to a statistically significant increase in flap thrombosis incidence (OR = 1.36, p = 0.375). The significant correlation between genetic variants and phenotypic expression suggests that FGG rs2066865 may serve as a biomarker for identifying patients at risk for hypercoagulable states. However, the absence of direct correlation between elevated fibrinogen levels and flap thrombosis in our regression analysis indicates that genetic predisposition to elevated fibrinogen may be necessary but not sufficient for thrombotic complications. This suggests that additional factors whether technical, mechanical, or patient-specific must converge to precipitate clinical thrombosis (Khouri, 1998; Pattani, 2010).

4.2.4 rs2227589 in *SERPINC1* gene

Antithrombin deficiency, first described by Egeberg in 1965, represents one of the earliest recognised inherited thrombophilias and among the most clinically significant, conferring 10–20-fold increased thrombosis risk substantially higher than Factor V Leiden or prothrombin G20210A (Zöller, 1999; Patnaik, 2008). This serpin superfamily member serves as the principal physiological inhibitor of multiple coagulation proteases, including thrombin, factors Xa, IXa, and XIa, with activity dramatically enhanced (up to 1000-fold) by heparin and endogenous heparan sulphate (Pike, 2005). The critical importance of antithrombin is underscored by embryonic lethality in homozygous-deficient mice, suggesting that complete deficiency is incompatible with mammalian life (Zöller, 1999).

Inherited antithrombin deficiency follows an autosomal dominant pattern with variable penetrance, classified into type I (quantitative deficiency, 10–15 % of cases) and type II (qualitative defects, subdivided into reactive site, heparin-binding site, and pleiotropic effect variants) (Kumar, 2015; Perry, 1996). The rs2227589 variant in *SERPINC1* (c.41+141G>A) represents a relatively common polymorphism whose functional significance and thrombotic risk association remain incompletely characterised, with conflicting evidence from population studies (Jiang, 2017).

Previous research has demonstrated that reduced antithrombin concentrations do not invariably result in thrombotic events. A familial study of inherited AT deficiency revealed multiple pedigrees exhibiting low AT plasma levels without significant thrombotic history (van der Meer, 1973). In studied population no significant differences in plasma AT levels were observed between patients carrying either heterozygous or homozygous variants of the rs2227589 in the *SERPINC1* gene, regardless of free flap thrombosis status.

In our studied population, no significant differences in plasma antithrombin activity were observed between patients carrying either heterozygous or homozygous variants of rs2227589, regardless of free flap thrombosis status. All measured antithrombin levels remained within normal reference ranges (75–125 %), providing no phenotypic evidence that this particular variant substantially impairs antithrombin function.

Notably, two out of five patients presenting with heterozygous genotype and subsequent free flap thrombosis were under 50 years of age. Data indicate that initial thrombotic events typically manifest by age 30, with approximately 50 % of AT-deficient patients experiencing a thrombotic episode, predominantly deep vein thrombosis by age of 50 (Patnaik, 2008; Hart, 2022).

4.2.5 rs1801133 in the MTHFR gene

The *MTHFR* C677T variant demonstrated the highest prevalence in our cohort (26.5 %), consistent with European population frequencies where homozygote rates range from 10–14 % (Wilcken, 1996; Botto, 2000). This variant results in an alanine-to-valine substitution at position 677 of methylenetetrahydrofolate reductase, the enzyme catalysing conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate – the methyl donor for homocysteine remethylation to methionine (Goyette, 1998). Homozygous variant carriers exhibit approximately 70 % reduction in enzyme activity, with heterozygotes showing intermediate reduction (Rozen, 1997).

The primary biochemical consequence of C677T homozygosity is mild to moderate hyperhomocysteinemia, particularly in individuals with suboptimal folate status (Klerk, 2002). Homocysteine, a sulphur-containing amino acid intermediate in methionine metabolism, has been implicated in both arterial and venous thrombotic disorders through multiple proposed mechanisms including endothelial dysfunction, oxidative stress, impaired nitric oxide bioavailability, and enhanced platelet reactivity (Welsch, 1997; Liew, 2015). However, the causal relationship between hyperhomocysteinemia and thrombosis remains controversial, with meta-analyses yielding conflicting conclusions (Ray, 2002; Clarke, 2012).

Among all patients with free flap thrombosis, ten were identified as carriers of the *MTHFR* gene rs1801133 G/A genotype and more than a half (n=8) exhibited elevated serum homocysteine levels (> 12 µmol/L). Three patients presented with isolated *MTHFR* gene variants, with one experiencing arterial free flap thrombosis and the other two developing venous thrombosis. This distribution involving both arterial and venous circulations aligns with literature suggesting that hyperhomocysteinemia, unlike classical venous thrombophilias, may predispose to thrombosis in both vascular beds (Falcon, 1994; Pathare, 2004).

The clinical implications of *MTHFR* C677T for microsurgical practice remain uncertain. Given the variant's high population prevalence (approximately one-quarter of Europeans carry at least one variant allele), the absence of strong thrombotic associations in our study, and conflicting evidence from broader literature, routine *MTHFR* genotyping cannot currently be recommended for preoperative risk assessment. If hyperhomocysteinemia is identified, the appropriate management whether through B-vitamin supplementation, modified thromboprophylaxis, or enhanced monitoring remains undefined. Future research should focus on whether homocysteine-lowering interventions improve microsurgical outcomes in genetically or biochemically high-risk patients, though the negative results of cardiovascular trials suggest limited potential for benefit (Bonaa, 2006).

4.3 Coagulation parameters

The coagulation parameter findings yield several important clinical implications for microsurgical practice, while simultaneously revealing the complex pathophysiology underlying free flap thrombosis.

The significant association between reduced activated protein C resistance (APCR) and thrombosis risk (OR = 2.45, $p = 0.043$) represents the most clinically actionable finding of this study. APCR values below 2.0, particularly the observed median of 1.83 in thrombosis patients versus 2.01 in successful cases, suggest that preoperative APCR testing could potentially identify high-risk patients who might benefit from enhanced thromboprophylaxis or more intensive postoperative monitoring (Dahlbäck, 1993; Castoldi, 2010).

The protein C anticoagulant pathway plays a critical role in regulating thrombin generation through the inactivation of factors Va and VIIIa (Segers, 2007). In the context of microvascular surgery, where small vessel calibre and low flow states create inherently prothrombotic conditions, even modest impairment of this regulatory mechanism may shift the haemostatic balance toward thrombosis (Furie, 2008). The finding that reduced APCR correlates with increased flap thrombosis risk, independent of Factor V Leiden genotype status, suggests that APCR measurement captures functional anticoagulant capacity more comprehensively than genetic testing alone. This observation has practical implications, as APCR can be influenced by acquired factors including pregnancy, oral contraceptive use, inflammatory states, and elevated factor VIII levels all conditions that may be present in surgical populations (Castoldi, 2010).

The clinical utility of routine APCR screening in microsurgical candidates warrants careful consideration. While the 2.45-fold increased thrombosis risk associated with APCR < 2.0 appears substantial, the absolute risk increase must be embedded within the overall 9 %

thrombosis rate observed in this cohort. Cost-effectiveness analysis would be necessary to determine whether universal APCR screening is justified, or whether testing should be reserved for patients with additional risk factors such as personal or family history of thrombosis, previous flap failure, or complex reconstructions requiring prolonged operative times. The absence of current consensus regarding thromboprophylaxis protocols in microsurgery (Khansa, 2013; Pan, 2014) further complicates the translation of APCR findings into clinical practice, as optimal management strategies for identified high-risk patients remain undefined.

The absence of significant associations between thrombosis risk and other coagulation parameters including prothrombin time, fibrinogen concentration, and antithrombin activity highlights the complex and multifactorial nature of microsurgical thrombosis. This finding contrasts with the well-established associations between these parameters and systemic venous thromboembolism, where elevated fibrinogen levels (Rosendaal, 2009), reduced antithrombin activity (Patnaik, 2008), and prothrombin elevation (Poort, 1996) constitute recognised risk factors.

Unlike spontaneous venous thromboembolism, where systemic hypercoagulability drives thrombotic risk through Virchow's classical triad of hypercoagulability, endothelial injury, and stasis (Kumar, 2010), microsurgical thrombosis may be more dependent on local factors at the anastomotic site. These include technical precision of vessel approximation, anastomotic tension, vessel wall trauma, calibre mismatch between donor and recipient vessels, and the unique haemodynamic environment created by the surgical reconstruction (Khouri, 1998; Pattani, 2010). The small vessel calibre typical of microsurgical anastomoses (1–3 mm) creates flow conditions distinct from larger venous systems, potentially minimising the impact of systemic coagulation abnormalities while amplifying the importance of local mechanical and haemodynamic factors.

The fibrinogen findings merit particular discussion given the robust genetic association between *FGG* rs2066865 and plasma fibrinogen levels demonstrated in this cohort. Despite A/A genotype carriers exhibiting significantly elevated fibrinogen concentrations (5.57 ± 1.81 g/L vs. 4.08 ± 1.32 g/L in G/G carriers, $p = 0.004$), this genetic predisposition to hyperfibrinogenaemia did not translate to statistically significant increases in clinical thrombosis risk. This apparent paradox may be explained by several mechanisms. First, fibrinogen exists as both a substrate for thrombin (generating fibrin) and an acute-phase reactant responsive to surgical stress (van Hylckama Vlieg, 2003). All patients in this cohort underwent major surgery with associated inflammatory responses, potentially elevating fibrinogen levels universally and obscuring baseline genetic differences. Second, the timing of measurement (preoperatively) may not capture peak fibrinogen elevations occurring during the critical

postoperative period when most thromboses develop (Schlimp, 2016; Tang, 2010). Third, fibrinogen's role in microsurgical thrombosis may be secondary to platelet activation and adhesion processes, which were not directly measured in this study.

The unexpected inverse association between homocysteine elevation (> 12 µmol/L) and thrombosis risk (OR = 0.35, p = 0.029) represents the most paradoxical finding requiring careful interpretation and validation. This observation directly contradicts established literature linking hyperhomocysteinemia to both arterial and venous thrombotic events (Falcon, 1994; Pathare, 2004; Liew, 2015), though meta-analyses have yielded conflicting conclusions regarding the strength and causality of this relationship (Ray, 2002; Clarke, 2012).

Several potential explanations warrant consideration. First, the small sample size for homocysteine measurements in thrombosis patients (n = 14) creates substantial potential for chance findings and Type I error. Second, the pathophysiology of microsurgical thrombosis may differ fundamentally from spontaneous thrombotic disorders. Homocysteine's proposed thrombogenic mechanisms including endothelial dysfunction, oxidative stress, and impaired fibrinolysis (Welsch, 1997) may be overwhelmed by the acute surgical trauma and its associated prothrombotic stimuli. Furthermore, the *MTHFR* C677T polymorphism, while associated with elevated homocysteine in some populations, demonstrates variable penetrance dependent on folate status (Klerk, 2002; Rozen, 1997). Patients with the variant genotype but adequate folate intake may not exhibit hyperhomocysteinemia, potentially explaining the absence of straightforward genetic associations with thrombosis risk.

The results suggest that comprehensive thrombophilia screening, beyond APCR measurement, may provide limited clinical value for predicting microsurgical thrombotic complications in unselected patient populations. This challenges the intuitive approach of extensive preoperative coagulation testing advocated by some practitioners (Biban, 2019; Pannucci, 2015) and supports more selective, targeted screening strategies. The poor discriminatory power of our combined coagulation-based predictive model (AUC = 0.65) reinforces this conclusion, indicating that laboratory parameters alone inadequately capture the complex, multifactorial determinants of flap success.

4.4 Predictive model and clinical utility

The relatively poor discriminatory power of all three predictive models (AUC: 0.54–0.63) highlights the multifactorial nature of flap thrombosis. The genetic-only model performed barely better than random chance (AUC = 0.61), while the coagulation-only model showed modest improvement (AUC = 0.65). The combined model, incorporating genetic, coagulation, and confounding factors, achieved the highest but still limited

discriminatory power (AUC = 0.69). These findings suggest that thrombotic complications in microvascular surgery result from complex interactions between multiple factors that extend beyond genetic predisposition and laboratory parameters. Technical factors, surgical experience, anastomotic tension, vessel quality, and postoperative management likely play equally or more important roles in determining outcomes.

The cumulative risk factor analysis revealed a modest trend towards higher thrombosis rates with multiple risk factors, although statistical significance was not achieved. Patients with three or more risk factors demonstrated a 20 % thrombosis rate compared to 13 % in those without identifiable risk factors. While this trend suggests potential clinical utility for risk stratification, the confidence intervals were wide, limiting definitive conclusions.

