

Research Article

A New Pharmacodynamic Test to Evaluate Effects of Oral Contraceptives on Coagulation

de Kruijf P^{1*}, Naji S¹, Krijnen C¹, Jespersen J², and Klufft C¹¹Good Biomarker Sciences, The Netherlands²Department of Public Health, University of Southern Denmark, Denmark

*Corresponding author

P. de Kruijf, Good Biomarker Sciences, Zernikedreef 8, 2333 CL Leiden, The Netherlands, Tel: 31 71 751 71 70; Fax: n/a ;

Email: p.dekruijf@gbsleiden.nl

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Keywords

- Combined oral contraceptives; Thrombodynamics; Clot growth; Fibrin; Thrombin

Abstract

The use of combined oral contraceptives has been associated with an increased risk of thromboembolic disease. Current *in vitro* tests are focusing on one or some sets of multiple factors but are not really pharmacodynamic tests. In this report we present thrombodynamics, which are an elegant *in vitro* analysis that resembles *in vivo* coagulation on a prothrombotic surface and gives insight in the spatial dynamics of the formation of a fibrin clot and thrombin generation. The effect of the use of three different types combined oral contraceptives, belonging to either the 2nd or 3rd generation, on the thrombodynamic parameters have been investigated. Data show that fibrin clot formation and thrombin generation are increased after 6 months treatment of combined oral contraceptives. Furthermore, with thrombodynamics it is possible to identify the 'high hemostatic responders' including multiple factors, which may have the highest risk to develop thrombogenic effects while using combined oral contraceptives.

ABBREVIATIONS

ATG: Activator Thrombin Generation; APTT: Activated Partial Thromboplastin Time; COC: Combined Oral Contraceptives; ETP: Endogenous Thrombin Potential; TF: Tissue Factor; TFPI: Tissue Factor Pathway Inhibitor; 30EELNG: COC with 30 µg Ethinylestradiol and 150 µg Levonorgestrel; 30EEDSG: COC with 30 µg Ethinylestradiol and 150 µg Desogestrel; 20EEDSG: COC with 20 µg Ethinylestradiol and 150 µg Desogestrel

INTRODUCTION

The increased risk of thromboembolic disease associated with the use of combined oral contraceptives (COC) [1-3], has induced extensive evaluation of the effects on *in vitro* coagulation tests, molecular markers and plasma factor levels.

Observations include an enhancement of coagulation in global clotting tests such as the APTT, increase in thrombin generation in specific tests, reduction in inhibition including activated protein C (APC resistance test), increase in specific coagulation factors and decrease in specific coagulation inhibitors most notably TFPI and antithrombin [4-9]. In addition, molecular markers reporting the continuous coagulation and fibrinolysis actions such as prothrombin fragment 1+2 and plasmin-antiplasmin complexes are elevated and increases in fibrin degradation products indicate increased fibrin formation and dissolution [10,11].

The data of all those specific effects indicate a procoagulant effect, but the critical contributions to thromboembolic disease are not exactly clear [12-14]. In practice most changes are being evaluated for each combined contraceptive but cannot be interpreted in an algorithm [15]. Although global tests incorporate

multiple factors, they are not really pharmacodynamic tests. Pharmacodynamic tests studies the effect of a drug on the body [16].

Recently, a new candidate for pharmacodynamic testing was developed. It was proposed that clotting is a system of waves starting from a procoagulant surface [17,18]. That model resembles *in vivo* coagulation on a prothrombotic surface. That is unlike current global clotting tests, which are based on homogeneous clotting.

The theory has been transferred to a test system with clotting starting from a tissue factor-immobilized surface and video recording with time lapse of the growth of the clot. The equipment is developed at Hemacore laboratories and is collectively called Thrombodynamics [18,19]. In this system the growth of the clot is a primary variable and in addition the thrombin formation during the growing of the clot is recorded. The latter is essential for programming the clot resistance against lysis [20].

