

Aus dem Lehrstuhl für Klinische Chemie und Laboratoriumsmedizin

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DER FAKULTÄT FÜR MEDIZIN

DER UNIVERSITÄT REGENSBURG

Thrombin Generation by Valproate

Inaugural-Dissertation

Zur Erlangung des Doktorgrades der Medizin

der

Fakultät für Medizin

der Universität Regensburg

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2019

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Tag der mündlichen Prüfung: 04.07.2019

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1 Introduction

1.1. Thrombin

Thrombin (factor 2a = F2a = IIa) plays key roles in different biological phenomena including hemostasis, thrombosis, inflammation, and cell proliferation [1,2]. It is a very important serine protease of human blood, cleaving substrates after basic residues, mainly after arginine. Thrombin is the central enzyme of mammalian blood coagulation: thrombin converts fibrinogen to fibrin, provides a feedback amplification of coagulation via generation of F5a and F8a, and activates platelets. Thrombin is generated from its precursor prothrombin (F2), which in turn is generated from the prepro-thrombin that is synthesized in the liver [3]. The posttranslational modifications of the prepro-thrombin in the endoplasmic reticulum are critical for forming a domain rich in γ -carboxyglutamate capable of binding calcium ions (Ca^{2+}), a condition that is essential for binding negatively charged phospholipid surfaces of injured vascular tissue and activated platelets at the site of injury [4,5]. Thrombin is generated from its precursor prothrombin (F2) by cleavage of two peptide bonds of F2 by F10a [6]. Briefly, F10a binds F5a, Ca^{2+} and phospholipids (PL) to form the “pro-thrombinase complex” which activates prothrombin (F2) to thrombin (F2a) (Figure 1). The intrinsic or extrinsic F10-ase and the pro-thrombinase PL- Ca^{2+} complexes are easily formed on negatively charged phospholipids, e.g. of the outer membrane of activated platelets [7].

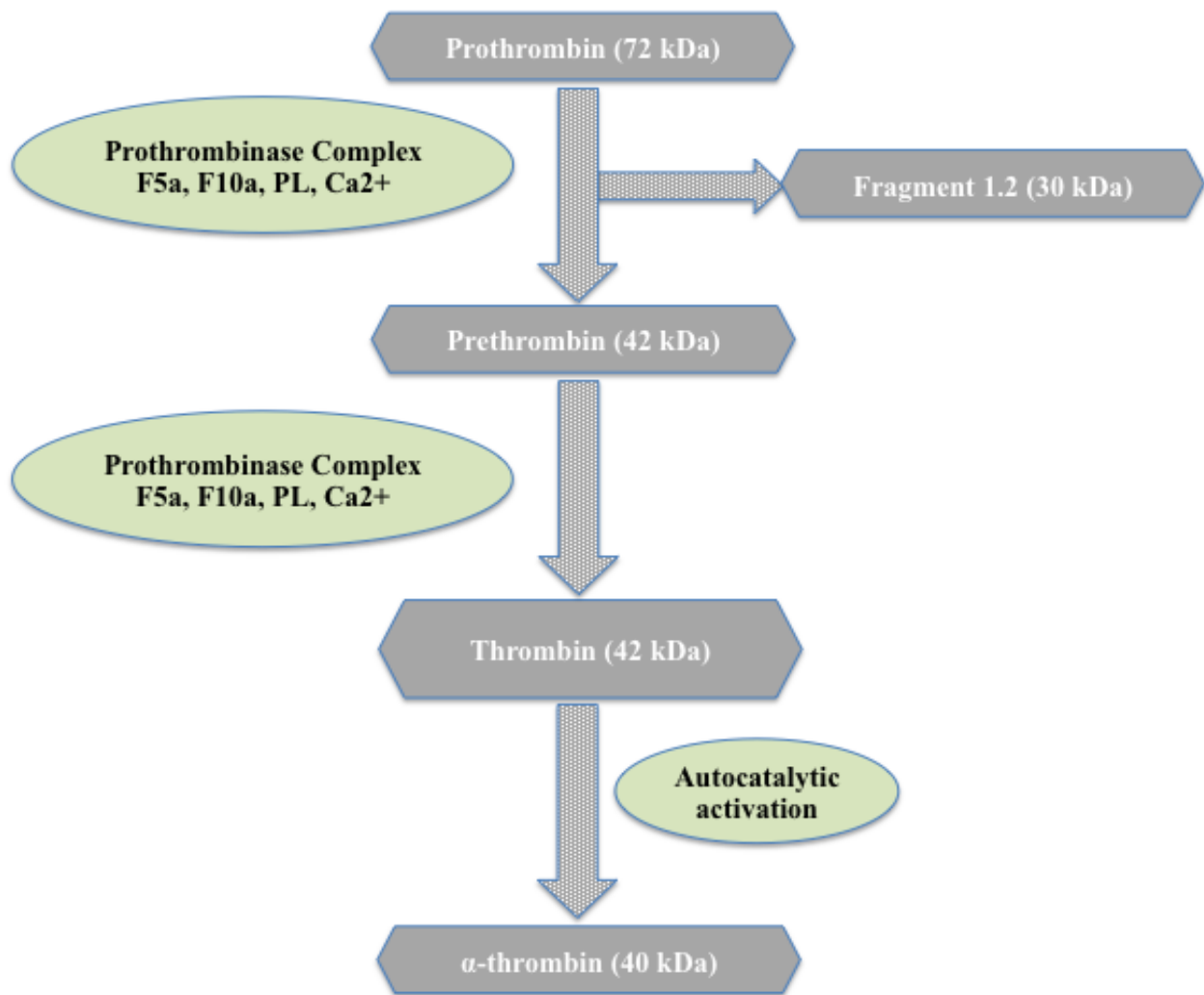


Figure 1. Activation of prothrombin. Prothrombinase complex activates prothrombin (72 kDa) to prethrombin-2a (42 kDa) which upon autocatalytic activation involving the splitting of 13 amino acid N-terminal of the A-chain generates α -thrombin (40 kDa) (adopted from Davie and Kulman [8]). Both F2a and α -thrombin are active serine proteases [8].

Negatively or delta-negatively charged groups can trigger intrinsic F2a generation, possibly by folding of F12 into F12a or pre-kallikrein (PK) into kallikrein (K) [9,10]. Briefly, upon its contact with negative charged molecules a small amount of F12 gets activated (into F12a). F12a cleaves PK into active K, which in turn reciprocally activates F12. F12a or K initiates fibrin formation through F11 activation. As such F11 is an essential factor, individuals with F11 deficiencies show variable bleeding disorders [11]. An overview of the intrinsic coagulation system is presented in Figure 2.

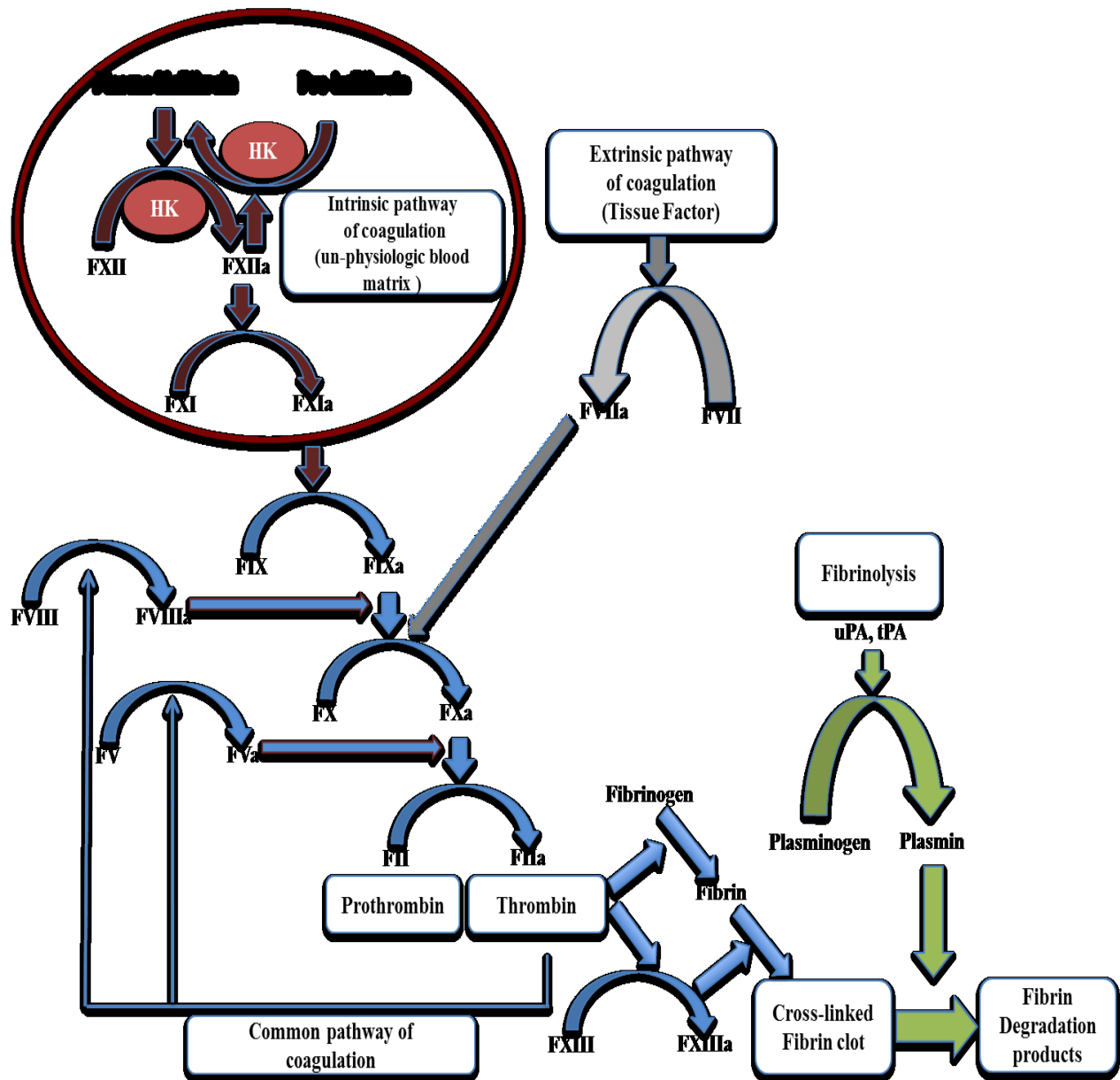


Figure 2. The hemostasis cascade. The intrinsic pathway (contact system) or the extrinsic pathway can activate the coagulation cascade. Both pathways lead to the activation of factor X and subsequently to thrombin. The factors II, VII, IX, and X are dependent on calcium ions and phospholipids. Thrombin converts fibrinogen into fibrin and activates FV, FVIII, FXIII, and platelets. FVa and FVIIIa accelerate thrombin generation. FXIIIa stabilizes the fibrin clot via cross-linking of the γ -chains. In fibrinolysis plasminogen is converted into plasmin that degrades the fibrin clot into soluble fibrin degradation products, such as D-dimer.

Abbreviations: HK: high molecular weight kininogen (a non-enzymatic pro-cofactor); F: Coagulation factor followed by a roman numeral; “a” denotes the activated form of F; uPA: urokinase (urinary plasminogen activator); tPA, tissue plasminogen activator. (Modified from Loof et al [12]).

1.2. Valproic acid

Valproic acid is a widely used drug against different conditions of seizure disorders such as against the manic episodes of bipolar disorder, against neuropathic pain, or other psychiatric illnesses [13–17]. It is also one of the most prescribed anti-epileptic drugs [18–20]. Furthermore, through its action as a histone deacetylase inhibitor or due to its pro-oxidative nature [21–25], valproic acid is also cytotoxic agent to many different cancer types [26]. Valproic acid is a branched short-chain fatty acid. As such it is subject to mitochondrial β -oxidation [27]; yet, due to the branched nature of the 5 main C-atoms, the β -oxidation cannot completely proceed to acetyl-coenzyme A which make it also a subject to enzymatic transformation by cytochrome P450 (CYP450). Several lines of evidence show that valproic acid can act as an inducer, a substrate, and an inhibitor of CYP450 [28–42]. The mechanism of valproic acid induced inhibition of CYP450 [43–45] is related to its ability to transform the enzyme into a generator of reactive oxygen species.

Different mechanisms of action can be attributed to valproate or its metabolites whereas they can act as neuronal membranes stabilizer (possibly comparable to acetone [46]), or disturb the mitochondrial hemostasis when available in toxic concentrations [47], or they could also induce the metabolic syndrome [48]. The ability of valproate to inhibit protein synthesis in hepatocytes and endothelium, results in decreased concentrations of fibrinogen or of von Willebrand factor [49–51]. It has been shown that valproate also decreases both the platelet number and the platelet aggregation [52–54]. Even though, it rarely causes serious bleeding complications, valproate may lead to atherosclerosis and thrombosis [54].

1.3. Tryptophan

Tryptophan is an essential amino acid necessary for protein synthesis (by animals) and has an important role in the production of other compounds such as nicotinic acid (vitamin) and serotonin (neuro-hormone) [55]. In this latter case tryptophan is first converted into 5-hydroxytryptophan (5-HTP), and then to serotonin [56]. As a result, the effectiveness of tryptophan for the treatment of a variety of conditions typically associated with low serotonin levels in the brain is reported [57]. For instance, tryptophan showed great promise as an antidepressant when used alone [58] and in combination with other antidepressant drugs [59]. In addition to the importance of tryptophan for the treatment of many conditions it is also widely used along with its metabolite 5-HTP as a safe and reasonably effective sleep aid [60]. As an over

the counter dietary supplement tryptophan and 5-HTP do not require prescription [60]. Tryptophan also seems to enhance platelet aggregation [61]. Tryptophan is composed of a Na^+ binding indol ($\text{C}_8\text{H}_7\text{N}$) side group [62] that is linked to the C-2 atom with the main amino (NH_2) group; the C-1 atom is the one with the main carboxylic acid (COOH) group [63]. At physiological blood pH the carboxyl group is negatively (-) and delta-negatively (δ^-) charged. Therefore, it can trigger intrinsic thrombin generation, possibly by folding of factor 12 into factor 12a (F12a) or pre-kallikrein into kallikrein [9,64]. An increase in tryptophan concentration is expected after meals, drugs, or infusions (parenteral amino acids or amino acids as stabilizer of drugs, e.g. N-acetyl-tryptophan in human albumin drugs) [65]. Consequently, an increase in the plasma concentration of tryptophan or of any other amino acid via the COO^- group or a lipophilic side chain [66] could pathologically trigger intrinsic thrombin generation [67].

1.4. Leucine

Leucine is an essential α -amino acid that also belongs to the branched chain amino acids (BCAA). In contrast to other amino acids which require hepatic gluconeogenesis to generate useful metabolic energy, BCAA are mainly found in muscle and therefore, may be used directly by skeletal or heart muscle [68]. Due to the chemical formula of leucine $\{\text{HO}_2\text{CCH}(\text{NH}_2)\text{CH}_2\text{CH}(\text{CH}_3)_2 = \text{C}_6\text{H}_{13}\text{NO}_2$ with a hydrocarbon side chain}, it is classified as a hydrophobic = lipophilic amino acid. Leucine has an isobutyl R group [69]. Since the carboxyl-group of leucine at physiological blood pH is negatively (-) and delta-negatively (δ^-) charged, it can trigger intrinsic thrombin (F2a) generation [9]. In addition, as a lipophilic amino acid leucine can also trigger thrombin generation. The chemical structure of leucine is similar to valproate (2-amino-4-methylpentanoic acid = $\text{HOOCCH}(\text{CH}_3\text{CH}_2\text{CH}_2)_2 = \text{C}_8\text{H}_{16}\text{O}_2$).

1.5. Glucose

Glucose, $\text{C}_6\text{H}_{12}\text{O}_6$ or $\text{H}-(\text{C}=\text{O})-(\text{CHOH})_5-\text{H}$, is a monosaccharide, with five hydroxyl (OH) groups and one aldehyde group along its six-carbon backbone. Interestingly, in addition to the role of glucose in health as a source of energy and in disease as in diabetes, it has been shown that it also triggers the contact phase of human blood coagulation both in vitro and in vivo [70,71]. In vitro, supplementation of plasma with glucose resulted in increased generation of F12a and of F2a [72–74]. Therefore, increased glucose levels can affect blood coagulation.

2 Aims of the Study

Valproate is a negatively charged and lipophilic drug; therefore, it might pathophysiologically trigger the intrinsic phase of blood coagulation, which could result in thrombosis/atherosclerosis. Consequently, the present study aimed to quantify thrombin generation in normal citrated plasmas supplemented with valproate, using the ultra-specific, ultra-sensitive thrombin generation assay of the newest generation RECA (recalcified coagulation activity assay) [67,75,76]. Additionally, the action of other substances that are lipophilic and/or (delta) negatively charged at physiological pH such as valproate, tryptophan, leucine, or glucose on intrinsic thrombin generation was quantified. All substances were tested at concentrations expected in patients following the specified treatment or in diabetes mellitus. The chemical structures of the aforementioned compounds are presented in Figure 3.

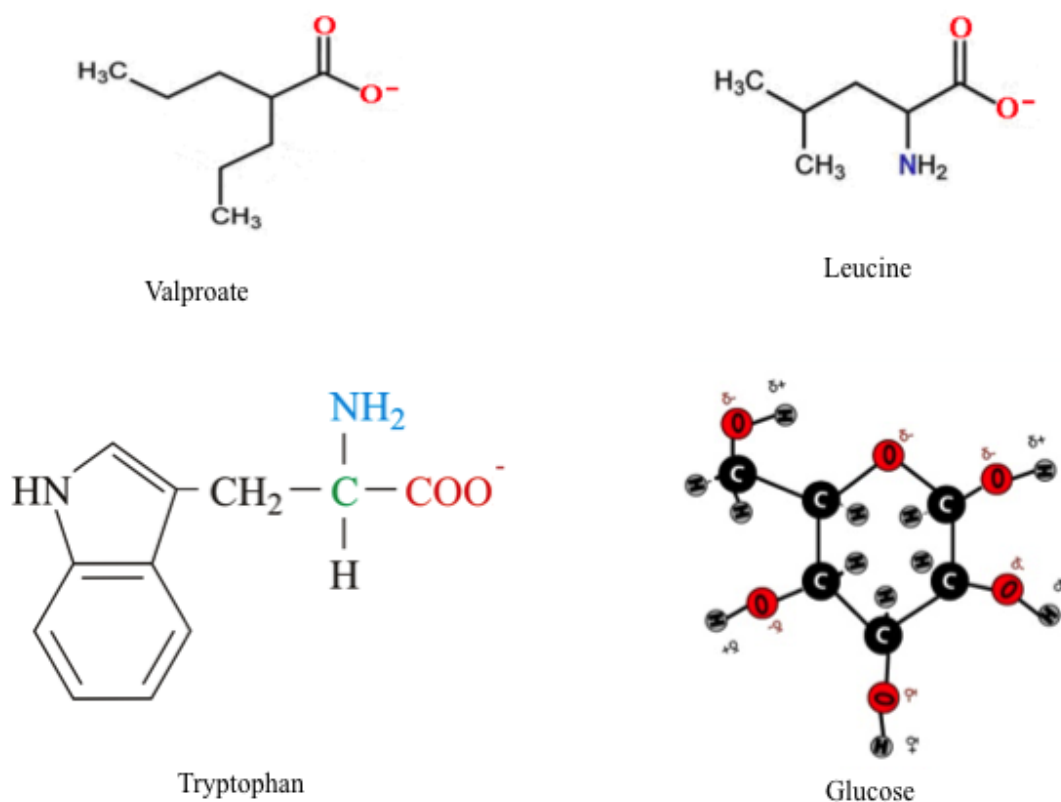


Figure 3. Chemical structures of valproate, leucine, tryptophan, and glucose

3 Materials and methods

3.1. Plasmas

Unfrozen normal platelet poor citrated plasma (1 part 106 mM sodium citrate + 9 parts venous blood; polypropylene monovettes from Sarstedt, Nümbrecht, Germany; centrifugation at 2800g for 10 min at 23°C; about 0.5-4h old, stored at 23°C) was from healthy individuals who gave written informed consent.

Additionally, pools in emptied polypropylene monovettes of this unfrozen normal plasma, consisting of 5-20 individual samples were used. In all the experiments 50 µl samples of plasma were supplemented with the different substances by immediate repetitive 1+1 dilution on high quality polystyrene U-wells microtiter plates (Brand, Wertheim, Germany; article nr. 701300 or 781600).

3.1.1. Valproate – Supplemented Plasmas

Normal plasma was from 235 individuals (freshest: < 1h 23°C old, fresh: 1-2h 23°C old, or 2-4h 23°C old), and 31 pools out of them. 50 µl of these samples were supplemented with 0-245 mg/l, 0-490 mg/l, or 0-1961 mg/l (final conc.) sodium valproate (Orfiril® 100 mg/ml vials; Desitin, Hamburg, Germany; 100 mg sodium valproate = 86.76 mg valproic acid).

Lyophilized and reconstituted commercial plasmas depleted in factors 12, 11, 9, 8, 10, 5, 2, or 7 or 100 % normal control plasma N® were from DadeBehring, Marburg, Germany. Other lyophilized plasmas depleted in factors 5 or 7 were from Instrumentation Laboratory (IL, Lexington, USA). 50 µl of these samples were supplemented with 0-245 mg/l, 0-490 mg/l, or 0-1961 mg/l (final conc.) sodium valproate (Orfiril® 100 mg/ml vials; Desitin, Hamburg, Germany; 100 mg sodium valproate = 86.76 mg valproic acid) by immediate repetitive 1+1 dilution on high quality polystyrene U-wells microtiter plates.

3.1.2. Tryptophan – Supplemented Normal Plasmas

Plasmas of 25 individual (about 4h 23°C old) and 3 different pools out of them were used. 50 µl of these samples were supplemented with 0-4.5 mM additional tryptophan ($C_{11}H_{12}N_2O_2$; MW = 204.23; normal plasma concentration = 15-65 µM).

3.1.3. Leucine – Supplemented Normal Plasmas

Plasmas of 19 individual (about 4h 23°C old) and 2 different pools out of them were used. 50 µl of these samples were supplemented with 0-9 mM additional leucine ($C_6H_{13}NO_2$; MW = 131.17; normal plasma concentration = about 0.1-0.2 mM).

3.1.4. Heparin – Supplemented Normal Plasmas

50 µl samples of 7 individual normal plasmas and one pooled normal plasma out of them were supplemented with 3.8 mIU/ml unfractionated heparin (UFH; Sarstedt), a poly-negatively charged molecule. 5 individual unfrozen normal plasmas and one pooled normal plasma out of them were supplemented with 0.5 IU/ml of the low-molecular-weight-heparin (LMWH) enoxaparin (Clexane®, Sanofi-Aventis, Frankfurt, Germany).

3.2. The recalcified coagulation activity assay (RECA)

50 µl unfrozen normal platelet poor plasma samples in U-wells high quality polystyrene microwells were supplemented by 1+1 dilutions on the plate with the different substances. 5 µl 250 mM $CaCl_2$ out of 4 ml frozen/thawed aliquots in siliconized glass (DadeBehring, Marburg, Germany; article nr. OVKE49) were added by an Eppendorf®multipipette with 0.9% NaCl-rinsed and completely emptied new disposable polypropylene-tips, and the plates were intensely shaken for 5s. After coagulation reaction times (CRT) of 0-30 min at 37°C (in a digitally controlled water bath) 100 µl arginine-reagent (2.5 M arginine, 0.16 % Triton X 100® (polyoxyethylene octyl phenyl ether), pH 8.6; Sigma, Deisenhofen, Germany) were added to stop thrombin generation and to depolymerize nascent un-polymerized fibrin. Thrombin generation = thrombin activity at a certain CRT minus basal plasmatic thrombin activity (at 0 min CRT). After 3 min (23°C) the plasmatic turbidity at 405 nm was determined by a microtiter plate photometer with a 1 mA resolution (Milenia; DPC, Los Angeles, USA). 25 µl aliquoted frozen/thawed 1 mM chromogenic thrombin substrate HD-CHG-Ala-Arg-pNA (Pentapharm, Basel, Switzerland; 100 mg article nr. 081-03) in 1.25 M arginine, pH 8.7 (CS) were added.

The $\Delta A_{405nm}/t$ was determined. The RECA was calibrated with 0.1 IU/ml purified bovine thrombin (DadeBehring) in 6 % human albumin (DadeBehring) that replaced the plasma sample and generated a specific $\Delta A_{405nm}/t$ of about 3 mA/min (37°C). The maximal ΔA_{405nm} with this lot of chromogenic substrate was about 1000 mA; 40 % = 400 mA (= upper limit of linear range).

Thrombin generation = thrombin activity generated in CRT minus basal thrombin activity in citrated plasma at 0 min CRT (addition of arginine reagent prior to addition of Ca^{2+} = 0.008 ± 0.002 IU/ml thrombin). The approximate 200 % stimulatory concentration (approx. SC200) of valproate on thrombin generation was determined in the ascending part of the incubation time vs. thrombin generation curve. Valid are only thrombin activities in this part of curve (F2a activity at time point 1 divided by F2a activity at time point 2 should be less than 1), i.e. where there are no significant amounts of nascent fibrin that entraps nascent thrombin. With supra-molar concentrations of arginine these thrombin generation assays of the newest generation get rid of the problems concerning nascent fibrin (= antithrombin-1) or kallikrein [77].

3.3. In vivo experiments

3.3.1. Action of glucose or valproate on systemic thrombin generation in mice

3.3.1.1. Optimized concentration of EDTA

260 μl or 130 μl 0.9 % NaCl of drug quality were added to 2.6 ml polypropylene monovettes which contain $2.6 \times 1.6 = 4.16$ mg sterile $\text{K}_3\text{-EDTA}$ (ethylene diamine tetra acetic acid, tripotassium salt from Sarstedt, Nümbrecht, Germany), resulting a $\text{K}_3\text{-EDTA}$ solution of 16 mg/ml or 32 mg/ml, respectively. Then 50 μl of this $\text{K}_3\text{-EDTA}$ -solution was given to a polypropylene syringe and 300 μl of murine blood were added. The final $\text{K}_3\text{-EDTA}$ -concentrations in murine blood were 2.3 or 4.6 mg/ml, respectively. The high EDTA concentration does not disturb the systemic F2a test but improves anticoagulation in complicated blood drawings.

3.3.1.2. Mice

Males and females Balb/c mice, 6-8 weeks old (average weight 25g), were obtained from Harlan Winkelmann (Borchen, Germany). Mice were housed under optimal conditions (6 mice per cage) in a 12/12 hour light/dark cycle with food and water available *ad libitum*. Mice were randomly distributed into treatment and control groups. Mice (n=2 or 3) received intraperitoneally (i.p.) different amounts of glucose or valproate, for control they received 0.9% NaCl. All experimental procedures were approved by the local animal ethics committee and met German and international guidelines.

3.3.1.3. Valproate intraperitoneal (i.p.) injection

Valproate: (Orfiril® 100 mg/ml vials; Desitin, Hamburg, Germany; 100 mg sodium valproate = 86.76 mg valproic acid), 100 μl (0 mg/ml, 20 mg/ml, or 100 mg/ml) were given i.p. to the mice

(each dosage for 2 mice), i.e. 0 mg, 2 mg, or 10 mg valproate equivalent to about 0, 60, or 300 mg/kg body weight. 1h after i.p. injection the murine blood was drawn by vena cava puncture as described above with K₃-EDTA in the syringe, resulting in final blood concentrations of 2.3 mg/ml K₃-EDTA. The experiment was repeated with 0, 0.05, 0.1, 0.2, or 0.4 mg valproate injected i.p. (each dosage for 2 mice).

3.3.1.4. Glucose intraperitoneal (i.p.) injection

300 µl of 0-1 M glucose (final amount was 0-0.3 mmoles glucose) were given i.p. to the mice (each dosage for 2 mice). Blood was withdrawn from the mice at different time points (0, 5, 30, 45, 60, 90, or 180 minutes) after i.p. injection, using vena cava puncture to avoid coagulation activation.

3.3.1.5. Assay for systemic F2a in murine blood induced by glucose

The freshly drawn 4.6 mg/ml EDTA-blood of treated or untreated (100% control) mice (usually n=3 for each glucose amount) in 1 ml polypropylene cups was centrifuged for 8 min at 3500 rpm (2200 g; 23°C). After centrifugation, 35 µl of the EDTA-plasma were mixed with 35 µl 2.6 M arginine (pH 8.6) in polystyrene half-area wells (Greiner, Frickenhausen, Germany; article nr. 675101). 35 µl of 0 mM (turbidity control) or 1 mM (main reaction) chromogenic thrombin substrate CHG-Ala-Arg-pNA in 1.25 M arginine (pH 8.7) were added. The increase in absorbance measured at 405 nm ($\Delta A_{405\text{nm}}/t$) was determined at each time point at 37°C by a microtiter plate photometer with a 1 mA resolution (Tecan Sunrise, Crailsheim, Germany). The $\Delta A/t$ values of the turbidity control were subtracted from the $\Delta A/t$ values of the main chromogenic reaction.

3.3.1.6. Assay for systemic F2a in murine blood induced by valproate

Blood from treated and control mice in 1 ml polypropylene cups was centrifuged for 8 min at 3500 rpm (2200 g; 23°C). 50 µl samples of this EDTA-plasma were mixed with 50 µl 2.5 M arginine, pH 8.6 in high quality polystyrene U-wells microtiter plate. Then 25 µl 0 mM (turbidity control reaction) or 2 mM CHG-Ala-Arg-pNA (main reaction; chromogenic thrombin substrate from Pentapharm, Basel, Switzerland) in H₂O were added and $\Delta A_{405\text{nm}}/t$ (37°C) was determined. The $\Delta A/t$ values of the turbidity control reaction were subtracted from the $\Delta A/t$ values of the main chromogenic reaction. 100 µl of the 100 % human standard = pooled normal EDTA-plasma of n=48 healthy donors after written informed consent, stabilized 1+1 with 2.5 M arginine,

pH 8.6, were incubated identically as the mice samples in the chromogenic reaction. The 100 % human standard had a $\Delta A/t$ of 70 mA/h (37°C).

3.3.1.7. Determination of glucose concentrations in mice plasma

Glucose in EDTA-plasma of mice was determined enzymatically, using the D Glucose/ D-Fructose determination kit from Boehringer Mannheim-R-Biopharm Roche (article nr. 10 139 106 035). The principle of the method is hexokinase-mediated phosphorylation of glucose to glucose-6-phosphat (G-6-P), followed by G-6-P dehydrogenase – mediated oxidation of G-6-P into gluconate-6-phosphate and generation of NADPH, that is monitored at 340 nm. 5 μ l EDTA-plasma were incubated in polystyrene F-wells (NUNC, Wiesbaden, Germany; article nr. 446140) with 100 μ l reagent 1 (64 mg NADP, 160 mg ATP, magnesium sulphate, triethanolamine, pH 7.6 in 27 ml H₂O), 2 μ l reagent 2 (\approx 200 U hexokinase, \approx 100 U G-6-P dehydrogenase in 0.7 ml) and 100 μ l H₂O for 15 min at 23°C. The specific increase of absorbance at 340 nm, determined by a microtiter plate photometer (Tecan Sunrise), was approximately 200 mA for the 0.5 g/l (50 mg/dl = 2.78 mM) glucose standard.