This finding underscores the challenge of developing reliable preoperative risk assessment tools based solely on genetic and laboratory parameters. The relatively small effect sizes and overlapping confidence intervals suggest that current genetic testing may have limited clinical utility for routine preoperative screening in microvascular surgery.

4.5 Study limitations and methodological considerations

Several important limitations must be acknowledged when interpreting these results. First, the relatively small sample size (n = 155) may have limited statistical power to detect modest genetic effects, particularly for rare variants like Factor V Leiden. Second, the retrospective design introduces potential selection bias and limits the ability to control for confounding variables systematically.

Third, the timing of coagulation parameter measurement in relation to surgery may have influenced results. Acute-phase reactions following surgical trauma can significantly alter coagulation parameters, potentially obscuring baseline genetic effects. Fourth, the absence of detailed perioperative anticoagulation protocols in our analysis may have confounded results.

To add, our findings must be interpreted in the context of complementary research from the same institution. As previously documented (Vanags et al., 2020; Stepanovs, 2025), technical and acquired variables are dominant predictors of thrombosis in our patient population. The fact that prolonged surgical duration and rotational thromboelastometry (RTE) detected hypercoagulability were shown to increase thrombosis risk by factors of 3.5 and 8.8, respectively, provides a compelling explanation for the absence of a detectable signal from the common genetic variants investigated in the present study. In our study this was a deliberate methodological choice to maintain focus on the primary hypothesis related to genetic and haemostatic parameters which did not include a systematic analysis of technical and surgical

variables such as anastomosis time, vessel calibre, or specific surgical techniques, though we admit that technical surgical variables remain a significant limiting factor in our study.

Finally, the focus on specific genetic polymorphisms may have overlooked other relevant variants or epigenetic factors that influence thrombotic risk. This observation aligns with the established understanding that populations harbouring multiple risk factors, whether genetic or acquired, demonstrate increased thrombotic risk, supporting the characterisation of VTE as a multigenetic and multifactorial disorder (Dahlbäck, 2005). The rapidly expanding field of pharmacogenomics suggests that multiple genetic variants likely interact in complex ways to influence coagulation function.

4.6 Future research

Despite the absence of strong genetic associations, this study provides valuable insights for the development of personalised approaches to microvascular surgery. The identification of fibrinogen polymorphisms as modulators of plasma fibrinogen levels suggests potential therapeutic targets for risk modification. Patients carrying high-risk genotypes might benefit from enhanced monitoring, modified surgical techniques, or targeted pharmacological interventions.

Furthermore, the complex interactions between genetic, acquired, and technical factors highlight the need for multidisciplinary approaches to risk assessment. Future research should integrate genetic information with comprehensive clinical, technical, and laboratory parameters to develop more accurate predictive models.

As well the findings of this study raise several important questions that warrant further investigation. First, larger multicentre studies are needed to validate these findings and provide adequate statistical power for detecting modest genetic effects. Such studies should incorporate standardised surgical techniques, anticoagulation protocols, and outcome measurements to minimise confounding variables.

Second, genome-wide association studies may identify novel genetic variants associated with microvascular thrombotic complications. The candidate gene approach used in this study, while hypothesis-driven, may have missed important genetic contributors outside of traditional coagulation pathways.

Third, functional studies examining the mechanistic relationships between genetic variants and microvascular thrombosis could provide insights into therapeutic targets. Understanding how specific polymorphisms affect coagulation function in the unique haemodynamic environment of microvascular anastomoses may reveal novel intervention strategies.

Finally, the development of comprehensive risk prediction models incorporating genetic, clinical, technical, and laboratory factors represents an important goal for personalised surgical care. The integration of genetic screening with traditional risk assessment methods, such as modified Caprini scores, may improve patient risk stratification. Such analyses could provide valuable insights into personalised risk assessment strategies for microsurgical procedures. As well as machine learning approaches may prove particularly valuable for identifying complex interactions between multiple variables.

4.7 Strengths and limitations

Study strengths

This investigation possesses several methodological strengths that enhance the validity and significance of its findings. The prospective observational design with standardised protocols minimised potential recall bias and ensured systematic data collection across all participants. All microvascular procedures were performed by highly experienced microsurgeons at a specialised centre, reducing technical variability and enhancing the generalizability of findings to similar tertiary care settings.

The comprehensive assessment approach represents a particular strength, integrating genetic analysis of five thrombophilia-associated variants with corresponding phenotypic measurements (fibrinogen, APCR, antithrombin, homocysteine, prothrombin time). This dual approach enabled robust genotype-phenotype correlation analysis, as evidenced by the significant association between *FGG* rs2066865 and plasma fibrinogen concentrations (de Willige, 2005; Grünbacher, 2007). Few previous studies have systematically examined both genetic predisposition and acquired haemostatic alterations in the specific context of microsurgical reconstruction.

The detailed clinical characterisation of patients, including standardised interviews, family history assessment, and perioperative monitoring, provided rich contextual data beyond simple genetic testing. The ethical rigor of the study, with appropriate informed consent procedures and ethics committee approval for genetic material donation, ensured participant protection and research integrity.

Furthermore, the focus on microsurgical thrombosis rather than spontaneous venous thromboembolism addresses a specific clinical question with direct surgical relevance, distinguishing this work from broader thrombophilia epidemiological studies. The examination of cumulative genetic burden and development of predictive models, despite their limited discriminatory power, represents an important attempt to translate genetic information into clinically applicable risk assessment tools.

Study Limitations

Despite these strengths, several important limitations must be acknowledged when interpreting the findings. The primary limitation concerns statistical power. With only 14 thrombotic events among 155 patients, the study lacks sufficient power to detect modest genetic effects, particularly for rare variants such as Factor V Leiden (n = 7 carriers) (Rosendaal, 1995; Kujovich, 2011) and Prothrombin G20210A (n = 3 carriers). The wide confidence intervals observed across all genetic associations reflect this fundamental constraint, precluding definitive conclusions regarding the absence of genetic effects.

The single-centre design, while ensuring protocol standardisation, limits generalizability across different surgical practices, patient populations, and healthcare systems. Ethnic homogeneity within the predominantly European population further restricts applicability to other ethnic groups with different baseline genetic variant frequencies and thrombotic risk profiles.

The candidate gene approach, focusing on five well-characterised variants, may have overlooked novel or less-studied genetic contributors to microsurgical thrombosis. Contemporary genome-wide association studies might identify additional relevant variants de (Haan, 2012) outside traditional coagulation pathways. Similarly, the absence of detailed pharmacogenomic analysis (e. g. variants affecting anticoagulant metabolism) represents a missed opportunity to address personalised thromboprophylaxis strategies.

Technical and surgical variables, despite being performed by experienced surgeons, remained incompletely characterised and controlled. Factors such as anastomosis time, vessel calibre, surgical complexity, and intraoperative complications were not systematically quantified, potentially confounding genetic associations. The study design could not distinguish between technical failure and biologically-mediated thrombosis.

The timing of coagulation parameter measurement warrants consideration. Preoperative samples may not reflect perioperative haemostatic stress, while surgical trauma induces acute-phase reactions that alter fibrinogen and other coagulation factors (van Hylckama Vlieg, 2003; Schlimp, 2016). This temporal constraint may have obscured true genetic effects on haemostatic function during the critical postoperative period.

The relatively short follow-up period (10 days for survival analysis) captured only early thrombotic complications. Late thrombotic events or long-term flap outcomes were not assessed, potentially underestimating the true complication rate and missing delayed manifestations of genetic thrombophilia (Hosein, 2016).

Finally, the absence of a validation cohort prevents external verification of findings. The observed associations, particularly the unexpected inverse relationship between

homocysteine and thrombosis risk, require confirmation in independent populations before clinical implementation.

These limitations collectively suggest that negative findings should be interpreted as insufficient evidence to exclude genetic associations rather than definitive proof of their absence. Future research should address these constraints through multi-centre collaborations, larger sample sizes, genome-wide approaches, and comprehensive perioperative characterisation to advance understanding of genetic contributions to microsurgical thrombotic complications.

Conclusions

1. Successful identification of patients carrying the targeted single nucleotide variants across all five genetic loci: rs6025 in *FV* (2.1 %), rs1799963 in *FII* (1.3 %), rs2066865 in *FGG* (22 %), rs2227589 in *SERPINC1* (9.7 %), and rs1801133 in *MTHFR* (26.5 %) was performed. Variant frequencies demonstrated excellent concordance with established European population distributions (fold change 0.91–1.33), confirming appropriate population sampling without selection bias. All genotype distributions maintained Hardy-Weinberg equilibrium, validating technical accuracy and population representativeness.
2. The study aim was achieved by determining that no individual genetic variant demonstrated a statistically significant association with thrombotic outcomes (all $p > 0.05$). This null finding is a significant scientific conclusion, suggesting that the thrombotic risk conferred by these common polymorphisms is of limited clinical penetrance in the context of microsurgery, where local haemodynamic, technical, and acquired factors may predominate.
3. Among acquired coagulation parameters, only activated protein C resistance (APCR) below 2.0 demonstrated significant association with thrombosis (OR = 2.45, 95 % CI: 1.02–5.89, $p = 0.043$). Conventional parameters including prothrombin time, fibrinogen concentration, and antithrombin activity showed no significant associations (all $p > 0.05$). Acquired thrombophilia factors, as traditionally measured preoperatively, demonstrate limited standalone predictive utility except for functional APCR assessment.
4. Integrated risk assessment models incorporating genetic variants, coagulation parameters, and confounders yielded insufficient discriminatory power for clinical application (AUC: 0.61–0.69). The overall flap success rate of 94.84 % with 9.0 % thrombosis incidence concentrated within 72 hours postoperatively aligns with contemporary microsurgical standards. These results indicate that the examined combination of common genetic variants and standard coagulation parameters cannot reliably predict individual thrombotic risk, highlighting the multifactorial nature of flap thrombosis where technical, mechanical, and local haemodynamic factors may predominate over systemic genetic predisposition.

Proposals

The limitations identified in this study point toward several important research priorities:

1. To address the primary limitation of statistical power multi-centre prospective studies with standardised protocols are warranted to validate these findings and provide adequate statistical power for detecting modest genetic effects, particularly for less common genetic variants like Factor V Leiden.
2. Longitudinal studies could be considered to track patients over time and assess the long-term impact of genetic and acquired factors on thrombotic outcomes and overall flap success.
3. Investigation of genetic variants affecting anticoagulant drug metabolism and efficacy could guide personalised anticoagulation strategies in high-risk patients.
4. Because our predictive models showed insufficient discriminatory power (AUC 0.61–0.69), the development of predictive models using machine learning approaches may better capture complex interactions between genetic, clinical, technical, and laboratory factors than traditional statistical methods.
5. Future research should also consider the ethical implications of genetic testing in surgical contexts, including patient consent, genetic discrimination, and the psychological impact of genetic risk information on patients and their families.

While this study did not demonstrate strong genetic associations with thrombotic complications, it emphasises the importance of continued research into the complex factors governing microvascular surgical outcomes. The pursuit of personalised medicine in reconstructive surgery remains a worthy goal, albeit one that requires more comprehensive approaches than genetic testing alone.

Lists of publications and reports on topics of the Thesis

Publications:

1. Drizlionoka, K., Zariņš, J., Ozoliņa, A., Ņikitina-Zake, L., Mamaja, B. 2019. Polymorphism rs2066865 in the Fibrinogen Gamma Chain (FGG) gene increases plasma fibrinogen concentration and is associated with an increased microvascular thrombosis rate. *Medicina*, 55, 563
2. Ozoliņa, A., Vanags, I., Ņikitina-Zaķe, L., Mamaja, B. 2021. Inherited Thrombophilias in Thrombosis Advancement in Microvascular Flap Surgery. *Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences*. Vol. 75, No. 2 (731), 113–120–567.

