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**ORIGINAL ARTICLE**

# Impact of progestagens on activated protein C (APC) resistance among users of oral contraceptives

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**Summary.** Oral contraceptive (OC) use is associated with an increased risk of venous thromboembolism. Previous data reported higher thrombotic risk in women using third-generation combined OC than in those using second generation OC. The difference could be explained by differential effects of progestagens on plasma sensitivity to activated protein C (APC). The main purpose of this cross-sectional study was to assess the influence of a progestagen-only OC (chlormadinone acetate) as well as the effect of several combined OC with different progestagen components on APC resistance. The effect of APC on endogenous thrombin potential (ETP) was investigated in the plasma of healthy women using either combined OC ($n = 82$) or progestagen-only OC ($n = 28$), and in non-users ($n = 64$). Carriers of factor V Leiden were excluded. Compared with non-users, there was no significant change in APC resistance in women using progestagen-only OC. Women who used combined OC were less sensitive to APC than non-users ($P < 0.001$) and the difference was significantly more pronounced in women using third-generation OC ($n = 41$) than in those who used second-generation OC containing levonorgestrel ($n = 22$) ($P < 0.05$). Compared with OC containing levonorgestrel, use of norethisterone-containing OC ($n = 9$) was associated with an increased resistance to APC ($P < 0.05$). Women who used cyproterone-containing OC ($n = 10$) were less sensitive to APC than those using third-generation OC ($P < 0.05$) or second-generation OC containing levonorgestrel ($P < 0.05$). Protein S, factor II and FVIII levels explained in part the OC-related changes in APC sensitivity variations. ETP-based APC resistance may contribute to explain why different brands of OC can be associated with different levels of thrombogenicity.

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## Introduction

The use of oral contraceptives (OC) is clearly associated with an increased incidence of thromboembolic disease [1,2]. This association depends on the estrogen dose [3] and on the type of progestagen. Thus, the risk of thrombosis is twofold higher in so-called third-generation OC which contain desogestrel or gestodene users than in second-generation OC containing levonorgestrel users [4,5]. Recent studies suggest that combined OC containing cyproterone acetate can substantially increase the risk of thrombosis [6,7]. Data assessing the impact of progestagen-only OC on the thrombotic process are scarce [8].

The elevated risk of venous thrombosis in OC users could be mediated throughout the hemostatic system. Changes in clotting factors first reported in combined OC users were increased plasma levels of procoagulant proteins [factor (F)VII, fibrinogen, FII, FVIII], decreased levels of coagulation inhibitors [antithrombin (AT), protein S (PS)] and fibrinolysis proteins (tPA, PAI1), and increased levels of coagulation and fibrinolysis activation markers (for review 9,10). A recent cross-over study indicated that second and third-generation OC have different effects on a large number of procoagulant, anticoagulant and fibrinolytic parameters [11–13]. However, whether the increase in coagulation potential could be balanced by the rise in fibrinolytic activity so preserving the hemostatic equilibrium remains unknown. Activated protein C (APC) resistance due to FV Leiden is an established risk factor for venous thrombosis [14]. APC resistance in the absence of FV Leiden also increases this risk [15,16]. OC use leads to acquired APC resistance [17,18] and women using third-generation OC are more resistant to the anticoagulant action of APC than users of second-generation OC [19]. This difference may reflect a progestagen-specific effect. Moreover, a significant influence of the dose of levonorgestrel among women using second-generation OC has been observed [20]. A differential effect on plasma APC sensitivity could be a plausible mechanism to

explain thrombotic risk differences associated with the use of different OC. The main purpose of this study was to gain more insight into the effect of progestagen-only OC on APC resistance and on the impact of different progestagen components in users of combined OC.

## Materials and methods

### *Study design*

This cross-sectional study took place in years 2000–01. Its main purpose was the comparison of the effects of different types of OC and hormone replacement therapy (HRT) currently used in France on APC resistance and hemostatic variables. The participants were healthy female volunteers aged 25–65 years consecutively recruited in a health care center (IPC, Paris, France).

All participants answered a standardized questionnaire concerning demographic background, medical history, drug use (including HRT and OC) and personal habits such as smoking and alcohol consumption. Information on pill day and menstrual periods was systematically recorded.

Blood pressure was measured three times on the right arm after 10-min rest. Height and weight of the subjects were systematically measured. Body mass index (BMI) was expressed as the ratio of the weight (kg) to the square of the height ($m^2$).