3.3.1.8. Determination of valproate concentration in mice blood

Mice were i.p. injected with 100 μ l 0.5 mg/ml (0.05 mg) or 10 mg/ml (1 mg) valproate in 0.9 % NaCl of drug quality. After 1h EDTA-blood was withdrawn as described above, centrifuged (2200 g; 23°C), and 50 μ l EDTA-plasma were analyzed 1+2 diluted with 0.9 % NaCl for the plasmatic valproic acid-concentration by a DXC-800 SN 1202 analyzer (Beckman, Krefeld, Germany). The principle of the method was kinetic interaction of microparticles in solution (KIMS), i.e. immobilized turbidity-causing valproate competes with clear plasmatic valproate for added antibodies. The resulting turbidity is indirectly proportional to the plasmatic valproate concentration: minimal therapeutic concentrations are 50-100 mg/l.

3.4. Data analysis

Thrombin generation is defined as thrombin activity generated in coagulation reaction time (CRT) minus basal plasmatic thrombin activity at 0 min CRT. 100% thrombin generation was defined as the thrombin generation in un-supplemented plasma. The approximate 200 % stimulatory concentration (approx. SC200) or the 50% inhibitory concentration (approx. IC50) of valproate on thrombin generation was determined in the ascending part of the time vs. thrombin generation curve. Only thrombin activities in this part of curve (F2a activity at time point 1 divided by F2a activity at time point 2 < 1) are valid, i.e. where there are no significant amounts of nascent fibrin that entraps nascent thrombin. With supra-molar concentrations of arginine these thrombin generation assays of the newest generation get rid of the problems concerning nascent fibrin, which is equivalent in effect to antithrombin-1 or kallikrein.

Mean values (MV) and standard deviations (SD) of duplicate determinations and the intra-assay coefficients of variation (CV) were calculated: $(SD)/(MV) \times 100\%$. The approx. SC200 values were reported as $MV \pm 1 \text{ SD}$.

4 Results

4.1. Thrombin generation by valproate

The effect of valproate on thrombin generation was tested in 1) 235 normal citrated plasma samples including (freshest: < 1h 23°C old, fresh: 1-2h 23°C old, or 2-4h 23°C old), 2) 31 normal plasma pools, and 3) lyophilized and reconstituted commercial citrated plasmas depleted in factors 12, 11, 9, 8, 10, 5, 2, or 7 or 100 % normal control plasma.

4.1.1. Thrombin generation in freshest plasmas by valproate

Freshest plasmas (about 0.5h old) from 10 individuals were supplemented with up to 490 mg/l sodium valproate. Nine of them had an approx. SC200 of 26 ± 22 mg/l (MV \pm 1 SD; range: 4-65 mg/l) valproate, while one individual freshest plasma had an approx. SC200 of 230 mg/l valproate (Table 1). The mean thrombin generations of these freshest plasmas gave an approx. SC200 of 10 mg/l valproate (Figure 4). Pooled normal plasma (out of these 10 freshest plasmas) had an approx. SC200 of 5 mg/l (Figure 5).

Table 1. Approx. SC200 in 10 freshest individual normal plasmas

Normal Plasma	SC200 [mg/l]
1	40
2	230
3	50
4	10
5	7
6	15
7	10
8	65
9	30
10	4

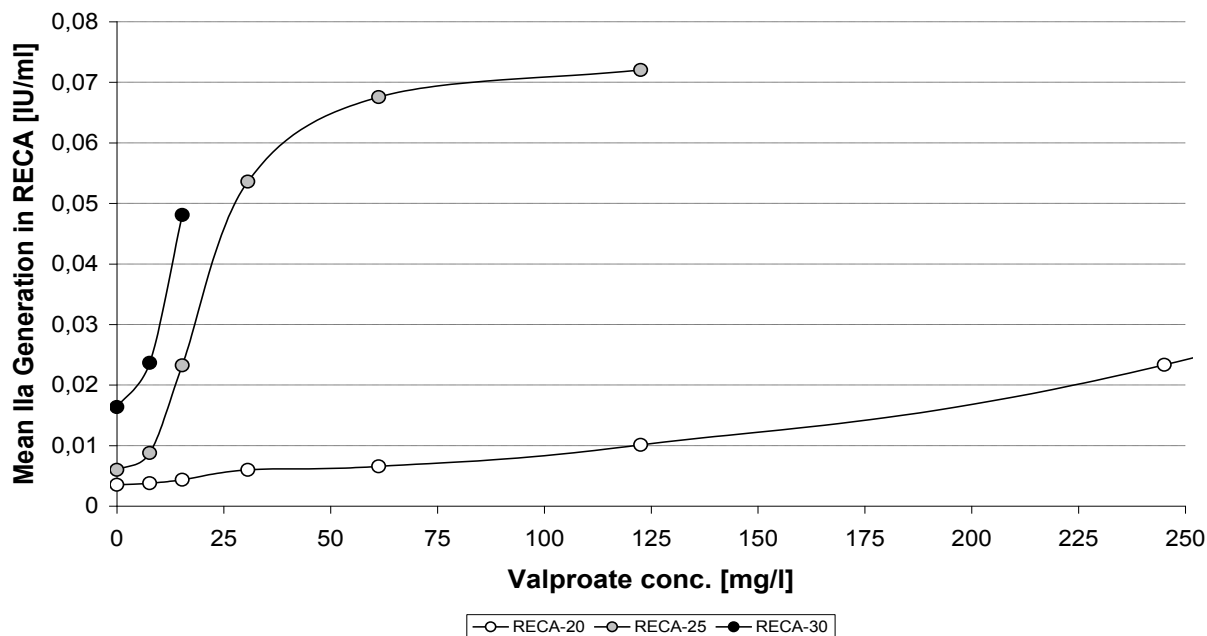


Figure 4. Mean F2a generation in dependence of valproate conc. in **freshest normal plasma**. The mean IU/ml values of freshest individual normal plasmas 1-10 (about 0.5h 23°C old) were calculated. The approx. SC200 was **10 mg/l**, determined in RECA-25 (25 min CRT) and RECA-30 (30 min CRT), being here more sensitive than the RECA-20 values.

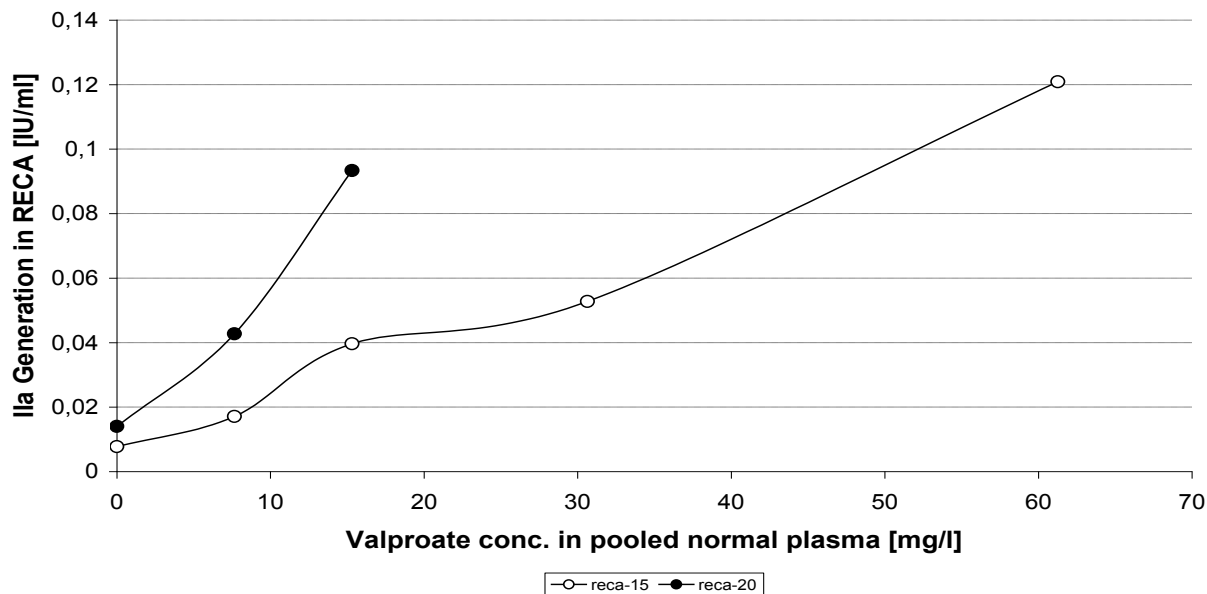


Figure 5. F2a generation in dependence of valproate conc. in **pooled normal plasma**. 50µl freshest pooled normal plasma (about 0.5h 23°C old) were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 15 or 20 min (RECA-15 or RECA-20). The approx. SC200 was 5 mg/l.

4.1.2. Thrombin generation in fresh plasmas by valproate

A total of 59 fresh plasmas obtained from healthy donors were randomly divided into 4 different sets (Set A (14), Set B (18), Set C (18), and Set D (9)) to measure thrombin generation in response to different plasma concentration - ranges of valproate.

Set A: 14 individual fresh plasmas were used to measure thrombin generation by valproate (0-245 mg/l, representing the typical approximate range of valproate in treated patients). 13 out of 14 (93%), the values of which are < 100 mg/l, had an approx. SC200 of 27 ± 23 mg/l (MV ± 1 SD; range: 2-70 mg/l) valproate. One of the 14 plasmas (7%) had an approx. SC200 of 120 mg/l valproate. The 14 individual fresh plasmas had an approx. SC200 of 33 ± 33 mg/l (MV ± 1 SD; range: 2-120 mg/l) valproate (Figure 6, Table 2). The initial trigger concentration in pooled normal plasma (out of these 14 individual plasmas) was 30 mg/l valproate (Figure 7).

Table 2. Approx. SC200 in 14 fresh individual normal plasmas

Normal Plasma	SC200 [mg/l]
1	40
2	70
3	120
4	25
5	2
6	4
7	60
8	50
9	35
10	8
11	15
12	6
13	2
14	30

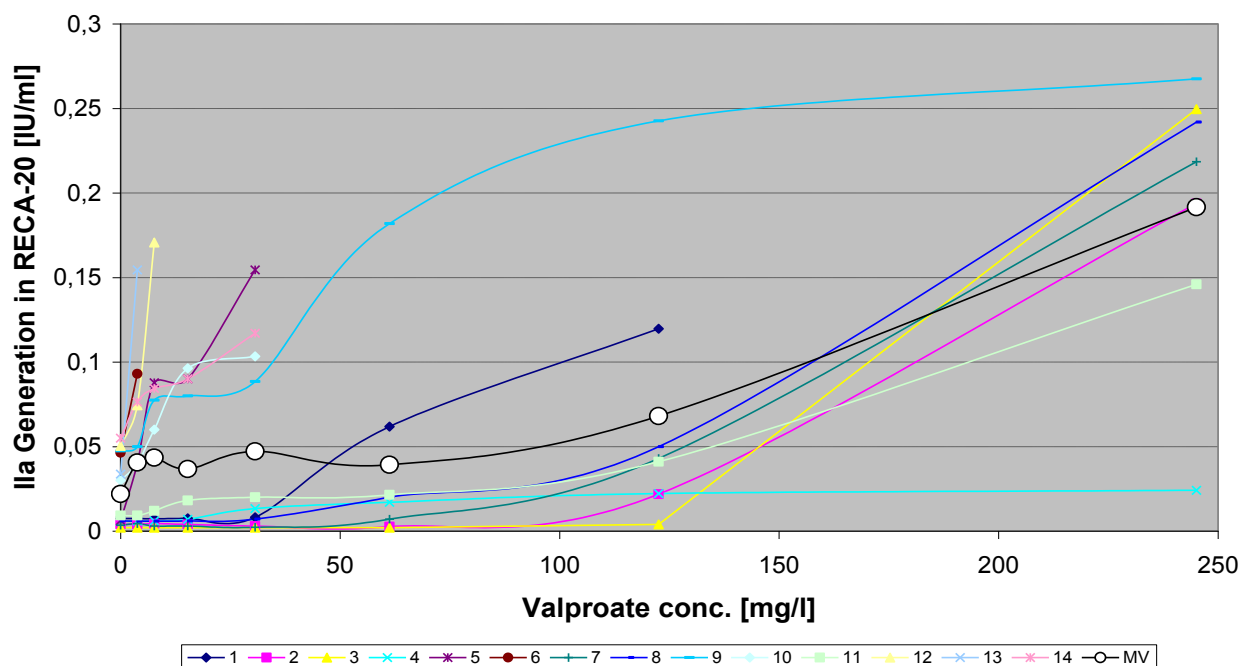


Figure 6. F2a generation in dependence of valproate conc. in **fresh normal plasmas** 50 μ l fresh individual normal plasmas 1-14 were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min (RECA-20). For approx. SC200 see table 2.

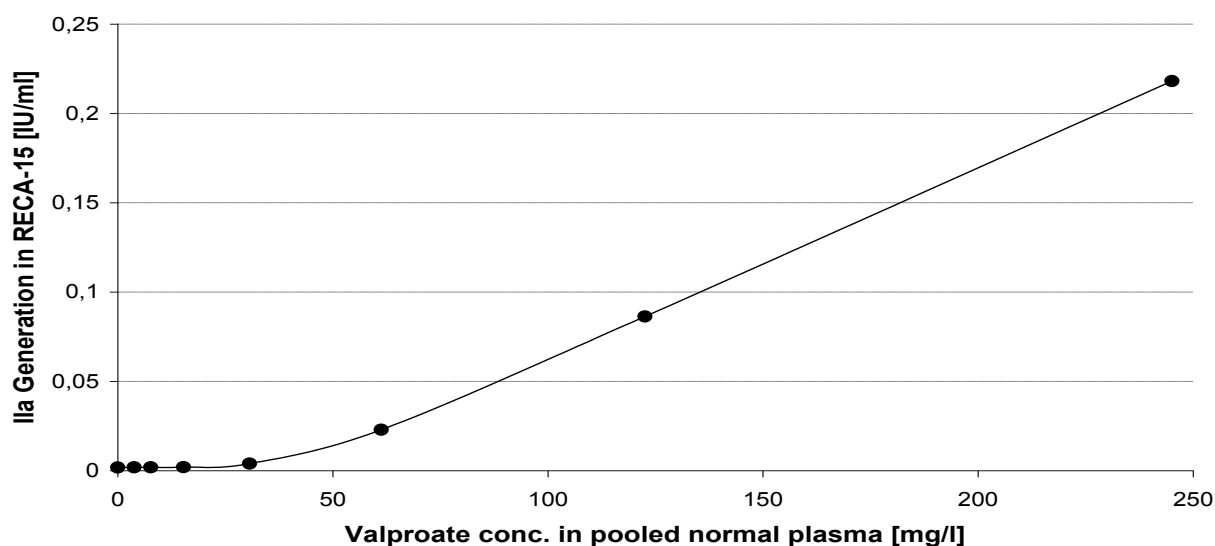


Figure 7. F2a generation in dependence of valproate conc. in **pooled normal plasma** 50 μ l pooled normal plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 15 min (RECA-15); approx. initial trigger concentration = **30 mg/l valproate**.

Set B: 18 individual fresh plasmas were supplemented with sodium valproate up to 245 mg/l. Similarly to Set A, 15 samples out of the 18 (83%) had an approx. SC200 of 35 ± 24 mg/l (MV \pm 1 SD; range: 3-70 mg/l). Three of the 18 plasmas (17%) had approx. SC200 values resistant ≥ 100 mg/l (Figure 8, Table 3). The approx. SC200 values of the 18 plasmas were 48 ± 38 mg/l (MV \pm 1 SD; range 3-120 mg/l). The mean thrombin generations of these fresh plasmas gave an approx. SC200 of 15 mg/l valproate [expressed in IU/ml thrombin (Figure 8) and in % of un-supplemented control sample (= 100 %) (Figure 9)], the approx. SC200 was **17 mg/l**. Pooled normal plasma (out of these 18 individual ones) had an approx. SC200 of 15 mg/l (Figure 10).

Table 3. Approx. SC200 in 18 fresh individual plasmas

Normal Plasma	SC200 [mg/l]
1	10
2	40
3	60
4	5
5	65
6	15
7	120
8	12
9	4
10	44
11	50
12	30
13	120
14	60
15	50
16	70
17	100
18	3

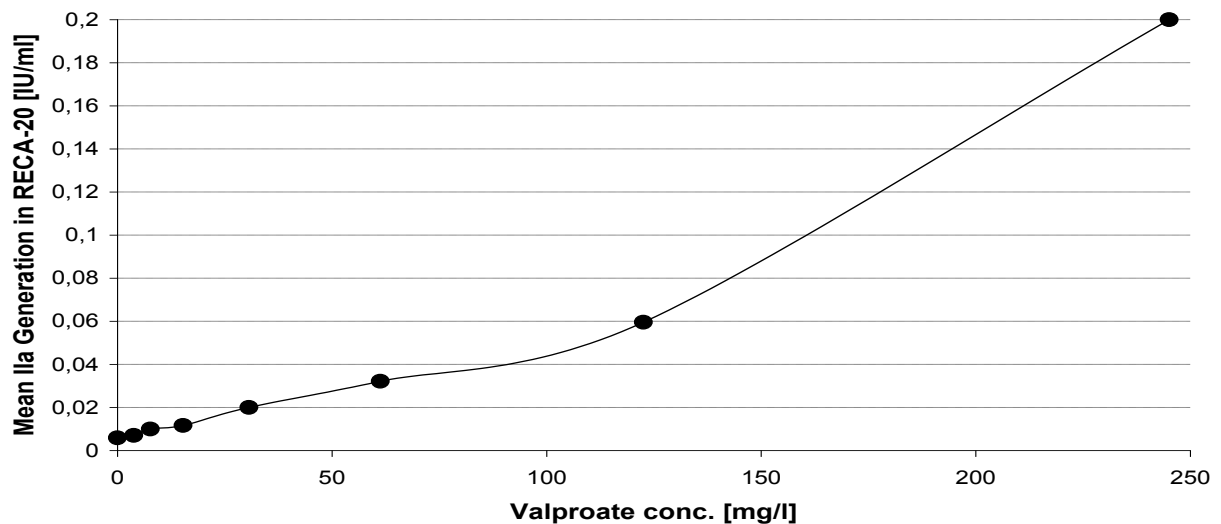
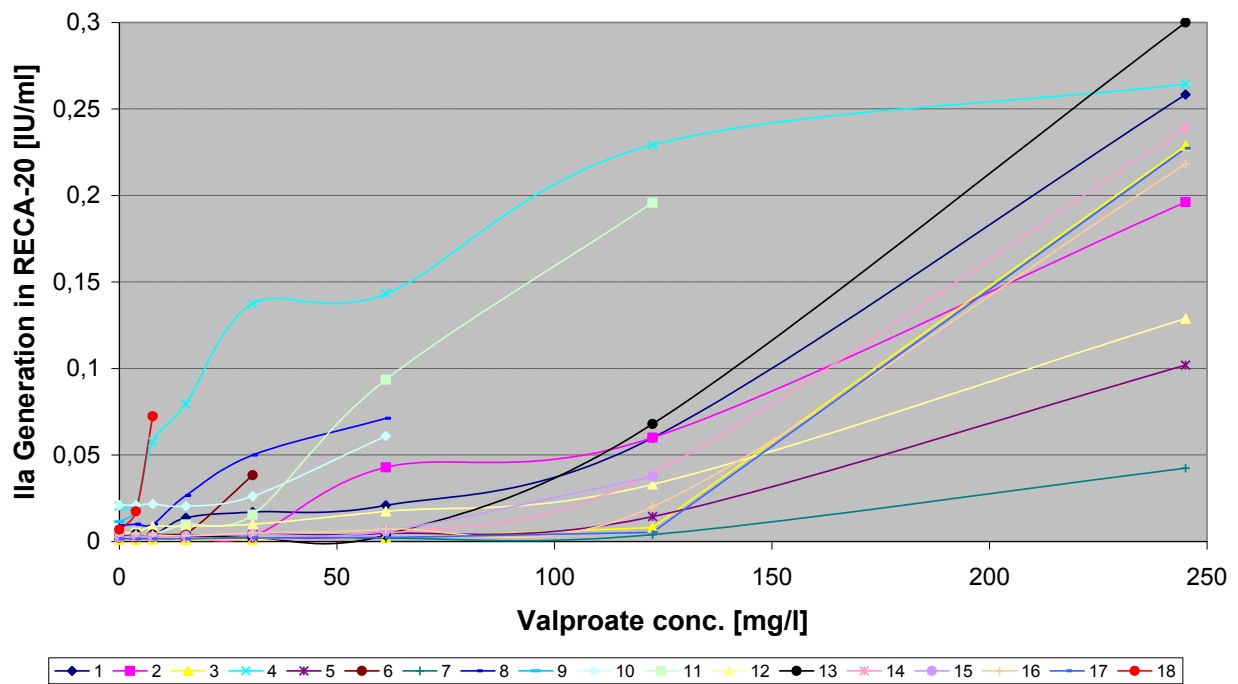


Figure 8. F2a generation in dependence of valproate conc. in **fresh normal plasmas** 50 μ l fresh individual normal plasmas 1-18 were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min (RECA-20). Figure 8a: Individual plasmas, for approx. SC200 see table 3; Figure 8b: **Mean F2a** generations of 18 individual plasmas; the approx. SC200 was **15 mg/l valproate**.

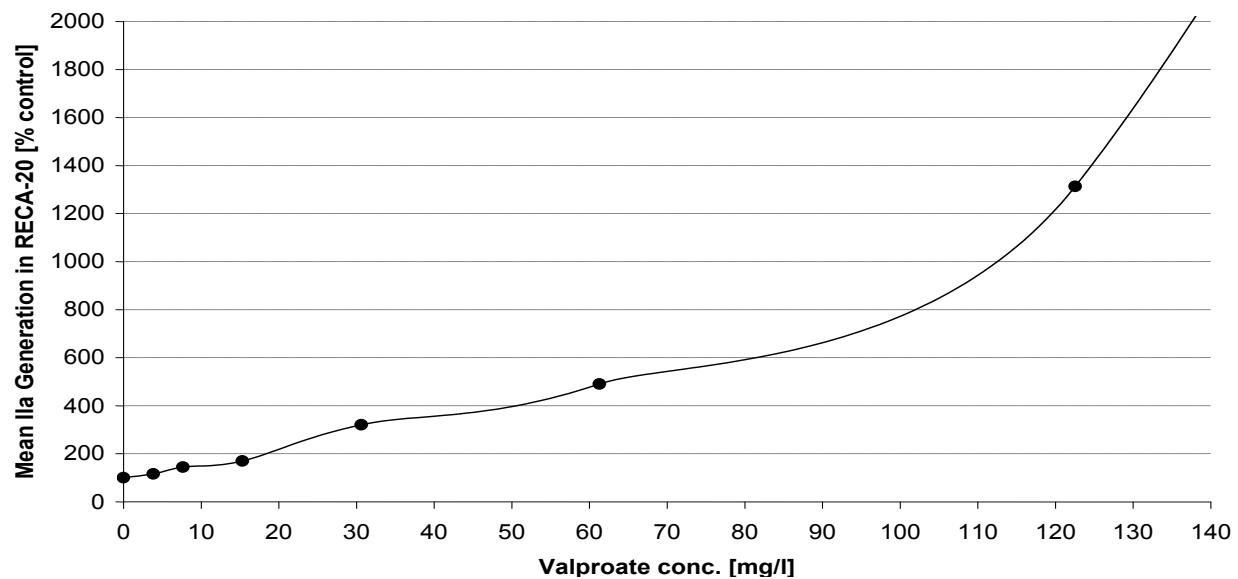
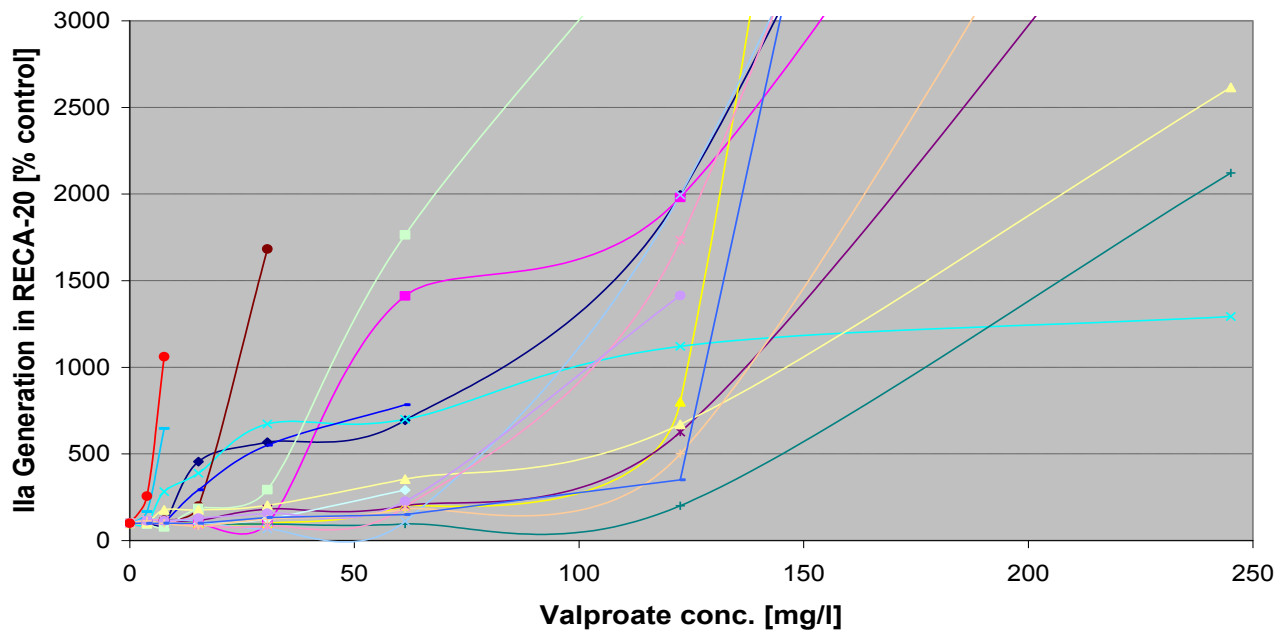


Figure 9. F2a generation in dependence of valproate conc. in fresh normal plasmas. The F2a generations were calculated in % of un-supplemented control. 50 μ l fresh normal plasmas 1-18 were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min (RECA-20). Figure 9a: Individual plasmas, Figure 9b: **Mean value** [% control] of individual plasmas, the approx. SC200 was **17 mg/l valproate**.

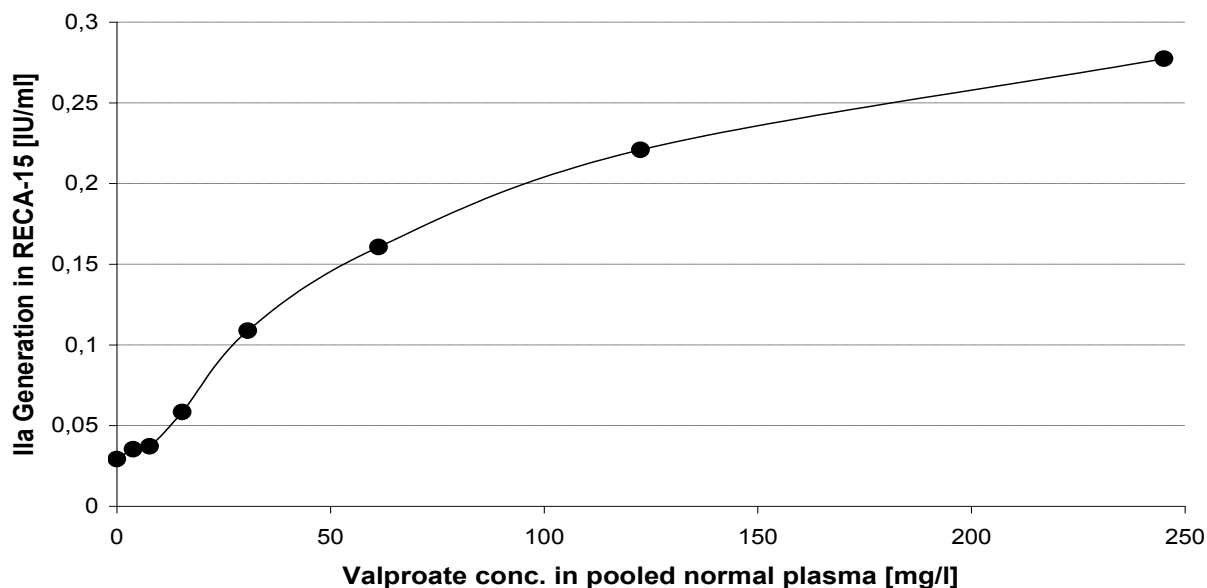


Figure 10. F2a generation in dependence of valproate conc. in pooled fresh normal plasma
 50 µl pooled normal plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was **15 mg/l valproate**.

- Set C: 18 fresh normal plasmas were supplemented with up to 1961 mg/l valproate. 12 of 18 (67%) had SC200 values < 100 mg/l [approx. SC200 of 26 ± 23 mg/l (MV \pm 1 SD); range: 5-60 mg/l. The mean thrombin generations of these fresh plasmas gave an approx. SC200 of 15 mg/l valproate (Figure 11, Table 4).

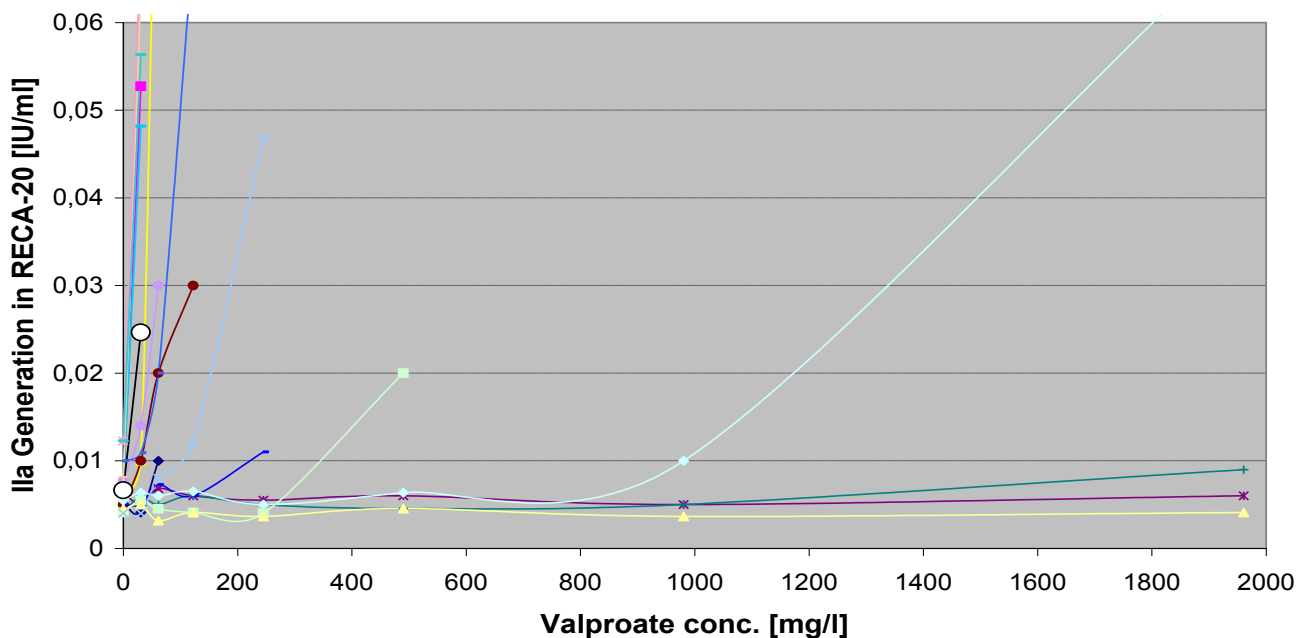


Figure 11. F2a generation in dependence of valproate conc. in fresh normal plasma 50 μ l fresh individual normal plasma 1-18 (about 2h 23°C old) were supplemented in U-microwells (Brand®701300) with 0-1961 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min (RECA-20). Each individual plasma has an individual symbol; the approx. SC200 of the mean thrombin generations (O) was 15 mg/l, the approx. SC200 values of the 18 plasmas are listed in table 4.

