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DOCTORAL THESIS

Title: Differences in hemocoagulation in patients with metabolic disorders

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Hradec Králové, 2024

STATEMENT OF AUTHORSHIP

I hereby declare that I am the sole author of this doctoral thesis and that this thesis is my original work generated independently under the guidance of my supervisor Prof. Přemysl Mladěnka and my consultant Dr. Alejandro Carazo. All the sources of information and literature I have used for the redaction of this doctoral thesis are cited in the text and properly listed in the "References" section of this thesis. This work has not been used to obtain another or the same degree.

I agree to make my thesis available through the information system of Charles University.

In Hradec Králové, Jaka Fadraersada, M.Sc.

Date: August 2024

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ABSTRACT

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Hyperglycemia, insulin resistance, and hyperlipidemia can enhance procoagulant activity which is strongly correlated with an increased risk of cardiovascular and cerebrovascular events. Thrombosis becomes the major culprit of these events. Antiplatelet drugs are mostly used to prevent arterial thrombotic events such as acute myocardial infarction and ischemic stroke, while venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE), is prevented and cured using anticoagulants.

Direct oral anticoagulants (DOACs) are now widely used in clinical praxis replacing vitamin K antagonists (VKAs) and partly heparins, since they are safer in terms of drug-drug and food-drug interactions, have fewer adverse effects, are administered in fixed doses, and mostly used orally. There are currently two groups of DOACs, factor Xa inhibitors (FXa-Is) and direct thrombin inhibitors (DTIs).

Our study tested *ex vivo* the effects of clinically relevant concentrations (1 μM) of two FXa-Is (xabans; rivaroxaban and apixaban) and two DTIs (gatrans; dabigatran and argatroban), anticoagulants that are commonly used in clinical praxis. The investigation was performed both in healthy individuals and patients with familial hypercholesterolemia (FH) or type 1 diabetes mellitus (DMT1) by measuring

their effect on blood coagulation using prothrombin time (PT; reported as international normalized ratio /INR/) and activated partial thromboplastin time (aPTT) assays. In addition, 143 compounds were screened for their potential prolongation in coagulation *ex vivo* in order to find novel anticoagulant scaffold(s) or warn about this property when not desired.

Based on the results and analyses in healthy populations, body mass index (BMI) and lipid serum levels were negatively correlated with PT/INR and aPTT, which means that coagulation is facilitated in persons with higher BMI and lipid levels. Interestingly, DOACs prolonged PT/INR and aPTT more extensively in FH and DMT1 patients than in generally healthy controls, although there were no significant differences in coagulation system activity between healthy donors and patients when no anticoagulant drug was added. Lower vitamin K levels in the patient group might be the reason behind this phenomenon since it is critical for the production of 7 coagulation and anticoagulation factors. Interestingly, as serum levels of lipids were well managed in these patients, also lower lipid levels can contribute to the observed phenomenon. These novel findings suggested that DOACs used in patients with metabolic diseases might have higher efficacy, on the other hand, they can increase the potential for adverse effects, mainly bleeding.

Unexpected bleeding as the adverse effects as well as the need for novel anticoagulants stimulated us to another series of experiments in the search for compounds having anticoagulant activities. We have tested different scaffolds including the class of (iso)flavonoids and their metabolites, alkaloids, catechol, and synthetic heterocyclic compounds, that have mostly also antiplatelet effects. A combination of antiplatelet and anticoagulant effects can be advantageous. However, none of the tested compounds showed strong anticoagulation effects. In a few cases, mild and clinically irrelevant activities were observed as they were found at a relatively high concentration (100 μM).

In conclusion, this study emphasizes that lipid and vitamin K levels, as well as BMI, are important determinants of the activity of the coagulation system and can affect the effects of clinically used DOACs. Importantly, these outcomes are coming from our *ex vivo* study, which indicates that *in vivo* confirmation is still missing. We implicate also the role of low-degree inflammation as the impact of BMI was observed notwithstanding the blood was incubated in all donors with the same concentration

of anticoagulants. The second conclusion is that anticoagulant activity seems to be a very rare phenomenon as none of 143 compounds tested by us can be considered as an active anticoagulant.

ABSTRAKT (v češtině)

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Název dizertační práce Rozdíly v hemokoagulaci u pacientů s metabolickými chorobami

Hyperglykémie, inzulinová resistence a hyperlipidemie mohou zvýšit prokoagulační aktivitu krve, která silně koreluje se zvýšeným rizikem kardiovaskulární nebo cerebrovaskulární události. Trombóza je v tomto případě hlavní příčinou. Protidestičková léčiva se používají hlavně k prevenci arteriálních trombotických událostí jako je akutní infarkt myokardu a ischemická cévní mozková příhoda, zatímco žilní tromboembolická nemoc (VTE, z angl. venous thromboembolism), zahrnující hlubokou žilní trombózu a plicní embolii, se léčí antikoagulancii, která se také podávají preventivně v tomto případě.

Přímá perorální antikoagulancia (DOACs, z angl. direct oral anticoagulants) široce nahradila v klinické praxi antagonisty vitaminu K a částečně i hepariny, a to z důvodu větší bezpečnosti, a to jak kvůli menšímu počtu interakcí léčivo-léčivo a léčivo-potravina, tak díky menšímu počtu nežádoucích účinků, a také podávání ve fixní dávce a většinou navíc perorálně. V současné době rozlišujeme 2 typy DOACs, inhibitory faktoru Xa (FXa-Is, známé také jako xabany) a přímé inhibitory trombinu (DTIs, angl. direct thrombin inhibitors, známé také jako gatrany).

V naší *ex vivo* studii jsme otestovali účinky klinicky relevantních koncentraci (1 μM) dvou FXa-Is (rivaroxaban a apixaban) a dvou DTIs (dabigatran a argatroban), tedy antikoagulancií, která se běžně používají v klinické praxi. Výzkum byl proveden jak u zdravých jedinců, tak u pacientů trpících familiární hypercholesterolemií (FH) nebo diabetes mellitus 1. typu (DMT1), a to pomocí změření jejich účinku na koagulaci krve přes protrombinový čas (PT; vyjádřený přesněji i jako INR – mezinárodní normalizovaný poměr, z angl. international normalised ratio) a aktivovaný parciální tromboplastinový čas (aPTT). Navíc bylo v rámci screeningu otestováno celkem 143 sloučenin, zda nemohou mít účinky na koagulaci krve z důvodu nalezení nových modelových antikoagulačních struktur nebo varování před antikoagulačními účinky, pokud nejsou u těchto látek vhodné.

Podle našich výsledků a analýz vzorků krve u zdravé populace, koreloval index tělesné hmotnosti (BMI, body mass index) a hladina lipidů v séru negativně s PT/INR a aPTT, což znamená, že při vyšším BMI a vyšší hladině lipidů dochází k urychlení koagulace. Zajímavé je, že DOACs prodloužily PT/INR a aPTT výrazněji u FH a DMT1 pacientů než u obecně zdravých dobrovolníků, zatímco bez přídavku antikoagulancií nebyly nalezeny významné rozdíly v koagulaci mezi zdravými jedinci a pacienty s těmito chorobami. Nižší hladina vitaminu K u pacientů může být jedním z důvodů těchto rozdílu, protože vitamin K je nezbytný pro tvorbu 7 koagulačních a antikoagulačních faktorů. Zajímavé také je, že hladiny lipidů v séru byly nižší u těchto pacientů, a tak použití hypolipidemik také pravděpodobně přispělo k pozorovanému jevu. Tyto nové nálezy tedy naznačují, že DOACs mohou mít u pacientů s metabolickými chorobami vyšší účinnost. Na druhé straně tím ale mohou také zvýšit riziko nežádoucích účinků, a to zejména krvácení.

A právě riziko nežádoucího neočekávaného krvácení ale také potřeba nalezení nových antikoagulancií nás stimulovalo k provedení série dalších experimentů s cílem nalézt látky s antikoagulanční aktivitou. Vyzkoušeli jsme různé chemické základní struktury jako (iso)flavonoidy a jejich metabolity, alkaloidy, katecholy a heterocyklické sloučeniny, které většinou měly také protidestičkové účinky. Kombinace protidestičkové a antikoagulační aktivity může být výhodná, avšak žádná z testovaných látek se neukázala mít silný antikoagulační účinek. V několika případech byly nalezeny mírné a klinicky nerelevantní účinky, protože byly pozorovány v relativně vysokých koncentracích (100 μM).

Závěrem lze zdůraznit, že tato studie ukázala, že hladiny lipidů a vitaminu K, stejně jako BMI, jsou důležitými faktory ovlivňujícími aktivitu koagulačního systému a mohu tak změnit účinky klinicky používaných DOACs. Je také potřeba připomenout, že tyto výsledky vycházejí z naší *ex vivo* studie, což ukazuje, že potvrzení v *in vivo* podmínkách bude potřebné. Domníváme se, že také nelze vyloučit vliv zánětu o nízké intenzitě, protože vliv BMI byl pozorován i přesto, že krev všech dárců byla inkubována se stejnou koncentrací antikoagulancií. Druhým hlavním závěrem je fakt, že antikoagulační aktivita bude relativně neobvyklým jevem, protože žádné ze 143 sloučenin, námi testovaných, nelze považovat za účinné antikoagulancium.

LIST OF ABBREVIATIONS

AMI	acute myocardial infarction
aPTT	activated partial thromboplastin time
CD	cluster of differentiation
DIC	disseminated intravascular coagulation
DMT1	type 1 diabetes mellitus
DMT2	type 2 diabetes mellitus
DOACs	direct oral anticoagulants
DTIs	direct thrombin inhibitors
FDP	fibrin degradation products
FH	familial hypercholesterolemia
FI / FIa	coagulation factor I (fibrinogen) / activated factor I (insoluble fibrin)
FII / FIIa	coagulation factor II (prothrombin) / activated coagulation factor II (thrombin)
FVa	activated factor V
FVIIa	activated factor VII
FVIIIa	activated factor VIII
FIXa	activated factor IX
FXa	activated factor X
FXIa	activated factor XI
FXIIa	activated factor XII
FXIIIa	activated factor XIII
FXa-Is	factor Xa inhibitors
GIT	gastrointestinal tract
HDL	high-density lipoprotein
HIT	heparin-induced thrombocytopenia

HMWK	high molecular weight kininogen
INR	international normalized ratio
ISI	international sensitivity index
LDL	low-density lipoprotein
LMWH	low molecular weight heparin
PAI-1	plasminogen activator inhibitor 1
PF4	platelet factor 4
PT	prothrombin time
ROS	reactive oxygen species
SOD	superoxide dismutase
TAFI	thrombin-activatable fibrinolysis inhibitor
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TPA	tissue plasminogen activator
UFH	unfractionated heparin
vWF	von Willebrand factor
VKAs	vitamin K antagonists
VKCOR1	vitamin K epoxide reductase complex subunit 1
VTE	venous thromboembolism

1. INTRODUCTION

Coagulation is a physiological complex response to bleeding caused by tissue injury, in which each step activates the next one and ultimately produces a blood clot, in order to stop bleeding [1]. There are two phases of hemostasis, primary and secondary. The primary hemostasis forms an unstable platelet plug at the site of injury and is followed by the secondary hemostasis, which is an activation of the coagulation cascade forming and stabilizing the plug, stopping the blood flow and minimizing blood loss [2].

Plasma clotting cascade consists of a series of reactions involving the activation of zymogens (inert precursors of enzymes) via specific proteolysis. The resulting enzymes are catalytically active serine proteases, yet they have low enzymatic activity *in vitro* as isolated proteins [3]. Contrarily, binding of a typical clotting protease to a specific protein cofactor on a suitable membrane surface markedly potentiates the enzymatic activity, often by as much as five orders of magnitude or more. Also, protein cofactors of the blood clotting cascade generally circulate in the plasma as inert pro-cofactors that must be converted into active cofactors, again through proteolysis [1]. Activated proteins known as coagulation factors are designed with “a” (e.g. Xa, Va, etc.)

Coagulation consists of three pathways, the extrinsic, intrinsic, and common pathways (Figure 1). The extrinsic pathway involves initiation by coagulation factor III (tissue factor /TF/) and its interaction with coagulation factor VII, whereas coagulation factors XII, XI, IX, and VIII are utilized in the intrinsic pathway. The common pathway uses coagulation factors X, V, II (prothrombin), I (fibrinogen), and XIII [4, 5].

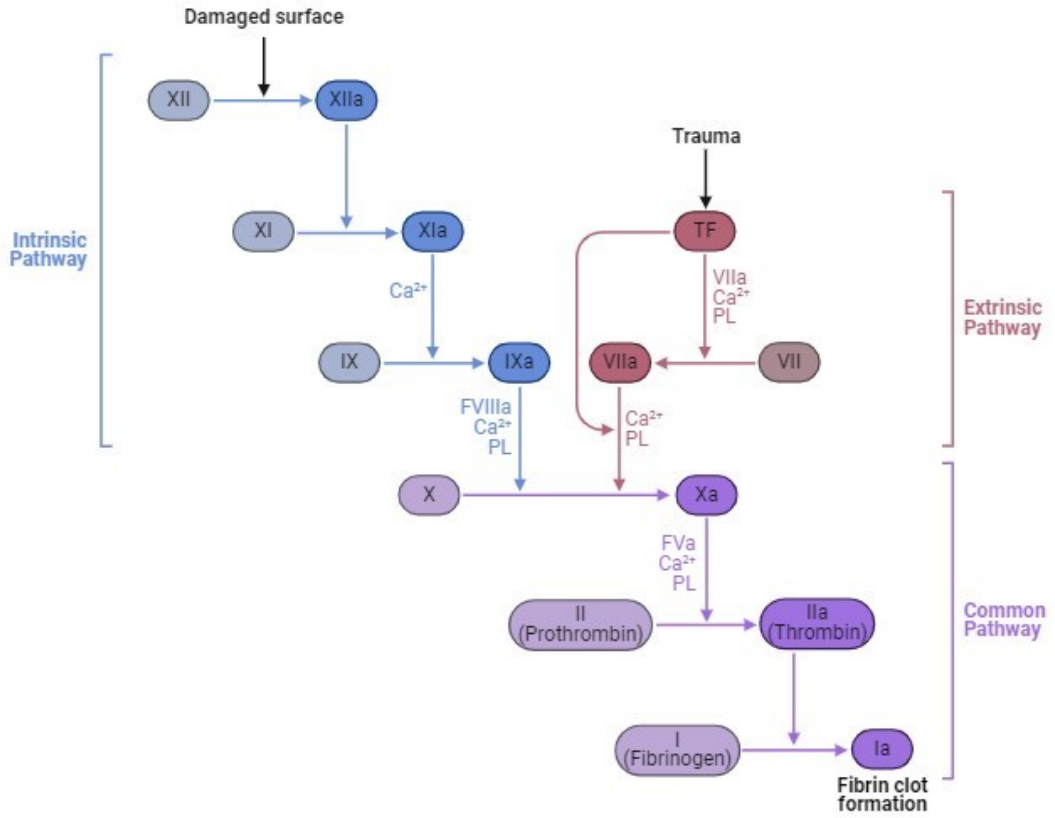


Figure 1. **Overview of the coagulation cascade**. Adopted and modified from Smith et al., 2015 [1].

1.1 Coagulation Pathways

1.1.1 The extrinsic pathway

The extrinsic pathway is initiated by an injury that causes blood to escape from the vascular system [4]. This pathway is triggered when TF is exposed to the bloodstream due to endothelial or more complex injury. TF, also sometimes known as thromboplastin, coagulation factor III, or CD 142, is constitutively expressed by certain cells within the vessel wall in the subendothelial layer and on cells surrounding blood vessels, such as vascular smooth muscle cells, pericytes, and adventitial fibroblasts. Hence, physiologically it is not in contact with blood [6-8].

Factor VII (FVII, known as well as proconvertin) needs to bind to TF before its activation to active enzyme FVIIa. FVII is synthesized in the liver and circulates in plasma at a concentration of about 10 nM [9]. FVII occurs in the plasma as an inert zymogen with approximately 1% of the active form FVIIa. The conversion of zymogen FVII into FVIIa happens through proteolysis of a single peptide

bond, resulting in two disulfide-linked polypeptide chains [10]. The reaction can be mediated by more enzymes (thrombin, activated coagulation factors Xa, IXa, XIIa, and the FVIIa-TF complex itself). The active forms of most coagulation serine proteases have extremely short plasma (seconds to minutes) half-lives because plasma contains high concentrations of protease inhibitors. However, free FVIIa is not susceptible to most of these inhibitors and consequently circulates with a half-life of approximately 2 hours. Inactive FVII has an even longer half-life of 5 hours [11, 12].

TF forms a catalytic complex with FVIIa (TF:FVIIa), the so-called extrinsic factor tenase complex, which remains on the phospholipid surface of the cell membrane. There are two ways to form the TF:FVIIa complex: direct capture of FVIIa by TF or by capture of FVII and subsequent conversion to FVIIa via proteolytic cleavage [13]. This complex activates the other zymogens, coagulation factors IX (FIX) and X (FX) [14, 15]. Activated FX (FXa), in the presence of activated coagulation factor Va (FVa), calcium ions (designed originally as coagulation factor IV), and phospholipids, forms the prothrombinase complex, which converts prothrombin (FII) into thrombin (FIIa) [16]. Activated FIX (FIXa) with its activated cofactor, FVIIIa, forms the intrinsic factor tenase complex (FIXa:FVIIIa) in the presence of calcium. The formation of the intrinsic tenase complex is essential for amplification of the clotting process required for sustained hemostasis, by increased FXa production and accelerated thrombin production [15, 16].

1.1.2 The intrinsic pathway

The intrinsic or contact pathway is initiated by factor XII (FXII, Hageman factor) activation, with two critical components of the contact system that include prekallikrein and high molecular weight kininogen (HMWK) [17]. FXII is synthesized in the liver and is activated *via* proteolysis by kallikrein, plasmin, and autoactivation by FXIIa [1]. Prekallikrein is also made in the liver and circulates in plasma bound from 75% to HMWK [18]. HMWK is produced by granulocytes, platelets, and endothelial cells, but the main source of plasma HMWK is also the liver [19]. The major contribution of HMWK to the

intrinsic pathway is the facilitation of negatively charged surface contact with FXIIa and it is required for the efficient formation of kallikrein in surface-activated plasma [1, 20].

Negatively charged molecules, including dextran sulfate and silica, are able to change the conformation of FXII, in a process so-called autoactivation, resulting in the generation of active FXII (FXIIa) [21-23]. The autoactivation of FXII can also be mediated by other physiological molecules, including RNA, DNA, and polyphosphate [24-26]; by the plasma membrane of activated platelets [27].

Activation of the intrinsic pathway can be enhanced by both prekallikrein and HMWK. During the process of contact activation, initially generated FXIIa can activate prekallikrein forming α-kallikrein that can itself activate FXII establishing a positive feedback loop [28]. Moreover, α-kallikrein-mediated activation of FXII is approximately 30 times more efficient than autoactivation mediated by contact with a negative surface [21]. The reciprocal activation of FXII and prekallikrein is further amplified through the cofactor activity of HMWK [29]. The produced FXIIa then activates its downstream substrate, FXI to FXIa. Limited proteolysis of FIX to FIXa by FXIa then allows for the formation of the intrinsic tenase complex (FIXa:FVIIa), which in turn activates FX to FXa and hence leads to thrombin generation [1].

The intrinsic pathway can also be activated by components of the extrinsic and common pathways. Cross-activation is important for the sustained activation of coagulation as the activity of the TF-FVIIa complex, which can efficiently activate FIX, is inhibited by tissue factor pathway inhibitor (TFPI) [30]. More recently, it has been shown that the TF-FVIIa and TF-FVIIa-FXa complexes can also activate FVIII. Through this mechanism, the extrinsic Xase, TF-FVIIa, and the product FXa can directly promote the formation of the active intrinsic Xase, FVIIIa-FIXa [31, 32]. Moreover, the terminal coagulation protease thrombin can also activate FXI leading finally to a marked amplification of the thrombin generation itself [33].

1.1.3 Common pathway

The common pathway of the coagulation cascade is the final sequence of events that leads to stable blood clot formation. This pathway begins with the activation of FX (Stuart-Prower factor) to FXa. This activation can occur, as mentioned above, through either the extrinsic pathway (via TF:FVIIa complex) or the intrinsic complex (via the intrinsic tenase complex consisting of FIXa, FVIIIa, calcium ions, and phospholipids) [34].

FXa, in the presence of its cofactor FVa, calcium ions, and phospholipids, forms the prothrombinase complex on the surface of activated platelets. The prothrombinase complex is a crucial enzymatic assembly that catalyzes the conversion of prothrombin to thrombin [35]. Thrombin generation is a pivotal step in the coagulation cascade. Thrombin is a serine protease that plays multiple roles not only in the coagulation process. Thrombin amplifies the coagulation cascade by a positive feedback. It activates several upstream clotting factors, including FV, FVII, FVIII, and FXI, enhancing the overall clotting response, but it also activates platelets and therefore further stimulates the whole process of hemostasis [36, 37].

Thrombin converts fibrinogen (FI), a soluble plasma protein, into insoluble fibrin (FIa). Fibrin strands form the structural framework of the clot. Fibrin monomers polymerize to form a fibrin mesh that traps blood cells and forms the basis of the clot [38]. Thrombin also activates FXIII (fibrin-stabilizing factor) to FXIIIa, a transglutaminase that cross-links fibrin fibers, stabilizing the fibrin clot and making it resistant to fibrinolysis [39, 40].

1.2 Diabetes mellitus, hypercholesterolemia, and their impact on coagulation

Diabetes mellitus (DM) is a metabolic disorder characterized by a resistance to the action of insulin, insufficient insulin secretion, or both. The clinical manifestation of these disorders is hyperglycemia. The vast majority of patients with DM are classified into one of two broad categories: type 1 DM (DMT1) resulting from the autoimmune destruction of the β-cells of the pancreas and leading

to an absolute deficiency of insulin, or type 2 DM (DMT2) defined by the presence of insulin resistance that leads to the inability to normalize plasma glucose levels. There is also a progressive loss of β-cell over time [41], as shown in Figure 2.

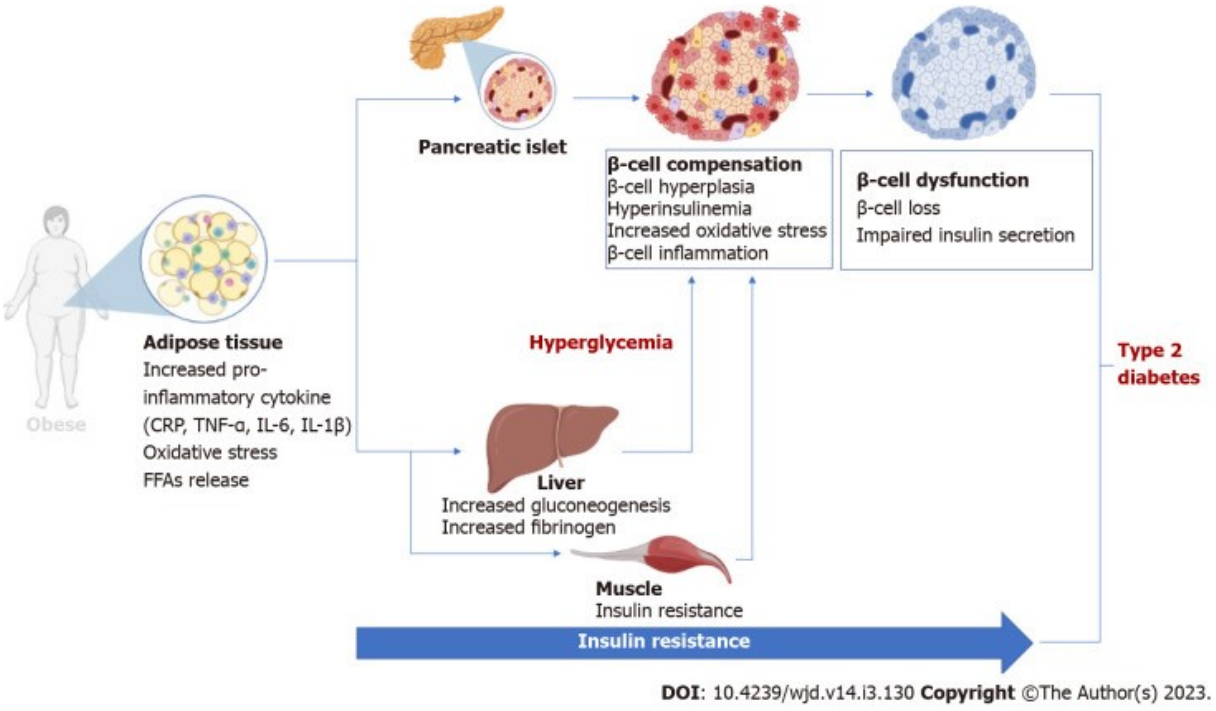


Figure 2. An overview of the pathological processes associated with progressive loss of β-cell function in DMT2. The figure was taken from Dludla et al., 2023 [42].

In patients with DM, metabolic impairment including hyperglycemia and/or insulin resistance disturb the physiological balance in coagulation and fibrinolysis. They can also cause alterations in platelet number and activation cascades, as well as qualitative and/or quantitative modification of coagulation and fibrinolytic factors [43]. An increase in mean platelet number and hypersensitivity is observed in patients with hyperglycemia [44-46]. A potential underlying mechanism for platelet hyperactivity in this condition could be an upregulated expression or increased formation of pro-aggregatory factors like P-selectin, thromboxane A_2, and von Willebrand factor (vWF), amplifying the aggregation and adhesion of platelets [47]. Furthermore, elevated concentrations of coagulation factors is also reported in DM patients, such as factor V [48], factor VIIa [48, 49], factor VIII [48, 50], factor X [48], factor XI [51], factor XIIa [50, 52], factor XIII [53], prothrombin [48], and fibrinogen [54, 55].

In addition, not only changes in pro-coagulation proteins can be found in patients with diabetes, but several anticoagulation proteins have also a reduced plasma concentration in both types of diabetes, including protein C [48, 56] and protein S [57]. Interestingly, ambiguous results were reported for the antithrombin III concentration in DMT2 patients. Whereas one study reported a reduced concentration [58], two other studies documented elevated concentrations of this protein [50, 59].

In terms of fibrinolysis disturbance, the availability of tissue plasminogen activator (TPA) is decreased because of the elevated concentration of plasminogen activator inhibitor 1 (PAI-1) associated with glucose intolerance both in healthy individuals [60] and patients with diabetes [61-63]. The concentration of other inhibitors of fibrinolysis, including thrombin-activatable fibrinolysis inhibitor (TAFI) [64, 65] and α2-macroglobulin [58, 66] are also elevated in patients with diabetes mellitus.

As DMT2 is associated with metabolic syndrome, it is frequently linked with abnormalities in blood lipids. Hyperlipidemia describes a condition in which there are elevated levels of serum lipids, including triglycerides and cholesterol or both (≥240 mg/dL) [67]. This condition is an independent risk factor for cardiovascular and cerebrovascular events since it can affect blood coagulation and might lead to thrombosis as well [68]. It has been shown that patients with hypercholesterolemia or hypertriglyceridemia had higher fibrinogen and FVII levels and shorter coagulation times than patients with lower cholesterol levels [69, 70]. Further, high total cholesterol and triglyceride levels correlated with increased procoagulant activity of factors II, VII, IX, and X, which impacted a shorter prothrombin time (PT) [71]. In addition, in a cross-sectional study, hypercholesterolemia and hypertriglyceridemia were associated with elevated serum prekallikrein levels [72], which is the precursor of α-kallikrein and hence contributes to blood clotting and shorter activated partial thromboplastin time (aPTT) [73].

1.3 Assessment of coagulation

In vitro coagulation tests- prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT)- measure the time elapsed between activation of the coagulation cascade and formation of the stable clot.

1.3.1 Prothrombin time (PT)

The PT measures in principle the time necessary to generate fibrin after activation of FVII. It measures the integrity of the extrinsic and common pathways (FVII, V, X, prothrombin, and fibrinogen) [74]. A prolonged PT may reflect either coagulation factor deficiency or enhanced circulating coagulation inhibitors [75]. The distinction is made by repeating the test after a 1:1 mix with normal plasma. The test is more sensitive than the aPTT for deficient levels of coagulation factors, and a relatively small drop in FVII levels may prolong the PT. Inherited deficiency of FVII is a rare bleeding disorder characterized by prolonged PT and a normal aPTT [76]. The PT completely corrects when mixed with normal plasma. Acquired deficiencies are usually related to liver disease, vitamin K antagonist therapy, or depletion secondary to consumptive coagulopathy, severe bleeding, or extensive transfusion [77]. Circulating inhibitors are most often directed at FX or thrombin. The most common are heparin or products of fibrinolysis [74].

In this assay (Figure 3), citrated plasma and an activating agent (usually thromboplastin) are incubated at 37°C. The plasma is recalcified and the time is measured until the clot is formed. Normal/reference values may slightly vary between laboratories, but usually, clot times between 12 and 15 seconds are considered as valid normal [78]. Thromboplastin reagent is an important component of the PT test, containing a combination of TF, in complex with lipid surface of procoagulant membrane and calcium chloride ions. Thromboplastin reagents can be synthesized as recombinant human TF enclosed in phospholipid vesicles, or they can be obtained from animal or human tissues (brain or placenta) [79, 80]. The PT results reported by various laboratories can vary also due to the use of different TF sources [81].

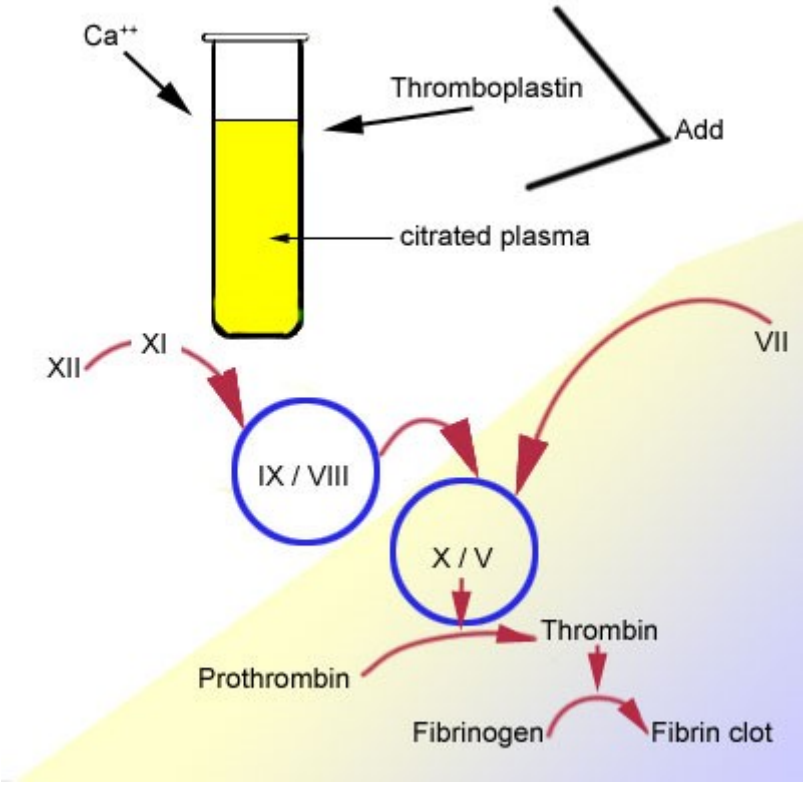


Figure 3. A short overview of the prothrombin test. The figure was taken from McGill University – Physiology Virtual Laboratory web page [82].

To standardize the PT results between different laboratories and reagents, the international normalized ratio (INR) is used. The INR is derived from PT which is calculated as a ratio of the patient's PT to a control PT standardized for the potency of the thromboplastin reagent [83, 84] using the following formula:

$$INR = \left(\frac{PTpatient}{PTcontrol}\right)^{ISI}$$

$PT_{patient}$: the PT of the patient's blood.

$PT_{control}$: the average PT of normal blood (control) from the same laboratory.

ISI: the International Sensitivity Index, which is a calibration value for the thromboplastin reagent used. It accounts for differences in the sensitivity of thromboplastin reagents.

1.3.2 Activated partial thromboplastin time (aPTT)

The aPTT measures principally the time necessary to generate fibrin from initiation of the intrinsic pathway. Activation of FXII is accomplished with an external agent (e.g. kaolin) capable of activating FXII without activating FVII. The test is conducted in the presence of a phospholipid emulsion, which substitutes for the platelet factors required for the cascade to operate normally [1]. The aPTT assay employs citrated plasma, an activating agent, and phospholipids that are added together and incubated at 37°C (Figure 4). Calcium is added, and the time necessary for the clumping formation is measured. The normal time is usually reported as less than 30 to 35 seconds and the normal range is usually considered to be about 10 seconds (e.g. 25 to 35) [85].

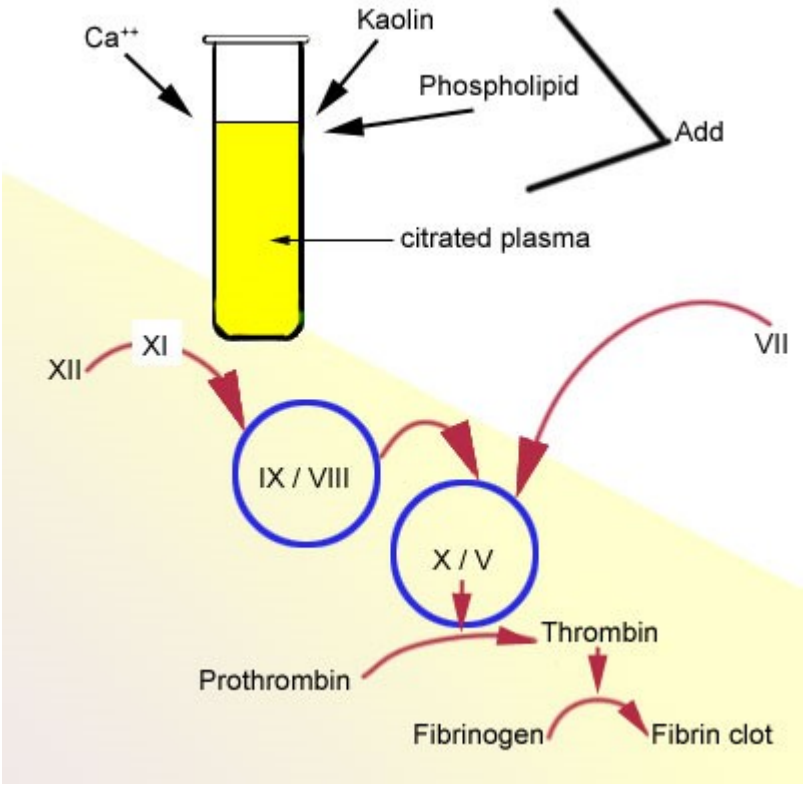


Figure 4. A short overview of activated partial thromboplastin time test. The figure was taken from McGill University – Physiology Virtual Laboratory web page [82].

The results of this test can be abnormal in the condition of reduced quantities of FXII, XI, IX, VIII, X, V, prothrombin, and fibrinogen (all integral parts of the intrinsic and common pathways). It is usually prolonged if a patient has less than approximately 30% normal activity of these coagulation

factors. It can also be abnormal in the presence of a circulating inhibitor to any of the coagulation factors in the intrinsic and common pathways [86].

The aPTT is a good screening test for inherited or acquired factor deficiencies. Inherited disorders including classic hemophilia A (FVIII deficiency) and hemophilia B (FIX deficiency, known also as Christmas disease) are well-known diseases in which the aPTT is prolonged [87]. A prolonged aPTT that cannot be completely normalized with the addition of normal plasma (mixed sample in a 1:1 ratio) can be explained only by the presence of a circulating coagulation inhibitor. The presence of these inhibitors is almost always acquired but their exact nature is not always apparent [88]. From a clinical point of view, the most common inhibitors should be considered, in particular antithrombins, which inhibit the activity of thrombin on the conversion of fibrinogen to fibrin. The two most common inhibitors are heparin, which acts through the naturally occurring protein antithrombin III, and fibrin degradation products (FDP), formed by the action of plasmin on the fibrin clot and usually present in elevated concentrations in disseminated intravascular coagulation (DIC) and primary fibrinolysis [89].

Occasionally the reported value of the aPTT will be lower than normal. This shortened time may reflect the presence of increased levels of activated coagulation factors in the context of a hypercoagulable state. This is observed in some patients in the early stages of DIC but should not be considered diagnostic for that condition [90].

1.3.3 Thrombin time (TT)

This test measures the time necessary to drive the reaction of fibrinogen to fibrin in the presence of thrombin. It measures the integrity of this reaction and shows an abnormality to either a decrease in normal fibrinogen or an inhibitor to its activation [91]. In the TT test, citrated plasma is incubated at 37°C and thrombin is added to the solution. Time is measured from the addition of thrombin to the generation of the clot. In this assay, calcium is not involved [92].

The results of the TT test can reveal coagulation abnormalities, which can be explained in one of three ways: 1. fibrinogen deficiency (<100 mg/dL), 2. abnormal fibrinogen formation, or 3. presence of an inhibitor to the reaction mediated by thrombin. As with other tests of the coagulation cascade, if a 1:1 mixing with normal plasma normalizes the prolonged time, factor deficiency is the culprit [93]. As

it pertains to fibrinogen, however, one must distinguish a decrease in normal fibrinogen from the production of an abnormal fibrinogen (dysfibrinogenemia) [93, 94].

Acquired deficiency of fibrinogen is usually due to a consumptive coagulopathy of, less often, severe liver disease [95, 96]. Hereditary deficiencies exist but with variable clinical presentations [97]. Dysfibrinogenemia can be acquired or inherited. The acquired form is usually found in association with severe liver disease but has been reported in other diseases. The congenital form is rare, usually autosomal dominant [98]. A discordance between immunologic and physiologic measurements of fibrinogen is the key to diagnosis. The most common acquired inhibitors of this reaction are heparin and FDP. The effect of heparin can be eliminated by catalyzing the reaction with reptilase (batroxobin). This compound is a snake serine protease similar to thrombin but which, unlike thrombin, is insensitive to heparin. FDP is commonly seen in consumptive coagulopathies and primary fibrinolytic states [94, 99].

1.4 Clinically used anticoagulants

One in four fatalities worldwide is caused by thrombosis [100], rendering it one of the major public health priorities. Thrombosis is the underlying cause of most acute myocardial infarctions (AMI), strokes, and venous thromboembolism (VTE) [101]. Antiplatelet drugs are mostly used to prevent arterial thrombotic events such as AMI and ischemic stroke, while VTE, including deep vein thrombosis (DVT) and pulmonary embolism (PE), is prevented and cured using anticoagulants. The different groups used in clinical praxis are summarized in Figure 5 [102, 103].

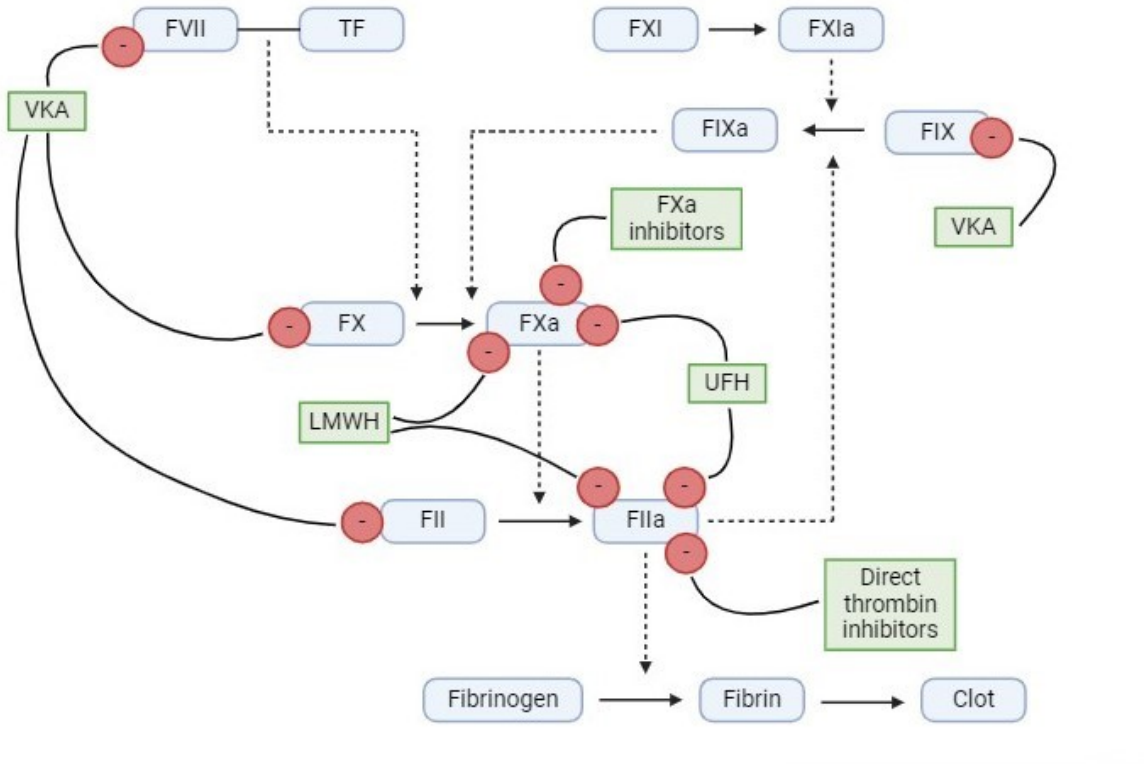


Figure 5. **Target of anticoagulant agents**. Adopted and modified from Franchini et al., 2016 [104].

