EXHIBIT 1015

Changes in Androgens During Treatment with Four Low-Dose Contraceptives

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The aim of the present study was to compare changes in the endogenous androgen environment in healthy women while on low-dose oral contraceptives (OCs). One-hundred healthy women were randomized to receive one of four OCs during six months: 21 tablets of Cilest®, Femodeen®, Marvelon®, or Mercilon®. During the luteal phase of the pretreatment cycle, body weight and blood pressure were recorded and the following parameters were measured: sex hormone-binding globulin (SHBG), corticosteroid-binding globulin (CBG), testosterone (T), free testosterone (FT), 5α-dihydrotestosterone (DHT), androstenedione (A), dehydroepiandrosterone-sulphate (DHEA-S) and 17α-hydroxy-progesterone (17OHP) while also the free androgen index (FAI) was calculated. Measurements were repeated during the 3rd week of pill intake in the 4th and the 6th pill month. There were no differences on body mass and blood pressure with the use of the four OCs. The mean serum DHEA-S decreased significantly in all groups though less in the Mercilon® group when compared to Cilest® and Marvelon® (approximately 20% vs 45%). Mean serum SHBG and CBG increased significantly in all four groups approximately 250% and 100%, respectively. In each group CBG also increased significantly but less in women taking Mercilon® (~75%) as compared to the others (~100%). Current low-dose OCs were found to have similar impact on the endogenous androgen metabolism with significant decreases of serum testosterone, DHT, A, and DHEA-S. They may be equally beneficial in women with androgen related syndromes such as acne and hirsutism. CONTRACEPTION 1996;53: 171–176

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Introduction

Oral contraceptives (OCs) have been described as a risk factor for the development of cardiovascular disease (CVD). At first this phenomenon was attributed to the estrogenic component of the OC.[1,2] Parallel with dose reduction of both the estrogenic and the progestogenic component and the introduction of new progestins, the incidence of CVD decreased significantly. At present there is doubt whether or not modern OCs increase the risk for cardiovascular disease.[3,4] The mechanism of a possible influence on CVD has also been attributed to the intrinsic androgenic potential of progestins used in OCs. This can at least in part be theoretically attributed to the influences on lipid metabolism.[5]

The androgen environment in individual women taking oral contraceptives depends on the eventual androgenic effect of the utilized progestin, the anti-gonadotropic effect of the estrogen-progestin combination altering ovarian androgen production and the effect of the OC on plasma proteins with sex steroid-binding properties.

The androgenic effect of progestins may be exerted in two different ways: first, through stimulating the binding of androgens to the androgenic receptors of the target organ and, second, to their affinity to sex hormone-binding globulin (SHBG) and other androgen binding proteins, thus displacing testosterone (T) from SHBG binding and consequently increasing the proportion of free testosterone (FT).[6] Therefore, the SHBG level is a useful parameter in the evaluation of the androgenicity of oral contraceptives.

Literature data examining the new progestins norgestimate, desogestrel and gestodene concentrate on their possible influence on lipid metabolism and clotting. Surprisingly, few reports systematically assess the changes in sex steroids exerting androgenic properties during treatment with OC.

The aim of the present study was to compare the effects of four modern low-dose OCs on sex steroids with androgenic properties and their binding proteins

in serum. Therefore, the present study specifically evaluated changes brought about in plasma concentrations of SHBG, CBG, albumin (Alb), T, FT, dihydrotestosterone (DHT), androstenedione (A), dehydroepiandrosterone-sulphate (DHEA-S) and 17α-hydroxyprogesterone (17OHP).

Material and Method

Study Design

In an open randomised study, 100 female volunteers were equally assigned to one of four different OC preparations during six cycles on therapy (Table 1).

The healthy volunteers ranging in age between 18 and 38 years, were not pregnant, not lactating, and had delivered at least six months before inclusion into the study. Their menstrual cycles had to be within a duration between 25 and 36 days.

