Fibrin clot structure - pro-fibrinolytic effect of oral contraceptives in apparently healthy women

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Summary
Fibrin metabolism is influenced by many factors. The velocity of fibrin formation, genetic polymorphisms, fibrinolytic features and the structure of the fibrin clot are determinants of fibrin turnover. Oral contraceptives (OCs) have significant impact on the haemostatic system, by increasing the concentration of coagulation factors, plasminogen and tissue plasminogen activator activity, and decreasing the concentration of haemostatic inhibitors. The present study addresses the influence of OCs on fibrin structure and fibrin metabolism. The study included 70 women treated with seven different OC-formulations. Blood was collected at baseline and after six months of OCs. The plasma concentration of fibrinogen, thrombin-antithrombin complex (TAT), plasminogen, plasmin-antiplasmin complex (PAP), D-Dimer and thrombin generation measures were determined. Fibrin structure measures and fibrin clot lysis not affected by the plasma concentration of plasminogen activators and inhibitors were determined. OCs increased the concentration of fibrinogen, TAT, plasminogen, PAP and D-dimer significantly and affected measures of thrombin generation (p<0.001). The maximal optical density of fibrin (p<0.001), the fibrin fibre density (p=0.03), fibrin fibre diameter (p=0.003), fibrin mass-length ratio (p<0.001) and lysis per hour (p<0.001) increased significantly upon OC-treatment. Lysis per hour was not correlated to the concentration of plasminogen. We conclude that the effect of OCs on the coagulation system is balanced by alterations in fibrin structure, facilitating clot lysis and contributing to the fibrinolytic susceptibility already present in women treated with OC. These alterations may counterbalance the OC-induced increased thrombin generation and reduced coagulation inhibitory potential, contributing to maintenance of the haemostatic balance in women receiving OCs.

Keywords
Oral contraceptives, coagulation, fibrinolysis, fibrin structure, clot lysis

Introduction
The processes leading to fibrin formation and fibrin degradation are important determinants of fibrin metabolism (1), but also the structure of the fibrin clot itself contributes significantly to the dynamics of fibrin modulation (2). Here, a number of factors are involved. The velocity of fibrin formation, polymorphisms in the genes coding for fibrinogen and coagulation factor XIII (3, 4), flow conditions, binding and function of fibrinolytic proteins and many other factors determine the structure and turnover of the fibrin clot (5–7). Alterations in fibrin structure can be related to thrombosis or bleeding (4, 7). Clinical studies have demonstrated that plasma clots from patients suffering from VTE are dense and composed of thin fibres with reduced fibrinolytic susceptibility (8, 9) and fibrin structure measures are suggested as important biomarkers for thrombosis, providing assessment of the clotting capability of plasma and subsequently the fibrinolytic susceptibility of the fibrin formed (7).

In this setting it is of interest that oral contraceptives (OCs) have significant impact on the haemostatic system (10–12). The plasma concentration of most coagulation factors increases significantly, whereas the concentration of inhibitory regulators of the coagulation system, such as antithrombin and protein S, is decreased upon OC-use. These alterations facilitate enhanced activation of coagulation, illustrated by increased plasma levels of thrombin-antithrombin complexes (TAT) and prothrombin fragment 1+2, and result in increased thrombin generation (13, 14). The use of OCs, however, affects also the fibrinolytic system, illustrated by increase in tissue-type plasminogen activator (t-PA) activity, increased concentrations of plasminogen, plasmin-antiplasmin complexes (PAP) and D-dimer and reduced concentration of plasminogen activator inhibitor 1 (PAI-1) as a consequence of OC-treatment (10–12). As OCs also increase the concentration of fibrinogen, these findings in combination indicate that fibrin structure and turnover may be influenced by use of OC.

The effect of OCs, however, on fibrin formation, structure and degradation has not been studied in detail so far. Thus, the present
intervention study addresses the influence of OCs on fibrin metabolism by comparing measures of the kinetics of fibrin formation, fibrin structure and fibrin degradation in a population of young women before and after six months of treatment with seven different OC-formulations.