Table 4. Approx. SC200 in individual plasmas supplemented up to 1961 mg/l valproate

Normal Plasma	SC200 [mg/l]	Normal Plasma	SC200 [mg/l]
1	60	10	1000
2	5	11	300
3	30	12	Resistant
4	5	13	60
5	Resistant	14	7
6	30	15	30
7	1800	16	5
8	200	17	60
9	6	18	15

- Set D: 9 fresh normal plasmas were supplemented with 0-245 mg/l sodium valproate. For 67 % of samples (6 out of 9), the respective approx. SC200 values were 42 ± 43 mg/l (MV \pm 1 SD; range 10-120 mg/l) valproate. Excluding the values ≥ 100 mg/l, the approx. SC200 was 26 ± 21 mg/l valproate; range: 10-60 mg/l, while the mean thrombin generations of these plasmas gave an approx. SC200 of 15 mg/l valproate (Figure 12, table 5). Pooled normal plasma (out of these 9 samples) had an approx. SC200 of 15 mg/l (Figure 13).

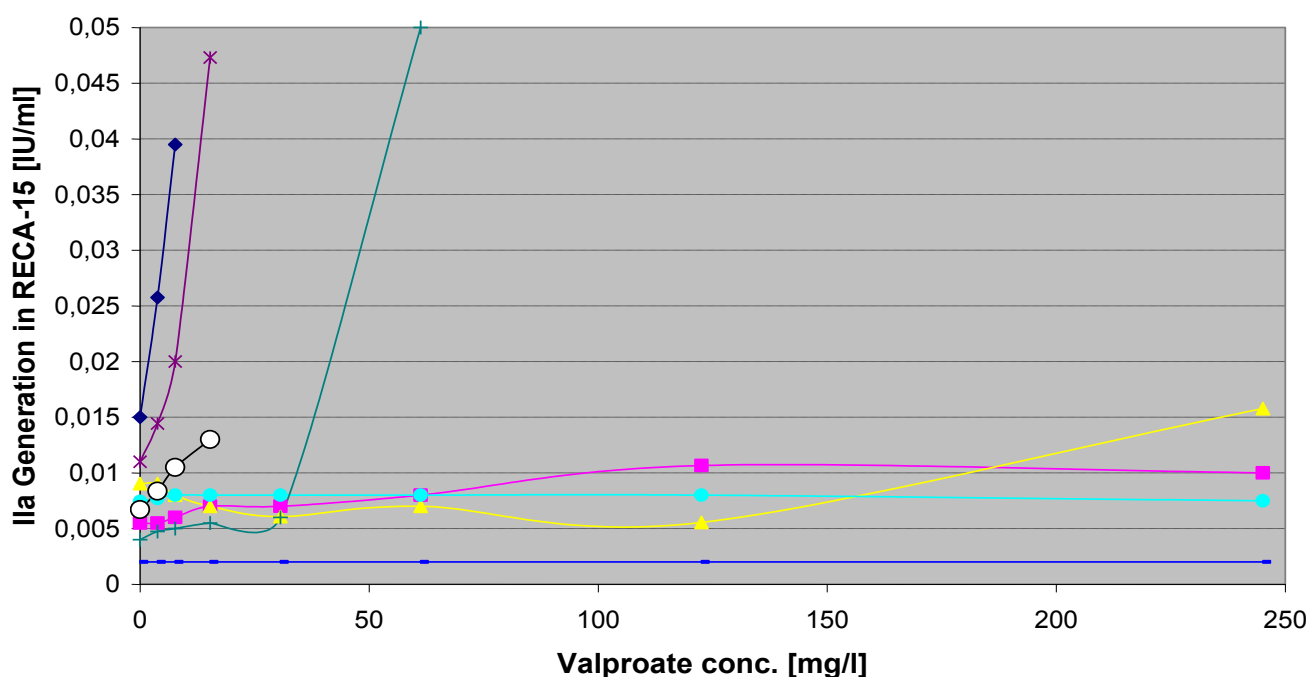


Figure 12. F2a generation in dependence of valproate concentration in normal plasma 50 μ l individual normal plasma 1-9 of healthy plasma donors (about 2-4h 23°C old) were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10 min (RECA-10; Figure 9a) or 20 min (RECA-20; Figure 9b). Each individual plasma has an individual symbol; the approx. SC200 of the mean thrombin generations (O) was 20 mg/l valproate in RECA-10 and 15 mg/l valproate in RECA-20. Since the most sensible value is the valid one, 15 mg/l valproate is the approx. SC200 of the mean F2a generations. The approx. SC200 values of the 9 plasmas are listed in table 5.

Table 5. Approx. SC200 in individual plasmas of healthy plasma donors

Normal Plasma	RECA-10 SC200 [mg/l]	RECA-15 SC200 [mg/l]
1	10	5
2	120	120
3	Resistant	Resistant
4	Resistant	Resistant
5	20	8
6	10	10
7	30	40
8	Resistant	Resistant
9	60	50
MV	42	39
SD	43	44

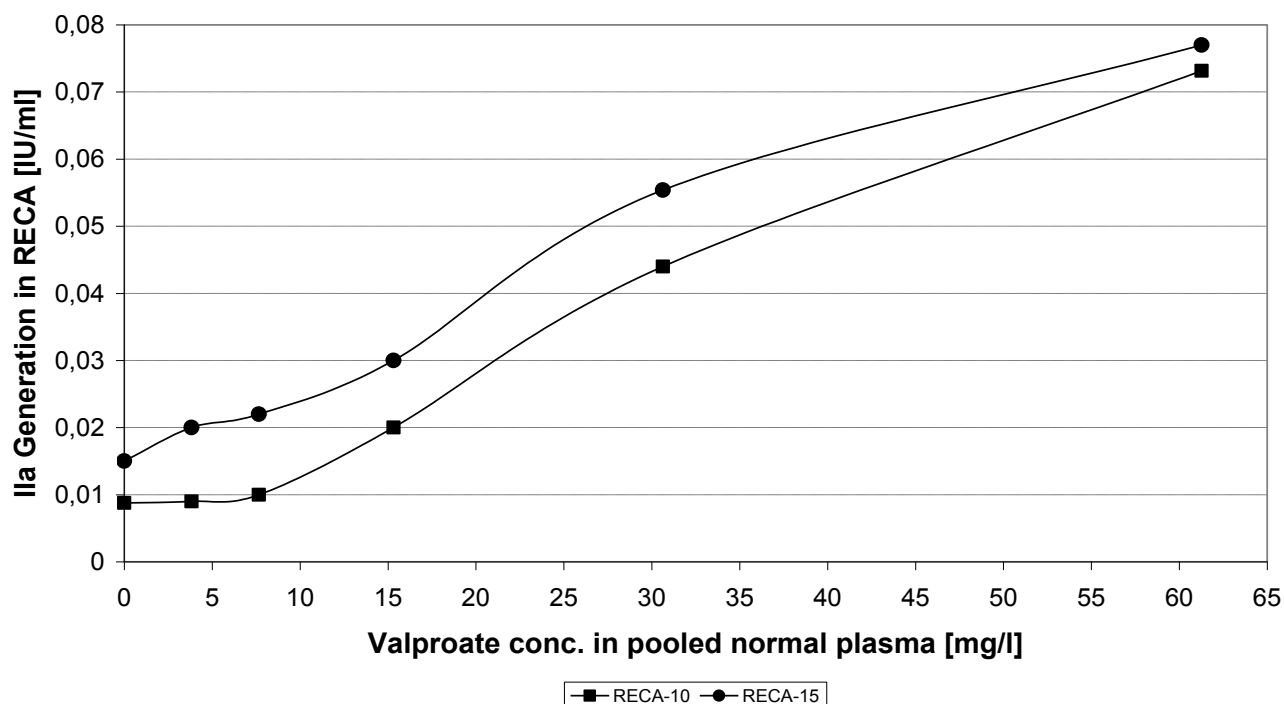


Figure 13. F2a generation in dependence of valproate conc. in pooled normal plasma 50 µl pooled normal plasma (out of the plasmas in figure 19) were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10 min (RECA-10) or 20 min (RECA-20). The approx. SC200 was 15 mg/l valproate in RECA-10 and in RECA-20.

4.1.3. Thrombin generation in normal plasmas by valproate.

In total 235 individual plasmas and 31 normal pools had been analysed. 214 individuals of 235 (91%) plasmas had an approx. SC200 < 245 mg/l, here the approx. SC200 values were 40 ± 47 mg/l (MV \pm 1 SD). 195 individuals of 235 (83 %) with approx. SC200 \leq 100 mg/l had an approx. SC200 value of 28 ± 27 mg/l (MV \pm 1 SD). Figures 14 -16 show thrombin generations in individual normal plasma. The 31 investigated normal pools had approx. SC200 values of 11 ± 9 mg/l (MV \pm 1 SD; range 2-35 mg/l). Figures 17-19 show further thrombin generations in pooled normal plasma.

The relatively wide distribution of the individual results is shown in figure 20. The pathologically low approx. SC200 values distribute with a Gaussian normal distribution peaking around 5 mg/l valproate.

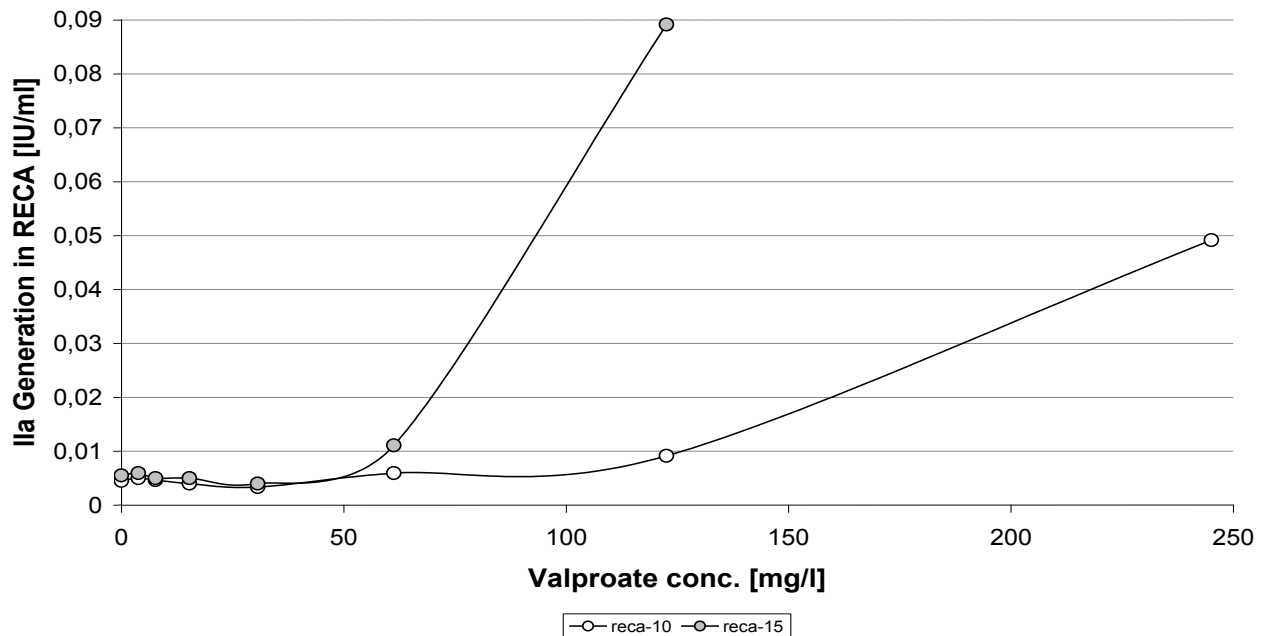


Figure 14. F2a generation in dependence of valproate concentration in **normal plasma** 50 μ l individual normal plasma were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10 min (RECA-10) or 15 min (RECA-15). The approx. SC200 was **60 mg/l valproate**, determined in RECA-15.

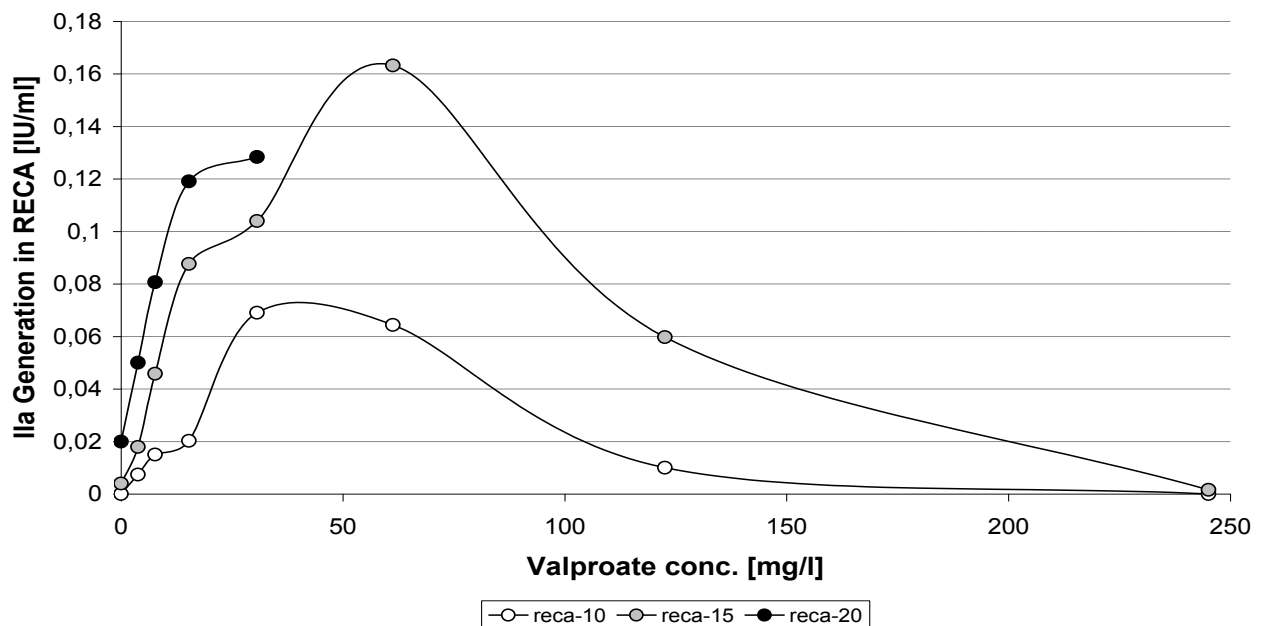


Figure 15. F2a generation in dependence of valproate concentration in **normal plasma** 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10-20 min (RECA-10, RECA-15, RECA-20). The approx. SC200 was **3 mg/l valproate**.

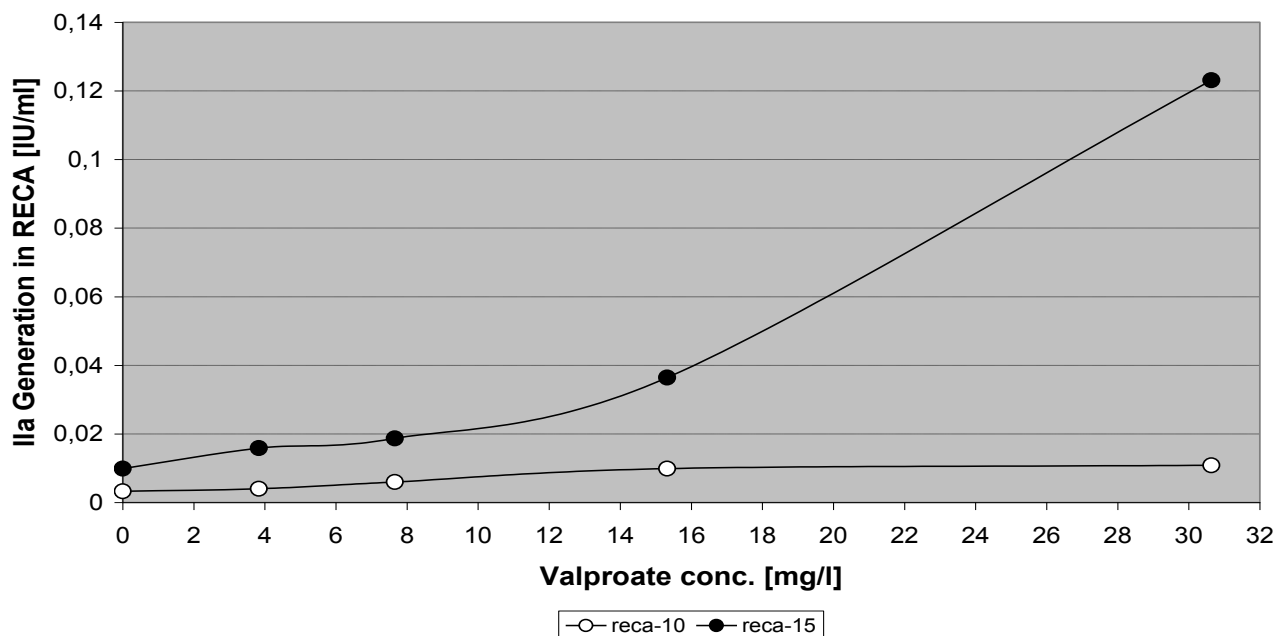


Figure 16. F2a generation in dependence of valproate in **individual normal plasma** 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10 min or 15 min (RECA-10 or RECA-15). The approx. SC200 was **8 mg/l valproate**.

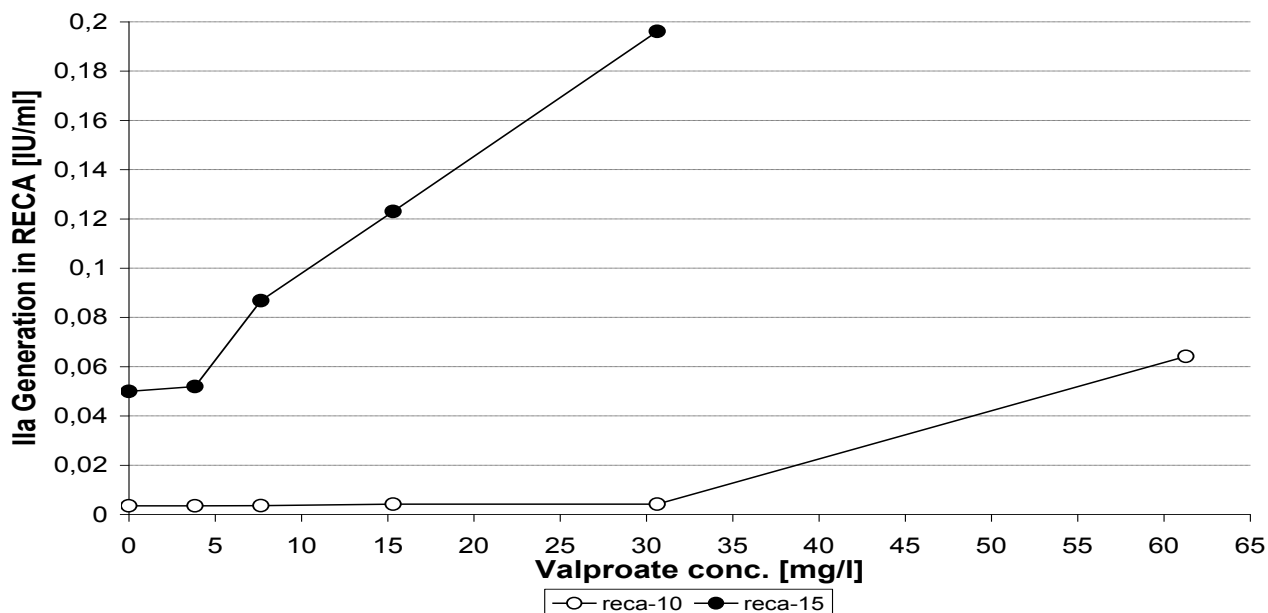


Figure 17. F2a generation in dependence of valproate conc. in **pooled normal plasma** 50 μ l pooled normal plasma (out of the plasmas of figures 30-33) were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10-15 min (RECA-10, RECA-15). The approx. SC200 was **10 mg/l valproate**.

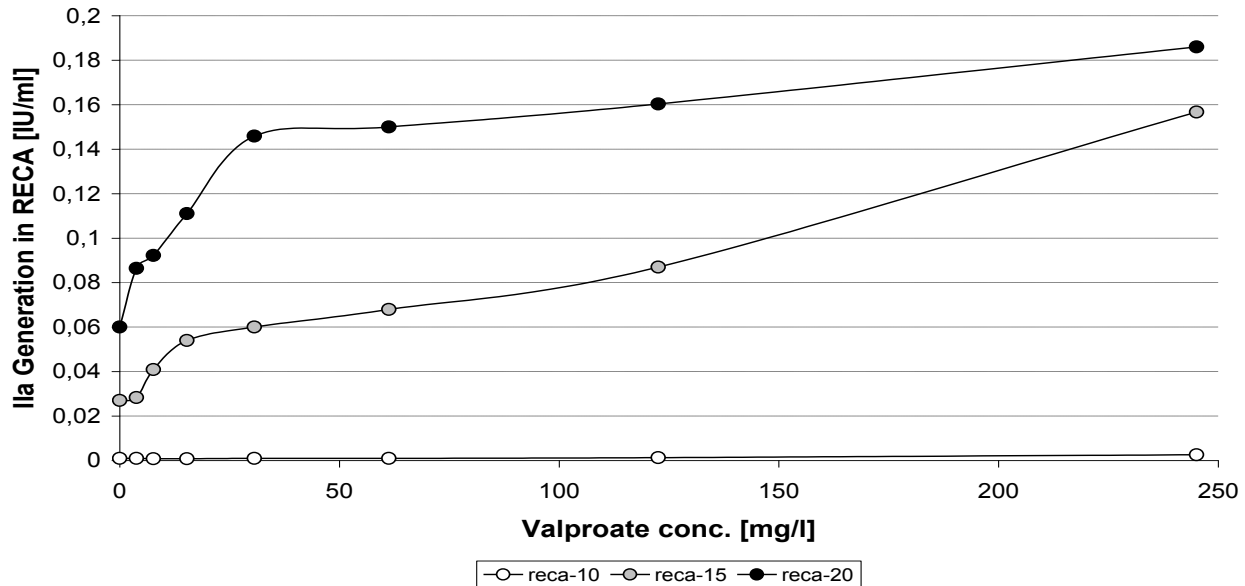


Figure 18. F2a generation in dependence of valproate in **pooled normal plasma** 50 μ l pooled normal plasma (out of 7 individual unfrozen plasmas) were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 10 min, 15 min, or 20 min (RECA-10, RECA-15 or RECA-20). The approx. SC200 was **15 mg/l valproate**.

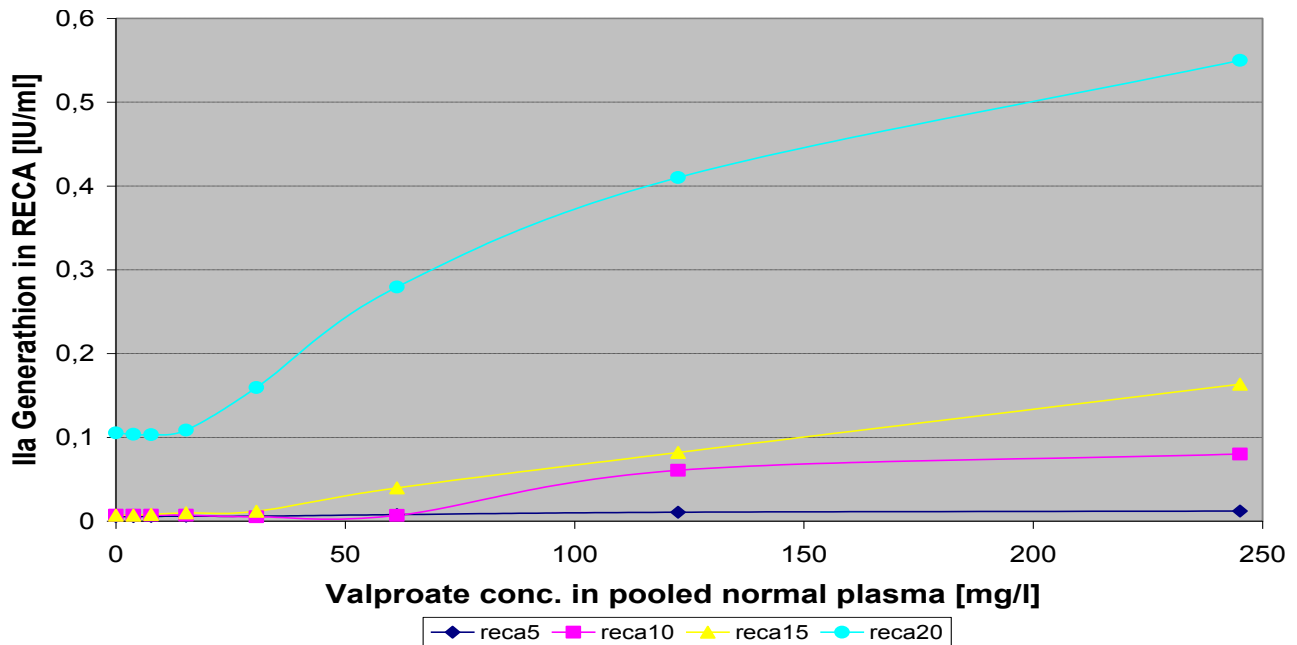


Figure 19. F2a generation in dependence of valproate in **pooled normal plasma** 50 μ l pooled normal plasma (out of 8 individual unfrozen plasmas) were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 5 min, 10 min, or 15 min (RECA-5, RECA-10 or RECA-15). The approx. SC200 was **35 mg/l valproate**.

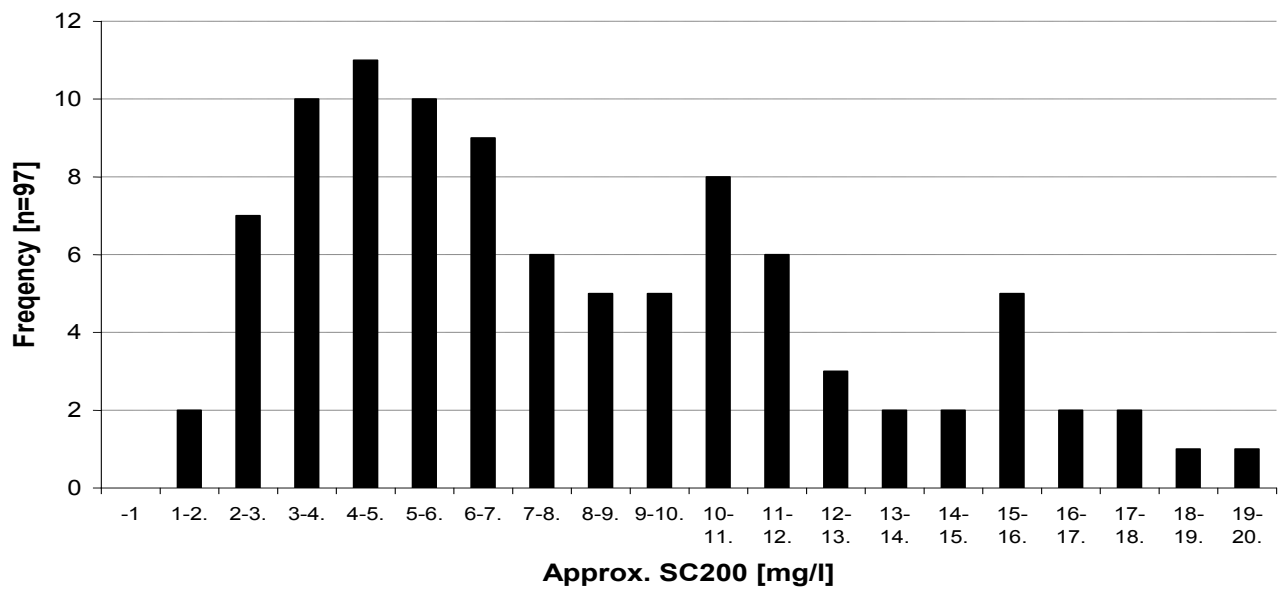
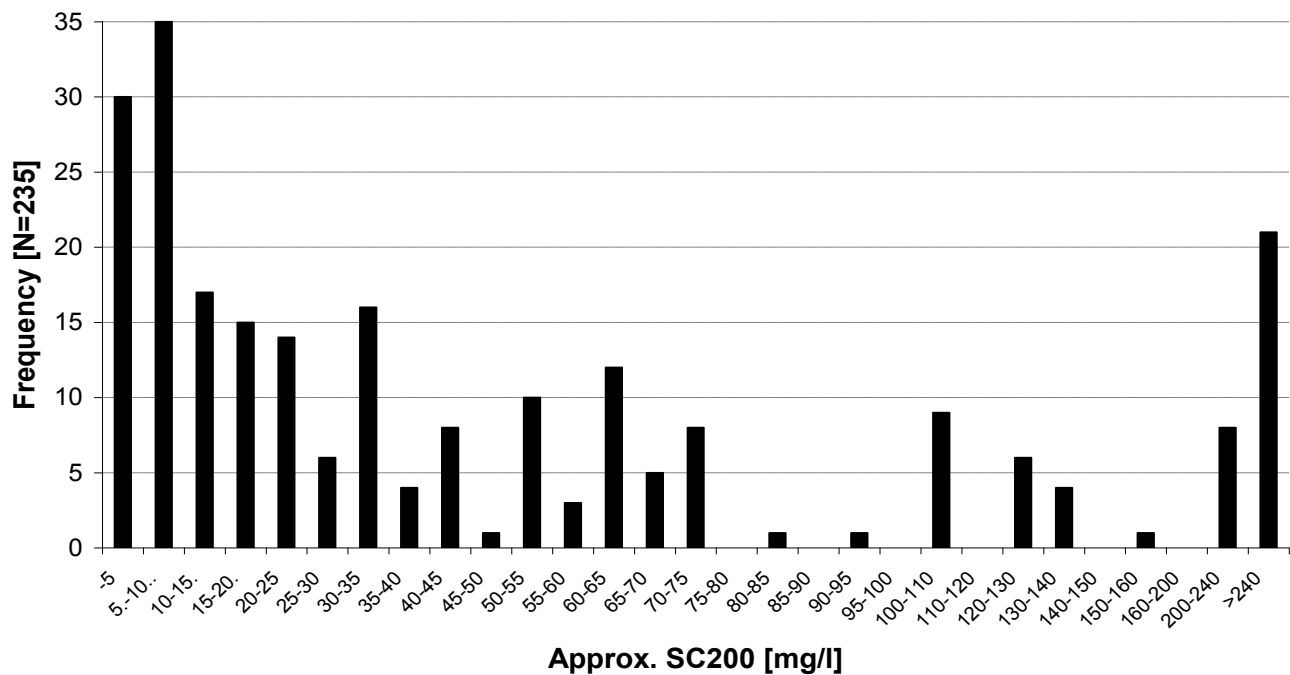


Figure 20. Relative wide distribution of the individual results. The pathologically low approx. SC200 values distribute with a Gaussian normal distribution peaking around 5 mg/l valproate.