Obese women were excluded [Body Mass Index (BMI)= weight in kg/height in m^2 > 28.0].[7] The use of steroid-containing medication or medication possibly interfering with steroid metabolism was discontinued for at least two months prior to treatment and the use of medication known to alter liver enzyme function was not allowed. Also, the well known relative contraindications to the use of OCs such as a history of thromboembolic disorder, hyperlipoproteinemia, known or suspected estrogen-dependent neoplasia, liver tumors and undiagnosed abnormal genital bleeding were reasons for exclusion. Finally, the volunteers were not allowed to smoke more than 10 cigarettes a day.

Each subject was seen before therapy during a control cycle between days 24–27 (luteal phase) and while on therapy during pill cycle 4 and 6 (between days 18–21 of pill intake). At every visit, body weight and blood pressure were recorded and blood collected for measurements of SHBG, CBG, Alb, T, FT, DHT, A, DHEA-S and 17OHP. Also, the free androgen index (FAI=T×100/SHBG)[8] was calculated. All blood samples were drawn between 08.00 and 10.30 am after a fasting period of at least 12 h. The blood was allowed to clot at room temperature, serum was col-

lected after centrifugation at 2000×g and kept frozen at –20°C until assayed. The volunteers were instructed to take the first tablet on the first or second day of the menstruation following the control cycle visit. Then one tablet was taken daily at a fixed time for 21 days followed by 7 tablet-free days.

The study was approved by the ethical committee of the hospital beforehand and was conducted according to the guidelines for Good Clinical Practice.[9] Written informed consent was obtained from all subjects at the beginning of the study.

Assays

SHBG was measured by a commercially available non-competitive coated-tube immunoradiometric assay (IRMA, Farmos Diagnostica, Orion Corporation, Turku, Finland) based on the method of Hammond et al.[10] The within-assay and between-assay coefficients of variation (CV_w and CV_b) ranged from 2.6% to 2.9% and from 3.5% to 4.6%, respectively. Serum concentrations of CBG were measured with a commercially available direct competitive liquid-phase RIA (Techland SA, Liège, Belgium); the CV_w ranged from 3.2% to 4.7% and the CV_b from 8.6% to 8.8%. Serum T was measured after diethylether extraction by a specific charcoal RIA as described by Dony et al.[11]; the CV_w and the CV_b were 5.6% and 6.9%, respectively. Serum FT concentrations were assayed with a commercially available direct competitive solid-phase RIA (Diagnostic Products Corporation, Los Angeles, CA, USA); CV_w and CV_b were 6.4% and 12.1%, respectively. DHT was measured with a specific in-house charcoal RIA. Purification of serum specimens by diethylether extraction and column chromatography with Sephadex LH20 were as described by Van Duren[12]; CV_w and CV_b calculated from a serum pool with a mean DHT level of 1.8 nmol/L were 6.5% and 11.7%, respectively. Androstenedione was measured after diethylether extraction and Sephadex LH20 chromatography by a charcoal RIA as described by Van Duren[12]; CV_w was 3.6% and CV_b was 7.0%. DHEA-S concentrations were measured with a highly specific charcoal RIA as described by Reijnders[13]; CV_w and CV_b were 3.7% and 6.9%, respectively. Concentrations of 17OHP in serum were determined after diethylether extraction followed by Sephadex LH20 chromatography with a specific charcoal RIA as described by Dony et al.[11]; CV_w and CV_b were 3.7% and 6.9%, respectively.

Statistics

All values are given as mean ± sd. P values less than

Table 1. Composition of the four monophasic contraceptives tested

Preparation	Ethinylestradiol per Tablet	Progestagen per Tablet
Cilest®	35 µg	250 µg norgestimate
Femodeen®	30 µg	75 µg gestodene
Marvelon®	30 µg	150 µg desogestrel

heteroscedasticity and skewness, all parameters were log-transformed.