Exclusion criteria were anticoagulant treatment, pregnancy, personal history of thrombotic events (self-reported history of deep venous thrombosis or pulmonary embolism), cardiovascular disease (self-reported history of myocardial infarction, coronary insufficiency, stroke, arterial occlusive disease) or malignancy.

The study protocol was approved by an ethical committee. Written consent was obtained from all participants.

Premenopausal women with a normal menstrual cycle were classified into several groups according to the use of OC and to the type of OC. Women who did not use OC during the last 3 months were included as non-users. Current users who used OC at the time of blood sampling or in whom treatment was interrupted less than 3 months ago were included as second-generation OC users, third-generation OC users or progestagen-only OC users. Second-generation OC refer to those containing levonorgestrel and third-generation OC are those containing either desogestrel or gestodene. Combined OC containing other progestogen components were studied separately. Progestagen-only OC users consisted of women who used chlormadinone acetate, a 17α-hydroxyprogesterone derivative.

### *Blood samples*

Venous blood was drawn between 08.00 h and 10.00 h after overnight fasting and 10 min rest. Cholesterol, triglycerides and glucose measurements were performed immediately. No attempt was made to control the day of the cycle in OC users and non-users.

For coagulation measurements, venous blood (nine volumes) was collected in 5 mL vacutainer tubes containing 0.105 mol $L^{-1}$ trisodium citrate (one volume). Platelet-poor plasma was obtained by two centrifugation steps at $2500 \times g$ for 15 min at 15 °C. Aliquots were transferred into plastic tubes, quickly frozen and stored at −40 °C. Venous blood was also collected in tubes containing 0.084 mL 15% EDTA for DNA extraction

### *Hemostasis measurements*

At the time of the assays, frozen plasma samples were thawed in a water-bath at 37 °C for 5 min and then handled at room temperature.

The APTT-based APC resistance assay was performed with a STA analyzer (Diagnostica Stago, Asnières, France), using the Coatest APC resistance kit (Biogenics, Maurin, France). Results of the test were expressed as the ratio of the clotting times of subject plasmas in the presence and absence of APC.

The effect of APC on the endogenous thrombin potential (ETP) initiated via the extrinsic coagulation pathway was measured as described elsewhere [21]. Briefly, thrombin generation was started in defibrinated plasma with 0.43 ng $mL^{-1}$ tissue factor (Innovin, Dade), 16 mmol $L^{-1}$ $CaCl_2$, and 15.2 μmol $L^{-1}$ phospholipid vesicles (DOPS/DOPE/DOPC, 20/20/60/M/M/M) either in the absence or presence of 40 nmol $L^{-1}$ APC (Hyphen). Concentrations are final concentrations in plasma mixtures. The APC concentration used was selected to give a residual activity of about 10% in normal plasma. After a 20-min incubation, the amidolytic activity of the $\alpha_2$-macroglobulin-thrombin complex ($\alpha_2$M-IIa) was used as an end-point marker to quantify thrombin generation. For each subject's plasma, the APC sensitivity ratio (APCsr) was defined as the ratio of $\alpha_2$M-IIa determined in the presence and in the absence of APC. The normalized APC sensitivity ratio (nAPCsr) was defined as the ratio of the APCsr obtained for the subject's plasma to the APCsr obtained for a normal plasma pool. The plasma pool was collected from 10 healthy subjects (before a planned surgery) in the same way as for women enrolled into the study.

Commercially available kits based on ELISA methods were used for measuring prothrombin fragment F1 + 2 (Enzygnost F1 + 2 micro, Behring), D Dimers (Fibrinostika FBDP, Organon Teknika), free TFPI (Asserachrom free TFPI, Diagnostica Stago) and tPA antigen (Imulyze tPA, Biopool) levels. The other parameters (FVIIIc, FVIIc, FII, fibrinogen, antithrombin activity, PS activity) were measured using reagents (FVIII, FVII or FII deficient plasmas and Neoplastine, STA fibrinogen, STA Stachrom ATIII, STA Staclot protein S) from Diagnostica Stago on a STA analyzer.

### *DNA isolation and genotyping*

Genomic DNA was extracted from EDTA blood samples using a standard method. The FV Leiden mutation was searched for as previously described [22].