Materials and methods

The present study is a sub-study of a previously published clinical trial which was designed as a randomised, open-label, parallel group, comparative study in seven study centers in five countries (the Netherlands, Germany, Belgium, Ireland, USA) (15). 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 OC for at least two complete menstrual 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 five years, no undiagnosed abnormal genital bleeding in the last six months, no history or presence of active thrombophlebitis or thromboembolic disorders, no family history of cardiovascular disease in first degree relatives <55 years, and no coagulation disorder, hyperlipidaemia, pregnancy or other contraindications to OC-use.

Originally 752 subjects were randomised, of whom 707 subjects were treated for a period of six cycles with one of seven monophasic OCs (Table 1), containing either levonorgestrel, gestodene, desogestrel or norgestimate as the progestogenic component (15). Assessments were performed before start of treatment (baseline) and during cycle three and six of treatment.

The present study consists of 70 subjects from one study center, i.e. 10 subjects receiving each of the seven OCs studied were consecutively enrolled in the study.

Collection and handling of blood

Baseline, citrate stabilised 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 minutes (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 °C in tightly capped cryotubes (Nunc, Roskilde, Denmark). Before analysis, the samples were thawed for 5 min at 37 °C, kept at room temperature, and analysed within 1 hour (h). Baseline samples and samples from cycle six were analysed.

Determination of markers of fibrinogen/fibrin turnover

Plasminogen was determined as previously described (16). The plasma concentration of PAP and TAT was determined with the Enzygnost PAP ELISA kit and Enzygnost TAT Elisa kit, respectively, from Siemens Healthcare Diagnostics (Marburg, Germany). Thrombin generation was analysed by the calibrated automated thrombin generation assay (Thrombinoscope BV, Maastricht, The Netherlands) employing the Fluoroscan Ascent microplate fluorometer from Thermo Fisher Scientific, Hvidovre, Denmark. The Thrombinoscope software was used for calculation of the various measures of TG: the lag time of the thrombin formation process (lag time), the time to the peak thrombin concentration was reached (time to peak), the peak thrombin concentration and the endogenous thrombin potential (ETP) recording the total amount of thrombin formed. Plasma fibrinogen was determined according to Clauss. D-Dimer was determined using the Coalize D-Dimer ELISA kit from Chromogenix, Mölndal, Sweden.

Fibrin structure analysis

The polymerisation, fibrin fibre properties and degradation of plasma clots were determined according to Sjöland et al. (17) using turbidity measurements and calculations according to Carr et al. (18, 19). In brief, polymerisation of plasma clots was investigated by mixing citrated plasma with thrombin (final concentration 0.11 IU/ml), CaCl₂ and Tween 80. Turbidity was followed for 30 min at 405 nm on a Sunrise plate reader (Tecan Austria, Grödig/Salzburg, Austria). The lag time for fibrin formation was recorded as the time from the first reading of optical density (OD) until an OD-value of 0.03 was achieved. The maximal OD recorded during fibrin polymerisation was determined. All clots were made in duplicate. To investigate fibrin fibre properties and subsequent clot lysis, another set of plasma clots were made. After overnight incubation the OD was read at 405, 540, 608 and 690 nm,

<table>
<thead>
<tr>
<th>Oestrogen</th>
<th>Progestogen</th>
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<tbody>
<tr>
<td>Ethinylestradiol 50 µg</td>
<td>Levonorgestrel 125 µg</td>
</tr>
<tr>
<td>Ethinylestradiol 30 µg</td>
<td>Levonorgestrel 150 µg</td>
</tr>
<tr>
<td>Ethinylestradiol 30 µg</td>
<td>Desogestrel 150 µg</td>
</tr>
<tr>
<td>Ethinylestradiol 20 µg</td>
<td>Desogestrel 150 µg</td>
</tr>
<tr>
<td>Ethinylestradiol 30 µg</td>
<td>Gestodene 75 µg</td>
</tr>
<tr>
<td>Ethinylestradiol 20 µg</td>
<td>Gestodene 75 µg</td>
</tr>
<tr>
<td>Ethinylestradiol 35 µg</td>
<td>Norgestimate 250 µg</td>
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Table 2: Baseline characteristics of the women included in the study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N=70</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>22.8 (18.35)*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.2 (18.0–27.5)*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>120 (95–139)*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>71 (53–85)*</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Asian n, (%)</td>
<td>3 (4.3 %)</td>
</tr>
<tr>
<td>Black n, (%)</td>
<td>1 (1.4 %)</td>
</tr>
<tr>
<td>Caucasian n, (%)</td>
<td>63 (90.0 %)</td>
</tr>
<tr>
<td>Other n, (%)</td>
<td>3 (4.3 %)</td>
</tr>
<tr>
<td>*Median and range.</td>
<td></td>
</tr>
</tbody>
</table>