In the primary analysis, changes from baseline in the four groups were compared using the Welch multiple range test.[14] In order to controll the overall error rate per parameter, this was done in a stepwise manner: first the changes over 6 months were evaluated, and only if these were (nominally) significant, the changes over the first 4 months were also evaluated (close testing procedure[15]). This method corresponds to the intuitive idea that one first investigates if there are any long-term differences after 6 months and then, only if this is the case, one investigates whether the differences already occur after 4 months.

In a secondary analysis, the changes from baseline within the groups were evaluated. In order to control the overall error rate, a pooled t-test was carried out: the changes were evaluated for the four groups simultaneously in an ANOVA model with no intercept. Again changes over 4 months were tested, only if those over 6 months were found significant.

Results

Population Characteristics
The characteristics of the study groups are given in Table 2.

Dropouts
Two women became pregnant during the control cycle and, therefore, dropped out prior to treatment. They were replaced according to the protocol.

There were twelve true dropouts, the reasons for which are summarized in Table 3. Subject number 81 (Ci-group) was not available during pill cycle 4, which is the reason why the second visit was planned during pill cycle 3. Subject number 85 (Fe-group) was not available during the third, fourth and fifth pill cycle; she was unable to attend the second visit. Hence, a total of 88 volunteers completed the study according to the protocol.

Vital Signs
Changes in body weight and blood pressure (systolic and diastolic) registered in the control cycle and in

Table 3. Drop-outs according to pill cycle, subject number, pill type and complaint(s)

Pill Cycle	Subject Number	Pill Type*	Complaint(s)
1	15	Ci	Headache of unknown origin
1	85	Fe	Spotting/breakthrough bleeding
2†	41	Me	Mastopathy
2†	43	Me	Tachycardia/hyperventilation
2†	68	Ci	Body weight and mood change
3	76	Fe	Headache during pill-free week
3†	81	Ci	Pruritis vaginalis
4	3	Fe	Sinusitis: antibiotics
4	16	Ci	Hepatitis A
4†	58	Ma	Protocol violation
4†	6	Me	Increase in migraine
Unknown	98	Me	Poor compliance

*Ci—Cilest®; Fe—Femodeen®; Ma—Marvelon®; Me—Mercilon®.
†Drop-out after pill day 21.

the pill cycles 4 and 6 are shown in Table 4. None of these parameters significantly changed throughout the study period.

Hormones and Binding Proteins
All the steroidal serum parameters tested (T, FT, DHT, A, DHEA-S, 17OHP, Alb) significantly decreased while on medication during 6 pill cycles (ratio of decrease between 1.3 and 3.0), irrespective of the OC preparation used (Tables 5 and 6). All changes were observed already after 4 pill cycles.

Comparing the decreases of the above mentioned variables after 6 months between the 4 pill groups, the only significant difference observed concerned DHEA-S: the mean decrease of DHEA-S was $21\pm18\%$ in the Me-group, significantly different compared to $43\pm18\%$ and $44\pm18\%$, respectively, in the Ci- and Ma-group, while the Fe-group was in between with a decrease of $34\pm14\%$.

Significant increases were observed in the levels of steroid binding proteins. Both SHBG and CBG increased significantly during pill-intake in all four

Table 2. Sample characteristics (mean ± sd)

Oral Contraceptive	Age (Years)	Cycle Length (Days)	Menses (Days)	BMI* (kg/m²)
Cilest®	26.9 (4.2)	28.3 (1.5)	5.0 (0.8)	22.2 (2.3)
Femodeen®	26.3 (4.9)	28.8 (2.1)	4.7 (1.2)	22.0 (2.4)
Marvelon®	27.1 (5.1)	28.6 (2.1)	5.0 (1.0)	22.0 (2.4)

Table 4. Changes in body mass and systolic and diastolic blood pressure of the four preparations tested after six pill cycles (mean ± sd)

	Change in Body Mass (%)	Change in Systolic Blood Pressure (%)	Change in Diastolic Blood Pressure (%)
Cilest®	+0.5 (3.9)	+1.6 (9.8)	+3.9
Femodeen®	+1.8 (3.9)	−1.4 (6.6)	+0.5
Marvelon®	+1.0 (2.8)	+0.0 (7.6)	−2.4
Mercilon®	+1.1 (2.8)	−0.5 (7.7)	−1.0

groups. At six months on therapy, the mean increase of SHBG was 263% (±119%) and 94% (±26%) in the case of CBG.