Presentations at international conferences

1. Drizlionoka, K., Stepanovs, J., Ozoliņa, A., Ņikitina-Zaķe, L., Mamaja, B. 2019. Markers for thrombosis prediction in free flap surgery. Euroanaesthesia, European Congress of European Society of Anaesthesiology, Vienna, Austria, 01.–03. June 2019.
2. Drizlionoka, K., Stepanovs, J., Ozoliņa, A., Ņikitina-Zaķe, L., Mamaja, B. 2019. Assessment of rotational thromboelastometry and standard coagulation profile in predicting thrombosis in microvascular flap surgery. Rīga Stradiņš University international conference on medical and health care sciences. 04.–05. April 2019. Riga, Latvia. Abstract Book 2019, 433
3. Drizlionoka, K., Stepanovs, J., Krustiņš, L., Ozoliņa, A., Ņikitina-Zaķe, L., Mamaja, B. 2018. The association of hereditary thrombophilia with clinically relevant hypercoagulation state and free flap thrombosis in microvascular surgery. Congress of International Society of Thrombosis and Haemostasis. ISTH SSC Dublin, Ireland, 17.–21. July 2018.
4. Drizlionoka, K., Ozoliņa, A., Ņikitina-Zaķe, L., Mamaja, B. 2018. Plasma fibrinogens increase due to polymorphism rs2066865 in FGG gene as a risk factor for thrombosis in microvascular surgery. Euroanaesthesia, Congress of European Society of Anaesthesiology, Copenhagen, Denmark, 02.–04. June 2018.
5. Drizlionoka, K., Stepanovs, J., Ozoliņa, A., Ņikitina-Zaķe, L., Mamaja, B. 2018. The association of increased plasma fibrinogen concentration due to polymorphism in FGG gene with free flap thrombosis in microvascular surgery. Rīga Stradiņš University Scientific Congress 16.–17. March 2018, Rīga, Latvia. https://www.rsu.lv/sites/default/files/imce/Zinātnes %20departaments/2018/RSU_zinatnis
6. Drizlionoka, K., Stepanovs, J., Ņikitina-Zaķe, L., Mamaja, B. 2017. Does inherited thrombophilia contributes thrombosis in microvascular free flap surgery: a pilot study. Euroanaesthesia, Congress of European Society of Anaesthesiology. Geneva, Switzerland, 03.–05. June 2017.
7. Drizlionoka, K., Stepanovs, J., Ņikitina-Zaķe, L., Mamaja, B. 2017. Genetic contribution towards thrombosis in free flap surgery: a pilot study. Rīga Stradiņš University Scientific Congress Rīga, Latvia, 5 April 2017, Abstracts, http://www.rsu.lv/images/ stories/zk_2017/genetic_contribution_thrombosis_ free_flap_surgery.pdf
8. Drizlionoka, K., Stepanovs, J., Ņikitina-Zaķe, L., Mamaja, B. 2016. The role of inherited thrombophilia in patients undergone free flap surgery: a systemic review of the literature. 8th International Baltic Congress of Anaesthesiology and Intensive Care. 1–3 December 2016. Tallinn, Estonia.

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Annexes

Research Ethics Committee and Ethics Committee approval

Centrālā medicīnas ētikas komiteja

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Rīgā

28.11.2016. Nr.1/28-11-16

„Rīgas Austrumu klīniskā universitātes slimnīca" SIA

Atzinums par pētījuma pieteikumu
„Iedzimto un iegūto trombofīliju nozīme mikrovaskulārajā brīvo lēveru ķirurģijā"

Centrālā medicīnas ētikas komiteja 2016.gada 8.septembrī ir izskatījusi „Rīgas Austrumu klīniskā universitātes slimnīca" SIA iesniegto pētījuma pieteikumu *„Iedzimto un iegūto trombofīliju nozīme mikrovaskulārajā brīvo lēveru ķirurģijā".*

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2016.gada 8.septembra sēdes protokola Nr.2016-4 punktu Nr.5 un iesniegtajiem papildinājumiem, tiek izsniegts atzinums, ka „Rīgas Austrumu klīniskā universitātes slimnīca" SIA iesniegtais pētījuma pieteikums *„Iedzimto un iegūto trombofīliju nozīme mikrovaskulārajā brīvo lēveru ķirurģijā"* nav pretrunā ar bioētikas normām.

Centrālās medicīnas ētikas komitejas priekšsēdētāja E.Pole

Strautiņš, 67876190

Annex 2

Template of questionnaire for study participant

VESELĪBAS UN IEDZIMTĪBAS ANKETA

(aizpilda genoma pētījumu dalībnieks kopā ar pētījuma darbinieku)

I daļa – INTERVIJA

1. Kāds ir jūsu dzimums? Sieviete ☐ Vīrietis ☐

2. Jūsu dzimšanas gads? Gads:___________ **Kāds ir jūsu šībrīža vecums gados?** Gadi:______________

3. Kur Jūs esat dzimis? Kurzeme ☐, Vidzeme ☐, Latgale ☐, Zemgale ☐, Rīga ☐, Cita valsts ________________

4. Kur dzimuši Jūsu tuvinieki (atzīmējiet tikai to, ko Jūs zināt, izmantojot augstākminētos reģionu apzīmējumus)?

Tēvs________________________Tēva tēvs________________________Tēva māte__________________

Māte________________________Mātes tēvs_______________________Mātes māte_________________

5. Kāda ir Jūsu un Jūsu tuvinieku tautība? Jūsu_______________

Tēva________________________Tēva tēva________________________Tēva mātes_________________

Mātes_______________________Mātes tēva_______________________Mātes mātes________________

6. Kāds ir Jūsu ģimenes stāvoklis? Precējies ☐, Neprecējies ☐, Šķīries ☐, Atraitnis ☐

7. Kādi ir jūsu dzīves apstākļi? Dzīvoju viens ☐ dzīvoju ar ģimeni ☐ dzīvoju ar partneri ☐ dzīvoju ar vecākiem ☐

8. Vai Jums ir brāļi vai māsas? Nē ☐ Jā ☐ māsas_____brāļi_____pusmāsas_____pusbrāļi_____

9. Vai Jums ir dvīņubrālis vai dvīņumāsa? Nē ☐ Jā ☐ brālis_____ māsa______

10. Vai Jums ir savi bērni? Nē ☐ Jā ☐ meitas_____ dēli______

11. Kāda ir Jūsu izglītība? Augstākā ☐, Vidējā ☐, Speciālā ☐, Pamatskolas ☐

12. Vai Jūs esat Černobiļas AES avārijas seku likvidators? Jā ☐, Nē ☐

13. Vai Jūs dzīves laikā esat bijis pakļauts kaitīgiem vides vai darba faktoriem? Nē ☐, Jā ☐.

Kādiem?____

14. Vai Jums ir alerģija? Nē ☐, Jā ☐, Iespējams ☐, Nezinu ☐, Ja zinat, tad pret ko?______________________

15. Vai Jums ir medikamentu nepanesamība? Nē ☐, Jā ☐, Iespējams ☐, Nezinu ☐,

Ja ir, tad kurus medikamentus nepanesat?

Novokaīns ☐ Hormoni ☐ Sirds zāles ☐ Vakcīnas ☐ Kontrastvielas ☐ Vitamīni ☐

Antibiotiķi ☐ Aspirīns. ☐ Pretsāpju ☐ Asinsspiediena ☐ Citi ☐_______________________

16. Vai Jūs regulāri lietojat medikamentus? Nē ☐, Jā ☐ Kādus?

Pret bezmiegu ☐ Pret sirdslēkmēm ☐ Pret sirds ritma traucējumiem ☐ Pret sāpēm ☐

Pret krampjiem ☐ Pret paaugstinātu asinsspiedienu ☐ Pret elpas trūkumu ☐ Antibiotiķus ☐

Hormonu preparātus ☐ Hormonālo kontracepciju ☐ Pret paaugstinātu glikozes līmeni ☐

Pret gremošanas traucējumiem ☐ Vitamīnus ☐ Citus ☐______________________________

17. Vai Jūs šobrīd smēķējat? Nē ☐, Jā ☐ **Cik gadus?** __________ **Cik cigaretes dienā?**___________

18. Vai Jūs dzīves laikā jebkad esat smēķējis un šobrīd atmetis? Nē ☐, Jā ☐

Cik gadus kopumā ir smēķēts?_______________Cik cigaretes dienā vidēji?___________________

19. Vai bieži esat uzturējies piesmēķētās telpās? Nē ☐, Jā ☐

20. Vai Jūs lietojiet alkoholu?

Nē ☐, izņēmuma gadījumos ☐, 1-2 reizes nedēļā ☐, vismaz 3 reizes nedēļā ☐, katru dienu ☐

1

21. Cik daudz vidēji vienā reizē lietojiet alkoholu? (1glāzīte = apmēram 50g stiprā alkohola,)

Vienu glāzīti ☐, dažas glāzītes ☐, līdz labsajūtai ☐, kamēr vairs nav ko dzert ☐,

22. Kāds ir Jūsu dzīvesveids šobrīd un agrāk?

Dzīvesveids	Pārsvarā sēžu, guļu, pārvietojos ar automašīnu, fiziska slodze ne biežāk kā reizi nedēļā. Fiziski neaktīvs,	Eju kājām iepirkties, pastaigāties, bet fiziska slodze ne biežāk kā 4 reizes nedēļā. Fiziski mazaktīvs,	Strādāju fiziski smagu darbu, bieži braucu ar velosipēdu, skrienu, peldu, sportoju vairāk kā 4 reizes nedēļā.Fiziski aktīvs,	Esmu profesionāls sportists
Agrāk	☐	☐	☐	☐
Pēdējā laikā	☐	☐	☐	☐

23. Cik ilgi ir dzīvojuši Jūsu tuvinieki?

Lūdzu ierakstiet mirušo tuvinieku vecumu atbilstošajā vietā.
Lūdzu neatzīmēt tos tuviniekus, priekšlaicīgi gājuši bojā - krituši karā, gājuši bojā izsūtījumā, nelaimes gadījumos un tamlīdzīgi.

	<20	21-30g.v	31-40.g.v	41-50g.v.	51-60g.v.	61-70.g.v.	71-80g.v.	>80.g.v.	nezinu
Tēvs									
Māte									
Tēva tēvs									
Tēva māte									
Mātes tēvs									
Mātes māte									
Brāļi									
Māsas									
Dēli									
Meitas									

24. Vai Jūsu ģimenē ir bijuši saslimšanas gadījumi ar ļaundabīgiem audzējiem?

Nē ☐ , Nezinu ☐, Jā ☐

Ja jā, tad lūdzu ierakstiet slimo tuvinieku skaitu un saslimšanas aptuveno vecumu atbilstošajā vietā!
Paskaidrojums: tantes ir tēva vai mātes māsas, onkuļi ir tēva vai mātes brāļi.

Onkoloģiskā slimība	Jums pašam	tēvam	mātei	Brāļiem, māsām	Tēva vecākiem	Mātes vecākiem	Tantēm, onkuļiem no tēva puses	Tantēm, onkuļiem no mātes puses
Krūts vēzis								
Plaušu vēzis								
Dzemdes kakla								
Olnīcu vēzis								
Barības vada								
Kuņģa vēzis								
Zarnu vēzis								
Aizkuņģa								
Prostatas vēzis								
Melanoma								
Nieru/urīnpūšļa								
Asinsrades sistēmas								
Ir, bet nezinat kāds audzējs								

Kāda cita onkoloģiska slimība? Lūdzu ierakstiet kāda un kam no ģimenes un asinsradiniekiem

____
____
____
____
____

2

25. Vai Jūsu ģimenē ir bijuši saslimšanas gadījumi ar sirds un asinsvadu sistēmas slimībām?

Nē ☐ , Nezinu ☐ , Jā ☐

Ja jā, tad lūdzu ierakstiet saslimšanas aptuveno vecumu, kā arī tuvinieku skaitu (ja vairāk par 1) atbilstošajā vietā!

Slimība	Jums pašam	tēvam	mātei	brālim	māsai	dēlam	meitai	kādam no vecvecākiem
Augsts asinsspiediens								
Stenokardija								
Miokarda infarkts								
Insults								
Paaugstināts holesterīns								
Sirds mazspēja								
Kāda cita, bet nezinat precīzi kāda?								

Kāda cita sirds asinsvadu sistēmas slimība? Lūdzu ierakstiet kāda un kam no ģimenes un asinsradiniekiem

____

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____

26. Vai Jūsu ģimenē ir bijuši saslimšanas gadījumi ar vielmaiņas un endokrīnām slimībām?

Nē ☐ , Nezinu ☐ , Jā ☐

Ja jā, tad lūdzu ierakstiet saslimšanas aptuveno vecumu, kā arī tuvinieku skaitu (ja vairāk par 1) atbilstošajā vietā!

Slimība	Jums pašam	tēvam	mātei	brālim	māsai	dēlam	meitai	kādam no vecvecākiem
1. tipa cukura diabēts								
2. tipa cukura diabēts								
Vairogdziedzera slimības								
Aptaukošanās								
Kāda cita, bet nezinat precīzi kāda?								