In the study we have investigated the fibrin clot formation as well as the thrombin generation of 30 apparently healthy women before and after 6 months COC treatment using the Thrombodynamic system. Ten women obtained either 2nd generation COC (Ethinylestradiol 30 µg – Levonorgestrel 150 µg) or a 3rd generation COC (Ethinylestradiol 30 µg – Desogestrel 150 µg; or Ethinylestradiol 20 µg – Desogestrel 150 µg).

MATERIALS AND METHODS

The present study is a sub-study of a previously published clinical trial which was designed as a randomized, open-label, parallel group, comparative study in seven study centers in

five countries (the Netherlands, Germany, Belgium, Ireland, USA [21,22]). The study was conducted in compliance with the Declaration of Helsinki, ICH Harmonized Tripartite Guideline: Guideline for Good Clinical Practice (ICH-GCP), and with the national regulations in the countries where the study was conducted.

Participants and intervention

Participants included in the study were healthy, nonsmoking, nulliparous women who had not used an oral contraceptive for at least two complete cycles preceding the start of intake of the study drug, regular menstrual cycles (mean cycle length ≥ 24 and ≤ 35 days) for the two cycles preceding the start of study medication, no malignancy or history of such in the last 5 years, no undiagnosed abnormal genital bleeding in the last 6 months, no history of presence of active thrombophlebitis or thromboembolic disorders, no family history of cardiovascular diseases in first degree relatives < 55 years, and no coagulations disorder, hyperlipidemia, pregnancy or other contraindications to OC-use. Originally 752 subjects were randomized, of whom 707 subjects were treated for a period of six cycles with one of seven monophasic OCs component [11,22].

The study population included in the present study consists of 30 subjects i.e. 10 subjects receiving Ethinylestradiol 30 μg - Levonorgestrel 150 μg (30EELNG), Ethinylestradiol 30 μg - Desogestrel 150 μg (30EEDSG) or Ethinylestradiol 20 μg - Desogestrel 150 μg (20EEDSG). Baseline is compared with six-month (six cycles) treatment.

Collection and handling of blood

Baseline, citrate stabilized blood samples were taken on or between cycle days 18 and 21, counted from the onset of the last menstrual bleeding. Study drug use started on the first day of the first menstrual bleeding following the baseline blood sampling. Blood samples during treatment cycles were taken on or between days 18 and 21 of tablet intake. Blood samples were taken after an overnight fast, before 10 AM after the subject had been sitting quietly for 20 min, and with an applied pressure to the arm of 15 mm Hg, using an inflatable cuff. Citrate plasma was isolated by centrifugation for 20 min at 2000g. The plasma was subsequently aliquoted and stored at -80° in tightly capped cryotubes (Nunc, Roskilde, Denmark), until analysis.

Thrombodynamic assay

The coagulation state of blood plasma samples was qualitative and quantitative evaluated using a Thrombodynamics Analyzer System T-2 (Hemacore LLC, Moscow, Russia). This system records the fibrin clot light scattering changes in a time-lapse video microscopy mode. Furthermore, it records the fluorescence signal during cleavage of synthetic AMC-based substrate by thrombin. The spatiotemporal dynamics of the fibrin clot formation and the thrombin formation are subsequently analyzed and numerical parameters are calculated describing the coagulation process, such as: lag time, rate of clot growth, clot size (fibrin formation), and amplitude thrombin peak and rate of thrombin peak propagation (thrombin formation). In addition, near the activator site, the thrombin generation parameters ETP, Cmax, Lag time and Tmax are calculated.

