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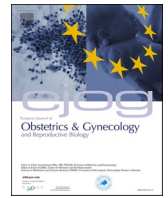
横田, 奈津子

<https://hdl.handle.net/2324/7363646>

出版情報 : Kyushu University, 2024, 博士 (医学) , 課程博士
バージョン :

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Full length article

The effects of dienogest and combined oral contraceptives on protein S-specific activity in endometriosis patients

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ARTICLE INFO

Keywords:

Protein S

Dienogest

Combined oral contraceptives

Thrombosis

Endometriosis

ABSTRACT

Objective: One serious side effect of combined oral contraceptives (COCs) is venous thromboembolism. Reduced activity in activated protein C-related coagulation pathways is attributable to low protein S activity in one-third of Japanese patients with deep vein thrombosis. Here, we quantified the behavior of protein S-specific activity in response to dienogest (DNG) and COCs using the protein S-specific activity assay system to explore its potential utility as a thrombosis marker.

Study Design: This was a prospective cohort study. Female patients aged 20–49 years who were starting drug treatment for endometriosis using DNG or COCs were enrolled. Blood samples were taken before treatment and at the first, third, and sixth months of treatment. To analyze the primary endpoints, changes in total protein S antigen levels, total protein S activity, and protein S-specific activity from baseline to each time point were estimated using a linear mixed-effects model. All statistical analyses were performed in the SAS software version 9.4 (SAS Institute, Cary, NC). A two-sided $P < 0.05$ was considered statistically significant.

Results: 64 patients took DNG and 34 patients took COCs. Protein S-specific activity did not change significantly from baseline in the six months after treatment started in either group. In the DNG group, total protein S activity and total protein S antigen levels increased slightly from baseline levels after the treatment. The means for total protein S activity and total protein S antigen levels in the COC group remained within reference limits, but they both decreased markedly in the first month and stayed low. Protein S-specific activity in four women remained below the reference limit throughout the whole study period, suggesting they may have potential protein S deficiencies.

Conclusion: The effects of DNG on protein S were negligible, though both total protein S activity and antigen levels decreased soon after COC treatment began and remained low. As there was no VTE event during the study, further studies with larger numbers of patients will be needed to confirm that protein S-specific activity can be a surrogate marker of VTE risk.

Introduction

Combined oral contraceptives (COCs) and progestin are major hormonal therapies for endometriosis [1]. One serious side effect of COCs is venous thromboembolism (VTE) [2–4]. Conversely, dienogest (DNG) has negligible effects on lipid metabolism and glucose level [5], but its

effects on coagulation pathways are not well known.

COC users exhibit a significantly decreased sensitivity to activated protein C (APC) [6,7]. Estradiol represses the production of protein S by down-regulating the protein S α gene promoter [8]. Reduced activity in APC-related coagulation pathways is attributable to low protein S activity in one-third of Japanese patients with deep vein thrombosis [9].

Abbreviations: APC, activated protein C; BL, baseline; BMI, body mass index; C4BP, C4b binding protein; COC, combined oral contraceptive; DNG, dienogest; EGF, epidermal growth factor; FSA, full set of analysis; UMIN, university hospital medical information network; VTE, venous thromboembolism; WHO, world health organization.

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<https://doi.org/10.1016/j.ejogrb.2024.01.028>

Received 26 August 2023; Received in revised form 15 January 2024; Accepted 21 January 2024

Available online 29 January 2024

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One protein S mutation, called protein S Tokushima, was detected in 1.6 – 1.8 % of healthy Japanese individuals, whereas its prevalence was 5 – 10 % in Japanese VTE patients [9–12]. Protein S Tokushima leads to a protein S type II deficiency characterized by low protein S activity relative to the level of protein S antigen [12–14]. Only free protein S can activate protein C, but this form constitutes only 40 % of the protein S present in plasma [15]; the other 60 % is bound to C4b binding protein (C4BP). The protein S-specific activity assay system offers superior accuracy superior to traditional assays, quantifying protein S activity as the sum of its free and bound forms. Protein S type II deficiency should be suspected when protein S-specific activity is low [12,16]. In this study, the behavior of protein S-specific activity in response to DNG and COCs was evaluated and its potential utility as a thrombosis marker was explored.