### *Statistical analysis*

Statistical analysis used procedures available in the Statistical Analysis System (SAS) software (SAS Institute, Inc., Cary, NC, USA). Distribution of ETP-based APC sensitivity was positively skewed and so log transformed values were used. Mean levels of ETP-based APC sensitivity are given in arithmetic form. Analysis of variance and the $\chi^2$ test were used to compare the baseline characteristics of subjects. Multiple comparison procedures including the Bonferroni test were used to assess the differences in hemostatic variables between OC groups and non-users. Stepwise multiple linear regressions were used to assess the relative contribution of hemostatic variables to the prediction of APC sensitivity. Finally, unadjusted and adjusted regression coefficients were used to estimate the contribution of selected hemostatic variables to the OC-related changes in APC resistance.

## Results

### *Baseline characteristics of the subjects*

Blood samples were obtained from 180 premenopausal women. Sixty-six were non-users. Among OC users, 23 women used second-generation OC, 43 women used third-generation OC and 29 women used progestagen-only OC. In addition, nine women used norethisterone-containing OC and 10 women used cyproterone-containing OC. Overall, mean duration of OC use was 170 months, ranging from 10 to 360 months.

The FV Leiden mutation was present at the heterozygous state in 3.3% subjects. Considering the known influence of FV Leiden on APC resistance, FV Leiden carriers (two non-users, one second-generation OC user, two third-generation OC users and one progestagen-only OC user) were excluded from the statistical analyzes.

Among users of second-generation OC, seven women used monophasic OC with 30 μg ethinylestradiol and 150 μg levonorgestrel. Nine women used biphasic OC with 30 μg/40 μg ethinylestradiol and 150 μg/200 μg levonorgestrel (mean daily dose 183 μg). Six women used triphasic OC with 30 μg/40 μg/30 μg ethinylestradiol and 50 μg/75 μg/125 μg levonorgestrel (mean daily dose 92 μg). Women who used third-generation OC received either 20 μg ($n = 26$) or 30 μg ($n = 15$) ethinylestradiol pills containing desogestrel (150 μg per day) or gestodene (75 μg per day). Women who used progestagen-only OC received 10 mg chlormadinone during 18–21 days per cycle.

Baseline characteristics of subjects are reported in Table 1. Women using cyproterone-containing OC were younger than non-users. No significant differences between groups were found in terms of BMI, blood pressure and cholesterol. Women who used third-generation or cyproterone-containing OC had significantly higher triglyceride levels than non-users.

### *Impact of OC on hemostatic variables*

Mean ETP-based nAPCsr was significantly higher in carriers of factor V Leiden than in non-carriers (2.39 vs. 0.94, respectively, $P < 0.001$). Mean values of nAPCsr by OC use are given in the Fig. 1. Compared with non-users, there was no significant change in nAPCsr in women using progestagen-only OC. Women who used combined OC were less sensitive to APC than non-users ($P < 0.001$) and the difference was significantly more pronounced in women using third-generation OC than in those who used second-generation OC containing levonorgestrel ($P < 0.05$). Compared with OC containing levonorgestrel, use of norethisterone-containing OC was associated with an increased resistance to APC ($P < 0.05$). Women who used cyproterone-containing OC were less sensitive to APC than those using third-generation OC ($P < 0.05$) or second-generation OC containing levonorgestrel ($P < 0.05$).

Mean levels of other hemostatic variables are given in Table 2. Combined OC use was associated with changes in most hemostatic variables. By contrast, minor changes in clotting tests were observed in progestagen-only OC users. In combined OC users, increased plasma concentrations of procoagulant factors, fibrinogen, FVIII, FII, and FVII, decreased plasma concentrations of coagulation inhibitors (AT, PS, TFPI) and tPA were observed. Changes in hemostatic variables were generally more pronounced in third-generation OC users than in second-generation OC users. There were significant differences in PS, FII, and factor VII between second- and third-generation OC. Interestingly, use of cyproterone-containing OC was associated with the strongest effects