Kāda cita vielmaiņas vai endokrīnā slimība? Lūdzu ierakstiet kāda un kam no ģimenes un asinsradiniekiem

____

____

____

____

____

27. Vai Jūs vai kāds no Jūsu tuviniekiem cieš no kādām citām hroniskām kaitēm?

Nē ☐, Nezinu ☐, Jā ☐

Ja jā, tad lūdzu ierakstiet kurš tuvinieks un aptuveni kādā vecumā

____

____

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____

3

28. Vai pēdējā gada laikā griezāties pēc palīdzības vai bijāt uz plānveida vizīti pie kāda no nosaukto specialitāšu ārstiem?

Infektologs ________ Jā ☐, Nē ☐

Asins vai asinsrades orgānu slimību speciālists (hematologs) ________ Jā ☐, Nē ☐

Uztura, vielmaiņas un endokrīno slimību speciālists (endokrinologs) ________ Jā ☐, Nē ☐

Nervu sistēmas slimību speciālists (neirologs) ________ Jā ☐, Nē ☐

Acu slimību speciālists (oftalmologs, okulists) ________ Jā ☐, Nē ☐

Ausu, kakla, deguna ārsts (LOR) ________ Jā ☐, Nē ☐

Plaušu slimību speciālists (pulmonologs) ________ Jā ☐, Nē ☐

Gremošanas slimību speciālists (gastroenterologs) ________ Jā ☐, Nē ☐

Ādas un zemādas slimību speciālists (dermatologs) ________ Jā ☐, Nē ☐

Skeleta, muskuļu, saistaudu slimību speciālists (reimatologs, traumatologs) ________ Jā ☐, Nē ☐

Uroģenitālās sistēmas slimību speciālists (urologs, nefrologs) ________ Jā ☐, Nē ☐

Ginekologs ________ Jā ☐, Nē ☐

29. Vai Jums šobrīd ir kādas veselības problēmas izņemot to, kuras dēļ griezāties pie ārsta?

Lūdzu minēt ārstu uzstādītas diagnozes, ja tās ir zināmas.

Nē ☐, Nezinu ☐, Jā ☐

Ja jā, tad lūdzu ierakstiet kādas

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II daļa – MĒRĪJUMI

1. Pētījuma dalībnieka augums __________cm. **svars** ____________kg.?

2. Pētījuma dalībnieka vēdera apkārtmērs ______________________________cm

3. Pētījuma dalībnieka arteriālais asinsspiediens pirms intervijas ______/______ mmHg

4. Pētījuma dalībnieka arteriālais asinsspiediens pēc intervijas ______/______ mmHg

5. Pētījuma dalībnieka pulsa frekvence __________________________________

PALDIES PAR ATBILDĒM ☺

4

Annex 3

Template of informed consent for study participant

Gēnu donora piekrišanas dokuments
(saistībā ar vienotu iedzīvotāju genoma datu bāzi)

I. (aizpilda gēnu donors, viņa aizbildnis vai aizgādnis)

1. Esmu saņēmis un iepazinies ar rakstisku informāciju par ģenētiskās izpētes projekta mērķi, saturu, ilgumu un iespējamiem riskiem. Uz visiem maniem jautājumiem esmu saņēmis saprotamas un izsmeļošas atbildes. Man bija pietiekami daudz laika, lai pārdomātu savu lēmumu piekrist kļūt par gēnu donoru.

2. Esmu informēts, ka man būs tiesības iepazīties ar datiem, kas par mani tiks iegūti un glabāti pēc ģenētiskās izpētes. Apzinos, ka man nebūs tiesību pieprasīt maksu par audu paraugu nodošanu, mana veselības stāvokļa apraksta vai ģenealoģisko datu sastādīšanu vai izpēti, kā arī par izpētes rezultātu izmantošanu.

3. Mana veselības stāvokļa apraksta papildināšanu, atjaunošanu vai pārbaudi genoma datu bāzē (vajadzīgo atzīmēt):

☐ atļauju,
☐ aizliedzu.

4. Mana genoma izpētes apjomu (vajadzīgo atzīmēt):

☐ neierobežoju,
☐ ierobežoju līdz noteiktam apjomam (norādīt izpētes ierobežojumus)

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5. Manu audu paraugu un veselības stāvokļa aprakstu nosūtīšanu ģenētiskajai izpētei ārpus Latvijas (vajadzīgo atzīmēt):

☐ atļauju,
☐ aizliedzu.

6. Ja, izpētot manu genomu, tiks atklāta informācija par man līdz šim nezināmu apdraudējumu manai vai manu radinieku veselībai (vajadzīgo atzīmēt):

☐ piekrītu, ka man tiek paziņota šāda informācija,
☐ piekrītu, ka man tiek paziņota šāda informācija tikai tādā gadījumā, ja risks veselībai ir novēršams,
☐ nevēlos, ka man tiek paziņota šāda informācija.

7. Piekrītu piedalīties genoma izpētes projektā brīvprātīgi, bez maksas. Piekrītu, ka ģenētiskajai izpētei tiek ņemti manu audu paraugi un sastādīts veselības stāvokļa apraksts un (vai) ģenealoģija. Apzinos, ka jebkurā brīdī bez paskaidrojumiem varu atsaukt savu piekrišanu. Šādā gadījumā manu audu paraugi, veselības stāvokļa apraksts un jebkura ar personas identificēšanu saistīta informācija tiek iznīcināta.

Gēnu donors

Vārds un uzvārds (drukātiem burtiem) ____
Personas kods ____
Adrese ____
Datums ____
(diena, mēnesis, gads)
Paraksts ____

II

Gēnu pētnieks/Gēnu donoru ārstējošais ārsts
Vārds un uzvārds (drukātiem burtiem) ____
Amats ____
Paraksts ____
Datums ____
(diena, mēnesis, gads)
Aizpildīšanas vietas nosaukums ____
____

Valsts iedzīvotāju genoma datubāzes kods

VIGDB kods

Audu paraugu un veselības stāvokļa apraksta transporta kods

____

$trkodbar$	
$trkod$	
Dat	
gr 29	$grkod$
$dockod$	$medkod$

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INHERITED THROMBOPHILIAS IN THROMBOSIS ADVANCEMENT IN MICROVASCULAR FLAP SURGERY

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Microvascular flap surgery is a reliable method for reconstructive surgery. To avoid and foresee free flap thrombosis advancement after microvascular flap surgery, patient assessment, flawless surgical technique, and eligible perioperative care are pivotal. In this prospective observational study, we aimed to elucidate the most common inherited single nucleotide polymorphisms (SNPs) attributable to free flap thrombosis. A total of 152 patients undergoing microvascular flap surgery during the study period of 2016–2019 were analysed for five SNPs: rs6025 in Factor V Leiden (FVL) *gene, rs1799963 in Factor II* (FII) *gene, rs2066865 in Fibrinogen Gamma Chain* gene (FGG), *rs2227589 in* SERPINC 1 *gene and rs1801133 in Methylene Tetrahydrofolate Reductase* (MTHFR) *gene. Activated protein C resistance (aPCR), prothrombin, antithrombin (AT), fibrinogen and homocysteine plasma levels were measured to determine association with the analysed SNPs and with free flap thrombosis advancement. Our preliminary results show that carriers of* FVL *mutation were associated with aPCR, as we observed significantly lower aPCR plasma levels in carriers of genotype C/T, as compared to C/C;* p *= 0.006 (CI 95%, 0.44 to 1.19). Additionally, mean fibrinogen plasma levels were higher in carriers of* FGC *gene rs2066865 genotype A/A (5.6 ± 1.81 g/l), as compared to G/A and G/G;* p *= 0.04 (CI 95%, 0.007 to 1.09);* p *= 0.004 (CI 95%, 0.48 to 2.49), respectively. The study group included 12 patients (7.9%) with free flap thrombosis. For one patient free flap thrombosis advancement might have been related to the rs6025T –* FVL *mutation with a PCR plasma level 1.21. Lower aPCR levels was associated with carriers of* FVL *rs6025 C/T and higher fibrinogen plasma levels with carriers of* FGG *rs2066865 A/A, suggesting that these genotypes might predict higher free flap thrombosis risk, but we found no significant association between analysed SNPs and free flap thrombosis advancement.*

Key words: *polymorphisms, free flap thrombosis, Leiden factor, hyperhomocisteinemia, antithrombin deficiency, fibrinogen, prothrombin gene mutation.*

INTRODUCTION

Microvascular flap surgery is a reliable method for reconstructive surgery. Preconditions for success are thoughtful patient assessment, flawless surgical technique, and eligible perioperative care.

Post-lesion damage of endothelial cells employs its procoagulant moiety combined with other factors of the classical Virchow (1845) triad. A hypercoagulable state is an obvious predictor for thrombosis in particular groups of patients, e.g., trauma, malignancy, etc. (Biben and Atmodiwirjo, 2019; Vanags *et al.*, 2020).

Regarding inherited thrombophilia, the majority of single nucleotide polymorphisms (SNPs) are associated with defects affecting anticoagulant mechanisms (Dählback, 2008). Recent evidence suggests that five SNPs (Table 1) might influence thrombosis advancement: rs6025 in the Factor V Leiden (*FVL*) gene, rs1799963 in the Factor II (*FII*) gene, rs2066865 in the Fibrinogen Gamma Chain (*FGG*) gene,

Table 1. Relationship between analysed SNPs, coded plasma proteins and effect on coagulation

SNP	Gene	Plasma proteins	Effect on coagulation
rs6025	*FVL*	aPCR	Leads to aPCR and inactivation of factor Va by aPC
rs1799963	*FII*	Thrombin	Leads to elevated prothrombin levels and an increase of production of thrombin and thrombin activable fibrinolysis inhibitor
rs2066865	*FGG*	Fibrinogen	Leads to increase in plasma fibrinogen concentration
rs2227589	*SERPINC1*	AT	Leads to AT deficiency with increase in thrombin and factor Xa activity
rs1801133	*MTHFR*	Homocysteine	Leads to reduced activity of the MTHFR enzyme and increased plasma homocysteine level, promoting inflammation and atherosclerosis

SNP, single nucleotide polymorphism; FVL, Factor V Leiden; FII, Factor II; FGG, Fibrinogen Gamma; MTHFR, methylene tetrahydrofolate reductase; AT, antithrombin; aPCR, activated protein C resistance; aPC, activated protein C

rs2227589 in the *SERPINC1* gene and rs1801133 in the Methylene Tetrahydrofolate Reductase (*MTHFR*) gene (Rees *et al.*, 1995; El-Galaly *et al.*, 2013; Pathare *et al.*, 2004). However, there is not much literature demonstrating associations of mentioned SNPs and occurrence of free flap thrombosis in microvascular flap surgery patients.

Therefore, the aim of our study was to analyse the most common inherited SNPs that might have an effect on free flap thrombosis advancement after microvascular flap surgery.

Factor V Leiden gene, a mutation of *FVL*, is the most widespread autosomal dominant genetic mutation (Rees *et al.*, 1995; Rosendaal *et al.*, 1995; Ridker *et al.*, 1997). *FVL* mutation is a missense mutation in the FV gene (NM_000130.4(F5):c.1601G>A (p.Arg534Gln), rs6025 (c.1691C>T) where in position R506 arginine is replaced by glutamine (Zoller and Dahlbäck, 1994; Friedman *et al.*, 2010). It is known as activated protein C resistance (aPCR), which is attributable to the *FVL* mutation in up to 95% of cases (Segers, 2007).

Factor II gene. *FII* or the prothrombin mutation (NM_000506.4(F2):c.97 G>A, rs1799963 G/A), was found to occur in 3–17 % of patients with VTE and about 1–8 % of healthy controls (Rosendaal *et al.*, 1998). Carriers of the rs1799963A allele have a higher plasma prot hrombin level and 2.8-fold higher risk of VTE (Poort *et al.*, 1996; Meltzer *et al.*, 2010).

Fibrinogen gamma chain gene. Fibrinogen is a plasma glycoprotein synthesised by hepatocytes. Fibrinogen molecules are comprised of two sets of three polypeptide chains — A alpha; B beta; and gamma (Henschen *et al.*, 1983). The three chains are encoded by three separate genes, fibrinogen alpha (FG>A), fibrinogen beta (*FGB*), and fibrinogen gamma (*FGG*). The rare allele T of rs20066865 polymorphism (NM_000509.5(FGG):c.216 GA, rs2066865G/A) in the *FGG* gene is described as a reason for alterations in the coagulation system (Drizlionoka *et al.*, 2019).