Material and methods

Study design

This was a prospective cohort study. We targeted female patients aged 20 – 49 years old with intact ovarian function who were starting drug treatment for endometriosis using DNG or COCs. The key exclusion criteria were: treatment with sex steroid hormones or GnRH agonists in the three months before enrollment, current use of anticoagulants or antiplatelet drugs, surgery in the four weeks before enrollment, or any conditions for which the study drugs were contraindicated. Informed consent for participation in the study was obtained from all patients before enrollment. This study was approved by the Institutional Review Board for Clinical Trials. In total, 111 patients were enrolled at 12 gynecology clinics and departments from May 2016 to December 2018. Patients prescribed DNG (the DNG group) took one 1 mg tablet orally twice daily for 24 weeks. Patients prescribed COCs (the COC group) took either 1 mg norethisterone / 0.035 mg ethinylestradiol, 1 mg norethisterone / 0.02 mg ethinylestradiol, or 3 mg drospirenone / 0.02 mg ethinylestradiol. The COC group patients took tablets orally for six cycles (i.e. 24 weeks) at the frequency specified in each drug's respective instructions for use. Blood samples were taken at four time points: baseline (BL), one month (1 M), three months (3 M), and six months (6 M). Whole-blood specimens were collected in 3.2 % sodium citrate blood collection tubes and centrifuged within one hour of acquisition to separate the plasma (3000 rpm, 5 – 10 min). Plasma specimens were frozen at – 80 °C for storage and thawed before evaluation using the protein S-specific activity assay system at Nagasaki International University.

Protein S-specific activity

Frozen plasma samples, which had not been thawed before analyses, were thawed in a water bath at 37 °C within 5 min. In the total protein S-specific activity assay system, total protein S activity and antigen levels were measured using the chromogenic and latex agglutination methods, respectively, using a Hitachi 7080 automated analyzer (Hitachi High-Technologies, Tokyo, Japan), as described by Tsuda and colleagues [12] with a slight modification to improve applicability. For the total protein S activity measurements, the protein S-C4BP complex in plasma was initially dissociated by diluting the samples 126 times with a dilution buffer containing APC and Factor Xa. Then, 3 µL of the diluted plasma was mixed with 150 µL of a reagent that contained Factor Va, liposome, and CaCl₂. After incubating for 5 min, 50 µL of another reagent that contained prothrombin and S-2238, a chromogenic substrate for thrombin, was added and the absorbance change was evaluated at the main wavelength of 405 nm with an auxiliary wavelength of 450 nm for 5 min.

For the total protein S antigen measurements, free protein S in plasma was initially combined with C4BP by incubating 3 µL of the samples with 100 µL of a reagent that contained an excess amount of

purified human C4BP. After incubating for 5 min, 100 µL of another reagent that contained monoclonal anti-protein S antibody (9H6)-bound latex particles was added, and the absorbance change was assessed at 700 nm for 5 min. Total protein S activity and antigen levels were expressed as IU/dL using the World Health Organization (WHO) Second International 394 Blood Coagulation and Fibrinolysis 2019, Vol 30 No 8 Standard for protein S (03/228, NIBSC, Hertfordshire, UK). The protein S-specific activity was then determined by calculating the ratio of total protein S activity to total protein S antigen levels.

Statistical methods

To examine the effects of DNG and COCs, the primary endpoints of this study were total protein S antigen levels, total protein S activity, and protein S-specific activity. Patients' initial characteristics were summarized and differences between the DNG and COCs groups were compared using t-tests or chi-squared tests. To analyze the primary endpoints, changes in total protein S antigen levels, total protein S activity, and protein S-specific activity from baseline to each time point were estimated using a linear mixed-effects model. The covariates included group (DNG|COC), time (BL|1M|3M|6M), a group–time interaction term, and four background variables: age, body mass index (BMI), smoking status and family history of thrombosis. Age was included because of the striking age difference between the groups; the latter three variables were included because they are known thrombosis risk factors that could potentially affect protein S-specific activity. To analyze the sensitivity of the main analysis, a multiple imputation method was performed that assumed the missing data was not random. All statistical analyses were performed in the SAS software version 9.4 (SAS Institute, Cary, NC). A two-sided $P < 0.05$ was considered statistically significant.