4.1.4. Thrombin generation in heparinised plasmas by valproate

Since the presence of heparin might modulate thrombin generation in susceptible valproate-supplemented plasmas [78], the action of valproate on thrombin generation in presence of either 3.8 mIU/ml unfractionated heparin (UFH; a polynegative molecule) (7 individual normal plasmas and 1 pooled normal plasma prepared out of them) or with 0.5 IU/ml of the low-molecular-weight-heparin (LMWH) enoxaparin (5 individual unfrozen normal plasmas and 1 pooled normal plasma out of them) was studied. Interestingly, the addition of 3.8 mIU/ml unfractionated heparin to normal plasmas can have differential effects on thrombin generation. For instance, in one sample the approx. SC200 was 50 mg/l valproate without UFH and 10 mg/l valproate with 3.8 mIU/ml UFH (Figure 21). A slight decrease from approx. SC200 of 90 mg/l valproate without UFH to 70 mg/l valproate with 3.8 mIU/ml UFH was also observed (Figure 22). On the other hand, in other samples an increase in the approx. SC200 occurred [from 25 mg/l valproate without UFH to 35 mg/l valproate with 3.8 mIU/ml UFH (Figure 23), and from 15 mg/l valproate without UFH to 70 mg/l valproate with 3.8 mIU/ml UFH (Figure 24). Interestingly, in one plasma where the approx. SC200 was only 7 mg/l valproate without UFH, the presence of 3.8 mIU/ml UFH made this plasma resistant against valproate-induced F2a generation (Figure 25). In pooled plasma, the resistance to thrombin generation increased from an approx. SC200 of 9 mg/l valproate without UFH to 35 mg/l valproate with 3.8 mIU/ml UFH (Figure 26).

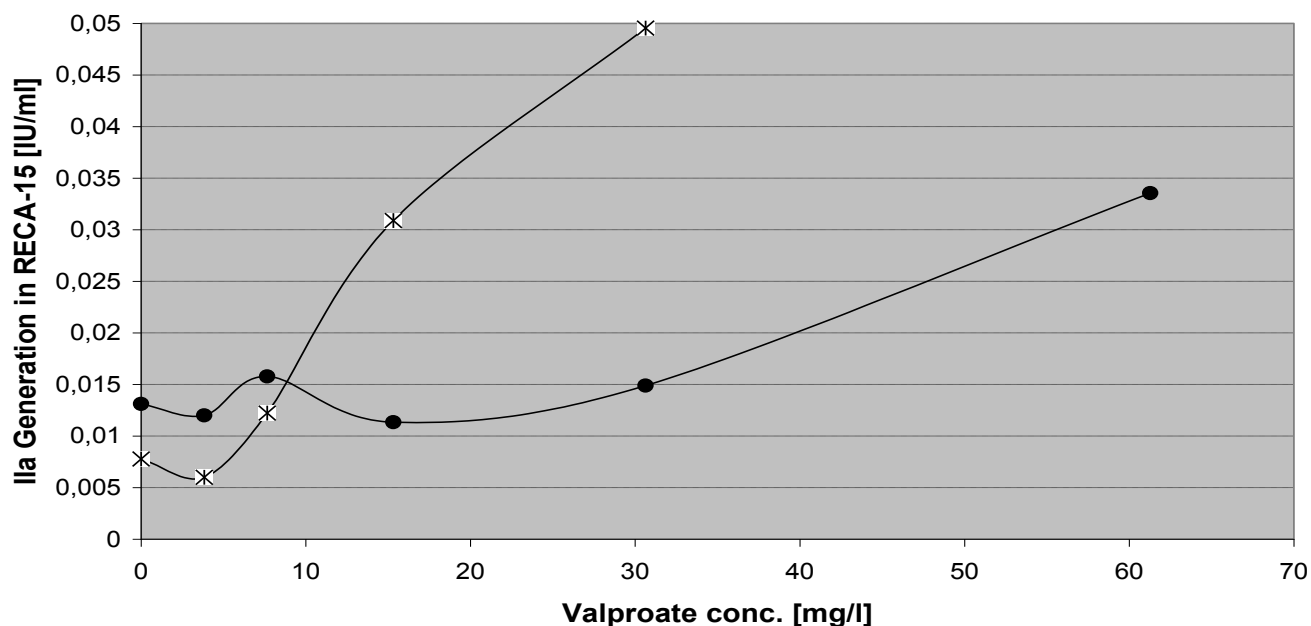


Figure 21. F2a generation in dependence of **valproate / heparin in normal plasma** 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 3.8 mIU/ml heparin (UFH; *). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 50 mg/l valproate without UFH and 10 mg/l valproate with 3.8 mIU/ml UFH.

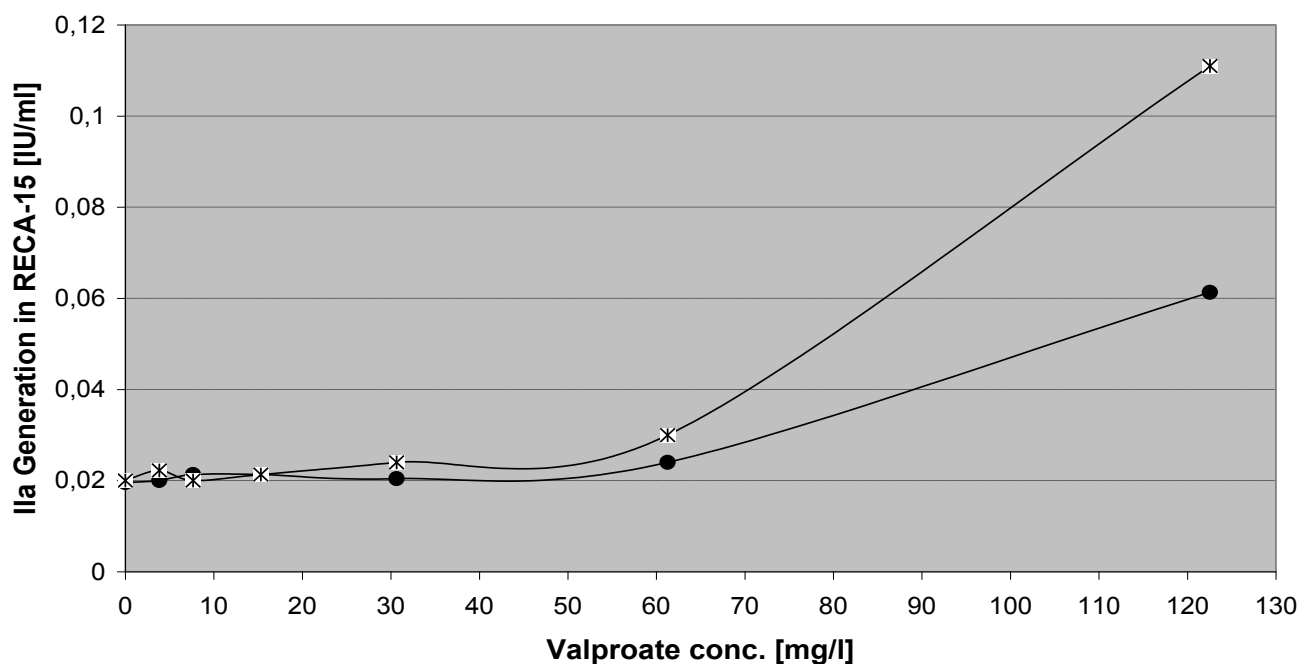


Figure 22. F2a generation in dependence of **valproate / heparin in normal plasma** 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 3.8 mIU/ml heparin (UFH; *). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 90 mg/l valproate without UFH and 70 mg/l valproate with 3.8 mIU/ml UFH.

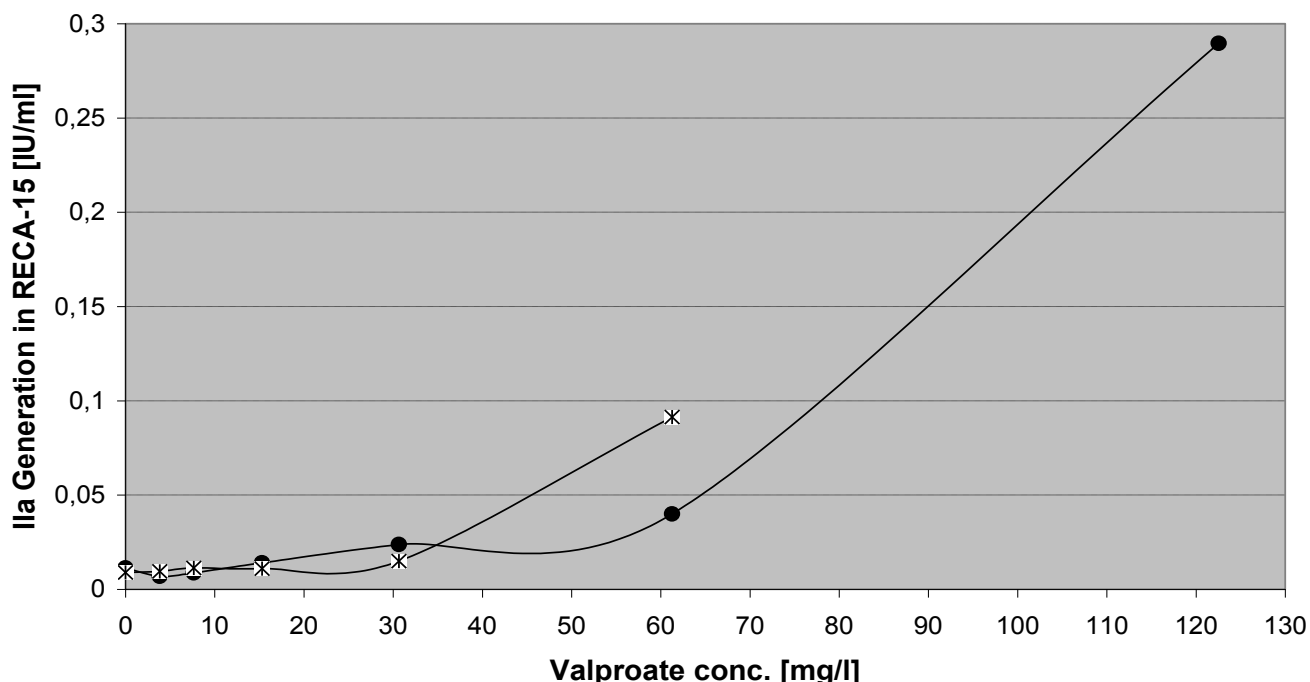


Figure 23. F2a generation in dependence of valproate / heparin in normal plasma 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 3.8 mIU/ml heparin (UFH; *). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 25 mg/l valproate without UFH and 35 mg/l valproate with 3.8 mIU/ml UFH.

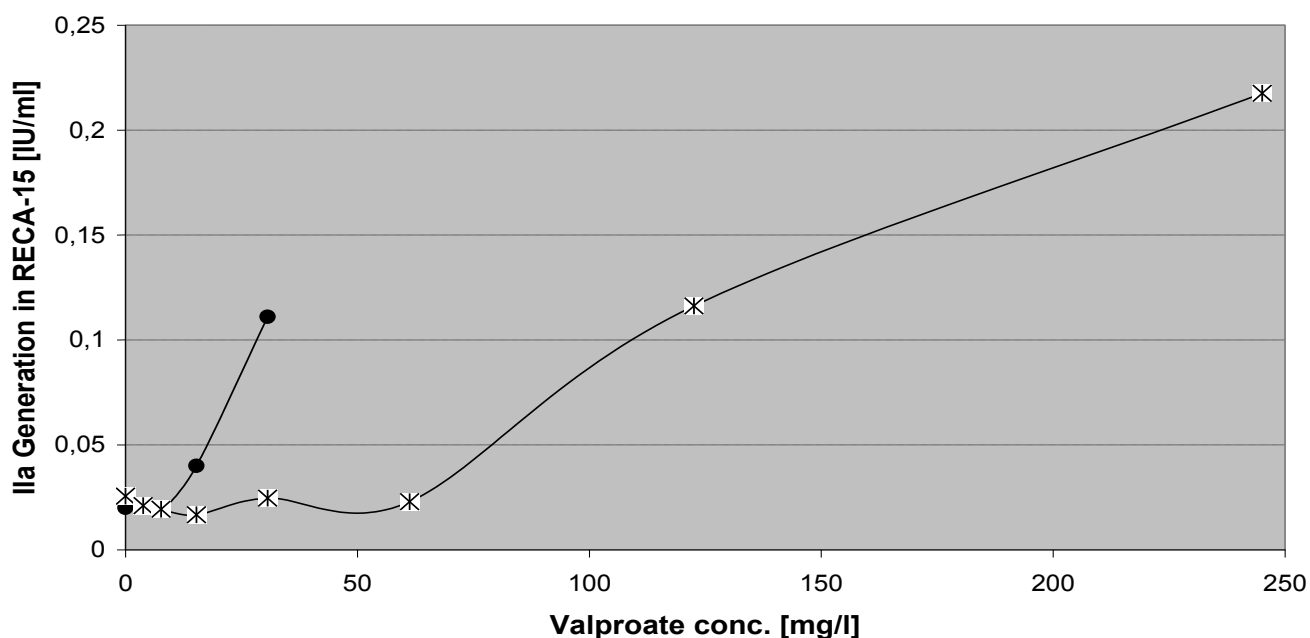


Figure 24. F2a generation in dependence of valproate / heparin in normal plasma 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 3.8 mIU/ml heparin (UFH; *). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 15 mg/l valproate without UFH and 70 mg/l valproate with 3.8 mIU/ml UFH.

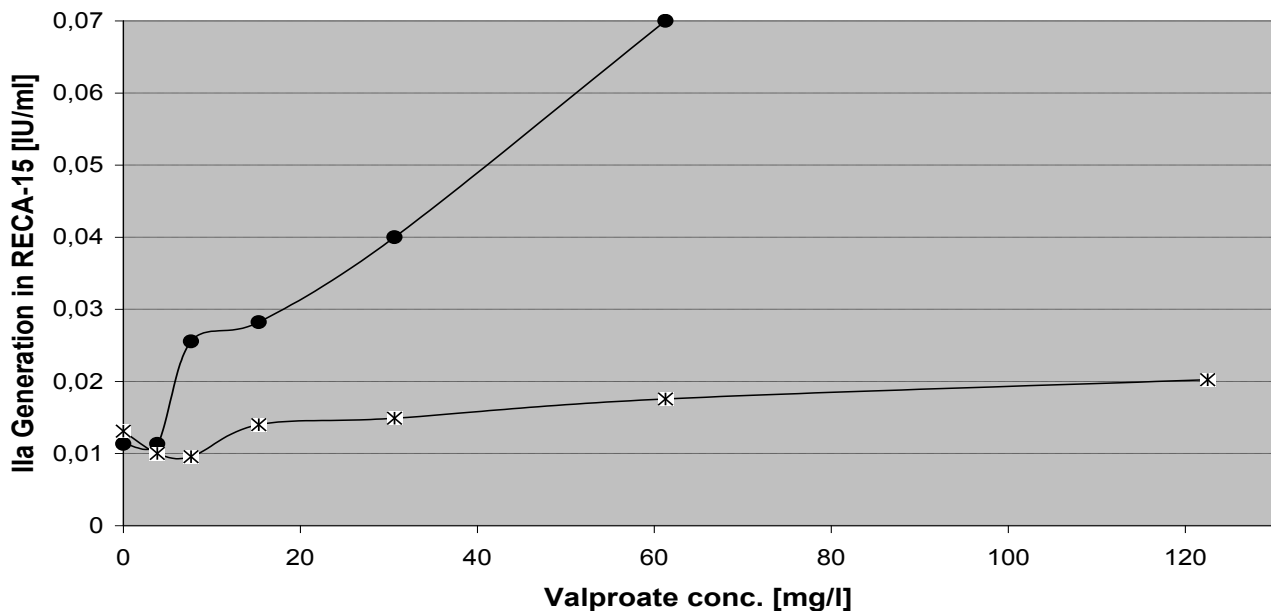


Figure 25. F2a generation in dependence of valproate / heparin in normal plasma 50 μ l individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 3.8 mIU/ml heparin (UFH; *). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 7 mg/l valproate without UFH, presence of 3.8 mIU/ml UFH made this plasma resistant against valproate-induced F2a generation.

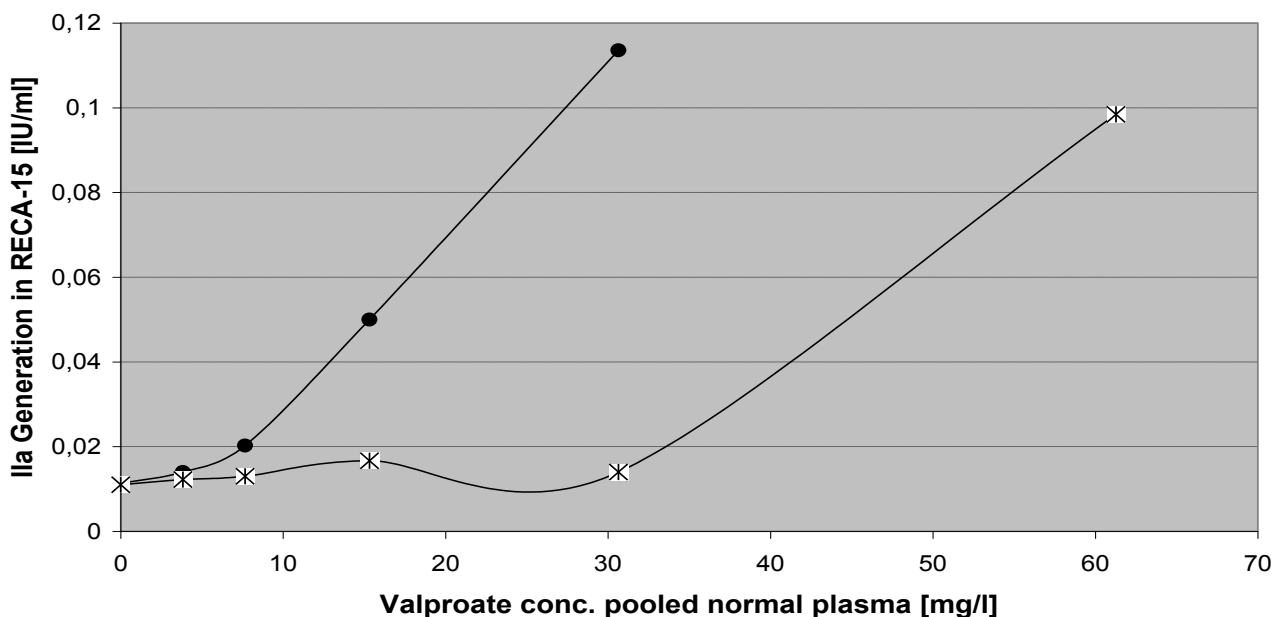


Figure 26. F2a generation in dependence of valproate / heparin in pooled normal plasma 50 μ l pooled normal plasma (out of the last individual 7 plasmas) were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 3.8 mIU/ml heparin (UFH; *). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 9 mg/l valproate without UFH and 35 mg/l valproate with 3.8 mIU/ml UFH.

Addition of 0.5 IU/ml LMWH neutralized the enhancement of thrombin generation by valproate (Figures 27-29). Plasma with an approx. SC200 of 4 mg/l valproate without LMWH became resistant against valproate-induced thrombin generation in presence of 0.5 IU/ml LMWH (Figure 27). The approx. SC200 also increased from 4 mg/l valproate without LMWH, to 150 mg/l valproate in presence of LMWH (Figure 28). The approx. SC200 increased from 8 mg/l valproate without LMWH, to 15 mg/l valproate with 0.5 IU/ml LMWH (Figure 29). Interestingly, in pooled plasma the approx. SC200 increased to > 250 mg/l valproate with 0.5 IU/ml LMWH (Figure 30, 31).

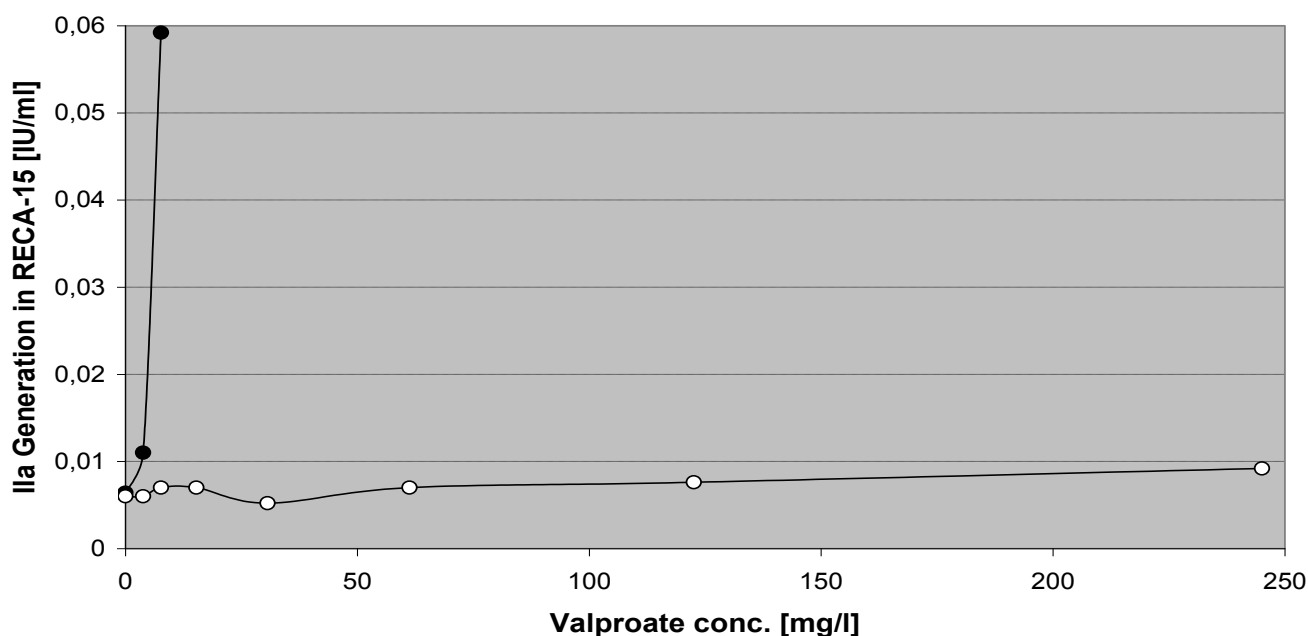


Figure 27. F2a generation in dependence of valproate / LMWH in individual normal plasma 50 µl individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 0.5 IU/ml enoxaparin (O). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 4 mg/l valproate without LMWH, the plasma became resistant against valproate-induced thrombin generation in presence of 0.5 IU/ml LMWH.

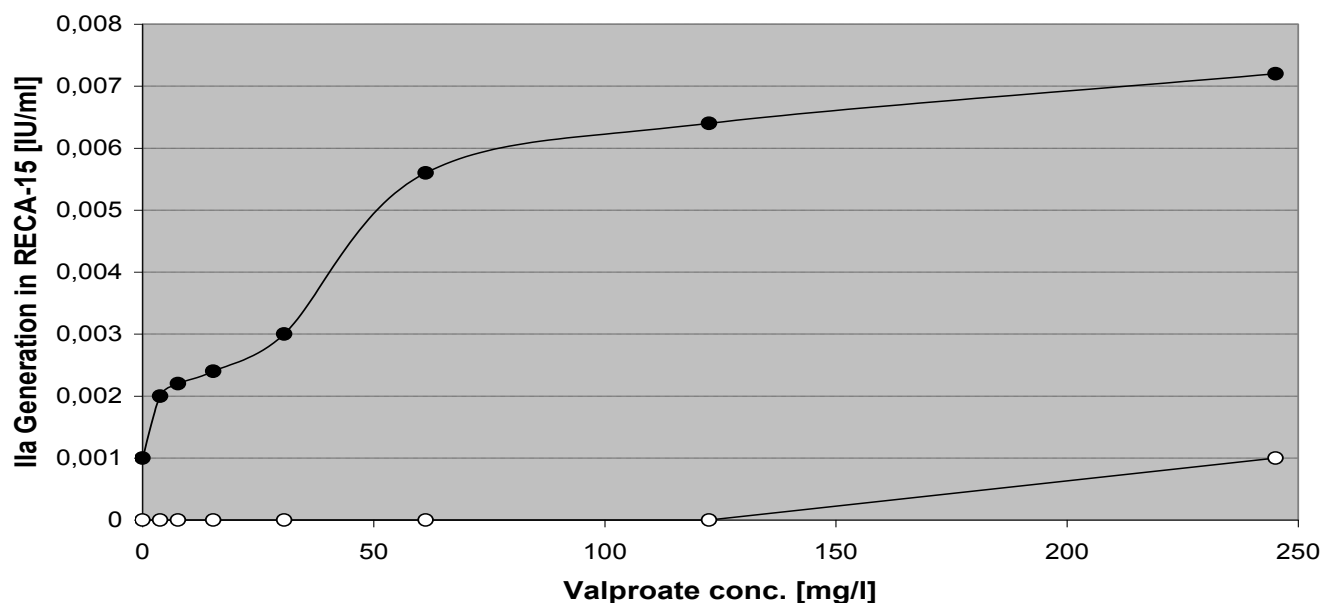


Figure 28. F2a generation in dependence of valproate / LMWH in individual normal plasma 50 µl individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 0.5 IU/ml enoxaparin (O). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 4 mg/l valproate without LMWH, the plasma became nearly resistant against valproate-induced thrombin generation in presence of 0.5 IU/ml LMWH. The approx. SC200 was 150 mg/l valproate.

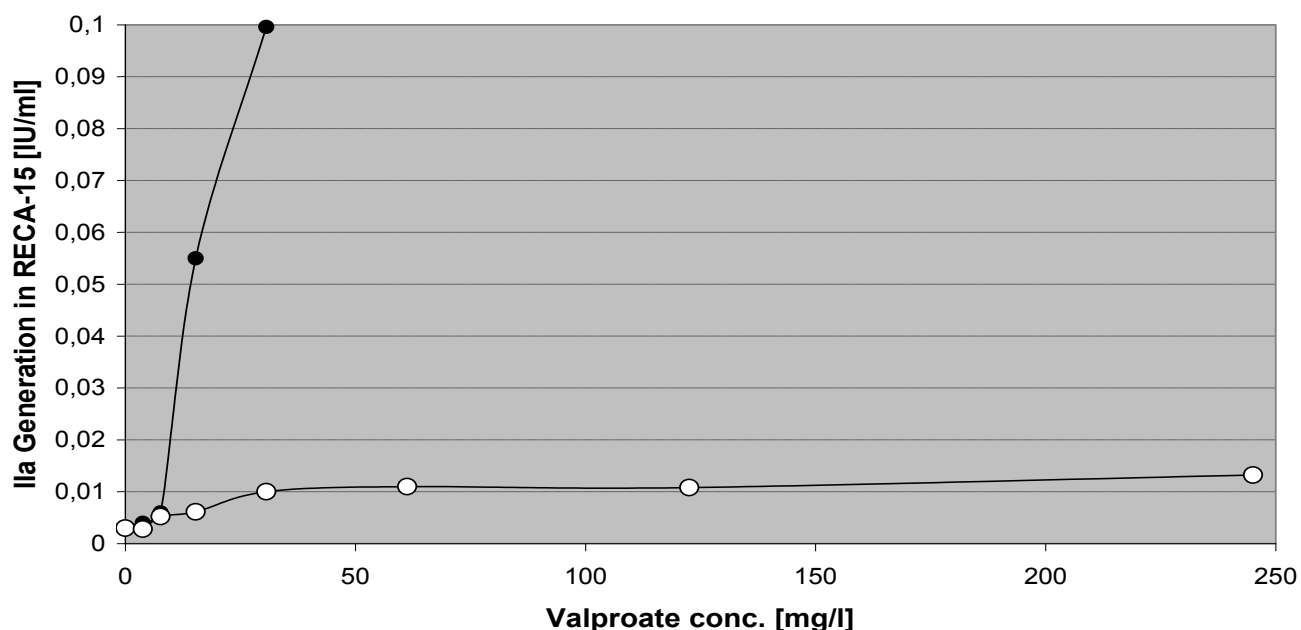


Figure 29. F2a generation in dependence of valproate / LMWH in individual normal plasma 50 µl individual normal plasma were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 0.5 IU/ml enoxaparin (O). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 8 mg/l valproate without LMWH, and 15 mg/l valproate with 0.5 IU/ml LMWH.

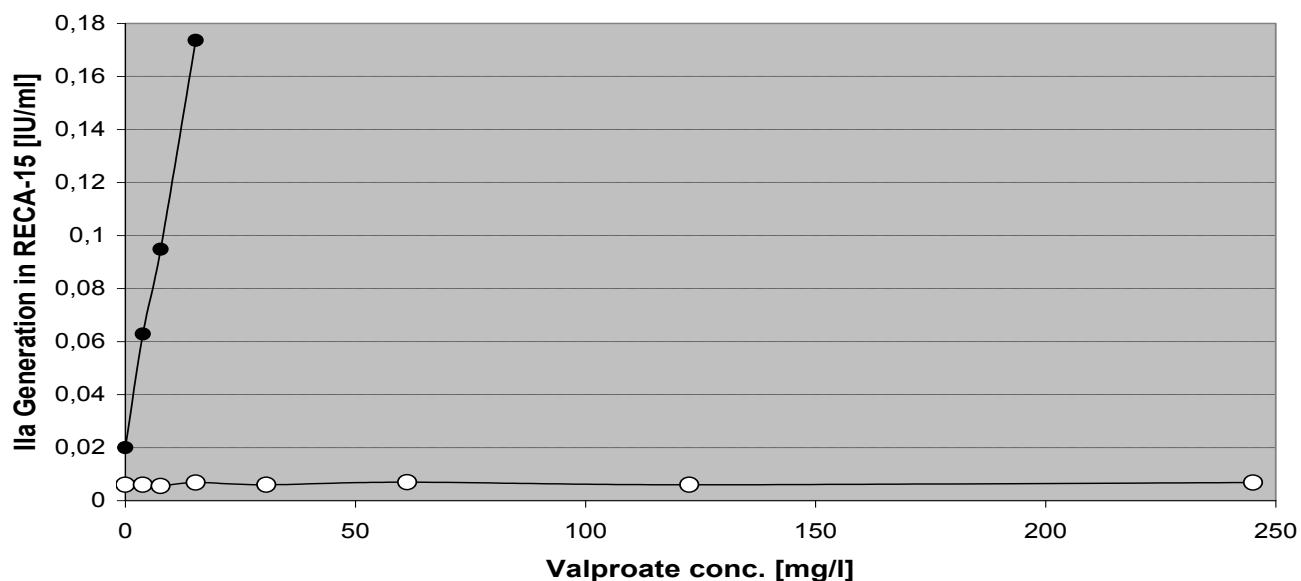


Figure 30. F2a generation in dependence of valproate / LMWH in pooled normal plasma 50 μ l pooled normal plasma (out of individual 4 plasmas) were supplemented in U-microwells (Brand®701300) with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml (●) or 0.5 IU/ml enoxaparin (○; the LMWH Clexane®). The RECA was performed with a coagulation reaction time of 15 min (RECA-15). The approx. SC200 was 3 mg/l valproate without LMWH, and > 250 mg/l valproate with 0.5 IU/ml LMWH.

