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Protein S levels are lower in women receiving desogestrel-containing combined oral contraceptives (COCs) than in women receiving levonorgestrel-containing COCs at steady state and on cross-over

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Summary. This study aimed to identify specific haemostatic changes that might account for previous observations of higher venous thromboembolic risk among users of combined oral contraceptives (COCs) containing desogestrel (DSG) than levonorgestrel (LNG). Sixty-three current users of monophasic 30 μg oestrogen COCs containing either LNG or DSG omitted one pill-free interval (PFI), switching immediately either to the opposite formulation for one cycle or continuing with the same pill. Venesection followed the initial PFI after one cycle (21 tablets) and two cycles (42 tablets) of continuous pill taking, and after the following PFI. Protein S was lower in users of DSG than LNG formulations after the first PFI (mean ± SD, 0·67 ± 0·09 vs 0·76 ± 0·10, $P < 0·001$) and after one cycle (0·61 ± 0·09 vs 0·76 ± 0·09, $P < 0·0001$). Protein S decreased when switching from LNG to DSG pills (0·77 ± 0·07–0·65 ± 0·06, $P < 0·0001$), mirrored by an increase at switching from DSG to LNG formulations (0·61 ± 0·08–0·73 ± 0·10, $P < 0·005$). Mean protein S levels remained within the normal range. Three different markers of thrombin generation remained unaltered. Potential explanations for COC-related thrombotic events are 'acquired resistance to activated protein C' or inhibition of fibrinolysis. A potential role has been described for protein S deficiency in both. A further triggering factor is a probable prerequisite for actual thrombosis, but pill-takers whose levels of protein S were in the lowest percentiles may be at greatest risk.

Keywords: Protein S, combined oral contraceptives, desogestrel, levonorgestrel, thrombin.

The use of the combined oral contraceptive pill (COC) has been associated with a three–sixfold increase in relative risk of venous thromboembolism (VTE) compared with non-COC users (for reviews, World Health Organization, 1998; Anon, 2000). The risk is highest in the first year of COC use, but the risk drops rapidly to the level of non-users after cessation of COCs. Several groups have suggested a doubling of the risk of VTE when COCs containing third-generation progestogens (specifically gestodene or desogestrel) were used compared with COCs containing second-generation progestogens (norethisterone or levonorgestrel). Some experts believe that the figures for incidence of VTE in users of any COCs are actually too low, particularly in the young and first-time users (Lawrenson *et al*, 1999). Others say that there are no true differences in the rate of VTE between COCs containing second- and third-generation progestogens, and that some studies may be flawed owing to selection bias and confounding variables (Farmer *et al*, 1999; Lewis, 1999; Suissa *et al*, 2000).

The incidence of VTE is higher in COC users with the factor V Leiden (FVL) mutation (Vandenbroucke *et al*, 1994), which is known to be associated with resistance to activated protein C (rAPC) (Rosing *et al*, 1997) and increased thrombin generation (Simioni *et al*, 1996). However, there are methodological variations in the measurement of rAPC. Desogestrel (DSG)-containing COCs may cause greater rAPC in the Rosing test than those containing levonorgestrel (LNG) (Rosing *et al*, 1999). Although this has been used to explain the reported increased risk of VTE in users of COCs with third-generation progestogens, Heinemann *et al* (1998), using a very similar methodology to Rosing, were not able to associate rAPC with VTE risk.

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Table I. Base line characteristics of study subjects.

Study group	AA	AB	BB	BA
Number of subjects	21	15	15	12
Age (years)	27·4 (20–35)	27·3 (22–35)	28·0 (22–33)	27·8 (22–36)
Smokers	28·6% (6/21)	26·7% (4/15)	26·7% (4/15)	8·3% (1/12)
BMI (Body Mass Index)	21·4 (18–25)	21·5 (19–26)	21·6 (18–25)	23·2 (21–25)
BP systolic	110 (90–130)	105 (90–120)	105 (90–120)	110 (90–130)
BP diastolic	71 (60–82)	70 (60–80)	70 (60–85)	71 (60–81)
Length COC use (months)	81 (4–180)	57 (12–102)	95 (24–180)	111 (33–240)
Length of current brand use (months)	39 (4–180)	33 (11–84)	70 (6–120)	54 (3–96)
Nulliparity	95% (1 Unknown)	100%	93% (1 Parous + 1 unknown)	100%

A, Microgynon 30 group at recruitment; B, Marvelon 30 group at recruitment; Parity, percentage nulliparity is shown for all study subjects in whom parity was known; one parity was unknown in groups AA and BB; volunteers might have had one or more abortions.