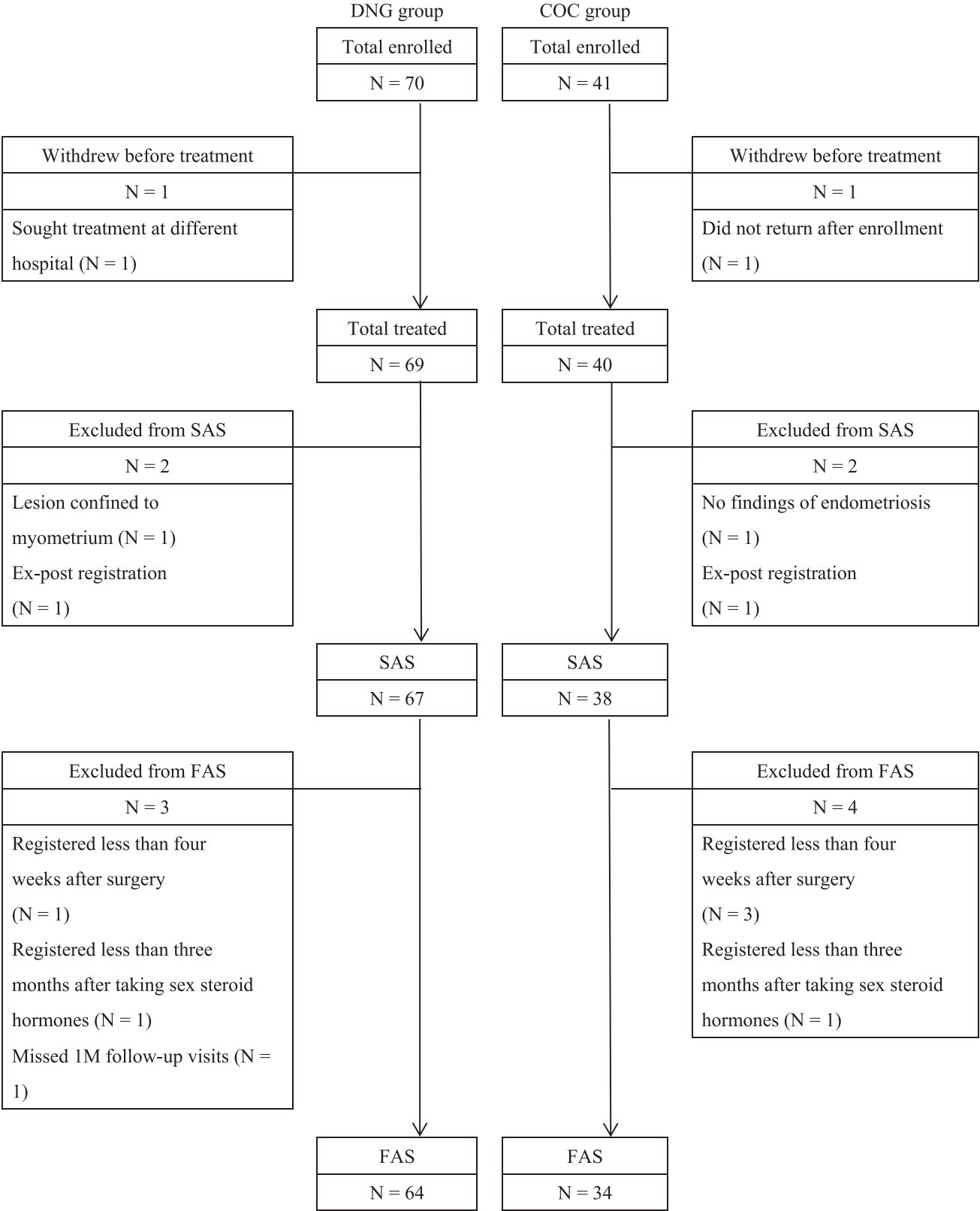
Results

Study population

In total, 111 patients were enrolled in the present study (DNG: 70, COC: 41; Fig. 1). Two women withdrew before the start of the study, ten were disqualified, and one dropped out. The full analysis set (FAS) consisted of the 98 subjects that remained (DNG: 64, COC: 34). Patients in the DNG group were significantly older than those in the COC group (Table 1). Common complications included uterine fibroids (DNG: 8, COC: 1), adenomyosis (5, 0), hypertension (4, 0), and migraine headaches (4, 0). Of the patients in the COC group, seven, nine, and 18 were prescribed 1 mg norethisterone / 0.035 mg ethinylestradiol, 1 mg norethisterone / 0.02 mg ethinylestradiol, and 3 mg drospirenone / 0.02 mg ethinylestradiol respectively.