... and the fibre mass-length ratio, fibre density and fibre diameter were calculated. To initiate fibrinolysis of the clots t-PA (50 µg/ml) and flufenamic acid were added onto the clots as previously described (17). The 405 nm OD was then followed every 5 min for 4 h at 25°C. The rate of fibrinolysis per hour was determined from the slope of the curve when the slope became constant, and was normalised with respect to the maximum absorbency value before lysis initiation.

Statistics

Changes between the results obtained at baseline and after six months of OC-use were evaluated by the Wilcoxon Signed Rank Test. Potential associations between variables were assessed by Spearman rank order correlation analysis.

Results

Study population characteristics

Demographic and anthropometric data of the study population are presented in Table 2. The majority (90%) of the women was lean and of Caucasian origin. All women were normotensive.

The effect of OCs on measures of fibrin turnover

OC-treatment induced significant increase in the plasma concentration of TAT, fibrinogen, plasminogen, PAP and D-dimer (P<0.001). Measures of thrombin generation were also affected by OC-treatment with significant decrease in lag time and time to peak and significant increase in peak thrombin concentration and ETP (p<0.001) (Table 3).

The effect of OCs on fibrin structure

Fibrin structure analyses revealed that the lag time for clot formation was not significantly affected by OC-treatment (15 s at baseline and after six months of OC-treatment (p>0.05, data not shown), but use of OC increased other measures of fibrin formation significantly. The maximal optical density (peak OD) recorded during fibrin formation rose from 0.457 (0.411 – 0.536) at baseline to 0.569 (0.507 – 0.633) after six months (p<0.001) (Figure 1A). A slight, but significant increase was recorded in fibrin fibre density increasing from 2.7 (2.4 – 3.0) x 10² Da/cm³ to 2.9 (2.6 – 3.2) x 10² Da/cm³ (p=0.03) (Figure 1B). Fibrin fibre diameter increased from 0.13 (0.12 – 0.15) µm at baseline to 0.14 (0.13 – 0.16) µm after six months (p=0.003). The mass-length ratio of the formed fibres was 3.8 (3.3 – 4.6) x 10¹² Da/cm at baseline, increasing to 4.4 (3.8 – 5.1) x 10¹² Da/cm after OC-treatment (p<0.001) (Figure 1C). The susceptibility to fibrinolysis measured as lysis per hour increased from 14.6 (12.1 – 16.0) % before treatment to 18.8 (16.2 – 19.9) % after six months of OCs, (p<0.001) (Figure 1D).

Correlation analysis demonstrated that the plasma level of plasminogen and the lysis per hour were not significantly associated at baseline (Spearman ρ=0.15, p=0.22) or after six months of OC-treatment (Spearman ρ=0.16, p=0.19).

Discussion

The current study demonstrates that use of OCs for six months alters fibrin structure significantly. The peak OD, fibre diameter, mass-length ratio, fibre density and fibre diameter were increased. These changes were associated with changes in fibrinolysis and measures of fibrin turnover, which were also increased by OC-treatment.

Table 3: Effect of six months of oral contraceptive use on plasma markers of fibrinogen/fibrin turnover (N=70).

<table>
<thead>
<tr>
<th></th>
<th>Baseline*</th>
<th>Six months*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin-antithrombin complex (µg/l)</td>
<td>2.5 (2.1–3.4)</td>
<td>3.0 (2.4–4.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thrombin generation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>3.3 (3.0–3.7)</td>
<td>2.7 (2.3–3.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>7.2 (6.3–7.7)</td>
<td>5.5 (5.0–6.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak thrombin concentration (nmol/l)</td>
<td>188 (150–213)</td>
<td>386 (333–429)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ETP (µmol/l x min)</td>
<td>1.38 (1.17–1.51)</td>
<td>2.27 (2.02–2.42)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen (gl/l)</td>
<td>3.0 (2.7–3.4)</td>
<td>3.6 (3.3–3.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasminogen (%)</td>
<td>86 (78–95)</td>
<td>121 (109–133)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasmin-antiplasmin complex (mg/l)</td>
<td>0.37 (0.29–0.53)</td>
<td>0.67 (0.53–0.86)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D-dimer (mg/l)</td>
<td>0.12 (0.10–0.17)</td>
<td>0.20 (0.16–0.26)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*The results are presented as median and interquartile range. ETP: endogenous thrombin potential.
density and mass length ratio of fibrin are increased by OC-treatment, and, of particular notice, the clot formed after OC-treatment is more readily lysed than the clot formed at baseline.