It is well documented that COC use is associated with increased levels of most coagulation factors, while levels of anti-thrombin III and protein S may be decreased, and fibrinolytic activity is increased (Campbell *et al*, 1993; Kluft & Lansink, 1997). In general, these changes begin within the first cycle of COC use and tend to plateau after two to three cycles. The haemostatic changes return to normal within 2–4 weeks of stopping COC use, depending on the protein under study, although some rebound effect may be seen before complete normalization (Robinson *et al*, 1991). It is probable that the levels of some coagulation factors revert towards normal levels during the pill-free interval (PFI), which may prevent the changes from becoming excessive. There is little detailed information from controlled studies about the influence of third-generation progestogens and the consequences of switching between COC types on sensitive activation markers for thrombin generation.

We hypothesized that removing the PFI might reduce this partial normalization and alter the haemostatic balance sufficiently to further increase the risk of thrombosis. This would have considerable clinical implications as there are a number of accepted medical and social indications for the omission of the PFI (Guillebaud, 1999). These include: women changing COC brand; epileptics and others on enzyme-inducing drugs; and postponement of the withdrawal bleed for social convenience, which is frequently practised by continuing pill taking for extra days or weeks (Thomas & Ellertson, 2000).

We therefore planned a pilot study to investigate the impact of omitting the PFI on a variety of haemostasis activation markers and inhibitors, and any differences in women receiving 30 μg of ethinyl estradiol (EE) COCs with either LNG (second generation) or DSG (third generation). Our preliminary results were presented at the 1st North Sea Conference on Thrombosis & Haemostasis, June 2000 (Mackie *et al*, 2000).

MATERIALS AND METHODS

Study structure. We performed a prospective, non-randomized, comparative study using healthy, established COC users, taking a constant 30 μg dose of EE, with either monophasic LNG (Microgynon®, Schering) or monophasic DSG (Marvelon®, Organon). Seventy-five women were enrolled, but 11 (nine on Microgynon® and two on Marvelon®) were unable to comply with study visits for personal reasons and were withdrawn. One woman, taking Marvelon®, was withdrawn because of migraine with focal aura, necessitating a different method of contraception. Specimens from these 12 subjects were discarded as it had been decided that a minimum of four samples had to be available for assay. Sixty-three women completed the study. Group A were taking Microgynon® and group B Marvelon®. Subgroups AA and BB remained on the same COC formulation, while in subgroups AB and BA participants agreed to cross-over to the opposite COC type. In subgroups AA and BB, after one monitored cycle of 21 pills followed by a 7-d PFI, the women were asked to omit the next PFI. Forty-two tablets were therefore taken continuously before having another PFI. In subgroups AB and BA, after one monitored normal COC cycle, the women were asked to omit the next PFI and start a packet of the opposite COC type immediately. They therefore received two packets, 21 tablets of each type, continuously before having another PFI (Fig 1). Blood samples were collected on five occasions during the study, beginning with 'd 1' of a pill packet. When two packets of pills were taken without a 7-d break, the first pill of the second packet was counted as 'd 22', the last pill of the second packet was on 'd 42' (Fig 1).

Volunteers were 20–36 years of age and had been taking their contraceptive pills normally for at least 3 months before entering the study (Table I). Other inclusion criteria were: Body Mass Index (BMI) 18–26, normotensive (BP $< 130/85$), alcohol intake $\leqslant 14$ units per week and no more than one unit each evening prior to blood taking. Subjects with a personal history of venous thrombosis, a family history of venous or arterial thrombosis in a first-degree relative below the age of 45 years, other chronic medical illnesses, prominent varicosities or within 6 weeks of surgery were excluded. Further exclusion criteria were smoking >10 cigarettes/d, all standard absolute contra-indications to combined oral contraception plus use of non-steroidal anti-inflammatory drugs (NSAIDs) within 7 d of blood taking. Informed consent was obtained from study