Changes in protein S-specific activity

Protein S-specific activity did not change significantly from baseline in the six months after treatment started in either group. Instead, mean activity fluctuated within a narrow range across the four observation points (DNG: 1.06 – 1.07, COC: 1.02 – 1.09; Fig. 2, Table 2). The reference range for protein S-specific activity is 0.86 – 1.18. A protein S type II deficiency can be suspected when protein S-specific activity is below 0.78, which is more than three standard deviations below the reference mean. The measured protein S-specific activity was below this limit throughout the whole study period in four women, suggesting potential protein S deficiency (DNG: 3, COC: 1; Table 3). Among these patients, all of their total protein S antigen levels were within normal limits, and two of the patients had total protein S activity levels that were within normal limits.



DNG, Dienogest; COC, combined oral contraceptive; SAS, Safety analysis set; FAS, Full analysis set.

Fig. 1. Study population. DNG, Dienogest; COC, combined oral contraceptive; SAS, Safety analysis set; FAS, Full analysis set.

Table 1
Patient characteristics.

Characteristic		FAS			P
		DNG group (N = 64)	COC group (N = 34)	All (N = 98)	
N (%) for categorical variables					
Age (at enrollment)	Mean (±S.D.)	40.4 (±4.94)	30.9 (±5.35)	37.1 (±6.82)	<0.001
	Median (min–max)	41.0 (27.0–49.0)	32.0 (20.0–38.0)	38.0 (20.0–49.0)	
BMI	N	63	34	97	0.023
	Mean (±S.D.)	21.9 (±3.33)	20.4 (±2.16)	21.3 (±3.05)	
	Median (min–max)	20.9 (15.4–31.4)	20.0 (16.5–25.3)	20.8 (15.4–31.4)	
Smoking status	Current or former	11 (17.2)	7 (20.6)	18 (18.4)	0.679
	Current	5 (45.5)	3 (42.9)	8 (44.4)	
Family history of VTE	None	57 (89.1)	31 (91.2)	88 (89.8)	0.381
Surgical history	Yes	25 (39.1)	17 (50.0)	42 (42.9)	0.298
History of sex steroid hormone therapy	Yes	11 (17.2)	3 (8.8)	14 (14.3)	0.260
Comorbid condition(s)	Yes	30 (46.9)	8 (23.5)	38 (38.8)	0.024
Lesion site of endometriosis	Ovary	54 (84.4)	31 (91.2)	85 (86.7)	0.345
	Recto-uterine pouch	14 (21.9)	1 (2.9)	15 (15.3)	0.013
	Vesico-uterine pouch	0 (0.0)	1 (2.9)	1 (1.0)	0.168
	Uterosacral ligament	1 (1.6)	1 (2.9)	2 (2.0)	0.646
	Sigmoid colon/rectum	3 (4.7)	2 (5.9)	5 (5.1)	0.798
	Other	6 (9.4)	1 (2.9)	7 (7.1)	0.239
Subjective pain (VAS: 0–10)	N	38	26	64	0.616
	Mean (±S.D.)	5.2 (±2.86)	5.5 (±2.39)	5.3 (±2.66)	
	Median (min–max)	5.0 (0.0–10.0)	6.0 (1.0–10.0)	5.0 (0.0–10.0)	

COC, combined oral contraceptive; DNG, dienogest; FAS, full analysis set; VAS, visual analogue scale; VTE, venous thromboembolism.