Plasma samples were defrosted for 5 minutes in a water bath at 37°C . Next, plasma was filtered over a 2 μm filter plates (Ceveron), to remove microparticles (reduction of spontaneously clot formation). Subsequently, 120 μl filtered plasma was added to Reagent tube I (Thrombodynamics-4D PLS kit; lyophilized protein-inhibitor of contact pathway (corn trypsin inhibitor), and fluorogenic substrate for thrombin) and incubated in the thermostat block of the T-2 system at 37°C for 3 min. During incubation, 5 μl of PLS reagent (Thrombodynamics-4D PLS kit; reconstituted lyophilized phospholipid vesicles), is added to Reagent tube I. Next, all sample of Reagent tube I was transferred to Reagent tube II (Thrombodynamics-4D PLS kit; lyophilized calcium salt), and after reconstitution of calcium salt the complete content of Reagent tube II was transferred to one channel of the measurement cuvette. This procedure was repeated for the 2nd channel in the measurement cuvette. The coagulation process in the thin layer of plasma present in the measurement cuvette is started when the activating insert (immobilized TF on plastic surface) is placed in the measurement cuvette (Thrombodynamics-4D PLS kit). The fibrin clot formation and thrombin generation were measured for 90 min.

Statistical analysis

Spatial clot growth parameters were analyzed using Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA).

For comparing 0- and 6-months data, the data were analyzed with a two-tailed paired-t-test using GraphPad Prism version 8.0. Data were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Fibrin clot formation

The fibrin clot formation was initiated by exposing the immobilized tissue-factor to a thin layer of plasma sample, and followed with a video microscopy system for 90 min. As shown in Figure 1 (A-B), clot formation starts on the activator (TF), and propagates into the bulk of plasma. Significant differences for clot size growth are observed of subjects before (0 months, Figure 1 A, C), and after 6 months of COC treatment (Figure 1B, C). Of note, 'high hemostatic responders' [4], can be identified (Figure 1C). The increased clot size after 90 min is more pronounced in subjects treated with 30EELNG and 30EEDSG compared with 20EEDSG: $31 \pm 28\%$, $31 \pm 11\%$ and $13 \pm 13\%$, respectively (average ± 1 SD (n=10), Table 1). This indicates a role of concentration of ethinylestradiol, which is in agreement with earlier publications in which a less pronounced effect on hemostatic variables such as D-dimer, (prothrombin) fragment 1+2, antithrombin III, protein S and fibrinogen was demonstrated for 20EEDSG compared with 30EEDSG [21,23,24].

The individual clot size growth curves of 30EELNG, 30EEDSG and 20EEDSG before and after 6 months treatment is presented in Figure 1 (D-F). The Lag time (time between clotting initiation and actual appearance of the fibrin clot) is for all conditions 1 min. Since this parameter is dependent on the tissue factor concentration present on the active insert (+ TF pathway proteins [25]), and the same type of insert is used, this is as expected. The initial clot growth rate (2-6 min), and stationary clot growth rate (15-25 min), are significant higher after 6 months treatment, except the initial clot growth rate for 20EEDSG (Table 1). Initial