**Table 1** Baseline characteristics of women by OC use

| | Non-user ($n = 64$) | Progestagen-only ($n = 28$) | 30 μg EE + levonorgestrel ($n = 22$) | 30 μg EE + norethisterone ($n = 9$) | Third generation OC ($n = 41$) | 35 μg EE + cyproterone ($n = 10$) |
|---|---|---|---|---|---|---|
| Age (years) | 39.7 (8.6) | 36.5 (11.7) | 36.8 (8.4) | 37.7 (9.8) | 39.4 (7.4) | 30.7 (9.5)* |
| Smokers (%) | 23.4 | 21.4 | 27.3 | 11.1 | 24.4 | 20 |
| BMI (kg $m^{-2}$) | 22.6 (4.2) | 21.9 (2.3) | 23.4 (4.1) | 22.7 (3.6) | 23.2 (3.2) | 22.7 (3.8) |
| SBP (mmHg) | 121.8 (13.9) | 127.0 (20.1) | 122.7 (15.0) | 129.8 (13.6) | 123.0 (13.8) | 116.3 (9.2) |
| DBP (mmHg) | 73.1 (8.9) | 78.4 (12.5) | 78.5 (12.2) | 80.6 (10.2) | 77.9 (9.9) | 70.2 (9.1) |
| Triglycerides (mg/100 mL) | 63.4 (29.3) | 56.6 (19.0) | 79.0 (24.5) | 90.2 (21.8) | 87.2 (28.4)* | 126.4 (72.2)* |
| HDL cholesterol (mg/100 mL) | 69.1 (16.2) | 63.5 (15.7) | 64.3 (15.1) | 68.9 (13.3) | 76.5 (12.4) | 81.7 (22.8) |
| LDL cholesterol (mg/100 mL) | 113.5 (31.3) | 128.6 (28.3) | 110.6 (26.9) | 116.4 (36.0) | 101.6 (22.1) | 105.7 (35.5) |

Mean values (SD). Comparison with non-user group: *$P < 0.05$.

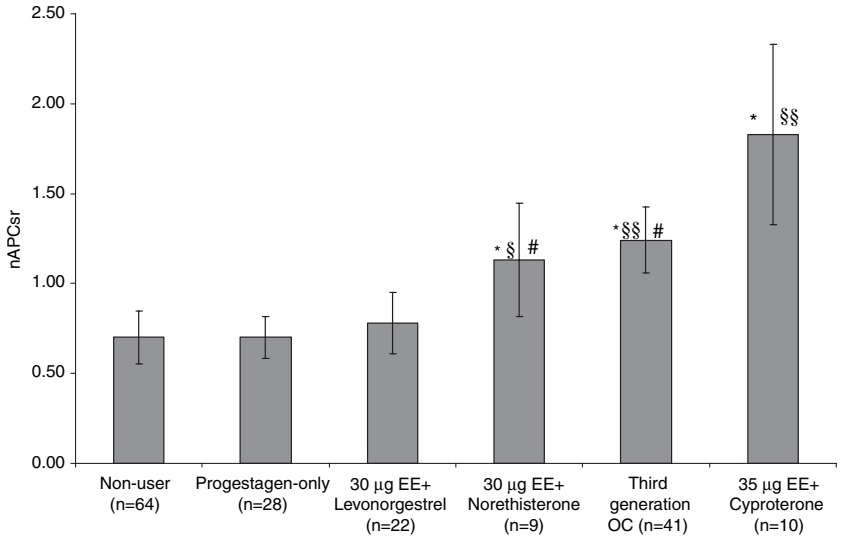


**Fig. 1.** Mean values of ETP-based nAPCsr after adjustment for age and BMI by OC use. Vertical lines are 95% confidence interval of the mean. Comparison vs. non-user: *:$P < 0.01$; comparison vs. 30 µg EE + levonorgestrel: §$P < 0.05$; §§$P < 0.001$; comparison vs. 35 µg EE + cyproterone: #$P < 0.05$.

on hemostatic variables. Combined OC use was associated with a small increase in APTT-based APC resistance but the difference was not significant in subgroups analysis.

PS activity, FII and FVIII concentrations were significant correlates of the ETP-based nAPCsr and explained about 20% of the total variance of this measurement (multiple correlation coefficient $R = 0.45$, $P < 0.001$). Taken together, PS, FII and FVIII explained 36% of the OC-related changes in ETP-based nAPCsr (results not shown).

Among combined OC users, there was a decrease in ETP-based nAPCsr during the OC-free days (day 22 to day 28) but the difference was not significant (1.03 vs. 1.25, respectively, $P = 0.40$). No clear change in ETP-based nAPCsr and other hemostatic variables were found in relation to phases of menstrual cycles. There was no significant association between any hemostatic tests and the duration of OC use. There was a non-significant inverse association between nAPCsr and the average daily dose of levonorgestrel among second-generation OC users. Results were similar in third-generation OC users who received either 20 or 30 µg ethinylestradiol and no significant difference was observed between OC containing desogestrel and those containing gestodene.

## Discussion

Our data show differential effects of OC on ETP-based APC resistance and emphasize the importance of progestagen components. Combined OC containing cyproterone have the strongest impact on APC resistance. Norethisterone-containing OC and third-generation OC have similar influence on ETP-based APC sensitivity. Progestagen-only OC has no effect on nAPCsr.