Historically, the first anticoagulant drugs to prevent VTE were unfractionated heparin (UFH) and vitamin K antagonists (VKAs) [84, 105]. In the following years, these drugs have been optimized to prevent or treat VTE more efficiently. More recently a novel generation of anticoagulant drugs has been introduced, which are designed to directly target coagulation factor Xa and thrombin [106, 107].

1.4.1 Unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH)

UFH and its derivative, LMWH, are anticoagulants that are administered parenterally, intravenously and subcutaneously, respectively [105]. Heparins, as indirect anticoagulants, promote the activity of the major natural inhibitor of coagulation, antithrombin III, which is a serine protease inhibitor that primarily deactivates FXa, one of the pivotal actors within the coagulation cascade. In addition, UFH also metabolizes and deactivates thrombin and FIXa via the same enzyme antithrombin III [108, 109]. Once antithrombin III binds to FXa or thrombin, they form a complex that is rapidly degraded by proteases in circulation [110]. The activity of heparins can be reversed by the sulfate protamine that binds to circulating heparins, forming a stable salt with diminished anticoagulant activity

[111]. LMWH has largely replaced UFH in clinical settings because of the lower risk of bleeding, selectivity toward FXa decreased binding to plasma and endothelial proteins, improved possibility of subcutaneous administration, and more precise dose-effect relationship enabling easier assessment of dosing [112].

One of the most important disadvantages of UFH and LMWH is that approximately 2.6% and 0.2% of treated individuals, respectively, develop potentially life-threatening heparin-induced thrombocytopenia (HIT) [113, 114]. In this condition, for an unknown reason, a small group of heparin-treated individuals can generate an immune response against heparin-platelet factor 4 (PF4) complexes [115]. Heparin-PF4-IgG-immune complexes subsequently activate platelets via their FcγIIa receptors, causing thrombocytopenia and thrombosis [116].

The active structure of heparin can be reduced to five carbohydrate units (Figure 6). Indeed, fondaparinux, a synthetic analog of the antithrombin-binding pentasaccharide which selectively inhibits factor Xa and does not inhibit thrombin. It has complete bioavailability after subcutaneous injection and exhibits a plasma half-life of approximately 17 hours with predictable pharmacokinetics [117]. Because of its attributes, fondaparinux is administered once daily at a fixed dose and routine coagulation monitoring is not required [104]. This anticoagulant is contraindicated in patients with renal dysfunction (GFR <30 mL/min) [118]. There is no specific antidote for fondaparinux-related hemorrhage, but recombinant activated factor VII (rFVIIa) can be administered to normalize coagulation times and thrombin generation [119].

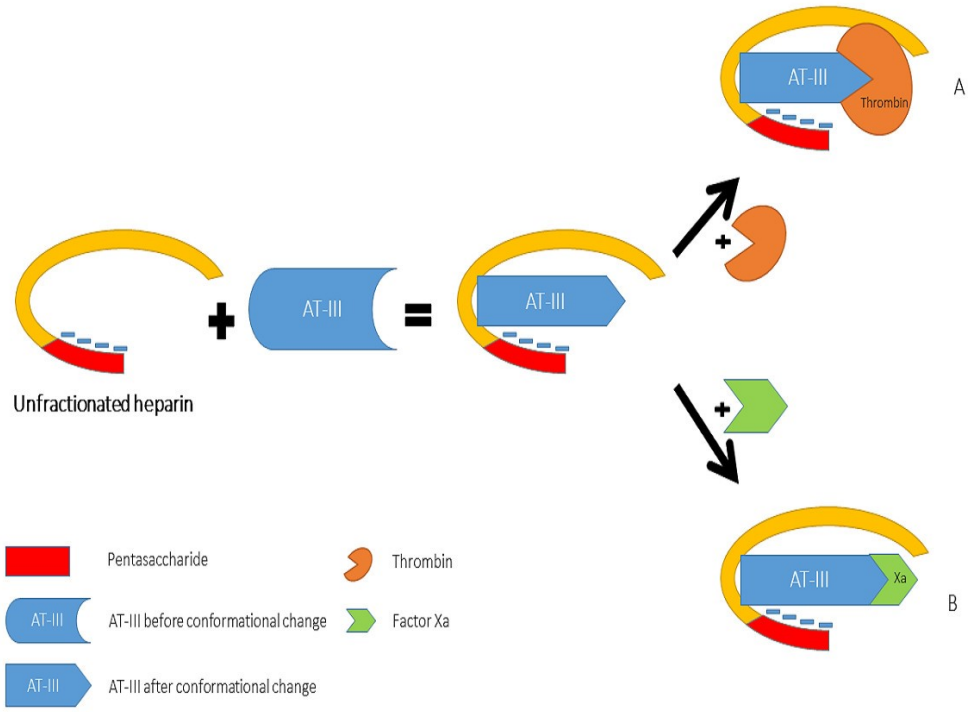


Figure 6. A short overview of the mechanism of action of heparin. (A) thrombin, (B) factor Xa. The figure was taken from Quaranta et al., 2015 [120].

1.4.2 Vitamin K antagonist (VKAs)

VKAs were the first generation of anticoagulants administered orally, a significant advantage over the parenterally administered heparins. Vitamin K is necessary for the biosynthesis of γ-carboxyglutamic acid residues in vitamin K-dependent coagulation factors (FII, FVII, FIX, and FX) that are essential for biological activity [121, 122]. VKAs inhibit vitamin K epoxide reductase (known as vitamin K epoxide reductase complex subunit 1, VKCOR1, Figure 7), an enzyme responsible for reducing vitamin K to its active form. As a result of VKA treatment, the pool of vitamin K in the circulation is gradually reduced and consequently, coagulation is revoked [123].

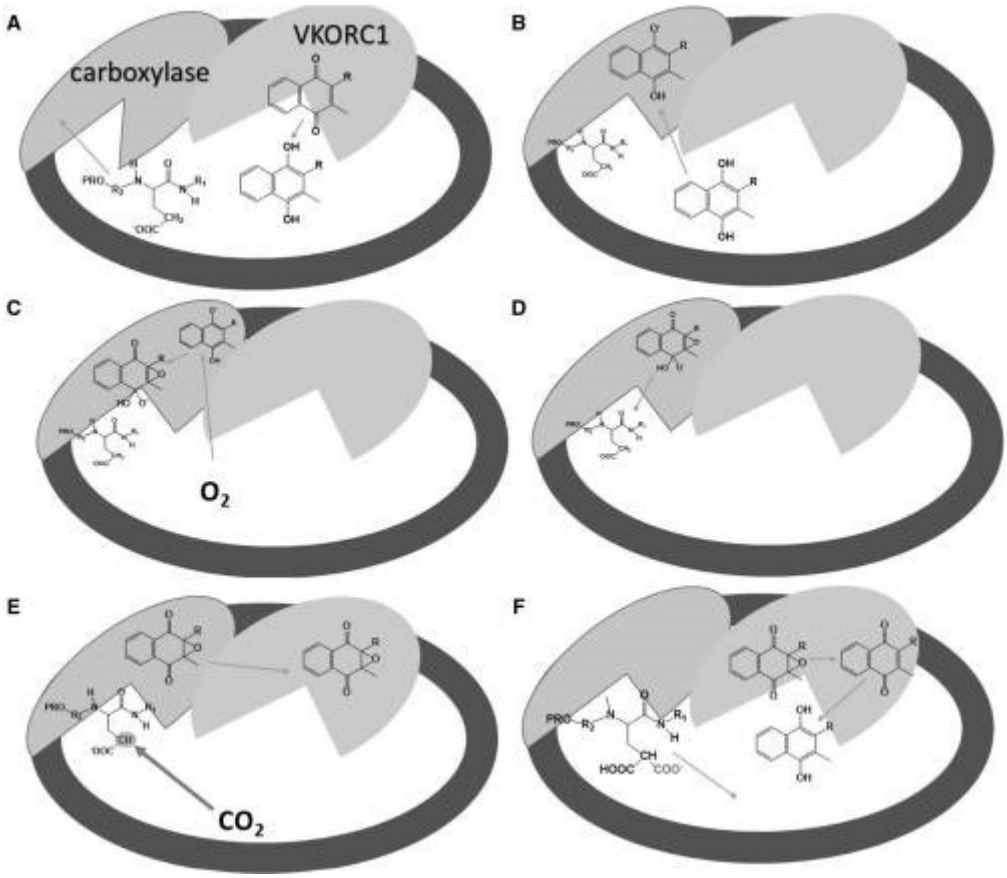


Figure 7. Probable steps in the carboxylation process mediated by vitamin K in the endoplasmic reticulum. A protein that contains a PRO-sequence is targeted and subsequently bound to the carboxylase in the first step (A). This binding markedly increases the enzymatic function of the carboxylase. The quinone form of vitamin K is reduced to hydroquinone by VKORC1. Hydroquinone is deprotonated by carboxylase in the next step (B). Oxygen reacts with deprotonated vitamin K hydroquinone to produce alkoxide (C). This strong base deprotonates the γ-carbon of glutamyl residue to form a carbanion, which reacts with carbon dioxide (D–E). At the same time, vitamin K epoxide is formed (E). γ-glutamyl carboxylation is accomplished and the formed protein is released from the enzyme and further transported to the Golgi apparatus (not shown), while vitamin K epoxide is converted first to vitamin K quinone and then to vitamin K hydroquinone (F) by VKORC1. The figure was taken from Mladěnka et al., 2022 [122].

Although oral admission is an advantage compared to parenterally administered heparins, VKAs present several flaws. Firstly, VKA transiently depletes the circulating vitamin K pool and impairs the

synthesis of vitamin K-dependent anticoagulation factors (proteins C, S, and Z) [122], VKA treatment reaches its optimal therapeutic action 3 to 7 days after the first admission but can cause transient hypercoagulability state. For this reason, a bridging period usually with the administration of an LMWH is required [124, 125]. Secondly, the dosage and admission of VKA must be monitored frequently with INR values to assess the risk of bleeding of the patients [126]. Finally, numerous interactions between VKAs and food or other drugs can significantly influence the efficacy of the treatment [84, 127]. The list of possible side effects of the most commonly used VKA, warfarin, is in Table 1.

Table 1. Adverse effects of warfarin

Adverse effects	Notes	References
Minor bleeding	Defined as skin hematoma >25 cm2, spontaneous nosebleed of > 5 minutes duration, gingival bleeding for > 5 minutes, any bleeding leading to hospitalization, any bleeding leading to transfusion < 3 units of blood product. The incidences are vary from 4 % to 20% of all patients.	[128, 129]
Major bleeding	Defined as macroscopic hematuria, gastrointestinal, retroperitoneal, cranial, and intra-abdominal bleeding, bleeding requiring hospitalization or an invasive procedure, bleeding causing a 2-unit decrease in the hemoglobin level, and bleeding requiring >3 units of blood product transfusion. The incidence of GI bleeding is 3.9-12 % and intracranial hemorrhage is 0.25-1.1 % patients per year.	[130-132]
Skin necrosis	A rare complication with a prevalence of 0.01-0.1 % of patients. Thrombosis, hypersensitivity, hemorrhage, factor VII deficiency, protein C deficiency, and a direct toxic effect of warfarin have all been suggested as potential pathophysiological mechanisms.	[133, 134]
Purple toe syndrome	An extremely uncommon complication with a prevalence of approximately 0.02% of patients. The proposed pathogenesis was due to direct and toxic insults to the capillaries, resulting in increased dilation and permeability of the vasculature.	[135, 136]
Osteoporosis & fractures	The osteoporotic fracture rates were 3.6 per 100 patient-years in men and 5.4 per 100 patient-years in women. The possible underlying mechanisms are warfarin antagonizes vitamin K-dependent processes and impairs the γ-carboxylation of osteocalcin and other bone matrix proteins, which are crucial in bone mineralization and formation.	[137-139]
Alopecia	The reported incidence of warfarin-induced alopecia was 30-40 % of patients. Two hypotheses are that anticoagulant-induced hair loss might be disseminated thrombosis in the microcirculation of capillaries that feed the roots of the hair or there may be distension of bundles of the dermis caused by bleeding with focal degeneration of collagen bundles, which would provoke strangulation of the hair root and injury to the connective tissue of the dermal papilla.	[140-142]
Hepatotoxicity	Warfarin is associated with a 0.8-1.2 % risk of transaminase elevation >3 upper limit of normal and 9.0 per 1000 person-years of liver injury hospitalization. The mechanism is unclear, but given that warfarin has been demonstrated to have pro-oxidant properties, exogenous warfarin dosages may have varying effects on the activity of catalase and superoxide dismutase (SOD) depending on the type of tissue. Changes in the activity of the antioxidant enzymes SOD and catalase observed in the tissue of rats given warfarin could be interpreted as an indication, rather than a direct cause, of the relationship between oxidative activity and inflammation.	[143-145]

1.4.3 Direct oral anticoagulants (DOACs)

The limitations of heparins and VKAs led to the development of new anticoagulant agents that selectively target specific steps in the coagulation cascade in order to have a high efficacy and safety profile. This enables a fixed dosage oral regimen generally not necessitating laboratory monitoring. Currently approved DOACs for use in thromboembolic disorders are factor Xa inhibitors (FXa-Is, xabans) and direct thrombin inhibitors (DTIs, gatrans) [146].

1.4.3.1 Factor Xa inhibitors (FXa-Is)

FXa-Is bind the active site of factor Xa, thereby preventing it from converting prothrombin to thrombin. Rivaroxaban and apixaban are two examples of drugs from this group. Compared to warfarin, these drugs also have fewer severe drug interactions, hence laboratory monitoring is mostly not required [107]. However, bleeding is still the most serious adverse effect of FXa-Is. To treat this condition andexanet alpha, a neutralizing agent of FXa, can be employed. Andexanet alpha is a recombinant protein that can serve as a decoy for FXa-Is, by mimicking the FXa active site without being able to engage in the formation of the prothrombinase complex [147]. Since andexanet alpha is an expensive product, prothrombin complex concentrates can alternatively be administered as a neutralizing agent [148].

1.4.3.2 Direct thrombin inhibitors (DTIs)

Dabigatran and argatroban are the most known drugs from this group. Dabigatran etexilate is the prodrug of the active moiety dabigatran. This drug directly inhibits the enzyme thrombin [149]. Argatroban is a drug derived from L-arginine and is able to specifically block the active site of thrombin, thus functioning like dabigatran also independently of antithrombin III [150]. Furthermore, argatroban is capable of upregulating the generation of nitric oxide [151], which can generate additional beneficial antithrombotic effects because of NO-mediated inhibition of platelet activation [152, 153].

Like other anticoagulants, the major adverse effect of DTIs is bleeding. Dabigatran should be used with caution in renal impairment or in patients over the age of 75, as the risk of bleeding is higher in these groups [154]. Like FXa-Is, the risk of drug interaction is lower than VKAs, but in particular

dabigatran etexilate is effluxed by P-glycoprotein in the gastrointestinal tract (GIT), so interaction at this level can affect the effectivity of the treatment [155]. The neutralizing agent for dabigatran is a humanized Fab fragment of an antibody (idarucizumab) that directly binds dabigatran and neutralizes its activity [156].

2. AIM OF THE WORK

The primary aim of this work was to examine and compare the *ex vivo* effect of indirect (heparin) and direct anticoagulants (rivaroxaban, apixaban, dabigatran, and argatroban) in healthy donors, patients with familial hypercholesterolemia (FH), and DMT1 by using PT/INR and aPTT.

The secondary aims were a) to investigate the correlation of coagulation values with anthropological and biochemical parameters from healthy individuals and patients, and b) to find out possible novel anticoagulant scaffold(s) or to warn about anticoagulation effects of tested compounds when not desired.

3. METHODOLOGY

3.1 Enrolment of healthy donors and patients

Blood samples from a total of 50 healthy individuals, 15 familial hypercholesterolemia, and 50 DMT1 patients were collected by venipuncture into disposable plastic syringes containing 3.2 % citrate sodium (BD Vacutainer, NJ, USA) or a clotting accelerator (for biochemical assessment of basic metabolic parameters). The blood sampling was always performed in the morning (7:30-8:30 AM). Alcohol was not allowed 24 hours before blood collection. All participants signed informed consent and this study was approved by the Ethical Committees from both the University Hospital of Hradec Králové (No. 201907 S04P from June 21, 2019 and extended by No. 202007 I04 from June 30, 2020) and the Faculty of Pharmacy, Charles University (No. UKFaF/92240/2021-2 from March 3, 2021). All experiments were performed according to the declaration of Helsinki.

The characterization of the donor in terms of illnesses and drug use is summarized in Table 2.

Blood was carefully transferred to the laboratory for analysis under standard conditions and immediately centrifuged at 2000*g* for 20 min using a Hettich Zentrifugen Universal 32 R (Tuttlingen, Germany) at room temperature to obtain plasma for coagulation assays. Experiments were initiated within 1 h after the blood draw. In addition, a part of the plasma from each donor or patient was stored at -80°C for future assays.

Table 2. Characterization of donors and patients by their illnesses and drug use

			Healthy donors	Familial Hypercholesterolemia		Diabetes Mellitus type 1
				Undergoing apheresis	Without apheresis	
	Total, n (%)		50 (100)	5 (41.7)	7 (58.3)	50 (100)
Health conditions	Hypertension		9 (18)	2 (16.7)	-	13 (26)
	Hypothyroidism		6 (12)	2 (16.7)	-	6 (12)
	Allergy		9 (18)	1 (8.3)	-	-
	Asthma		4 (8)	1 (8.3)	-	-
	GERD		3 (6)	-	-	-
Drugs	Antidyslipidemics	Cholesterol absorbtion inhibitors	-	3 (25)	7 (58.3)	-
		HMG-CoA reductase inhibitors	-	3 (25)	7 (58.3)	11 (22)
		Fibrates	-	1 (8.3)	1 (8.3)	1 (2)
		PCSK9 inhibitors	-	4 (33.3)	6 (50)	-
		MTP inhibitors	-	2 (16.7)	-	-
		Combination[a]	-	1 (8.3)	-	1 (2)
	Antidiabetics	Insulin	-	-	1 (8.3)	50 (100)
		Biguanides	-	2 (16.7)	3 (25)	-
		Sulfonylureas	-	1 (8.3)	-	-
		GLP-1 receptor agonists	-	1 (8.3)	-	-
		SGLT-2 inhibitors	-	-	1 (8.3)	2 (4)
	Antihypertensives	ACE-inhibitors	-	2 (16.7)	2 (16.7)	10 (20)
		AT-II antagonists	4 (8)	-	-	-
		Ca^{2+}-channel blockers	5 (10)	-	-	4 (8)
		β-blockers	4 (8)	2 (16.7)	1 (8.3)	-
		Diuretics	-	1 (8.3)	-	2 (4)
		Combination[b]	-	2 (16.7)	1 (8.3)	4 (8)
	Antiplatelet	ASA	-	2 (16.7)	2 (16.7)	-
		P2Y12 receptor inhibitors	-	1 (8.3)	-	-

	Combination[c]	-	2 (16.7)	-	-
Antiasthmatics	β agonist + corticosteroid	-	1 (8.3)	-	1 (2)
Hormones	Thyroid hormone	6 (12)	2 (16.7)	-	10 (20)
Other	Corticosteroid	4 (8)	-	-	-
	Antihistamine	6 (12)	-	-	1 (2)
	Gabapentinoid	-	-	-	7 (14)
	Hormonal contraceptive	5(10)	-	-	5 (10)
	Antidepressant	-	-	-	3 (6)

ACE: angiotensin-converting enzyme; ASA: acetylsalicylic acid; AT-II: angiotensin II; GERD: gastroesophageal reflux disease; GLP-1: glucagon-like peptide-1; HMG-CoA: 3-hydroxy-3-methyl-glutaryl Co-A; MTP: microsomal triglyceride transfer protein; PCSK9: proprotein convertase subtilisin/kexin type 9; SGLT-2: sodium-glucose linked transporter-2.

[a]: Cholesterol absorption inhibitor + HMG-CoA reductase inhibitor

[b]: ACE inhibitor +β-blocker, ACE inhibitor + Ca2+- channel blocker, β-blocker + Ca2+- channel blocker

[c]: ASA + P2Y12 receptor inhibitor, ASA + glycine

3.2 Coagulation assays

PT (reported as INR) and aPTT were measured using a semi-automated 4-channel Ceveron® coagulometer (Technoclone, Vienna, Austria). For PT assay, 100 µL of normal control or plasma-containing vehicle or anticoagulant solution was incubated at 37°C for one minute. After the incubation period was finished, 200 µL of Technoplastin®-HIS (containing rabbit brain thromboplastin with calcium) was added and the coagulation time was recorded. For aPTT, 100 µL of plasma-containing vehicle or anticoagulant was incubated together with the same amount of Dapttin® (containing silica/sulfatide-phospholipids) and incubated at 37°C for 2 minutes prior addition of 100 µL of 25 mM $CaCl_2$. Experiments were performed at least in duplicates for each condition. In thrombin time (TT) assay, 200 µL of mixed plasma and solvent/compound was initially incubated for 1 min at 37 °C. After incubation, 200 µL of 3.3 IU of thrombin reagent (containing bovine thrombin, Technoclone) was added and TT was recorded. All reagents were purchased from Technoclone.

3.3 Biochemical analysis

Biochemical markers (glucose, serum, creatinine, LDL, HDL, total cholesterol, triglycerides, and non-HDL) were measured in serum. All biomarkers except creatinine were measured using commercial enzymatic kits by Cobas® and detected by Cobas 8000 system (Roche Diagnostic, Basel, Switzerland). Creatinine urine levels were determined from urine samples using an SPD-M20A Shimadzu diode array detector (Kyoto, Japan). The complete procedure has already been described in detail in a previous publication [157]. A stationary phase consisting of two connected RP-18e monolithic columns (4.6 mm × 50mm, 3.0 mm × 100mm) in combination with a 15 mM phosphate buffer as the mobile phase was used. Creatinine was detected at a wavelength of 235 nm. Glycated hemoglobin (HbA1c) was determined using fully automated Arkray Adams HA-8180 (Arkray Inc., Japan). The principle is ion-exchange HPLC with gradient elution and VIS detection. Vitamin K was determined by the LC-MS/MS method [158]. The chromatographic separation was carried out on a Kinetex™ C18 column using a mobile phase consisting mainly of methanol. The target analytes were detected by electrospray ionization mass spectrometry. The simple sample preparation technique based on miniaturized liquid-liquid extraction was used for serum samples.

3.4 Chromogenic assays of coagulation enzymes

Two types of chromogenic substrates, S-2222® for factor Xa and S-2238® for thrombin were used (both Sigma-Aldrich, St. Louis, MO, USA). For the measurement of FXa, DMSO (final concentration of 0.1%, as a vehicle, i.e. negative control), rivaroxaban solution (final concentration of 10 nM, as a positive control), and TRIS-HCl buffer solution were used. Briefly, 75 µL of test compounds, rivaroxaban, or solvent were added into a well of the microplate, subsequently 75 µL of FXa solution (final concentration of 2 U/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added. After 1 minute of incubation in the dark and at 37°C, 50 µL of S-2222® was added and the absorbance was measured every minute at 405 nm and 37°C for 30 minutes.

For the measurement of thrombin, a final concentration of 0.1% of DMSO, a final concentration of 10 nM of dabigatran, and a TRIS-PBS buffer solution were used. A volume of 75 µL of test compounds, dabigatran, or solvent were added into the microplate, subsequently, 75 µL of thrombin solution (final concentration of 0.75 U/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added. After 10 minutes of incubation at 37°C, 40 µL of S-2238® solution was added into the mixtures and the absorbance was measured for 30 minutes at 405 nm and 37°C. The samples were measured using Hidex® Sense Beta Plus for both assays (Hidex Oy, Turku, Finland).

3.5 Standards and tested compounds

Apixaban was purchased from Toronto Research Chemical corp. (Ontario, Canada). Argatroban was bought from Eubio (Vienna, Austria). Dabigatran, rivaroxaban, and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich. Heparin was purchased from Zentiva (Prague, Czech Republic). Saline (0.9%) was bought from B.Braun (Melsungen, Germany).

Flavone, 5-hydroxyflavone, 7-hydroxyflavone, chrysin, apigenin, luteolin, baicalein, baicalin, diosmin, 3-hydroxyflavone, kaempferol, quercetin, morin, myricetin, rutin, trihydroxyethyl rutin, naringenin, naringin, hesperetin, hesperidin, taxifolin, epicatechin, catechin, daidzein, and genistein (all minimal purity of 95%) were purchased from Sigma-Aldrich. Genistin and apigenin-7-glucoside were purchased from Extrasynthese (Lyon, France). Negletein and mosloflavone were synthesized by a convergent synthesis starting from chrysin at the Sapienza University of Rome (Italy) [159].

3,3,8-Trimethyl-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3a); 3,3-Dimethyl-8-ethyl-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3b); 3,3-Dimethyl-6-oxo-8-propyl-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3c); 3,3-Dimethyl-8-isopropyl-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3d); 8-Butyl-3,3-dimethyl-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3e); 3,3-Dimethyl-6-oxo-8-phenyl-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3f); 3,3-Dimethyl-8-(2-methylphenyl)-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3g); 3,3-Dimethyl-8-(4-methoxyphenyl)-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3h); 3,3-Dimethyl-8-(2-furyl)-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c]pyridine-5-carbonitrile (3i); 3,3-Dimethyl-6-oxo-8-(2-thienyl)-3,4,6,7-tetrahydro-1H-pyrano [3,4-c] pyridine-5-carbonitrile (3j) were synthesized at Institute of Fine Organic Chemistry of A. L. Mnjoyan (Yerevan, Armenia). The complete structure of these compounds is presented in Figure 8.

5-(4-nitrophenyl)-10-phenyl-5,10-dihydropyrido[2,3-d:6,5-d']dipyrimidine-2,4,6,8(1H,3H,7H,9H)-tetraone (1-A); 10-(p-tolyl)-5-(4-(trifluoromethyl)phenyl)-5,10-dihydropyrido[2,3-d:6,5-d']dipyrimidine-2,4,6,8(1H,3H,7H,9H)-tetraone (1-B); 4-(10-(4-bromophenyl)-2,4,6,8-tetraoxo-1,2,3,4,5,6,7,8,9,10-decahydropyrido[2,3-d:6,5-d']dipyrimidin-5-yl)benzonitrile (1-C); 5,10-bis(4-(trifluoromethyl)phenyl)-5,10-dihydropyrido[2,3-d:6,5-d']dipyrimidine-2,4,6,8(1H,3H,7H,9H)-tetraone (1-D); 10-(4-(trifluoromethyl)phenyl)-5-(3,4,5-trimethoxyphenyl)-5,10-dihydropyrido[2,3-d:6,5-d']dipyrimidine-2,4,6,8(1H,3H,7H,9H)-tetraone (1-E); 4-(10-(4-hydroxyphenyl)-2,4,6,8-tetraoxo-1,2,3,4,5,6,7,8,9,10-decahydropyrido[2,3-d:6,5-d']dipyrimidin-5-yl)benzaldehyde (1-F); 5-(furan-2-yl)-10-(4-methoxyphenyl)-2,8-dithioxo-2,3,5,8,9,10-hexahydropyrido[2,3-d:6,5-d']dipyrimidine-4,6(1H,7H)-dione (1-G); 10-(3,5-bis(trifluoromethyl)phenyl)-5-(1-methyl-1l4-pyran-4-yl)-2,8-dithioxo-2,3,5,8,9,10-hexahydropyrido[2,3-d:6,5-d']dipyrimidine-4,6(1H,7H)-dione (1-H); 2-amino-6-bromo-4-(5-methoxy-1H-indol-3-yl)-4H-chromene-3-carbonitrile (2-F); 2-amino-4-(1H-indol-3-yl)-6-nitro-4H-chromene-3-carbonitrile (2-N); 2-amino-6-nitro-4-(1H-pyrrolo[2,3-b]pyridin-3-yl)-4H-chromene-3-carbonitrile (2-O); 6-amino-2-mercapto-5-((1-methyl-1H-indol-3-yl)(4-(trifluoromethyl)phenyl)methyl)pyrimidin-4-ol (3-B); 4-((6-amino-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)(1H-indol-3-yl)methyl)benzonitrile

(3-S); 6-amino-5-((4-(dimethylamino)phenyl)(1H-indol-3-yl)methyl)pyrimidine-2,4(1H,3H)-dione (3-T); 2-((6-amino-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)(5-bromo-1H-indol-3-yl)methyl)benzoic acid (3-U); 2-(9-(4-methoxyphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-2,3,4,5,6,7,8,9-octahydroacridin-10(1H)-yl)succinic acid (4-M); 2-(3,3,6,6-tetramethyl-9-(4-nitrophenyl)-1,8-dioxo-2,3,4,5,6,7,8,9-octahydroacridin-10(1H)-yl)succinic acid (4-N); 3-mercapto-2-(3,3,6,6-tetramethyl-9-(4-nitrophenyl)-1,8-dioxo-2,3,4,5,6,7,8,9-octahydroacridin-10(1H)-yl)propanoic acid (4-O); 3-(1H-imidazol-4-yl)-2-(3,3,6,6-tetramethyl-1,8-dioxo-9-(p-tolyl)-2,3,4,5,6,7,8,9-octahydroacridin-10(1H)-yl)propanoic acid (4-P); 5-(4-methoxyphenyl)-1,5-dihydro-2H-benzo[6,7]chromeno[2,3-d]pyrimidine-2,4,6,11(3H)-tetraone (5-A); 5-(4-(trifluoromethyl)phenyl)-1,5-dihydro-2H-benzo[6,7]chromeno[2,3-d]pyrimidine-2,4,6,11(3H)-tetraone (5-B); 4-(2,4,6,11-tetraoxo-1,3,4,5,6,11-hexahydro-2H-benzo[6,7]chromeno[2,3-d]pyrimidin-5-yl)benzonitrile (5-C); 3-(3-(4-amino-6-hydroxy-2-mercaptopyrimidin-5-yl)-2-(4-hydroxy-3-methoxyphenyl)propyl)-4-hydroxy-2H-chromen-2-one (6-A); 6-amino-5-(3-(4-hydroxy-2-oxo-2H-chromen-3-yl)-2-(4-(trifluoromethyl)phenyl)propyl)pyrimidine-2,4(1H,3H)-dione (6-B); 6-amino-5-(3-(4-hydroxy-2-oxo-2H-chromen-3-yl)-2-(3,4,5-trimethoxyphenyl)propyl)-1-methylpyrimidine-2,4(1H,3H)-dione (6-C); 7-(3,4-dihydroxyphenyl)-6H,7H-chromeno[4,3-d]pyrido[1,2-a]pyrimidin-6-one (7-A); 11-methyl-7-(4-(trifluoromethyl)phenyl)-6H,7H-chromeno[4,3-d]pyrido[1,2-a]pyrimidin-6-one (7-B); 11-methyl-7-(4-(methylamino)phenyl)-6H,7H-chromeno[4,3-d]pyrido[1,2-a]pyrimidin-6-one (7-C); 3,3'-(2-(4-nitrophenyl)propane-1,3-diyl)bis(4-hydroxy-2H-chromen-2-one) (8-A); 3,3'-(2-(3-hydroxyphenyl)propane-1,3-diyl)bis(4-hydroxy-2H-chromen-2-one) (8-B); 3,3'-(2-(4-(trifluoromethyl)phenyl)propane-1,3-diyl)bis(4-hydroxy-2H-chromen-2-one) (8-C); 5-((5-bromo-3-((3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-2-oxoindolin-3-yl)methyl)pyrimidine-2,4,6(1H,3H,5H)-trione (9-B); 5-((5-chloro-3-((3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-2-oxoindolin-3-yl)methyl)pyrimidine-2,4,6(1H,3H,5H)-trione (9-C); 5-((3-((3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methyl)-5-iodo-2-oxoindolin-3-yl)methyl)pyrimidine-2,4,6(1H,3H,5H)-trione (9-D) were synthesized at Department of chemistry, Visva-Bharati (Central University), Santiniketan (India). The structure of these compounds is presented in Figure 9.

4-ethylguaiacol (2-methoxy-4-ethylphenol), o-toluidine, 4-methylcatechol, 3-methoxycatechol, 1,2-dimethoxybenzene, 2-methoxy-4-methylphenol, 2,4-dimethoxytoluene, 4-allyl-1,2-dimethoxybenzene, o-cresol, pyrocatechol, 3,5-dichlorocatechol, 4,5-dichlorocatechol, 4-tert-butylcatechol, 4-nitrocatechol and 2-aminophenol were purchased from Sigma-Aldrich (Sigma-Aldrich, Sant-Louis, MO, USA). 3-aminocatechol, 4-aminocatechol, 4-etylcatechol, 3-fluorocatechol, 4-fluorocatechol, 4-chlorocatechol, 3-isopropylcatechol and 3-methylcatechol were purchased from Toronto Research Chemicals (Toronto, ON, Canada).

Berberine chloride, isocorydine, and papaverine hydrochloride were purchased from Sigma Aldrich (Prague, Czech Republic), glaucine was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA) and boldine was purchased from Carl Roth (Karlsruhe, Germany). All other alkaloids were isolated by members of the ADINACO research group at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmacy, in Hradec Králové, Charles University, Czech Republic. Bulbocapnine, corycavamine and corydine were isolated from *Corydalis cava*, scoulerine from *Eschscholzia californica*, cryptopine, parfumine and sinactine from *Fumaria officinalis*, and allocryptopine and protopine from *Chelidonium majus*.

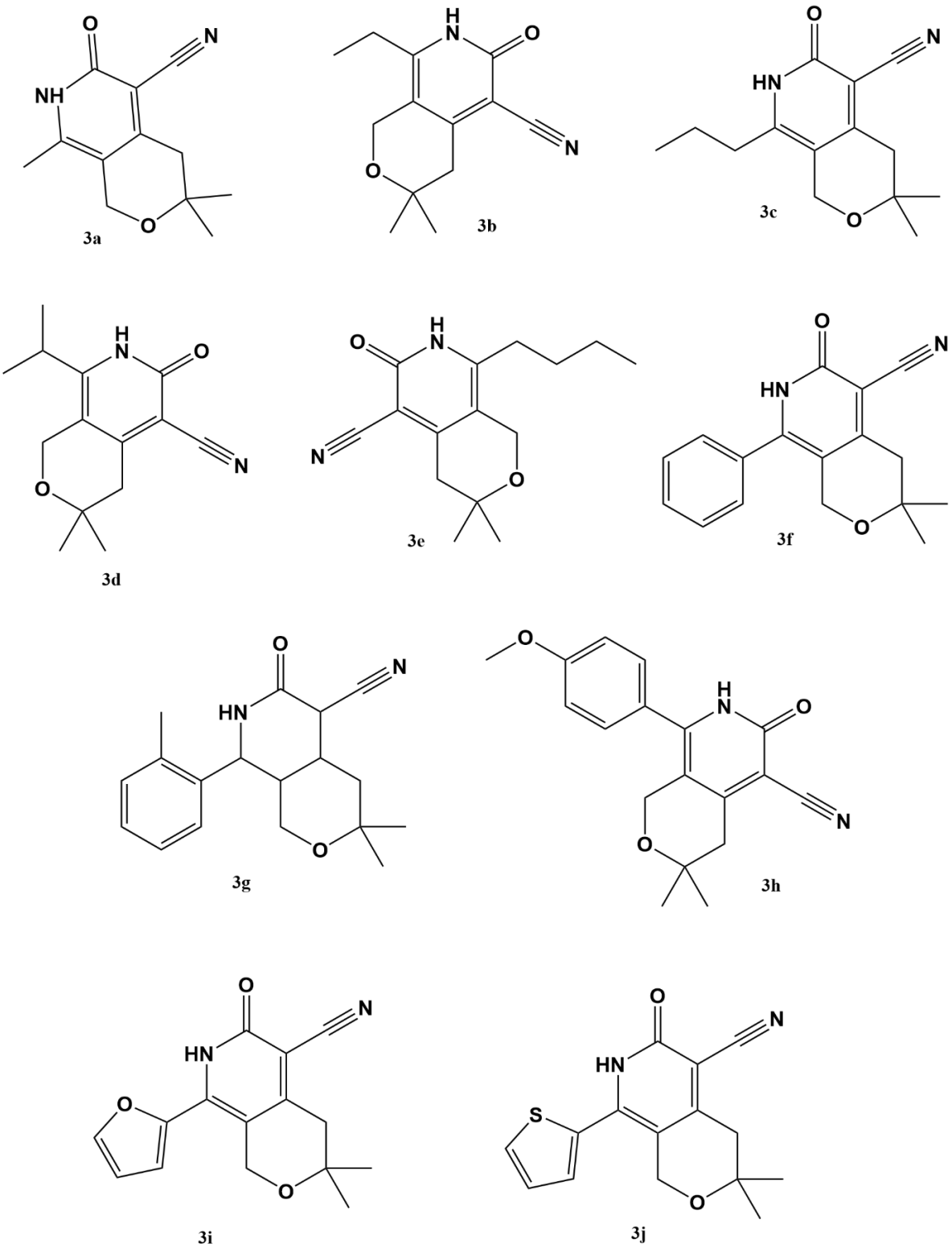
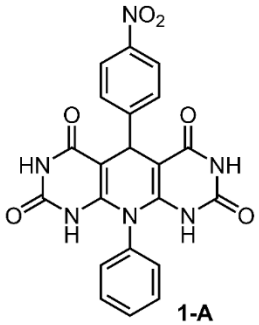
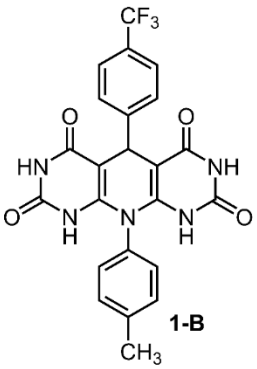


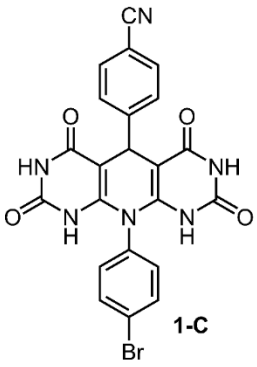
Figure 8. The chemical structure of the pyridine compounds.