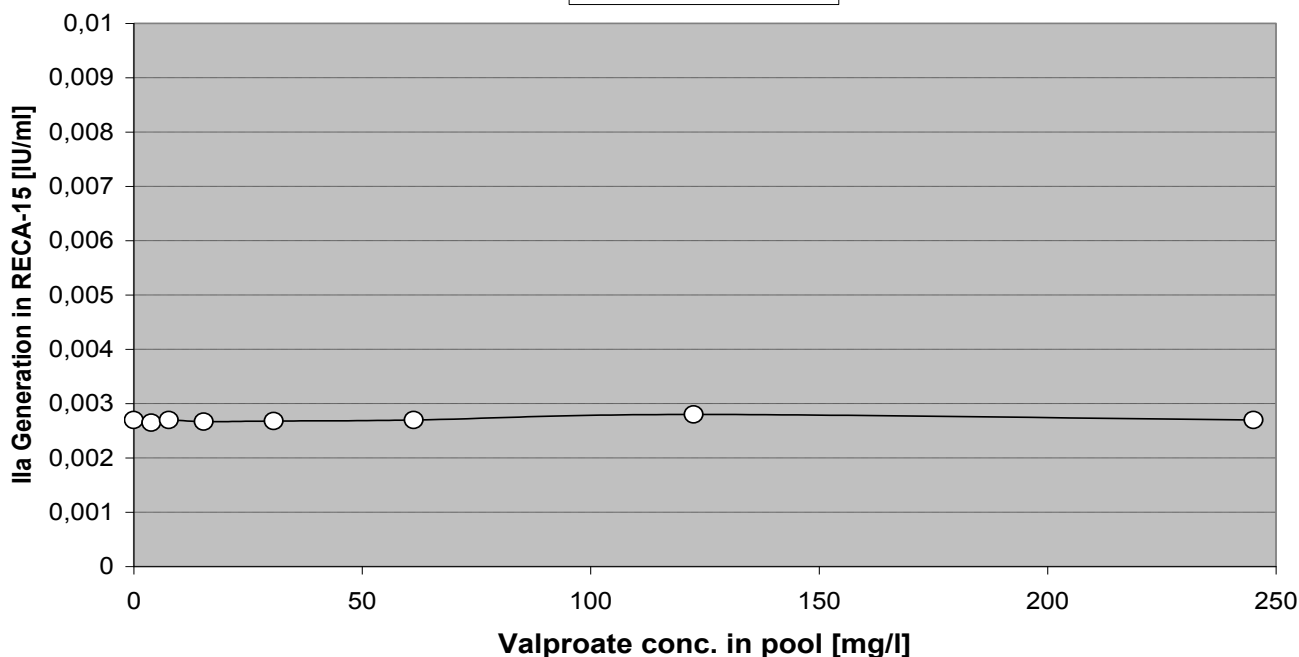
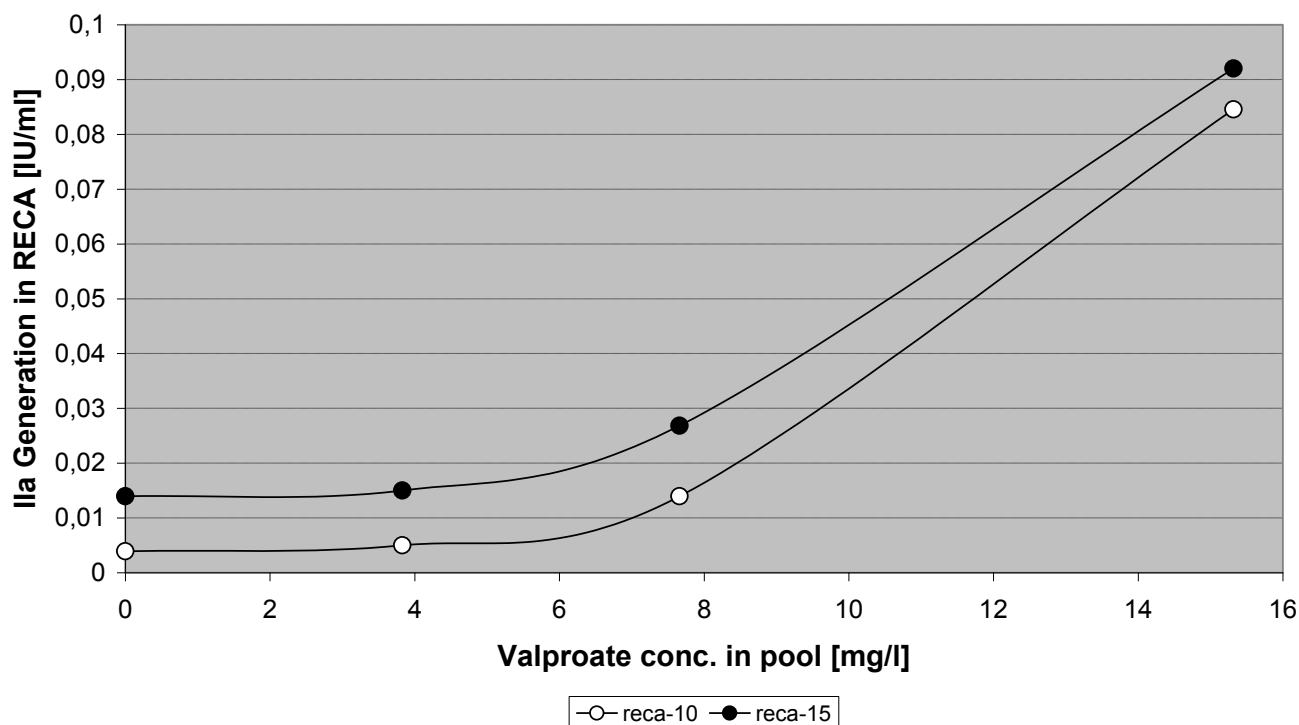


Figure 31. F2a generation in dependence of valproate / LMWH in pooled normal plasma 50 µl pooled normal plasma (out 8 individual plasmas) were supplemented in U-wells with 0-245 mg/l sodium valproate using repetitive 1+1 dilution and with 0 mIU/ml or 0.5 IU/ml enoxaparin. The RECA was performed with a coagulation reaction time of 10 min or 15 min (RECA-10 (O) or RECA-15 (●)). The approx. SC200 was 7 mg/l valproate without LMWH (Figure 31a), and > 250 mg/l valproate with 0.5 IU/ml LMWH (Figure 31b).

4.1.5. Thrombin generation in lyophilized plasma by valproate

The determination of the approx. SC200 of valproate on intrinsic thrombin generation was also evaluated in commercial lyophilized normal plasma and in plasma depleted for different factors. Figure 32 shows that that in reconstituted 100 % normal control plasma the approx. SC200 is 20 mg/l valproate (Figure 32). The RECA with 20 min coagulation reaction time (CRT) in un-supplemented plasma gave a thrombin generation of about 0.09 IU/ml. The F2a generation range 0.01-0.1 IU/ml is of eminent importance in RECA tests, because here thrombin activity is easily detectable and it is not too high to generate significant amounts of antithrombin-1 (nascent fibrin). Thus, in order to avoid antithrombin-1 interferences, the thrombin generation range of 0.01-0.1 IU/ml is best interpretable.

In un-supplemented F12 deficient lyophilized plasma only about 6.5 mIU/ml thrombin was generated in RECA-20 (Figure 33). About 200 mg/l valproate were needed to increase this basal thrombin generation by 100 %, which underlines the enormous importance of F12 in contact phase triggered thrombin generation.

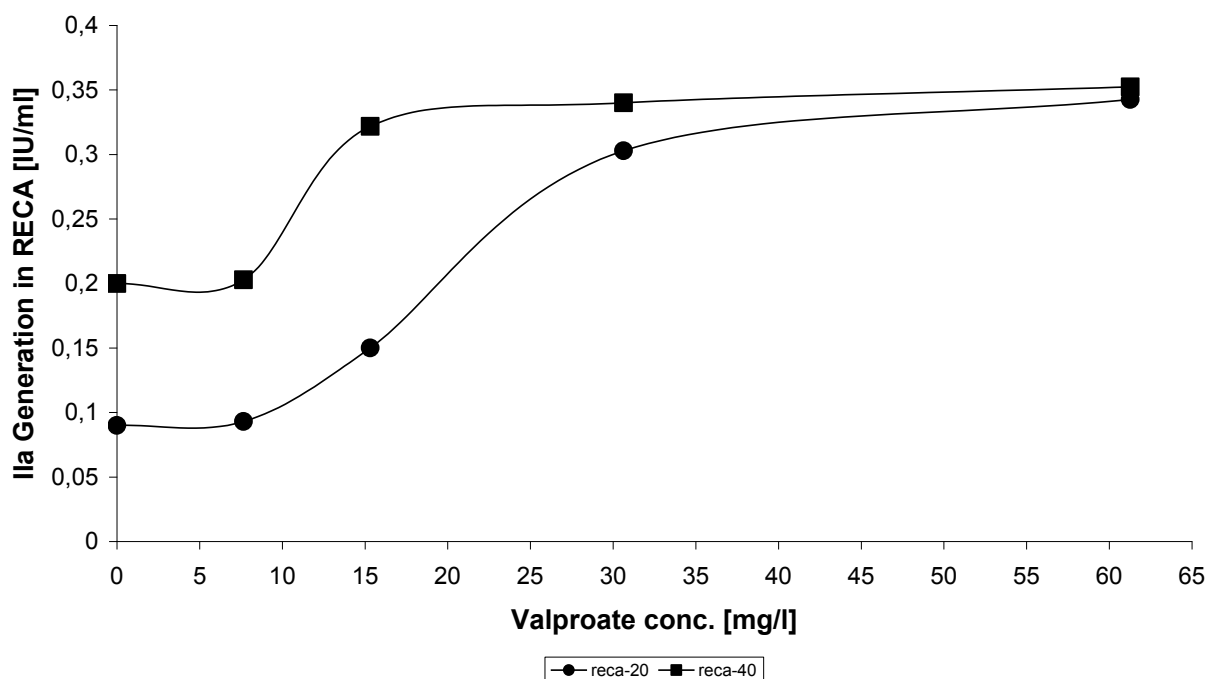


Figure 32. Thrombin (F2a) generation in dependence of valproate conc. in normal plasma 50 µl reconstituted 100 % normal control plasma N® were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min (RECA-20); approx. SC200 = 20 mg/l valproate.

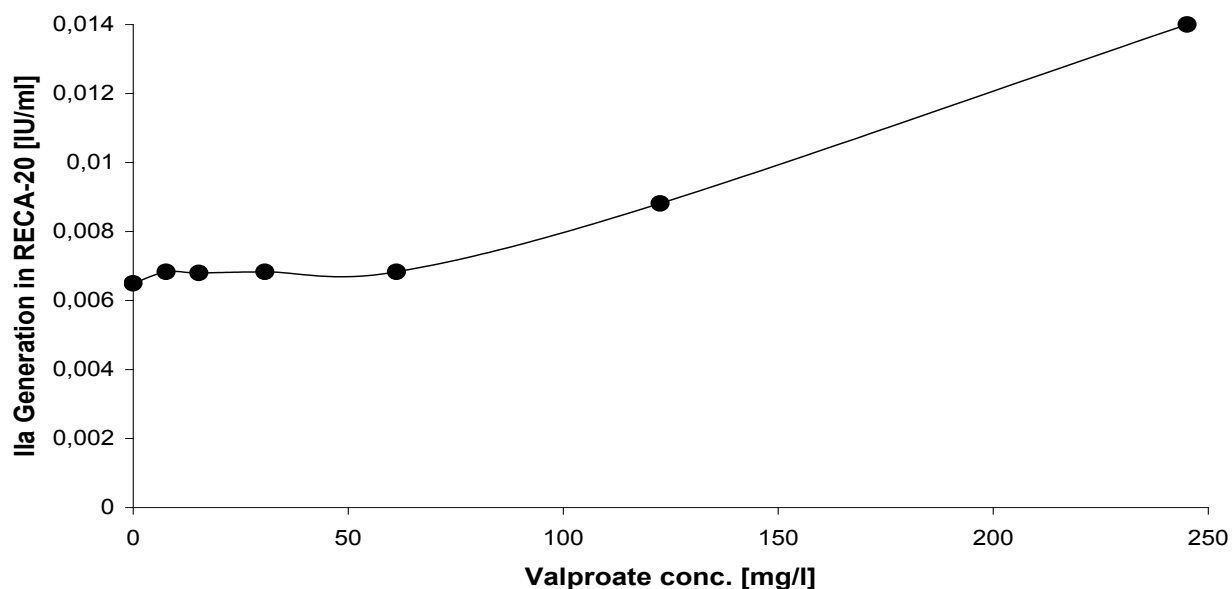


Figure 33. F2a generation in dependence of valproate concentration in F12 deficient plasma 50 μ l reconstituted F12 deficient plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min (RECA-20). The approx. SC200 was 200 mg/l valproate.

In F9, F8, F10 deficient commercial (stabilized) plasma the approx. SC200 was 120 mg/l, 150 mg/l, or 180 mg/l valproate, respectively (Figures 34-36). All factor deficient plasmas are not 100 % factor depleted, a remaining concentration of about 1 % might be enough to generate small activities of thrombin.

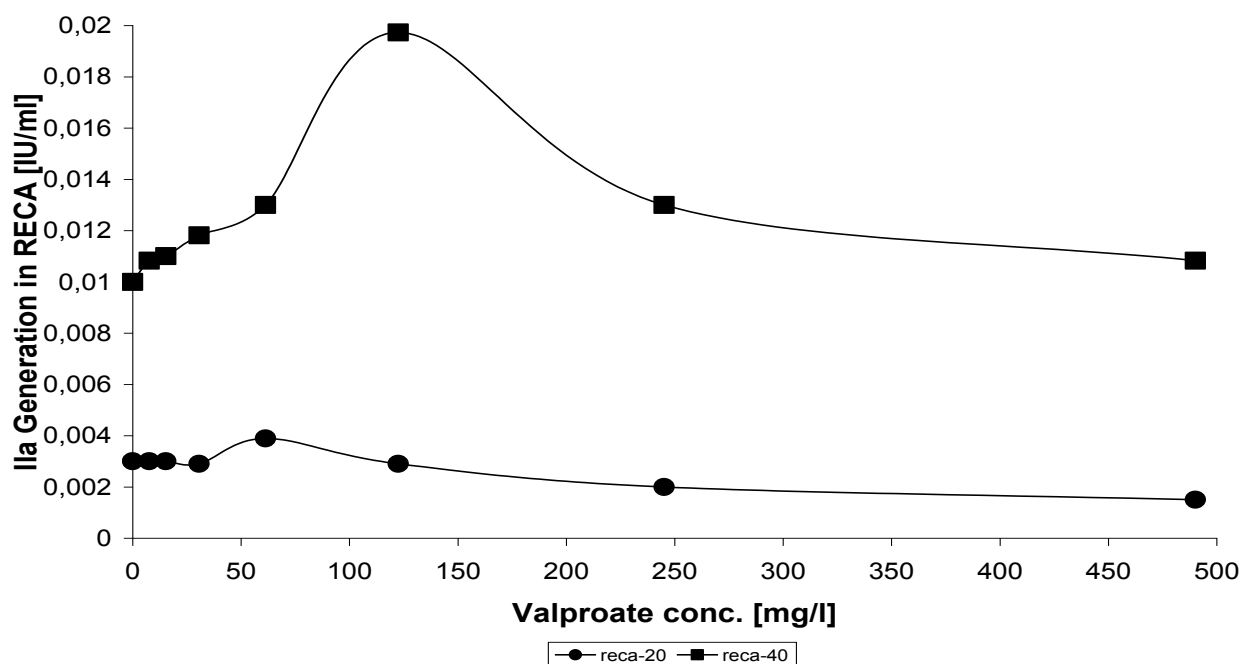


Figure 34. F2a generation in dependence of valproate concentration in F9 deficient plasma 50 µl reconstituted F9 deficient plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min or 40 min (RECA-20 or RECA-40). The approx. SC200 was 120 mg/l valproate.

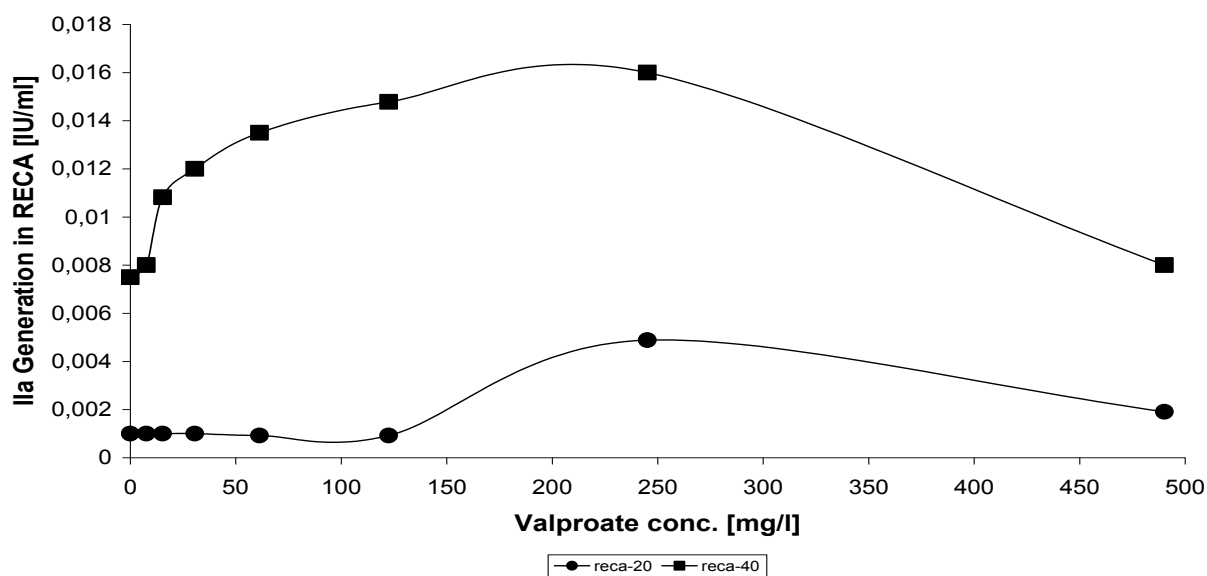


Figure 35. F2a generation in dependence of valproate concentration in F8 deficient plasma 50 µl reconstituted F8 deficient plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min or 40 min (RECA-20 or RECA-40). The approx. SC200 was 150 mg/l valproate.

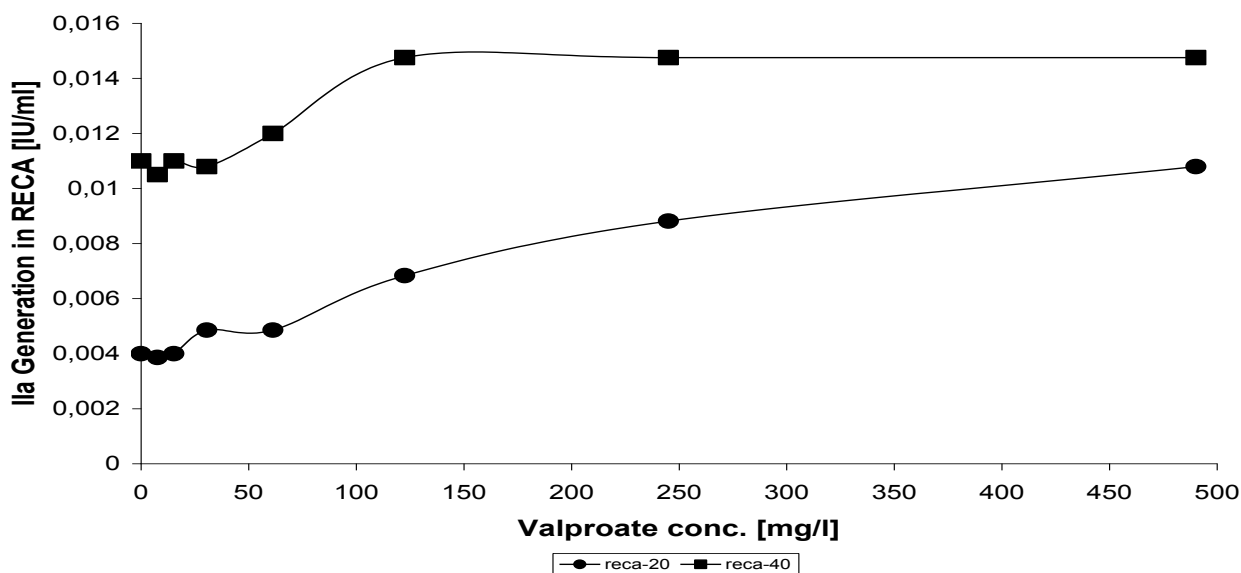


Figure 36. F2a generation in dependence of valproate concentration in F10 deficient plasma 50 µl reconstituted F10 deficient plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min or 40 min (RECA-20 or RECA-40). The approx. SC200 was 180 mg/l valproate.

In F5 deficient plasmas the approx. SC200 were 30 mg/l or 15 mg/l valproate (Figure 37). Very high valproate concentrations can decrease the thrombin generation (Figures 34,35,37).

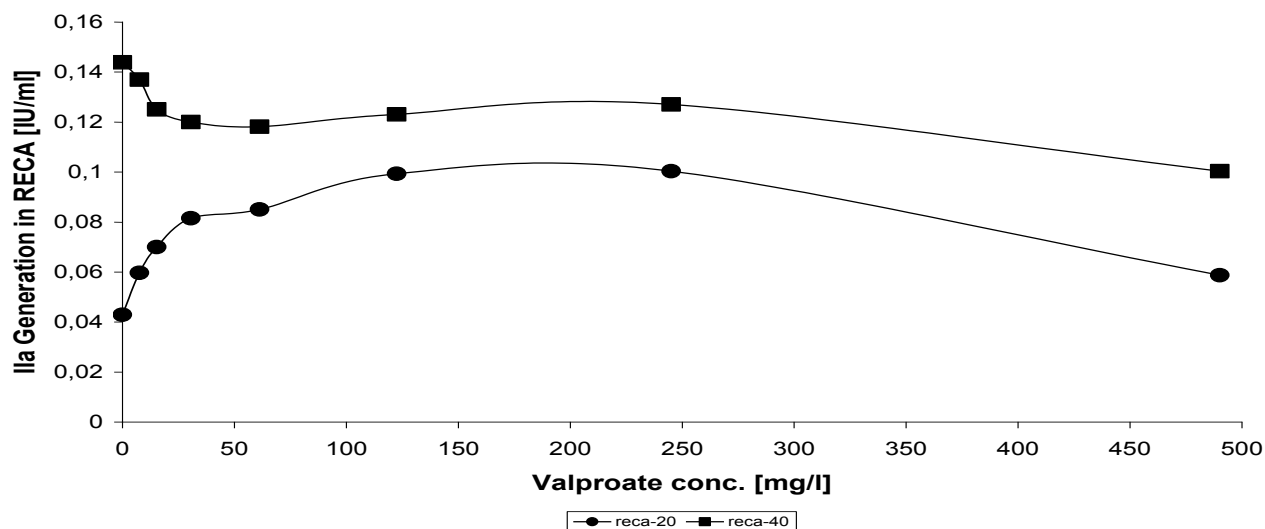


Figure 37. F2a generation in dependence of valproate concentration in F5 deficient plasma 50 µl reconstituted F5 deficient plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min or 40 min (RECA-20 or RECA-40). The approx. SC200 was 30 mg/l or 15 mg/l valproate, respectively.

F2 deficient plasma had an approx. SC200 of 120 mg/l valproate (Figure 38), while in F7 deficient plasma only 6 mg/l valproate is needed, presumably because in F7 deficient plasma the whole intrinsic clotting cascade is nearly not affected (Figure 39).

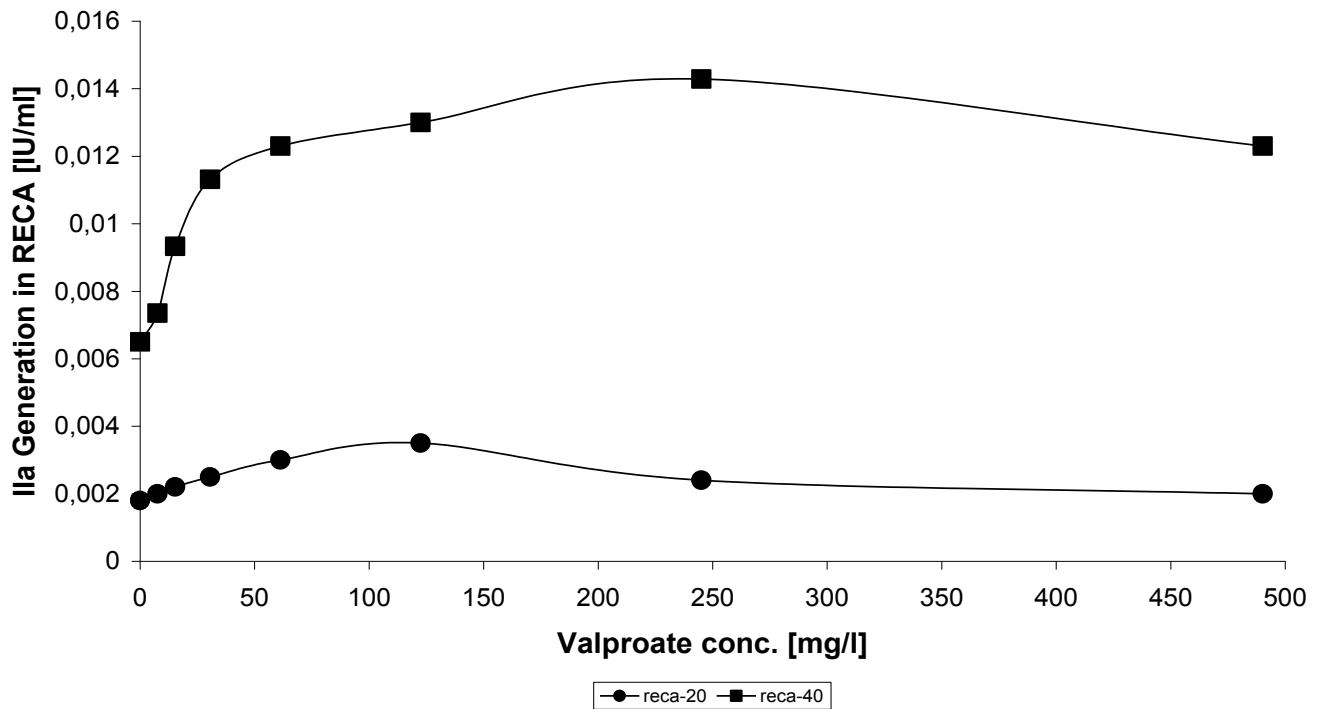


Figure 38. F2a generation in dependence of valproate concentration in F2 deficient plasma 50 µl reconstituted F2 deficient plasma were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min or 40 min (RECA-20 or RECA-40). The approx. SC200 was 120 mg/l.

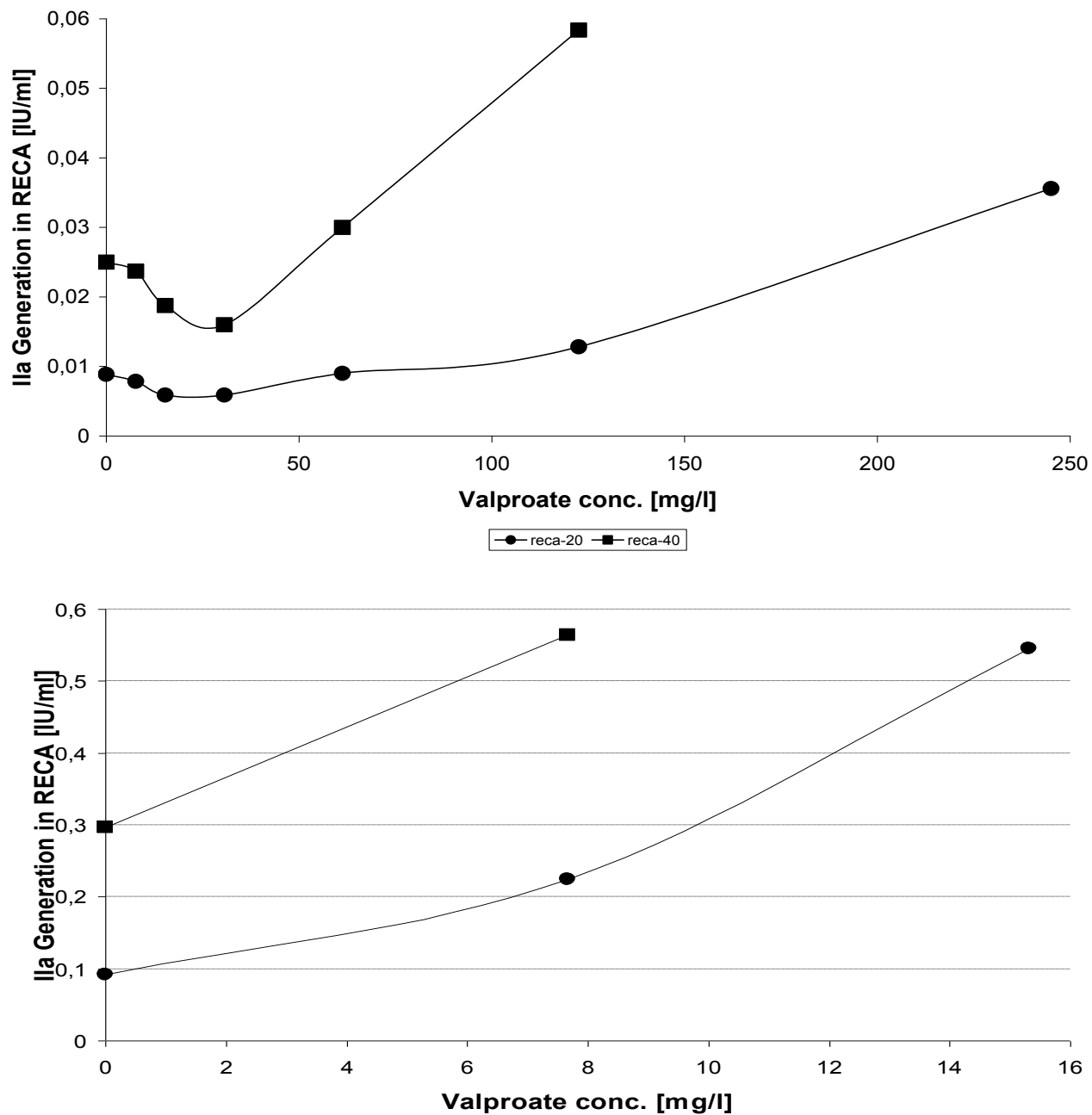


Figure 39. F2a generation in dependence of valproate concentration in F7 deficient plasma 50 μ l reconstituted F7 deficient plasma (DadeBehring = Figure 39a; IL = Figure 39b) were supplemented in U-microwells (Brand®701300) with sodium valproate using repetitive 1+1 dilution. The RECA was performed with a coagulation reaction time of 20 min or 40 min (RECA-20 or RECA-40). The approx. SC200 was 6 mg/l.

4.2. Thrombin generation in plasma by Tryptophan

Tryptophan is an amino acid that is negatively charged and therefore can also stimulate thrombin generation. Therefore, we also studied the effect of tryptophan on thrombin generation using 25 normal individual normal plasmas.

21 of 25 (84 %) individual normal plasmas had an approx. SC200 of 1.3 ± 1.0 mM tryptophan (mean value ± 1 standard deviation; $MV \pm 1SD$) (Figures 40-44). Interestingly, 4 of 25 (16 %) individual normal plasmas were resistant against added tryptophan when added to less than 4 mM (Figure 45). Furthermore, in 3 pools of normal plasmas the approx. SC200 is of 0.2 ± 0.1 mM added tryptophan (Figure 46). Collectively, this data indicate that tryptophan has differential effects on thrombin generation.