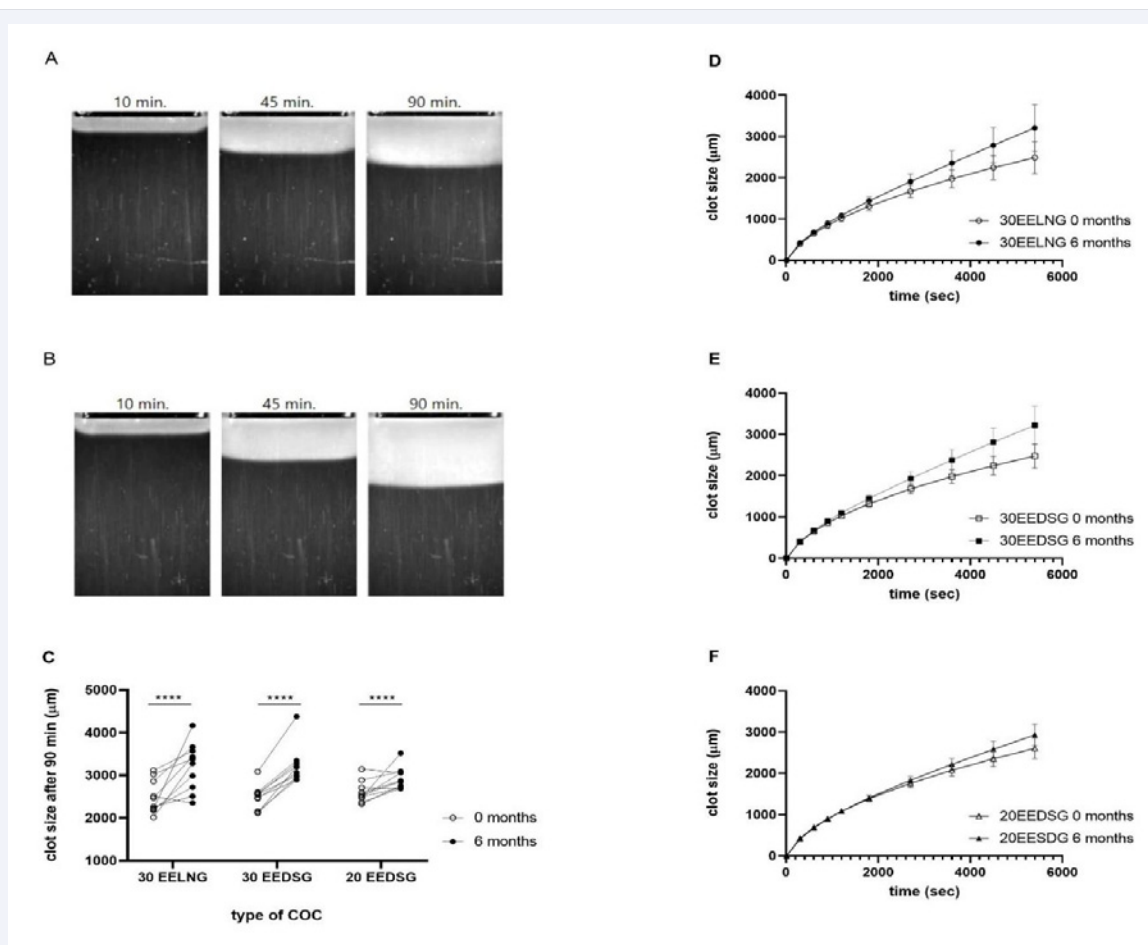


Figure 1 Fibrin clot growth. Typical images of a growing fibrin clot before COC treatment (A) and after 6 months COC treatment (B). Clot size after 90 min before (○) and after (●) 6 months treatment of the 3 different types of combined oral contraceptives (COC) investigated in this study (C). The clot size versus time plot calculated from the images of 10 subjects before and after 6 months treatment of 30EELNG (D), 30EEDSG (E) or 20EEDSG (F).

Table 1: Thrombodynamic parameters (fibrin clot growth) of n=10 subjects treated for 6 months with either 30EELNG, 30EEDSG or 20EEDSG. Initial rate of clot growth is between 2-6 min and stationary rate of clot growth is between 15-25 min. Average values \pm SD are shown.

			30EELNG (n=10)		30EEDSG (n=10)		20EEDSG (n=10)	
			0 months	6 months	0 months	6 months	0 months	6 months
Lag time	min	Tlag	1 \pm 0	1 \pm 0	1 \pm 0	1 \pm 0	1 \pm 0	1 \pm 0
Initial rate of clot growth	$\mu\text{m}/\text{min}$	Vi	59 \pm 3	62 \pm 3	60 \pm 3	63 \pm 3	63 \pm 3	62 \pm 4
Stationary rate of clot growth	$\mu\text{m}/\text{min}$	Vst	32 \pm 3	36 \pm 4	32 \pm 2	37 \pm 3	33 \pm 2	35 \pm 2
Clot size after 90 min	μm	CS	2488 \pm 386	3209 \pm 563	2473 \pm 292	3222 \pm 437	2604 \pm 250	2931 \pm 260
Clot density	a.u.	D	19244 \pm 3182	22596 \pm 3394	20720 \pm 2554	25185 \pm 3440	19373 \pm 2689	22562 \pm 2091