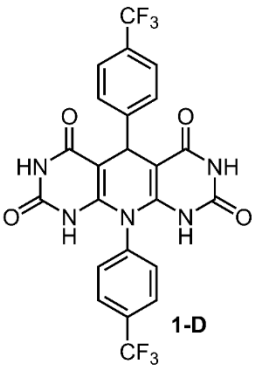
1-A



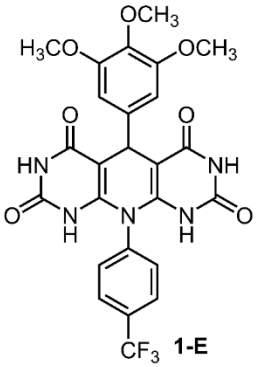
1-B



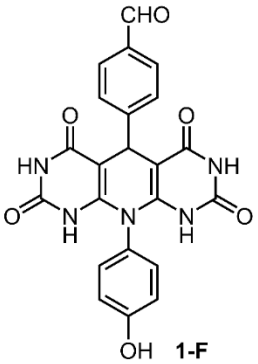
1-C



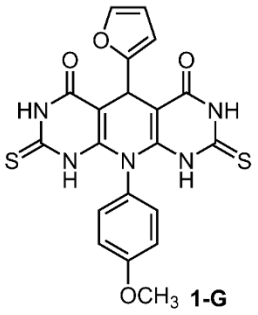
1-D



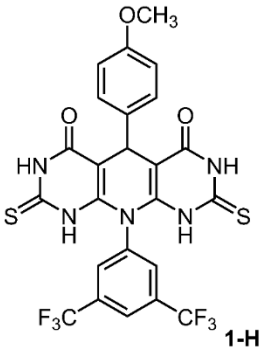
1-E



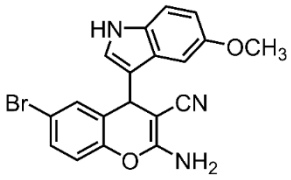
1-F



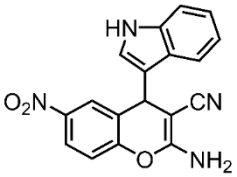
1-G



1-H



2-F



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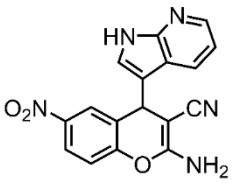
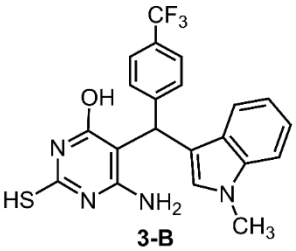
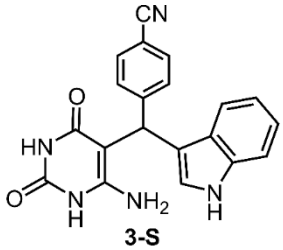


2-O



3-B



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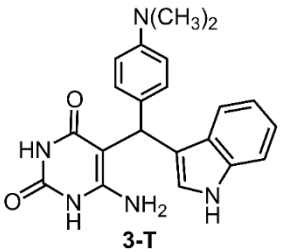

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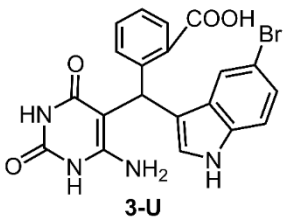
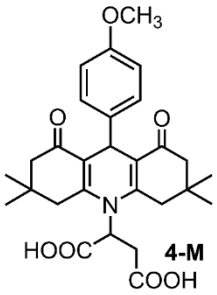
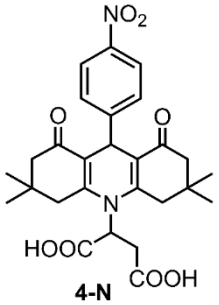


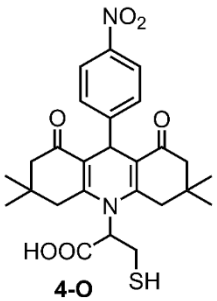
3-U



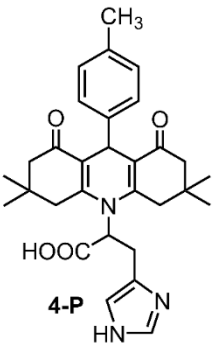
4-M



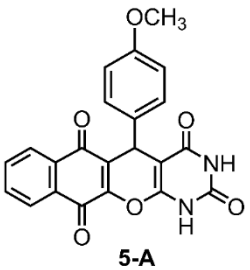
4-N



4-O



4-P



5-A

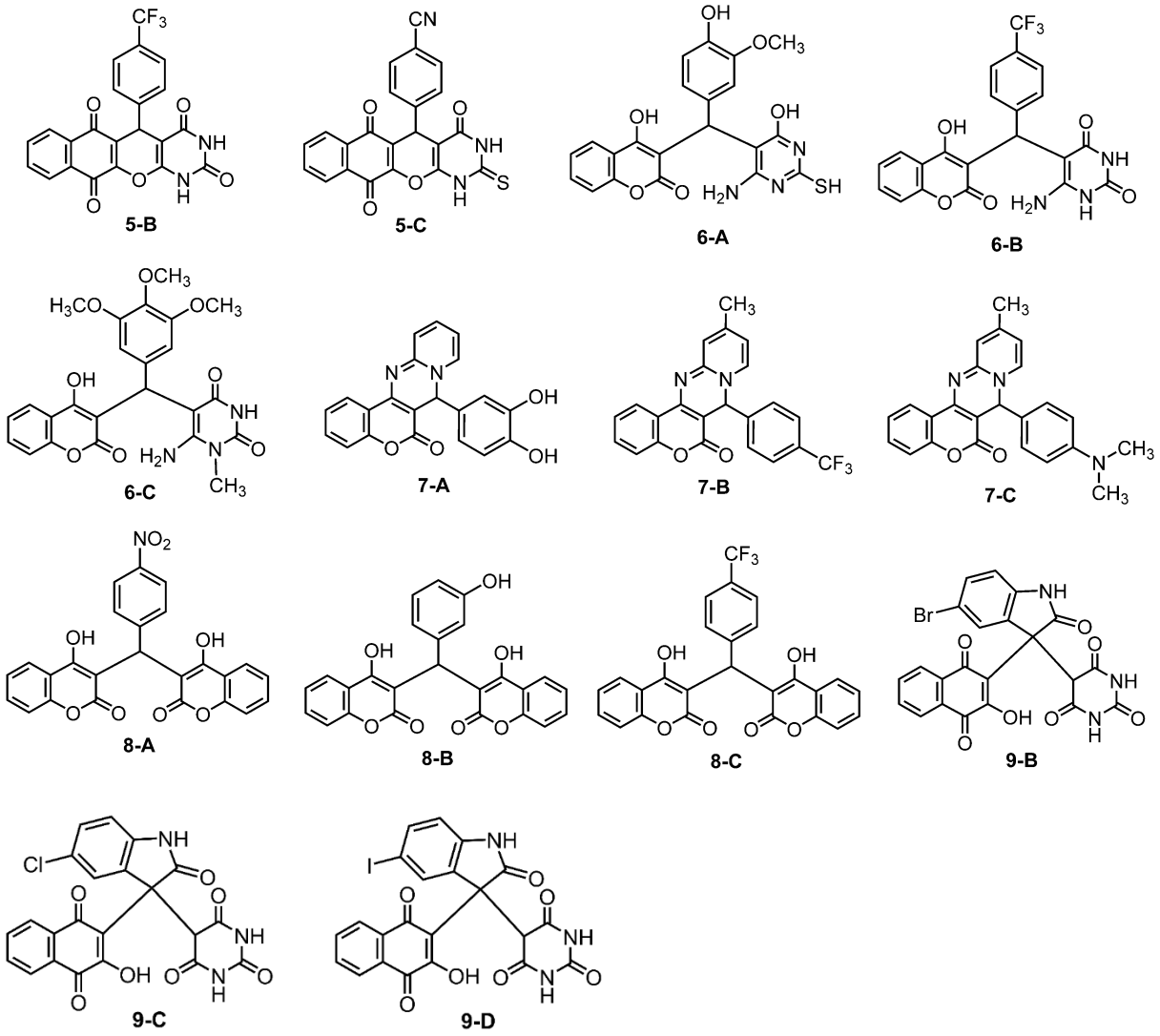


Figure 9. The chemical structures of heterocyclic compounds. The figure was taken from Hrubša et al., 2022 [160].

3.6 Statistical analysis

All data are presented as mean ± SD. Coagulation data were compared and analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test or Student's t-test or Mann-Whitney test depending on the number of variables and normality of the data. Normality data was checked by the Kolmogorov-Smirnov test (if the samples were ≥ 50) and the Shapiro-Wilk test (if the samples were < 50). Correlations were tested using Pearson's or Spearman's correlation test again depending on the normality of the distribution of the data. All statistical studies were performed using GraphPad v. 10.1.2 software (GraphPad Software, San Diego, CA, USA).

4. RESULTS

4.1 Healthy donors

The first series of head-to-head comparisons of clinically used direct anticoagulant drugs was performed in the 50, generally, healthy individuals enrolled. Two FXa-Is, rivaroxaban and apixaban, and two DTIs, dabigatran and argatroban were employed at an equimolar concentration of 1 μM and compared to vehicle control (DMSO, a final concentration of 1%) and positive control (heparin, a final concentration of 5 IU/mL and 0.5 IU/mL for PT and aPTT assays, respectively).

In the PT test, rivaroxaban was the most active compound, increasing INR from 0.97 ± 0.07 basal value (CI 95% 0.96-0.99) to 2.49 ± 0.34 (CI 95% 2.42-2.56, $p<0.001$). INR comparison revealed no difference between FXa-I, apixaban, and argatroban; however, when the calculation was performed by calculating the percentage of PT in every individual (100% was the individual vehicle control), apixaban was significantly more active than argatroban, and both anticoagulants were more active than dabigatran in both types of calculations (Figure 10A & B). In the aPTT test, DTIs were clearly more active than FXa-Is, with argatroban being the most active anticoagulant, followed by dabigatran and rivaroxaban; apixaban was the least active (Figure 10C & D).

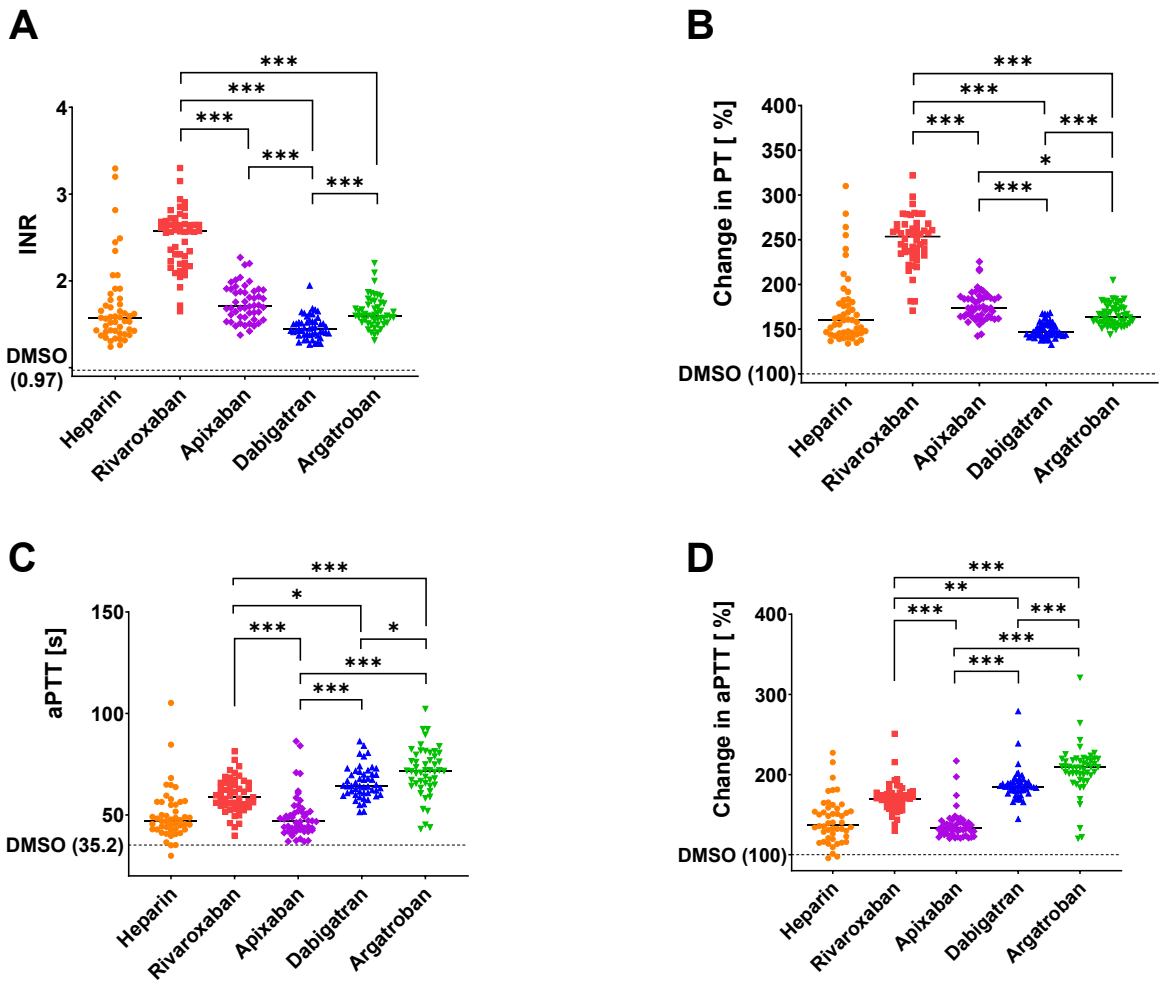


Figure 10. Comparison of the tested direct anticoagulants in healthy volunteers. (A) International normalized ratio (INR) calculated from prothrombin time (PT), (B) percent changes in PT calculated in every volunteer individually (i.e. solvent in every donor was set to 100%), (C) activated partial thromboplastin time (aPTT), (D) percent changes in aPTT calculated in every donor individually. Final concentrations were DMSO 1%, heparin 5 IU/mL in PT and 0.5 IU/mL in aPTT, whereas those of rivaroxaban, apixaban, dabigatran, and argatroban 1 μM. *p<0.05; **p<0.01; ***p<0.001. Statistical comparison to heparin is not shown, as it was used solely for control reasons.

Next, possible associations between the obtained INR and aPTT values with all anthropometric and biochemical parameters of the donor were analyzed (Table 3). The most significant correlations were observed between the effects of all direct anticoagulants on INR and BMI values (Figure 11). In all cases, a higher BMI value meant a lower anticoagulant response, however, a similar effect was not observed in aPTT (Figure 12). Other significant correlations were found only in individual compounds, such as heparin - INR and argatroban – aPTT in relation to age (Figures 13 & 14). No sex-related differences (Figures 15 & 16) were observed. There were also no differences when smoking habit was considered (Figures 17 & 18). No changes were observed despite the fact that females and smokers present mostly numerically lower coagulation times.

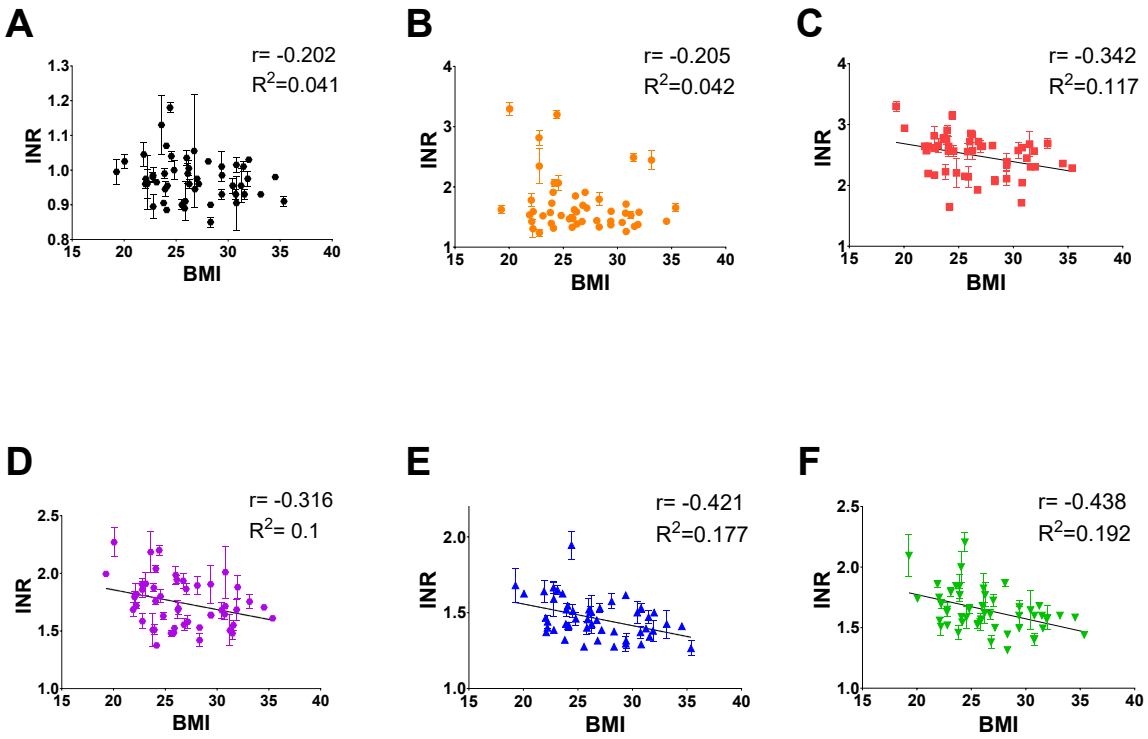


Figure 11. Relationship between international normalized ratio (INR) and body mass index (BMI) in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

Table 3. Correlation of anticoagulant activity with selected anthropological and biochemical parameters

	Parameters	DMSO		Heparin		Rivaroxaban		Apixaban		Dabigatran		Argatroban	
		r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
INR	Age	-0.06	0.681	-0.326	**0.022**	0.091	0.528	-0.158	0.273	-0.206	0.152	-0.048	0.74
	Height	0.125	0.388	0.174	0.232	0.101	0.487	0.031	0.832	0.099	0.494	0.086	0.551
	Weight	-0.126	0.382	-0.071	0.629	-0.244	0.088	-0.273	0.055	-0.326	**0.021**	-0.348	**0.013**
	BMI	-0.202	0.159	-0.205	0.158	-0.342	**0.015**	-0.316	**0.025**	-0.421	**0.002**	-0.438	**0.002**
	Serum glucose	-0.225	0.116	-0.306	**0.03**	-0.22	0.126	-0.425	**0.002**	-0.267	0.061	-0.257	0.072
	Serum LDL	-0.141	0.328	-0.224	0.122	0.042	0.771	-0.079	0.587	-0.294	**0.038**	-0.151	0.295
	Serum HDL	-0.184	0.2	0.094	0.52	-0.184	0.2	0.117	0.418	0.074	0.61	-0.006	0.969
	Serum TG	-0.221	0.124	-0.037	0.803	-0.16	0.266	-0.329	**0.02**	-0.222	0.122	-0.258	0.07
	Total cholesterol	-0.175	0.224	-0.15	0.305	-0.043	0.765	-0.081	0.578	-0.285	**0.045**	-0.176	0.222
	Serum nonHDL	-0.177	0.22	-0.188	0.196	0.03	0.834	-0.127	0.379	-0.314	**0.026**	-0.173	0.229
	Serum creatinine	-0.16	0.271	-0.345	**0.017**	0.002	0.988	-0.116	0.426	-0.142	0.331	-0.028	0.847

	Parameters	DMSO		Heparin		Rivaroxaban		Apixaban		Dabigatran		Argatroban	
		r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
aPTT	Age	-0.141	0.33	-0.262	0.072	-0.205	0.154	0.061	0.674	-0.203	0.158	-0.329	**0.02**
	Height	0.013	0.927	0.045	0.762	-0.073	0.615	-0.153	0.289	0.056	0.699	0.1	0.488
	Weight	-0.077	0.598	-0.066	0.658	-0.181	0.208	-0.089	0.537	-0.11	0.449	-0.077	0.593
	BMI	-0.097	0.505	-0.099	0.501	-0.156	0.279	0.002	0.986	-0.148	0.304	-0.155	0.284
	Serum glucose	-0.144	0.318	-0.028	0.849	-0.145	0.315	-0.234	0.102	-0.186	0.197	-0.138	0.339
	Serum LDL	-0.277	0.051	-0.407	**0.004**	-0.338	**0.016**	0.121	0.404	-0.346	**0.014**	-0.424	**0.002**
	Serum HDL	-0.063	0.664	0.047	0.749	-0.097	0.505	-0.079	0.585	-0.025	0.863	0.111	0.443
	Serum TG	-0.266	0.062	-0.33	**0.022**	-0.404	**0.004**	-0.239	0.095	-0.356	**0.011**	-0.275	0.053
	Total cholesterol	-0.338	0.016	-0.419	**0.003**	-0.433	**0.002**	0.02	0.893	-0.427	**0.002**	-0.404	**0.004**
	Serum nonHDL	-0.312	**0.028**	-0.44	**0.002**	-0.393	**0.005**	0.051	0.724	-0.416	**0.003**	-0.448	**0.001**
	Serum creatinine	-0.182	0.211	-0.215	0.147	-0.1	0.493	-0.252	0.081	-0.137	0.347	-0.056	0.704

BMI=body mass index, LDL=low-density lipoproteins, HDL=high-density lipoproteins, TG=triglyceride

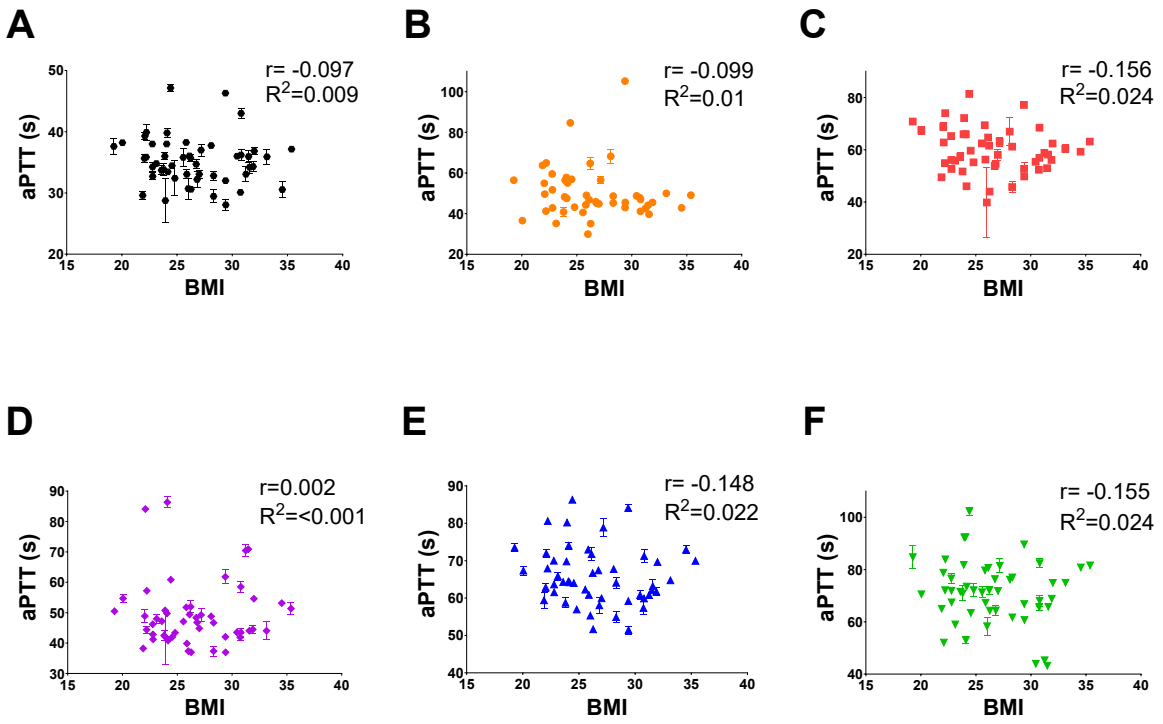


Figure 12. Relationship between activated partial thromboplastin time (aPTT) and BMI. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM).

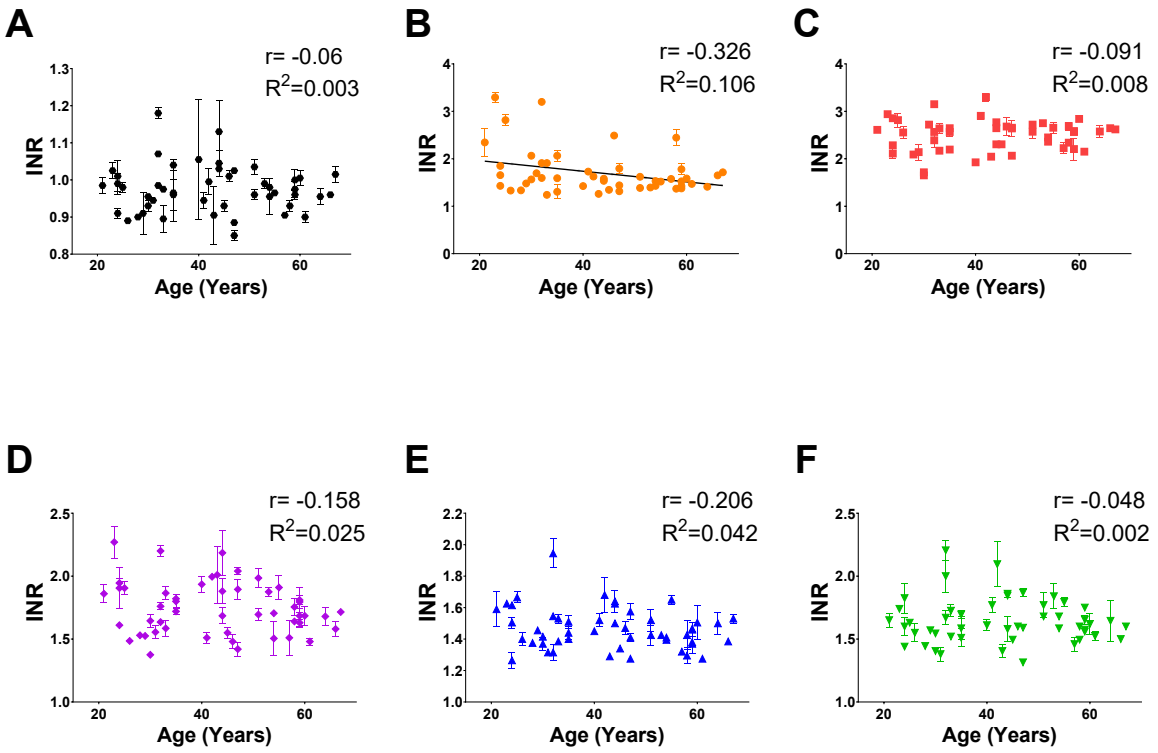


Figure 13. Relationship between INR and age in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

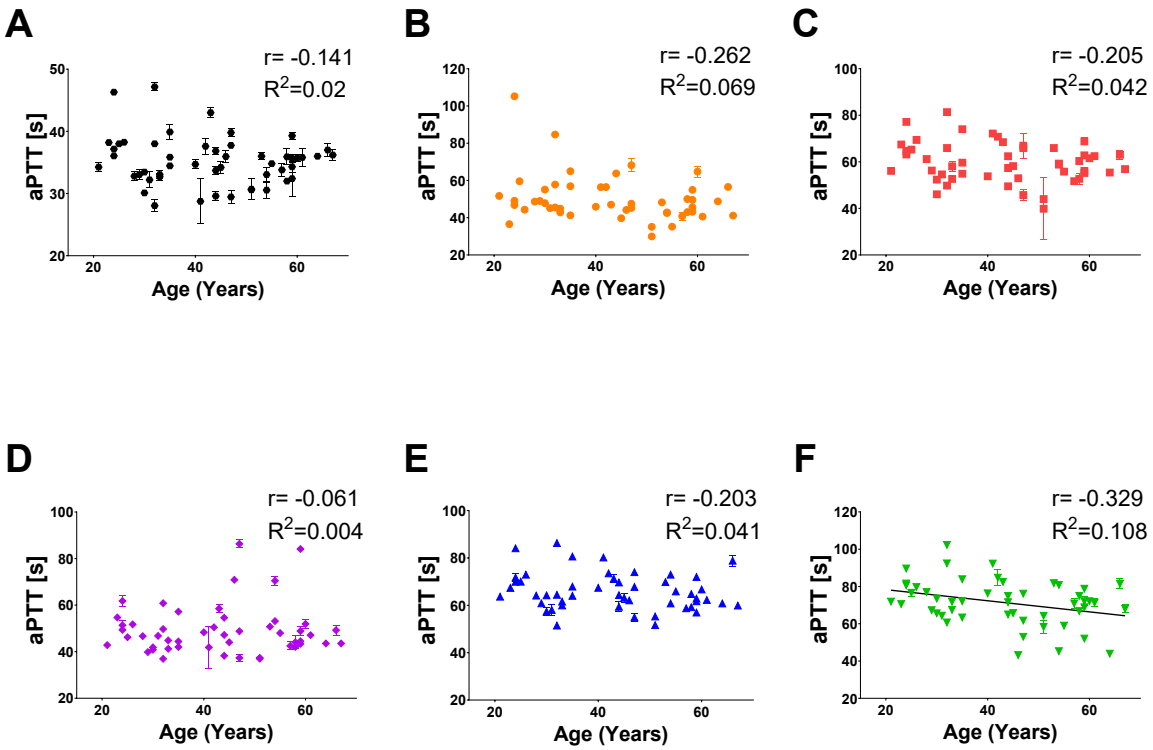


Figure 14. Relationship between aPTT and age in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

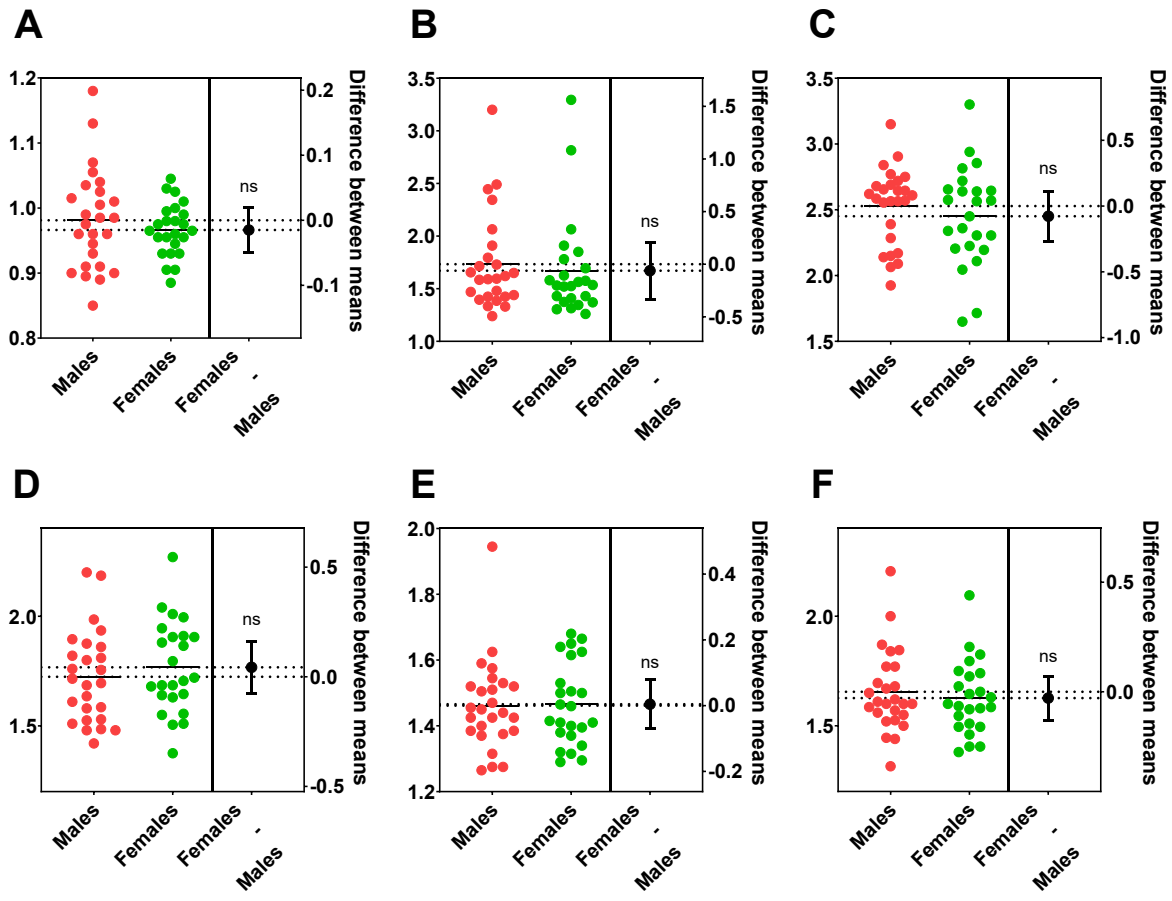


Figure 15. Comparison of INR values between healthy male and female donors. (A) negative control (DMSO), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). No significant differences were found.

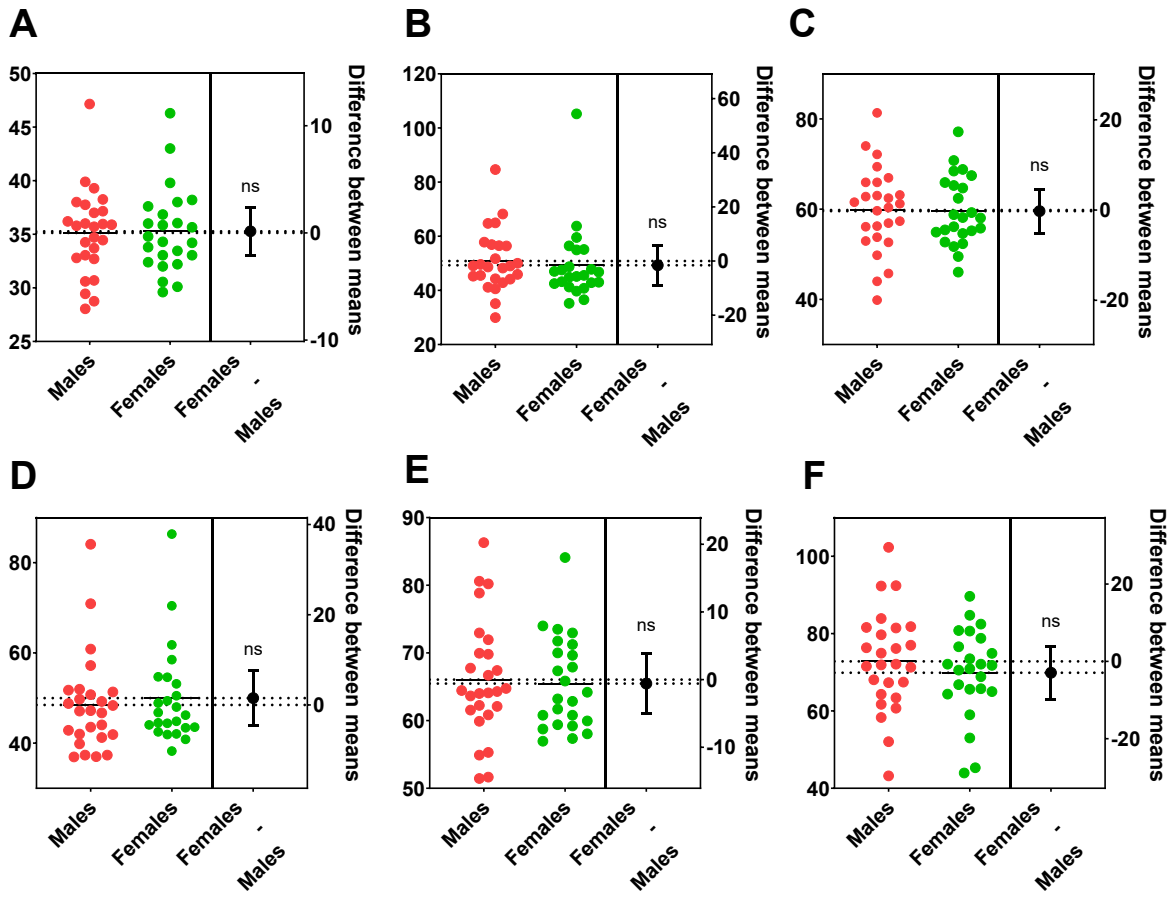


Figure 16. Comparison of aPTT values between healthy male and female donors. (A) negative control (DMSO), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). No significant differences were found.

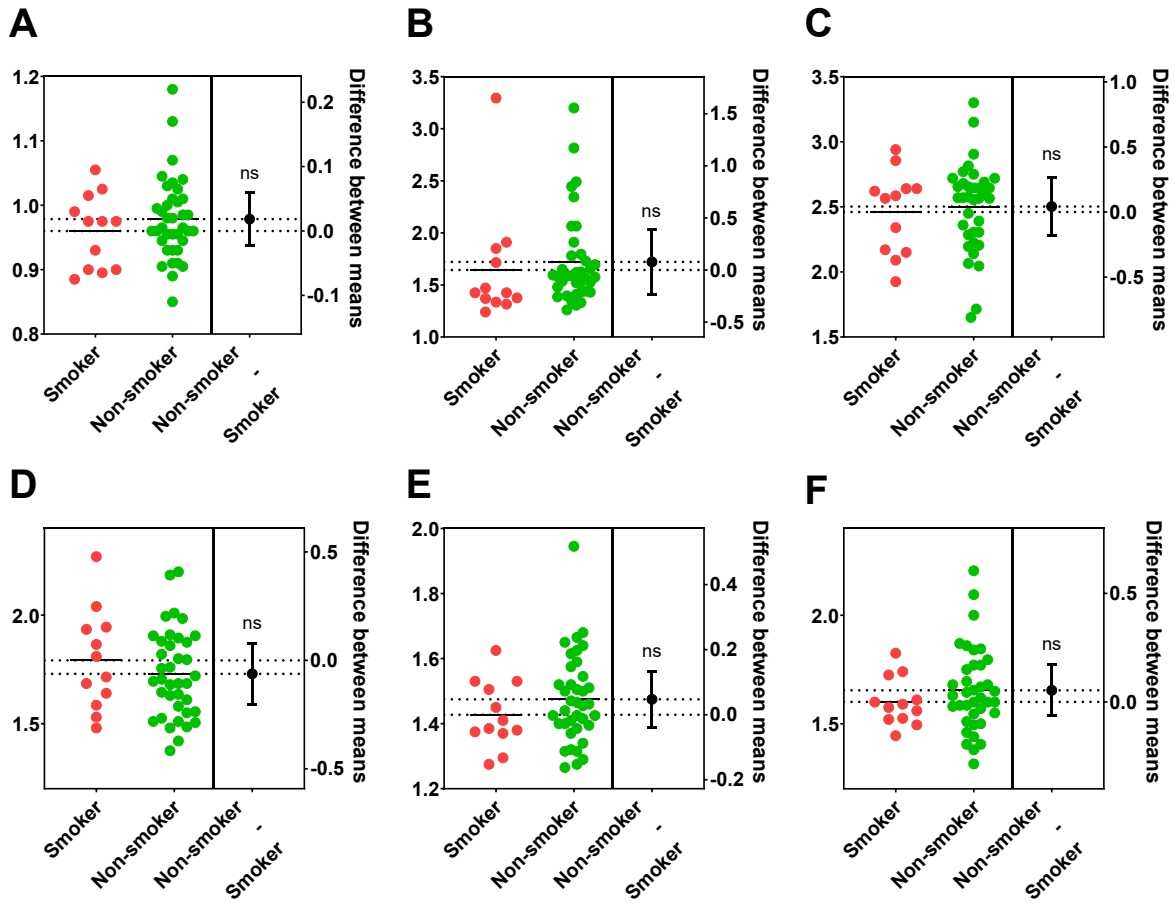


Figure 17. Comparison of INR values between smoker and non-smoker persons in healthy donors. (A) negative control (DMSO), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). No significant differences were found.

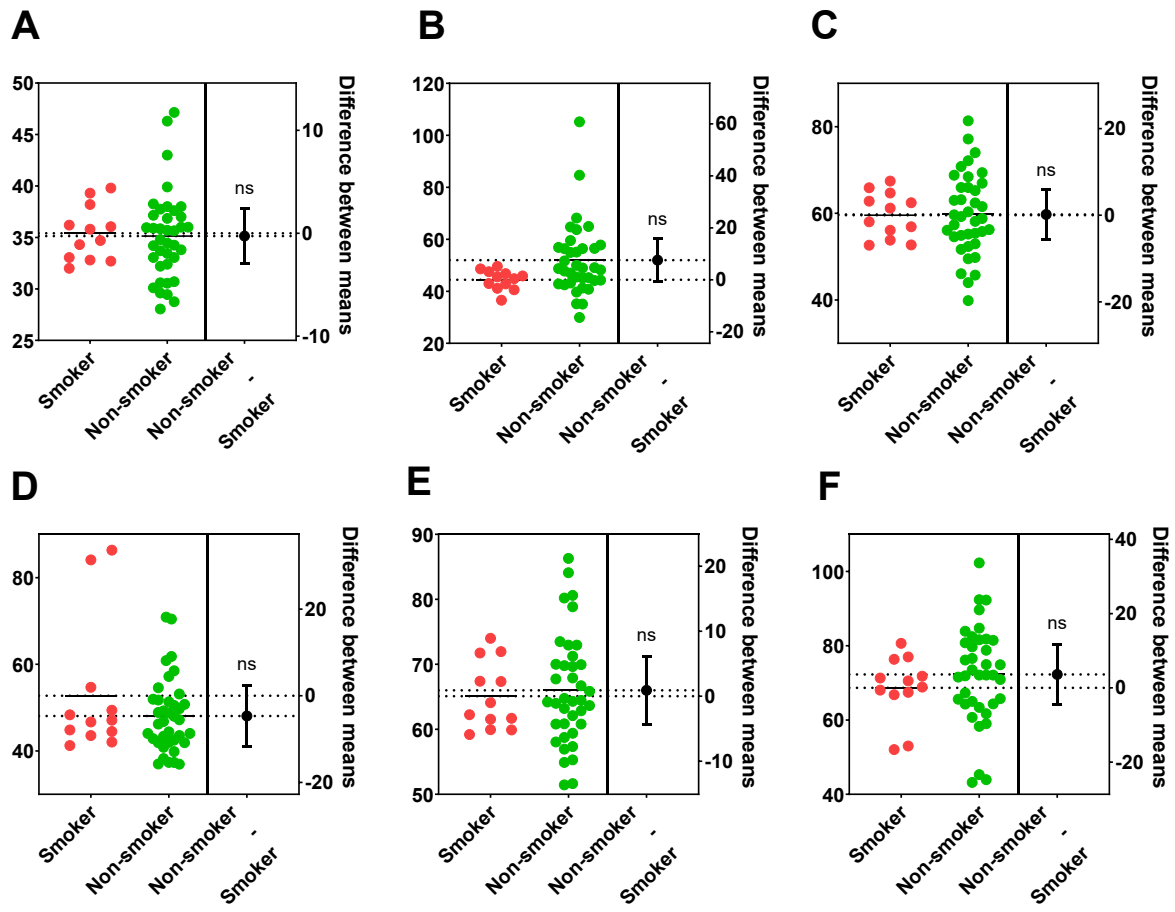


Figure 18. Comparison of aPTT values between smoker and non-smoker persons in healthy donors. (A) negative control (DMSO), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). No significant differences were found.