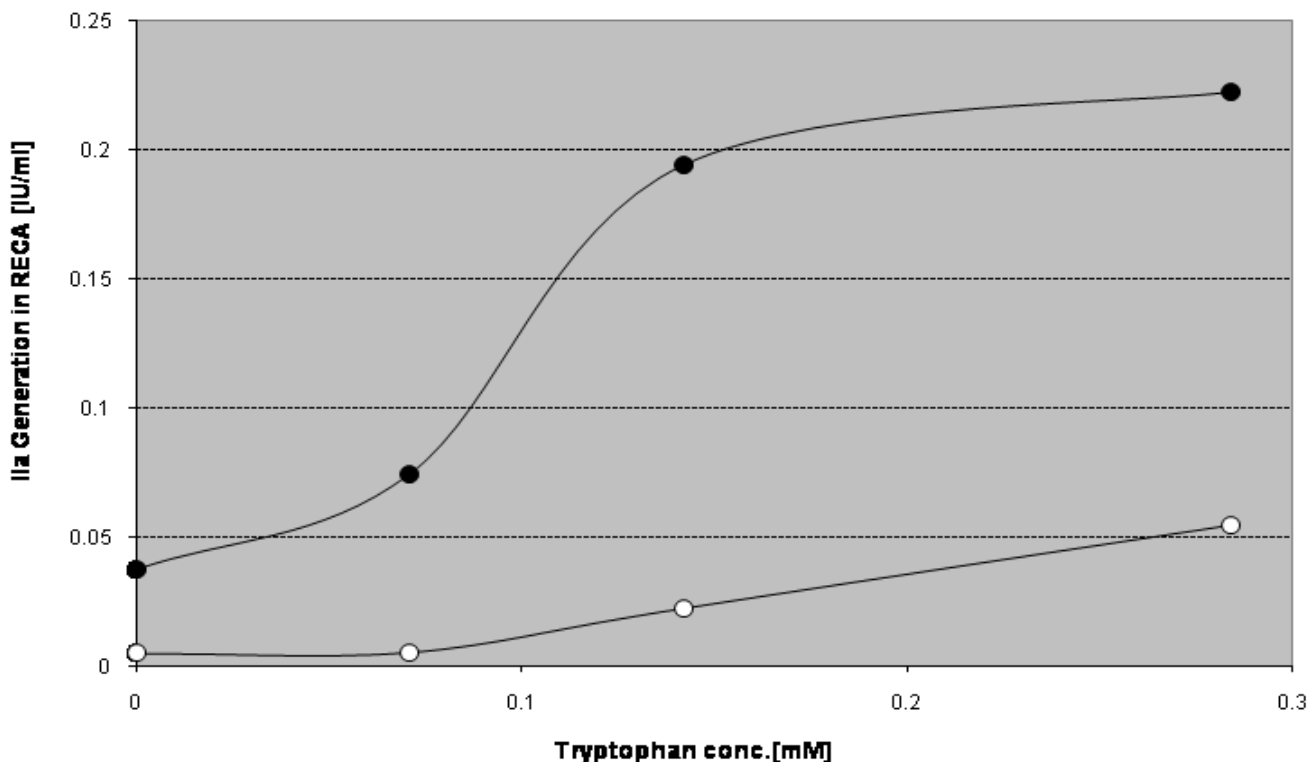


Figure 40. F2a generation in tryptophan – supplemented normal plasmas 50 μ l samples of 2 unfrozen normal citrated plasmas (O, ●) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20 (20 min CRT). The approx. SC200 values were 0.1 mM added tryptophan for both of them.

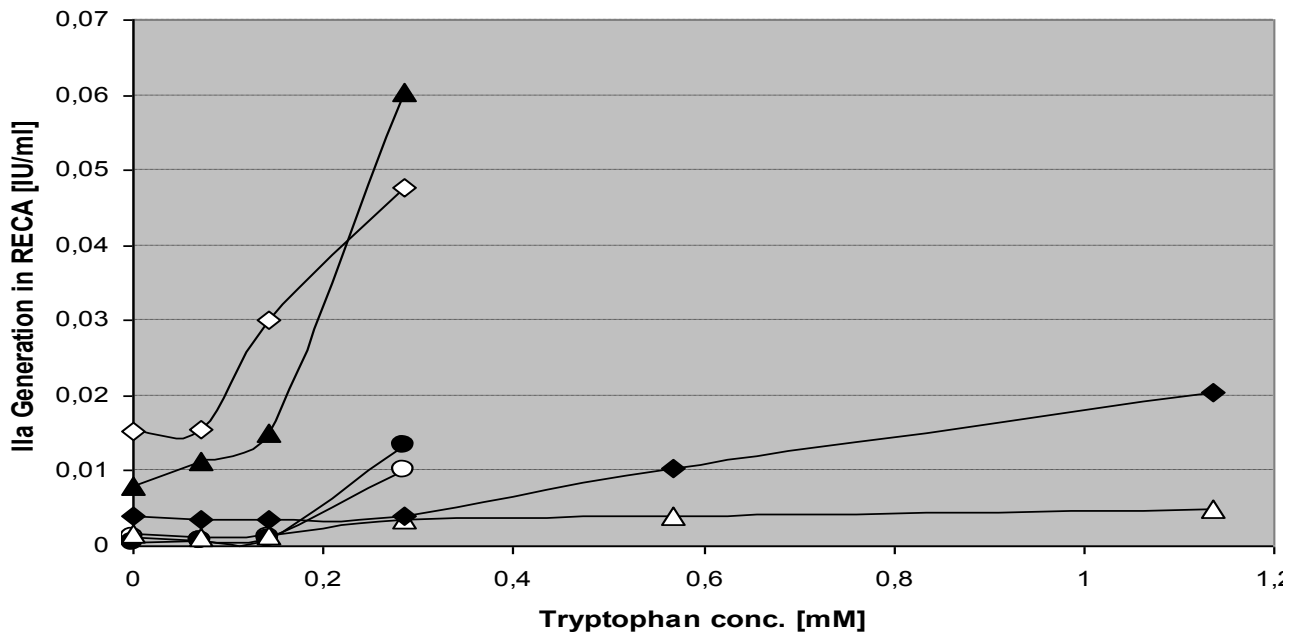


Figure 41. F2a generation in tryptophan – supplemented normal plasmas 50 μ l samples of 6 unfrozen citrated plasmas (O,●,Δ,▲,◇,◆) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20. The approx. SC200 values were between 0.2 and 0.5 mM added tryptophan.

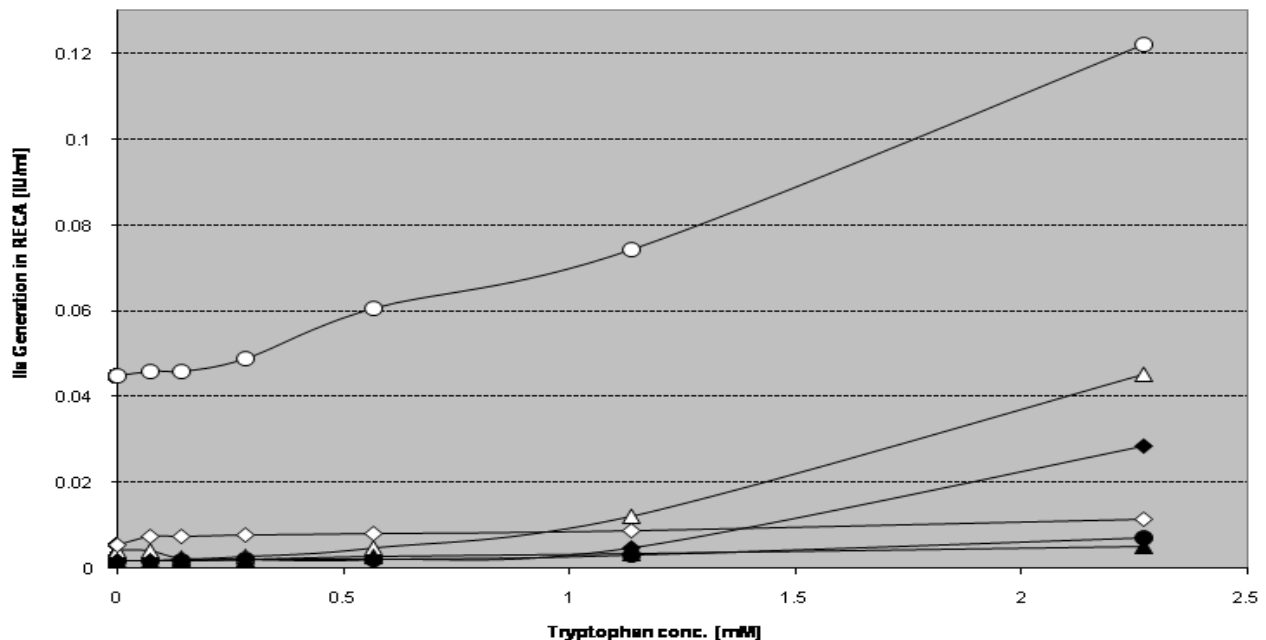


Figure 42. F2a generation in tryptophan – supplemented normal plasmas 50 μ l samples of 6 unfrozen normal citrated plasmas (O,●,Δ,▲,◇,◆) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20. The approx. SC200 values were between 1 and 1.5 mM added tryptophan.

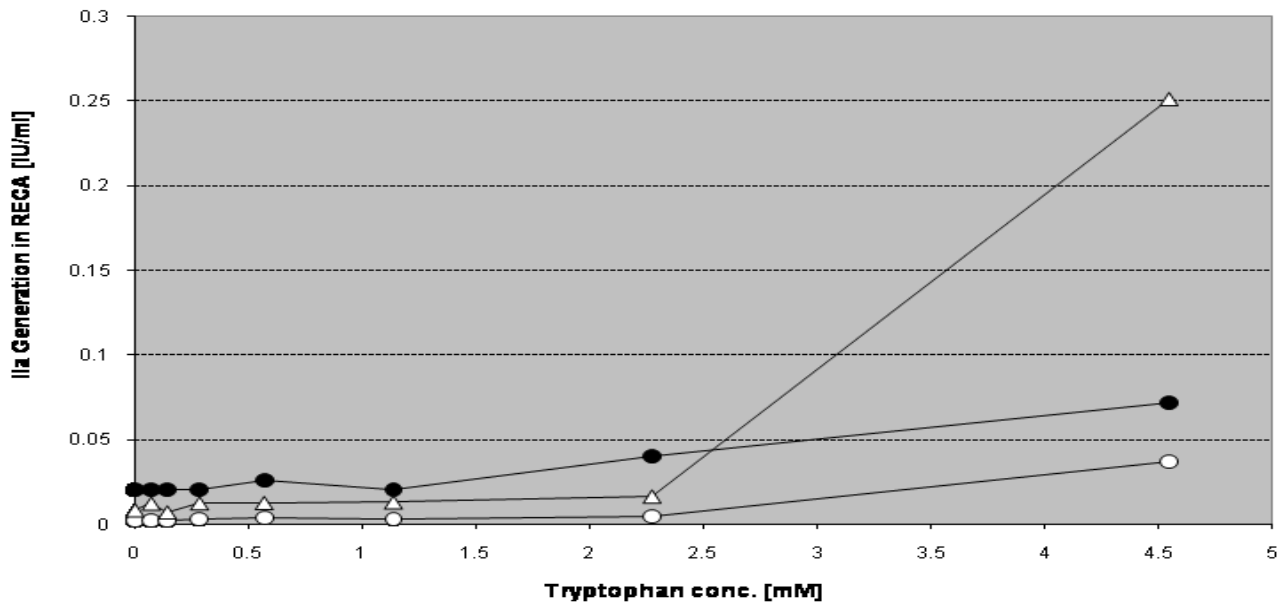


Figure 43. F2a generation in tryptophan – supplemented normal plasmas 50 μ l samples of 3 unfrozen normal citrated plasmas (O,●,Δ) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20. The approx. SC200 values were between 2 and 2.3mM added tryptophan.

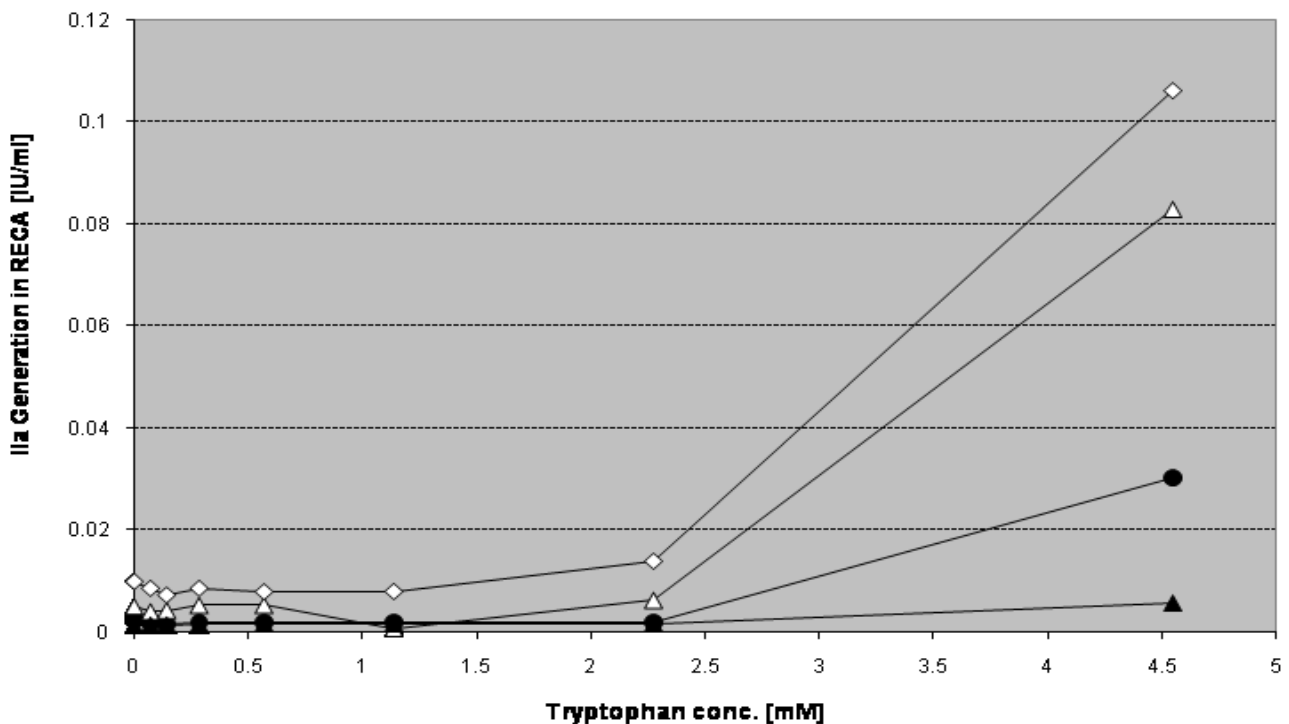


Figure 44. F2a generation in tryptophan – supplemented normal plasmas 50 μ l samples of 4 unfrozen normal citrated plasmas (●,Δ,▲,◇) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20. The approx. SC200 values were between 2.5 and 2.8 mM added tryptophan.

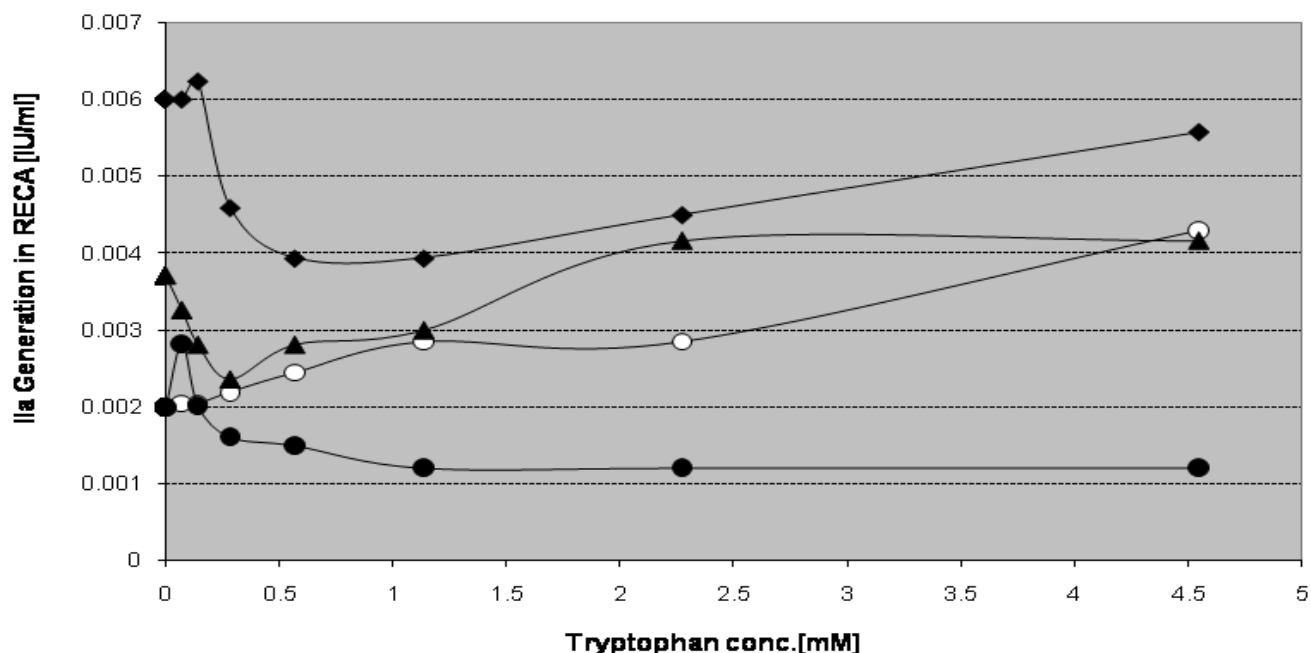


Figure 45. F2a generation in tryptophan – supplemented normal resistant plasmas 50 μ l samples of 4 unfrozen normal citrated plasmas (O, ●, ◆, ▲) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-30. The approx. SC200 values were 4 mM added tryptophan for the first one and the others were resistant up to 4.5 mM added tryptophan.

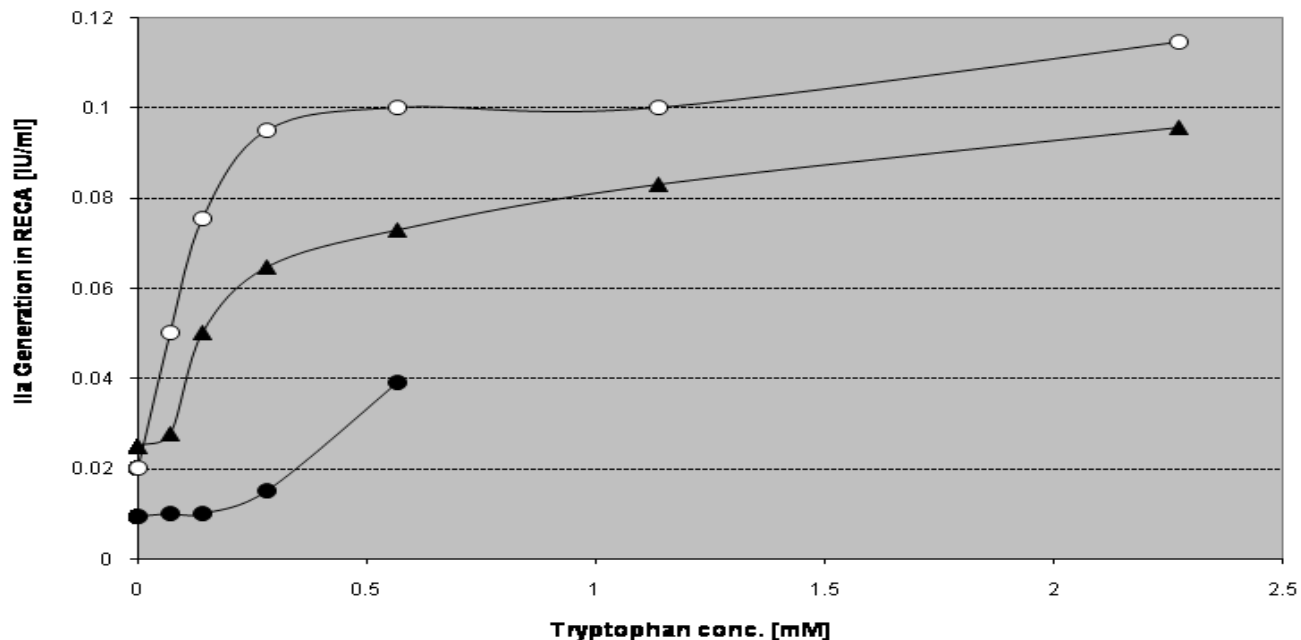


Figure 46. F2a generation in tryptophan – supplemented pools of normal plasmas 50 μ l samples of 3 unfrozen pools of normal citrated plasmas (O, ●, ▲) were supplemented with 0-4.5 mM additional tryptophan by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-10 (10 min CRT). The approx. SC200 values were 0.1, 0.3, and 0.2 mM added tryptophan, respectively.

4.3. Thrombin generation in plasmas by leucine

Leucine which is also negatively charged and very similar in structure to valproate can also induce thrombin generation. Here, the effect of leucine on thrombin generation was also evaluated in 19 normal plasma, as previously described. The data shows that in 13 of 19 (68 %) individual normal plasmas the approx. SC200 of added leucine is 0.4 ± 0.4 mM (MV \pm 1SD) (Figures 47-49). Interestingly, 4 of 19 (21 %) individual normal plasmas were resistant against added leucine when added to less than 9 mM (Figure 50). When tested against 2 different normal pools the approx. SC200 was about 0.4 ± 0.2 mM added leucine (Figure 51). 2 of 19 (11 %) plasmas had a different reaction pattern: the first one was sample 12 with an approx. SC200 value of 0.2 mM added leucine and an IC₅₀ value of 2 mM (Figure 52). And the second one was sample 8 which had no approx. SC200 but an approx. IC₅₀ value of 0.1 mM added leucine (Figure 53). Collectively, this data indicate that leucine has also differential actions on thrombin generation.

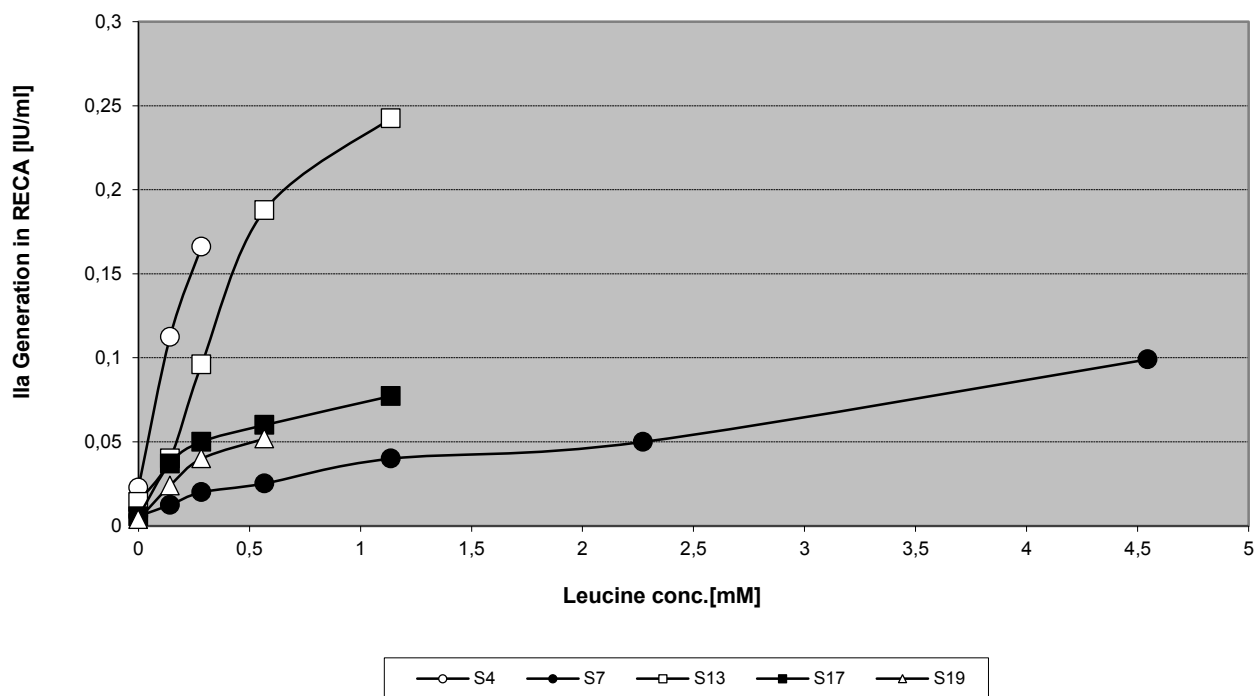


Figure 47. F2a generation in leucine – supplemented normal plasmas. 50 μ l samples of 5 unfrozen normal citrated plasmas (\circ , \bullet , \square , \blacksquare , \triangle) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20 (20 min CRT). The approx. SC200 values were 0.1 mM added leucine for all of them.

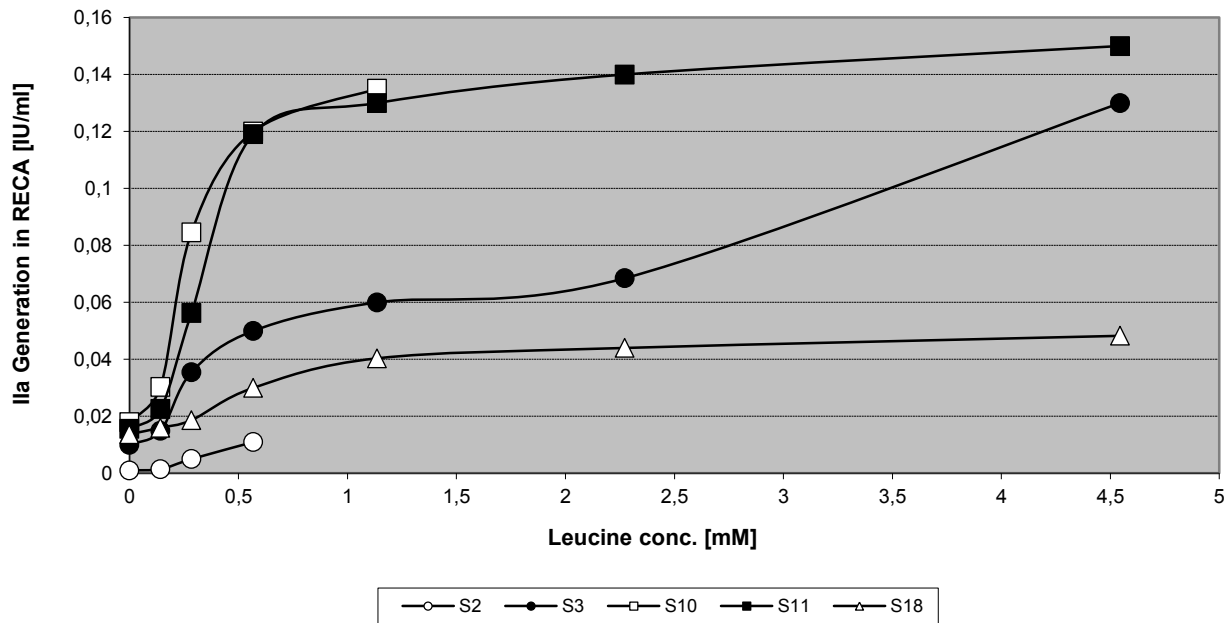


Figure 48. F2a generation in leucine – supplemented normal plasmas 50 μ l samples of 5 unfrozen citrated plasmas (○, ●, □, ■, △) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20. The approx. SC200 values were between 0.2 and 0.5 mM added leucine.

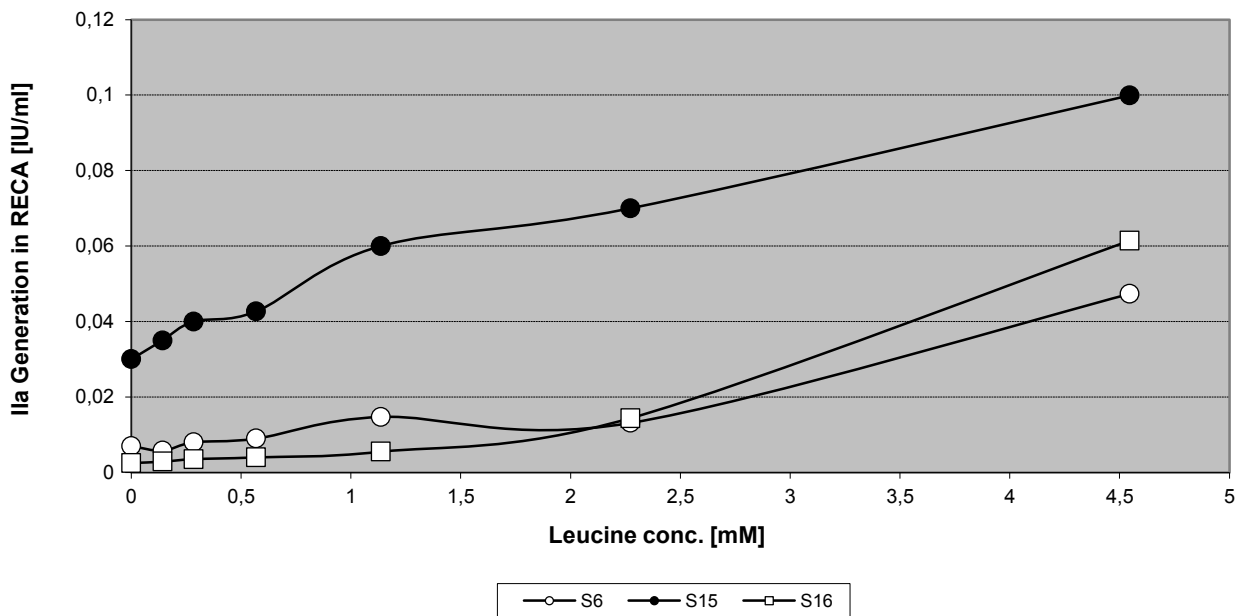


Figure 49. F2a generation in leucine – supplemented normal plasmas 50 μ l samples of 3 unfrozen normal citrated plasmas (○, ●, □) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-20. The approx. SC200 values were around 1 mM added leucine.

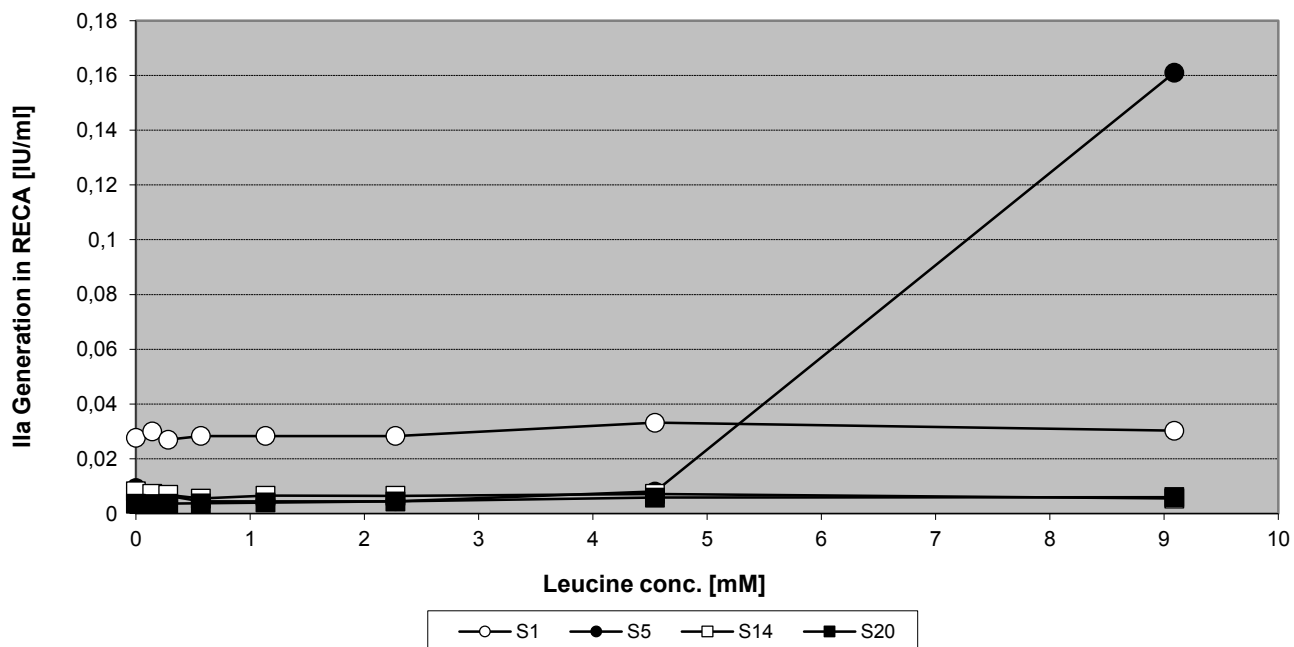


Figure 50. F2a generation in leucine – supplemented normal resistant plasmas 50 μ l samples of 4 normal citrated plasmas (\circ , \bullet , \square , \blacksquare) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-30. The approx. SC200 values were 5 mM added leucine for the second one and the others were resistant up to 9 mM added leucine.