A polymorphism in *SERPINC1* gene (NM_000488.3(*SERPINC1*):c.41+141C>T, rs2227589 C/T) is recognised as causing antithrombin (AT) deficiency and is associated with the highest risk of VTE of all known inherited thrombophilias. Inherited AT deficiency is an uncommon autosomal dominant disorder. Prevalence in general population is 1 : 2000 to 1 : 5000 and is associated with a 10-fold to 20-fold increased risk of VTE (Patnaik and Moll, 2008). Most of the VTE episodes are unprovoked for those with AT deficiency (Patnaik and Moll, 2008).

Methylene Tetrahydrofolate Reductase gene, a polymorphism in *MTHFR* gene (NM_005957.4(*MTHFR*):c.665 G>A (p.Ala222Val), results in reduced activity of this enzyme (Rosenberg *et al.*, 2002). An increased level of homocysteine has been reported as a risk factor for coronary artery disease (Rozen, 1997; Stanger *et al.*, 2004), especially in young/middle aged Caucasians with an increased risk for myocardial infarction (Xuan *et al.*, 2011), cerebral and peripheral artery disease (Welsch *et al.*, 1997).

MATERIALS AND METHODS

Subject and perioperative management. In an observational prospective case series study, we enrolled 152 adult consecutive patients who had microvascular flap surgery in the Centre of Plastic and Reconstructive Microsurgery of Latvia during 2016–2019.

All patients undergoing microvascular flap surgery were enrolled during the study period after exclusion criteria were applied. The informed consent form including the request to donate genetic material and the protocol was approved by the Latvian Central Ethics Committee (No. 1/28-11-16). All patients provided written, informed consent.

The exclusion criteria were: pregnancy, peripartum period; transfusion of allogeneic blood components, and/or coagulation factors within 72 h perioperatively, proven left ventricular failure, allogenic bone marrow transplantation, liver failure, liver transplantation, and end-stage kidney disease.

General anaesthesia (GA) was provided for all patients according to the guidelines for free flap transfer microsurgery. Peripheral nerve blocks under ultrasound guidance were performed for analgesia requirement when applicable. For those without a peripheral nerve block, continuous intravenous infusion of fentanyl 0.5–1 mcg/kg/h was administered. Fluid management, oxygen supply and transfusion manage-

ment were provided in the post-anaesthesia care unit with tight haemodynamic monitoring and followed local guidelines for microvascular flap surgery post-operative care.

During the study, a total of 152 microvascular flap surgeries were performed by highly trained specialists. The venue of free flap thrombosis (i.e., arterial, venous, or both) was assured by direct visualisation during the revision of anastomosis.

For patients on direct oral anticoagulants, medication was stopped 72 h prior to sample collection to avoid any inaccuracy in testing. For patients taking a vitamin K antagonist, medication was stopped according to the patient's international normalised ratio (INR) level, 4 to 5 d prior to sample collection. Patients with a high thrombosis risk were switched to low-molecular-weight heparin (LMWH) and use was discontinued 12 h prior to the surgery.

Each patient was genotyped to determined SNPs: rs6025 in the *FVL* gene, rs1799963 in the *FII* gene, rs2066865 in the *FGG* gene, rs2227589 in the *SERPINC1* gene and rs1801133 in the *MTHFR* gene. Plasma concentrations of aPCR, prothrombin, fibrinogen, AT and serum homocysteine were measured one time pre-operatively.

Laboratory workup and genotyping. Blood samples were drawn on the day of microsurgery prior to the induction of GA and any crystalloid infusion. All tests were processed within an hour.

To determine potential alteration in plasma protein levels associated with the analysed SNPs, the following laboratory measurements were performed: aPCR was measured using the clotting method with a Sysmex CS2100i, UK LTD (reference range ≥ 1.8). Prothrombin was measured using the clotting time method (for prothrombin added tissue thromboplastin ISI < 1.5) with a Sysmex system, CS-2100i, UK LTD (reference range 70–130%). Plasma AT was measured using a chromogenic method with a Sysmex CS-2100i, UK LTD (reference range 75–125%). Total plasma fibrinogen concentration was measured by Clauss method in citrated plasma using a STA-R COMPACT Diagnostika Stago, Asnières-sur-Seine, France (reference range 2–4 g/l). Homocysteine was measured using the immunochemical luminescence method on a ADVIA, Centaur, Siemens Healthcare GmbH, Germany (reference range 5.00–12.00 mkmol/l).

Genomic DNA was extracted using the standard phenol-chloroform extraction protocol. Extracted DNA was dissolved in water. Genotyping was performed by Taqman Pre-Designed SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) on a Viia7 Real-Time polymerase chain reaction (PCR) system (Applied Biosystems) (Livak, 1999) according to the supplier's recommendations.

In addition to genetic and coagulation laboratory workout, patients were interviewed to register co-morbidities, particularly focusing on previous thrombotic events. We also registered demographic data, localisation of soft tissue defect, type of free flap transfer and the duration of surgery.

Statistical analysis. We compared variables with independent-sample (unpaired) t-tests using SPSS 23 Statistics software (IBM Korea, Seoul, Korea). The Kolmogorov–Smirnov test was used to check whether the variables followed a normal distribution. Normally distributed, continuous variables were presented as means ± standard deviation (M ± SD) and categorical variables as percentages (%). The frequency of alleles was tested against Hardy–Weinberg equilibrium. Odds ratios and 95% confidence intervals were calculated to evaluate factor impacts between groups. Comparisons between genotype groups were performed with Kruskal–Wallis H tests for nonparametric variables and with ANOVA for parametric variables. Pearson's and two-tailed Fisher exact tests were used for data comparison, depending on the number of cases. The Spearman's rank correlation coefficient was used where applicable. Statistical significance was assumed as two-tailed with $p < 0.05$.

RESULTS

We analysed 152 patients scheduled for microvascular flap surgery (35 female and 117 males), who met the inclusion criteria. Table 2 displays demographic and clinical variables of the studied group. History of personal or family thrombosis was observed in 5.2% and 13.8% of female and male cases, respectively. Most patients had surgery after more than 30 days post-traumatic injury and defect localisation was mostly on a lower extremity. Correlation was not observed between SNPs and metabolic disturbances and smoking.

Relationships between SNPs and coded protein plasma levels. To analyse each SNP, patients were classified by their *FVL* gene C/T, *FII* gene G/A, *FGG* gene G/A, *SERPINCI1* gene C/T and *MTHFR* gene G/A characteristics and subdivided into three groups, according to the genotype of each gene polymorphism. The genotype results of analysed SNPs were all in Hardy-Weinberg equilibrium. Table 3 shows protein plasma levels in relation to the analysed SNPs.

rs6025 *FVL* gene. 96% of the studied population had the C/C genotype of the *FVL* gene rs6025 polymorphism. We observed significantly a lower aPCR plasma level in carriers of genotype C/T, as compared to C/C; $p = 0.006$ (CI 95%, 0.44 to 1.19). None of the analysed patients had genotype T/T.

rs2066865 *FGG* gene. Analysis of the association between *FGG* gene rs2066865 polymorphism and fibrinogen plasma levels showed that mean fibrinogen plasma levels were higher in carriers of the genotype A/A, compared to G/A and G/G; $p = 0.04$ (CI 95%, 0.007 to 1.09); $p = 0.004$ (CI 95%, 0.48 to 2.49), respectively. The largest number of patients were carriers of G/G and A/G genotypes (in 60% and 34% of cases) and only 6% of the studied patients had the

Table 2. Characteristics of the studied group

n = 152	n (%)
Age, years (mean ± SD)	45.1 (14.9)
Sex, female	35 (23)
History of thrombosis	10 (5.2)
Family history of thrombosis	21(13.8)
Smoking	64 (42.1)
Metabolic disturbances?	15 (9.9)
Defect aetiology:	
Trauma	70 (46.1)
Recent trauma, (< 30 days)	25 (16.4)
Polytrauma	12 (7.9)
Chronic inflammation	34 (22.4)
Malignancy	30 (19.7)
Combustion	6 (3.9)
Defect localisation:	
Lower extremity	82 (53.9)
Upper extremity	36 (23.6)
Trunk	3 (2.00)
Head/orofacial	29 (19.07)
Abdomen	2 (1.3)

Data are presented as mean ± SD or number (n) and percentage (%).

*Diabetes mellitus; adiposities (BMI > 25)

A/A genotype, which was associated with a significantly higher mean fibrinogen plasma level (5.6 ± 1.81 g/l).

rs2227589 *SERPINC1* gene. Although carriers with the C/C genotype (81%) of the *SERPINC1* gene rs2227589 polymorphism had a tendency to have a lower AT plasma level, we did not observe a significant difference of AT plasma level between homo- and heterozygous allele carriers; $p = 0.09$ (CI 95%, 1.37 to 18.02).

rs1799963A in *FII* gene. As for the above gene, mean prothrombin plasma level displayed no significant difference between *FII* gene rs1799963A polymorphism genotype groups; $p = 0.8$ (CI 95%, 20.31 to 26.23 between G/G and G/A genotype carriers).

rs1801133 *MTHFR* gene. Carriers of the *MTHFR* gene rs1801133 genotype G/A and A/A presented with slightly higher plasma levels of homocysteine, as compared with carriers of genotype G/G, but this difference did not reach significance; $p = 0.07$ (CI 95%, 0.26 to 5.6) between G/G and G/A; $p = 0.26$ (CI 95%, 2.39 to 8.26) between G/G and A/A.

Case series. In Table 4, we demonstrate data for 12 patients (7.89%) of 152 who presented with free flap thrombosis. Only one of the twelve patients had all wild type alleles of analysed SNPs. The other eleven had one or a combination of two of the analysed SNPs. Polymorphism rs1801133 in the *MTHFR* gene G/A genotype was most often observed in combination or alone (Table 4). For all of those patients, the homocysteine plasma level was higher than normal, with a mean value 13.3 ± 0.74 mKmol/l. Two patients had solely a in *MTHFR* gene. One presented with free flap thrombosis in

Table 3. Determined SNPs and coded protein plasma levels

Type of SNP	Coded protein plasma levels, normal range (min.-max.)	*p*
rs6025 *FVL* (C > T)	aPCR (>1.8)	
C/C (n = 146)	2.02 ± 0.32*	*0.006
C/T (n = 6)	1.19 ± 0.17*	
T/T (n = 0)	–	
rs1799963 *FII* (G > A)	Prothrombin (70–130%)	
G/G (n = 148)	96.38 ± 20.23	NS
G/A (n = 3)	99.33 ± 15.94	
A/A (n = 1)	106.00	
rs2066865 *FGG* (G > A)	Fibrinogen (2–4 g/l)	
G/G (n = 91)	4.1 ± 1.3 #	* 0.04
G/A (n = 52)	4.6 ± 1.7 *	# 0.004
A/A (n = 9)	5.6 ± 1.8 *#	
rs2227589 *SERPINC1* (C > T)	AT (75–125%)	
C/C (n = 123)	89.56 ± 14.14	NS
C/T (n = 28)	97.89 ± 14.51	
T/T (n = 1)	92.10	
rs1801133 *MTHFR* (G > A)	Homocysteine (5.00–12.00 mkmol/lL)	
G/G (n = 81)	10.56 ± 4.05	NS
G/A (n = 61)	13.23 ± 5.55	
A/A (n = 10)	13.50 ± 5.81	

Values are presented as mean, ± SD; sig. 2- tailed $p < 0.05$. SNP, single nucleotide polymorphism; FVL, Factor V Leiden; FII, Factor II; FGG, Fibrinogen Gamma; MTHFR, methylene tetrahydrofolate reductase; AT, antithrombin; aPCR, activated protein C resistance; NS, nonsignificant; n, number; G, guanine; A , adenine; T, thymine; C, cytosine

arterial and the other in venous flow. The rs2066865 polymorphism in the *FGG* gene G/A genotype was most often detected, and associated with a higher mean fibrinogen plasma level (4.7 ± 0.35 g/l). Only one patient had a homozygote form of A/A genotype, with a fibrinogen plasma level of 4.7 g/l. None, except one, had perioperative thromboprophylaxis. Most of the patients (10 of 12, 83%) with free flap thrombosis had a defect with localisation on a lower extremity.

The first patient in Table 4 was a carrier of the *FVL* gene rs6025 polymorphism C/T genotype who presented with a markedly lower aPCR plasma level (1.21) and underwent microvascular flap surgery twice in two years. Both of these transfers were complicated by free flap thrombosis. Free flap thrombosis might have been related to the rs6025T *FVL* mutation in this case.

When comparing patients with (n = 12) and without (n = 140) free flap thrombosis, we were not able to find any specific factor or their combinations that independently affected advancement of free flap thrombosis.