The relationships between coagulation values and biochemical parameters, particularly regarding cholesterol levels, are more pronounced in aPTT compared to that of PT (INR). Higher levels of LDL-cholesterol were associated with shorter aPTT in 4 of the 5 tested anticoagulants, meanwhile, a shortened INR was only found in dabigatran (Figures 16 & 20). Similar conditions were observed in other parameters, INR correlated only with triglycerides after apixaban treatment (Figure 21). Total cholesterol and non-HDL-cholesterol INR correlated solely with PT dabigatran-treated samples (Figures 23 & 25). Shorter aPTT times were observed in 3 anticoagulants (heparin, rivaroxaban, and dabigatran) when the levels of triglycerides increased (Figure 22). Identical relationships were found in total cholesterol and non-HDL as shorter aPTT was associated with higher total cholesterol and non-HDL cholesterol in samples treated with four anticoagulants and the negative control as well (Figures 23 & 25). Among all cholesterol parameters, only HDL-cholesterol did not correlate with neither INR nor aPTT (Figures 26 & 27). In addition, an increase in glucose levels shortened INR in heparin and apixaban-treated samples, but not in the case of aPTT (Figures 29 & 30).

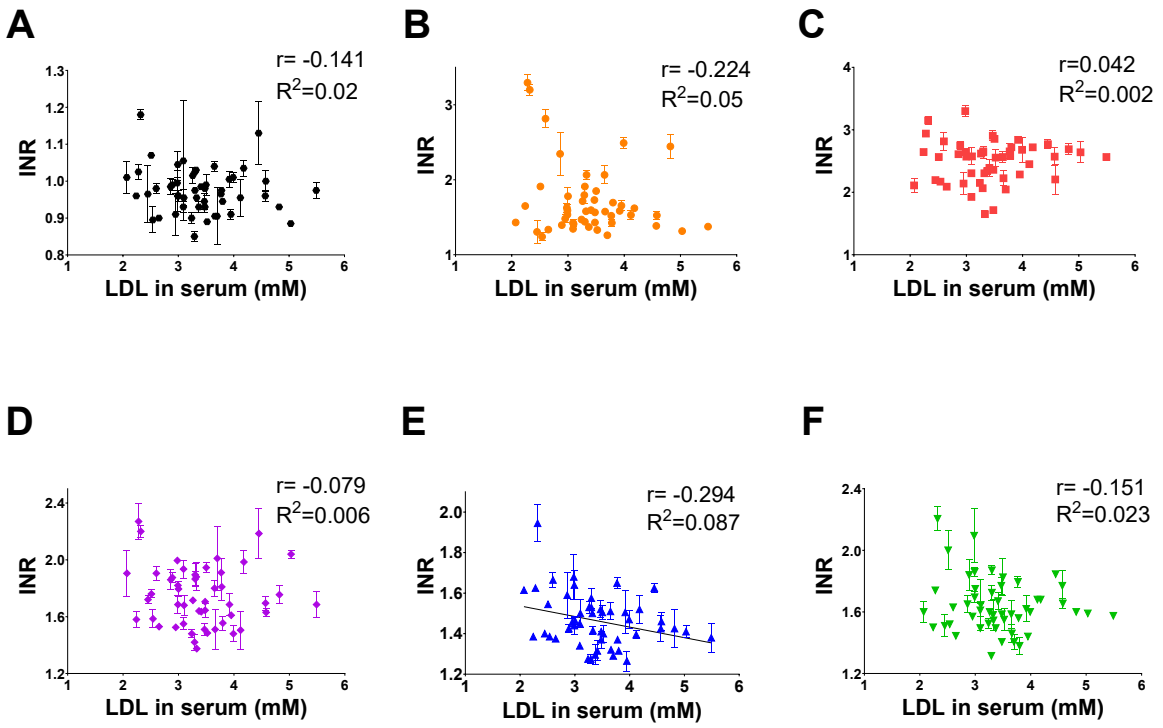


Figure 19. Relationship between INR and LDL-cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

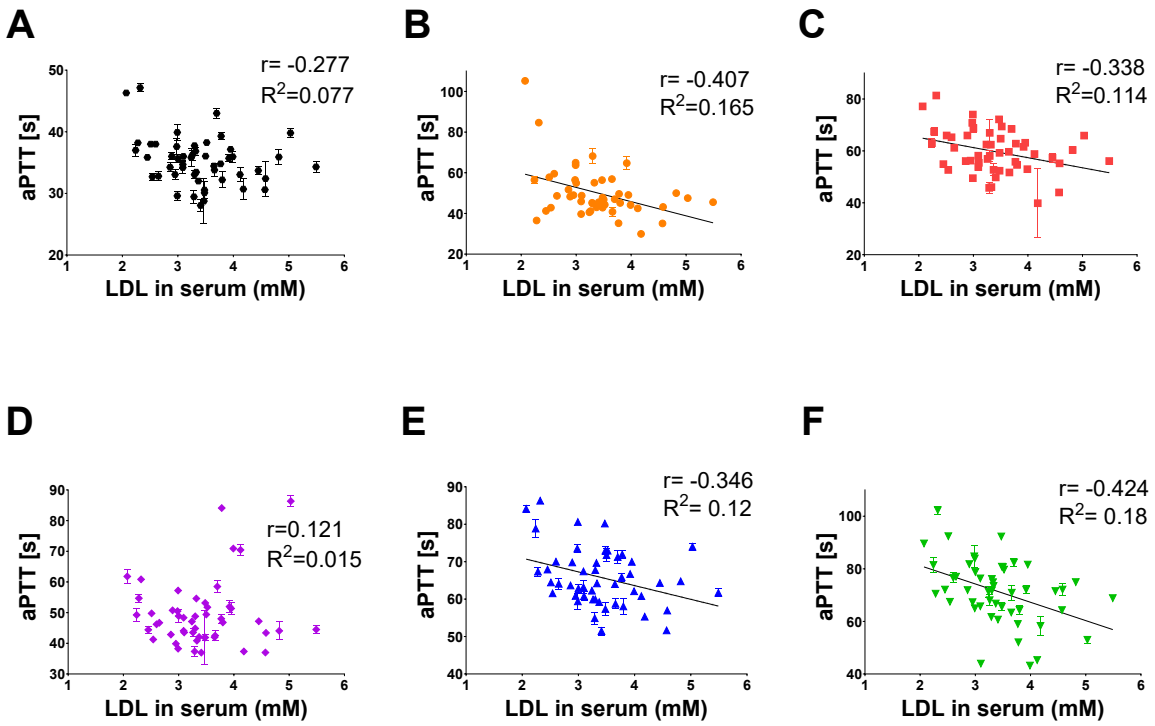


Figure 20. Relationship between aPTT and LDL-cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

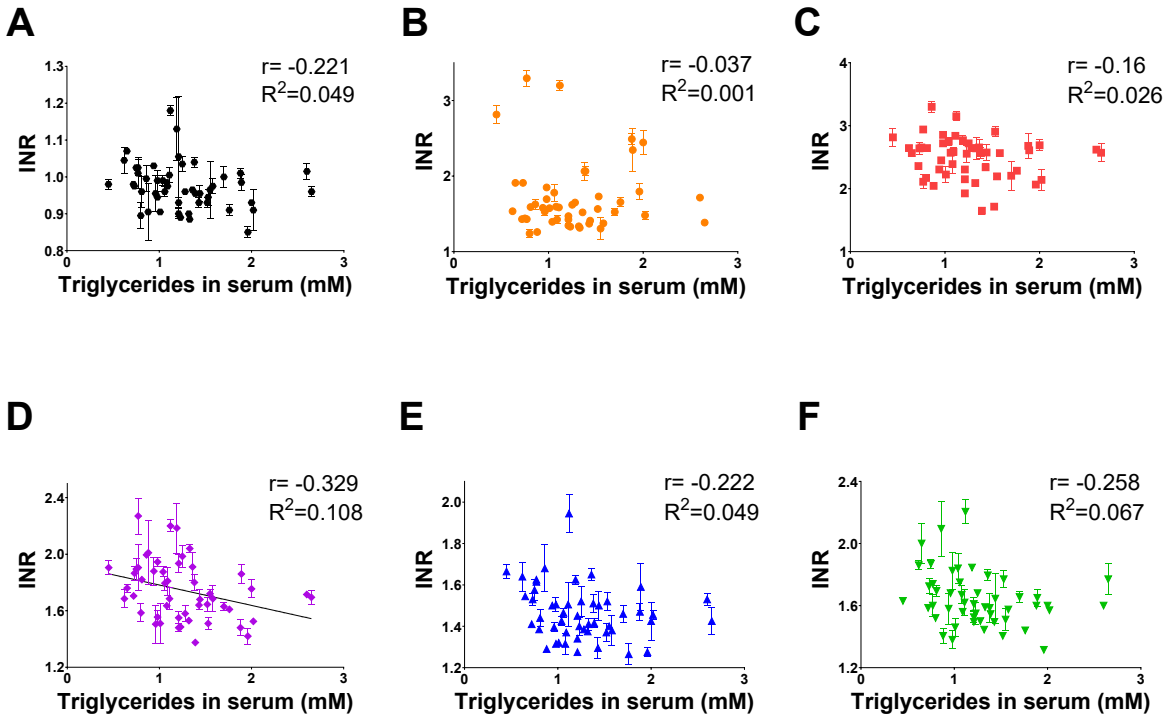


Figure 21. Relationship between INR and triglyceride levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

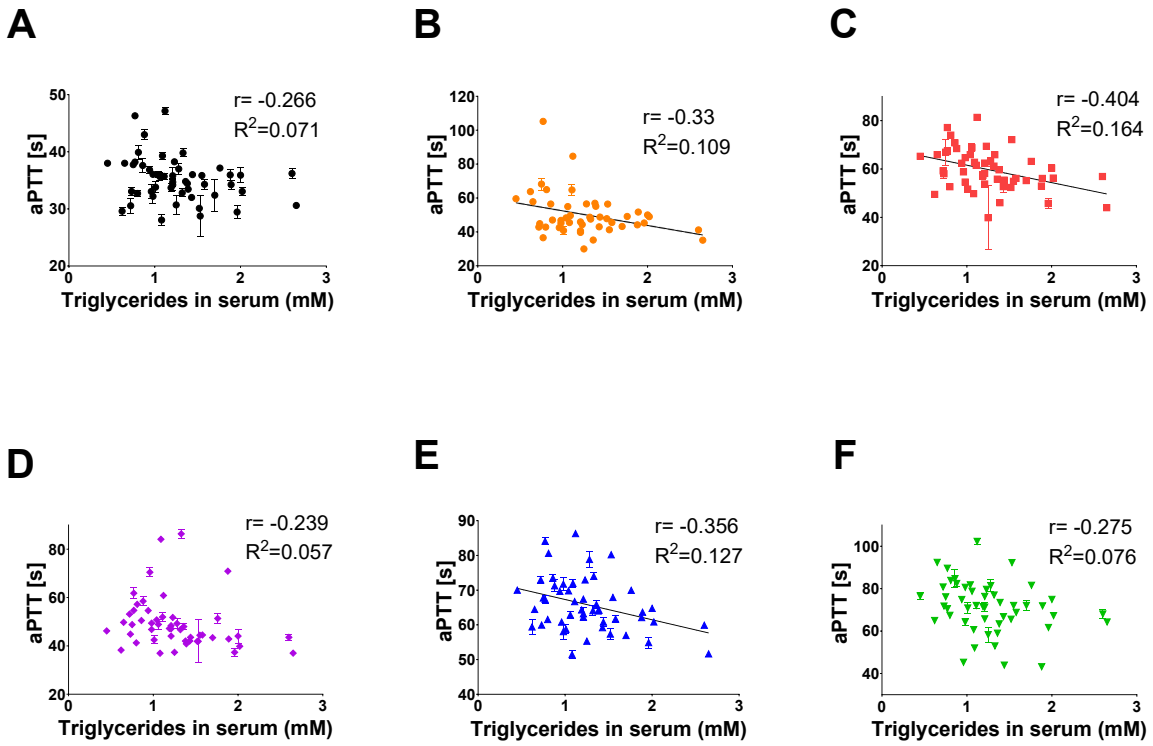


Figure 22. Relationship between aPTT and triglyceride levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

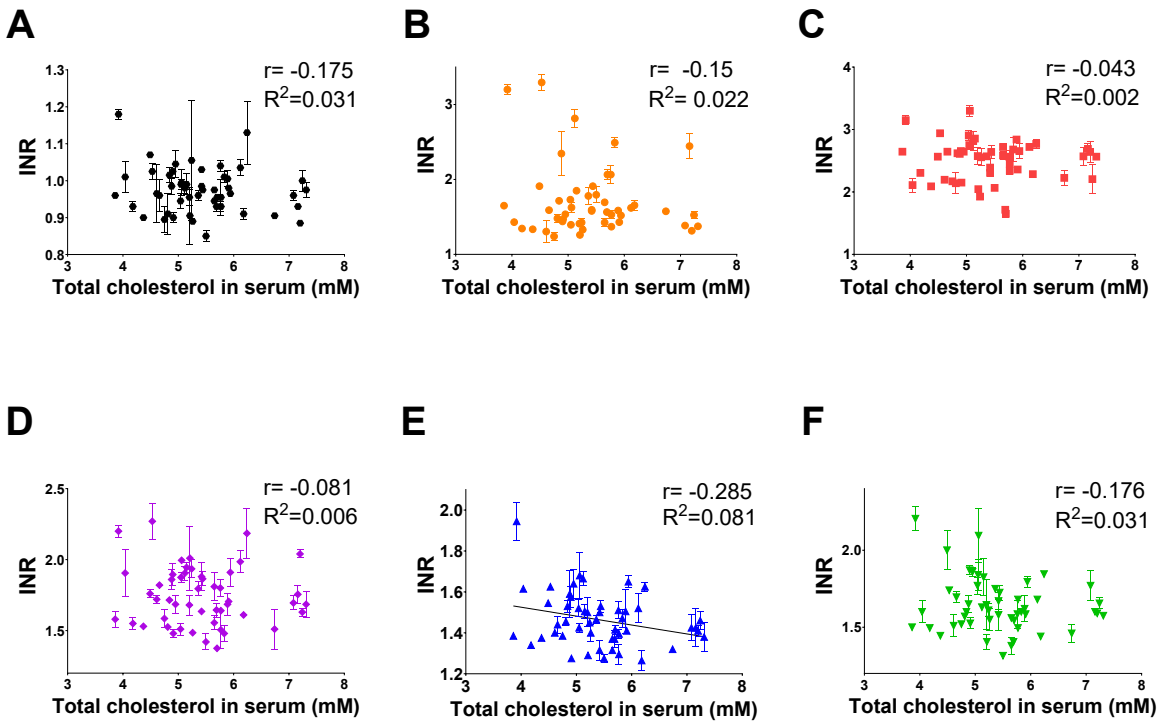


Figure 23. Relationship between INR and total cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

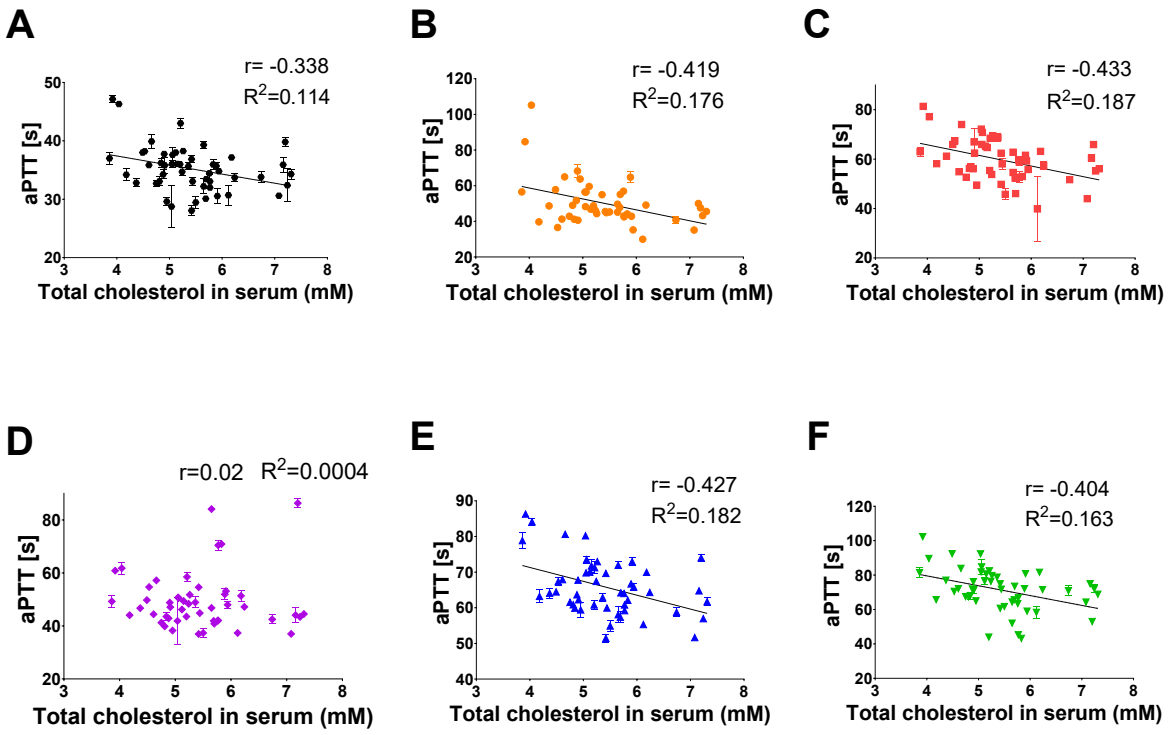


Figure 24. Relationship between aPTT and total cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

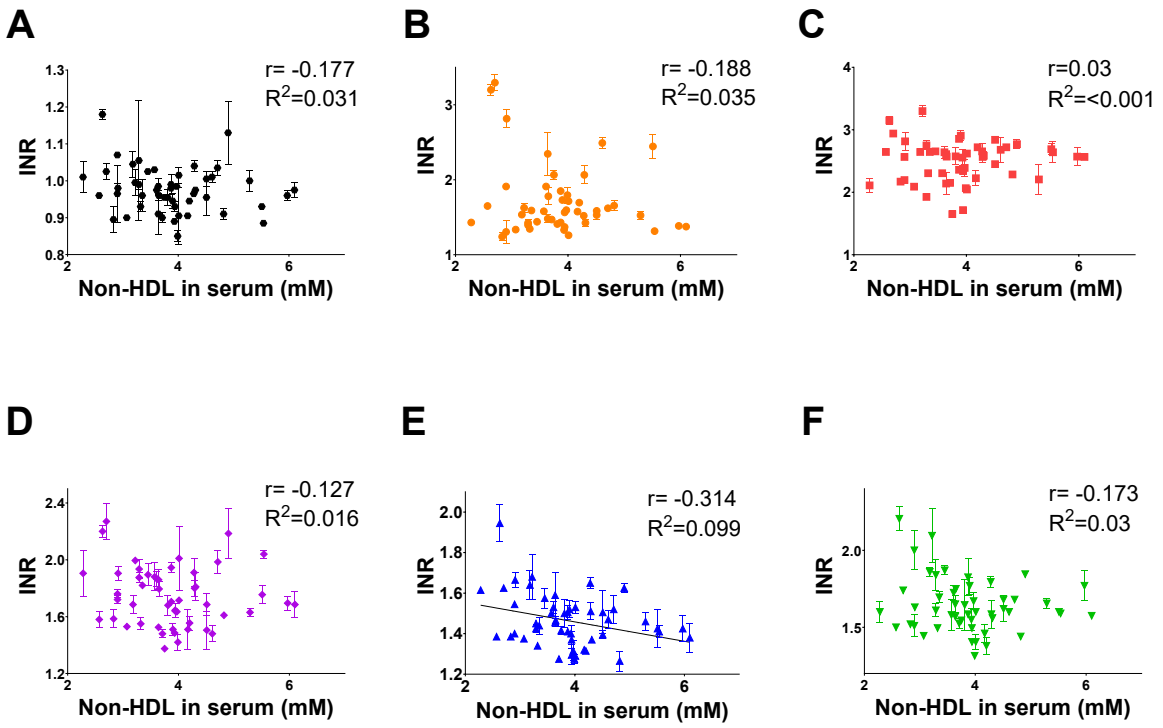


Figure 25. Relationship between INR and non-HDL-cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

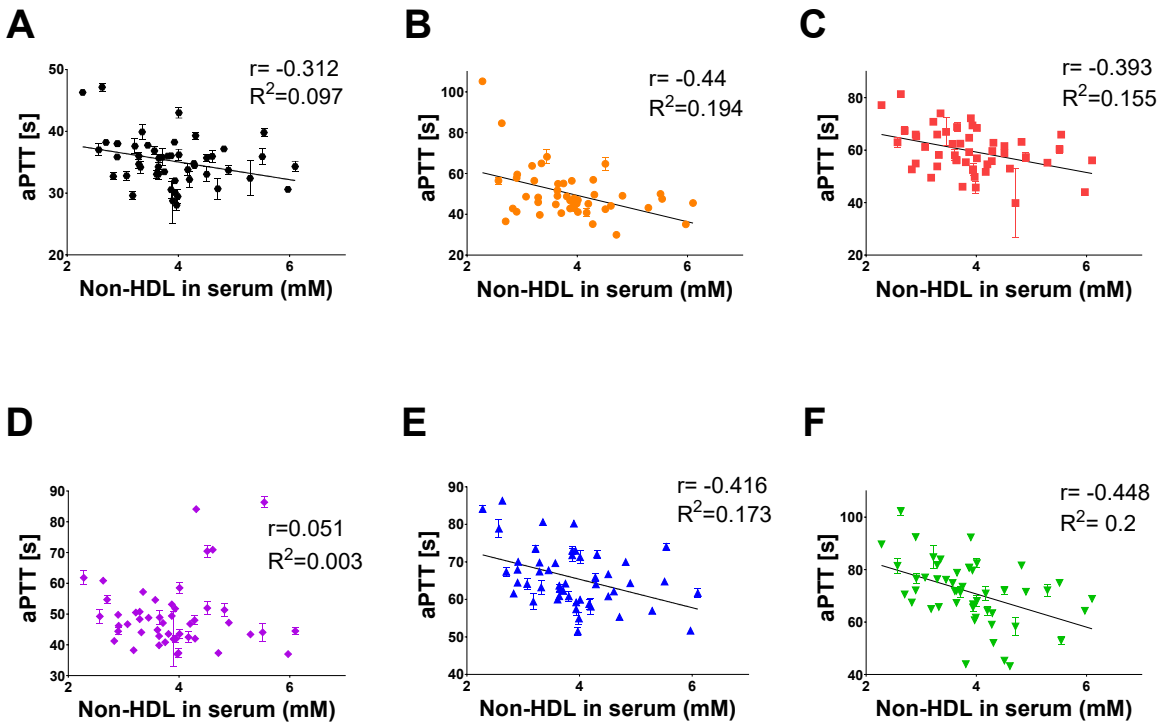


Figure 26. Relationship between aPTT and non-HDL-cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

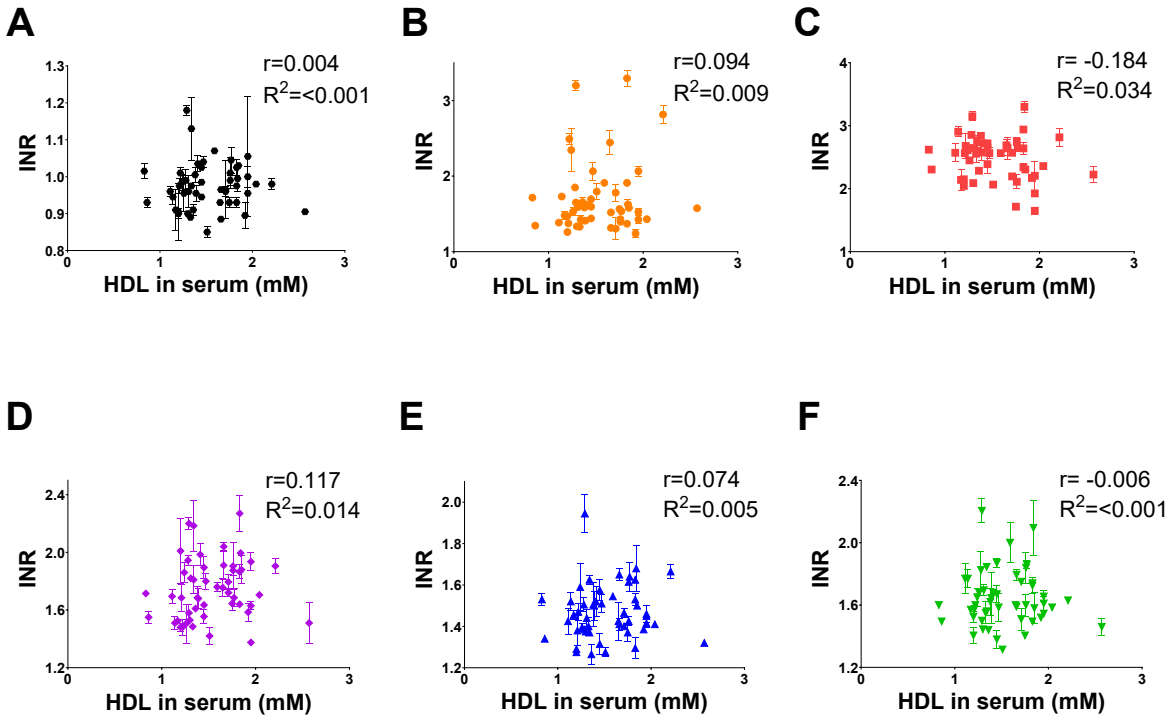


Figure 27. Relationship between INR and HDL-cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM).

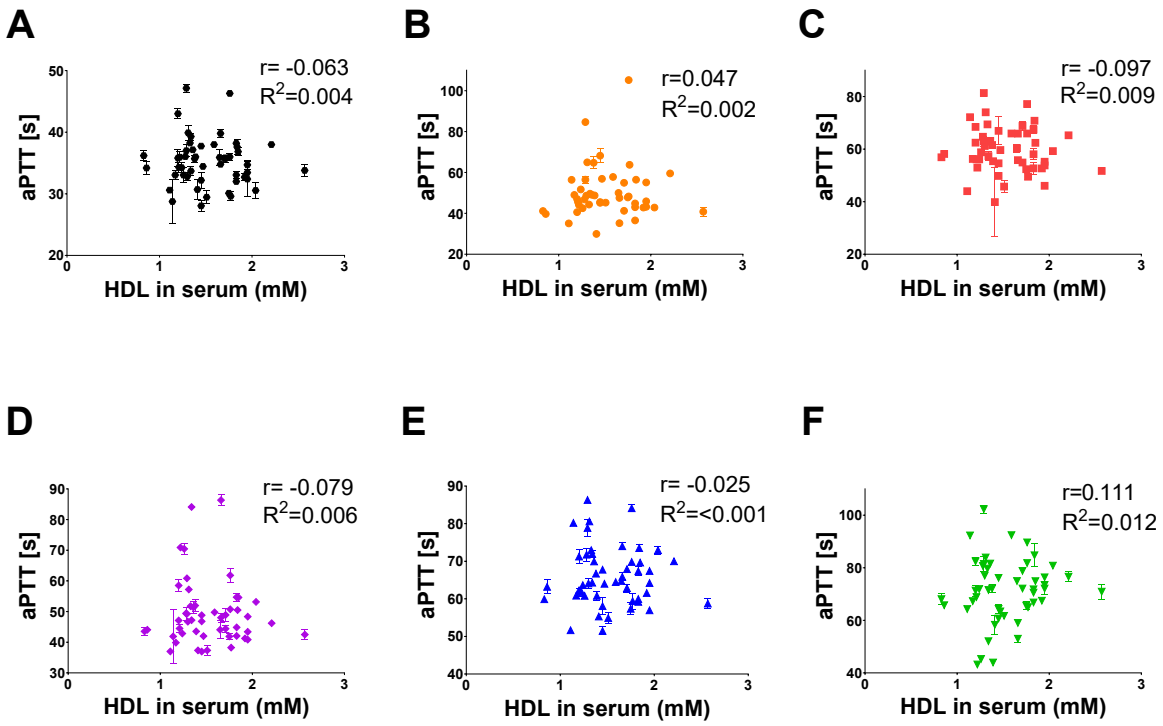


Figure 28. Relationship between aPTT and HDL-cholesterol levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM).

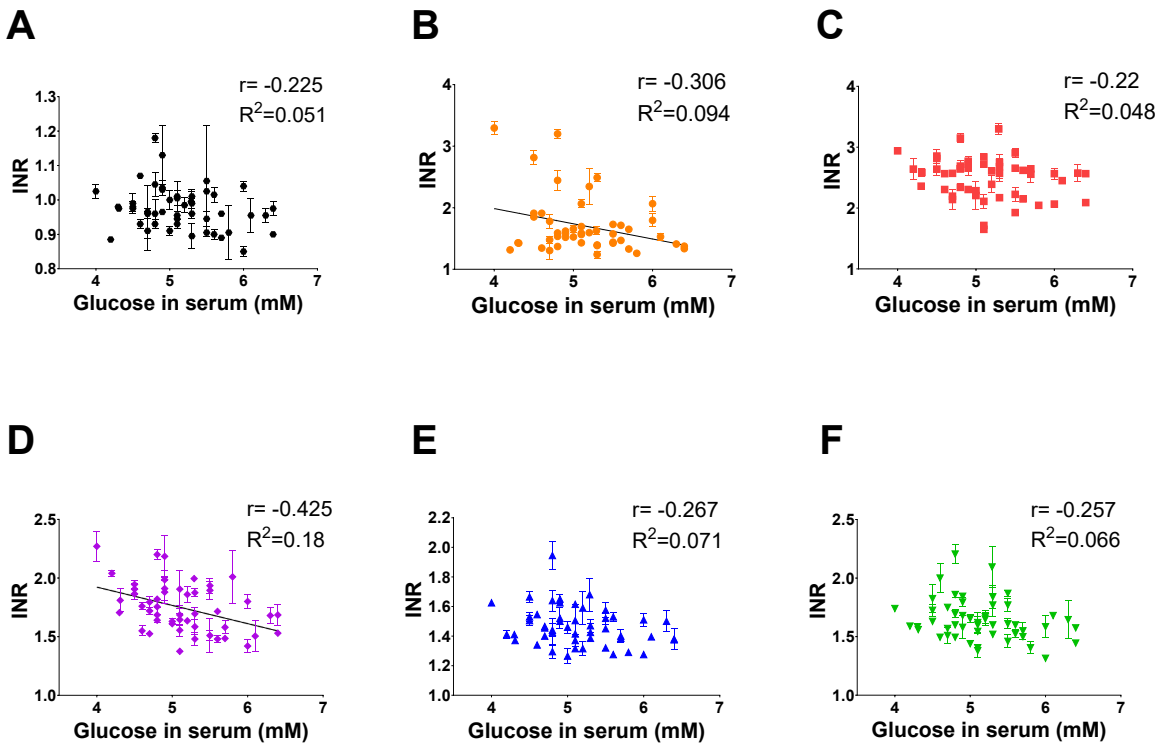


Figure 29. Relationship between INR and glucose levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 5 IU/mL), (C) rivaroxaban (1 µM), (D) apixaban (1 µM), (E) dabigatran (1 µM), and (F) argatroban (1 µM). In case of significant relationships, also linear regression was performed.

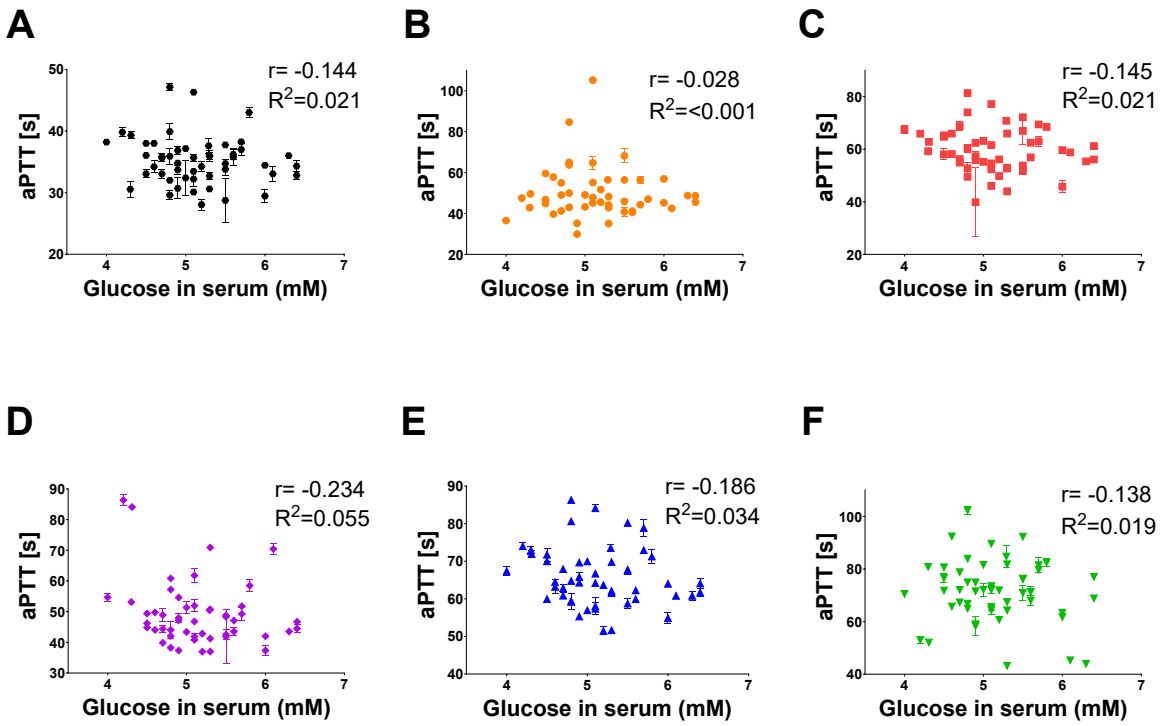


Figure 30. Relationship between aPTT and glucose levels in healthy donors. (A) Negative control (solvent) (DMSO 1%), (B) positive control (heparin 0.5 IU/mL), (C) rivaroxaban (1 μM), (D) apixaban (1 μM), (E) dabigatran (1 μM), and (F) argatroban (1 μM).

4.2 Familial Hypercholesterolemia (FH)

As cholesterol plays an important role in coagulation (chapter 1.2), further experiments were carried out with severe cases of FH patients. Fifteen patients were enrolled in this study. Although it is a small number, this is the number of all FH patients treated at University Hospital in Hradec Králové. Of the fifteen patients, only twelve patients were finally analyzed in our study since three of them had been administered anticoagulants on a long-term basis.

First, the effect of clinically used direct anticoagulants, negative (DMSO) and positive (heparin) controls were compared between healthy individuals and FH patients, to see the possible differences between them (Figure 31). When PT (INR) was analyzed, there were no differences in basal coagulation

between FH and controls. There were however differences in aPTT as FH had longer aPTT than controls in solvent-treated samples (Figure 31B).

As expected, all tested anticoagulants extended coagulation time in FH patients as it did with healthy donors (Figure 10). Dabigatran treatment, however, prolonged coagulation in FH patients to a significantly higher extent when compared to controls in both PT and aPTT assays. Interestingly, the effect of rivaroxaban was only significant in the case of aPTT, while there were no differences between FH and controls for apixaban or argatroban.

In the next step, the possible differences in three homozygous and nine heterozygous patients were investigated. Even if the number of homozygous patients was very low, there did not appear to be differences in coagulation based on PT and aPTT tests as well as in the effects of anticoagulants (Figure 32).

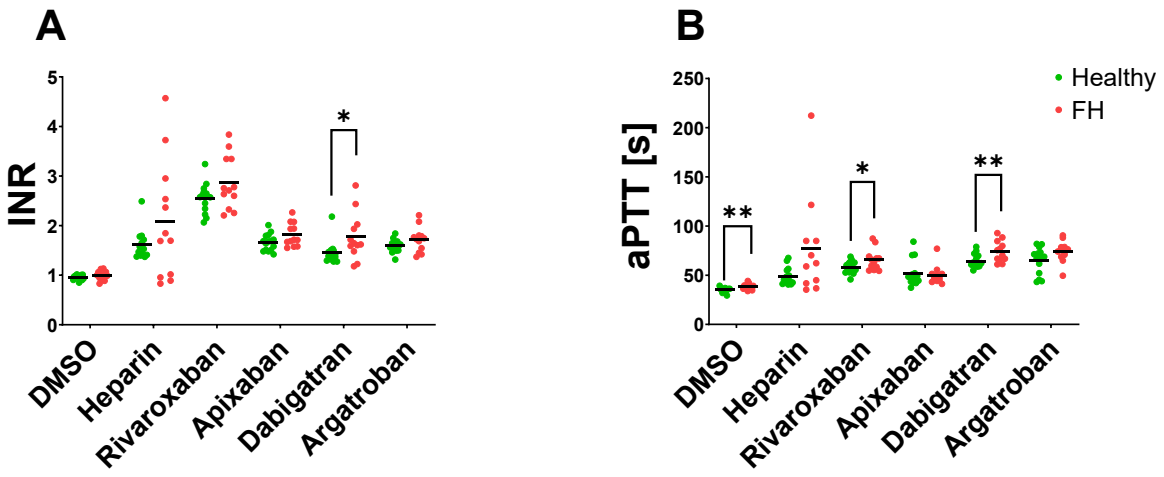


Figure 31. Coagulation differences between generally healthy donors and familial hypercholesterolemia (FH) patients. (A) prothrombin time (PT) values expressed as the international normalized ratio (INR), (B) activated partial thromboplastin time (aPTT). DMSO was used as vehicle control with a final concentration of 1% and heparin as positive control with a final concentration of 5 and 0.5 IU/mL for PT and aPTT assays, respectively. The final concentration of 1 µM was employed for all tested direct anticoagulants. In patients treated with apheresis, solely pre-apheresis values were considered. *$p<0.05$; **$p<0.01$

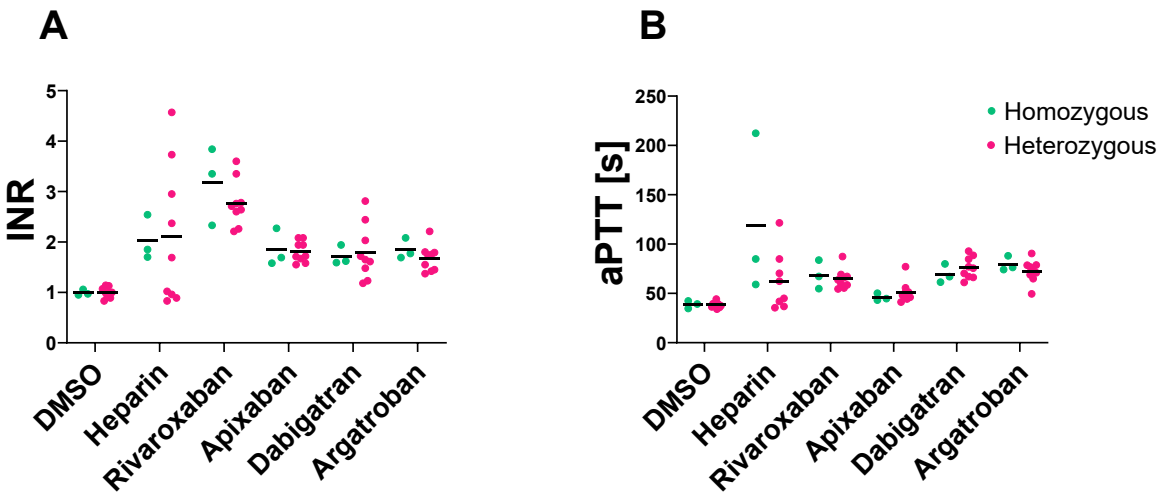


Figure 32. Coagulation in homozygous and heterozygous FH patients. (A) PT values are expressed as INR and (B) aPTT. DMSO was used as vehicle control with a final concentration of 1% and heparin as positive control with a final concentration of 5 and 0.5 IU/mL for PT and aPTT assays, respectively. The final concentration of 1 μM was employed for all tested direct anticoagulants. In patients treated with apheresis, solely pre-apheresis values were considered. No significant differences were found.