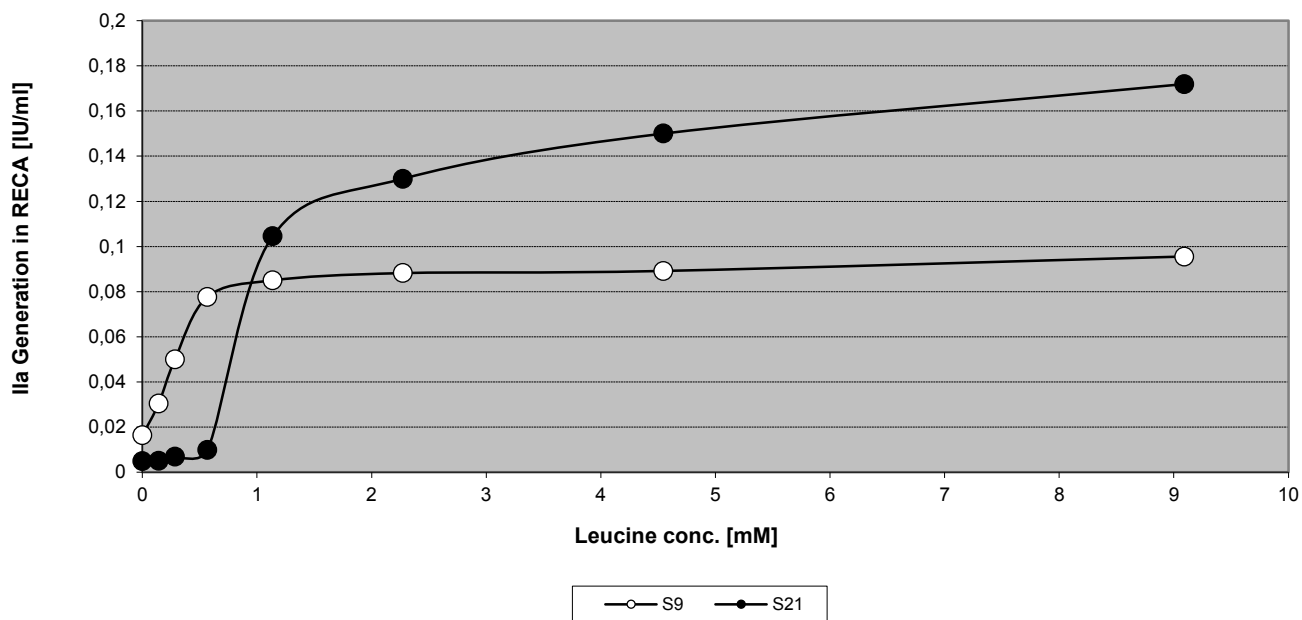


Figure 51. F2a generation in leucine – supplemented pools of normal plasmas 50 μ l samples of 2 unfrozen pools of normal citrated plasmas (\circ , \bullet) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in RECA-10 (10 min CRT). The approx. SC200 values were 0.2 and 0.6 mM added leucine, respectively.

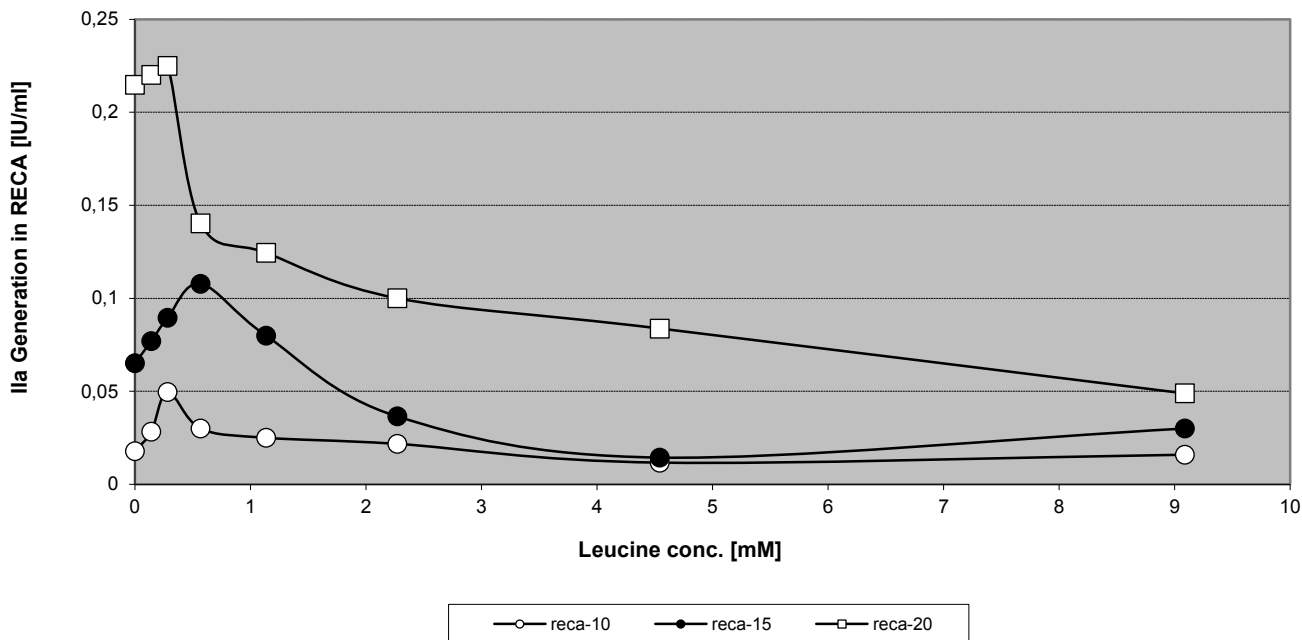


Figure 52. F2a generation in leucine – supplemented normal plasmas 50 μ l samples of 1 unfrozen normal citrated plasma (S12) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in (RECA-10, 15 and 20). The plasma had approx. SC200 value of 0.2 mM added leucine and then IC50 values of 2 mM.

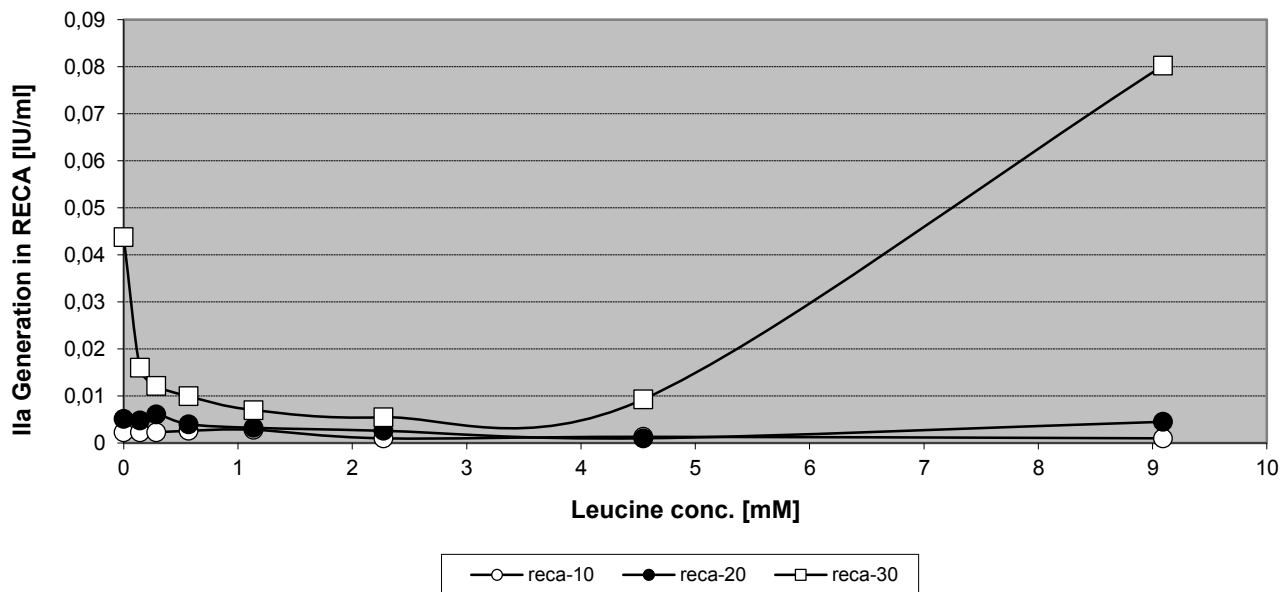


Figure 53. F2a generation in leucine – supplemented normal plasmas 50 μ l samples of 1 unfrozen normal citrated plasma (S8) were supplemented with 0-9 mM additional leucine by repetitive 1+1 dilution on the microtiter plate (Brand®701300). Immediately thereafter they were analyzed in (RECA-10, 20 and 30). The plasma had approx. IC50 values of 0.1 mM added leucine.

4.4. Thrombin generation by glucose

Thrombin generation by the delta-negative charge of glucose was also tested in individual citrated plasmas. The approx. SC200 for added glucose on intrinsic thrombin generation in individual citrated plasmas was 0.3 ± 0.1 mM (MV \pm 1SD) (Figure 54). The approx. SC200 of the mean values of the individual thrombin generations [% un-supplemented control] was 0.3 mM added glucose (Figure 55). Pooled normal plasma had also an approx. SC200 of 0.3 mM added glucose (Figure 56). Freshest normal plasma had an approx. SC200 of 0.3 mM or 0.1 mM added glucose, when glucose was added up to 20 mM (Figure 57a) or up to 2 mM (Figure 57b), respectively.

Addition of glucose up to about 1 mM to plasma always resulted into a proportional increase of F2a generation. However, in some samples the results demonstrate that addition of glucose to plasma may not result into a strict increase of individual F2a generation: there might be glucose concentrations where the individual F2a generation decreases, e.g. around 2-3 mM, 5 mM, or around 8-12 mM added glucose.

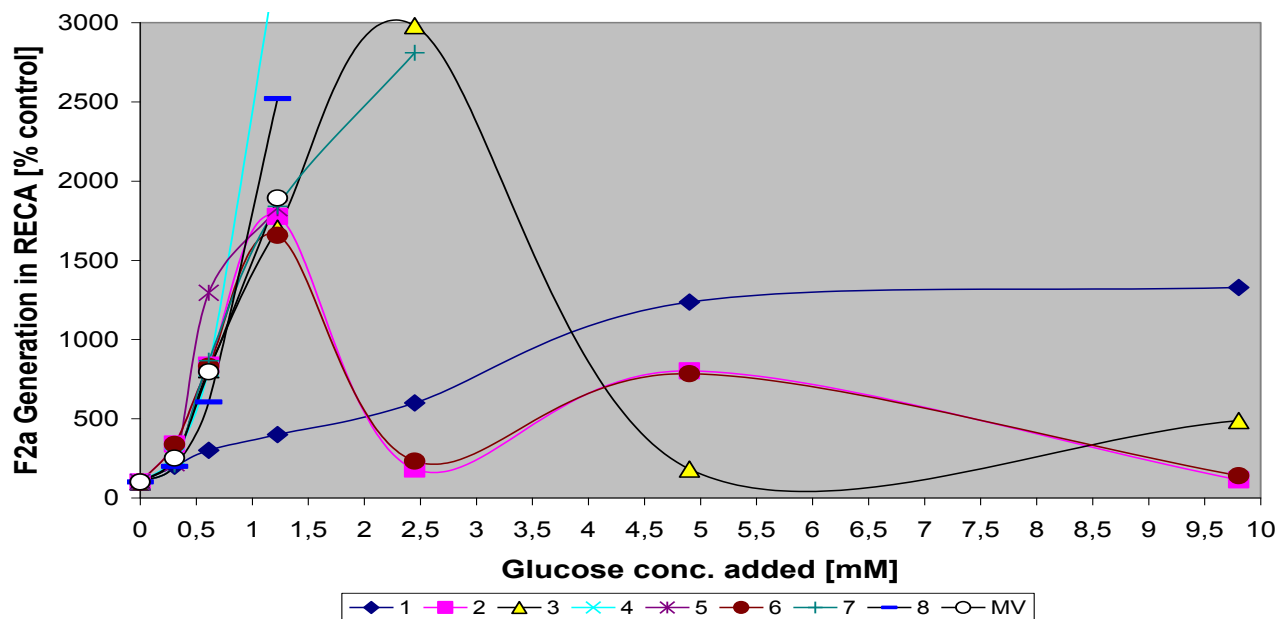


Figure 54. Thrombin (F2a) generation by glucose. 50 μ l samples of 8 normal human citrated plasmas (individual symbols) in high quality polystyrene wells (Brand®781600) were supplemented with additional 0-19.6 mM glucose. The RECA was immediately started. Valid and shown are only the most sensible thrombin generations in the ascending part of the CRT vs. F2a generation – curve. Mean thrombin generations (O); the approx. SC200 values on intrinsic thrombin generation were 0.3 ± 0.1 mM (MV \pm 1SD) added glucose.

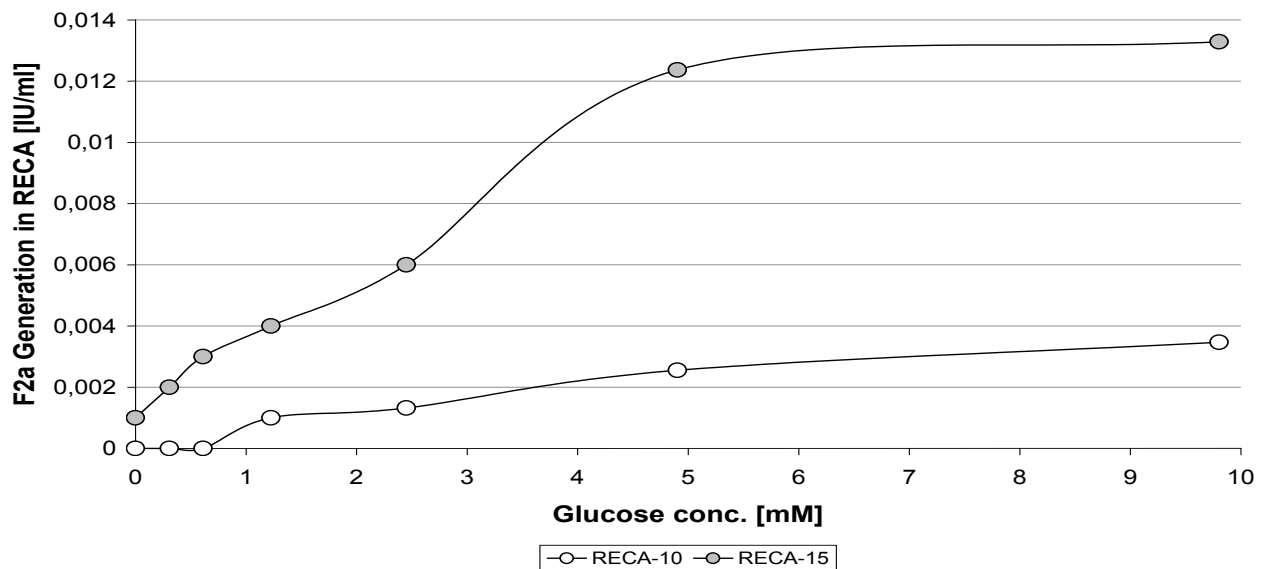


Figure 55. F2a generation by glucose. 50 μ l samples of normal human citrated plasma 1 in high quality polystyrene wells (Brand®781600) were supplemented with additional 0-19.6 mM glucose. The RECA was immediately started. The approx. SC200 value on intrinsic thrombin generation was 0.3 mM added glucose.

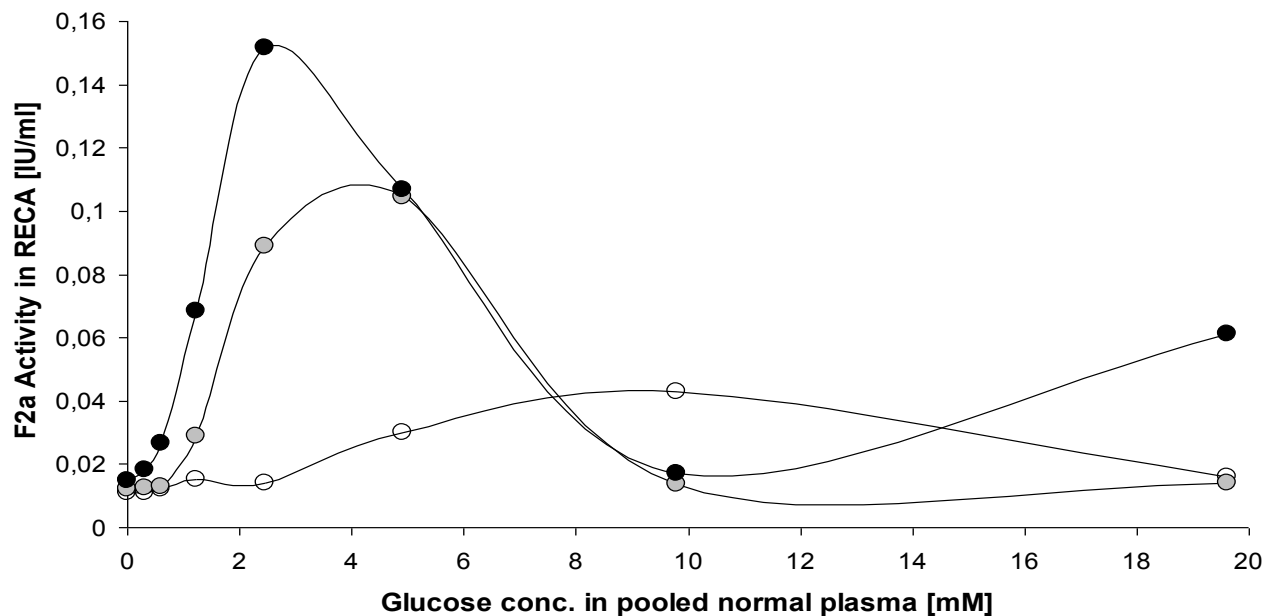


Figure 56. F2a generation by glucose. 50 μ l samples of pooled normal human citrated plasma in high quality polystyrene wells (Brand®781600) were supplemented with additional 0-19.6 mM glucose. The RECA was immediately started (RECA-15 = O, RECA-20 = greyO, RECA-25 = ●). The approx. SC200 value on intrinsic thrombin generation was 0.3 mM added glucose.

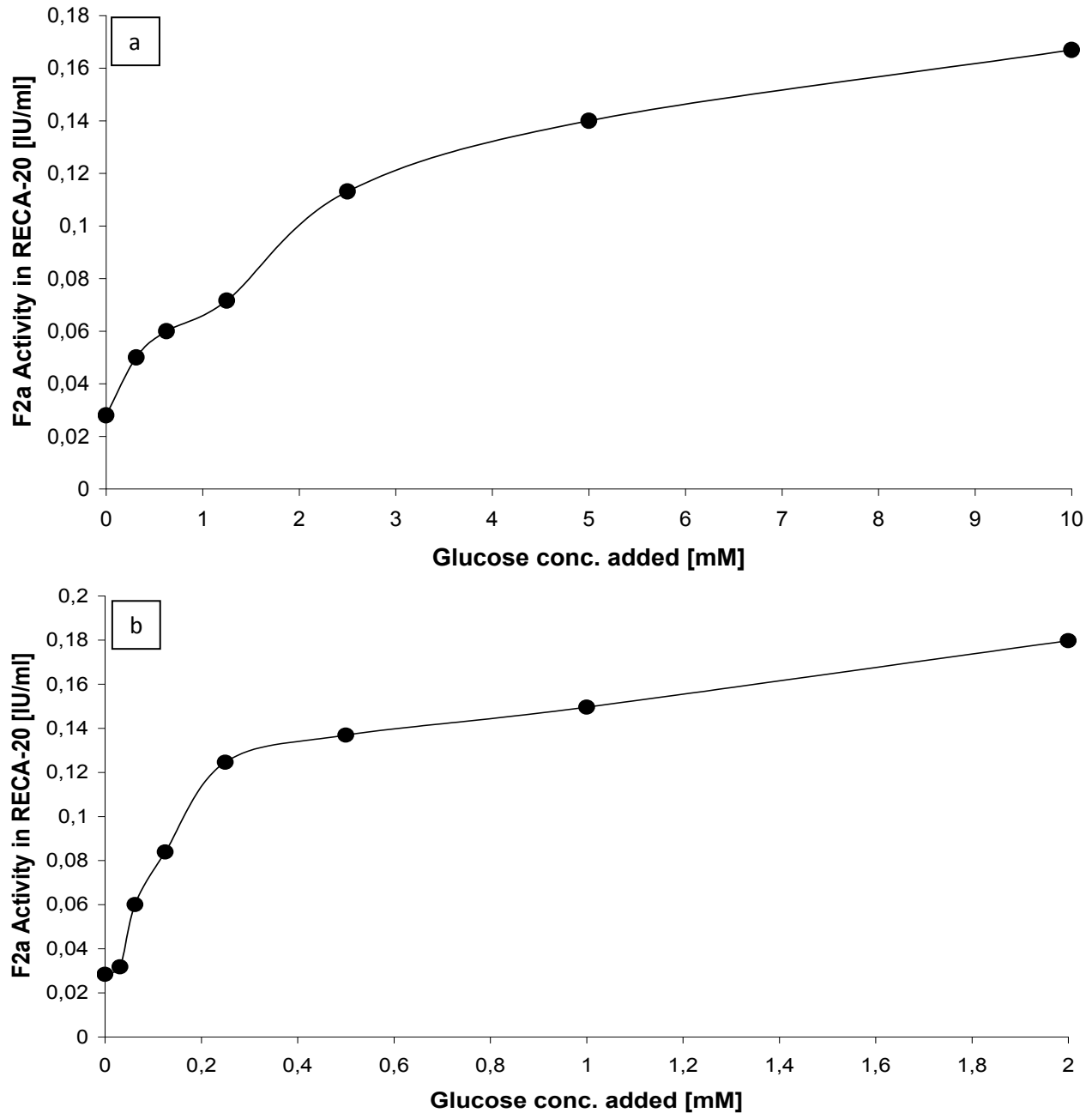


Figure 57. Thrombin generation in freshest normal plasma. 50 μ l samples of freshest normal plasma (<1h 23°C) in high quality untreated U-wells polystyrene microwells (Brand®781600) was supplemented with up to 20 mM additional glucose or up to 2 mM additional glucose. The RECA was performed with 20 min coagulation reaction time (RECA-20). The approx. SC200 on intrinsic thrombin generation were 0.3 mM for addition up to 20 mM glucose (Figure 57a) or 0.1 mM for addition up to 2 mM glucose (Figure 57b).

4.5. Systemic thrombin generation in mice

In this last part thrombin generation was evaluated in mice following i.p. injection of either valproate or glucose as detailed in materials and methods. The F2a activity in normal murine EDTA-plasma was 654 ± 109 % (MV \pm 1SD) of normal, when compared with the human 100% of normal standard, or 100 ± 17 % of normal, when calculated normal murine MV \pm 1SD. This is equivalent to 36 ± 6 mIU/ml F2a.

4.5.1. Thrombin generation in mice blood after i.p. valproate

Mice were i.p. injected with 0-10 mg valproate. 2 mg valproate i.p. enhanced the circulating F2a activity by about 2 fold, while 10 mg valproate i.p. enhanced F2a about 3 fold (Figure 58). Furthermore, 0.05 mg valproate resulted in about 120% increase of normal F2a, whereas 0.2 mg valproate induced an increase of about 150% of normal F2a (Figure 59). 120% of normal F2a is the value, where the normal human intravascular coagulation (NIC) changes into pre-pathologic disseminated intravascular coagulation (PIC-0) (pre-PIC). 150% of normal is the value, where PIC-0 changes into PIC-1 (typical PIC). 200% of normal would be the value, where PIC-1 changes into PIC-2 (consumption PIC).

An i.p. injection of 100 μ l 0.5 mg/ml (0.05 mg) or 10 mg/ml (1 mg) valproate resulted in 9 mg/l or 204 mg/l valproic acid in EDTA-plasma. Thus, the 120% F2a level was reached with plasma concentrations of about 10 mg/l valproate.

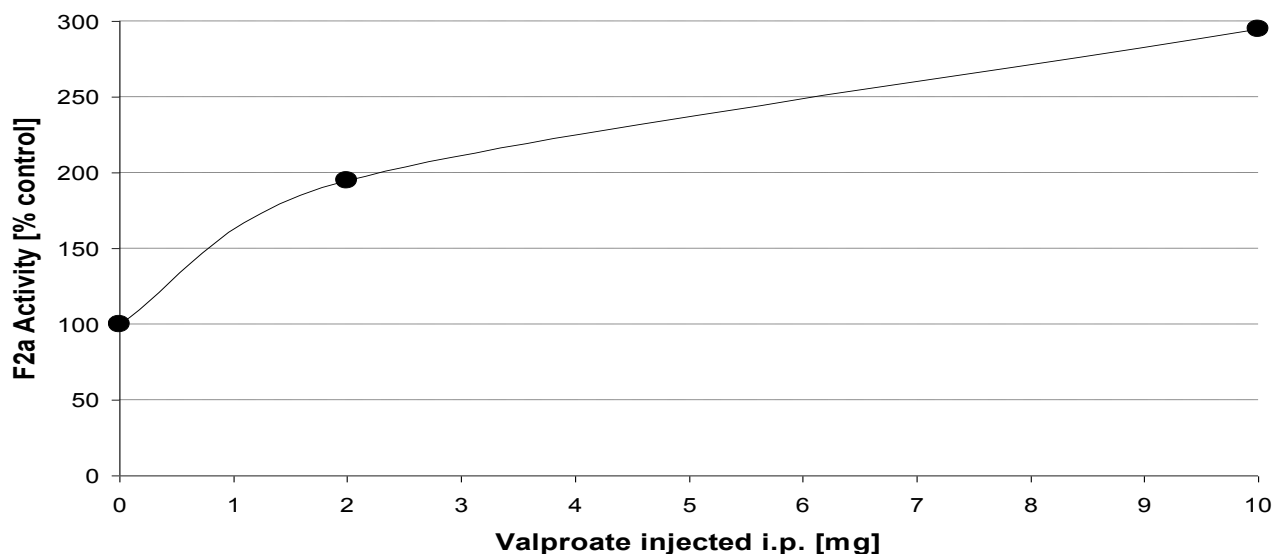


Figure 58. *In vivo* thrombin generation by valproate. Mice were i.p. injected with 0-10 mg valproate. After 1h EDTA-blood was withdrawn, stabilized 1+1 with 2.5 M arginine, pH 8.6, and analyzed for circulating thrombin (F2a) activity. Mice with 0 mg valproate were the control mice (100 % of murine norm; mean values; CV values < 10%).

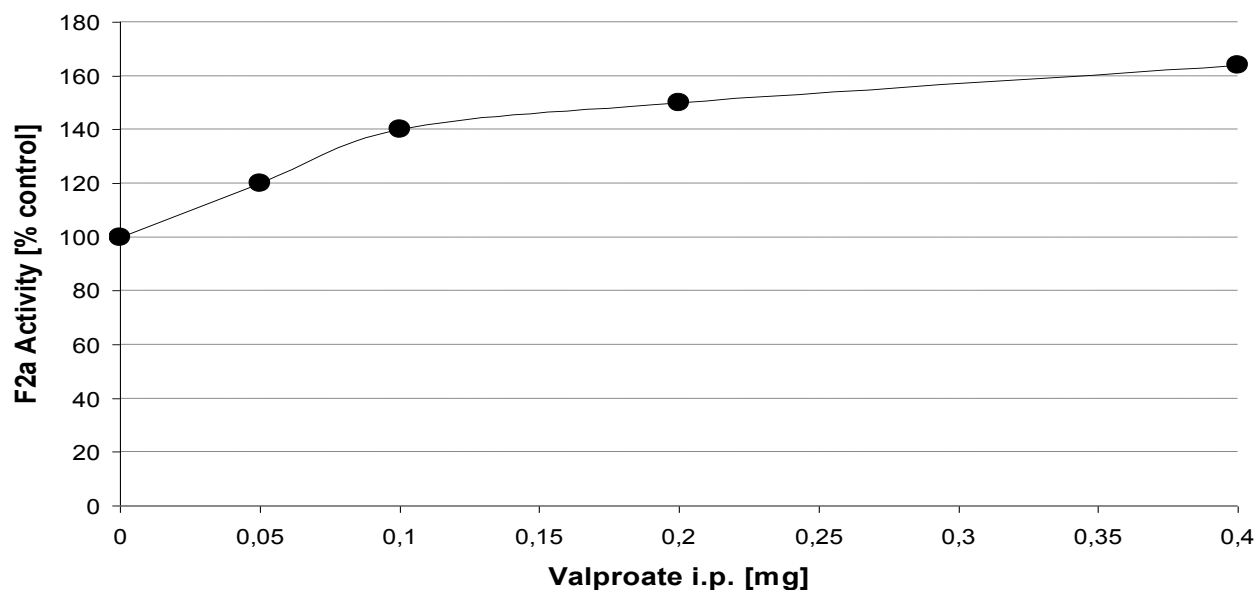


Figure 59. *In vivo* thrombin generation by valproate. Mice were i.p. injected with 0-0.4 mg valproate. After 1h EDTA-blood was withdrawn, stabilized 1+1 with 2.5 M arginine, pH 8.6, and analyzed for systemic thrombin (F2a) activity. Mice with 0 mg valproate were the control mice (100 % of murine norm; mean values, CV values < 10 %).

4. 5. 2. Thrombin generation in mice blood after i.p. glucose

In this part of the study the mice were *i.p.* injected with different amounts of glucose (100 μ l and 300 μ l 1 M). The control group was injected with 0.9% NaCl. After 0.5 h there was a proportional linear increase of systemic thrombin activity with the increase in the amount of glucose injected whereby 0.15 and 0.3 mmoles glucose *i.p.* resulted in approximately 1.4 and 1.9 fold systemic thrombin activity, respectively (Figure 60). As shown in another experiment (Figure 61(a)), systemic F2a activity increased significantly time and dose dependently. 1 h after *i.p.* injection of 0.15 - 0.3 mmoles glucose resulted in about 1.4 fold increase of plasmatic glucose and in about 2.5 fold increase of systemic F2a activity (Figure 61(b)). This implies a continuous systemic generation of thrombin over time parallel to the increase of plasmatic glucose.