Abbreviations: **30EELNG**: COC with 30 μg Ethinylestradiol and 150 μg Levonorgestrel; **30EEDSG**: COC with 30 μg Ethinylestradiol and 150 μg Desogestrel; **20EEDSG**: COC with 20 μg Ethinylestradiol and 150 μg Desogestrel

clot growth is dependent on the diffusion of active factor of the active insert, whereas stationary growth is occurring far from activating surface, thus determined by plasma protein properties [18]. Therefore, based on the data of current study, we conclude that TF-pathway proteins and other plasma proteins are involved in increase clot growth rate for 30EELNG and 30EEDSG after 6

months, whereas for 20EEDSG there is only a role for non TF-pathway proteins. The clot density, a parameter that reflects the firmness and structure of formed fibrin clot, is for all three COCs higher (16-21% increase), after 6 months treatment (Table 1). Consequently, these fibrin clots are may be less susceptible for fibrinolysis indicating a higher risk for thrombotic disease.

Thrombin formation within the clot

Thrombin formation is started when the activating insert is exposed to a thin layer of plasma. Thrombin cleaves a synthetic AMC-based substrate and the fluorescence signal of AMC, which is proportional to thrombin concentration, is followed for 90 min with a video microscopy system. As shown in Figure 2 (A-B), thrombin moves from activation site into the plasma sample. In more detail, the thrombin peak moves as a wave into space as illustrated in Figure 2 for both 0 month (C) and 6 months treatment (D). Of note, in the area near the (high concentration) TF-immobilized surface, the concentration of thrombin rapidly increases [26], therefore these peaks are off scale at < 0.2 mm distance.

Two parameters are defined: stationary amplitude (A_{st} ; Figure 2E), which reflects the maximal activity of thrombin in the clot and the rate of thrombin peak propagation (V_t ; Figure 2F). There is a significant increase in both the A_{st} and V_t for subjects treated 6 months with 30EELNG, 30EEDSG or 20EEDSG (Figure 2E-F), and like in Figure 1C, the 'high hemostatic responders' [4], can be recognized. The increased A_{st} is more pronounced in subjects treated with 30EELNG and 30EEDSG compared with 20EEDSG: $48 \pm 19\%$, $51 \pm 14\%$ and $36 \pm 11\%$, respectively (average $\pm 1SD$ (n=10), Table 2), again indicating that a lower

concentration of ethinylestradiol in COC may lead to a lower risk of thrombotic disease.

Thrombin formation near activator site

In addition to the above discussed parameters, near the activator site (0.05-0.2 mm) thrombodynamics analyze the 'traditional' thrombin generation test parameters. Included are: the endogenous thrombin potential (ETP; which is the net amount thrombin that can be generated, *area under the curve*), maximal thrombin concentration that can be generated (C_{max}), the time that thrombin activity reaches 20 AU/L (Lag time) and the time to reach peak concentration (T_{max}), which reflects the velocity of thrombin formation. The near activator thrombin concentration curves of subjects treated with 30EELNG, 30EEDSG and 20EEDSG are shown in Figure 3. For all, there is a significant increase of both the total amount of thrombin (ETP), and the maximal concentration thrombin formed (C_{max}), after 6 months treatment compared with 0 months. There is no significant difference for the Lag time and T_{max} probably due to high concentration of TF on activating insert. Of note, the maximal thrombin concentration near the activator site is 7-12 times higher than the thrombin concentration in the stationary amplitude (Table 2). The phenomenon was earlier seen by Kuprash *et al*, which saw a 5-fold difference in their tested plasma samples [26].