In these patients, pharmacotherapy was not the only approach, since eight patients also underwent apheresis, therefore the long-term impact of this treatment modality needed to be investigated. As an anticoagulant was used within the apheresis procedure, the prolongation of post-apheresis coagulation values was expected (Figure 33). For this reason, we could compare coagulation parameters solely in before-apheresis samples with samples from patients not treated with this procedure. No significant differences were obtained between patients not treated and those treated with apheresis (Figure 34AB). As most of the patients were also treated with PCSK9Ab, the differences between patients treated with apheresis and PCSK9Ab *vs.* patients treated solely with PCSK9Ab were compared. However, there were no differences in coagulation parameters observed between these two groups (Figure 34CD).

Since vitamin K is a cornerstone for the synthesis of many coagulation factors (chapter 1.4.2, [122]), the most important forms of vitamin K (K_1, MK-4, MK-7, and MK-9) were measured in the

serum of both healthy and FH patients (Figure 35). MK-9 was under the limit of detection in all samples and therefore was not further evaluated. There were no differences in basal levels between healthy donors and patients with FH, but apheresis significantly reduced the levels of MK-4 and there was a tendency for other forms to drop as well.

Further, the possibility of an association between biochemical parameters with coagulation patterns was studied. The most important correlations were found in lipid levels with aPTT (Figure 36). The inverse correlation coefficients for LDL, non-HDL, and total cholesterol with aPTT indicate that elevated serum cholesterol levels are linked to shorter coagulation times, potentially accelerating the coagulation process. It is noteworthy that a negative correlation was also found between HDL-cholesterol and aPTT; however, this phenomenon was only observed in the samples that contained solvent and the addition of anticoagulants did not produce the same effect.

Complete correlation analyses of INR and aPTT with biochemical parameters are available in Tables 4 & 5.

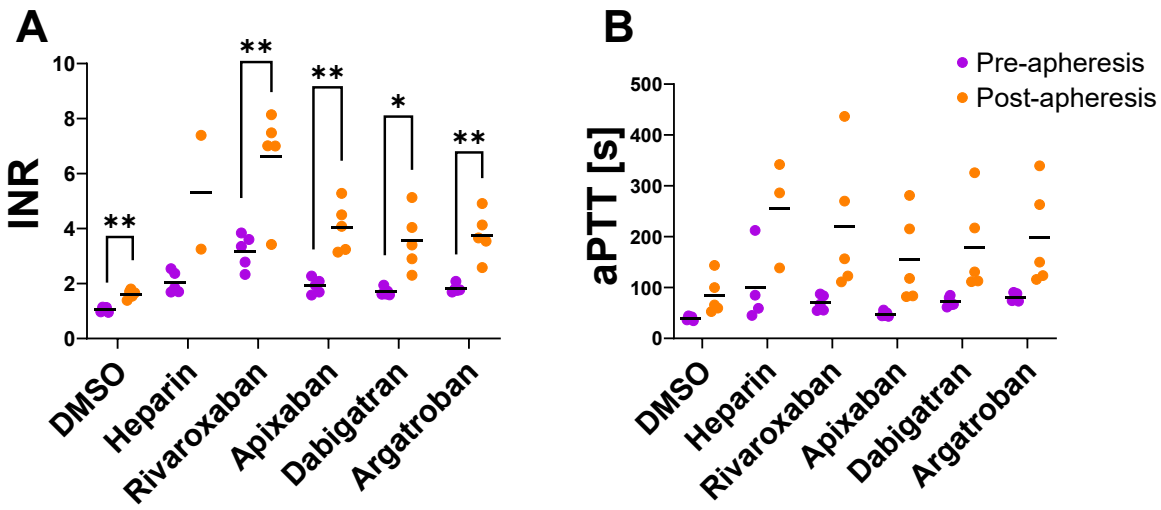


Figure 33. Coagulation results in FH patients pre- and post-apheresis. (A) PT values expressed as INR and (B) aPTT values in 5 included FH patients undergoing apheresis. *p<0.05; **0<0.01

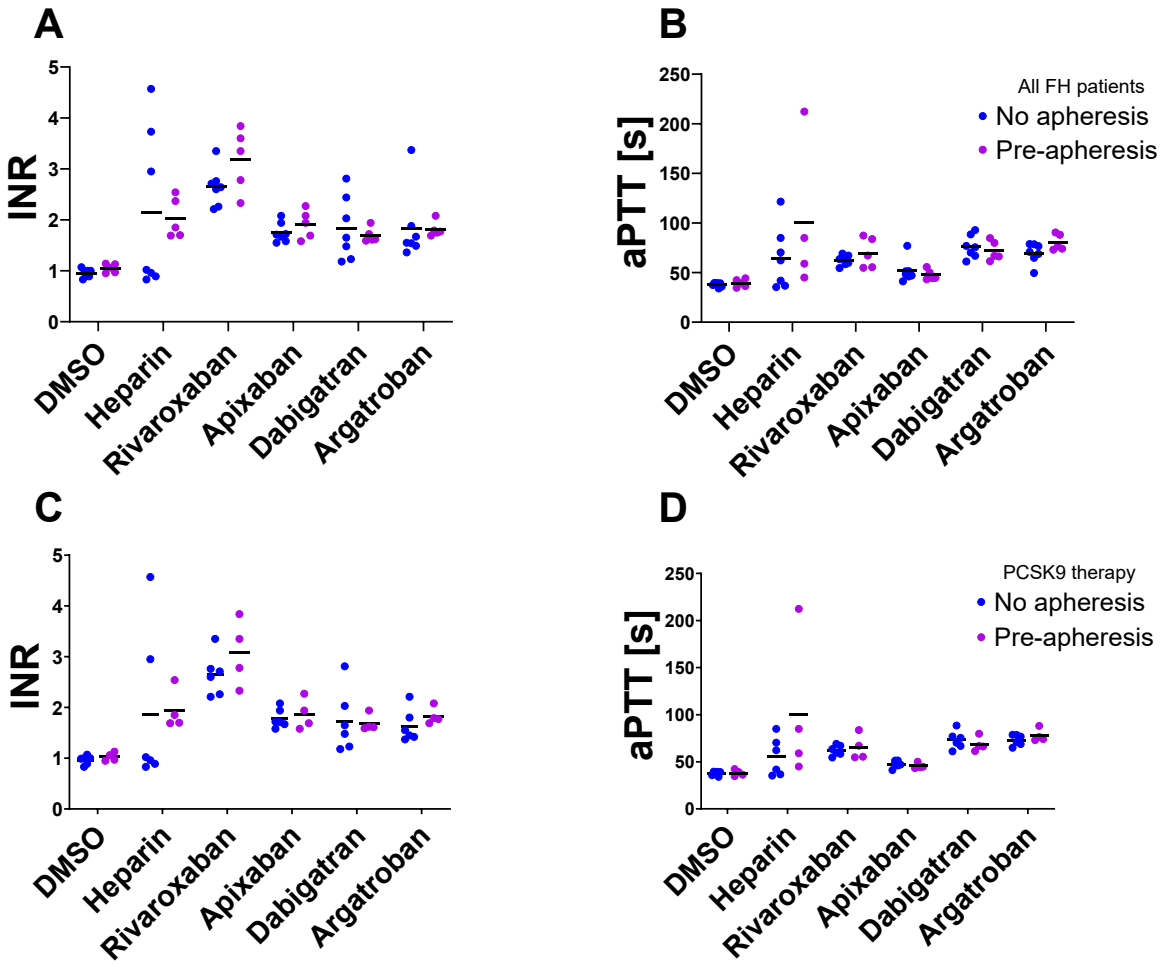


Figure 34. Coagulation results of FH patients without apheresis and pre-apheresis. (A) PT values expressed as INR, (B) aPTT, (C) PT expressed as INR in PCSK9Ab-treated patients, and (D) aPTT in PCSK9Ab-treated patients. In total, 10 FH patients were treated with PCSK9Ab, 4 of them underwent apheresis. No apheresis means morning blood samples of patients not treated with apheresis (7 patients) while pre-apheresis means morning blood samples before apheresis procedure (5 patients). No significant differences were found.

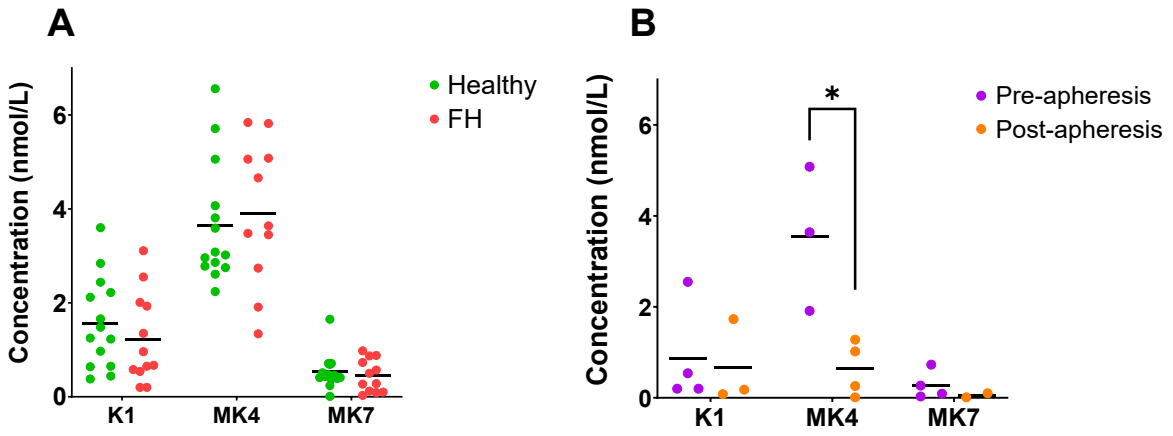


Figure 35. Detection of vitamin K forms: vitamin K_1 (K1) and vitamin K_2 forms (MK-4 and MK-7) in plasma samples. (A) healthy donors vs. all FH patients and (B) FH patients pre- vs. post-apheresis. Complete vitamin K data for pre- and post-apheresis were available solely for 3 FH patients not using anticoagulants. *p<0.05

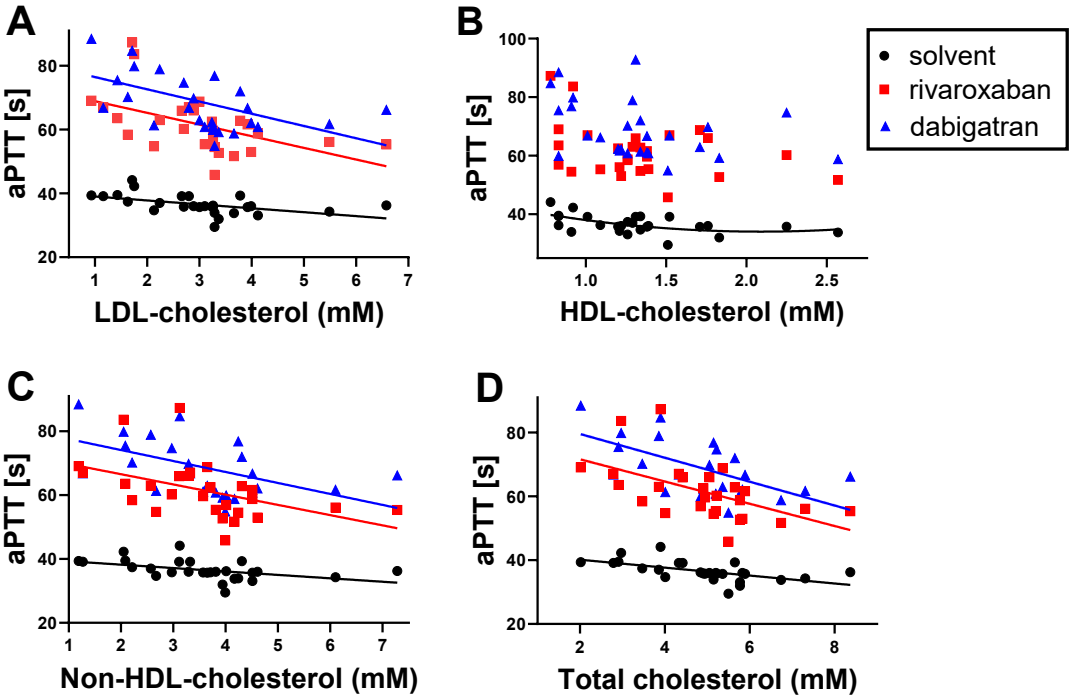


Figure 36. Correlation of lipidic parameters with aPTT. (A) correlation between LDL-cholesterol and aPTT, (B) correlation between HDL-cholesterol and aPTT – significant correlation was found solely for solvent, (C) correlation between non-HDL-cholesterol and aPTT, and (D) correlation between total cholesterol and aPTT.

Table 4. Pearson correlations between coagulation data and biochemical parameters detected in FH patients or volunteer blood samples

	Parameters	DMSO		Heparin		Rivaroxaban		Apixaban		Dabigatran		Argatroban	
		r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
INR	Glucose	**0.491**	**0.009**	0.112	0.593	0.24	0.229	0.201	0.316	0.181	0.368	0.091	0.651
	LDL	0.003	0.988	-0.239	0.25	**-0.412**	**0.033**	-0.264	0.183	**-0.389**	**0.045**	-0.325	0.098
	HDL	**-0.424**	**0.028**	-0.212	0.309	-0.276	0.164	-0.219	0.274	-0.225	0.259	-0.277	0.163
	TG	0.362	0.064	-0.083	0.693	0.032	0.873	0.156	0.438	-0.085	0.645	-0.191	0.339
	Total cholesterol	-0.059	0.769	-0.285	0.167	**-0.448**	**0.019**	-0.269	0.174	**-0.43**	**0.025**	**-0.386**	**0.047**
	Non-HDL	0.072	0.72	-0.248	0.232	**-0.392**	**0.043**	-0.219	0.273	**-0.389**	**0.045**	-0.326	0.097
	Vit. K_1	0.094	0.647	-0.197	0.344	-0.187	0.361	-0.122	0.553	-0.221	0.279	-0.204	0.317
	Vit. MK4	-0.143	0.497	-0.255	0.219	-0.301	0.144	-0.069	0.744	-0.255	0.22	-0.377	0.063
	Vit. MK7	-0.108	0.601	-0.252	0.225	-0.234	0.25	-0.119	0.563	-0.191	0.351	-0.18	0.379
	Total vitamin K	-0.034	0.868	-0.284	0.169	-0.369	0.064	-0.096	0.642	-0.264	0.193	-0.357	0.073
aPTT	Glucose	0.219	0.273	**0.648**	**0.0003**	0.232	0.245	-0.116	0.408	0.289	0.144	0.184	0.359
	LDL	**-0.499**	**0.008**	**-0.45**	**0.021**	**-0.501**	**0.007**	0.073	0.716	**-0.488**	**0.010**	**-0.400**	**0.04**
	HDL	**-0.479**	**0.012**	-0.310	0.123	-0.371	0.057	-0.121	0.549	-0.367	0.060	-0.094	0.640
	TG	0.151	0.452	**0.471**	**0.015**	0.096	0.634	-0.062	0.756	0.078	0.699	0.068	0.735
	Total cholesterol	**-0.571**	**0.002**	**-0.411**	**0.037**	**-0.547**	**0.03**	0.013	0.949	**-0.540**	**0.004**	-0.379	0.051
	Non-HDL	**-0.459**	**0.016**	-0.342	0.088	**-0.468**	**0.014**	0.053	0.794	**-0.462**	**0.015**	-0.377	0.053
	Vit. K_1	-0.135	0.512	0.074	0.724	-0.07	0.735	-0.036	0.862	-0.186	0.363	-0.091	0.658
	Vit. MK4	-0.072	0.732	-0.085	0.693	-0.235	0.258	-0.059	0.778	-0.228	0.272	-0.265	0.201
	Vit. MK7	-0.095	0.644	0.033	0.875	-0.087	0.674	-0.073	0.724	-0.141	0.491	-0.293	0.146
	Total vitamin K	-0.16	0.434	-0.054	0.797	-0.21	0.304	-0.024	0.907	-0.214	0.295	-0.264	0.193

LDL= low-density lipoprotein; HDL= high-density lipoprotein; TG: triglyceride

Table 5. Spearman correlations between coagulation data and biochemical parameters detected in FH patients or volunteer blood samples

	Parameters	DMSO		Heparin		Rivaroxaban		Apixaban		Dabigatran		Argatroban	
		r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
INR	Glucose	0.284	0.152	0.071	0.736	0.057	0.776	-0.002	0.994	0.144	0.475	-0.004	0.986
	LDL	-0.092	0.648	-0.093	0.659	-0.37	0.057	-0.363	0.063	**-0.462**	**0.015**	**-0.407**	**0.035**
	HDL	**-0.538**	**0.004**	-0.21	0.315	-0.309	0.117	-0.272	0.17	-0.351	0.073	-0.192	0.337
	TG	0.154	0.444	-0.032	0.878	-0.16	0.426	-0.014	0.946	-0.085	0.675	-0.379	0.051
	Total cholesterol	-0.101	0.617	-0.102	0.629	-0.325	0.099	-0.287	0.147	**-0.39**	**0.045**	-0.317	0.108
	Non-HDL	-0.003	0.989	-0.051	0.81	-0.324	0.1	-0.309	0.117	**-0.387**	**0.046**	**-0.381**	**0.049**
	Vit. K_1	0.000	0.999	-0.179	0.391	-0.203	0.32	-0.195	0.341	-0.228	0.264	-0.149	0.469
	Vit. MK4	-0.175	0.402	-0.104	0.621	-0.302	0.142	-0.192	0.357	-0.14	0.504	-0.352	0.084
	Vit. MK7	-0.287	0.155	-0.357	0.08	-0.317	0.115	-0.187	0.361	-0.259	0.202	-0.194	0.343
	Total vitamin K	-0.091	0.658	-0.202	0.334	-0.34	0.089	-0.189	0.354	-0.243	0.231	-0.331	0.098
aPTT	Glucose	-0.012	0.951	0.065	0.754	-0.186	0.353	-0.213	0.287	0.026	0.898	-0.065	0.746
	LDL	**-0.622**	**0.0005**	**-0.43**	**0.028**	**-0.629**	**0.0004**	-0.103	0.611	**-0.589**	**0.001**	**-0.6**	**0.001**
	HDL	**-0.525**	**0.005**	-0.114	0.58	-0.286	0.148	-0.188	0.347	**-0.421**	**0.029**	-0.164	0.415
	TG	-0.039	0.846	-0.261	0.198	**-0.397**	**0.041**	-0.279	0.158	-0.145	0.47	-0.301	0.127
	Total cholesterol	**-0.664**	**0.0002**	-0.334	0.096	**-0.619**	**0.0006**	-0.123	0.541	**-0.573**	**0.002**	**-0.512**	**0.006**
	Non-HDL	**-0.557**	**0.003**	-0.383	0.054	**-0.606**	**0.001**	-0.097	0.631	**-0.549**	**0.003**	**-0.6**	**0.0009**
	Vit. K_1	-0.139	0.5	**-0.466**	**0.019**	-0.185	0.367	-0.021	0.921	-0.261	0.199	-0.183	0.372
	Vit. MK4	0.013	0.952	**-0.49**	**0.015**	-0.297	0.149	-0.113	0.591	-0.253	0.222	-0.396	0.051
	Vit. MK7	-0.154	0.452	-0.39	0.054	-0.093	0.65	-0.023	0.911	-0.219	0.282	-0.165	0.421
	Total vitamin K	-0.138	0.501	**-0.563**	**0.003**	-0.326	0.104	-0.142	0.488	-0.338	0.091	-0.318	0.113

LDL= low-density lipoprotein; HDL= high-density lipoprotein; TG: triglyceride

4.3 Diabetes mellitus type 1

Also, as a part of this study, coagulation activity between healthy donors and DMT1 patients was evaluated using PT (as INR) and aPTT (Figure 37). Plasma treated solely with DMSO as the vehicle control at a final concentration of 1% did not differ between generally healthy individuals and DMT1 patients in both assays. However, heparin as the positive control prolonged coagulation time more intensively in both PT and aPTT tests in patients than in the generally healthy group. Similar results were obtained with dabigatran as it prolonged coagulation more extensively in DMT1 patients than in generally healthy controls in both tests. However, in the case of xabans (FXa-Is), this phenomenon was only found when PT values were compared. The second DTI, argatroban, produced the same effect both in healthy and DMT1 patients.

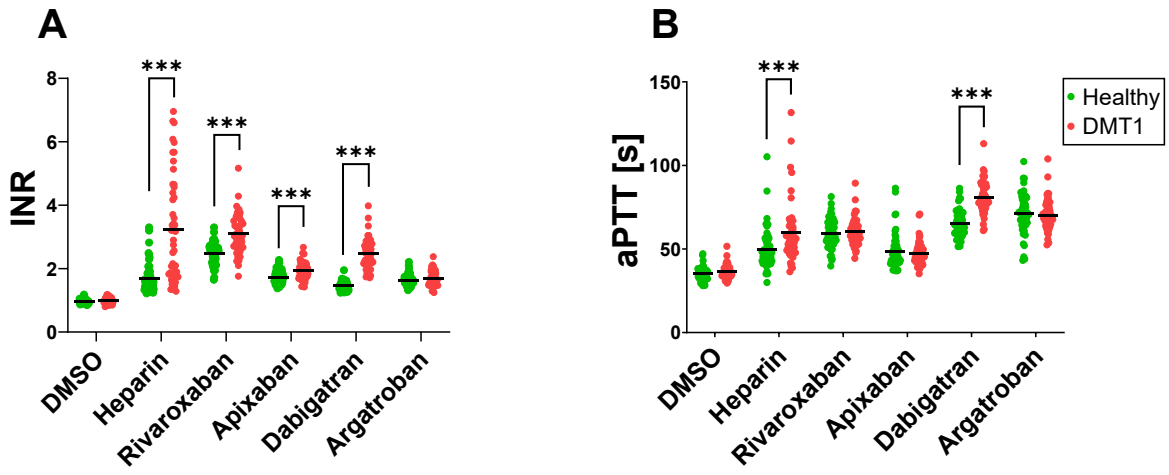


Figure 37. Coagulation differences between healthy donors and diabetes mellitus type 1 (DMT1) patients. (A) prothrombin time (PT) expressed as international normalized ratio (INR) and (B) activated partial thromboplastin time (aPTT). DMSO was used as vehicle control with a final concentration of 1% and heparin (indirect anticoagulant and positive control) with a final concentration of 5 and 0.5 IU/mL for PT/INR and aPTT assays, respectively. The final concentration for all tested direct anticoagulants was 1 µM. ***$p<0.001$

In the next step, again, the potential influence of anthropological parameters on blood coagulation was evaluated. For the age, enrolled subjects were divided into groups of 10-year ranges. Heparin yielded significant differences in all age groups except for the 30-39 years group (Figure 38A). Interestingly, there was a trend that the effect of heparin dropped linearly with age in the control group but not in the DMT1 patients (Figure 38F). The activity of rivaroxaban was significantly different in coagulation profiles between healthy and DMT1 samples in age ranges between 30 and 59 years of age, whereas the second xaban, apixaban, was active only in the elderly individuals (50-59 and 60+) (Figure 38BC). In dabigatran-treated samples, the differences between sample groups were observed in all age categories (Figure 38D), whereas the effect of argatroban was much weaker than dabigatran. Regardless, the significant differences in argatroban samples were observed solely in the oldest patients enrolled (Figure 38E). Additionally, the effect of dabigatran, argatroban, and rivaroxaban followed an increasing trend with age in DMT1 patients but not in healthy persons (Figure 38F).

The effects of heparin and anticoagulants were less pronounced in aPTT than in PT test (Figure 39). Significant differences in aPTT between DMT1 patients and healthy individuals in different age groups were found only with dabigatran (Figure 39D). For both groups, there was a modest but non-significant trend toward shorter coagulation times. The samples treated with heparin were different in aPTT only in the age range of 50-59 (Figure 39A). In healthy volunteers, coagulation times showed an intriguing downward trend with age for all anticoagulants used; however, this inclination was significant only in samples treated with heparin and argatroban. Although not consistent, this tendency was noted also in DMT1 samples.

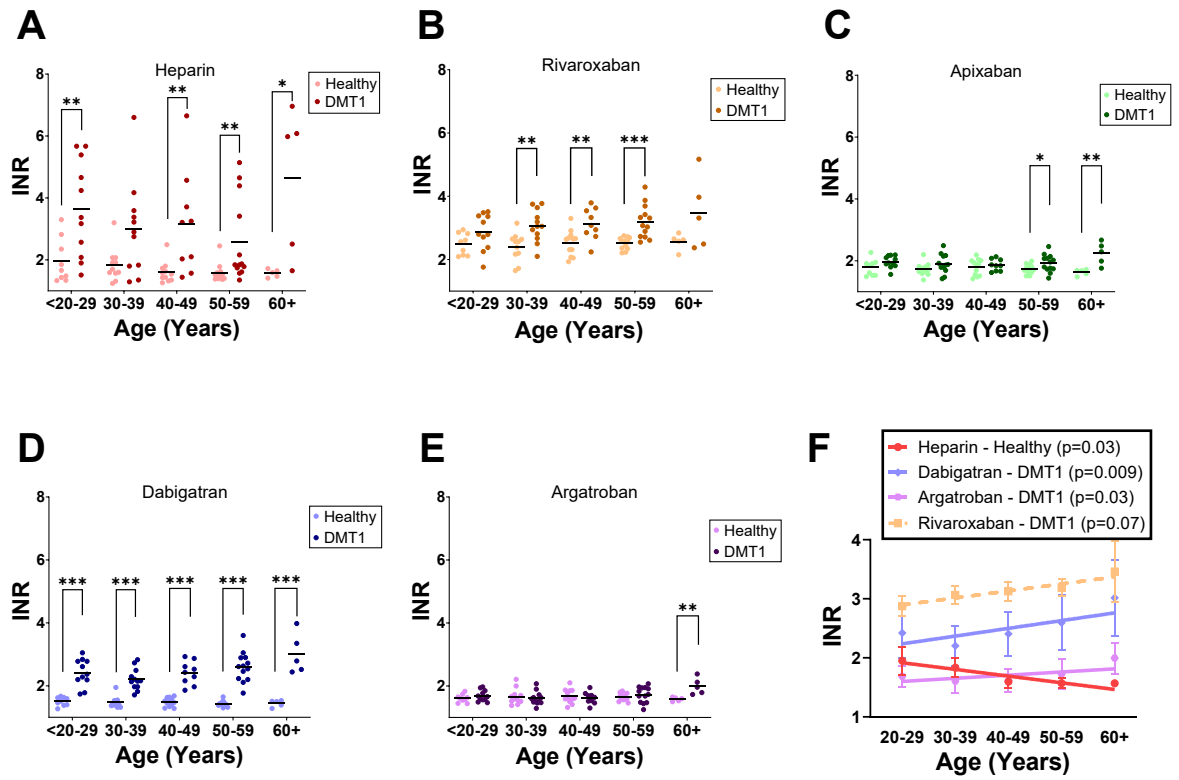


Figure 38. Differences in PT (expressed as INR) based on the age ranges in healthy donors and DMT1 patients after treatment with anticoagulants. (A) heparin, (B) rivaroxaban, (C) apixaban, (D) dabigatran, (E) argatroban, and (F) linear regression of each anticoagulant in case significant or nearly significant compared to healthy subjects or DMT1 patients. *p<0.05; **p<0.01; ***p<0.001

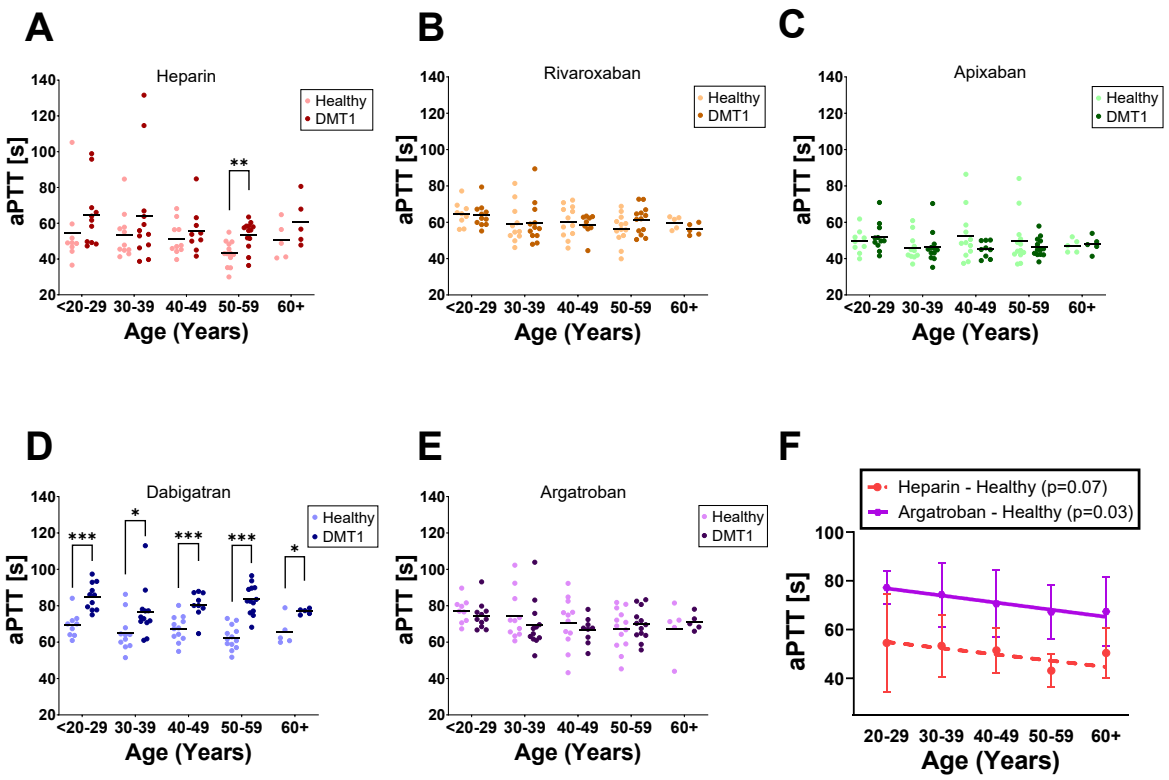


Figure 39. Differences in aPTT based on the age ranges in healthy donors and DMT1 patients after treatment with anticoagulants. (A) heparin, (B) rivaroxaban, (C) apixaban, (D) dabigatran, (E) argatroban, and (F) linear regression of each anticoagulant in case significant or nearly significant compared to healthy subjects or DMT1 patients. *p<0.05; **p<0.01; ***p<0.001

A comparable data analysis was carried out using BMI (Figures 40 & 41). Based on the international BMI index criteria, BMI was classified into three groups: 18.5-24.9 (normal weight), 25-29.9 (overweight), and 30+ (obesity). With the exception of argatroban, there were notable PT differences between the DMT1 patient groups and the healthy volunteer groups in every BMI category (Figure 40). Generally, lower anticoagulant effects were correlated with higher BMI (Figure 40). Similar to the age-effect analysis, aPTT (Figure 41) showed few significant differences. They were only observed for dabigatran across all BMI categories, and for heparin, only in two BMI categories (18.5-24.9 and 30+).

In a complementary analysis, coagulation results were split into two groups based on glycemia (below or above 7 mM) and glycated hemoglobin (below or above 53 mmol/mol) in individuals with diabetes. Nearly no differences were obtained. The one exception was heparin, which, according to the PT test, was more active in DMT1 patients whose glycated hemoglobin level was greater than 53 mmol/mol (Figures 42 & 43).

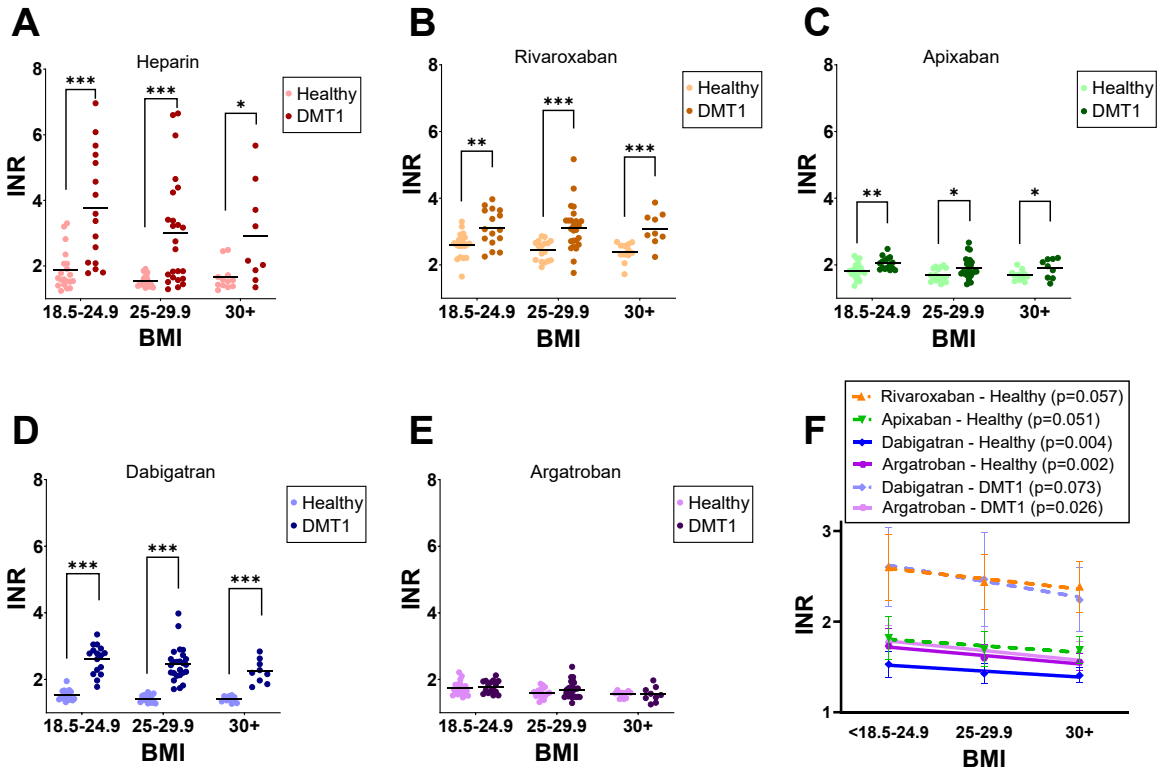


Figure 40. Differences in PT (expressed as INR) based on the body mass index (BMI) in healthy donors and DMT1 patients after treatment with anticoagulants. (A) heparin, (B) rivaroxaban, (C) apixaban, (D) dabigatran, (E) argatroban, and (F) linear regression of each anticoagulant in case significant or nearly significant compared to healthy subjects or DMT1 patients. *p<0.05; **p<0.01; ***p<0.001

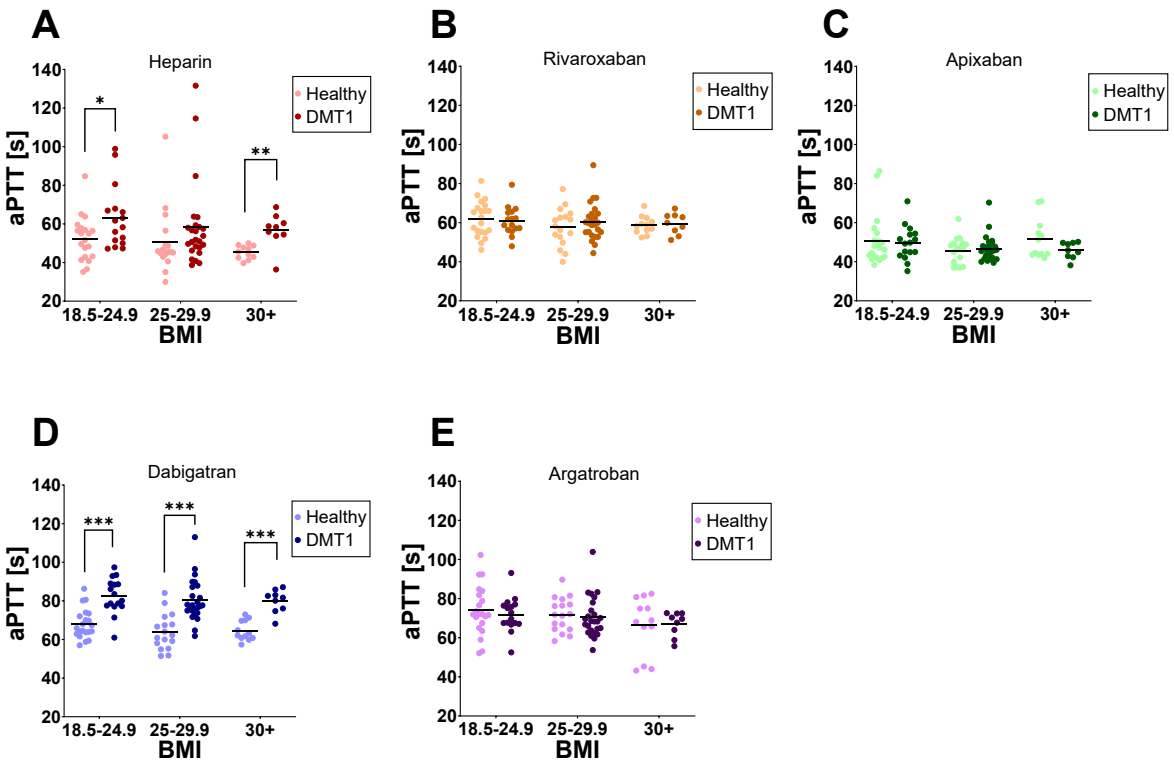


Figure 41. Differences in aPTT based on the BMI in healthy donors and DMT1 patients after treatment with anticoagulants. (A) heparin, (B) rivaroxaban, (C) apixaban, (D) dabigatran, and (E) argatroban. *p<0.05; **p<0.01; ***p<0.001

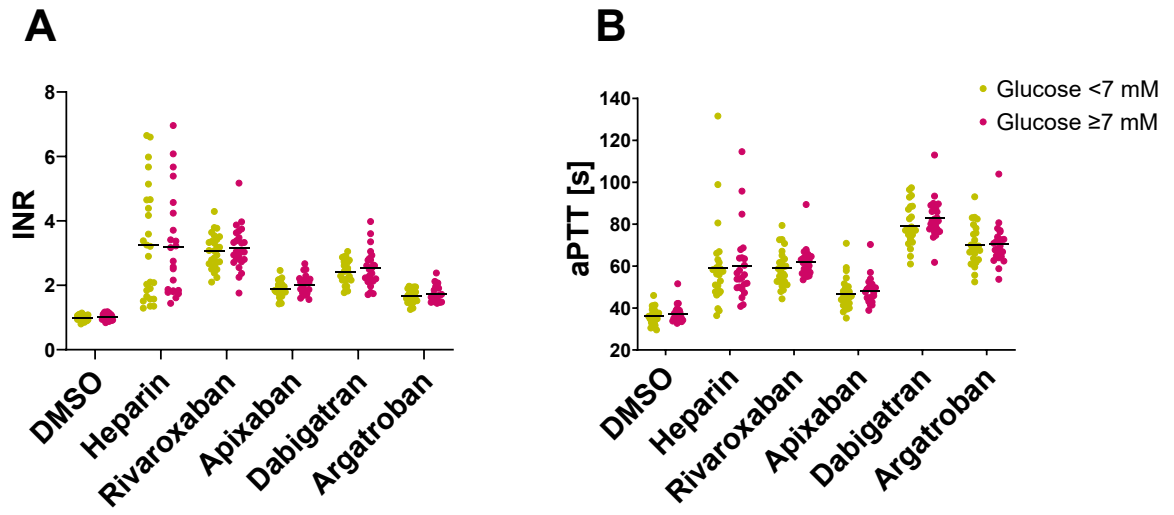


Figure 42. Comparison of (A) INR and (B) aPTT values of solvent and tested indirect and direct anticoagulants based on the glucose levels of DMT1 patients. No significant differences were found.