DISCUSSION

In this observational case series study we evaluated the association of hereditary thrombophilia with free flap thrombosis advancement in microvascular flap surgery. We fo-

Table 4. Case series of patients with free flap thrombosis

Free flap transfer	Cause	Defect localisation	Thrombosis venue	SNP and genotype	Laboratory parameters
Scapular/ Parascapular	osteomyelitis	lower extremity	venous *	rs6025 *FVL* C/T rs2066865 in *FGG* G/A	aPCR 1.21 Fibrinogen 4.24 g/l
Medial plantar artery	osteomyelitis	lower extremity	arterial	rs2227589 in *SERPINC1* C/T rs1801133 in *MTHFR* G/A	AT 76% Homocysteine 13.3 mkmol/l
Lateral arm	trauma	lower extremity	venous	rs2066865 in *FGG* G/A	Fibrinogen 4.4 g/l
Scapular/ Parascapular	osteomyelitis	lower extremity	venous	rs1801133 in *MTHFR* G/A	Homocysteine 12.8 mkmol/l
Scapular/ Parascapular	polytrauma	lower extremity	venous	rs2066865 in *FGG* G/A	Fibrinogen 4.7 g/l
Serratus anterior muscle	trauma	lower extremity	arterial and venous	rs2066865 in *FGG* G/A rs2227589 in *SERPINC1* C/T rs1801133 in *MTHFR* G/A	Fibrinogen 5.1 g/l
Osteocutaneus	trauma	lower extremity	arterial and venous	rs2227589 in *SERPINC1* C/T rs1801133 in *MTHFR* G/A	AT 81% Homocysteine 14.1 mkmol/l
Osteocutaneus	osteomyelitis	lower extremity	arterial and venous	rs2066865 in *FGG* G/A rs1801133 in *MTHFR* G/A	Fibrinogen 5.1 g/l Homocysteine 13.2 mkmol/l
Serratus anterior muscle	osteomyelitis	lower extremity	arterial and venous	rs2227589 in *SERPINC1* C/T	AT 111.8%
Radial forearm	malignancy	orofacial	arterial	rs1801133 in *MTHFR* G/A	Homocysteine 12.2 mkmol/l
Lateral arm	trauma	upper extremity	venous	rs2066865 in *FGG* A/A rs1801133 in *MTHFR* G/A	Fibrinogen 4.5 g/l Homocysteine 14.4 mkmol/l
Scapular/ Parascapular	trauma	lower extremity	venous	-	no alteration in laboratory data

SNP, single nucleotide polymorphism; FVL, Factor V Leiden; FII, Factor II; *FGG*, Fibrinogen Gamma; MTHFR, methylene tetrahydrofolate reductase; AT, antithrombin; aPCR, activated protein C resistance; NS, nonsignificant; n, number; G, guanine; A, adenine; T, thymine; C, cytosine; * patient with noticed association between SNP rs6025 in *FVL* and aPCR and free flap thrombosis

cused on five SNPs, which were mentioned in literature most frequently in relation with their coded plasma protein levels and free flap thrombosis advancement of diverse localisation, either venous or arterial origin, in 152 microvascular flap surgery patients. Our study group was relatively young (mean age 45.1 years), and only 5.2 per cent had a history of previous thrombotic events and 13.8 per cent had a positive family history of thrombosis. This study revealed that a significantly lower aPCR plasma level occurred in carriers of *FVL* gene genotype C/T in the analysed polymorphism. We also noticed a highest fibrinogen plasma level in carriers of *FGG* gene genotype A/A in the analysed polymorphism. Correspondingly, for one patient, advancement of free flap thrombosis was most likely associated with one of our analysed SNP where aPCR was detected.

In microvascular flap surgery both arterial and venous circulation systems are involved. Therefore, there is still lack of a haemostatic tool that would allow evaluating who would benefit from perioperative thromboprophylaxis without increasing risk of haemorrhage. Although has not yet been evaluated for microvascular flap surgery, the 2005 Caprini Risk Assessment Model could be applicable for those patients (Pannuci *et al*., 2015).

It has been mentioned that approximately 5 per cent of aPCR could occur by different mechanisms (Segers, 2007), particulary in FVL gene rs6025T C/T genotype carriers. We recognised only one patient with aPCR who had a C/T genotype combined with a markedly lower aPCR plasma level and who underwent microvascular flap surgery twice in two years' time period, both with eventual free flap thrombosis. aPCR can be detected in 20% of patients wih VTE and in more than a half of selected families with thrombophilia (Rees *et al*.,1995; Rosendaal *et al*., 1995). The prevalence of the homozygous T/T genotype form is 1.5 per cent of the general population (Rees *et al*., 1995). Thus, we can explain why none of the patients in our study group had this genotype of the FVL gene for the analysed SNP.

None of our patients with a mutation in the *FII* gene had free flap thrombosis. All patients had prothrombin plasma levels within the normal range. It has been observed that when the prothrombin cut off value reached 95.19%, risk of thrombosis increased by 2.34 times (Drizlionoka *et al*., 2019). This is consistent with observations made by previous investigators. Patients with a prothrombin plasma level higher than 1.15 U/ml had a 2.1-fold higher risk of VTE than those in the reference category < 0.95 U/ml (Poort *et al*., 1996).

We believe that increased risk of free flap thrombosis might be associated with increased plasma levels of fibrinogen. Carriers of the FGG gene rs2066865 genotype A/A had sig-

nificantly higher levels of fibrinogen, compared with carriers of genotype G/G. We also noticed that plasma levels of fibrinogen are higher for FGG gene heterozygotes A/G. The latter genotype was found in 5 of 12 patients with free flap thrombosis, as shown in Table 4. Undas and Casini (2019) previously reported that patients with hereditary dysfibrinogenemia are highly heterogeneous in clinical manifestation. Correlations between genotype and phenotype may sometimes not be established, mainly due to involvement and overlapping of a variety of mechanisms like changes in fibrin network stability, strength, architecture and impairment in fibrinogen binding sites, resulting in qualitative and quantitative alteration. Therefore, we speculate that A/G genotype carriers with consequently higher plasma fibrinogen levels might have an association with an increased free flap thrombosis incidence and could be considered as a genetic marker associated with free flap thrombosis development (Drizlionoka *et al.*, 2019).

Interestingly, lower AT concentration does not inevitably ensure thrombosis. In a study of a family with inherited AT deficiency, there were few cases of pedigrees with low AT plasma levels and with no convincing history of thrombosis (van der Meer *et al.*, 1973). We did not recognise a difference in plasma AT level either in patients carrying a heterozygous or homozygous form of SNP rs2227589 in the *SERPINC1* gene with or without free flap thrombosis. Both patients with the heterozygous form of SNP and free flap thrombosis had age under 50 years, which agrees with data showing that the first thrombotic event usually occurs by the age of 30 years and by the age of 50 years, and that 50% of patients having AT deficiency will have had thrombosis episode, generally DVT.

Seven out of the 12 analysed patients in the free flap thrombosis case series were carriers of the *MTHFR* gene rs1801133 G/A genotype and had a higher serum homocysteine plasma level (> 12 mkmol/l). Two patients had solely a mutation in the *MTHFR* gene. One eventually had free flap thrombosis in arterial and the other in venous flow. There have been controversies about whether an increase in serum homocysteine levels is only associated with arterial thrombosis, or also has an effect on VTE (Falcon *et al.*, 1994; Ray *et al.*, 2002; Pathare *et al.*, 2004). Thus, although no general agreement has been reached, we support the idea that a higher homocysteine plasma levels might be associated with higher risk of free flap thrombosis, taking into account that both arterial and venous blood flow are involved.

There were few limitations in the study. The main limitation was the rather small sample size; therefore, we were not able to obtain strong findings by means of genotype intergroup analysis. In the Centre of Plastic and Reconstructive Microsurgery of Latvia about forty surgeries are performed per year, limiting the sample size. Moreover, the analysed clinical outcome in our study was free flap thrombosis. The average incidence of free flap thrombosis varies between 8 and 10% (Vanags *et al.*, 2020). In a recent study (Vanags *et al.*, 2020), free flap thrombosis developed in 15.5% of microvascular flap surgery patients. It explains the rather small sample size of patients with free flap thrombosis in our study. Therefore, we were not able to find statistical significant differences in coded plasma proteins and SNPs between those with and without free flap thrombosis, most likely due to incomparable unequal sample size groups and unequal distribution of genotype groups for each analysed polymorphism.

Additionally, we were not able to assess the function of coded plasma proteins related with analysed SNPs. Most importantly for AT and fibrinogen, it would be advisable to distinguish between qualitative or quantitative inherited thrombophilia disorders. AT function in order to understand whether patients are carrying SNP rather than I type AT deficiency would be essential.

As reported previously (Vanags *et al.*, 2020), although all patients in our study received standardised treatment, and received operations by experienced plastic surgeons who routinely perform microvascular flap surgery, we consider that technical surgical factors are one of most important limitations. We were also not able to avoid the multifactorial nature of a thrombotic event and the human factor in surgery and post-operative care. Moreover, for patients that had more than one SNPs, it was almost impossible to evaluate the impact on one particular SNP in such a small study population. Inherited thrombogenic factors and acquired thrombogenic factors overlap, making it infeasible to distinguish the role of the SNP *per se.* Population bearing more than one risk factor, either genetic or acquired, are at higher risk and VTE is considered as a multigenetic/multifactorial disease (Dahlbäck, 2005).

Finally, low incidence of rare allele SNPs and free flap thrombosis rate revealed a rather wide confidence interval.

CONCLUSIONS

Our preliminary results showed that a lower aPCR level was associated with *FVL* rs6025 C/T and higher fibrinogen plasma levels with *FGG* rs2066865 A/A, suggesting that these genotypes might predict higher free flap thrombosis risk. However, we found no significant association between the analysed SNPs and free flap thrombosis advancement.

We further aim to increase the study group to explore the associations between the determined SNPs and free flap thrombosis rate.

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IEDZIMTO TROMBOFĪLIJU LOMA TROMBOZES ATTĪSTĪBĀ MIKROVASKULĀRĀ LĒVERU ĶIRURĢIJĀ

Mikrovaskulārajā ķirurģijā defektu slēgšanai no muskuļaudiem, kaulaudiem un/vai saistaudiem tiek veidots brīvais lēveris. Neraugoties uz uzkrātām zināšanām, prasmēm un labu tehnisko nodrošinājumu, brīvā lēvera trombozēšanās joprojām ir aktuāla problēma. Lēveru trombozēšanos, iespējams, būtiski varētu ietekmēt iedzimtās trombofīlijas, kuru loma ir aprakstīta citu trombožu — kā venozo, tā arteriālo — attīstībā. Šajā prospektīvajā pētījumā mēs 152 pacientiem, kuriem veica mikrovaskulāro lēveru ķirurģiju, noteicām piecus biežākos literatūrā aprakstītos polimorfismus: rs6025 FV Leidena (*FVL*) gēnā, rs1799963 FII gēnā, rs2066865 Fibrinogēna Gamma (*FGG*) gēnā, rs2227589 *SERPINC1* gēnā un rs1801133 *MTHFR* gēnā, kā arī novērtējām šo polimorfismu ietekmi uz to regulēto proteīnu izmaiņām plazmā. Dati tika vākti laika posmā no 2016. līdz 2019. gadam Latvijas Mikroķirurģijas centrā Rīgā. Pirmie rezultāti uzrādīja, ka pacientiem ar C/T genotipu rs6025 *FVL* gēnā ir ievērojami zemāks rezistentā aktivētā proteīna C (aPCR) līmenis plazmā, nekā tiem ar C/C genotipu; $p = 0.006$ (CI 95%, 0.44–1.1). Papildus novērojām, ka pacientiem ar A/A genotipu rs2066865 *FGG* gēnā ir salīdzinoši augstāks fibrinogēna līmenis plazmā (1.81 ± 5.6 g/l), nekā tiem ar G/A un G/G genotipiem; $p = 0.04$ (CI 95%, 0.007–1.09); $p = 0.004$ (CI 95%, 0.48–2.49). Brīvā lēvera trombozi konstatēja 12 pacientiem (7.89 %). Tikai vienam no tiem varēja atrast sakarību starp brīvā lēvera trombozēšanos un zemu aPCR līmeni plazmā, pieņemot, ka lēvera trombozēšanos noteica mutācija rs6025 *FVL* gēnā. Secinājums: kaut gan zemāks aPCR līmenis plazmā korelēja ar C/T genotipu rs6025 *FVL* gēnā un augstāks fibrinogēna līmenis — ar A/A genotipu rs2066865 *FGG* gēnā, kas varētu liecināt par augstāku trombozēšanās risku, mēs neatradām ticamu saistību starp noteikto gēnu polimorfismiem un brīvā lēvera trombozēšanos.