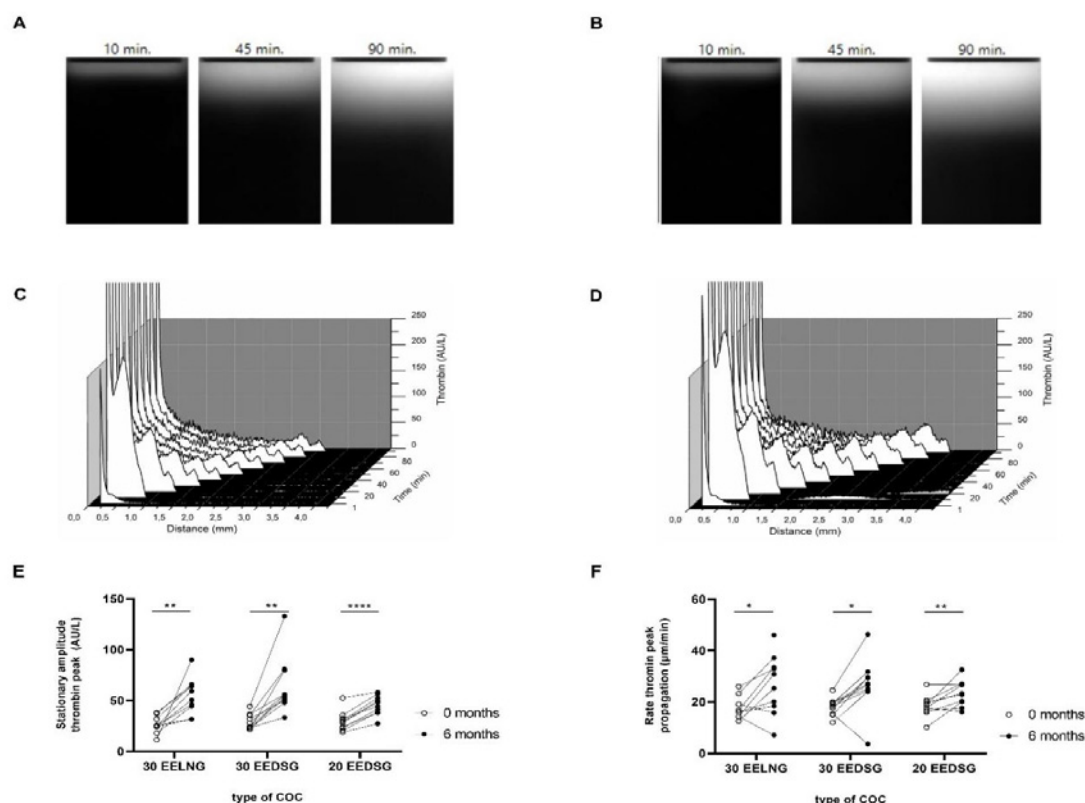


Figure 2 Thrombin formation. Typical images of AMC spatial distribution before COC treatment (A) and after 6 months COC treatment (B). Typical graph of spatial thrombin formation before COC treatment (C) and after 6 months COC treatment (D). Stationary amplitude after 60 min (E) and rate thrombin peak propagation 45-55 min (F) before (○) and after (●) 6 months treatment of the 3 different types of combined oral contraceptives (COC) investigated in this study.

Table 2: Thrombodynamic parameters (thrombin formation) in spatial distribution or near activator site (ATG) of n=10 subjects treated for 6 months with either 30EELNG, 30EEDSG or 20EEDSG. Average values \pm SD are shown.

