PLASMA LIPID AND LIPOPROTEIN LEVELS IN REGULARLY MENSTRUATING FEMALES WITH OVULATORY AND ANOVULATORY MENSTRUAL CYCLES

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Abstract

Introduction: Impaired ovarian hormone secretion affects plasma lipoprotein profile which in postmenopausal women is less favorable than in premenopausal ones. Similarly, in females engaged in vigorous physical activity with depressed ovary function such as oligomenorrhea or amenorrhea plasma lipoprotein profile is unfavorable in comparison with their regularly menstruating counterparts. However, data concerning plasma lipoprotein levels in females with subtle disturbances in ovarian hormone secretion resulting in anovulatory menstrual cycles are not available.

Aim of the study: The aim of the present study was the comparison of plasma lipoprotein, free fatty acid, and glycerol levels in regularly menstruating active females with ovulatory and anovulatory menstrual cycles. Additionally, we looked for associations between subjects' hormonal status (plasma insulin, cortisol and testosterone levels) and plasma lipids and lipoproteins.

Methods: A total of 37 women participated in the study. On the basis of 3 months basal body temperature measurements and the determination of plasma levels of progesterone and 17β-estradiol they were classified as ovulating (n=21) or non-ovulating (n=16). Total cholesterol (TC), HDL-cholesterol (HDL-C), triacylglycerols (TG), free fatty acids (FFA) and glucose were determined in fasting blood plasma using colorimetric methods and commercial kits (Randox Laboratories, UK). Insulin, cortisol and testosterone were measured by a standard radioimmunoassay and commercial kits (POLATOM, Poland).

Results: There were no between-group differences in plasma TC, HDL-C, LDL-C, TG, glycerol, insulin, cortisol and testosterone levels. In non-ovulating women plasma FFA levels between days 5-8 and 19-22 of the menstrual cycle were significantly higher (P<0.01 and P<0.001, respectively) than in ovulating counterparts. Elevated plasma FFA concentrations may be possibly recognized as a first sign of impaired lipid metabolism due to depressed ovary function.

Conclusion: Subtle disturbances in ovarian hormone secretion promote changes in plasma FFA levels, what has to be recognized as a first sign of impaired ovary function.

Key words: Lipids, lipoproteins, females, ovulation, anovulation

Introduction

Numerous studies have indicated the associations between ovarian hormone secretion and lipoprotein metabolism (1,2). In postmenopausal women decreased levels of endogenous estrogen unfavorably affect the lipoprotein profile with significant increase in plasma total cholesterol and decrease in HDL-cholesterol levels observed as early as during transition from perimenopausal status to menopause (3-5). Additionally, elevated lipoprotein levels have also been demonstrated in premenopausal women with hypoestrogenic conditions due to hypocaloric diet (e.g. in anorexia nervosa) and in polycystic ovary syndrome (PCOS) (6-9).

Data concerning the effect of ovarian hormone deficiency on plasma lipoprotein in female athletes are scarce. Kaiserauer et al. (10) have indicated that in amenorrheic runners plasma LDL-cholesterol levels were higher than in regularly menstruating ones. Furthermore, Lamon-Fava et al. (11) have demonstrated slightly higher plasma total cholesterol and triacylglycerol concentrations in amenorrheic runners than in their regularly menstruating counterparts.

Recently, Rickenlund et al. (12) have found that amenorrheic female athletes engaged in different endurance sports (distance running, marathon, cross-country skiing, triathlon and orienteering) are characterized by higher plasma total cholesterol and LDL-cholesterol concentrations in comparison with both oligomenorrheic and regularly menstruating ones. In addition, in oligomenorrheic athletes plasma LDL-cholesterol levels were inversely correlated with the number of menstruations during the last year. Additionally, it should be stressed that even slight disturbances

in lipoprotein profile in amenorrheic female athletes of the above-cited study were associated with decreased flow-mediated dilatation (FMD),which is considered to increase the risk of atherosclerosis.

Unfavorable effects of vigorous physical activity on plasma lipoprotein in females have also been shown in physical education students, with females characterized by higher plasma total cholesterol and LDL-cholesterol levels in comparison with their male counterparts (13).

On the contrary, there is a wealth of studies indicating that hormone replacement therapy in menopausal women improves plasma lipoprotein profile (14,15,16). Similarly, oral contraceptive use in physical education female students brings about lower plasma LDL-cholesterol and higher plasma HDL-cholesterol levels in comparison with women not using oral contraceptives (13).