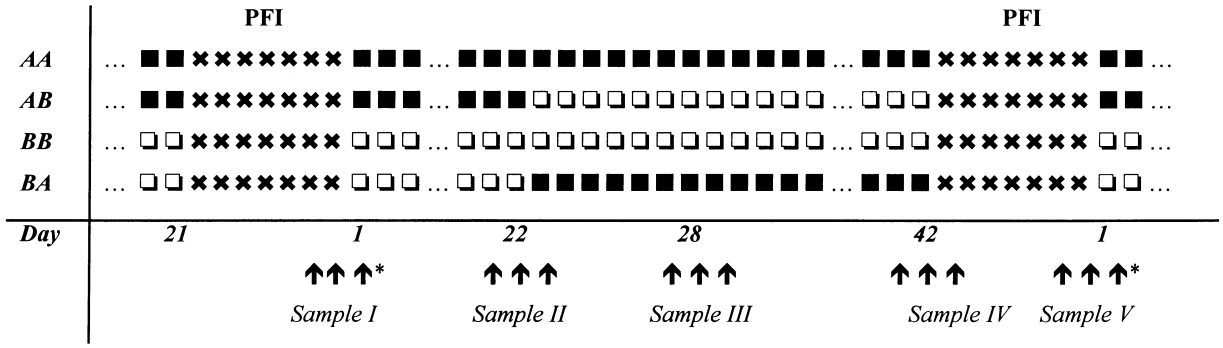

Fig 1. Diagram of pill-taking routine during study and timing of blood samples. AA/AB/BB/BA, study groups; ×, pill-free interval; ■, Microgynon®; □, Marvelon®. *If samples I and V were obtained on this day, it was before the first pill was taken.

participants and the study was approved by Camden & Islington Community Health Services NHS Trust Research Ethics Committee.

Blood samples. Non-fasting blood samples were collected between 07·30am and 10·00am on weekdays. After a short rest of 20 min, blood was obtained by clean venepuncture with minimal stasis. The first 5 ml blood was collected into EDTA for full blood count, followed by 5 ml clotted blood and 4 × 5 ml into 0·106 mol/l tri-sodium citrate using vacutainers (Becton Dickinson, Cowley, UK). Citrated blood was double centrifuged at 2000 ***g*** for 15 min to ensure platelet depletion. Plasma aliquots were stored at −80°C, then completely thawed at 37°C and mixed, before testing the full set of samples from each individual subject in the same assay batch.

Assays. Protein S was assayed using three methods: a functional, coagulation assay (PSact) (Bioclot Protein S, Biopool), and two enzyme linked immunosorbent assays (ELISAs), using either a monoclonal antibody specific for the free form of protein S (Asserachrom Free Protein S, Diagnostica Stago) or the supernatant plasma after polyethylene glycol (PEG) precipitation and polyclonal antisera (Dako). National or International standard plasmas (98/734 or 93/590; NIBSC, UK) were used as appropriate, with free protein S expressed according to the amount of free PS in the standard. C4b-binding protein (C4b-BP) (Kordia bv, Leiden), D-dimer (Dimertest Gold kit, Agen), thrombin:antithrombin complex (TAT),- and prothrombin fragment F1·2 (F1·2) (Enzygnost kits, Dade Behring), were measured by ELISA, and protein C was measured by amidolytic assay (Unicorn Diagnostics, London). Anti-phospholipid antibodies were sought by lupus anticoagulant, anti-cardiolipin and anti-β_2-glycoprotein I-tests; FV Leiden (FVL) and prothrombin mutations (G20210A) were assessed using polymerase chain reaction (PCR) and electrophoresis.

Statistics. The results were analysed using one way analysis of variance (ANOVA) with the significance level as $P < 0{\cdot}05$ and the Pearson coefficient of correlation.

RESULTS

The 63 subjects studied were distributed in the subgroups as follows: 20 AA, 16 AB, 15 BB, 12 BA. Samples were not available from four subjects at sample III (2AA, 2BB) and two subjects at sample IV (2AA), otherwise complete study compliance was achieved.

Five subjects had a weak anti-cardiolipin antibody on a single occasion, but this was not persistent; lupus anticoagulant and anti-β_2-glycoprotein-I were negative in all subjects. Three subjects were heterozygous for FVL; two were in group AA, one in AB. These subjects showed no elevation in thrombin generation markers TAT, F1·2) and, although one subject had decreased levels of functional protein S activity, this was probably an artefact due to FVL (Faioni *et al*, 1993). Six subjects were heterozygous for the G20210A prothrombin gene mutation (three group AA, one AB, two BB) and one of these was also heterozygous for FVL. There was no overall impact of any of these cases on the statistics for any of the measured variables and they were therefore retained in the analysis shown below.