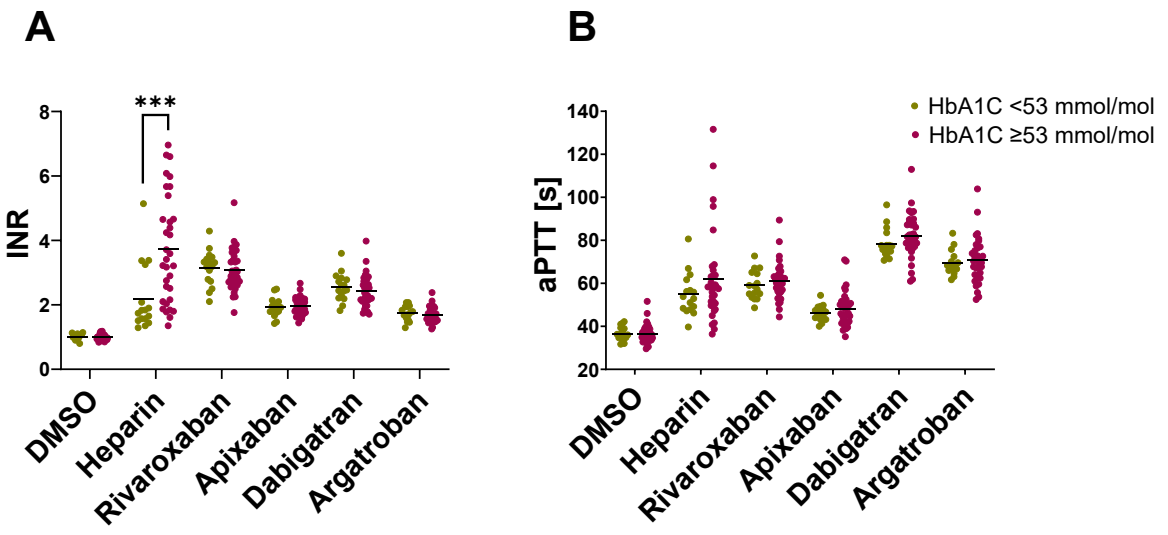


Figure 43. Comparison of (A) INR and (B) aPTT values of solvent and tested indirect and direct anticoagulants based on the glycated hemoglobin levels of DMT1 patients. ***p<0.001

Next, correlations between the obtained coagulation results and the measured biochemical values were analyzed (Table 6). All of the data – both DMT1 patients and healthy controls – were included in this analysis. There were several important correlations between PT values and a number of biochemical parameters. PT and aPTT were generally correlated with LDL, non-HDL, and total cholesterol (Figures 44 & 45). Higher levels of lipids in serum, specifically of LDL or total cholesterol shortened the coagulation time and, as a result, lessened the effect of tested anticoagulants. The relationship between glycemia and coagulation was not found. Similarly, there were no associations found in the entire group between glycated hemoglobin and coagulation. Figure 47 reveals some correlations that were discovered in a subgroup analysis, but glycated hemoglobin did not differ according to age, gender, BMI, or smoking habits (Figure 46). Higher concentrations of vitamin K resulted in longer coagulation times because total vitamin K values were mostly correlated negatively with PT. Figures 48 & 49, which present a sub-analysis of vitamin K forms, indicate that vitamin K_1 and MK-4, a major isoform of vitamin K_2, were the main drivers of the results. No correlation was found between the MK-7 form of vitamin K and any of the determined parameters. Nevertheless, with the exception of untreated (solvent) samples, the same correlations were not seen with aPTT.

Table 6. Correlations of selected biochemical profiles with the coagulation values

	Parameters	DMSO		Heparin		Factor Xa inhibitors				Thrombin Inhibitors			
						Rivaroxaban		Apixaban		Dabigatran		Argatroban	
		r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
INR	Glucose	-0.053	0.6	0.194	0.055	0.08	0.427	0.046	0.647	**0.335**	**<0.001**	-0.068	0.503
	gHb	-0.095	0.512	0.271	0.06	0.057	0.695	0.005	0.973	**-0.143**	**0.323**	-0.122	0.398
	LDL	**-0.227**	**0.023**	**-0.363**	**<0.001**	**-0.232**	**0.02**	**-0.452**	**<0.001**	**-0.515**	**<0.001**	**-0.335**	**<0.001**
	Total cholesterol	**-0.23**	**0.021**	**-0.313**	**0.002**	**-0.253**	**0.011**	**-0.465**	**<0.001**	**-0.479**	**<0.001**	**-0.321**	**0.001**
	HDL	0.071	0.482	-0.114	0.264	-0.161	0.109	0.007	0.942	-0.051	0.614	0.175	0.081
	Triglycerides	**-0.237**	**0.017**	-0.036	0.723	**-0.223**	**0.026**	**-0.308**	**0.002**	**-0.31**	**0.002**	**-0.441**	**<0.001**
	Non-HDL	**-0.246**	**0.014**	**-0.282**	**0.005**	**-0.215**	**0.032**	**-0.474**	**<0.001**	**-0.485**	**<0.001**	**-0.39**	**<0.001**
	Vitamin K_1	**-0.257**	**0.011**	0.046	0.657	**-0.255**	**0.012**	-0.188	0.066	**-0.347**	**<0.001**	-0.165	0.107
	Vitamin MK-4	**-0.298**	**0.003**	-0.149	0.145	**-0.37**	**<0.001**	**-0.334**	**<0.001**	**-0.477**	**<0.001**	-0.135	0.183
	Vitamin MK-7	-0.108	0.288	0.023	0.822	0.046	0.652	-0.115	0.255	0.17	0.093	-0.117	0.249
	Total vitamin K	**-0.357**	**<0.001**	-0.07	0.496	**-0.384**	**<0.001**	**-0.37**	**<0.001**	**-0.458**	**<0.001**	**-0.225**	**0.024**
	Creatinine serum	-0.065	0.521	-0.181	0.075	0.011	0.915	-0.093	0.36	-0.019	0.854	-0.036	0.719
	Creatinine urine	0.035	0.732	-0.096	0.346	0.047	0.644	-0.034	0.739	0.036	0.723	0.092	0.362
aPTT	Glucose	0.163	0.105	**0.252**	**0.012**	-0.106	0.296	-0.189	0.06	-0.098	0.33	-0.06	0.556
	gHb	-0.159	0.269	0.079	0.585	-0.032	0.825	-0.01	0.944	0.134	0.353	-0.025	0.863
	LDL	**-0.353**	**<0.001**	**-0.415**	**<0.001**	-0.142	0.16	-0.074	0.466	-0.137	0.175	-0.179	0.074
	Total cholesterol	**-0.403**	**<0.001**	**-0.399**	**<0.001**	-0.19	0.058	-0.111	0.271	-0.181	0.072	**-0.199**	**0.048**

HDL	0.007	0.947	0.013	0.899	-0.049	0.63	0.036	0.722	-0.05	0.622	-0.014	0.889
Triglycerides	**-0.317**	**0.001**	**-0.246**	**0.015**	-0.168	0.095	**-0.256**	**0.01**	-0.128	0.206	-0.123	0.222
Non-HDL	**-0.379**	**<0.001**	**-0.393**	**<0.001**	-0.148	0.143	-0.138	0.171	-0.159	0.113	-0.187	0.063
Vitamin K_1	-0.097	0.344	-0.1	0.923	0.026	0.801	-0.126	0.219	-0.021	0.841	0.092	0.368
Vitamin MK-4	**-0.269**	**0.007**	-0.188	0.065	-0.173	0.087	-0.193	0.055	-0.144	0.154	-0.165	0.103
Vitamin MK-7	-0.144	0.154	-0.039	0.707	-0.049	0.63	-0.114	0.263	0.097	0.341	0.091	0.368
Total vitamin K	**-0.276**	**0.006**	-0.149	0.144	-0.09	0.371	-0.185	0.066	-0.108	0.284	-0.081	0.426
Creatinine serum	0.067	0.508	-0.094	0.359	-0.058	0.57	-0.18	0.073	-0.102	0.312	-0.029	0.775
Creatinine urine	-0.025	0.802	-0.06	0.558	0.155	0.124	0.048	0.633	0.144	0.153	0.092	0.361

Data were analysed for both healthy and DMT1 patients together.

gHb - glycated hemoglobin (HbA1c); LDL – low-density lipoprotein; HDL – high-density lipoprotein

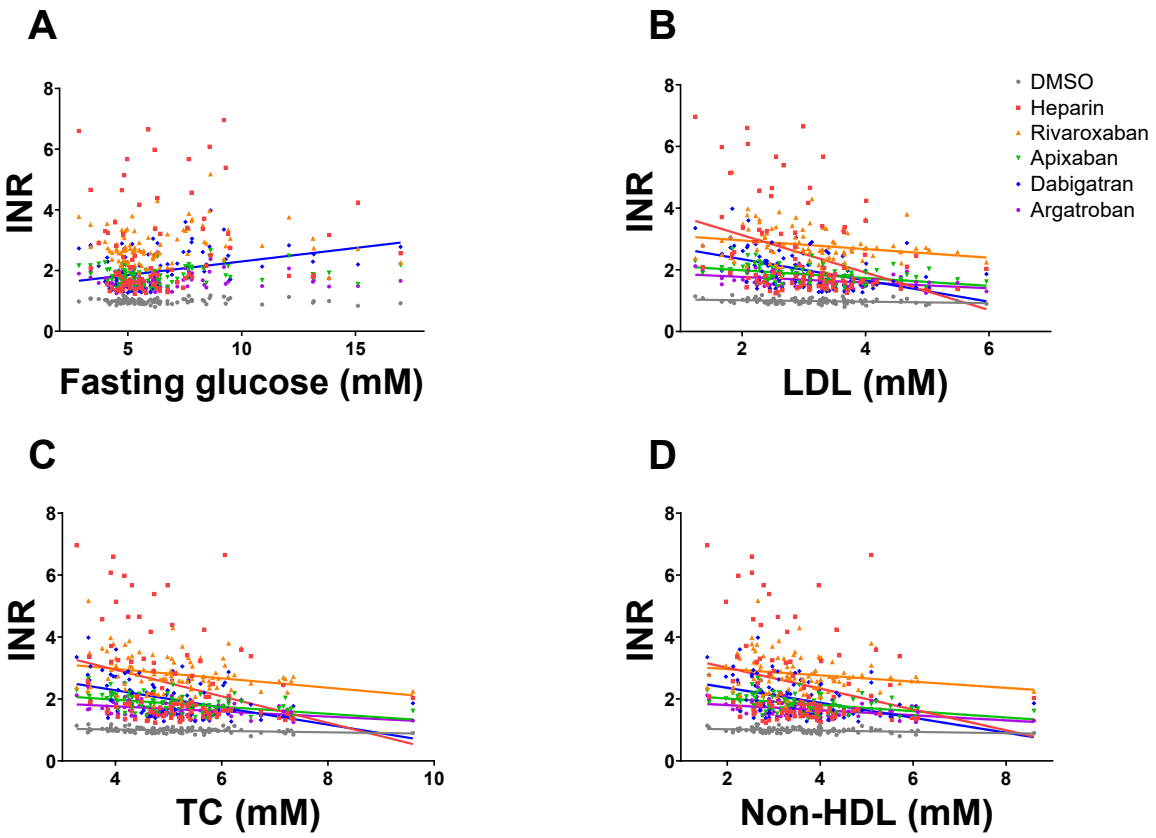


Figure 44. Correlation between PT (expressed as INR) and selected biochemical parameters (A) fasting glucose, (B) LDL, (C) total cholesterol (TC), and (D) non-HDL; after treatment with solvent (DMSO, at a final concentration of 1%), heparin (final concentration of 5 IU/mL) and direct anticoagulants (rivaroxaban, apixaban, dabigatran, and argatroban, all at a final concentration of 1 µM).

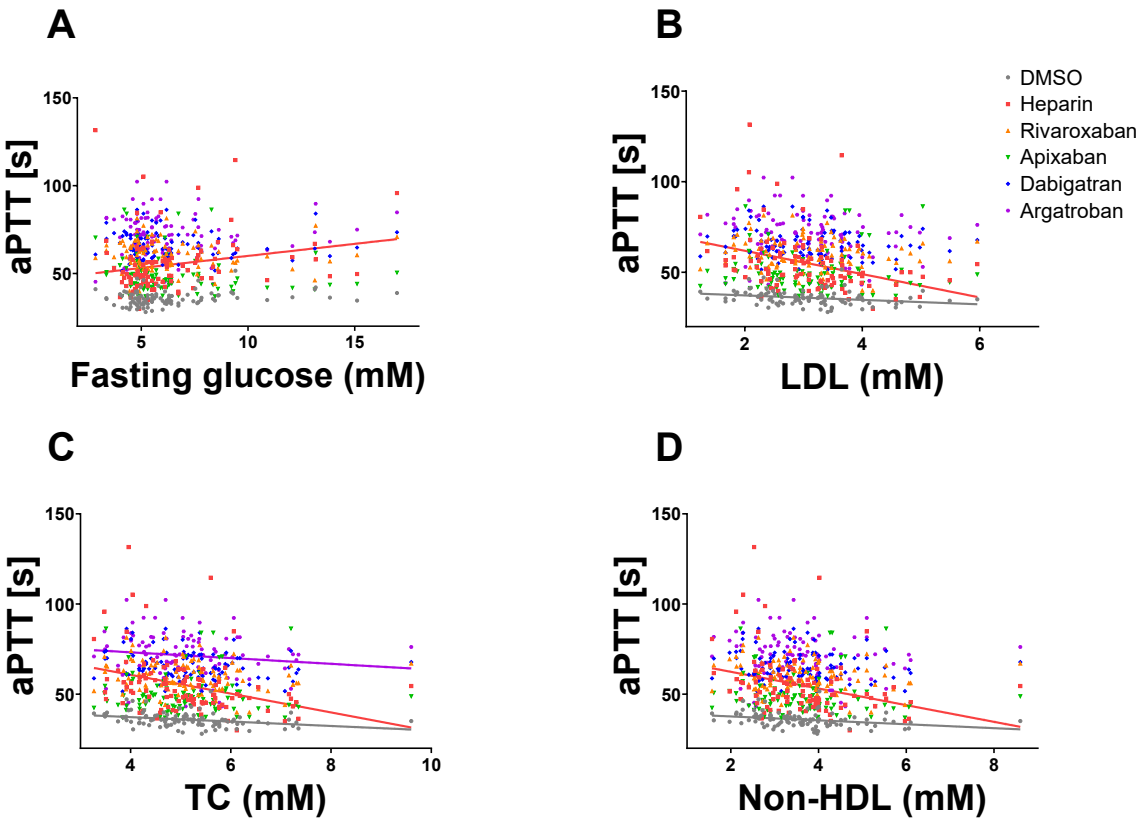


Figure 45. Correlation between aPTT and selected biochemical parameters (A) fasting glucose, (B) LDL, (C) total cholesterol (TC), and (D) non-HDL; after treatment with solvent (DMSO, final concentration of 1%), heparin (final concentration of 0.5 IU/mL) and direct anticoagulants (rivaroxaban, apixaban, dabigatran, and argatroban, all at a final concentration of 1 μM).

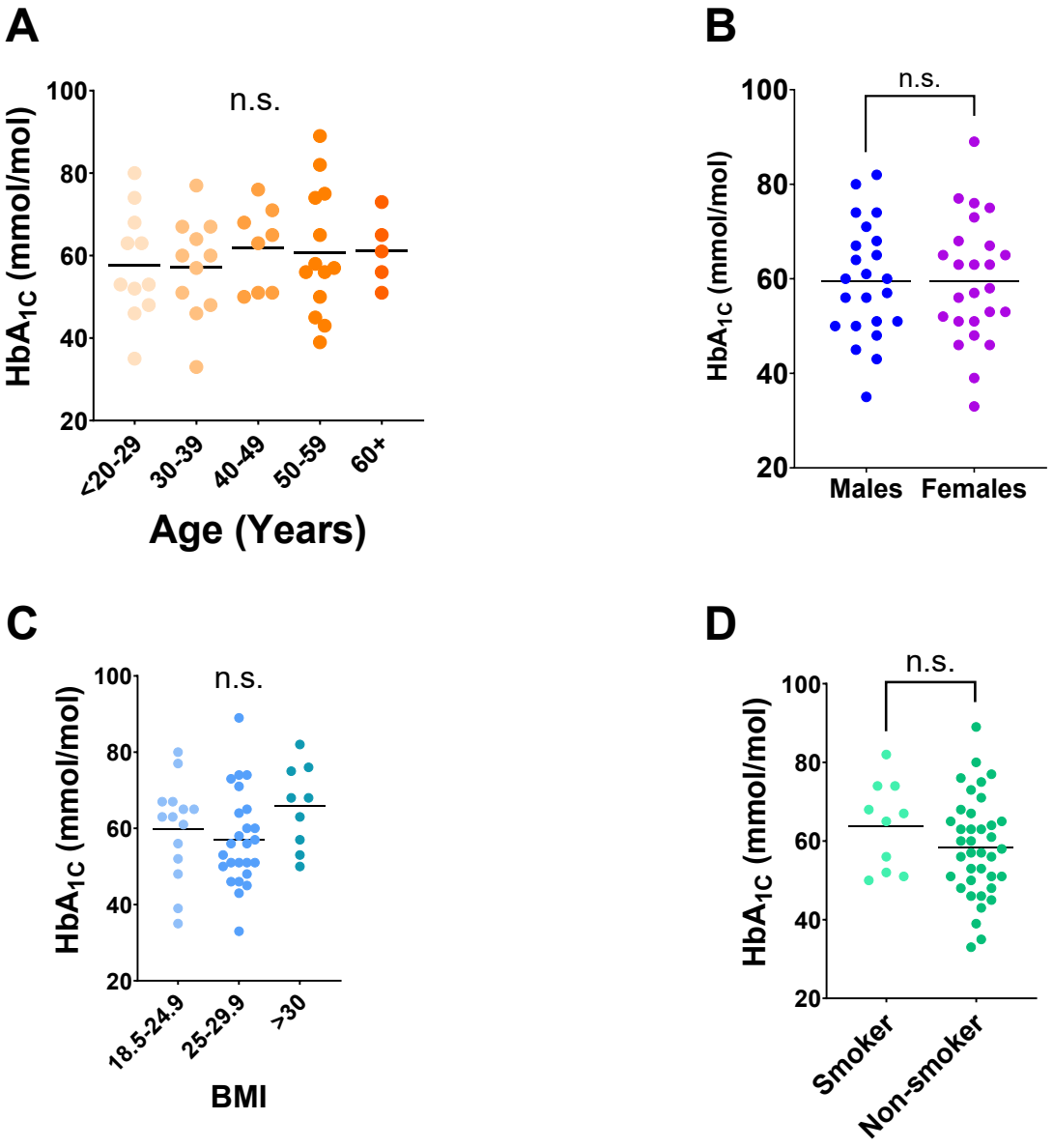


Figure 46. Comparison of glycated hemoglobin values from DMT1 patients in different subgroups.

(A) age groups, (B) gender, (C) BMI, and (D) smoking habit. No significant differences were found.

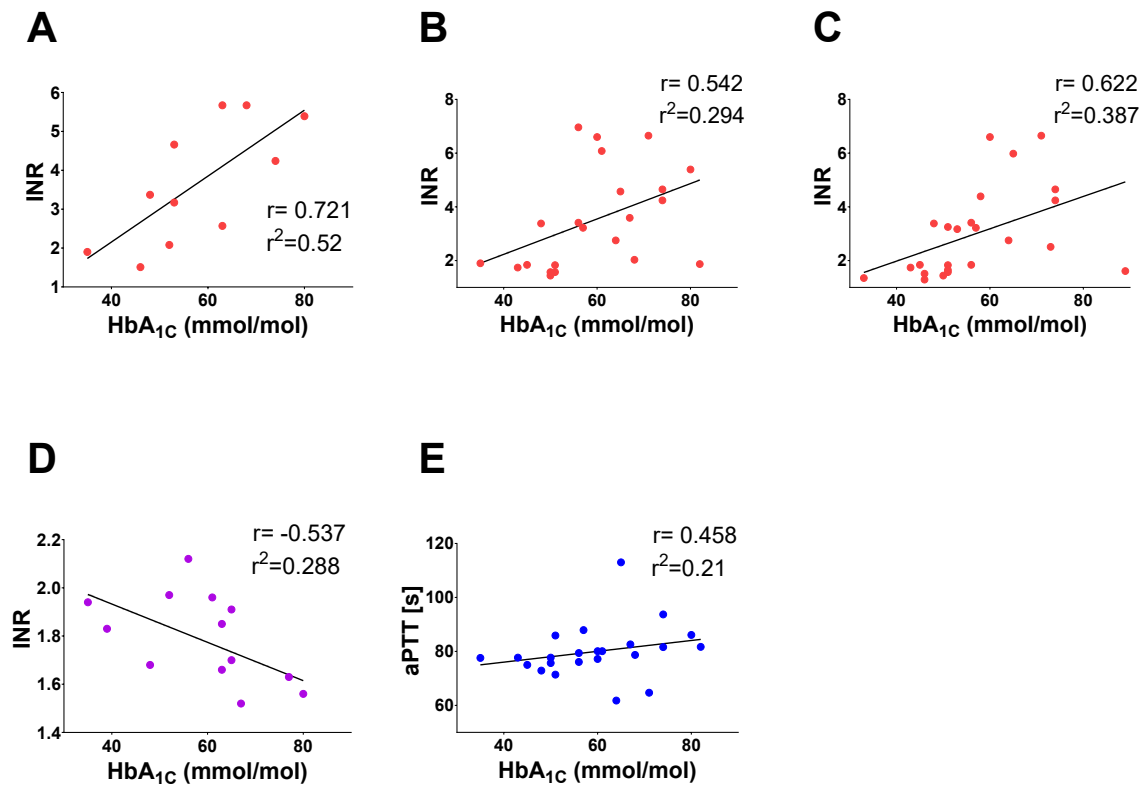


Figure 47. Correlations of tested anticoagulants (reported as INR or aPTT) with HbA1c in DMT1 patients. (A) INR of heparin in age group <20-29 years old, (B) INR of heparin in males, (C) INR of heparin in BMI 25-29.9, (D) INR of argatroban in BMI 18.5-24.9, and (E) aPTT of dabigatran in males.

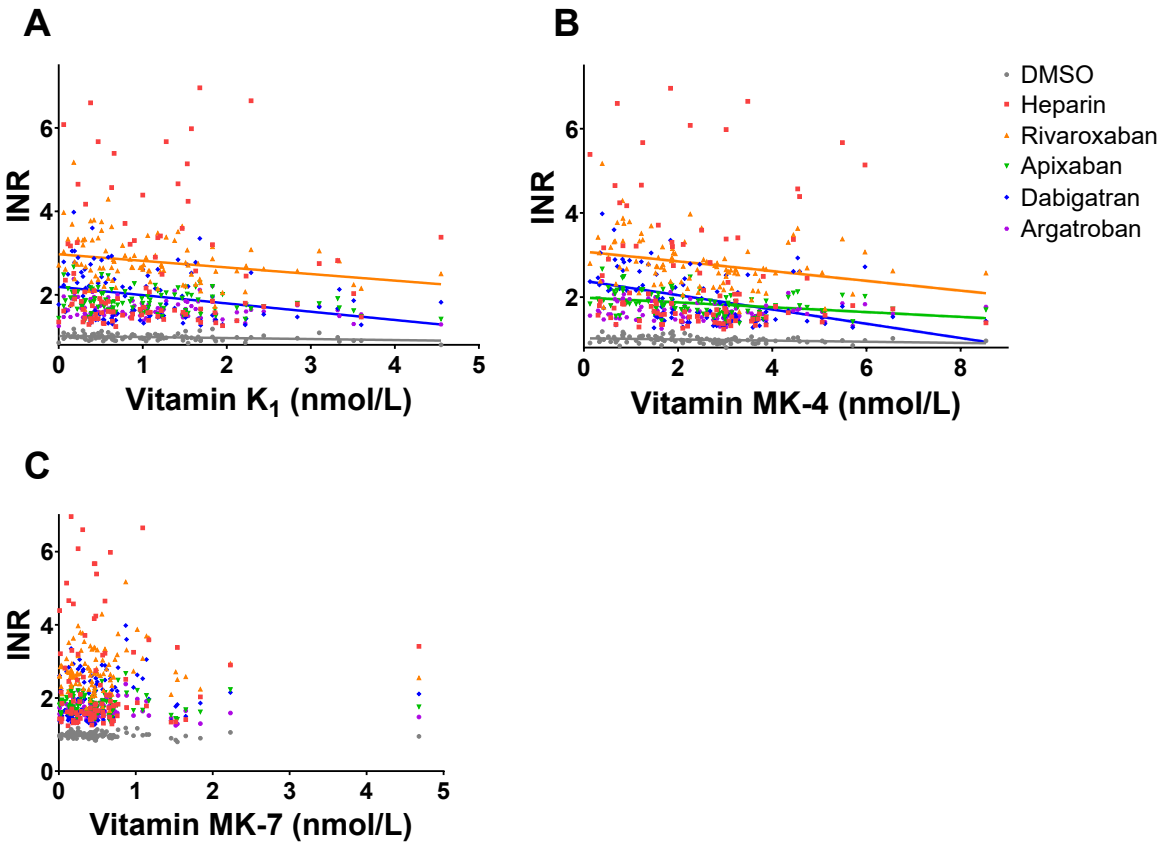


Figure 48. Correlation between prothrombin time (PT, expressed as INR: international normalized ratio) and vitamin K forms (A) vitamin K_1, (B) vitamin MK-4, and (C) vitamin MK-7 after treatment with solvent (DMSO, final concentration 1%), heparin (final concentration 5 IU/mL) and direct anticoagulants (rivaroxaban, apixaban, dabigatran, and argatroban, all at a final concentration of 1 μM)

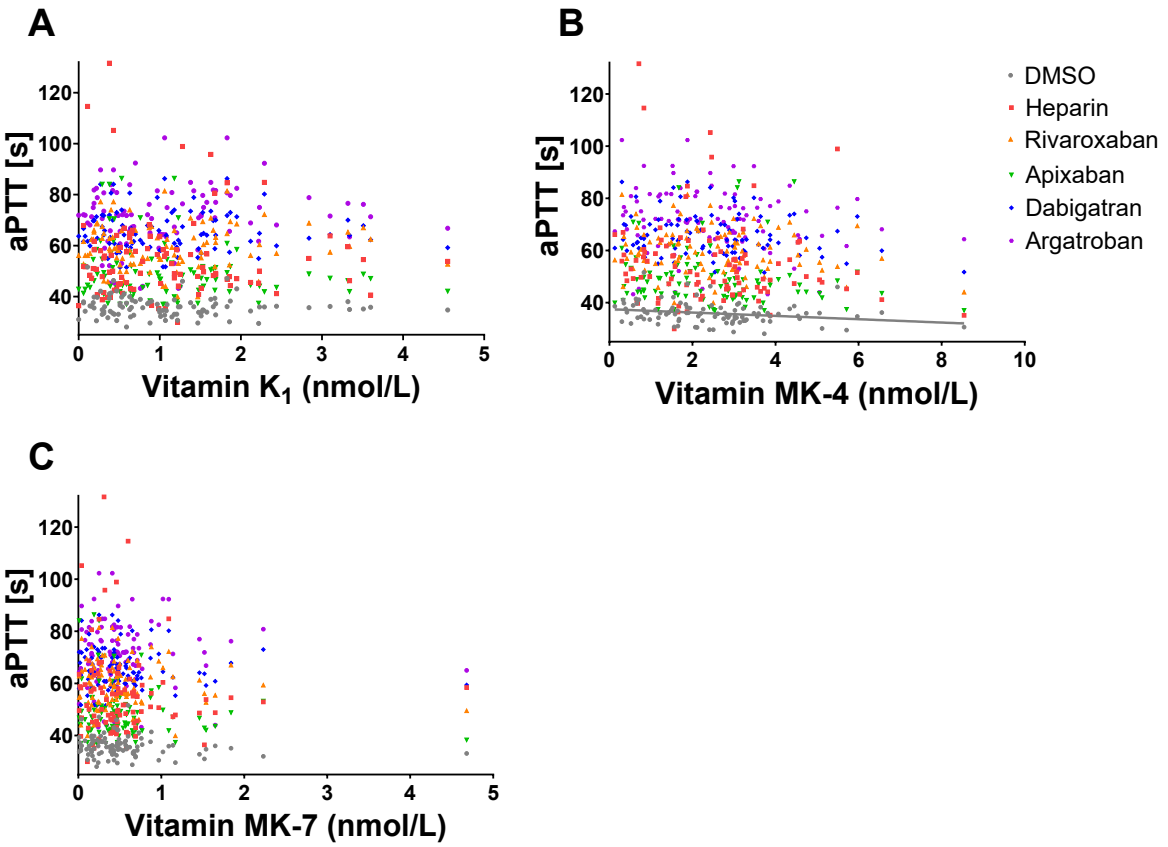


Figure 49. Correlation between aPTT and vitamin K forms (A) vitamin K_1, (B) vitamin MK-4, and (C) vitamin MK-7 after treatment with solvent (DMSO, final concentration of 1%), heparin (final concentration of 0.5 IU/mL) and direct anticoagulants (rivaroxaban, apixaban, dabigatran, and argatroban, all at a final concentration of 1 µM)

4.4 Testing of potentially novel anticoagulants

As flavonoids and their metabolites have an effect on platelet aggregation [161, 162] and some studies suggested their anticoagulant properties [163, 164], the potential impact of these natural compounds on blood coagulation was tested as well. None of the tested flavonoids affected PT (Figures 50A and 51A). Solely 3 flavonoids (diosmin, epicatechin, and naringin) had a mild significant effect on aPTT but contrary to what was expected, aPTT was slightly shortened. Therefore, these 3 compounds acted as weak procoagulatory agents. Moreover, this effect was observed at a high final concentration of 100 μM. Similar outcomes were observed with flavonoid metabolites formed by gut microbiota. None of them had an effect on PT and 4 isoflavonoid-specific metabolites mildly shortened aPTT at the concentration of 100 μM (Figures 50B and 51B). As these effects were observed solely at this high concentration, no future testing was performed.

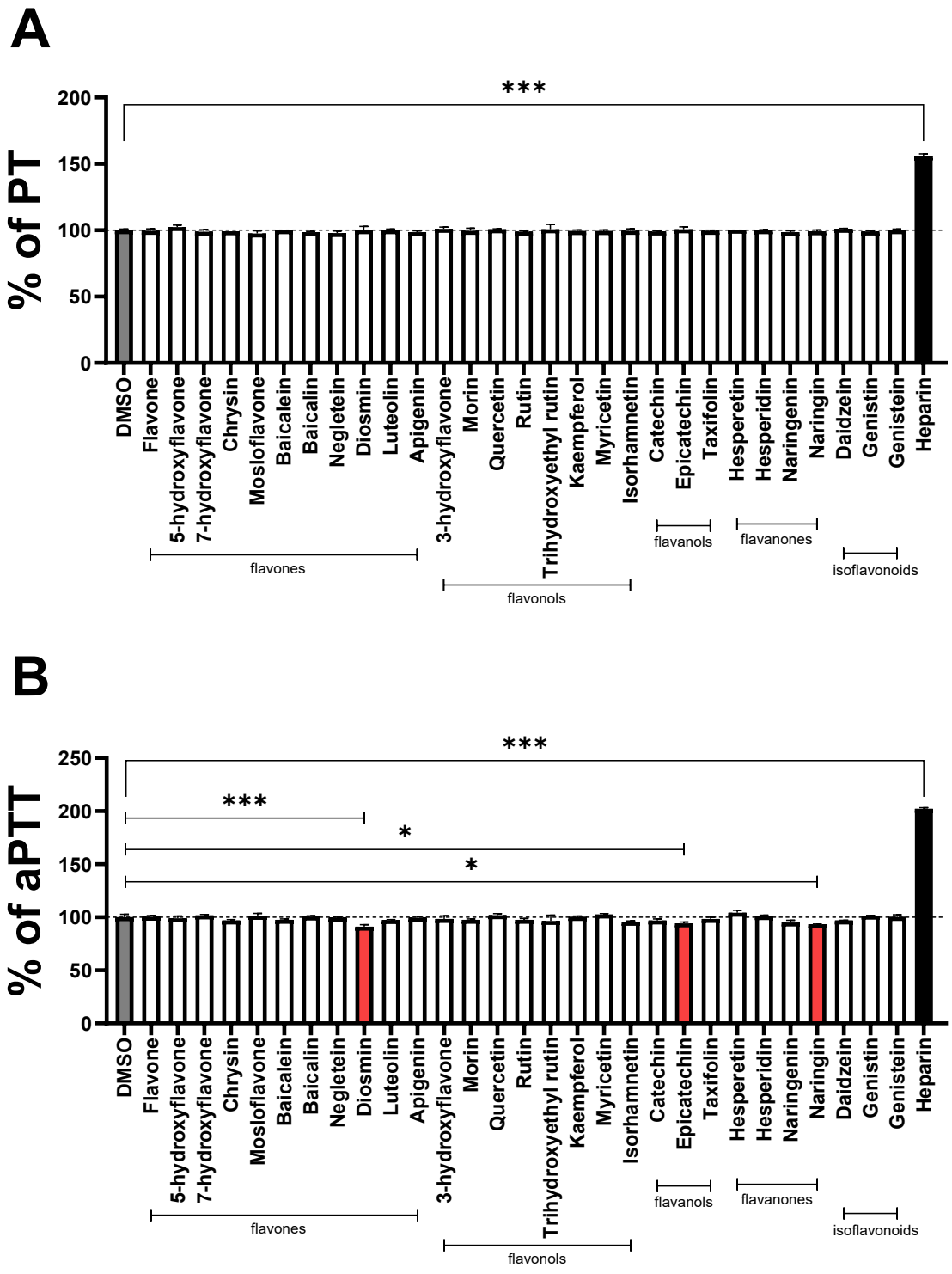


Figure 50. Effect of flavonoids on blood coagulation. Reported as a percentage change on (A) PT and (B) aPTT compared to vehicle (DMSO) values. The final concentration of DMSO was 1%, for heparin was 5 IU/mL and 0.5 IU/mL in PT and aPTT assays, respectively; and each tested compound was 100 µM. *p<0.05; ***p<0.001

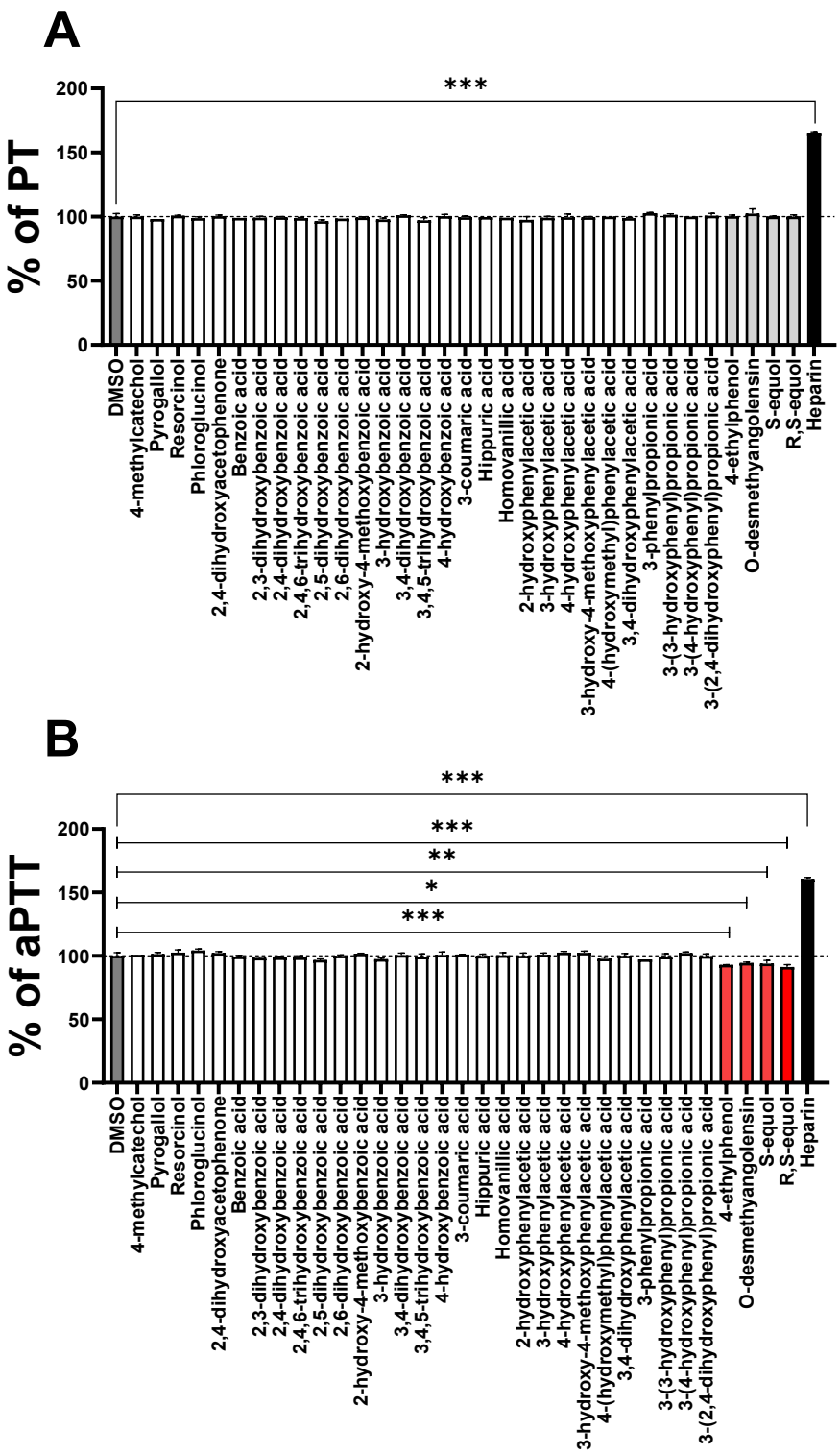


Figure 51. Effect of (iso)flavonoids metabolites on blood coagulation. Reported as a percentage change on (A) PT and (B) aPTT compared to vehicle (DMSO) values. The final concentration of DMSO was 1%, for heparin was 5 IU/mL and 0.5 IU/mL in PT and aPTT assays, respectively; and each tested compound was 100 µM. *p<0.05; **p<0.01; ***p<0.001

In another experiment, synthetic complex heterocyclic compounds as well as complex pyrano[3,4-c]pyridine derivatives, were investigated. From 34 synthetic heterocyclic compounds, only one compound (4-N; 2-(3,3,6,6-tetramethyl-9-(4-nitrophenyl)-1,8-dioxo-2,3,4,5,6,7,8,9-octahydroacridin-10(1H)-yl)succinic acid) significantly prolonged aPTT coagulation time but had no effect on PT. Since this compound has a potential anticoagulant effect, further concentration-effect study was conducted. The lowest concentration with a significant effect was 75 μM (Figure 52). As the effect of this compound was mild (about 12%) and observed solely in a relatively high concentration, no additional testing was performed.

Similar results were obtained with pyrano[3,4-c]pyridine. Of 10 compounds, only one compound (3h; 3,3-dimethyl-8-(4-methoxyphenyl)-6-oxo-3,4,6,7-tetrahydro-1H-pyrano[3,4-c] pyridine-5-carbonitrile) showed a potential effect. Interestingly, compound 3h significantly but mildly shortened aPTT, suggesting instead of anticoagulant, this compound had rather a procoagulant effect (Figure 53).

We also screened the potential for an anticoagulation effect in the case of 14 alkaloids and 23 catechol compounds with the same final concentration of 100 μM, but none of these compounds affected PT and aPTT (Figures 54 and 55).