It is well known that in physically active females more subtle disturbances in ovarian hormones secretion exist leading to anovulatory menstrual cycles (17). However, their diagnosis needs time-consuming procedures (at least three months measurements of basal body temperature and subsequent ovarian hormone determination) (18). Thus, there are no studies concerning plasma lipoprotein profile in non-ovulating females in comparison with ovulating counterparts.

The aim of the present study was the comparison of plasma lipoprotein, free fatty acid, and glycerol levels in regularly menstruating active females with ovulatory and anovulatory menstrual cycles. Additionally, we looked for associations between subjects' hormonal status (plasma insulin, cortisol and testosterone levels) and plasma lipids and lipoproteins.

Materials and Methods
Subjects

The participants were recruited on the basis of advertisements in the student's dormitories. All the subjects were physically active with the mean physical activity approximately 8 h/week. All the women were regularly menstruating, healthy non-smokers and not taking any medication on a regular basis. They were advised to follow their habitual diet and lifestyle throughout the study and to refrain from physical activity at last 48 h before blood sampling. The experimental protocol was accepted by the Ethics Commission at the Academy of Physical Education. All the females gave their written consent prior to participation. The prospective subjects monitored their basal body temperature (BBT) for 3 months before the study (19). Plasma 17ß-estradiol and progesterone concentrations were determined twice per cycle – between days 5 and 8 and again between 19 and 22 of the cycle. In 21 females with biphasic BBT patterns plasma progesterone levels exceeded 19 nmol/l between days 19

and 22 of the cycle. These participants were classified as ovulating (20). In 16 women, a lack of increase in BBT was observed, and plasma progesterone levels between days 19 and 22 of the cycle did not differ from those determined between days 5 and 8 of the cycle. Those subjects were classified as non-ovulating. The sequence of the study events is presented in Table 1.

Table 1. *The sequence of the study events*

Months	BBT*	Blood samples
I	+	-
II	+	-
III	+	-
IV	+	-

* Basal body temperature measurements

Blood samples

The participants were instructed to take their last meal no later than 10:00 – 10:30 p.m on the day preceding blood sampling. Blood was drawn between 8:30 and 9:00 a.m. into EDTA-containing tubes for free fatty acid (FFA) determination or into lithium heparin tubes for the assay of other biochemical variables using disposable syringes and needles under aseptic conditions. Erythrocytes and plasma fraction were separated by centrifugation (15 min/4000 rpm) and plasma was stored at -70°C until analyzed. All analyses on plasma obtained from one subject were performed on the same day. Blood withdrawal and processing were performed in the laboratory of the Department of Biochemistry in the Academy of Physical Education.

Biochemical analysis

Plasma levels of glucose, total cholesterol (TC), HDL-cholesterol (HDL-C), triacylglycerol (TG), FFA and glycerol were measured colorimetrically using Randox commercial kits (Randox Laboratories, United Kingdom). The coefficients of variations (CV) for the above-mentioned variables did not exceed 5%. Plasma LDL-cholesterol (LDL-C) concentrations were calculated according to Friedewald et al. (21). Plasma progesterone, 17ß-estradiol, cortisol, testosterone and insulin levels were determined by standard radioimmunoassay and commercial kits (POLATOM, Poland). Intra-and inter-assay coefficients of variation (CV) for 17ß-estradiol were 3.8% and 7.9%, for progesterone - 4.3% and 5.0%, for insulin - 6.8% and 9.3%, for testosterone -5.3% and 5.4% and for cortisol- 3.6% and 6.8%, respectively. C-peptide was assayed using ELISA and IBL commercial kits (Germany). Intra- and inter-assay coefficient of variations (CV) for C-peptide was 8.2% and 9.8%, respectively. All assays were run in duplicate.

Anthropometric measurements

Three skinfolds (abdominal, supscapular, triceps) were determined using Harpenden caliper (United Kingdom). Each measurement was repeated twice and the mean values were used for calculation of body fat (22).

Satistical analysis

The variable distribution was tested using the Shapiro-Wilk W test. Non-parametric Kruskal-Wallis ANOVA was used to establish the effect of ovulatory status as an independent variable (0-ovulatory cycles, 1-anovulatory cycles) on plasma insulin, C-peptide, 17β-estradiol, progesterone, testosterone, glycerol, FFA, LDL- C, TG levels determined between days 5-8 and 19-22 and recognized as dependent variables. Mann-Whitney U-test and Wilcoxon matched pairs test were used for comparison of means with non-Gaussian distribution. The effect of ovulatory status of normally distributed variables (plasma TC, HDL-C, cortisol) was established using two-ways ANOVA and post-hoc Tukey test. Spearman rank correlation coefficients between circulating FFA and hormones were also calculated for collected data of both follicular and luteal phases of the menstrual cycle. All calculations were performed using STATISTICA 6.0 (StatSoft, Inc. USA). Significance was set at P < 0.05. The results were expressed as the mean ± SD.