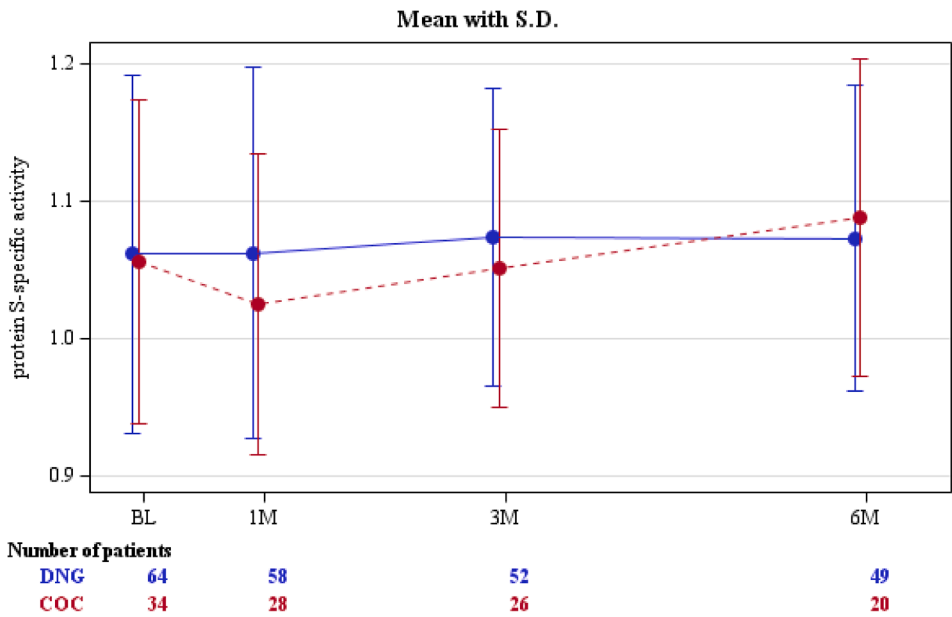


Fig. 2. Changes in protein S-specific activity.

Changes in total protein S activity

The mean total protein S activity remained within reference limits (0.74 – 1.34 IU/mL) in both groups, fluctuating within the ranges of 1.06 – 1.12 IU/mL among women taking DNG and 0.85 – 1.02 IU/mL among women taking COC. In the DNG group, the mean total protein S activity was slightly increased, with statistically significant increases at three and six months. Still, there was a recognizable pattern in the COC group: after dropping in the first month, total protein S activity varied little and remained low for the remainder of the observation period (BL: 1.02 IU/mL, 1 M: 0.85 IU/mL, 3 M: 0.85 IU/mL, 6 M: 0.87 IU/mL). The first-month decline in the COC group was confirmed to be statistically significant (Fig. 3; Table 2, 4). There were seven patients whose total protein S activities were below the reference limit two or more times during the treatment period, all were in the COC group. No VTE events

were observed during this study.

Changes in total protein S antigen levels

The mean total protein S antigen levels of both groups remained within reference limits (0.74 – 1.27 IU/mL), fluctuating within the ranges of 1.01 – 1.05 IU/mL (DNG) and 0.79 – 0.97 IU/mL (COC). In the DNG group, the mean total protein S antigen increased slightly after the treatment. Similar to total protein S activity, this measure decreased markedly in the first month among women taking COC, but continued to decrease steadily thereafter (BL: 0.97 IU/mL, 1 M: 0.83 IU/mL, 3 M: 0.81 IU/mL, 6 M: 0.79 IU/mL). The first-month decline in total protein S antigen levels in the COC group was confirmed to be statistically significant (Fig. 4; Tables 2, 4), just as it was for total protein S activity.

Protein S-specific activity (total protein S activity / total protein S

Table 2
Plasma concentration of protein S-specific activity, total protein S activity and total protein S antigen in baseline, first, third and sixth month of treatment.

Visit	DNG						-	COC					
	N	Mean	S.D.	Median	Min.	Max.		N	Mean	S.D.	Median	Min.	Max.
Protein S-specific activity													
BL	64	1.06	0.13	1.09	0.60	1.29		34	1.06	0.12	1.07	0.68	1.29
1 M	58	1.06	0.13	1.09	0.57	1.28		28	10.2	0.11	1.02	0.79	1.21
3 M	52	1.07	0.11	1.08	0.68	1.25		26	1.05	0.10	1.06	0.87	1.22
6 M	49	1.07	0.11	1.08	0.72	1.43		20	1.09	0.12	1.10	0.87	1.39
Total protein S activity (IU/mL)													
BL	64	1.06	0.18	1.02	0.67	1.54		34	1.02	0.15	0.99	0.71	1.41
1 M	58	1.07	0.19	1.04	0.59	1.47		28	0.85	0.14	0.86	0.65	1.24
3 M	52	1.12	0.21	1.08	0.74	1.67		26	0.85	0.13	0.87	0.56	1.10
6 M	49	1.11	0.20	1.08	0.74	1.54		20	0.87	0.15	0.86	0.56	1.32
Total protein S antigen (IU/mL)													
BL	64	1.01	0.19	0.96	0.73	1.85		34	0.97	0.11	0.94	0.79	1.22
1 M	58	1.02	0.22	0.97	0.72	2.08		28	0.83	0.11	0.83	0.60	1.10
3 M	52	1.05	0.20	1.00	0.74	1.73		26	0.81	0.10	0.80	0.62	1.03
6 M	49	1.04	0.20	1.00	0.71	1.59		20	0.79	0.09	0.78	0.62	0.95

DNG, dienogest; COC, combined oral contraceptive; BL, baseline; 1 M, first month; 3 M, third months; 6 M, sixth months.