			30EELNG (n=10)		30EEDSG (n=10)		20EEDSG (n=10)	
			0 months	6 months	0 months	6 months	0 months	6 months
Stationary amplitude of thrombin peak	AU/L	Ast	26 \pm 8	55 \pm 18	29 \pm 8	64 \pm 28	30 \pm 10	45 \pm 9
Rate of thrombin peak propagation	μ m/min	Vt	16 \pm 7	27 \pm 12	18 \pm 3	27 \pm 10	18 \pm 4	23 \pm 5
Thrombin potential of activator thrombin generation	AU*min/L	ETP_ATG	1654 \pm 173	2116 \pm 232	1683 \pm 325	2560 \pm 436	1815 \pm 384	2361 \pm 630
Maximum concentration of activator thrombin generation	AU/L	Cmax_ATG	290 \pm 36	369 \pm 29	310 \pm 71	422 \pm 65	346 \pm 53	413 \pm 62
Lag time of activator thrombin generation	min	Lag_ATG	0.2 \pm 0.1	0.1 \pm 0.0	0.2 \pm 0.1	0.1 \pm 0.1	0.1 \pm 0.0	0.1 \pm 0.0
Time to thrombin peak in activator thrombin generation	min	Tmax_ATG	1.9 \pm 0.3	1.8 \pm 0.3	1.8 \pm 0.2	1.9 \pm 0.4	1.8 \pm 0.3	1.8 \pm 0.3

Abbreviations: ATG: activator thrombin generation; **30EELNG:** COC with 30 μ g Ethinylestradiol and 150 μ g Levonorgestrel; **30EEDSG:** COC with 30 μ g Ethinylestradiol and 150 μ g Desogestrel; **20EEDSG:** COC with 20 μ g Ethinylestradiol and 150 μ g Desogestrel

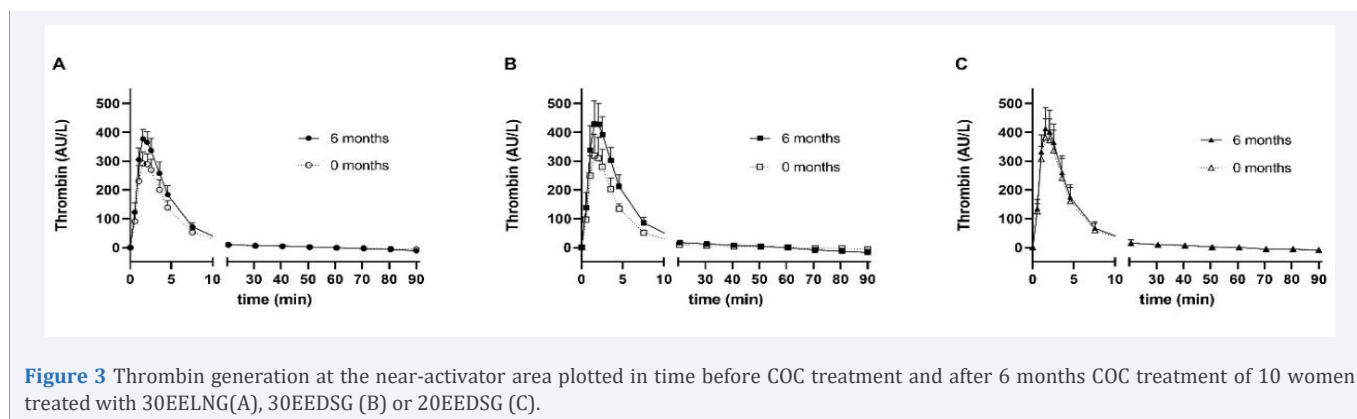


Figure 3 Thrombin generation at the near-activator area plotted in time before COC treatment and after 6 months COC treatment of 10 women treated with 30EELNG(A), 30EEDSG (B) or 20EEDSG (C).

CONCLUSION

Thrombodynamics is an elegant *in vitro* analysis that resembles *in vivo* coagulation on a prothrombotic surface which gives insight in the spatial dynamics of the formation of a fibrin clot. The data presented in the current study demonstrated that the use of three types of COC (30EELNG, 30EEDSG and 20EEDSG) might be involved in the increased risk of thromboembolic disease since both the fibrin clot formation and thrombin generation increased after 6 months treatment of COC. The read outs are consequences of multiple factors unless reductionistic approaches of other tests. Furthermore, with thrombodynamics the 'high hemostatic responders' can be identified who may have the highest risk to develop thrombogenic effects while using COCs.

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