The two methods for free protein S antigen assay showed similar trends throughout the study, except that greater variability was observed in the PEG precipitation assay. Therefore, unless otherwise specified, the free protein S assay (fPS) using monoclonal antibodies is featured below. There were no significant differences in fPS level between samples collected after the first monitored PFI and after one cycle of pill taking within each COC type (groups AA and BB, Fig 2A). There were also no significant differences with duration of pill taking, when the levels at 28 d (not shown) or 42 d were compared with baseline (first PFI) for each brand (either AA or BB). However, protein S activity (Fig 2B) was significantly reduced in subjects receiving Marvelon® (BB/BA), after d 21 of COC taking (mean ± SD, sample II vs I, 0·56 ± 0·12 vs 0·69 ± 0·14 IU/ml, $P < 0{\cdot}001$), whereas no difference was observed in those receiving Microgynon® (AA/AB).

Prior to any switching of products, subjects using Marvelon® (BB/BA) tended to have lower protein S levels, by both antigen and activity assays (Table II, Fig 2A and B), than subjects using Microgynon® (AA/AB) after the first PFI and at the end of the first COC cycle. When subjects were switched from one COC type to another (groups AB and BA), there were marked, mirror-image changes in protein S levels (by all assays) between sample II and sample IV. Protein S levels decreased significantly when changing from Microgynon® to Marvelon® and increased when

Table II. Differences in protein S antigen (fPS) and activity (PSact) between subjects receiving Microgynon® 30 (AA and AB) and Marvelon® 30 (BB and BA), at the end of the first PFI and after one cycle of COC use (samples I and II, Fig 1), before any switching of brands.

	fPS (IU/ml)		PSact (IU/ml)	
Sample	AA/AB	BB/BA	AA/AB	BB/BA
I (End of PFI)	0·76 ± 0·10	0·67 ± 0·09*	0·85 ± 0·18	0·69 ± 0·14*
II (After 21 pills)	0·76 ± 0·09	0·61 ± 0·09**	0·81 ± 0·15	0·56 ± 0·12**

*$P < 0·001$; **$P < 0·0001$.

changing from Marvelon® to Microgynon® (Fig 2A and B, Table III). There was a good correlation between protein S antigen ($r = 0·60$, $P < 0·0001$) and protein S activity ($r = 0·73$, $P < 0·0001$) levels between the PFI and two continuous cycles of COC use.

C4b-BP levels tended to decrease after the first monitored cycle of COC use, regardless of pill type, but with high intersubject variability this did not achieve statistical significance. There was no significant influence of missing the PFI on C4b-BP and levels increased after the PFI. Total PS values (not shown) generally showed a similar pattern to fPS. Neither total protein S, nor fPS antigen showed any correlation with C4b-BP levels; however, these protein S measurements correlated with one another at each sample time (e.g. sample IV $r = 0·64$, $P < 0·0001$). The mean PS antigen level remained within the laboratory normal reference range for non-pregnant females (0·55–1·09 IU/ml) at each time interval. Protein C levels were higher in Marvelon® than Microgynon® users in the first monitored PFI (mean ± SD 1·09 ± 0·16 vs 0·98 ± 0·15 IU/ml, $P < 0·01$) and after one cycle of pill taking (mean ± SD 1·10 ± 0·15 vs 0·98 ± 0·16 IU/ml, $P < 0·005$). There was no significant effect of missing the PFI or of changing COC type.

TAT and F1·2 were elevated in some individuals, but there was no obvious effect of COC type or PFI on the frequency of abnormality or level of the marker. D-dimer levels were increased after a cycle of COC use (sample I vs II) in Microgynon® users [groups AA/AB, geometric mean (95% CI): 41·4 (35·3–48) vs 32·6 (31·9–33·3) ng/ml, $P < 0·005$], but not Marvelon® users [groups BB/BA, 33·1 (31·5–34·7) vs 33·9 (30·7–37·5) ng/ml]. D-dimer levels were also significantly higher in Microgynon® than Marvelon® users after a cycle of COC use (sample II, $P < 0·02$), but not after the PFI. There was no statistical association between any activation marker and changing the COC type, or between activation markers and low protein S levels. Data on other haemostatic variables and activation markers will be presented elsewhere.