Table 3
Data of patients who had low protein S-specific activity throughout the whole study period.

Visit	Patient 1 (DNG)	Patient 2 (DNG)	Patient 3 (DNG)	Patient 4 (COC)
BL	0.71 (0.67 / 0.95)	0.76 (0.78 / 1.03)	0.60 (1.11 / 1.85)	0.68 (0.71 / 1.05)
1 M	0.63 (0.59 / 0.93)	0.77 (0.99 / 1.29)	0.57 (1.18 / 2.08)	0.80 (0.67 / 0.84)
3 M	0.80 (0.74 / 0.93)	0.76 (0.81 / 1.07)	0.68 (1.18 / 1.73)	0.81 (0.65 / 0.80)
6 M	0.81 (0.79 / 0.97)	NA	0.83 (1.32 / 1.59)	0.78 (0.63 / 0.81)

antigen level, reference range: 0.86 – 1.18, –3.0SD = 0.78); BL, base-line; DNG, dienogest; COC, combined oral contraceptive; NA, not available; 1 M, 1 month; 3 M, 3 months; 6 M, 6 months.

Results of analysis using a linear mixed-effects model. Missing values are assumed to be missing at random. CFB, changes from baseline; DNG,

Dienogest; COC, combined oral contraceptive; BL, base line; 1 M, 1 month; 3 M, 3 months; 6 M, 6 months.

Discussion

The mean protein S-specific activity remained within the reference range in both the DNG and COC groups for the duration of the observation period. In the DNG group, total protein S activity and antigen levels increased slightly. This was expected, as progestin has been shown to increase protein S, although the degree of this effect varies with the type of progestin [17]. Conversely, in the COC group, both total protein S activity and antigen levels declined significantly immediately after the treatment began, remaining at attenuated levels for at least six months. According to data provided by the protein S-specific activity assay system [12], the amount of total protein S activity below the reference value includes VTE cases. Although the mean total protein S activity was within the reference range, there were several cases where levels were below the reference limits. Patients with low total protein S activity might have VTE, although there were no VTE patients in this study. VTE

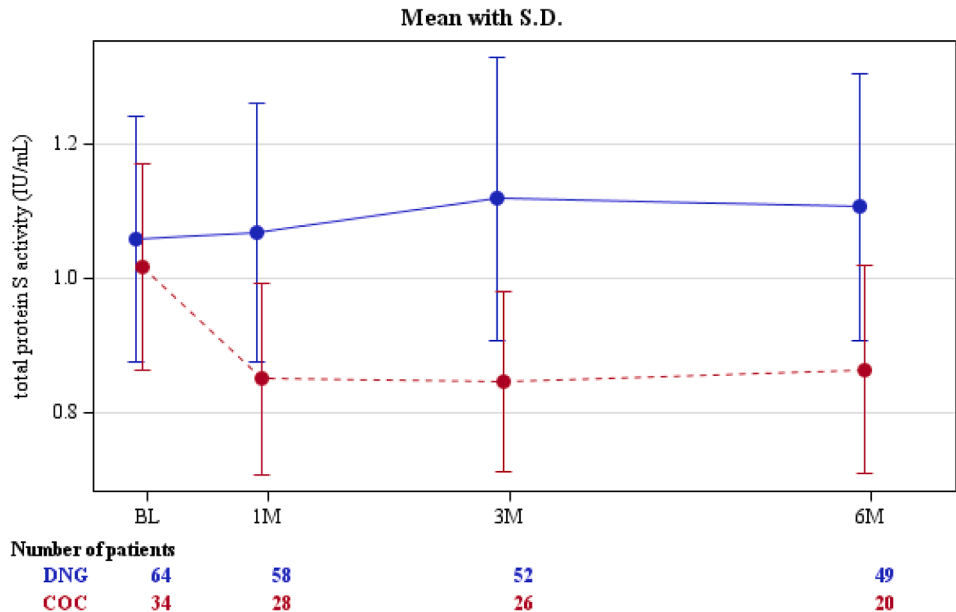


Fig. 3. Changes in total protein S activity.

Table 4
Changes from baseline by group and time.