Using a different method (chromogenic assay), we tested iso(flavonoids) and their metabolites for direct inhibition of FXa and thrombin. None of these compounds affected thrombin although dabigatran inhibited 70% and more than 96% at final concentrations 1 and 10 μM, respectively (Figure 56B). A potential effect was found for another test, these natural compounds inhibited 40-60% of FXa at the highest concentration tested (100 μM) meanwhile, rivaroxaban inhibited more than 98% of FXa at a final concentration of 0.05 μM (Figure 56A)

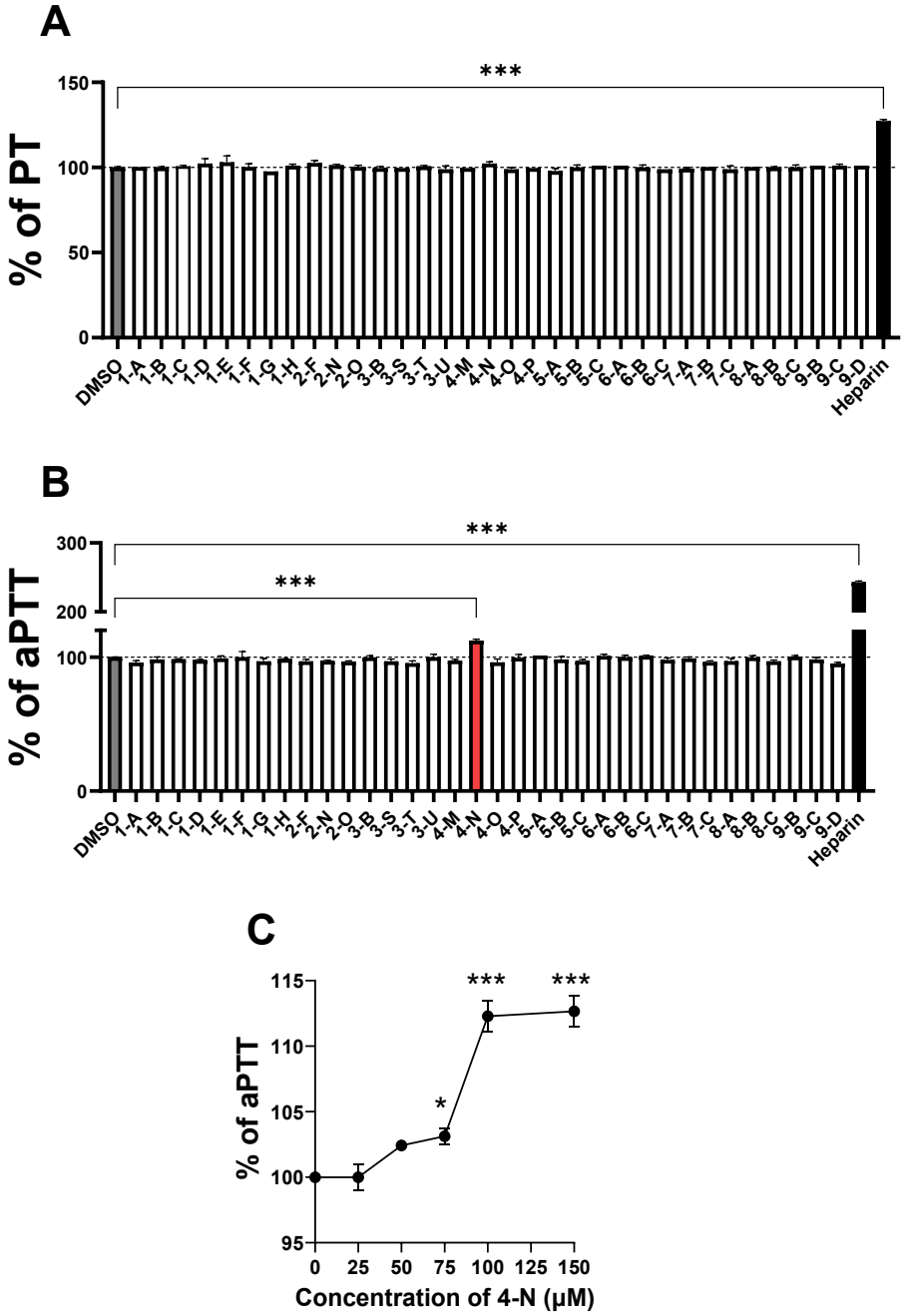


Figure 52. The anticoagulant effect of synthetic heterocyclic compounds. (A) prothrombin time (PT) and (B) activated partial thromboplastin time (aPTT). All tested compounds were incubated at a concentration of 100 μM. DMSO was used as the solvent and negative control. Positive control was heparin: 5 IU/mL and 0.5 IU/mL for PT and aPTT assays, respectively. (C) anticoagulation effect of compound 4-N on aPTT in different concentrations. The compound was tested between concentrations 25 – 150 μM. Concentrations 75 – 150 μM shown significant results compared with DMSO as a negative control. *$p<0.05$; *** $p<0.001$.

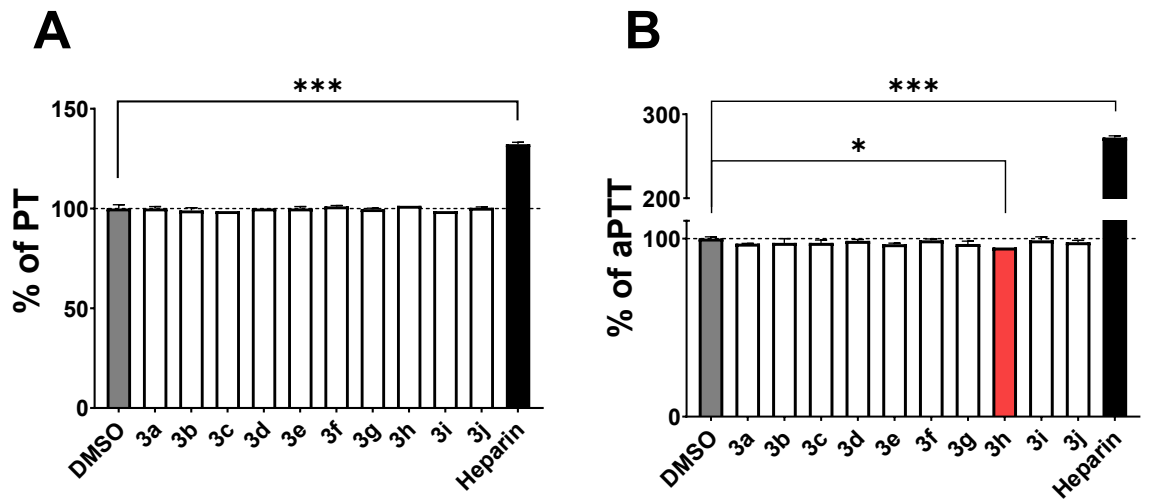


Figure 53. Anti/procoagulant effect of the pyridine and pyrano derivatives. (A) PT and (B) aPTT values of tested compounds, heparin (positive control), and DMSO (negative control). The final concentration for tested compounds was 100 µM in both assays, while heparin was 5 IU/mL for PT and 0.5 IU/mL for aPTT assay. *p<0.05; ***p<0.001

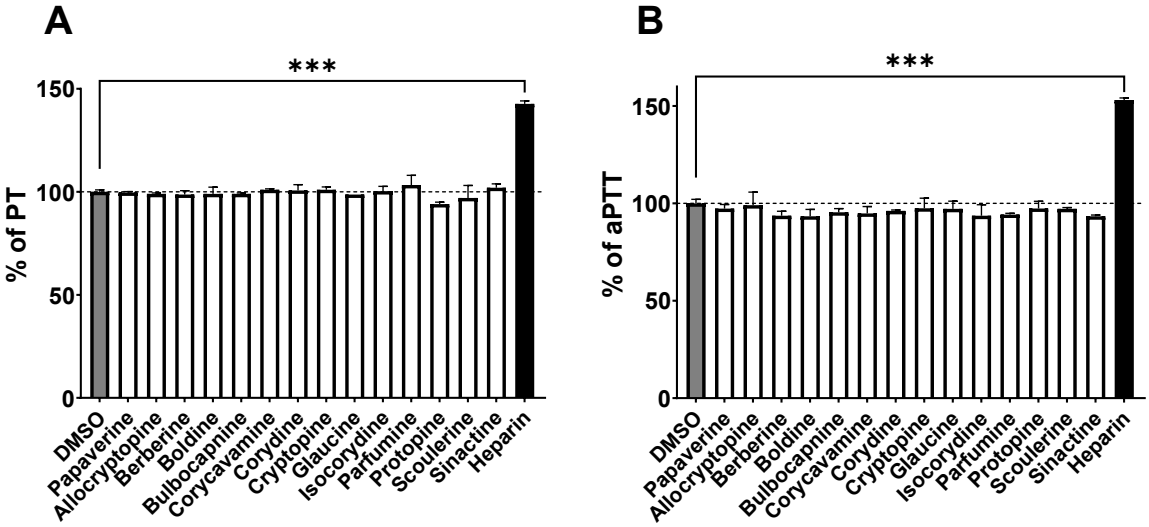


Figure 54. The anticoagulation effect of alkaloid compounds. (A) PT and (B) aPTT values of tested compounds, heparin (positive control), and DMSO (negative control). The final concentration for tested compounds was 100 µM in both assays, while heparin was 5 IU/mL for PT and 0.5 IU/mL for aPTT assay, respectively. ***p<0.001

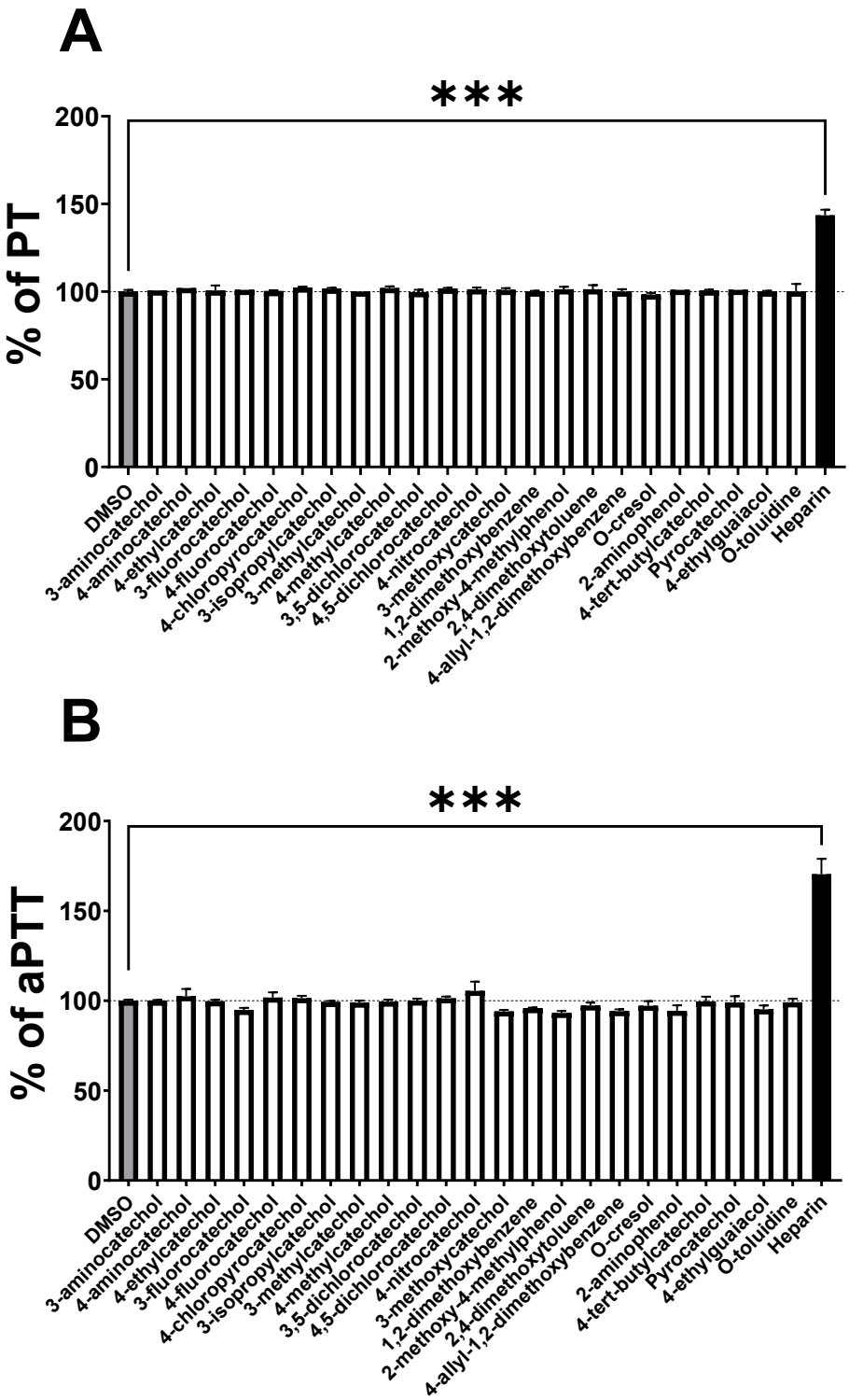


Figure 55. The anticoagulation effect of catechol compounds. (A) PT and (B) aPTT values of tested compounds, heparin (positive control), and DMSO (negative control). The final concentration for tested compounds was 100 μM in both assays, while heparin was 5 IU/mL for PT and 0.5 IU/mL for aPTT assay, respectively. ***$p<0.001$

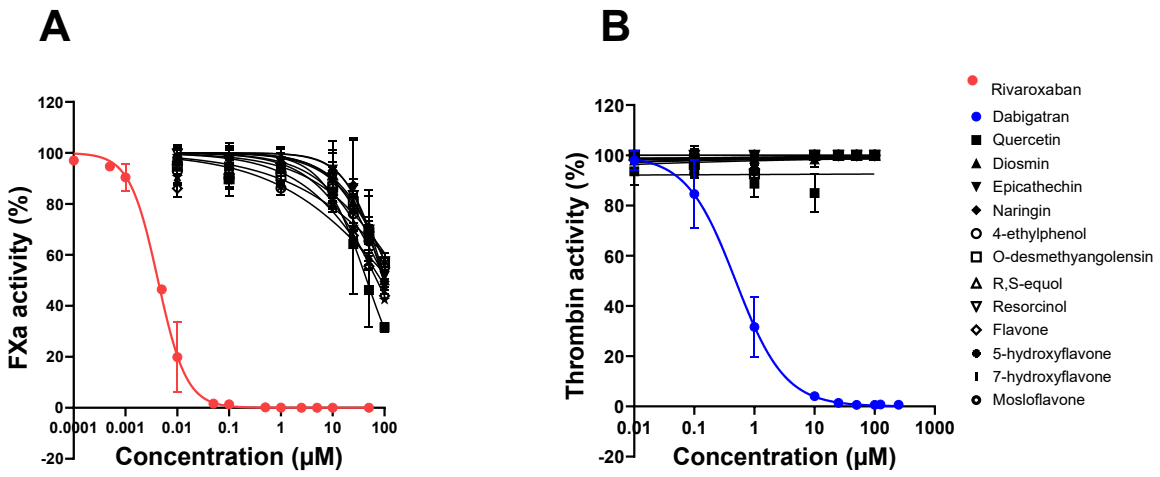


Figure 56. The inhibition activity of (iso)flavonoid and their metabolites of (A) FXa and (B) thrombin. The used concentrations for tested compounds were 0.01 – 100 μM. Rivaroxaban and dabigatran were used as the positive control for FXa and thrombin, respectively.

5. DISCUSSION

Blood coagulation is an essential physiological mechanism that stops excessive bleeding when a blood vessel is damaged. Physiologically there is an intricate equilibrium between coagulation and anticoagulation factors, so clots form only when required and are restricted to the site of injury, preserving regular blood flow and preventing conditions like thrombosis [165]. However, this process can be altered in individuals with metabolic disorders such as hypercholesterolemia and diabetes mellitus, and lead to a hypercoagulable state which is consistent with the higher risk of thrombotic events such as AMI, stroke, and VTE [166, 167]. Hypolipidemic, antiplatelet, and/or antihypertensive medication is thought to be essential in preventing such cardiovascular events. Anticoagulant therapy is also used for the prevention of VTE including atrial fibrillation. Currently, DOACs, or direct oral anticoagulants, comprise most of this therapy [168, 169].

DOACs are clinically proven to overcome the disadvantages of conventional anticoagulants (VKAs and heparins) such as high interindividual variability, drug-drug or food-drug interactions, and inconvenient administration. In addition, they are administered in fixed doses [170]. However, our head-to-head study using four clinically used DOACs, consisting of two FXa inhibitors: rivaroxaban and apixaban, and two DTIs: dabigatran and argatroban, revealed that anthropological (BMI) and biochemical (glucose and lipid levels) parameters might influence their pharmacodynamic effects. BMI and lipid levels in serum correlated negatively with coagulation values (PT/INR and aPTT) [171]. This principally means that untreated or poorly treated hyperlipidemic and obese persons can be endangered by VTE notwithstanding anticoagulation treatment. The data on glycemia from our study are not such clear, glycemia or higher glycated hemoglobin rather prolonged coagulation times but the results were not unambiguous.

One important detail to emphasize is that this study was an *ex vivo* study, so we have not administered the drugs to the patients, but we only treated the collected blood with equal concentrations of anticoagulants. The tested concentrations of DOACs were always the same at 1 μM. In clinical situation, when fixed doses are used in patients with different BMIs, differences in plasma levels must

be observed. This in addition to observing the difference in pharmacodynamic effects creates variability in clinical outcomes. We intentionally selected a concentration of 1 μM, which is clinically relevant. This concentration based on our preliminary experiments, resulted in a clear and significant prolongation of aPTT and/or PT values after being treated with all tested anticoagulants. Moreover, this concentration was not excessively higher than the maximal plasma levels detected for these DOACs in clinical practice since similar levels can be detected in the plasma of treated patients: rivaroxaban 571 nM, apixaban 372 nM, dabigatran 278 nM [172], and argatroban 1671 nM [173].

In this project, other factors accompanying obesity such as proinflammation which contributes to increased blood coagulation or inhibits the effect of anticoagulation drugs [174], were not studied. However, in the future, the study of inflammatory markers will be included in the portfolio of our research group.

In our study, PT and aPTT assays were utilized since they are frequently used in clinical practice to determine the level of coagulation in individuals receiving anticoagulant therapy. Moreover, these assays are able to detect inhibition in the common coagulation pathway, therefore for both thrombin and factor Xa inhibitors [74]. In addition, these tests are feasible, precise, and not costly. To enable comparison with other studies, INR values were reported instead of PT [175].

We aimed to evaluate the effect of all four relatively novel anticoagulants in both healthy persons and patients presenting metabolic disorders (FH and DMT1) to determine a) their relative potency, and b) detect differences in their effect on healthy persons and the mentioned patients. The enrolled FH patients were not only treated pharmacologically but some of them were also undergoing lipid apheresis. As the apheresis procedure employs anticoagulants [176, 177] we could not compare the INR and aPTT from healthy individuals and DMT1 patients with FH patients post-apheresis, therefore the comparison was only for FH patients pre-apheresis and without apheresis.

Of the four direct anticoagulants used, rivaroxaban was the most active compound based on PT assay in all groups, whereas dabigatran provided the highest activity in aPTT. When both FXa-Is were compared, the prolongation effect of rivaroxaban was superior to apixaban. This result was quite

expected since rivaroxaban binds free factor Xa with a 4-fold higher than apixaban [178]. These data also seem to be consistent with clinical situations, as previous analyses found that rivaroxaban was significantly more likely than apixaban to cause major bleeding during primary prevention [179, 180]. Concerning DTIs, on average, dabigatran prolonged aPTT slightly more than argatroban, despite the incidence of major bleeding was higher in argatroban (± 7%; 20 out of 269) [181] than in dabigatran (± 3.1%; 168 out of 5358) [154]. This can be however confounded by higher clinically achievable levels of argatroban, which is given parenterally and reaches higher concentrations as reported above.

Nearly no differences were found in the basal coagulation levels of healthy individuals, FH, and DMT1 patients, especially for INR values. This is despite the fact that FH patients were reported to have higher levels of fibrinogen and coagulation factor VIII [182] and patients with diabetes mellitus have insulin resistance and high glucose plasma levels which can increase pro-coagulatory activity through an enhanced FVII conversion to its activated form (FVIIa) [183]. The difference was only shown between healthy and FH patients in aPTT (Table 7). The most plausible explanation is that the patients were well-treated pharmacologically for major cardiovascular risk factors. It is noticeable from the lipid levels, which were lower in patients than in healthy volunteers. As higher cholesterol values are related to accelerated blood coagulation [71] and we have found a similar relationship in our study (Figures 19-26, 36, 44, and 45), this can be an important determinant of our results. The same is not true for the glucose levels of our group of patients (Table 2, Table 8). In addition, there was a difference in BMI levels between healthy individuals and patients with FH (Table 8).

Additional differences were found when the coagulation results were compared after the addition of anticoagulants. The prolongation of INR and aPTT was more profound in almost all anticoagulants in FH and DMT1 patients than in healthy individuals (Table 7). Again, lower levels of lipids might have an important role here. These outcomes are quite paradoxical, as the disease seems to cause by itself a pro-coagulatory environment [43, 48, 50, 68], but anticoagulant treatment might be more efficient than in controls (Figures 37 and 45) and its effect seems to be stronger in higher serum lipid levels at least in persons with diabetes (Figures 44 and 45).

Another very novel finding is the impact of vitamin K. It needs not to be emphasized again that vitamin K is a key player in the synthesis of 7 coagulation and anticoagulation factors [122] and its levels were altered in patients suffering from DMT1. This can be another factor contributing to observed coagulation differences.

Although novel direct anticoagulants from the class FXa-I and DTI have largely replaced older oral anticoagulants, particularly VKA, there is still a search for newer anticoagulants. This research is driven by two crucial aspects. FXa-Is and DTIs still pose important risks of bleeding and although much safer, they still present relevant drug interactions. There is currently a novel investigation part for new anticoagulants, as inhibition of some coagulation factors, e.g. XI, XII is burdened by a lower risk of bleeding [184, 185]. In this study, relatively many compounds were screened by PT and aPTT tests to evaluate their potential anticoagulant effects. This effect can be positive under certain situations such as additive effect to antiplatelet potential, but also negative, if unexpected as they can lead to bleeding. It would be highly interesting to find a compound with both antiplatelet and anticoagulant effects, as compounds with these properties are usually used together in patients with coronary heart disease and atrial fibrillation. However, such research has not yet been successful. Apparently, the anticoagulant effect requires much more specific structural feature compared to the antiplatelet effect. Considering that the antiplatelet effect can happen after the interaction of the compounds with any of the receptors present on the platelet surface or some intracellular cascade [186], this dual effect is logically hard to find in any drug. Although the coagulation cascade is complicated, the number of involved enzymes is quite low. In fact, all 143 compounds tested by us, failed to show any significant anticoagulation effects. Somehow surprising was this failure with flavonoids as some of them were formerly described to prolong the coagulation [186]. Based on our limited experimental data, we suppose that this can be caused by the fact, that flavonoids can really inhibit FXa (Figure 56A), but this effect is observed solely at high concentrations and is hence relatively mild and likely without clinical importance.

Table 7. Coagulation comparison between healthy individuals, FH, and DMT1 patients

Parameters	INR			Significance	aPTT			Significance
	H	FH	DMT1		H	FH	DMT1	
DMSO	0.97 ± 0.06	0.99 ± 0.09	1 ± 0.08	n.s.	35.2 ± 3.9	38.4 ± 2.7	36.6 ± 3.9	*
Heparin	1.7 ± 0.46	2.09 ± 1.14	3.23 ± 1.64	+++ ; &	50.1 ± 12.5	77.6 ± 47.1	59.6 ± 18.1	** ; ++
Rivaroxaban	2.49 ± 0.33	2.87 ± 0.52	3.1 ± 0.6	** ; +++	59.7 ± 8.4	65.5 ± 9.8	60.4 ± 7.9	*
Apixaban	1.74 ± 0.21	1.82 ± 0.23	1.95 ± 0.27	+++	49.2 ± 10.5	50 ± 8.7	47.5 ± 6.9	n.s.
Dabigatran	1.46 ± 0.13	1.77 ± 0.45	2.47 ± 0.47	*** ; +++ ; &&&	65.8 ± 7.7	74.2 ± 9.7	81 ± 9.4	** ; +++ ; &
Argatroban	1.64 ± 0.17	1.72 ± 0.24	1.69 ± 0.23	n.s.	71.4 ± 12	74.1 ± 9.8	70.3 ± 9.2	n.s.

INR: international normalized ratio; aPTT: activated partial thromboplastin time; H: healthy; FH: familial hypercholesterolemia; DMT1: type 1 diabetes mellitus; n.s.: not significant in all comparisons

H vs FH: *$p<0.05$; **$p<0.01$; ***$p<0.001$

H vs DMT1: $^{++}p<0.01$; $^{+++}p<0.01$

FH vs DMT1: $^{\&}p<0.05$; $^{\&\&\&}p<0.001$

Table 8. Anthropological and biochemical parameters of healthy individual, FH and DMT1 patients

Parameters	Healthy	Familial hypercholesterolemia			DMT1	Significance
		Before + without apheresis	After apheresis	v.s.		
Age	43.14 ± 13.23	49.83 ± 9.39		-	42.14 ± 13.81	n.s.
BMI	26.58 ± 3.82	29.9 ± 4.34		-	27.11 ± 4.29	*
Glucose	5.1 ± 0.6	6.2 ± 1.5	8.1 ± 2.9	n.s.	7.4 ± 3.1	*** ; ¶¶¶ ; +++
Vit. K_1	1.29 ± 0.77	1.23 ± 0.92	0.79 ± 0.69	n.s.	0.95 ± 0.97	n.s.
Vit. MK-4	3.15 ± 1.37	3.91 ± 1.45	0.71 ± 0.49	ΦΦΦ	1.82 ± 1.39	¶¶¶ ; +++ ; ¤¤¤
Vit. MK-7	0.43 ± 0.36	0.45 ± 0.33	0.1 ± 0.08	n.s.	0.7 ± 0.73	+
Total vit. K	4.81 ± 1.83	5.27 ± 2.67	1.4 ± 1.17	Φ	3.4 ± 2.21	¶¶¶ ; +++ ; ¤
LDL	3.41 ± 0.73	2.44 ± 1.46	0.57 ± 0.55	Φ	2.77 ± 0.91	** ; ¶¶¶ ; +++ ; §§§
HDL	1.53 ± 0.34	1.1 ± 0.24	0.83 ± 0.2	n.s.	1.47 ± 0.44	*** ; ¶¶¶ ; ¤¤ ; §§
TG	1.26 ± 0.47	1.58 ± 1.02	0.74 ± 0.55	n.s.	1.43 ± 1.14	¶
TC	5.42 ± 0.85	4.11 ± 1.57	1.58 ± 0.48	ΦΦ	4.82 ± 1.11	*** ; ¶¶¶ ; ++ ; §§§
Non-HDL	3.89 ± 0.85	3.01 ± 1.56	0.74 ± 0.58	ΦΦ	3.36 ± 1.24	* ; ¶¶¶ ; + ; §§§

Before + without apheresis vs after apheresis: Φ $p<0.05$; ΦΦ $p<0.01$; ΦΦΦ $p<0.001$

Healthy vs FH before + without apheresis: * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Healthy vs FH after apheresis: ¶ $p<0.05$; ¶¶¶ $p<0.001$

Healthy vs DMT1: + $p<0.05$; ++ $p<0.01$; +++ $p<0.001$

FH before + without apheresis vs DMT1: ¤ $p<0.05$; ¤¤ $p<0.01$; ¤¤¤ $p<0.001$

FH after apheresis vs DMT1: §§ $p<0.01$; §§§ $p<0.001$

n.s.: all comparisons were not significant

DMT1: type 1 diabetes mellitus; BMI: body mass index; LDL: low-density lipoprotein: HDL: high-density lipoprotein; TG: triglycerides; TC: total cholesterol

6. STUDY LIMITATION

There are 4 major study limitations:

1) The use of aPTT and PT test is easy and precise but might not be able to detect small differences and in clinical praxis, the values are not always increased after administration of direct anticoagulants in contrast to heparins and VKA. In particular, rivaroxaban was shown to increase PT by 150-200%, while DTIs increased aPTT by 50-100% when compared to basal values (solvent control).
2) Our collaborators from the University Hospital at Hradec Králové prepared a method for detection of vitamin K_1 and several forms of vitamin K_2 (MK-4, MK-7, and MK-9). They were not able due to several reasons (e.g. lack of standards) to detect other vitamin K_2 forms which could particularize the relations between coagulation responses observed and vitamin K.
3) The number of severe cases of FH in our study was relatively low. Although the prevalence of FH is relatively high (1:313 individuals worldwide), particularly for the less serious heterozygous form. However, this condition is generally highly underdiagnosed. The problem could be overcome by the inclusion of patients being treated at other centers, but this was not possible at the time this project was taking place.
4) Our data should be confirmed in real *in vivo* clinical conditions since this was an *ex vivo* study.

7. CONCLUSION AND FUTURE PERSPECTIVES

In summary, there were no significant differences in PT/INR and aPTT values between healthy individuals and patients with metabolic diseases (FH or DMT1). However, when FXa-Is (rivaroxaban and apixaban) and DTIs (dabigatran and argatroban) were added *ex vivo* to the blood samples of healthy donors and patients, the prolongation of PT/INR and aPTT in patients was more extensive than in the healthy group. Correlation analyses revealed that BMI and lipid levels were critical for these results, moreover, vitamin K (K_1 and K_2), an important player in blood coagulation, was lower in DMT1 patients and partly in FH after lipid apheresis, compared to healthy individuals. These novel findings suggested that the efficacy of the anticoagulation effect of DOACs can be enhanced in patients with metabolic diseases, which are well pharmacologically treated for cardiovascular risk factors, particularly with hypolipidemic agents. Regardless, this can be potentially also associated with increased bleeding risk if these patients are treated with anticoagulants.

Although now DOACs are considered the safest anticoagulant therapy compared to VKA and heparins, as mentioned above, the risk of bleeding and certain drug-drug interactions cannot still be avoided. Based on this background, this study also covered the search for alternative anticoagulants from both natural and synthetic sources. Although we included a very large number of compounds with very different chemical structures, none of them possessed a clinically relevant anticoagulant effect. This fact suggests that it is much more complicated to find active novel anticoagulants than antiplatelet drugs.

Future studies in clinical praxis are needed to evaluate in detail the efficacy and adverse effects of DOACs in patients with metabolic disease. In particular, the impact of low-grade inflammation should be analyzed as they can contribute to enhanced coagulation and this analysis was not performed although such a situation can be observed particularly in obese individuals. It would be also of interest to study the impact of clinically used anticoagulants in real biological conditions by measuring their plasma levels and markers of the activity of the coagulation system. This is the only way how our *ex vivo* data can be confirmed *in vivo* and hence their clinical impact can be deciphered. Such analysis can answer

the question, of whether tailored anticoagulant therapy will not be better than currently used fixed doses regimen.

As the FH is much more common in the population than previously thought, more detailed data based on genetic analysis with analysis of their coagulation system can bring future specifications for the use of anticoagulants in these patients.

8. REFERENCES

1. Smith SA, Travers RJ, Morrissey JH. How it all starts: Initiation of the clotting cascade. Crit Rev Biochem Mol Biol. 2015;50(4):326-36. doi: 10.3109/10409238.2015.1050550.
2. Gale AJ. Continuing education course #2: current understanding of hemostasis. Toxicol Pathol. 2011;39(1):273-80. doi: 10.1177/0192623310389474.
3. Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. Nature. 1964;202:498-9. doi: 10.1038/202498a0.
4. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol. 2007;27(8):1687-93. doi: 10.1161/ATVBAHA.107.141911.
5. van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. Nat Rev Cardiol. 2019;16(3):166-79. doi: 10.1038/s41569-018-0110-0.
6. Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. Am J Pathol. 1989;134(5):1087-97.
7. Fleck RA, Rao LV, Rapaport SI, Varki N. Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. Thromb Res. 1990;59(2):421-37. doi: 10.1016/0049-3848(90)90148-6.
8. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, et al. Release of active tissue factor by human arterial smooth muscle cells. Circ Res. 2000;87(2):126-32. doi: 10.1161/01.res.87.2.126.
9. Fair DS. Quantitation of factor VII in the plasma of normal and warfarin-treated individuals by radioimmunoassay. Blood. 1983;62(4):784-91. doi: 10.1182/blood.V62.4.784.784.
10. Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. Blood. 1993;81(3):734-44. doi: 10.1182/blood.V81.3.734.bloodjournal813734.
11. Seligsohn U, Osterud B, Brown SF, Griffin JH, Rapaport SI. Activation of human factor VII in plasma and in purified systems: roles of activated factor IX, kallikrein, and activated factor XII. J Clin Invest. 1979;64(4):1056-65. doi: 10.1172/JCI109543.

12. Kondo S, Kisiel W. Regulation of factor VIIa activity in plasma: evidence that antithrombin III is the sole plasma protease inhibitor of human factor VIIa. Thromb Res. 1987;46(2):325-35. doi: 10.1016/0049-3848(87)90294-5.

13. Nemerson Y, Repke D. Tissue factor accelerates the activation of coagulation factor VII: the role of a bifunctional coagulation cofactor. Thromb Res. 1985;40(3):351-8. doi: 10.1016/0049-3848(85)90270-1.

14. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci U S A. 1977;74(12):5260-4. doi: 10.1073/pnas.74.12.5260.

15. Mann KG, Brummel-Ziedins K, Orfeo T, Butenas S. Models of blood coagulation. Blood Cells Mol Dis. 2006;36(2):108-17. doi: 10.1016/j.bcmd.2005.12.034.

16. Butenas S, Mann KG. Blood coagulation. Biochemistry (Mosc). 2002;67(1):3-12. doi: 10.1023/a:1013985911759.

17. Grover SP, Mackman N. Intrinsic Pathway of Coagulation and Thrombosis. Arterioscler Thromb Vasc Biol. 2019;39(3):331-8. doi: 10.1161/ATVBAHA.118.312130.

18. Mandle R, Jr., Kaplan AP. Hageman factor substrates. Human plasma prekallikrein: mechanism of activation by Hageman factor and participation in hageman factor-dependent fibrinolysis. J Biol Chem. 1977;252(17):6097-104.

19. Schmaier AH, Kuo A, Lundberg D, Murray S, Cines DB. The expression of high molecular weight kininogen on human umbilical vein endothelial cells. J Biol Chem. 1988;263(31):16327-33.

20. Griffin JH, Cochrane CG. Mechanisms for the involvement of high molecular weight kininogen in surface-dependent reactions of Hageman factor. Proc Natl Acad Sci U S A. 1976;73(8):2554-8. doi: 10.1073/pnas.73.8.2554.

21. Wiggins RC, Cochrane CC. The autoactivation of rabbit Hageman factor. J Exp Med. 1979;150(5):1122-33. doi: 10.1084/jem.150.5.1122.

22. van der Graaf F, Keus F, Vlooswijk R, Bouma B. The contact activation mechanism in human plasma: activation induced by dextran sulfate. Blood. 1982;59(6):1225-33. doi: 10.1182/blood.V59.6.1225.1225.

23. Tankersley DL, Alving BM, Finlayson JS. Activation of factor XII by dextran sulfate: the basis for an assay of factor XII. Blood. 1983;62(2):448-56. doi: 10.1182/blood.V62.2.448.448.

24. Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci U S A. 2006;103(4):903-8. doi: 10.1073/pnas.0507195103.

25. Kannemeier C, Shibamiya A, Nakazawa F, Trusheim H, Ruppert C, Markart P, et al. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. Proc Natl Acad Sci U S A. 2007;104(15):6388-93. doi: 10.1073/pnas.0608647104.

26. Noubouossie DF, Whelihan MF, Yu YB, Sparkenbaugh E, Pawlinski R, Monroe DM, Key NS. In vitro activation of coagulation by human neutrophil DNA and histone proteins but not neutrophil extracellular traps. Blood. 2017;129(8):1021-9. doi: 10.1182/blood-2016-06-722298.

27. Bendapudi PK, Deceunynck K, Koseoglu S, Bekendam RH, Mason SD, Kenniston J, Flaumenhaft RC. Stimulated Platelets but Not Endothelium Generate Thrombin Via a Factor XIIa-Dependent Mechanism Requiring Phosphatidylserine Exposure. Blood. 2016;128(22):258-. doi: 10.1182/blood.V128.22.258.258.

28. Muller F, Gailani D, Renne T. Factor XI and XII as antithrombotic targets. Curr Opin Hematol. 2011;18(5):349-55. doi: 10.1097/MOH.0b013e3283497e61.

29. Motta G, Rojkjaer R, Hasan AA, Cines DB, Schmaier AH. High molecular weight kininogen regulates prekallikrein assembly and activation on endothelial cells: a novel mechanism for contact activation. Blood. 1998;91(2):516-28. doi: 10.1182/blood.V91.2.516.516_516_528.

30. Mast AE. Tissue Factor Pathway Inhibitor: Multiple Anticoagulant Activities for a Single Protein. Arterioscler Thromb Vasc Biol. 2016;36(1):9-14. doi: 10.1161/ATVBAHA.115.305996.

31. Soeda T, Nogami K, Matsumoto T, Ogiwara K, Shima M. Mechanisms of factor VIIa-catalyzed activation of factor VIII. J Thromb Haemost. 2010;8(11):2494-503. doi: 10.1111/j.1538-7836.2010.04042.x.

32. Kamikubo Y, Mendolicchio GL, Zampolli A, Marchese P, Rothmeier AS, Orje JN, et al. Selective factor VIII activation by the tissue factor-factor VIIa-factor Xa complex. Blood. 2017;130(14):1661-70. doi: 10.1182/blood-2017-02-767079.

33. Kravtsov DV, Matafonov A, Tucker EI, Sun MF, Walsh PN, Gruber A, Gailani D. Factor XI contributes to thrombin generation in the absence of factor XII. Blood. 2009;114(2):452-8. doi: 10.1182/blood-2009-02-203604.

34. Monroe DM, Hoffman M. What does it take to make the perfect clot? Arterioscler Thromb Vasc Biol. 2006;26(1):41-8. doi: 10.1161/01.Atv.0000193624.28251.83.

35. Schreuder M, Reitsma PH, Bos MHA. Blood coagulation factor Va's key interactive residues and regions for prothrombinase assembly and prothrombin binding. J Thromb Haemost. 2019;17(8):1229-39. doi: 10.1111/jth.14487.

36. Huntington JA. Molecular recognition mechanisms of thrombin. J Thromb Haemost. 2005;3(8):1861-72. doi: 10.1111/j.1538-7836.2005.01363.x.

37. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiol Rev. 2013;93(1):327-58. doi: 10.1152/physrev.00016.2011.

38. Hsieh K. Thrombin interaction with fibrin polymerization sites. Thromb Res. 1997;86(4):301-16. doi: 10.1016/s0049-3848(97)00073-x.

39. Hethershaw EL, Cilia La Corte AL, Duval C, Ali M, Grant PJ, Ariens RA, Philippou H. The effect of blood coagulation factor XIII on fibrin clot structure and fibrinolysis. J Thromb Haemost. 2014;12(2):197-205. doi: 10.1111/jth.12455.

40. Anokhin BA, Dean WL, Smith KA, Flick MJ, Ariens RAS, Philippou H, Maurer MC. Proteolytic and nonproteolytic activation mechanisms result in conformationally and functionally different forms of coagulation factor XIII A. FEBS J. 2020;287(3):452-64. doi: 10.1111/febs.15040.

41. Kim JW, Ko SH, Cho JH, Sun C, Hong OK, Lee SH, et al. Loss of beta-cells with fibrotic islet destruction in type 2 diabetes mellitus. Front Biosci. 2008;13:6022-33. doi: 10.2741/3133.

42. Dludla PV, Mabhida SE, Ziqubu K, Nkambule BB, Mazibuko-Mbeje SE, Hanser S, et al. Pancreatic beta-cell dysfunction in type 2 diabetes: Implications of inflammation and oxidative stress. World J Diabetes. 2023;14(3):130-46. doi: 10.4239/wjd.v14.i3.130.

43. Li X, Weber NC, Cohn DM, Hollmann MW, DeVries JH, Hermanides J, Preckel B. Effects of Hyperglycemia and Diabetes Mellitus on Coagulation and Hemostasis. J Clin Med. 2021;10(11). doi: 10.3390/jcm10112419.

44. Ozder A, Eker HH. Investigation of mean platelet volume in patients with type 2 diabetes mellitus and in subjects with impaired fasting glucose: a cost-effective tool in primary health care? Int J Clin Exp Med. 2014;7(8):2292-7.

45. Malachowska B, Tomasik B, Szadkowska A, Baranowska-Jazwiecka A, Wegner O, Mlynarski W, Fendler W. Altered platelets' morphological parameters in children with type 1 diabetes - a case-control study. BMC Endocr Disord. 2015;15:17. doi: 10.1186/s12902-015-0011-8.

46. Venkatesh V, Kumar R, Varma DK, Bhatia P, Yadav J, Dayal D. Changes in platelet morphology indices in relation to duration of disease and glycemic control in children with type 1 diabetes mellitus. J Diabetes Complications. 2018;32(9):833-8. doi: 10.1016/j.jdiacomp.2018.06.008.