Even glucose amounts of less than 0.15 mmoles *i.p.* resulted in a significant increase of systemic F2a. So a glucose amount was chosen (300 μ l 0.125 M = 0.038 mmoles) that has a clear but still not too pronounced action on systemic thrombin generation: here an increase of the plasmatic glucose of about 1.5 fold could result in about 4 fold increase in systemic thrombin activity, depending on the time interval between glucose *i.p.* injection and blood drawing. The optimal time interval to withdraw blood was after about 45 min that reflected well the action of glucose on systemic thrombin activity (Figure 62).

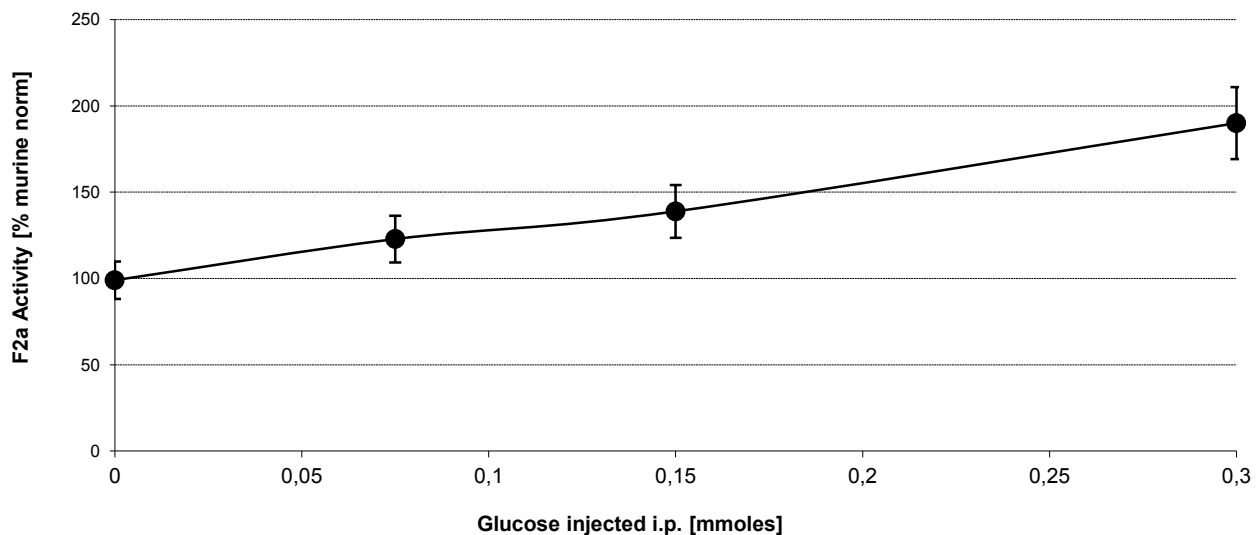


Figure 60: *In vivo* Thrombin generation by glucose. Mice were i.p injected with 300 μ l of glucose (0-1M). After 0.5 h EDTA-blood was withdrawn, centrifuged and the plasma stabilized with 1+1 with 2.5M arginine (pH 8,6) and analyzed for systemic thrombin (F2a) activity. Mice treated with NaCl were the 100% control mice.

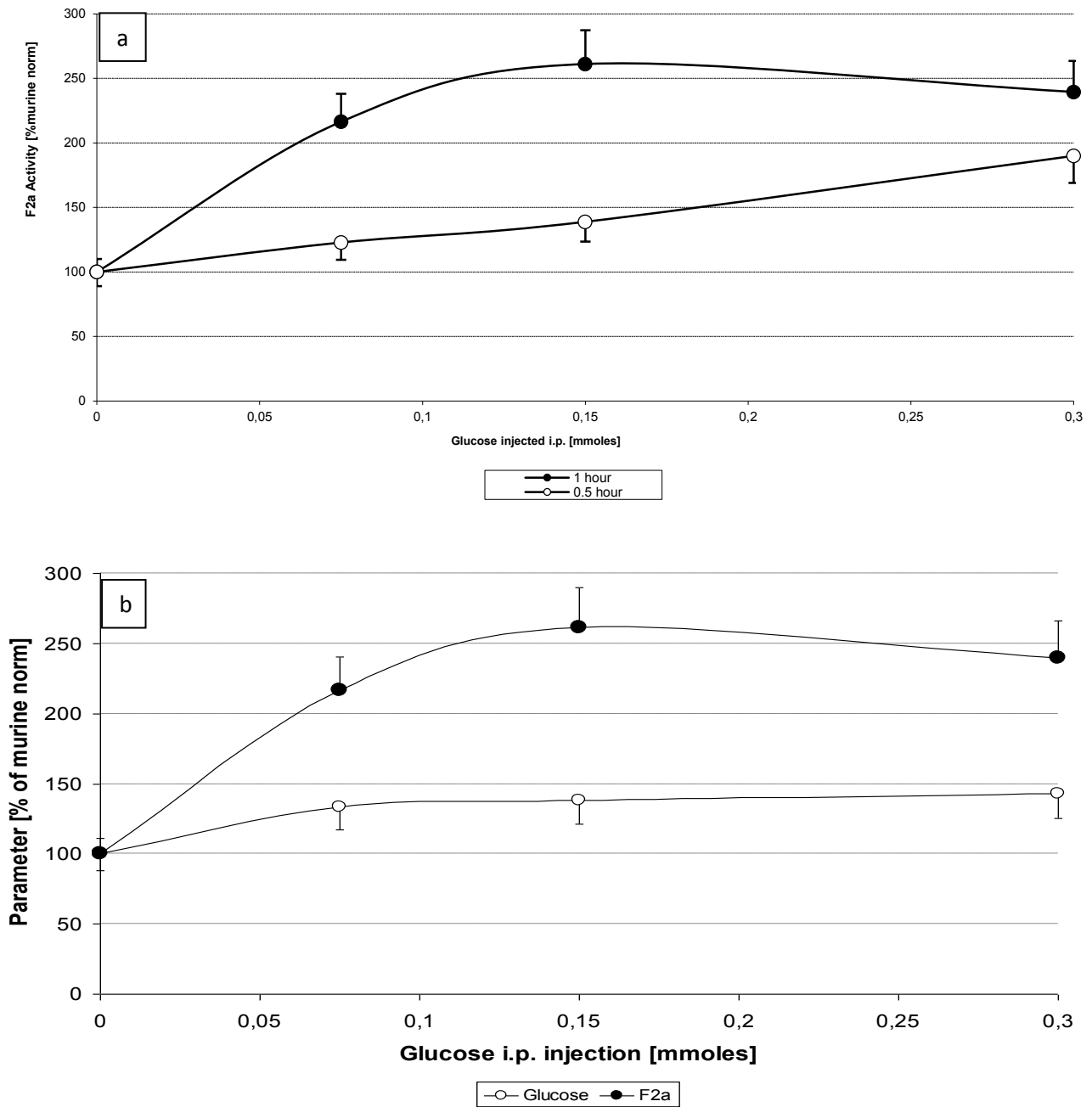


Figure 61: In vivo thrombin generation by glucose. Mice were i.p. injected with 300 μ l 0-1 M glucose. After 0.5, 1 h EDTA-blood was withdrawn, centrifuged, the plasma stabilized 1+1 with 2.5 M arginine, pH 8.6, and analyzed for circulating thrombin (F2a) activity (Fig. 2a). The plasmatic glucose concentrations are shown in figure 2b. Mice with 0 mg glucose were the control mice (100 % of murine norm F2a = 583 % of human norm F2a [95]; 100 % of murine norm glucose = 110 mg/dl = 6.1 mM; mean values).

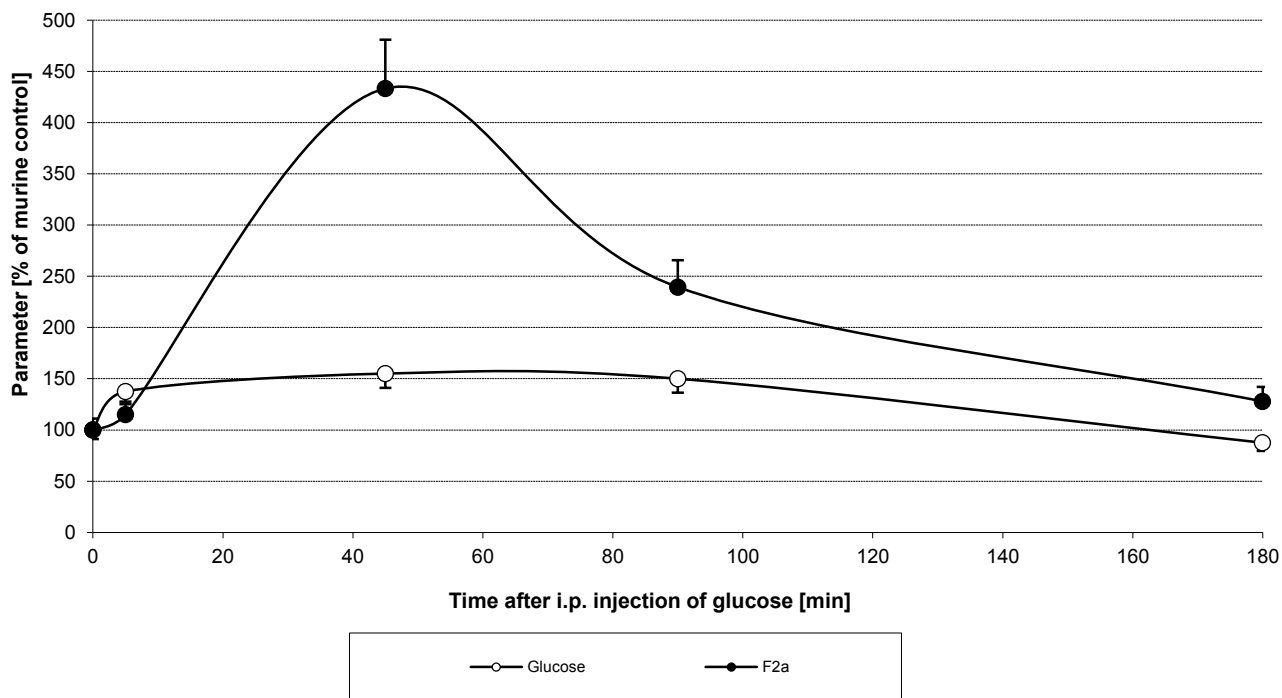


Figure 62: *In vivo* thrombin generation by glucose. Mice were i.p. injected with 300 μ l 0.125 M = 0.038 mmoles glucose. After 0-3h EDTA-blood was withdrawn, centrifuged, the plasma stabilized 1+1 with 2.5 M arginine, pH 8.6, and analyzed for systemic thrombin (F2a) activity. Mice with 0 mg glucose injected i.p. were the control mice.

Collectively, thrombin generation can be induced by the tested substances. Prophylactic treatment with LMWH is recommended for susceptible patients. Proposed scheme showing thrombin generation by valproate and glucose are presented in figure 63, respectively.

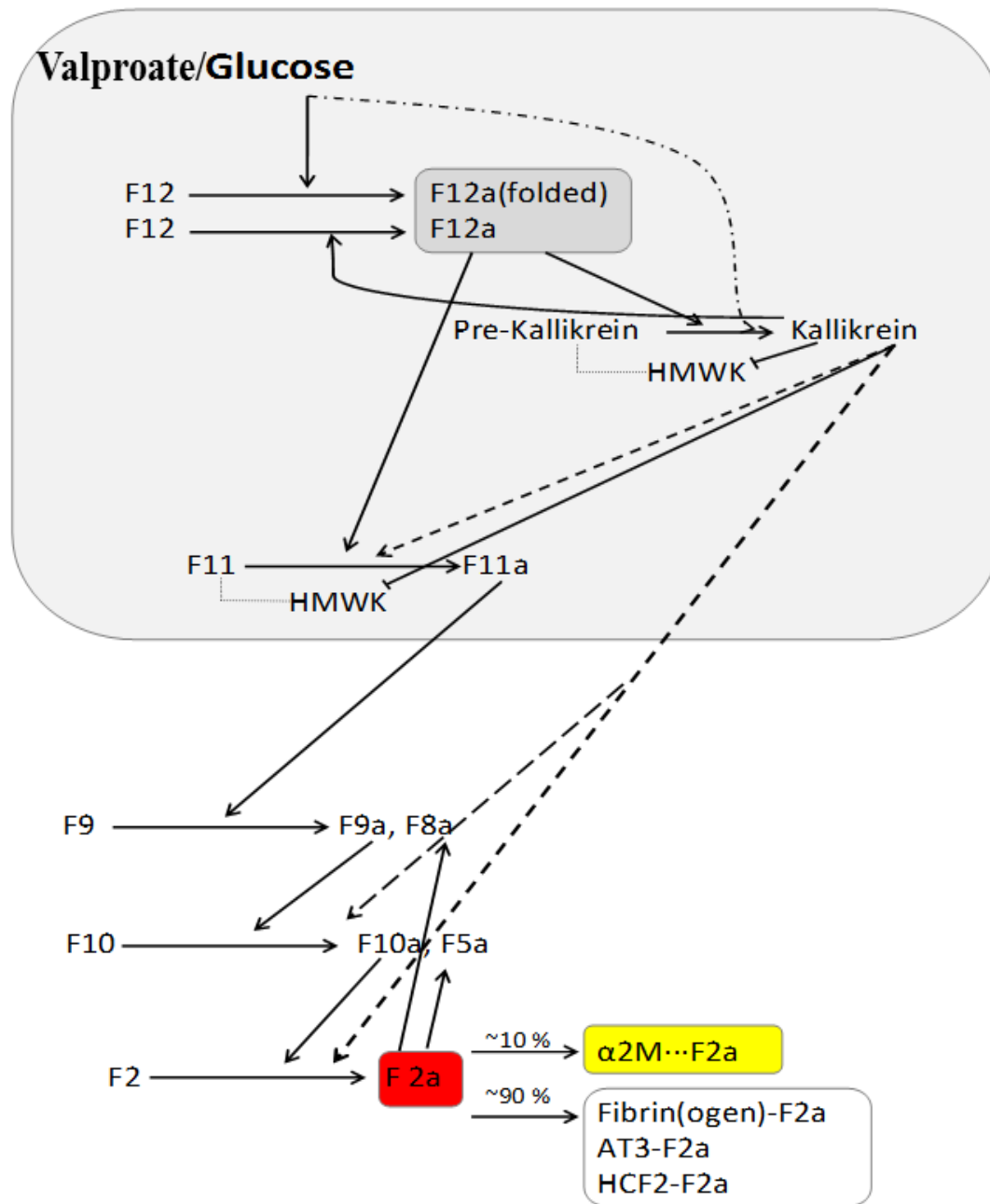


Figure 63. Thrombin (F2a) generation by valproate/glucose. Plasmatic valproate or increased plasmatic concentrations of glucose fold F12 and/or pre-kallikrein into the respective activated form. F12a and/or kallikrein are the main initiators of intrinsic coagulation. The intrinsic F10-ase is generated (F9a·F8a·Ca²⁺·PL) that generates F10a and F2a. About 10% of the generated F2a is entrapped in α2M. The amidolytic activity of α2M--F2a is measured photometrically [79,80]. picture modified according to [81].

5 Discussion

Pathological thrombin generation results in many complications such as thrombotic/atherosclerotic diseases. Lipophilic or negatively charged substances trigger the contact phase of blood coagulation at sub-millimolar plasma concentrations [66,82].

Patients susceptible to intrinsic thrombin generation can be identified by the new ultra-specific, ultra-sensitive chromogenic thrombin generation assays such as the recalcified coagulation activation activity assay (RECA), which detects any plasmatic pro-thrombotic tendency, prior drug administration to avoid unwanted complications of hemostasis. Due to its lipophilic and negatively charged nature of valproate this drug has the potential to trigger intrinsic thrombin generation. Interestingly, literature on the effect of valproate on thrombin generation is lacking. Therefore, this thesis focused mainly on investigating the effect of valproate on thrombin. Here it is shown that 83% of all normal citrated plasmas had an SC200 \leq 100 mg/l valproate. Their SC200 MV \pm 1 SD was 28 ± 27 mg/l. 31 normal plasma pools had SC200-values of 11 ± 9 mg /l (MV \pm 1 SD; range: 2-35 mg / l). The mean thrombin generations of the freshest plasmas (about 30 min old, stored at 23°C) gave an approx. SC200 of 10 mg/l valproate, while pooled normal plasma (out of 10 freshest plasmas) had an approx. SC200 of 5 mg/l. The analysis of about 10 fresh individual plasmas reflects similar results as the analysis of 235 individual normal plasmas. Moreover, in commercial lyophilized normal plasma the approx. SC200 was 20 mg/l valproate. It is worth mentioning that the concentration of valproate leading to thrombin generation often is lower than the inferior limit of the therapeutic range of valproate (50 mg/l), which emphasizes the importance of these findings. Furthermore, to evaluate the role played by different factors of the cascade on thrombin generation, the effect of valproate was evaluated using commercial stabilized plasma depleted for different factors (12, 11, 9, 8, 10, 5, 2, or 7). Differential effect of valproate on thrombin generation was observed. For instance, the approx. SC200 for the different depleted plasma were: 120 mg/l (F9 and F2), 150 mg/l (F8), 180 mg/l (F10), and 15/30 mg/l (F5). For F7 deficient plasma the approx. SC200 is 6 mg/l valproate, presumably because in F7 deficient plasma the whole intrinsic clotting cascade is nearly not affected. About 200 mg/l valproate were needed to increase the basal thrombin generation by 100 % in F12 deficient plasma, which underlines the enormous importance of F12 in contact phase triggered thrombin generation.

Previous data shows that addition of 3.8 mIU/ml (polynegatively charged) UFH to normal plasmas can further stimulate thrombin generation in susceptible valproate-supplemented plasmas [78] or it can inhibit thrombin generation (Data obtained from this thesis). Addition of 0.5 IU/ml LMWH neutralized the enhancement of thrombin generation by valproate. Yet, if the approx. SC200 is low, even 0.5 IU/ml LMWH does not completely inhibit enhanced thrombin generation. Collectively, based on the obtained results and in order to avoid thrombotic/atherosclerotic complications anticoagulant prophylaxis should be considered in chronic valproate administration, especially in patients with an approx. SC200 far below 50 mg/l valproate, which is the inferior limit of the therapeutic range of valproate. LMWH might be prophylactically or therapeutically dosed according to the EXCA (extrinsic coagulation activity assay [83]) or the INCA (intrinsic coagulation activity assay [84]), which are new sensitive assays to monitor plasmatic anticoagulation.

For control assay thrombin generation in murine vs. human plasma was compared. The F2a activity in the normal murine EDTA-plasma is equivalent to 36 ± 6 mIU/ml while the normal human EDTA-plasma contains 5.5 ± 1.1 mIU/ml F2a [85]. Furthermore, similar difference is in the level of $\alpha 2$ -macroglobulin ($\alpha 2$ M) whereas the murine plasma contains about 3-4 fold more $\alpha 2$ M than human plasma, which contains about 2 g/l $\alpha 2$ M [86,87]. Such a comparison is even more complicated for rats due to the fact that their normal blood contains only about 0.1 g/l $\alpha 2$ M; yet, in rats the $\alpha 2$ M is a strong acute phase reactant and can increase up to about 100 fold in inflammation [88,89]. On the other hand, murine $\alpha 2$ M is only a minor acute phase reactant and increases only up to about 2 fold [87]. Nevertheless, mice might have a different plasmatic thrombin generation / thrombin inactivation than humans. Murine thrombin-- $\alpha 2$ M could be more reactive than human thrombin-- $\alpha 2$ M under the used assay conditions (supra-molar conc. of arginine and chromogenic thrombin substrate). Therefore, translation of the results of the experiments with mice/rats in hemostasis into the physiology/pathophysiology of human hemostasis is difficult, e.g. a drug that needs a high concentration in mice/rats to trigger or inhibit coagulation might need a much lower concentration in humans [90–95]. Therefore, results obtained from ultra-specific, ultra-sensitive thrombin generation assays of the newest generation such as the RECA, INCA, or EXCA that are performed in human plasma [76,96] are more reliable than the *in-vivo* results obtained from mice/rats [81,97–99]. Consequently, whenever

possible, an animal experiment should be replaced by a good *in-vitro* thrombin generation test in individual (or pooled) human plasma.

Valproate-blood as a strange matrix (= environment of valproate including both the liquid plasma and the surfaces of blood cells / vessel cells) when compared to normal blood stimulates intrinsic coagulation.

Interestingly, leucine has also shown similar approx. SC200 values on recalcified plasmatic thrombin generation as glycine [9] or valproate. This is not surprising considering the similar chemistry of the molecule that results into a similar biochemistry with respect to triggering intrinsic thrombin generation. This action of leucine should also be taken into consideration if amino acids such as leucine are infused or if it is planned to add leucine to the galenical composition of a drug.

Tryptophan effect on thrombin generation was also evaluated since in addition to its effectiveness for treatment of a variety of conditions typically associated with low serotonin levels in the brain [57], it seems to act also on thrombocytes. A diet enriched with tryptophan enhanced platelet aggregation due to an increase in serotonin and its metabolites, demonstrated by the increased urinary excretion of 5-hydroxyindole acetic acid [100]. It has been shown that dietary tryptophan supplementation increases serotonin biosynthesis. The fact that amine is taken up by platelets it enhances platelet aggregation and usually acts as a vasoconstrictor [61]. In this part tryptophan induced intrinsic thrombin generation in pooled plasma with approx. SC200 values that are far lower than the approx. SC200 (10 mM) of imidazole (a molecule that is similar to a part of the indole side group) [64]. It is likely that the lipophilic benzene structure in the side group of tryptophan might contribute to a lower approx. SC200 value of tryptophan when compared with glycine [9]. Briefly, the present data demonstrate that tryptophan may also change plasmatic coagulation whereas the coagulation system of many patients will be susceptible to an infusion of only about 0.1-2.5 mM (final conc.) of an amino acid such as tryptophan [101]. If such an infusion is clinically required, the infusion should be accompanied by LMWH prophylaxis that protects against pathologic increases of plasmatic thrombin activity (IIa). Typically a target value of about 20 % of normal EXCA could be the adequate prophylactic – therapeutic anticoagulation [77,83]. Severe pro-thrombotic changes might even require EXCA-values of about 10 % of the normal.

The effect of glucose as a trigger for thrombin generation was also tested. Addition of glucose to plasma at a concentration up to 1 mM always resulted into a proportional increase of F2a

generation. However, glucose addition may not result into a strict increase of individual F2a generation since there might be glucose concentrations where the individual F2a generation decreases, e.g. around 2-3 mM, 5mM, or around 8-12 mM. The decrease of thrombin generation at about 10 mM added glucose seen when compared RECA-10 with RECA-15 is possibly related to a kallikrein-mediated opening of the α 2M cage, releasing entrapped thrombin that is then irreversibly inactivated by antithrombin-3 (AT3). For instance, the higher the amount of added glucose to plasma, the greater is the approx. SC200. Briefly, 200 mM added glucose resulted in an approx. SC200 of about 3 mM added glucose, while 20 mM added glucose resulted in an approx. SC200 of about 0.3 mM added glucose. The delta-negatively charged molecule glucose seems to fold the main inactivator of human thrombin = AT3 into an activated form. Therefore, glucose triggers as usual the intrinsic coagulation but the triggering action is antagonized by synchronously activated AT3. In conclusion, spurious increases of plasmatic glucose can pathologically enhance plasmatic thrombin generation: even an increase of just 0.1 mM, i.e. only 2% of the normal glucose concentration, can double plasmatic intrinsic coagulation. Glucose has to be considered as an important pathophysiologic contact trigger. Interestingly, pathologically accelerated coagulation has been suspected as a pathogenic factor that induces mesangial proliferation [96,97,102], as seen in diabetic glomerulopathy, one of the typical complications in diabetes. Furthermore, it has been shown that an enhancement of F10a generation can also cause diabetic nephropathy [85,96]. This is evidenced by the fact that the use of F10a inhibitors, such as fondaparinux, suppresses both glomerular hypertrophy and hypervascularity in db/db mice [103]. Interestingly, F10a generation might be triggered both intrinsically (via glucose itself) or extrinsically (via tissue factor = TF). Since the mesangium is rich in monocytes/macrophages, which are important generators of TF, therefore, upon inflammation, mesangial cells seem to produce large amounts of TF and of F5 [104]. Patients with type 2 diabetes mellitus are indeed in a hypercoagulable state that can only be determined by a thrombin generation assay, preferably without addition of phospholipids (PL) or TF [105], an assay version that is comparable to the RECA. The conventional plasmatic coagulation tests, such as PT, APTT, AT3, protein C, factors 2 and 8 are too blunted to detect the diabetic hypercoagulability. Diabetic patients have 5041/ μ l circulating micro-particles (mean value), 91 % of them bearing platelet antigens, while healthy controls have 1763/ μ l circulating micro-particles (mean value), 88 % of them seem to be platelet - derived. The best correlation ($r=0.66$) was found between the total number of micro-particles and the thrombin peak in a thrombin generation assay without PL and without TF. Finally,

contact triggers increase and can also decrease, intrinsic coagulation [106]. These findings strongly suggest that LMWH prophylaxis should be considered in the chronic phase of diabetes mellitus to avoid the generation of micro- and macro-thrombi [83,98,105,107].

Based on the data obtained from this thesis work and using an innovative thrombin generation test it is obvious that in order to avoid thrombotic/atherosclerotic complications anticoagulant prophylaxis might be considered in 1) chronic valproate administration, especially in patients with an approx. SC200 below 50 mg/l valproate, which is the inferior limit of the therapeutic range of valproate, 2) if an administration of about 1-2 mM (final plasma conc.) of an amino acid such as tryptophan or leucine is clinically required, and 3) in diabetic patients since even spurious increases of plasmatic glucose can pathologically enhance plasmatic thrombin generation.

6 Zusammenfassung

Pathologisch-erhöhte Aktivität von Thrombin in Blut kann zu Komplikationen führen, wie zum Beispiel zu thrombotischen/ atherosklerotischen Erkrankungen. Negativ geladene oder lipophile Substanzen können bereits in niedrigen Plasma-Konzentrationen die intrinsische Blutgerinnung (Starterenzyme: F12a und/oder Kallikrein) aktivieren. Es besteht eine große individuelle Variabilität bezüglich des Starts der intrinsischen Hämostase (z. B. durch unterschiedliche Proteinglykosylierung, unterschiedliche Plasmamatrix). Daher sind einige Personen besonders anfällig für gewisse Medikamente, die eine pathophysiologische Thrombinbildung auslösen können. Um unerwünschte Komplikationen zu vermeiden, könnten diese Patienten vor der Medikamentengabe durch den *Recalcified Coagulation Activity Assay* (RECA) mittels Bestimmung der 200% stimulierenden Konzentration (SC200) identifiziert werden. Dieser neue hochspezifische Thrombinbildungstest erkennt jede prothrombotische Tendenz im Blutplasma. Da Valproat, ein einfaches lipophiles und negativ geladenes Molekül, ein weit verbreitetes Medikament zur Behandlung verschiedener Krankheiten wie Epilepsie ist, konzentrierte sich diese Arbeit hauptsächlich auf die Wirkung von Valproat auf die Thrombinbildung. Andere Substanzen, die aufgrund ihrer lipophilen oder negativ geladenen Bestandteile die Thrombinbildung auch auslösen können, wie Tryptophan, Leucin oder Glucose, wurden zum Vergleich ebenfalls untersucht.

195 von 235 normalen Plasmen mit $SC200 \leq 100$ mg/l Valproat hatten eine Thrombin-Generierung von 28 ± 27 mg/l ($MV \pm 1$ SD). 31 normale Citratplasma-Pools hatten SC200-Werte von ca. 11 ± 9 mg / l ($MV \pm 1$ SD; Gesamtbereich: 2-35 mg / l). Sehr frisches (<1 Stunde altes) normales humanes Plasma ergab ähnliche Ergebnisse wie normales humanes Plasma, das einige Stunden bei 23°C gelagert wurde. Valproat induzierte die Thrombinbildung auch in verschiedenen lyophilisierten Mangel-Plasmen. Die Thrombinbildung durch Valproat wurde durch prophylaktische Konzentrationen (0.5 IU/ml) von niedermolekularem Heparin (LMWH) stark gehemmt.

In ähnlicher Weise führen Tryptophan und Leucin in klinisch verwendeten Konzentrationen sowie zu Plasma zugesetzte Glukose zur pathologischen Thrombinbildung. Die in vitro-Ergebnisse zu Valproat und Glucose in humanen Plasmen konnten in vivo bei Mäusen bestätigt werden. Um Patienten (insbesondere mit weiteren pro-thrombotischen Risikofaktoren), die sich in einem hoch-spezifischen und -sensitiven Thrombin-Generierungstest als empfindlich für

Valproat erweisen, vor unerwünschten thrombotischen Komplikationen zu schützen, könnte eine Antikoagulans-Prophylaxe in Betracht gezogen werden.

Die vorliegende Arbeit liefert anhand verschiedener in-vitro- und in-vivo-Modelle den Nachweis, dass in klinisch relevanten Konzentrationen eingesetztes Valproat die Thrombinbildung induziert.

Schlüsselwörter: Thrombin-Generierung, RECA, Valproat, Tryptophan, Leucin, Glukose

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8 List of Abbreviations

$\Delta A/t$	Increase in absorbance per time
5-HTP	5-hydroxytryptophan
$\alpha 2M$	$\alpha 2$ –macroglobulin
approx. SC200	approximate 200 % stimulatory concentration
AT3	Antithrombin-3
BCAA	Branched chain amino acids
CRT	Coagulation reaction time
CPA	Contact phase activity assay
CPT	Contact phase test
CYP450	cytochrome P450
EXCA	extrinsic coagulation activity assay
F2a	Factor 2a
F2a-Test	circulating thrombin activity test
i.p.	intra peritoneal
INCA	intrinsic coagulation activity assay
IU/m ²	international units per square meter
IU/mg	international units per milligram
K ₃ -EDTA	Ethylene diamine tetra acetic acid, tri-potassium salt
LMWH	low molecular weight heparin
MV	mean value
mM	Millimol per liter = mmol/l
MPS	mononuclear phagocytes-system
NIC	normal human intravascular coagulation
PL	Phospholipids
RECA	recalcified coagulation activity assay
SD	Standard deviation
TAT	Thrombin-anti-thrombin complex
TF	Tissue factor
UFH	unfractionated heparin

9 Danksagung

Allen, die mich im Laufe der Zeit, in der diese Arbeit entstanden ist, begleitet haben, möchte ich an dieser Stelle Dank sagen.

Mein Dank gilt Herrn PD. Dr. Thomas Stief, für die Betreuung dieser Arbeit, der freundlichen Hilfe und die zahlreichen Gespräche auf intellektueller und persönlicher Ebene. Ohne Ihre Unterstützung meine Arbeit hätte nicht gelingen können. Gleichzeitig danke ich Herrn PD. Dr. Nobert Ahrens, für Ihre mühevollen Geduld und liebevolles Verständnis und Unterstützung in dieser beschwerlichen Zeit. Mit Ihrer Unterstützung ich könnte meine Arbeit zu Ende zu bringen.

Mein außerordentlicher Dank geht auch an meine liebe Frau PD. Dr. Nahed Elnajjar. Sie hat unzählige Stunden ihrer freien Zeit geopfert, um mich beim Schreiben der Dissertation zu unterstützen. Danke für ihr Verständnis und Geduld in anstrengenden Zeiten bei der Anfertigung dieser Doktorarbeit.

Mein ganz besonderer Dank aber gilt meinen Eltern, die mir meinen bisherigen Lebensweg ermöglichten und denen ich diese Arbeit widme.