Measure	Group	Time	CFB	95 %CI		P
				Lower	Upper	
Protein S-specific activity	DNG	1 M	−0.00215	−0.02796	0.02365	0.8697
		3 M	0.00526	−0.02161	0.03214	0.6998
		6 M	0.01368	−0.01375	0.04111	0.3269
	COC	1 M	−0.02843	−0.06459	0.00774	0.1228
		3 M	−0.01595	−0.05319	0.02128	0.3995
		6 M	0.01397	−0.02700	0.05494	0.5023
Total protein S activity (IU/ mL)	DNG	1 M	0.00385	−0.03315	0.04085	0.8378
		3 M	0.04729	0.00876	0.08581	0.0164
		6 M	0.04488	0.00557	0.08420	0.0254
	COC	1 M	−0.17419	−0.22605	−0.12233	<0.0001
		3 M	−0.17807	−0.23145	−0.12469	<0.0001
		6 M	−0.15601	−0.21472	−0.09730	<0.0001
Total protein S antigen (IU/ mL)	DNG	1 M	0.00896	−0.02271	0.04064	0.5777
		3 M	0.03614	0.00315	0.06913	0.0319
		6 M	0.02611	−0.00757	0.05978	0.1280
	COC	1 M	−0.14397	−0.18837	−0.09957	<0.0001
		3 M	−0.15754	−0.20326	−0.11183	<0.0001
		6 M	−0.16236	−0.21265	−0.11207	<0.0001

due to COCs typically develops within a year of starting COCs and is especially likely in the first three months [18]. The prolonged reduction in total protein S activity lasting at least six months after initiating treatment led us to conclude that vigilant monitoring should be maintained for longer than six months.

Four of the women were suspected of having protein S deficiencies. Their protein S-specific activity values were low throughout the study period. The frequency of low protein S-specific activity in our study population (4/111 = 3.6 %) exceeded previously reported estimates of the prevalence of protein S deficiency in the Japanese population [8–12]. Protein S deficiency is a type of congenital thrombophilia that is common in Asian populations but rare in Western populations [19–21]. Protein S type II deficiency, the most common form in the Japanese population, manifests as reduced protein S activity without an accompanying decline in protein S antigen levels. The causative mutation is usually protein S Tokushima (K196E), which results in a Lys/Glu substitution at the 196th residue within the protein’s second EGF-like

domain [14]. However, while plasma protein S activity in women with protein S Tokushima was 16 % lower than in the wildtype counterpart and tended to be even lower during COC use, protein S activity alone is not sufficient for identifying protein S Tokushima [22,23]. Protein S assays measure the free and bound forms of protein S separately. However, since some of the bound protein S dissociates (i.e. becomes free protein S) during the measurement process [24], none of these conventional assay systems are truly quantitative in nature. Genetic testing, on the other hand, lacks broad applicability because it requires patient consent, making it infeasible as a screening tool for VTE risk. In contrast, the protein S-specific activity assay system is superior to traditional assays because it quantifies total protein S activity and total protein S antigen as the sum of its free and bound forms, respectively [12]. The protein S-specific activity was determined by calculating the ratio of total protein S activity to total protein S antigen levels, which can be used to rate protein S dysfunction. This assay system has even shown good agreement with the presence of mutant protein S alleles [12,16]. A study of protein S-specific activity in Japanese individuals showed that patients with venous thrombosis had a significantly lower protein S-specific activity than patients with arterial thrombosis and non-thrombotic patients [16]; monitoring protein S activity and protein S-specific activity may be useful for the prevention of venous thrombosis. Since protein S deficiency is observed as frequently in China and Taiwan as in Japan [13], the protein S-specific activity assay system is likely to be useful not only in Japan but all of Asia.

We believe that endometriosis could be treated more safely if providers made a habit of checking protein S-specific activity before prescribing hormonal therapies. If low protein S-specific activity is present, which raises a suspicion of protein S deficiency, providers should recommend DNG. Furthermore, measuring total protein S activity in women on COCs could help prevent or detect VTEs at an early stage. One critical requirement of endometriosis interventions is that patients can continue their treatments safely over extended periods. We believe that a system for quantifying protein S-specific activity would be especially useful as a screening tool in VTE risk assessments for ethnically Asian patients who are particularly prone to protein S deficiency. If the results allow VTE to be avoided or diagnosed early, quantifying protein S-specific activity as a part of VTE risk screening alongside the traditional interview could improve women’s quality of life.