47. Zaccardi F, Rocca B, Rizzi A, Ciminello A, Teofili L, Ghirlanda G, et al. Platelet indices and glucose control in type 1 and type 2 diabetes mellitus: A case-control study. Nutr Metab Cardiovasc Dis. 2017;27(10):902-9. doi: 10.1016/j.numecd.2017.06.016.

48. Kim HK, Kim JE, Park SH, Kim YI, Nam-Goong IS, Kim ES. High coagulation factor levels and low protein C levels contribute to enhanced thrombin generation in patients with diabetes who do not have macrovascular complications. J Diabetes Complications. 2014;28(3):365-9. doi: 10.1016/j.jdiacomp.2014.01.006.

49. Singh A, Boden G, Homko C, Gunawardana J, Rao AK. Whole-blood tissue factor procoagulant activity is elevated in type 1 diabetes: effects of hyperglycemia and hyperinsulinemia. Diabetes Care. 2012;35(6):1322-7. doi: 10.2337/dc11-2114.

50. Barillari G, Fabbro E, Pasca S, Bigotto E. Coagulation and oxidative stress plasmatic levels in a type 2 diabetes population. Blood Coagul Fibrinolysis. 2009;20(4):290-6. doi: 10.1097/MBC.0b013e328329e49b.

51. Patrassi GM, Vettor R, Padovan D, Girolami A. Contact phase of blood coagulation in diabetes mellitus. Eur J Clin Invest. 1982;12(4):307-11. doi: 10.1111/j.1365-2362.1982.tb02237.x.

52. Song DY, Gu JY, Yoo HJ, Kim YI, Nam-Goong IS, Kim ES, Kim HK. Activation of Factor XII and Kallikrein-Kinin System Combined with Neutrophil Extracellular Trap Formation in Diabetic Retinopathy. Exp Clin Endocrinol Diabetes. 2021;129(8):560-5. doi: 10.1055/a-0981-6023.

53. Mansfield MW, Kohler HP, Ariens RA, McCormack LJ, Grant PJ. Circulating levels of coagulation factor XIII in subjects with type 2 diabetes and in their first-degree relatives. Diabetes Care. 2000;23(5):703-5. doi: 10.2337/diacare.23.5.703.

54. Fattah MA, Shaheen MH, Mahfouz MH. Disturbances of haemostasis in diabetes mellitus. Dis Markers. 2003;19(6):251-8. doi: 10.1155/2004/797458.

55. Klein RL, Hunter SJ, Jenkins AJ, Zheng D, Semler AJ, Clore J, et al. Fibrinogen is a marker for nephropathy and peripheral vascular disease in type 1 diabetes: studies of plasma fibrinogen and fibrinogen gene polymorphism in the DCCT/EDIC cohort. Diabetes Care. 2003;26(5):1439-48. doi: 10.2337/diacare.26.5.1439.

56. Reverter JL, Reverter JC, Tassies D, Rius F, Monteagudo J, Rubies-Prat J, et al. Thrombomodulin and induced tissue factor expression on monocytes as markers of diabetic microangiopathy: a prospective study on hemostasis and lipoproteins in insulin-dependent diabetes mellitus. Am J Hematol. 1997;56(2):93-9. doi: 10.1002/(sici)1096-8652(199710)56:2<93::aid-ajh4>3.0.co;2-w.

57. Yasuma T, Yano Y, D'Alessandro-Gabazza CN, Toda M, Gil-Bernabe P, Kobayashi T, et al. Amelioration of Diabetes by Protein S. Diabetes. 2016;65(7):1940-51. doi: 10.2337/db15-1404.

58. Ceriello A, Giugliano D, Quatraro A, Stante A, Dello Russo P, Torella R. Increased alpha 2-macroglobulin in diabetes: a hyperglycemia related phenomenon associated with reduced antithrombin III activity. Acta Diabetol Lat. 1989;26(2):147-54. doi: 10.1007/BF02581366.

59. Borsey DQ, Prowse CV, Gray RS, Dawes J, James K, Elton RA, Clarke BF. Platelet and coagulation factors in proliferative diabetic retinopathy. J Clin Pathol. 1984;37(6):659-64. doi: 10.1136/jcp.37.6.659.

60. Meigs JB, Mittleman MA, Nathan DM, Tofler GH, Singer DE, Murphy-Sheehy PM, et al. Hyperinsulinemia, hyperglycemia, and impaired hemostasis: the Framingham Offspring Study. JAMA. 2000;283(2):221-8. doi: 10.1001/jama.283.2.221.

61. Verkleij CJ, Bruijn RE, Meesters EW, Gerdes VE, Meijers JC, Marx PF. The hemostatic system in patients with type 2 diabetes with and without cardiovascular disease. Clin Appl Thromb Hemost. 2011;17(6):E57-63. doi: 10.1177/1076029610384112.

62. Sommeijer DW, Hansen HR, van Oerle R, Hamulyak K, van Zanten AP, Meesters E, et al. Soluble tissue factor is a candidate marker for progression of microvascular disease in patients with Type 2 diabetes. J Thromb Haemost. 2006;4(3):574-80. doi: 10.1111/j.1538-7836.2005.01763.x.

63. Juhan-Vague I, Roul C, Alessi MC, Ardissone JP, Heim M, Vague P. Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients--relationship with plasma insulin. Thromb Haemost. 1989;61(3):370-3.

64. Hori Y, Gabazza EC, Yano Y, Katsuki A, Suzuki K, Adachi Y, Sumida Y. Insulin resistance is associated with increased circulating level of thrombin-activatable fibrinolysis inhibitor in type 2 diabetic patients. J Clin Endocrinol Metab. 2002;87(2):660-5. doi: 10.1210/jcem.87.2.8214.

65. Sherif EM, Elbarbary NS, Abd Al Aziz MM, Mohamed SF. Plasma thrombin-activatable fibrinolysis inhibitor levels in children and adolescents with type 1 diabetes mellitus: possible relation to diabetic microvascular complications. Blood Coagul Fibrinolysis. 2014;25(5):451-7. doi: 10.1097/MBC.0000000000000080.

66. Yoshino S, Fujimoto K, Takada T, Kawamura S, Ogawa J, Kamata Y, et al. Molecular form and concentration of serum alpha(2)-macroglobulin in diabetes. Sci Rep. 2019;9(1):12927. doi: 10.1038/s41598-019-49144-7.

67. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation. 2013;127(1):e6-e245. doi: 10.1161/CIR.0b013e31828124ad.

68. Kim MK, Han K, Kim HS, Park YM, Kwon HS, Yoon KH, Lee SH. Cholesterol variability and the risk of mortality, myocardial infarction, and stroke: a nationwide population-based study. Eur Heart J. 2017;38(48):3560-6. doi: 10.1093/eurheartj/ehx585.

69. Chan P, Tomlinsoin B, Tsai CW, Pan WH, Lee YS. Thrombophilia in patients with hypercholesterolemia. Metabolism. 1996;45(8):966-9. doi: 10.1016/s0026-0495(96)90264-4.

70. Chan P, Huang TY, Shieh SM, Lin TS, Tsai CW. Thrombophilia in Patients with Hypertriglyceridemia. J Thromb Thrombolysis. 1997;4(3/4):425-9. doi: 10.1023/a:1008857618659.

71. Kim JA, Kim JE, Song SH, Kim HK. Influence of blood lipids on global coagulation test results. Ann Lab Med. 2015;35(1):15-21. doi: 10.3343/alm.2015.35.1.15.

72. Shu Y, Zhao X, Yang C, Yan Y, Zheng Y, Wang X, Qiu C. Circulating prekallikrein levels are correlated with lipid levels in the chinese population: a cross-sectional study. Lipids Health Dis. 2023;22(1):79. doi: 10.1186/s12944-023-01849-5.

73. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. J Thromb Haemost. 2016;14(1):28-39. doi: 10.1111/jth.13194.

74. Winter WE, Flax SD, Harris NS. Coagulation Testing in the Core Laboratory. Lab Med. 2017;48(4):295-313. doi: 10.1093/labmed/lmx050.

75. Thachil J. Dispelling myths about coagulation abnormalities in internal medicine. Clin Med (Lond). 2014;14(3):239-44. doi: 10.7861/clinmedicine.14-3-239.

76. Sevenet PO, Kaczor DA, Depasse F. Factor VII Deficiency: From Basics to Clinical Laboratory Diagnosis and Patient Management. Clin Appl Thromb Hemost. 2017;23(7):703-10. doi: 10.1177/1076029616670257.

77. Neuenfeldt FS, Weigand MA, Fischer D. Coagulopathies in Intensive Care Medicine: Balancing Act between Thrombosis and Bleeding. J Clin Med. 2021;10(22). doi: 10.3390/jcm10225369.

78. Ignjatovic V. Prothrombin time/international normalized ratio. Methods Mol Biol. 2013;992:121-9. doi: 10.1007/978-1-62703-339-8_9.

79. Flanders MM, Crist R, Rodgers GM. Comparison of five thrombin time reagents. Clin Chem. 2003;49(1):169-72. doi: 10.1373/49.1.169.

80. Poller L. International Normalized Ratios (INR): the first 20 years. J Thromb Haemost. 2004;2(6):849-60. doi: 10.1111/j.1538-7836.2004.00775.x.

81. Dorgalaleh A, Favaloro EJ, Bahraini M, Rad F. Standardization of Prothrombin Time/International Normalized Ratio (PT/INR). Int J Lab Hematol. 2021;43(1):21-8. doi: 10.1111/ijlh.13349.

82. Lab TMPV. Hemostasis: PT and PTT tests [cited 2024 31 July]. Available from: https://www.medicine.mcgill.ca/physio/vlab/bloodlab/PT_PTT.htm.

83. Ansell J, Hirsh J, Dalen J, Bussey H, Anderson D, Poller L, et al. Managing oral anticoagulant therapy. Chest. 2001;119(1 Suppl):22S-38S. doi: 10.1378/chest.119.1_suppl.22s.

84. Hirsh J, Dalen J, Anderson DR, Poller L, Bussey H, Ansell J, Deykin D. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 2001;119(1 Suppl):8S-21S. doi: 10.1378/chest.119.1_suppl.8s.

85. Ignjatovic V. Activated partial thromboplastin time. Methods Mol Biol. 2013;992:111-20. doi: 10.1007/978-1-62703-339-8_8.

86. Barbosa ACN, Montalvao SAL, Barbosa KGN, Colella MP, Annichino-Bizzacchi JM, Ozelo MC, De Paula EV. Prolonged APTT of unknown etiology: A systematic evaluation of causes and laboratory resource use in an outpatient hemostasis academic unit. Res Pract Thromb Haemost. 2019;3(4):749-57. doi: 10.1002/rth2.12252.

87. Wagenman BL, Townsend KT, Mathew P, Crookston KP. The laboratory approach to inherited and acquired coagulation factor deficiencies. Clin Lab Med. 2009;29(2):229-52. doi: 10.1016/j.cll.2009.04.002.

88. Favaloro EJ. Coagulation mixing studies: Utility, algorithmic strategies and limitations for lupus anticoagulant testing or follow up of abnormal coagulation tests. Am J Hematol. 2020;95(1):117-28. doi: 10.1002/ajh.25669.

89. Castellone D. Inhibitor Identification in the Coagulation Laboratory. Laboratory Medicine. 2013;44(4):e118-e20. doi: 10.1309/lmpm7fpi28vqgvlk.

90. Suzuki K, Wada H, Matsumoto T, Ikejiri M, Ohishi K, Yamashita Y, et al. Usefulness of the APTT waveform for the diagnosis of DIC and prediction of the outcome or bleeding risk. Thromb J. 2019;17:12. doi: 10.1186/s12959-019-0201-0.

91. Mackie I, Casini A, Pieters M, Pruthi R, Reilly-Stitt C, Suzuki A. International council for standardisation in haematology recommendations on fibrinogen assays, thrombin clotting time and related tests in the investigation of bleeding disorders. Int J Lab Hematol. 2024;46(1):20-32. doi: 10.1111/ijlh.14201.

92. Undas A. Determination of Fibrinogen and Thrombin Time (TT). Methods Mol Biol. 2017;1646:105-10. doi: 10.1007/978-1-4939-7196-1_8.

93. May JE, Wolberg AS, Lim MY. Disorders of Fibrinogen and Fibrinolysis. Hematol Oncol Clin North Am. 2021;35(6):1197-217. doi: 10.1016/j.hoc.2021.07.011.

94. Kattula S, Byrnes JR, Wolberg AS. Fibrinogen and Fibrin in Hemostasis and Thrombosis. Arterioscler Thromb Vasc Biol. 2017;37(3):e13-e21. doi: 10.1161/ATVBAHA.117.308564.

95. Bolliger D, Gorlinger K, Tanaka KA. Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. Anesthesiology. 2010;113(5):1205-19. doi: 10.1097/ALN.0b013e3181f22b5a.

96. Arai S, Kamijo T, Takezawa Y, Sugano M, Nakazawa H, Yanagisawa R, et al. Acquired dysfibrinogenemia: monoclonal lambda-type IgA binding to fibrinogen caused lower functional plasma fibrinogen level and abnormal clot formation. Int J Hematol. 2020;112(1):96-104. doi: 10.1007/s12185-020-02874-1.

97. Casini A, de Moerloose P. Fibrinogen concentrates in hereditary fibrinogen disorders: Past, present and future. Haemophilia. 2020;26(1):25-32. doi: 10.1111/hae.13876.

98. Cunningham MT, Brandt JT, Laposata M, Olson JD. Laboratory diagnosis of dysfibrinogenemia. Arch Pathol Lab Med. 2002;126(4):499-505. doi: 10.5858/2002-126-0499-ldod.

99. Hayes T. Dysfibrinogenemia and thrombosis. Arch Pathol Lab Med. 2002;126(11):1387-90. doi: 10.5858/2002-126-1387-DAT.

100. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380(9859):2095-128. doi: 10.1016/S0140-6736(12)61728-0.

101. Diseases GBD, Injuries C. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2020;396(10258):1204-22. doi: 10.1016/S0140-6736(20)30925-9.

102. Lippi G, Franchini M. Pathogenesis of venous thromboembolism: when the cup runneth over. Semin Thromb Hemost. 2008;34(8):747-61. doi: 10.1055/s-0029-1145257.

103. Lippi G, Franchini M, Targher G. Arterial thrombus formation in cardiovascular disease. Nat Rev Cardiol. 2011;8(9):502-12. doi: 10.1038/nrcardio.2011.91.

104. Franchini M, Liumbruno GM, Bonfanti C, Lippi G. The evolution of anticoagulant therapy. Blood Transfus. 2016;14(2):175-84. doi: 10.2450/2015.0096-15.

105. Hirsh J, Warkentin TE, Shaughnessy SG, Anand SS, Halperin JL, Raschke R, et al. Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. Chest. 2001;119(1 Suppl):64S-94S. doi: 10.1378/chest.119.1_suppl.64s.

106. Yeh CH, Gross PL, Weitz JI. Evolving use of new oral anticoagulants for treatment of venous thromboembolism. Blood. 2014;124(7):1020-8. doi: 10.1182/blood-2014-03-563056.

107. Weitz JI, Jaffer IH, Fredenburgh JC. Recent advances in the treatment of venous thromboembolism in the era of the direct oral anticoagulants. F1000Res. 2017;6:985. doi: 10.12688/f1000research.11174.1.

108. Garcia DA, Baglin TP, Weitz JI, Samama MM. Parenteral anticoagulants: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest. 2012;141(2 Suppl):e24S-e43S. doi: 10.1378/chest.11-2291.

109. Rezaie AR, Giri H. Anticoagulant and signaling functions of antithrombin. J Thromb Haemost. 2020;18(12):3142-53. doi: 10.1111/jth.15052.

110. Heestermans M, Poenou G, Hamzeh-Cognasse H, Cognasse F, Bertoletti L. Anticoagulants: A Short History, Their Mechanism of Action, Pharmacology, and Indications. Cells. 2022;11(20). doi: 10.3390/cells11203214.

111. Sokolowska E, Kalaska B, Miklosz J, Mogielnicki A. The toxicology of heparin reversal with protamine: past, present and future. Expert Opin Drug Metab Toxicol. 2016;12(8):897-909. doi: 10.1080/17425255.2016.1194395.

112. Young E, Wells P, Holloway S, Weitz J, Hirsh J. Ex-vivo and in-vitro evidence that low molecular weight heparins exhibit less binding to plasma proteins than unfractionated heparin. Thromb Haemost. 1994;71(3):300-4. doi: 10.1055/s-0038-1642434.

113. Bloemen A, Testroote MJ, Janssen-Heijnen ML, Janzing HM. Incidence and diagnosis of heparin-induced thrombocytopenia (HIT) in patients with traumatic injuries treated with unfractioned or low-molecular-weight heparin: a literature review. Injury. 2012;43(5):548-52. doi: 10.1016/j.injury.2011.05.007.

114. Marchetti M, Zermatten MG, Bertaggia Calderara D, Aliotta A, Alberio L. Heparin-Induced Thrombocytopenia: A Review of New Concepts in Pathogenesis, Diagnosis, and Management. J Clin Med. 2021;10(4). doi: 10.3390/jcm10040683.

115. Amiral J, Bridey F, Dreyfus M, Vissoc AM, Fressinaud E, Wolf M, Meyer D. Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin-induced thrombocytopenia. Thromb Haemost. 1992;68(1):95-6.

116. Ahmed I, Majeed A, Powell R. Heparin induced thrombocytopenia: diagnosis and management update. Postgrad Med J. 2007;83(983):575-82. doi: 10.1136/pgmj.2007.059188.

117. Petitou M, Duchaussoy P, Herbert JM, Duc G, El Hajji M, Branellec JF, et al. The synthetic pentasaccharide fondaparinux: first in the class of antithrombotic agents that selectively inhibit coagulation factor Xa. Semin Thromb Hemost. 2002;28(4):393-402. doi: 10.1055/s-2002-34309.

118. Aursulesei V, Costache, II. Anticoagulation in chronic kidney disease: from guidelines to clinical practice. Clin Cardiol. 2019;42(8):774-82. doi: 10.1002/clc.23196.

119. Bijsterveld NR, Moons AH, Boekholdt SM, van Aken BE, Fennema H, Peters RJ, et al. Ability of recombinant factor VIIa to reverse the anticoagulant effect of the pentasaccharide fondaparinux in healthy volunteers. Circulation. 2002;106(20):2550-4. doi: 10.1161/01.cir.0000038501.87442.02.

120. Quaranta M, Erez O, Mastrolia SA, Koifman A, Leron E, Eshkoli T, et al. The physiologic and therapeutic role of heparin in implantation and placentation. PeerJ. 2015;3:e691. doi: 10.7717/peerj.691.

121. Nelsestuen GL, Zytkovicz TH, Howard JB. The mode of action of vitamin K. Identification of gamma-carboxyglutamic acid as a component of prothrombin. J Biol Chem. 1974;249(19):6347-50. doi: 10.1016/S0021-9258(19)42259-X

122. Mladěnka P, Macakova K, Kujovska Krcmova L, Javorska L, Mrstna K, Carazo A, et al. Vitamin K - sources, physiological role, kinetics, deficiency, detection, therapeutic use, and toxicity. Nutr Rev. 2022;80(4):677-98. doi: 10.1093/nutrit/nuab061.

123. Whitlon DS, Sadowski JA, Suttie JW. Mechanism of coumarin action: significance of vitamin K epoxide reductase inhibition. Biochemistry. 1978;17(8):1371-7. doi: 10.1021/bi00601a003.

124. Siegal D, Yudin J, Kaatz S, Douketis JD, Lim W, Spyropoulos AC. Periprocedural heparin bridging in patients receiving vitamin K antagonists: systematic review and meta-analysis of bleeding and thromboembolic rates. Circulation. 2012;126(13):1630-9. doi: 10.1161/CIRCULATIONAHA.112.105221.

125. Investigators BS. Bridging anticoagulation: is it needed when warfarin is interrupted around the time of a surgery or procedure? Circulation. 2012;125(12):e496-8. doi: 10.1161/CIRCULATIONAHA.111.084517.

126. Hirsh J, Dalen JE, Anderson DR, Poller L, Bussey H, Ansell J, et al. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 1998;114(5 Suppl):445S-69S. doi: 10.1378/chest.114.5_supplement.445s.

127. Wang M, Zeraatkar D, Obeda M, Lee M, Garcia C, Nguyen L, et al. Drug-drug interactions with warfarin: A systematic review and meta-analysis. Br J Clin Pharmacol. 2021;87(11):4051-100. doi: 10.1111/bcp.14833.

128. Wysowski DK, Nourjah P, Swartz L. Bleeding complications with warfarin use: a prevalent adverse effect resulting in regulatory action. Arch Intern Med. 2007;167(13):1414-9. doi: 10.1001/archinte.167.13.1414.

129. Ozturk M, Ipekci A, Kiyak SK, Akdeniz YS, Aydin Y, Ikizceli I, Sogut O. Bleeding Complications in Warfarin-Treated Patients Admitted to the Emergency Department. J Clin Med Res. 2019;11(2):106-13. doi: 10.14740/jocmr3669.

130. Yung D, Kapral MK, Asllani E, Fang J, Lee DS, Investigators of the Registry of the Canadian Stroke N. Reinitiation of anticoagulation after warfarin-associated intracranial hemorrhage and mortality risk: the Best Practice for Reinitiating Anticoagulation Therapy After Intracranial Bleeding (BRAIN) study. Can J Cardiol. 2012;28(1):33-9. doi: 10.1016/j.cjca.2011.10.002.

131. Witt DM, Delate T, Hylek EM, Clark NP, Crowther MA, Dentali F, et al. Effect of warfarin on intracranial hemorrhage incidence and fatal outcomes. Thromb Res. 2013;132(6):770-5. doi: 10.1016/j.thromres.2013.10.024.

132. Chen WC, Chen YH, Hsu PI, Tsay FW, Chan HH, Cheng JS, Lai KH. Gastrointestinal hemorrhage in warfarin anticoagulated patients: incidence, risk factor, management, and outcome. Biomed Res Int. 2014;2014:463767. doi: 10.1155/2014/463767.

133. Chan YC, Valenti D, Mansfield AO, Stansby G. Warfarin induced skin necrosis. Br J Surg. 2000;87(3):266-72. doi: 10.1046/j.1365-2168.2000.01352.x.

134. Pourdeyhimi N, Bullard Z. Warfarin-induced skin necrosis. Hosp Pharm. 2014;49(11):1044-8. doi: 10.1310/hpj4911-1044.

135. Talmadge DB, Spyropoulos AC. Purple toes syndrome associated with warfarin therapy in a patient with antiphospholipid syndrome. Pharmacotherapy. 2003;23(5):674-7. doi: 10.1592/phco.23.5.674.32200.

136. Cakebread HE, Knight HM, Gajendragadkar PR, Cooper JP. Warfarin-induced purple toe syndrome successfully treated with apixaban. BMJ Case Rep. 2014;2014. doi: 10.1136/bcr-2014-205320.

137. Gage BF, Birman-Deych E, Radford MJ, Nilasena DS, Binder EF. Risk of osteoporotic fracture in elderly patients taking warfarin: results from the National Registry of Atrial Fibrillation 2. Arch Intern Med. 2006;166(2):241-6. doi: 10.1001/archinte.166.2.241.

138. Signorelli SS, Scuto S, Marino E, Giusti M, Xourafa A, Gaudio A. Anticoagulants and Osteoporosis. Int J Mol Sci. 2019;20(21). doi: 10.3390/ijms20215275.

139. Huang HK, Peng CC, Lin SM, Munir KM, Chang RH, Wu BB, et al. Fracture Risks in Patients Treated With Different Oral Anticoagulants: A Systematic Review and Meta-Analysis. J Am Heart Assoc. 2021;10(7):e019618. doi: 10.1161/JAHA.120.019618.

140. Watras MM, Patel JP, Arya R. Traditional Anticoagulants and Hair Loss: A Role for Direct Oral Anticoagulants? A Review of the Literature. Drugs Real World Outcomes. 2016;3(1):1-6. doi: 10.1007/s40801-015-0056-z.

141. Marques MA, Porto CLL, Milhomens ALM, Vieira JM, Gomes CCA, Rocha ATC, Miller CDS. Alopecia in anticoagulated patients. J Vasc Bras. 2020;19:e20190018. doi: 10.1590/1677-5449.190018.

142. Hull KL, Gooding R, Burton JO. Resolution of warfarin-induced alopecia with conversion to apixaban. BMJ Case Rep. 2021;14(3). doi: 10.1136/bcr-2020-240579.

143. Arora N, Goldhaber SZ. Anticoagulants and transaminase elevation. Circulation. 2006;113(15):e698-702. doi: 10.1161/CIRCULATIONAHA.105.603100.

144. Alonso A, MacLehose RF, Chen LY, Bengtson LG, Chamberlain AM, Norby FL, Lutsey PL. Prospective study of oral anticoagulants and risk of liver injury in patients with atrial fibrillation. Heart. 2017;103(11):834-9. doi: 10.1136/heartjnl-2016-310586.

145. Atia MM, Mahmoud HAA, Wilson M, Abd-Allah EA. A comprehensive survey of warfarin-induced hepatic toxicity using histopathological, biomarker, and molecular evaluation. Heliyon. 2024;10(4):e26484. doi: 10.1016/j.heliyon.2024.e26484.

146. Weitz JI, Eikelboom JW, Samama MM. New antithrombotic drugs: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest. 2012;141(2 Suppl):e120S-e51S. doi: 10.1378/chest.11-2294.

147. Connolly SJ, Milling TJ, Jr., Eikelboom JW, Gibson CM, Curnutte JT, Gold A, et al. Andexanet Alfa for Acute Major Bleeding Associated with Factor Xa Inhibitors. N Engl J Med. 2016;375(12):1131-41. doi: 10.1056/NEJMoa1607887.

148. Cuker A, Burnett A, Triller D, Crowther M, Ansell J, Van Cott EM, et al. Reversal of direct oral anticoagulants: Guidance from the Anticoagulation Forum. Am J Hematol. 2019;94(6):697-709. doi: 10.1002/ajh.25475.

149. Eisert WG, Hauel N, Stangier J, Wienen W, Clemens A, van Ryn J. Dabigatran: an oral novel potent reversible nonpeptide inhibitor of thrombin. Arterioscler Thromb Vasc Biol. 2010;30(10):1885-9. doi: 10.1161/ATVBAHA.110.203604.

150. Fitzgerald D, Murphy N. Argatroban: a synthetic thrombin inhibitor of low relative molecular mass. Coron Artery Dis. 1996;7(6):455-8.

151. Jeske WP, Fareed J, Hoppensteadt DA, Lewis B, Walenga JM. Pharmacology of argatroban. Expert Rev Hematol. 2010;3(5):527-39. doi: 10.1586/ehm.10.53.

152. Major TC, Handa H, Brisbois EJ, Reynolds MM, Annich GM, Meyerhoff ME, Bartlett RH. The mediation of platelet quiescence by NO-releasing polymers via cGMP-induced serine 239 phosphorylation of vasodilator-stimulated phosphoprotein. Biomaterials. 2013;34(33):8086-96. doi: 10.1016/j.biomaterials.2013.07.041.

153. Bellomo TR, Jeakle MA, Meyerhoff ME, Bartlett RH, Major TC. The Effects of the Combined Argatroban/Nitric Oxide-Releasing Polymer on Platelet Microparticle-Induced Thrombogenicity in Coated Extracorporeal Circuits. ASAIO J. 2021;67(5):573-82. doi: 10.1097/MAT.0000000000001256.

154. Zhou Y, Yao Z, Zhu L, Tang Y, Chen J, Wu J. Safety of Dabigatran as an Anticoagulant: A Systematic Review and Meta-Analysis. Front Pharmacol. 2021;12:626063. doi: 10.3389/fphar.2021.626063.

155. Udomnilobol U, Dunkoksung W, Sakares W, Jianmongkol S, Prueksaritanont T. Assessing the relative contribution of CYP3A-and P-gp-mediated pathways to the overall disposition and drug-drug interaction of dabigatran etexilate using a comprehensive mechanistic physiological-based pharmacokinetic model. Front Pharmacol. 2024;15:1356273. doi: 10.3389/fphar.2024.1356273.

156. Pollack CV, Jr., Reilly PA, Eikelboom J, Glund S, Verhamme P, Bernstein RA, et al. Idarucizumab for Dabigatran Reversal. N Engl J Med. 2015;373(6):511-20. doi: 10.1056/NEJMoa1502000.

157. Krcmova L, Solichova D, Melichar B, Kasparova M, Plisek J, Sobotka L, Solich P. Determination of neopterin, kynurenine, tryptophan and creatinine in human serum by high throuput HPLC. Talanta. 2011;85(3):1466-71. doi: 10.1016/j.talanta.2011.06.027.

158. Mrstna K, Matousova K, Krcmova LK, Carazo A, Pourova J, Mladenka P, et al. Analysis of vitamin K(1) and major K(2) variants in rat/human serum and lipoprotein fractions by a rapid, simple, and sensitive UHPLC-MS/MS method. J Chromatogr A. 2024;1714:464548. doi: 10.1016/j.chroma.2023.464548.

159. Righi G, Antonioletti R, Silvestri IP, D'Antona N, Lambusta D, Bovicelli P. Convergent synthesis of mosloflavone, negletein and baicalein from crysin. Tetrahedron. 2010;66(6):1294-8. doi: https://doi.org/10.1016/j.tet.2009.12.021.

160. Hrubsa M, Nurjamal K, Carazo A, Nayek N, Karlickova J, Applova L, et al. Screening of Synthetic Heterocyclic Compounds as Antiplatelet Drugs. Med Chem. 2022;18(5):536-43. doi: 10.2174/1573406417666211026150658.

161. Karlickova J, Riha M, Filipsky T, Macakova K, Hrdina R, Mladenka P. Antiplatelet Effects of Flavonoids Mediated by Inhibition of Arachidonic Acid Based Pathway. Planta Med. 2016;82(1-2):76-83. doi: 10.1055/s-0035-1557902.

162. Applova L, Karlickova J, Riha M, Filipsky T, Macakova K, Spilkova J, Mladenka P. The isoflavonoid tectorigenin has better antiplatelet potential than acetylsalicylic acid. Phytomedicine. 2017;35:11-7. doi: 10.1016/j.phymed.2017.08.023.

163. Gunnarsson GT, Riaz M, Adams J, Desai UR. Synthesis of per-sulfated flavonoids using 2,2,2-trichloro ethyl protecting group and their factor Xa inhibition potential. Bioorg Med Chem. 2005;13(5):1783-9. doi: 10.1016/j.bmc.2004.11.060.

164. Mozzicafreddo M, Cuccioloni M, Eleuteri AM, Fioretti E, Angeletti M. Flavonoids inhibit the amidolytic activity of human thrombin. Biochimie. 2006;88(9):1297-306. doi: 10.1016/j.biochi.2006.04.007.

165. De Pablo-Moreno JA, Serrano LJ, Revuelta L, Sánchez MJ, Liras A. The Vascular Endothelium and Coagulation: Homeostasis, Disease, and Treatment, with a Focus on the Von Willebrand Factor and Factors VIII and V. Int J Mol Sci. 2022;23(15). doi: 10.3390/ijms23158283.

166. Klem I, Wehinger C, Schneider B, Hartl E, Finsterer J, Stollberger C. Diabetic atrial fibrillation patients: mortality and risk for stroke or embolism during a 10-year follow-up. Diabetes Metab Res Rev. 2003;19(4):320-8. doi: 10.1002/dmrr.386.

167. Lemkes BA, Hermanides J, Devries JH, Holleman F, Meijers JC, Hoekstra JB. Hyperglycemia: a prothrombotic factor? J Thromb Haemost. 2010;8(8):1663-9. doi: 10.1111/j.1538-7836.2010.03910.x.

168. Movahed MR, Hashemzadeh M, Jamal MM. Diabetes mellitus is a strong, independent risk for atrial fibrillation and flutter in addition to other cardiovascular disease. Int J Cardiol. 2005;105(3):315-8. doi: 10.1016/j.ijcard.2005.02.050.

169. Newman JD, Schwartzbard AZ, Weintraub HS, Goldberg IJ, Berger JS. Primary Prevention of Cardiovascular Disease in Diabetes Mellitus. J Am Coll Cardiol. 2017;70(7):883-93. doi: 10.1016/j.jacc.2017.07.001.

170. Schulman S. Advantages and limitations of the new anticoagulants. J Intern Med. 2014;275(1):1-11. doi: 10.1111/joim.12138.

171. Fadraersada J, Alva-Gallegos R, Skorepa P, Musil F, Javorska L, Matousova K, et al. Head-to-head ex vivo comparison of clinically used direct anticoagulant drugs. Naunyn Schmiedebergs Arch Pharmacol. 2024;397(6):4461-70. doi: 10.1007/s00210-023-02891-x.

172. Dunois C. Laboratory Monitoring of Direct Oral Anticoagulants (DOACs). Biomedicines. 2021;9(5). doi: 10.3390/biomedicines9050445.

173. Keyl C, Zimmer E, Bek MJ, Wiessner M, Trenk D. Argatroban pharmacokinetics and pharmacodynamics in critically ill cardiac surgical patients with suspected heparin-induced thrombocytopenia. Thromb Haemost. 2016;115(6):1081-9. doi: 10.1160/TH15-11-0847.

174. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. Arch Med Sci. 2017;13(4):851-63. doi: 10.5114/aoms.2016.58928.

175. Levy JH, Szlam F, Wolberg AS, Winkler A. Clinical use of the activated partial thromboplastin time and prothrombin time for screening: a review of the literature and current guidelines for testing. Clin Lab Med. 2014;34(3):453-77. doi: 10.1016/j.cll.2014.06.005.

176. Lee G, Arepally GM. Anticoagulation techniques in apheresis: from heparin to citrate and beyond. J Clin Apher. 2012;27(3):117-25. doi: 10.1002/jca.21222.

177. Blaha V, Blaha M, Solichova D, Krcmova LK, Lanska M, Havel E, et al. Antioxidant defense system in familial hypercholesterolemia and the effects of lipoprotein apheresis. Atheroscler Suppl. 2017;30:159-65. doi: 10.1016/j.atherosclerosissup.2017.05.002.

178. Jourdi G, Siguret V, Martin AC, Golmard JL, Godier A, Samama CM, et al. Association rate constants rationalise the pharmacodynamics of apixaban and rivaroxaban. Thromb Haemost. 2015;114(1):78-86. doi: 10.1160/TH14-10-0877.

179. Rasmussen LH, Larsen TB, Graungaard T, Skjoth F, Lip GY. Primary and secondary prevention with new oral anticoagulant drugs for stroke prevention in atrial fibrillation: indirect comparison analysis. BMJ. 2012;345:e7097. doi: 10.1136/bmj.e7097.

180. Fralick M, Colacci M, Schneeweiss S, Huybrechts KF, Lin KJ, Gagne JJ. Effectiveness and Safety of Apixaban Compared With Rivaroxaban for Patients With Atrial Fibrillation in Routine Practice: A Cohort Study. Ann Intern Med. 2020;172(7):463-73. doi: 10.7326/M19-2522.

181. Hursting MJ, Verme-Gibboney CN. Risk factors for major bleeding in patients with heparin-induced thrombocytopenia treated with argatroban: a retrospective study. J Cardiovasc Pharmacol. 2008;52(6):561-6. doi: 10.1097/FJC.0b013e3181926928.

182. Huijgen R, Kastelein JJ, Meijers JC. Increased coagulation factor VIII activity in patients with familial hypercholesterolemia. Blood. 2011;118(26):6990-1. doi: 10.1182/blood-2011-10-386227.

183. Boden G, Rao AK. Effects of hyperglycemia and hyperinsulinemia on the tissue factor pathway of blood coagulation. Curr Diab Rep. 2007;7(3):223-7. doi: 10.1007/s11892-007-0035-1.

184. Weitz JI, Fredenburgh JC. Factors XI and XII as Targets for New Anticoagulants. Front Med (Lausanne). 2017;4:19. doi: 10.3389/fmed.2017.00019.

185. Xia Y, Hu Y, Tang L. Factor XIa Inhibitors as a Novel Anticoagulation Target: Recent Clinical Research Advances. Pharmaceuticals (Basel). 2023;16(6). doi: 10.3390/ph16060866.

186. Saboor M, Ayub Q, Ilyas S, Moinuddin. Platelet receptors; an instrumental of platelet physiology. Pak J Med Sci. 2013;29(3):891-6. doi: 10.12669/pjms.293.3497.

The list of candidate's papers on the topics of this dissertation and the specification of his role in these papers

P1. Fadraersada J, Alva-Gallegos R, Skořepa P, Musil F, Javorská, Matoušova K, Krčmová LK, Paclíkova M, Carazo A, Blaha V, Mladěnka P. ***Head-to-head ex vivo comparison of clinically used direct anticoagulant drugs***. Naunyn-Schmiedeberg's Arch. Pharmacol. 2024; 397(6): 4461-4470. https://doi.org/10.1007/s00210-023-02891-x [IF_{2023}=3.6, $Q2_{IF}$ and $Q3_{AIS}$ in Pharmacology & Pharmacy]

- The first author, performed the experimental research, mathematical and statistical data analysis, writing – original draft preparation, and writing – review and editing.

P2. Hrubša M, Nurjamal K, Carazo A, Nayek N, Karlíčková J, Applová L, Karmakar I, Parvin S, **Fadraersada J**, Macáková K, Mladěnka P, Brahmachari G. ***Screening of Synthetic Heterocyclic Compounds as Antiplatelet Drugs***. Med Chem 2022;18(5):536-543 [IF_{2020}= 2.7, $Q3_{IF}$ and $Q3_{AIS}$ in Chemistry, Medicinal]

- The co-author, responsible for the anticoagulation experiment and data analysis, writing – original draft preparation

P3. Sirakanyan SN, Hrubša M, Spinelli D, Dias P, Kartsev V, Carazo A, Hovakimyan AA, Pourová J, Hakobyan EK, Karlíčková J, Parvin S, **Fadraersada J**, Macáková K, Geronikaki A & Mladěnka P. ***Synthesis of 3,3-dimethyl-6-oxopyrano[3,4-c] pyridines and their antiplatelet and vasodilatory activity***. J Pharm Pharmacol 2022;74(6): 887–895 [IF_{2019}= 2.6, $Q2_{IF}$ and $Q3_{AIS}$ in Pharmacology & Pharmacy)

- The co-author, responsible for the anticoagulation experiment and data analysis, writing – original draft preparation

P4. Hrubša M, Alva-Gallegos R, Parvin MS, Macáková K, Karlíčková J, **Fadraersada J**, Konečný L, Moravcová M, Carazo A, Mladěnka P. ***Comparison of Antiplatelets Effects of Phenol Derivatives in Humans***. Biomolecules 2022;12(1):117 [IF_{2020}=4.9, $Q1_{IF}$ and $Q2_{AIS}$ in Biochemistry & Molecular Biology]

- The co-author, responsible for anticoagulation experiment and data analysis, writing – original draft preparation

P5. Parvin MS, Hrubša M, **Fadraersada J**, Carazo A, Karlíčková J, Cahlíková L, Chlebek J, Macáková K, Mladěnka P. ***Can Isoquinoline Alkaloids Affect Platelet Aggregation in Whole Human Blood?*** Toxins 2022; 14(7):491 [IF_{2021}=5.0, $Q1_{IF}$ and $Q2_{AIS}$ in Toxicology, $Q2_{IF}$ and $Q2_{AIS}$ in Food Science & Technology)

- The co-author, responsible for anticoagulation experiment and data analysis, writing – original draft preparation

Other publications of the candidate with no direct relationship to the topic of this dissertation

- Carazo A, Hrubša M, Konečný L, Gunaseelan C, **Fadraersada J**, Skořepa P, Paclíková M, Musil F, Karlíčková J, Javorská L, Matoušová K, Kujovská-Krčmová L, Šmahelová A, Blaha V & Mladěnka P. ***Correlations among different platelet aggregation pathways in a group of healthy volunteers***. Platelets 2024; 35(1): 2336093 (IF_{2023} 3.3; $Q2_{IF}$ and $Q2_{AIS}$ in Hematology)
  - The co-author, responsible for the optical aggregation experiment, writing – original draft preparation
- Tvrdý V[#], Dias P[#], Nejmanová I, Carazo A, Jirkovský E, Pourová J, **Fadraersada J**, Moravcová M, Peterlin Mašič L, Sollner Dolenc M & Mladěnka P. ***The effects of bisphenols on the cardiovascular system ex vivo and in vivo***. Chemosphere 2023;313: 137565 [IF_{2021} 8.9, $Q1_{IF}$ and $Q2_{AIS}$ in Environmental Sciences]
  - The co-author, responsible for in vivo experiment, writing – original draft preparation