In the present analytical setting a constant thrombin concentration is added to plasma samples collected before and after OC-treatment. Measures of peak OD demonstrate that more fibrin is formed after OC-treatment. This finding is in agreement with the significant increase in fibrinogen concentration induced by the OC-treatment. Notably, experimental studies have demonstrated that an increase in the fibrinogen concentration at a constant thrombin concentration facilitates formation of more dense and highly branched fibrin networks composed of thin fibrin fibres (5, 17, 20, 21). Such fibrin characteristics are associated with reduced susceptibility to fibrinolysis (17, 22, 23). In line with these findings we observe that the fibrin formed after OC-treatment has increased fibre density. However, the fibrin possesses unique and quite opposite characteristics with respect to fibre diameter and fibrin mass length ratio, which both are significantly augmented. Moreover, the fibrin formed after six months of OC-treatment is more readily lysed than fibrin formed at baseline illustrated by the significant increase in lysis/h. Thus, despite that an increased amount of fibrin with slightly increased fibre density is formed after OC-treatment compared to baseline, the fibrin clot is lysed quicker than the clot formed at baseline. This finding is in agreement with the fibrin diameter recorded, as fibrin with thick fibres is more susceptible to lysis than thin stranded fibrin (17, 22, 24, 25).

It should be noted, however, that other factors may determine the fibrinolysis rate in women treated with OCs. We have pre-

Figure 1: Fibrin structure characteristics at baseline and after use of oral contraceptives for six months (n = 70). A) Maximal optical density at 405 nm. B) Fibrin fibre density. C) Fibrin mass-length ratio. D) Fibrin lysis per hour. The results are presented as median and interquartile range. *P<0.001, **P = 0.03.
Oral contraceptives have significant impact on the turnover of fibrin. The structure of fibrin contributes significantly to the dynamics of thrombin generation and the reduced coagulation inhibition potential characterising women treated with OCs, and thereby maintain the haemostatic balance and ensure sufficient fibrin turnover.

The design of our study does not allow drawing conclusions regarding the clinical consequences of the change in fibrin structure induced by OC-treatment. The fibrinolytic susceptibility of the fibrin clot, however, is increased by more than 25% and this may contribute to reduction in the risk of thrombosis induced by the effect of OC on the coagulation system. It would be of interest to implement fibrin structure analysis in large epidemiological studies on OC and risk of thrombosis in healthy women and in women with latent thrombophilia in particular.

The present study includes 70 subjects treated with seven different OCs. The risk of thrombosis differs according to the formulation of the oral contraceptives (32), and it might be tempting to investigate whether the oestrogen content or the progestogen component of the OC formulations influence the fibrin structure significantly. Preliminary statistical evaluations were performed after subdivision of the individuals according to the oestrogen content of the OCs (20 μg, 30–35 μg and 50 μg) and according to the progestogen component (levonorgestrel vs the other progestogens) (data not shown) demonstrating no difference in neither measures of coagulation activation nor fibrin structure measures in relation to the treatment groups. These preliminary observations, however, are based on small groups of women, and the power of the calculations is not sufficient to allow firm conclusions. Thus, the present study demonstrates that OC-use in general increases the fibrinolytic susceptibility and the turnover of fibrin significantly.

**What is known about this topic?**
- Oral contraceptives have significant impact on the turnover of fibrin.
- Fibrin structure measures assess the clotting capability of plasma and the fibrinolytic susceptibility of fibrin.
- The structure of fibrin contributes significantly to the dynamics of fibrin modulation.

**What does this paper add?**
- Use of oral contraceptives induces unique changes in fibrin structure.
- Oral contraceptives augment fibrin fibre diameter and fibrin mass length ratio.
- Use of oral contraceptives increases the fibrinolytic susceptibility of fibrin.

**Acknowledgements**
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**Conflicts of interest**
None declared.
References

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