DISCUSSION

One potential explanation of the observed excess risk of thrombosis with COCs containing third- compared with second-generation progestogens, if real, is that an acquired resistance to activated protein C develops, as measured in

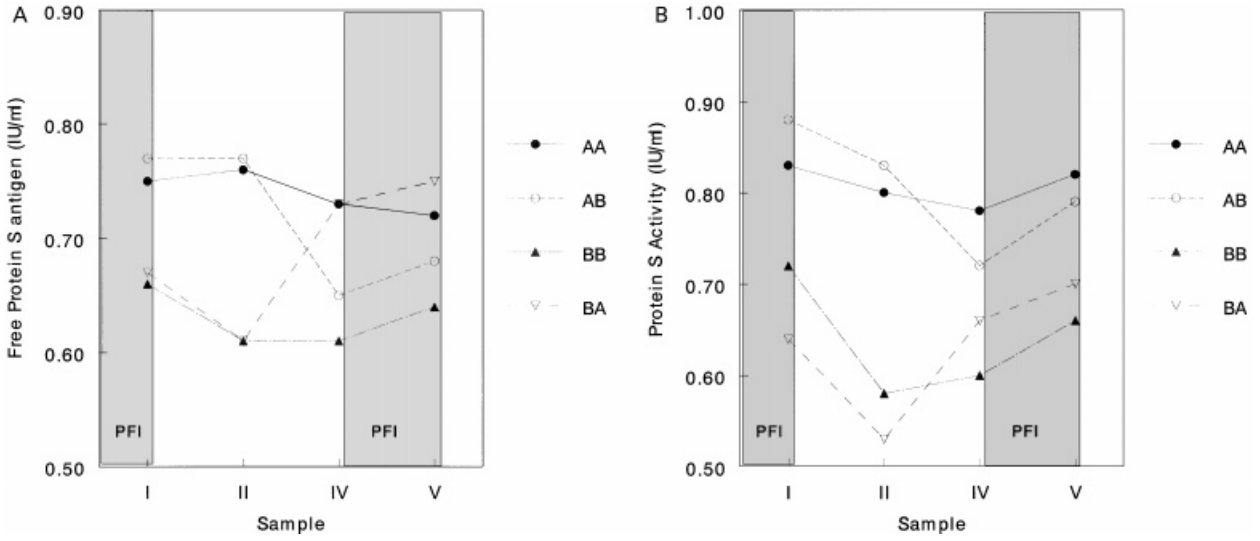

Fig 2. Free Protein S (fPS), Protein S Activity (PSact), C4b-BP and Protein C activity. Data is shown for fPS antigen measured using a specific monoclonal antibody (A) and protein S activity (B). The standard deviations showed little variability between the groups and sample times, and are omitted for clarity, but ranged between the following values: fPS 0·09–0·11 IU/ml; PSact 0·1–0·2 IU/ml. PFI, pill-free interval.

Table III. Changes in protein S antigen (fPS) and activity (PSact) when changing COC type.

		II	IV
	Sample	After 21 pills	After 42 pills
fPS (IU/ml)	AB	0·77 ± 0·07	0·65 ± 0·06***
	BA	0·61 ± 0·08	0·73 ± 0·10**
PSact (IU/ml)	AB	0·83 ± 0·14	0·72 ± 0·13*
	BA	0·53 ± 0·10	0·66 ± 0·11**

*$P < 0·05$; **$P < 0·005$; ***$P < 0·0001$.

AB, changing from Microgynon® 30 to Marvelon® 30; BA, changing from Marvelon® 30 to Microgynon® 30.

endogenous thrombin potential assays (Rosing *et al*, 1999), and that this results in excess thrombin generation. However, Heinemann *et al* (1998) did not find a link between rAPC and VTE, the methods are technically difficult to perform and not all reagents are readily available. Kluft & de Maat (1999a) suggested that LNG reduces the increase in resistance to APC as a result of oestrogen, in a dose-dependent manner, and that this effect may be specific for LNG. The hypothesis of an acquired rAPC defect requires independent confirmation and, if true, the defect requires biochemical explanation.

In sample I of our study, protein S levels were lower in users of Marvelon® (containing third-generation DSG) than Microgynon® (containing second-generation LNG). A number of previous studies have shown that protein S is decreased in women receiving COC pills (Granata *et al*, 1991; Archer *et al*, 1999; Winkler *et al*, 1999). The effect is probably mediated by oestrogen, as high-dose EE alone in young girls of tall stature resulted in a marked decrease in protein S, which took at least 4 weeks to reverse (Van Ommen *et al*, 1999). As there had only been the duration of one PFI prior to collection of sample I, the difference in initial protein S levels between groups A and B is probably as a result of insufficient time having elapsed since the therapy, i.e. 7 d. Our observation of a significant decline in protein S activity following one cycle of Marvelon®, but not Microgynon® (Fig 2B – sample II vs I), is also entirely congruent with Kluft's proposal (Kluft *et al*, 1999a) that LNG acts in an anti-oestrogenic way with respect to the procoagulant effects of EE.