Results

Characteristics of the participants are presented in Table 2. There were no differences between ovulating and non-ovulating women with respect to mean values of anthropometric data. However, the mean menstrual cycle was markedly longer (p<0.01) in non-ovulating women than in their ovulating counterparts.

Plasma 17β-estradiol levels between days 5 and 8 were significantly lower that between days 19 and 22 of the menstrual cycle in both of the groups (Table 3). However it was markedly lower in both phases of the cycle in non-ovulating participants (p<0.001 and p<0.01 between days 19 and 22 of the cycle, respectively).

In ovulating subjects plasma progesterone levels between days 19 and 22 increased 17.5 times (p<0.001) in comparison to days 5-8 of the cycle. In contrast, plasma progesterone concentrations between days 19 and 22, while markedly higher than between days 5 and 8 (p<0.02), were significantly lower (p<0.001) in non-ovulating than in ovulating women.

Table 2. *Subject characteristics (mean ± SD)*

	Ovulating women (n=21)	Non-ovulating women (n=16)
Age (years)	20.5 ± 1.2	20.3 ±1.0
Body mass (kg)	63.3 ± 6.8	62.1 ± 8.2
Body height (cm)	172.4 ± 6.7	169.8 ± 7.0
Fat (kg)	15.4 ± 3.0	15.5 ±3.5
Fat (%)	24.2 ± 2.0	23.5 ± 6.1
FFM (kg)*	47.8 ± 5.2	46.8 ± 5.2
Menstrual cycle length (days)	27.8 ± 1.9	30.2 ± 3.5[a]

*Fat free mass; [a] significantly longer than in ovulating women (P < 0.01)

Table 3. *Hormonal status of ovulating and non-ovulating subjects (mean ± SD)*

	Ovulating women (n=21)		Non-ovulating women (n=16)	
	Days 5-8	Days 19-22	Days 5-8	Days 19-22
17β-estradiol (pmol·L⁻¹)	206.5 ± 103.9	525.8 ± 122.3[a]	94.9 ± 22.0	395.5 ±172.0[a,c]
Progesterone (nmol·L⁻¹)	2.2 ± 0.6	38.5 ± 13.1[a]	2.1 ± 0.6	5.1 ± 3.8[b,d]
Testosterone (nmol·L⁻¹)	2.7 ±0.8	2.7 ±1.1	2.7 ±1.1	2.8 ±1.1
Cortisol (nmol·L⁻¹)	439.4 ±106.3	416.5 ±143.3	472.9 ±152.8	415.5 ±102.6
Insulin (pmol·L⁻¹)	51.6 ± 21.6	55.7 ± 22.4	43.4 ± 21.7	45.2 ± 20.7
C-peptide (pmol·L⁻¹)	521 ± 197	572 ± 169	481 ± 144	564 ±220

significantly different vs. days 5-8, [a] P < 0.001, [b] P < 0.02
significantly different vs. respective values in ovulating women, [c] P < 0.001, [d] P <0.05

Table 4. *Plasma lipoprotein profile, free fatty acid, glycerol and glucose concentrations in ovulating and non-ovulating women in respect to menstrual cycle phases (mean ± SD)*

	Ovulating women (n=21)		Non-ovulating women (n=16)	
	Days 5-8	Days 19-22	Days 5-8	Days 19-22
TC (mmol·L^{-1})*	5.5 ± 1,0	5.4 ± 1.0	5.4 ± 0.8	5.3 ± 0.9
LDL-C (mmol·L^{-1})	3.7 ± 0.8	3.7 ± 1.0	3.7 ±0.7	3.6 ± 0.9
HDL-C (mmol·L^{-1})	1.6 ± 0.5	1.6 ± 0.4	1.4 ±0.3	1.5 ± 0.3
TG (mmol·L^{-1})	0.66 ± 0.20	0.58 ± 0.21	0.76 ± 0.28	0.72 ± 0.31
FFA (mmol·L^{-1})	0.322 ± 0.162	0.304 ± 0.202	0.373 ± 0.223[a]	0.386 ± 0.182[b]
Glycerol (µmol·L^{-1})	78.9 ± 19.9	74.5 ± 25.1	75.8 ±28.2	76.5 ±31.2

*TC–total cholesterol; LDL–C – LDL–cholesterol. HDL–C– HDL–cholesterol; TG– triacylglycerols; FFA – free fatty acids;
Significantly different vs. respective values in ovulating women ([a] P< 0.01; [b] P < 0.001)

There were no differences between groups in plasma testosterone, cortisol, C-peptide and insulin concentrations. However, plasma insulin levels in ovulating women in both cycle phases tended to be higher than in their ovulating counterparts (by 18.9% and by 23.2% between days 5 and 8 and between days 19 and 22, respectively).