There were several limitations to the present study. First, patients in the two groups were not randomly assigned and physicians selected the

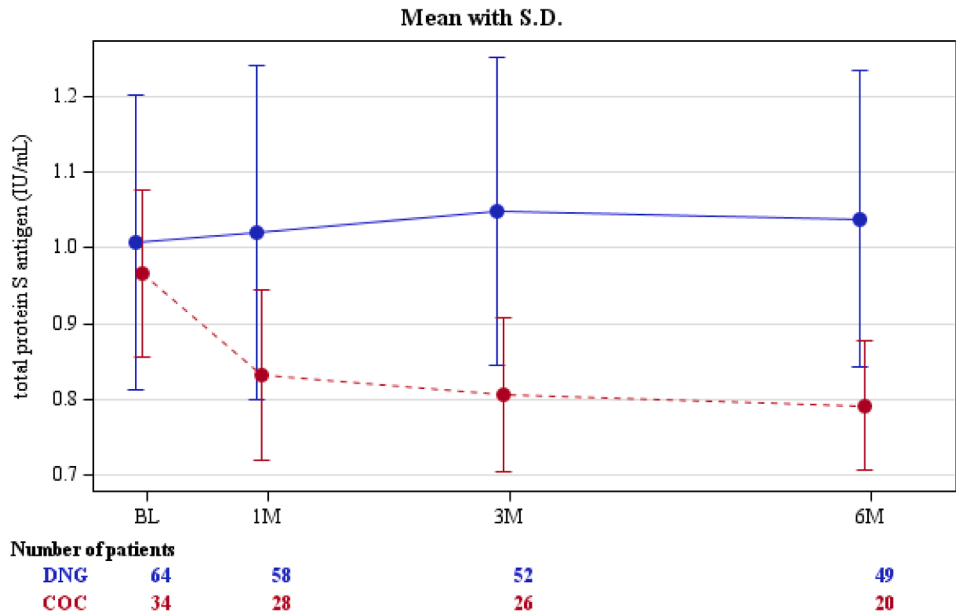


Fig. 4. Changes in total protein S-specific antigen.

appropriate medicine for the patients. This led to different numbers of cases between the two groups. Second, the sample size was relatively small and the participant group included no VTE events. To further verify this marker's usefulness in predicting thrombosis, it should therefore be monitored over a longer period alongside genetic testing in a larger sample of women on COCs.

Conclusion

The effect of DNG on protein S was negligible. COCs resulted in significant decreases in both total protein S activity and antigen levels immediately after the women started taking them and the attenuated levels persisted for at least the six month study period. However, the means of total protein S activity and antigen levels in the COC group remained within reference limits. Further study with a larger number of patients is required to confirm that protein S-specific activity can serve as a surrogate marker of VTE risk.

Declarations

Ethics approval and consent to participate: This study was approved by Kyushu University Institutional Review Board for Clinical Trials. Informed consent to participate in the study was obtained from all patients before enrollment. All methods were carried out in accordance with the Declaration of Helsinki.

Consent for publication: Consent for publication of this study is obtained from all patients.

Availability of data and materials: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Availability of date and materials

The data are not publicly available due to them containing information that could compromise research participant consent but are available from the corresponding author on reasonable request.

Funding: MOCHIDA PHARMACEUTICAL CO., LTD is only funding and had no influence on the design of the study and collection analysis, or interpretation of date and writing in the manuscript.

Authors' contributions

NY, KE, HK and KK planned the study, supervised the analysis, and interpreted the results. NY wrote the manuscript. HK did measured samples. All authors discussed and approved the final manuscript.

Trial registration: This study has registered in UMIN Clinical Trials Registry. The registry number is UMIN000022266, and the date of enrolment is May 10th, 2016.

CRediT authorship contribution statement

Natsuko Yokota: Writing – original draft, Visualization, Project administration, Formal analysis, Data curation, Conceptualization. **Reiko Inoue:** Resources. **Keiko Kawamura:** Resources. **Katsuko Ega-shira:** Writing – original draft, Visualization, Project administration, Formal analysis, Data curation, Conceptualization. **Hiroyuki Kuma:** Writing – original draft, Project administration, Conceptualization. **Kiyoko Kato:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Mr. Shimose conducted the data analysis and created the tables and figures. We thank Dr. Hiasa, Dr. Amada, Dr. Kato, Dr. Kitade, Dr.

Matsumoto, Dr. Nakahara, Dr. Sakai, Dr. Uchida, Dr. Ueoka, Dr. Watanabe and Dr. Yuki for data collection. We also thank Ms. Kanemitsu and Ms. Soda at Clinical Research Support Center Kyushu for their assistance. Financial support is provided by MOCHIDA PHARMACEUTICAL CO., LTD.

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