Switching from Microgynon® to Marvelon® caused a significant decrease in protein S antigen and activity, mirrored when switching COCs in reverse fashion. Tans *et al* (2000) have shown a similar decrease in protein S in association with DSG. Inherited protein S deficiency has been associated with an increased risk of thrombosis, and family studies (Simioni *et al*, 1999) have shown a high relative risk of VTE in congenital protein S or C deficiency compared with non-carriers. However, the exact relationship between protein S deficiency and thrombosis has recently been questioned (Liberti *et al*, 1999). Approximately 60% of circulating protein S is bound to C4b-BP and is inactive. The observed changes in protein S were independent of C4b-BP concentration. Protein C levels were higher in Marvelon® users, with no significant effect upon changing COC type. Similar increases in protein C with third-generation COCs have been observed by others (Granata *et al*, 1991; Winkler *et al*, 1999) and they have no known clinical consequence.

This was not a double-blind randomized trial because the Committee on Safety of Medicines (CSM) had recently declared, in their letter to all doctors in October 1995, that pills containing third-generation progestogens should be used – or continue to be used – only by those who were 'intolerant' of second-generation progestogen products. In the context of national newspaper headlines, which exaggerated the risk out of all proportion, we felt that advance agreement to randomization would greatly inhibit recruitment and also impede best practice; ie. our individualized counselling and discussion for those randomized to cross-over to, or continuation with, pills containing third-generation progestogens. In the event, the comparability in baseline characteristics of the recruited subjects was acceptable (Table I).

Protein S influences thrombin generation in tissue factor-activated, amidolytic rAPC tests when the phospholipid concentration is low (Van't Veer *et al*, 1999). Our observations would thus potentially explain the relatively increased rAPC results linked with desogestrel (Rosing *et al*, 1999). However, it is unclear whether the data generated using reconstituted systems directly applies to Rosing's methodology (Rosing *et al*, 1997) in which the phospholipid concentration appears to be high. There are probably other differences in native plasmas, including variations in different lipoproteins. Tissue factor pathway inhibitor (TFPI) affects the initiation phase of rAPC (Butenas *et al*, 1999) and reduced TFPI levels have been reported in gestodene and desogestrel users (Kluft *et al*, 1999b). This variable has not yet been measured in our study, but may also have influenced the results.

Other potential physiological roles for protein S include providing a molecular anchor for proteins at the surface of blood cells. The thrombin-activated fibrinolysis inhibitor (TAFI) is activated by thrombin bound to thrombomodulin. TAFI activity is increased in protein S-depleted plasma, at least partly as a result of an APC-independent function of protein S (Mosnier *et al*, 1999). In a cycle-controlled cross-over study, TAFI was increased in LNG-containing COC users and showed a further increase in DSG-containing COC users (Meijers *et al*, 2000). The increased TAFI activity therefore suggested a decreased fibrinolytic potential. Fibrinolysis is generally increased by COC pills and is thought to offset their prothrombotic effects (Campbell *et al*, 1993). Any such beneficial effects may be relatively less with third-generation COCs with which protein S is decreased and, by implication, TAFI activity increases and inhibits fibrinolysis. There is some evidence that elevated TAFI levels form a mild risk factor for venous thrombosis (Van Tilburg *et al*, 2000).

We could find no associated increase in activation

markers (TAT, F1·2, D-dimer) on switching between LNG- and DSG-containing COCs, suggesting that there was not an increase in thrombin generation. A recent study (Middeldorp *et al*, 2000) found increased F1·2, but not TAT or D-dimer, after two cycles of COCs, but no difference between DSG- or LNG-containing COCs. In conclusion, if protein S levels are implicated in the pathophysiology of thrombosis in COC users, whether using second- or third-generation progestogens, it is probable that pill takers whose protein S levels are in the lowest percentiles of change may be at greatest risk, but a further triggering factor may be necessary before actual thrombosis occurs.

POTENTIAL CONFLICTS OF INTEREST

Professor Machin has previously received research grants from Schering, and Professor Guillebaud has received fees for lectures and *ad hoc* consultancy work, as well as research grants from both Schering and Organon. Details of the sources of funding for the current study are provided in the Acknowledgments section of the manuscript.

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