There were no differences in plasma concentrations of TC, LDL-C, HDL-C, glycerol and TG between ovulating and non-ovulating women. (Table 4). But it should be stressed that plasma TG levels in non-ovulating participants were slightly higher than in ovulating ones (by 15% and 24% between days 5-8 and 19-22 of the cycle, respectively).

However, both between days 5 and 8 and 19 and 22 plasma FFA concentrations were markedly higher in non-ovulating females in comparison with ovulating subjects (p<0.001).

In non-ovulating women plasma FFA levels were not correlated with plasma glycerol concentrations, but in ovulating females there was a significant association between both variables (p<0.001) (Fig. 1 and 2).

Discussion

Despite marked differences in circulating ovarian hormones between ovulating and non-ovulating subjects, they did not differ with respect to the mean testosterone and cortisol plasma levels. Assuming that both cortisol and testosterone participate in the regulation of lipid metabolism – cortisol by stimulation of both hormone-sensitive and lipoprotein lipase in the adipose tissue, and testosterone by reduction of the expression of hormone-sensitive lipase and ß$_2$ –adrenoceptors (23-25), our results suggest that they are not responsible for differences in plasma lipid levels observed between ovulating and non-ovulating women.

However, the most important finding of the present study concerns significant difference in plasma FFA levels, markedly higher in non-ovulating women both
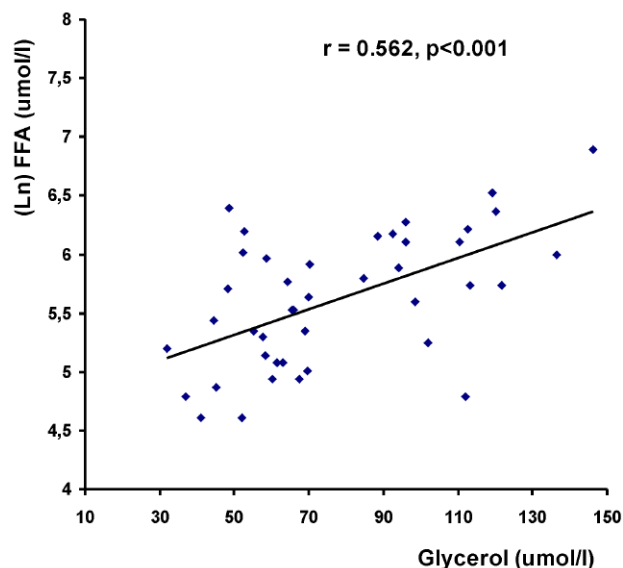

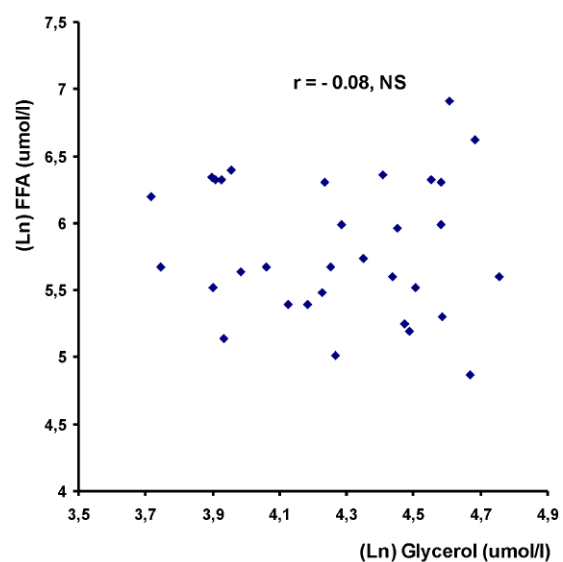Fig. 1. *The relationship between plasma FFA and glycerol levels in ovulating women*

Fig. 2. *The relationship between plasma FFA and glycerol levels in non-ovulating women*

between days 5-8 and 19-22 of the menstrual cycle in comparison with their ovulating counterparts. On the contrary, there were no between-group differences in plasma glycerol concentrations. Additionally, in ovulating, but not in non-ovulating subjects, a significant correlation was noted between plasma FFA and glycerol levels.