**Table 2** Hemostatic variables by OC use

| | Non-user ($n = 64$) | Progestagen-only ($n = 28$) | 30 µg EE + levonorgestrel ($n = 22$) | 30 µg EE + norethisterone ($n = 9$) | Third generation OC ($n = 41$) | 35 µg EE + cyproterone ($n = 10$) |
|---|---|---|---|---|---|---|
| nAPCsr | 0.70 (0.58) | 0.70 (0.31) | 0.78 (0.38) | 1.13 (0.41)*†‡ | 1.24 (0.58)*†‡ | 1.83 (0.70)*† |
| APTT-based APCsr | 2.44 (0.22) | 2.54 (0.24) | 2.43 (0.21) | 2.29 (0.16) | 2.35 (0.16) | 2.29 (0.20) |
| Fibrinogen (g $L^{-1}$) | 2.76 (0.50) | 3.07 (0.52)* | 2.94 (0.64) | 3.11 (0.22) | 2.85 (0.58) | 3.20 (0.63)* |
| PS activity (%) | 101.3 (18.6) | 108.2 (25.2)* | 108.9 (20.0) | 84.3 (26.1) | 88.3 (15.3)*† | 71.1 (19.2)* |
| FVIII (%) | 121.6 (30.9) | 108.9 (33.5) | 128.6 (27.6) | 160.5 (44.2)* | 132.4 (32.8) | 151.9 (25.9)* |
| FII (%) | 101.9 (10.1) | 111.5 (12.6)* | 104.6 (7.0) | 113.4 (13.6)* | 110.4 (12.9)*† | 116.7 (11.0)* |
| F1 + 2 (nmol $L^{-1}$) | 1.03 (0.30) | 0.87 (0.23) | 1.14 (0.39) | 1.21 (0.21) | 1.17 (0.29)* | 1.12 (0.21) |
| D-Dimers (ng $mL^{-1}$) | 176.5 (59.8) | 133.7 (37.3) | 219.7 (132.8) | 230.1 (104.3) | 216.0 (143.7) | 173.1 (72.5) |
| FVII (%) | 96.7 (24.4) | 95.7 (15.4) | 83.6 (19.9) | 102.9 (21.6) | 106.0 (23.7)† | 131.4 (18.1)* |
| AT (%) | 100.9 (6.9) | 99.9 (10.3) | 95.9 (7.1) | 94.5 (8.7) | 95.8 (7.7)* | 94.5 (12.4) |
| Free TFPI (%) | 8.2 (3.2) | 7.1 (1.6) | 6.3 (3.7)* | 6.1 (3.8) | 5.2 (1.1)* | 4.7 (0.9)* |
| tPA (ng $mL^{-1}$) | 5.5 (2.7) | 6.0 (3.1) | 3.9 (2.2)* | 3.9 (1.9) | 3.8 (2.2)* | 4.1 (2.3) |

Mean values (SD) adjusted for age and BMI. Comparison with non-user group: *$P < 0.05$. Comparison with levonorgestrel-containing OC: †$P < 0.05$. Comparison with cyproterone-containing OC: ‡$P < 0.05$.

The influence of combined OC on plasma APC sensitivity was previously reported and consistent data showed that acquired APCR was more pronounced in women receiving third-generation OC than in women using second-generation OC containing levonorgestrel [19,23,24]. Our results are in accordance with this finding. However, use of second-generation OC was not significantly associated with increased APC resistance. This may be due to the cross-sectional nature of the study and the small number of women. High concentrations of levonorgestrel in second-generation OC may also be relevant to this negative finding [20].

We found more significant changes in the ETP-based assay in which the coagulation process is triggered by a physiological pathway (addition of diluted tissue factor) than in the APTT-based assay. That the first test is more sensitive than the second one to OC and HRT effects is well-known [21,25]. The ETP-based APC resistance test has been recently related to the risk of venous thrombosis [16]. Our results therefore suggest that women using third-generation OC and those using OC containing either norethisterone or cyproterone are exposed to a higher risk of thrombosis than both levonorgestrel-containing OC users and non-users.