Second publication

Article

Polymorphism rs2066865 in the *Fibrinogen Gamma Chain* (*FGG*) Gene Increases Plasma Fibrinogen Concentration and Is Associated with an Increased Microvascular Thrombosis Rate

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Abstract: *Background and Objective*: Thrombosis due to inherited hypercoagulability is an issue that has been raised in microvascular flap surgery previously. We analyzed the association of a single nucleotide polymorphism (SNP) in rs2066865 in the *fibrinogen gamma chain* (*FGG*) gene, alteration in plasma fibrinogen concentration, and presence of microvascular flap thrombosis. *Materials and Methods*: A total of 104 adult patients with microvascular flap surgery were subjected to an analysis of the presence of SNP rs2066865 in the *FGG* gene. Alterations in plasma fibrinogen concentration according to genotype were determined as a primary outcome, and flap thrombosis was defined as a secondary outcome. *Results*: Flap thrombosis was detected in 11.5% of patients ($n = 12$). Successful revision of anastomosis was performed in four patients, resulting in a microvascular flap survival rate of 92.3%. We observed an increase in plasma fibrinogen concentration in genotype G/A and A/A carriers (G/G, 3.9 (IQR 4.76-3.04); G/A, 4.28 (IQR 5.38-3.18); A/A, 6.87 (IQR 8.25-5.49) (A/A vs. G/A, $p = 0.003$ and A/A vs. G/G, $p = 0.001$). Within group differences in microvascular flap thrombosis incidence rates were observed—G/G 6/79 (7.59%); G/A 5/22 (22.7%); A/A 1/3 (33.3%) (OR 0.30 95%; CI 0.044 to 0.57), $p = 0.016$; RR 3.2—when G/G versus G/A and A/A were analyzed respectively. *Conclusions*: A/A and G/A genotype carriers of a single nucleotide polymorphism in rs2066865 in the *fibrinogen gamma chain* gene had a higher plasma fibrinogen concentration, and this might be associated with an increased microvascular flap thrombosis incidence rate. Determined polymorphism could be considered as a genetic marker associated with microvascular flap thrombosis development. To confirm the results of this study, the data should be replicated in a greater sample size.

Keywords: fibrinogen; *fibrinogen gamma chain* gene; polymorphism; microvascular flap thrombosis; free flap failure; free tissue transfer

1. Introduction

Microvascular flap surgery poses an ability to cover a broad range of tissue defects for reconstructive purposes, fostering functional and aesthetic recovery. Flap thrombosis is still the leading cause of flap loss, resulting in patient and surgeon disaffection as well as increased hospital stay and costs. Overall, thrombotic events are attributable to 3% to 12% of flap complications. Although technical factors are of constant concern, inherited thrombophilia has been mentioned as a potential risk factor. Factor V Leiden, prothrombin gene mutation, protein C and protein S deficiencies, antithrombin

deficiency, and mutation of the *methylene tetrahydrofolate reductase* gene causing hyper-homocysteinemia are mentioned in literature as contributing factors leading to flap thrombosis; therefore, preoperative thrombophilia screening could be a cost-effective tool for the prevention of microvascular flap thrombotic complications [1–3].

Thrombosis is a heterogeneous disorder [4]. Microcirculation in the flap is affected by a variety of factors. Hypercoagulation and thrombosis due to increased plasma fibrinogen concentration are still unclear. Both structural and functional defects of fibrinogen have been reported as risk factors for deep venous thrombosis [5], and few studies have described an increased risk of arterial thrombosis caused by fibrinogen abnormalities [6,7]. Fibrinogen also is viewed as an acute phase reactant. The fibrinogen level rises in relation to aging [8] and pathophysiological changes such as inflammation conditions [9], trauma, and malignancy [10–12].

Fibrinogen is a plasma glycoprotein with a molecular weight of 340 kDa and is synthesized by hepatocytes. Fibrinogen molecules are elongated structures 45 nm long, which are comprised of two sets of three polypeptide chains—A alpha; B beta; and gamma [13]. The three chains are encoded by three separate genes, *fibrinogen alpha* (*FGA*), *fibrinogen beta* (*FGB*), and *fibrinogen gamma* (*FGG*), clustered in a region of approximately 50 kb on chromosome 4q31.3 [14]. Polymorphism rs2066865 in the *FGG* gene is described as a reason for alterations in the coagulation system. Particularly, fibrinogen gamma H2 haplotype-tagging SNP (FGG 10034C > T) has been demonstrated as a risk factor for deep venous thrombosis [15]. Haplotype H2 is associated with reduced plasma fibrinogen ' levels, thus promoting resistance to fibrinolysis through unique binding sites for coagulation factor XIIIB. The γ' chain is also reported to protect from inactivation by antithrombin, which is thought to facilitate heparin resistance to clot-bound thrombin and further contributes to thrombin activity on the clot surface, thus increasing the time for thrombus formation [16].

Therefore, we tested the hypothesis that SNP rs2066865 in the *FGG* gene alters the level of plasma fibrinogen concentration and could associate with an increase in the microvascular flap thrombosis rate.

2. Materials and Methods

2.1. Subjects

In this observational case series, we included a total of 104 patients who underwent microvascular flap transfer in The Centre of Plastic and Reconstructive Microsurgery of Latvia from 2016–2018.

We enrolled all adult patients undergoing microvascular flap surgery during the study period. The protocol and the informed consent form, including the request to donate genetic material, were approved by the Latvian Central Ethics Committee (Nr.1/28-11-16). All patients provided written, informed consent.

The exclusion criteria were: pregnancy, peripartum period; transfusion of allogeneic blood components, and/or coagulation factors within 72 h perioperatively; proven left ventricular failure; allogenic bone marrow transplantation; liver failure, liver transplantation; and end-stage kidney disease.

For patients on direct oral anticoagulants, medication was stopped 72 h prior to sample collection to avoid any inaccuracy in testing. For patients taking a vitamin K antagonist, medication was stopped, according to the patient's international normalized ratio (INR) level, 4 to 5 d prior to sample collection. Patients with a high thrombosis risk were switched to low-molecular-weight heparin (LMWH) and discontinued use 12 h prior to the surgery.

We determined SNP rs2066865 (G > A) in the *FGG* gene and collected laboratory data, including total plasma fibrinogen concentration, platelets, white blood count, and C-reactive protein.

An interview was performed to register patient histories, particularly with interest in any previous thrombotic event, use of antithrombotics, regular medication (including oral contraceptives), family history of thrombotic events, and any previously diagnosed inherited thrombophilias (factor V Leiden, prothrombin gene mutation, antithrombin deficiency, protein C deficiency, protein S deficiency, etc.)

We registered all tissue injury causative factors and defined 30 d as a recent trauma period in case of trauma or polytrauma etiology.

In regards to demographics, history, and family history collection, patient interviews were performed by the same clinician. A positive history of thrombosis was defined as any thrombotic event with either arterial or venous origin. Group malignancy consisted of patients with orofacial tumors in different stages (i.e., active malignancy) after treatment with actinotherapy and palliation.

All patients received standardized general anesthesia. According to our local guidelines, administration of 10 mL/kg of Dextran 40 (Fresenius Kabi, Polska Sp. Warsaw, Poland) intravenously, at the time the anastomoses were assured, was performed. Patient temperatures were monitored during the surgery. We measured axillar and nasopharyngeal temperatures, and Δt 1 °C was considered as the optimal difference between core and peripheral temperature. Infusion of warm fluids and a warming blanket were used to prevent a drop in the core temperature. During the study, a total of 104 microvascular flap transfers were performed by highly trained specialists. The venue of thrombosis (e.g., arterial, venous, or both) was assured by direct visualization during the revision of anastomosis.

2.2. Laboratory Workup

Blood samples were drawn on the day of surgery prior to the induction of anesthesia and any crystalloid infusion. All tests were processed within an hour.

Total plasma fibrinogen concentration was measured by the Clauss method [17] (normal range 2–4 g/L) in citrated plasma using the STA-R COMPACT (Diagnostika Stago, Asnières-sur-Seine, France). Platelet count (normal range 150–400 × 10^9/L) was measured using the Sysmex XP-300 (Sysmex Corporation, Chouku, Kobe, Japan). C-reactive protein was measured in serum with radial immunodiffusion with fixed-point immune rate methodology (with normal range < 5 mg/L).

2.3. Genotyping

Genomic DNA was extracted using standard phenol-chloroform extraction protocol. Extracted DNA was dissolved in water. We used SNP genotyping assay C_11503414_10 for rs2066865. Genotyping was performed by Taqman Pre-Designed SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) [18] on a Viia7 Real-Time polymerase chain reaction (PCR) system (Applied Biosystems) according to the supplier's recommendations.

2.4. Data Statistical Analysis

We compared variables with independent-sample (unpaired) *t*-tests by using the SPSS 23 Statistics software (IBM Korea, Seoul, Korea). The Kolmogorov–Smirnov test was used to check whether the variables followed a normal distribution. Normally distributed, continuous variables were presented as mean ± standard deviation (M ± SD) and categorical variables as percentages (%). In case values did not follow a normal distribution, the medians and interquartile ranges (IQRs) were presented. Odds ratios and 95% confidence intervals were calculated to evaluate factor impacts between groups. Comparisons between genotype groups were performed with Kruskal–Wallis H tests for nonparametric variables and with ANOVA for parametric variables. Pearson's x^2 correlation coefficient and p values were calculated, and Spearman's rank correlation coefficient was used where applicable. Statistical significance was assumed as two-tailed $p < 0.05$.

3. Results

3.1. Clinical Course

In total, 104 consecutive patients scheduled for microvascular flap surgery were subjected to analysis after inclusion and exclusion criteria were met. We analyzed 86 males and 18 females classified according to rs2066865 in the *FGG*-carrying genotype: G/G, $n = 79$; G/A, $n = 22$; and A/A, $n = 3$. The genotype results of rs2066865 in the *FGG* A/G polymorphism were all in Hardy–Weinberg

equilibrium. The characteristics of the studied group are listed in Table 1 according to the carried genotype of rs2066865 in *FGG*.

Table 1. Group characteristics according to the presence of the determined SNP (*n*, %).

***SNP* rs2066865 (G > A) In the *fibrinogen gamma chain* (*FGG*) Gene**	**G/G *n* = 79**	**G/A *n* = 22**	**A/A *n* = 3**
Age (M ± SD)	39.62 (13.03)	46.21 (12.49)	52.0 (14.39)
Sex, female	7 (8.8)	4 (18.1)	1 (33.3)
Free flap thrombosis	6 (7.59)	5 (22.7)	1 (33.3)
History of thrombosis:	2 (2.53)	3 (13.6)	2 (66.7)
arterial (MI; CI)	2 (2.53)	-	
venous (DVT; PATE)	-	3 (13.6)	2 (66.7)
Family history of thrombosis	7 (8.9)	3 (13.6)	2 (66.7)
arterial (MI; CI)	5 (6.37)	-	-
venous (DVT; PATE)	2 (2.53)	3 (13.6)	2 (66.7)
Medication			
antithrombotics	2 (2.53)	1 (4.5)	3 (100)
oral contraceptives	-	1 (4.5)	-
Smoking	22 (27.8)	15 (68.1)	3 (100)
Metabolic disturbances *	8 (10.1)	2 (9.1)	1 (33.3)
Alcohol abuse	6 (7.6)	1 (4.54)	1 (33.3)
Defect etiology: trauma	32 (41)	12 (54.5)	43(100)
recent trauma, (<30 d)	22 (27.8)	8 (36.3)	2 (66.7)
polytrauma	6 (7.6)	2 (9.1)	-
chronic inflammation	15 (19)	10 (45.5)	1 (33.3)
malignancy	4 (5.1)	4 (18.2)	1 (33.3)
burn	1 (1.3)	1 (4.5)	-

* Diabetes mellitus; adipositas (BMI > 25); MI—myocardial infarction; CI—cerebral infarction; DVT—deep vein thrombosis; and PATE—pulmonary artery thromboembolism.

Flap thrombosis was detected in 11.5% of patients (*n* = 12). After unsuccessful salvage, re-anastomosis total flap necrosis eventuated in 7.7% (*n* = 8) of patients, and partial flap necrosis occurred in 4.8% (*n* = 5) of patients, resulting in a microvascular flap survival rate of 92.3% (Table 2).

Table 2. Microvascular flap thrombosis needing readmission to the operating theater (*n* = 12).