Again, there were no differences between the different pill groups except for CBG: this protein shows a significantly less increase in women taking Mercilon® (74±21%) as compared to the three other contraceptives, Cilest® 96±31%, Femodeen® 101±21% and Marvelon® 102±22%.

Discussion

Progestins, especially those with intrinsic androgenic activity, have an opposite effect on lipoprotein metabolism as compared to estrogens. They especially stimulate hepatic lipase, thereby accelerating metabolism and clearance of high density lipids (HDL) and lowering HDL levels. Low HDL levels are thought to increase the risk for CVD.[16]

The binding of a given progestin to SHBG is an important determinant of its androgenicity. The progestins used in this study may, in this respect, however, differ from others.[17,18] These new progestins have been developed because of their small or absent

intrinsic androgenic properties. Many studies have indeed indicated that they have no negative effect on lipid metabolism. In this study the suppression of plasma hormone concentrations in individuals on four different OCs has been evaluated.

All androgen parameters investigated in the present study decreased while taking the new OCs regardless of the type. At the same time, the androgen binding proteins increased. The net effect of this was an even more substantial decrease of biologically active testosterone. The concentration of the most active androgen in the human, 5α-dihydrotestosterone, is directly related to that of free testosterone. Therefore, during intake of these OCs the endogenous androgen environment changed in the direction of hypoandrogenism. It is speculated that even if the applied progestins exert an intrinsic androgen action, this is negligible as concerned to the changes in the endogenous environment. It seems safe to postulate that all these four OCs are equally beneficial with regard to seborrhoea, acne and hirsutism, but the clinical effects of the four preparations regarding to the skin have not been investigated in our study. They should also give rise to a favourable change in lipid metabolism and an increase in the high density/low density lipids (HDL/LDL) ratio has indeed been reported for all of them.[19,20]

Norgestimate, desogestrel and gestodene all bind to progesterone and androgen receptors. The binding to the androgen receptor is significantly reduced, however, as compared to the older progestins. In view of our findings and the reports on HDL/LDL, therefore, a further attempt to reduce the progestin content of these OCs does not seem necessary. It may even be unwarranted, as a less effective cycle control may be the only achieved result.[21] All OCs tested in the present study decreased the concentration of DHEA-S

Table 5. Serum concentrations of albumin (Alb), sex hormone-binding globulin (SHBG), and corticosteroid-binding globulin (CBG); values are given as mean ± sd

Serum Variable		Control Cycle (n = 100)	Pill Cycle 4 (n = 90)	Pill Cycle 6 (n = 88)
Alb (g/L)	Ci	47.40 (2.63)	42.73 (2.73)	43.10 (1.97)
	Fe	47.76 (2.79)	43.95 (2.50)	43.41 (2.91)
	Ma	46.80 (2.53)	42.56 (1.76)	42.50 (2.02)
	Me	47.60 (2.45)	43.00 (2.56)	43.43 (2.60)
SHBG (nmol/L)	Ci	55 (20)	173 (48)	174 (64)
	Fe	51 (17)	163 (49)	162 (47)
	Ma	61 (31)	201 (68)	217 (88)
	Me	48 (20)	157 (52)	156 (49)
CBG (mg/L)	Ci	44.0 (5.6)	85.5 (7.9)*	84.5 (7.9)*
	Fe	43.8 (4.5)	86.2 (8.1)*	88.3 (7.9)*
	Ma	41.2 (5.5)	82.7 (6.4)*	82.4 (9.6)*

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