A part of OC-related changes in nAPCsr was explained through protein S, FII and FVIII levels. However, about 60% of this variation remains unexplained. The contribution of free PS in the ETP-based test was previously reported in healthy subjects [26], in women receiving females hormones (OC or HRT) [11,27] and in other clinical situations [28,29]. By contrast, Post *et al*. observed HRT-related changes in ETP-based nAPCsr, but did not find any contribution of PS or FII to these changes [30]. In accordance with our data, a contribution of high FII levels to acquired APC resistance was previously reported in both *in vitro* and *in vivo* studies [31–33]. The contribution of FVIII in the APTT-based APC sensitivity test is well-known [34]. However and by contrast with our results, Niziel *et al*. [26] previously reported no influence of FVIII on the ETP-based APC sensitivity test.

Other mechanisms can modify the hemostatic equilibrium and favor thrombotic process. TFPI is an inhibitor that regulates the tissue factor induced coagulation pathway. TFPI in complex with FXa is a potent inhibitor of the tissue factor/FVIIa complex [35]. Plasma TFPI deficiency can therefore theoretically increase the risk of thrombosis. Recently, low plasma TFPI levels were indeed associated with venous thrombosis [36] and striking lowering effect of both OC and HRT on TFPI levels were reported [27,36]. In accordance with Dahm *et al*. [36], we found 30–40% lower free TFPI levels in combined OC users than in non-users. In contrast with Hoibrateen *et al*. [27], we found no involvement of free TFPI in the ETP-based APC sensitivity assay. Whether low TFPI levels may contribute to the increased risk of venous thrombosis in OC users requires further study.

Our results are consistent with other studies showing the effects of second-generation and third-generation OC on hemostasis parameters [11–13,37,38]. We found significant differences in PS, FII and FVII levels between second- and third-generation OC. Changes in other hemostatic variables (FVIII, TFPI, tPA, F1 + 2 and D-dimers) were milder in second-generation OC users than in third-generation OC users but the differences did not reach the level of significance. The number of women included in our study could not be large enough to detect small differences between groups. The intra-individual variations in ETP-based nAPCsr and other hemostatic variables due to menstrual cycle may have also resulted in an underestimation of OC effects [19]. In a randomized cross-over study Middeldorp *et al*. [12], Meijers *et al*. [13] and Tans *et al*. [11] found OC-related changes in FII and PS but failed to detect differences between groups in coagulation activation markers levels, FVIII and tPA. Longitudinal data are more adequate than cross-sectional associations to assess the OC-related changes in hemostatic variables and differences in study design may also in part explain the discrepancies between studies.

In our study, 10 women were treated with a combination of ethinylestradiol (35 μg) and cyproterone acetate (2 mg). This drug is approved only as a therapy for androgen-sensitive skin conditions. However, this preparation provides also effective birth control. Interestingly, we found that the effects of cyproterone-containing OC and third-generation OC on hemostasis are very similar. This result might explain in part why cyproterone acetate confer a substantially increased risk of venous thrombosis, even higher than the risk associated with third-generation OC use [6]. These findings underline the importance of reserving cyproterone-containing OC for temporary use in women with serious acne.

Our study is the first to investigate extensively the influence of chlormadinone acetate, a progestagen-only OC, on hemostatic variables. Hemostasis parameters were similar in progestagen-only OC users and in non-users, and plasma levels of activation coagulation markers prothrombin F1 + 2 and D-dimers were even lower, suggesting a possible beneficial effect of progestagen-only OC. In addition, there was no significant change in ETP-based nAPCsr in women using chlormadinone acetate. Our data are consistent with early studies showing only minor changes in hemostasis among women using chlormadinone acetate alone [39]. However, chlormadinone acetate is a 17α-hydroxyprogesterone derivative structurally related to progesterone and our data may not be relevant to other progestogens used in OC, especially 19-nortestosterone derivatives.

The impact of progestagen-only OC on hemostasis has not been a mainstream topic for clinical investigations. No putatively detrimental effect was reported in women using the 19-norpregane derivative nomegestrol acetate [40,41]. A recent study demonstrated that progestagen-only preparations containing the same dose of levonorgestrel or desogestrel (150 μg) as present in combined OC induce changes in anticoagulant parameters opposite to those of combined OC [42]. Our results support the possibility that use of progestagen-only OC containing chlormadinone acetate may be safe with respect to thromboembolic risk. However, clinical trials are needed to demonstrate the safety of this

preparation, especially in women with a history of venous thrombosis.

In conclusion, despite being limited by its observational design, our study shows the importance of progestagen components of OC on plasma sensitivity to APC and other hemostatic variables. Our data may contribute to explain why different brands of OC can be associated with different levels of thrombogenicity.

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