Flap	**Etiology**	**Defect Localization**	**Time to Thrombosis**	**Thrombosis Venue**	**Re-Anastomosis**	**Flap Necrosis**	**Salvage Measure**
Scapular/Parascapular	osteomyelitis	lower extremity	18 h	venous	yes	no	additional vein anastomosis
Medial plantar artery flap	osteomyelitis	lower extremity	no data	arterial	yes	no	additional vein anastomosis
LAF	trauma	lower extremity	23 h 55 min	venous	yes	no	additional vein anastomosis
Scapular/Parascapular	osteomyelitis	lower extremity	23 h 30 min	venous	yes	no	-
Scapular/Parascapular	polytrauma	lower extremity	23 h 15 min	venous	yes	yes	additional vein anastomosis
Sartorius free flap	trauma	lower extremity	no data	arterial and venous	no	yes	NPWT; STSG
Osteocutaneus FF	trauma	lower extremity	120 h	arterial and venous	yes	yes	local muscle flap and STSG
Osteocutaneus FF	osteomyelitis	lower extremity	144 h	arterial and venous	yes	yes	local flap
Serratus anterior muscle flap	osteomyelitis	lower extremity	74 h 20 min	arterial and venous	yes	yes	NPWT; STSG
RFF	malignancy	orofacial	13 h 10 min	arterial	yes	yes	local flap
LAF	trauma	upper extremity	no data	venous	yes	yes	ALT flap
Scapular/Parascapular	trauma	lower extremity	49 h	venous	no	yes	local flap; STSG

NPWT—negative pressure wound therapy; STSG—split thickness skin graft; LAF—lateral arm flap; FF—fibula flap; RFF—radial forearm flap; and ALT—anterolateral thigh flap.

3.2. The relationship between single nucleotide polymorphism rs2066865 in the FGG gene and free flap thrombotic complications.

A higher incidence of flap thrombosis was detected in homozygous A/A and heterozygous A/G genotypes of SNP rs2066865 in *FGG* carriers compared to the G/G genotype carrier (33.3%; 22.7% vs. 7.59%) respectively (OR 0.3 CI, 95% 0.044 to 0.57, p = 0.016, Table 3). A risk ratio analysis showed that patients carrying A/A and G/A genotypes were 3.2 times more likely to have flap thrombosis compared to G/G genotype holders.

Table 3. Association of rs2066865 in the *FGG* gene with free flap thrombosis.

SNP (Gene)	Genotypes	Thrombosis Group	Non-Thrombosis Group	OR (CI, 95%) [a]	*p*
rs2066865 in *FGG*	GG/GA/AA	6/5/1	73/17/2	0.30 (0.044 to 0.57)	0.016

[a] OR and p value were calculated using logistic regressions adjusted for sex and age, history of smoking, and metabolic disturbances.

3.3. The Relationship between Single Nucleotide Polymorphism rs2066865 in the FGG gene and Inflammatory Parameters and Platelet Count.

Although we observed a positive association between C-reactive protein and increased plasma fibrinogen concentrations (r = 0.580, p = 0.002), white blood count, C-reactive protein, and platelet count parameters did not differ between G/G, G/A, and A/A genotype carriers. A/A carriers showed a tendency to have higher plasma C-reactive protein levels without reaching significance (G/G 18.39 vs. G/A 17.62 vs. A/A 31.00 (mg/L); p = 0.372).

3.4. The Relationship between Single Nucleotide Polymorphism Rs2066865 in the FGG Gene and the Plasma Fibrinogen Level.

Patients with SNP rs2066865 in *FGG* gene carriers of A/A and G/A genotypes had higher levels of plasma fibrinogen concentrations (G/G 3.9 (IQR 4.76–3.04); G/A 4.28 (IQR 5.38–3.18); A/A 6.87 (IQR 8.25–5.49); A/A vs. G/A, p = 0.003; and A/A vs. G/G, p = 0.001), as shown in Figure 1.

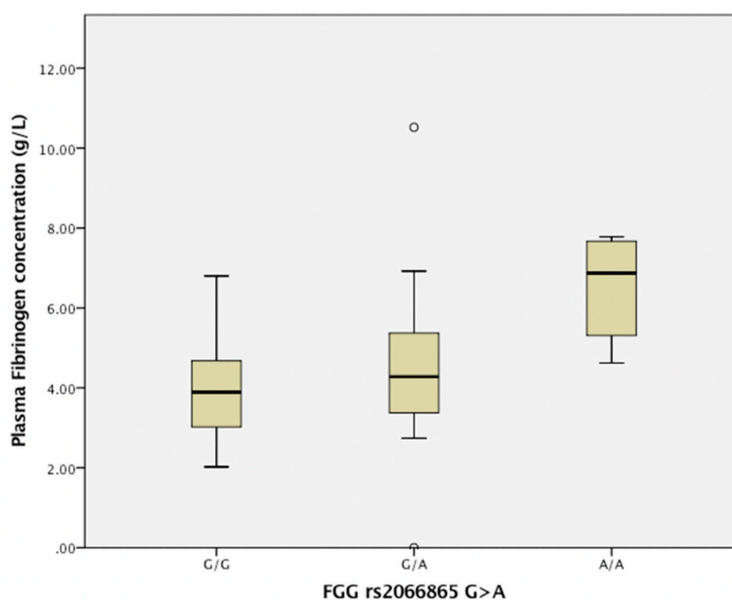

Figure 1. Comparison of plasma fibrinogen concentration in patients with single nucleotide polymorphism rs2066865 in the *FGG* gene (G > A); values are median (IQR range). Genotype A/A carriers had a higher plasma fibrinogen concentration compared to G/A genotype carriers (p = 0.003) and compared to G/G genotype carriers (p = 0.001).

4. Discussion

We evaluated the association between a single nucleotide polymorphism rs2066865 in the *FGG* gene, alteration in plasma total fibrinogen concentration, and thrombotic events in microvascular flap surgery.

In our studied group, patients with a variety of injury-causing factors underwent microvascular flap surgery. Overall, our results are within the range of microvascular flap survival rates reported in literature. Shechter et al. analyzed two groups of patients with breast cancer and radiation therapy who underwent postmastectomy breast reconstruction with a deep inferior epigastric artery perforator (DIEP) flap. They reported a total of 8.3% flap loss and vascular anastomosis failure in 5.6% of patients with additional boost radiation compared to zero percent for standard post mastectomy radiation therapy. They concluded that added radiation therapy potentially increased the risk for surgical complication [19]. Bendon et al. retrospectively analyzed patients with lower limb trauma and acute lower limb reconstruction. They had 3 out of 48 (6.25%) patients with flap failure. After revision of arterial and venous anastomosis, the primary complication was due to venous thrombosis in two cases within the first postoperative day [20].

An increased plasma fibrinogen concentration has been proposed to reflect the inflammatory state [11], and inflammation is a well-established risk factor for arterial thrombosis [4]. Particularly, γ' fibrinogen, one of the fibrinogen molecule chains, has shown a strong association with inflammation. Studies showed that γ' fibrinogen was highly associated with C-reactive protein levels. In a similar pattern, γ' fibrinogen levels were elevated during the acute phase and decreased with convalescence time [9]. We found an overall positive correlation between elevated plasma fibrinogen concentration and C-reactive protein, except in homozygous (A/A) SNP carriers, where high fibrinogen levels were significantly associated with increased levels of white blood count, whereas no association with C-reactive protein levels was established.

Interestingly, the reduced levels of fibrinogen γ'/γ and reduction in the γ'/γ ratio were strongly associated with an increase in venous thrombosis. In a study by Uitte de Willige et al., five haplotypes were identified in the *FGG* gene. None of the haplotypes was associated with alterations in the total fibrinogen level, although one of the haplotypes, H_2, was strongly associated with reduced fibrinogen γ' levels and a reduced γ'/γ ratio, and both markers were associated with increased risk for venous thrombosis. This indicates that only one *FGG* H_2 haplotype increases the risk of venous thrombosis by reducing fibrinogen γ' levels [5]. In our study, because of a lack of resources, we could not perform haplotype reconstructions for the determined polymorphism to compare the data.

Rosendaal et al. found correlation between increased age and changes in fibrinogen levels and an increased risk for thrombosis mainly of venous origin [8]. We found an increase in mean age in patients with homozygosity (A/A) and high plasma fibrinogen levels, though no correlation with an increase in flap thrombosis rate was observed within this group.

The study by Hollenbeck et al. presented data about the role of preoperative platelet count as a predictor of free flap thrombosis. They evaluated a total of 565 acute trauma patients who underwent lower extremity free tissue transfer, and they concluded that acute trauma patients with elevated preoperative platelet counts were at an increased risk for lower extremity free flap thrombotic complications [21]. In contrast, we did not find a significant association between an increase in platelet count and the rate of free flap thrombosis neither in homozygous nor heterozygous SNP carriers.

There were a few limitations in this study. First, as this is a clinical, observational case series study, our sample size was rather small for genetic study. In the Centre of Plastic and Reconstructive Microsurgery of Latvia, around forty microvascular flap surgeries are performed per annum; therefore, we were not able to strengthen our finding by means of genotype intergroup analysis, demonstrating that A/A carriers were associated with an increased risk of microvascular flap thrombosis. In addition, development of thrombosis is multifactorial, including a patient's related factors and other gene mutations, such as factor V Leiden and prothrombin gene mutation in the heterozygous state, which both present the most common hereditary thrombophilias but possess a relatively low risk

for venous thrombosis. Risk increases in the presence of other risk factors (e.g., trauma, major surgery, immobilization, etc.). Deficiencies in antithrombin, protein C, protein S, factor V Leiden, and prothrombin gene in the homozygous state hold greater risk for venous thrombosis, but these mutations are considerably rarer. The observed patients and their family members had a positive history of thrombotic events. As we were focusing on a particular SNP, we were not able to exclude the role of other gene mutations. To add, many patients harbor one or more hereditary or biological risk factors that are not recognized with available methods, and venous thrombosis due to biological causes is found in up to 16.93% of unselected patients [22]. It would be essential to observe gene combinations and possible interactions, which should be part of further investigation.

Secondly, our local perioperative guideline suggests administration of Dextran 40 because of its rheological properties in all patients at the time anastomosis is assured. Thus, its contribution to outcomes in our studied group should be kept in mind. The multiplicity of Dextran 40 effects is governed either by the ability to expand the volume of plasma and, therefore, reduce the hematocrit, which in turn lowers blood viscosity, and, on the other hand, by inhibiting the formation of erythrocyte aggregates as reported by Rosenblum in *Nature* (1968) [23]. In the study reported by Robles et al. [24], Dextran 40 was used to investigate its antithrombotic properties, particularly on platelet function in patients with peripheral artery disease (PAD). They found no difference in spontaneous platelet aggregation and agonist-induced platelet aggregation in response to an increasing Dextran 40 concentration in vitro; however, in patients with known PAD, collagen-induced platelet aggregation and adenosine diphosphate induced aggregation were significantly lower. Whether it plays a role in inhibiting the function of fibrinogen is also not clear. Notably, it has an effect in reducing the density of the fibrinogen network by means of blood volume expansion, thereby modifying blood rheology. In addition, recent data show that the molecular weight of the volume expanders mainly determines the duration of intravascular persistence, and it is not the determining factor in comprising coagulation [25].

Another limitation of the study is the multifactorial nature of the thrombotic event and the difficulty in analyzing the variables separately. Patient-related factors for both arterial and venous thrombosis are described in arterial and venous risk assessment scores, CHAD2S2-VASc and Caprini scores, respectively. In microvascular flap thrombosis, both arterial and venous pools play a role; therefore, we were not able to define an appropriate score for patient thrombotic risk assessment. However, it has been mentioned that the 2005 Caprini Risk Assessment Model could also be applied to patients undergoing microvascular flap surgery, although its ability to predict microvascular thrombosis has not been evaluated [26].

And finally, the patients were localized in the trauma department, and during the postoperative period, a lack of critical clinical monitoring of flap perfusion provoked late readmission to the operating theater, resulting in a 40% salvage rate after revision of anastomosis.

5. Conclusions

The results demonstrate that A/A and G/A genotype carriers of a single nucleotide polymorphism in rs2066865 in the *FGG* gene have higher plasma fibrinogen concentrations, and this might be associated with an increased free flap thrombosis incidence rate.

The determined polymorphism could be considered as a genetic marker associated with microvascular flap thrombosis development. Patients that have the determined polymorphism could benefit from perioperative thromboprophylaxis. To confirm the results of this study, the data should be replicated in a greater sample size.

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