Empirical evidence has shown that fasting plasma FFA levels result from complicated balance between, on the one hand, fatty acid release from adipose tissue TG lipolysis as well as from intravascular lipolysis of TG-rich lipoproteins (VLDL), on the other hand - from FFA uptake by peripheral tissues such as striated and heart muscles and the liver (26).

Both adipose tissue and intravascular lipolysis and in consequence FFA release are precisely regulated by hormonal (adrenaline, testosterone, cortisol, insulin) and neural (noradrenaline) factors affecting enzymes contributing to TG hydrolysis as well as ß-adrenoceptor expression (27,28,29,30). Furthermore, peripheral FFA uptake is at least partially under control of the expression of FFA transporters, which are sensitive to insulin action (31,32). Moreover, both FFA gradient between blood and tissues and energetic demands are known to affect FFA transport from central to peripheral compartment (33).

In addition, adipose tissue re-estrification of FFA originated from lipolysis seems to play a substantial role in the regulation of plasma FFA concentrations. Van Harmelen et al. (34) have indicated that both insulin and acylation-stimulating protein (ASP) inhibit basal and noradrenaline-induced FFA release from adipose tissue in vitro by stimulation of FFA re-estrification. Similarly, Kalderon et al. (35) have found that in the fasting rat in vivo 57% of lipolyzed FFA is re-estrified back into adipose tissue. In addition, about two-thirds of FFA re-estrification is carried out before FFA release into circulation and only 36% of FFA is oxidized in the muscle, whereas only a minor fraction is channeled into TG-containing hepatic very low density lipoprotein (VLDL). Diraison et al. (36) have revealed that extrahepatic re-estrification and peripheral FFA oxidation each contribute about one-half to whole-body FFA utilization in the postabsorptive state.

Assuming that fasting plasma glycerol concentrations are a good measure of both adipose tissue and intravascular lipolysis (37,38) and taking into account the lack of differences between circulating glycerol between ovulating and non-ovulating subjects, it could be presumed that the differences in plasma FFA levels between ovulating and non-ovulating women are due either to impaired FFA re-estrification in adipose tissue or to their peripheral uptake.

Research has well documented ovarian hormone effects on substrate availability and utilization. In

ovariectomized rats significant reduction in muscle oxidative phosphorylation has been noted (39). More detailed studies have indicated that ovariectomy reduces the activity of a key enzymes of FFA oxidation (CPT I and HAD) in the muscle (40). Moreover, research has well documented that adipocyte insulin sensitivity and in consequence antilipolytic activity is impaired in females with PCOS and hypogonadotrophic hypogonadism (HH) (41,42).

Thus, it could not be excluded that at least 3 month anovulatory cycles and depressed ovarian hormone secretion in non-ovulating subjects brought about elevated plasma FFA levels due to decreased FFA oxidation in the muscle and/or re-estrification in the adipose tissue.

The above-mentioned assumption seems feasible and is supported by lack of correlation between plasma FFA and glycerol levels in non-ovulating women, indicating that lipolytic rate plays a minor role in the regulation of circulating FFA in subjects with disturbed ovarian hormone secretion. However, research has shown that 17β-estradiol exerts a direct, non-genomic action on endocrine pancreas stimulating insulin secretion (43,44). In the current study circulating plasma insulin did not differ in ovulating and non-ovulating women, however both between days 5-8 and 19-22 hormone concentrations in non-ovulating women were lower by 15.9% and by 18.8%, respectively in comparison with ovulating ones. Therefore, it could not be excluded that it was due to low circulating 17β-estradiol levels and slightly depressed endocrine pancreas function.

Considering the no differences observed between non-ovulating and ovulating women in plasma C-peptide concentrations, that is recognized as a good indicator of insulin secretion from the endocrine pancreas, it could be postulated that insulin secretion was similar in both groups (45).

Therefore, slightly lower circulating insulin in non-ovulating women was probably due to elevated hepatic insulin uptake (44). In turn, lower circulating insulin in non-ovulating women may promote adipose tissue lipolysis resulting in higher circulating FFA (46). This was not the case in the current study, since plasma glycerol levels did not differ between ovulating and non-ovulating women, supporting the above-mentioned hypothesis that higher plasma FFA in non-ovulating women is probably due to depressed peripheral uptake and/or adipose tissue re-estrification. However, the reason for the elevated plasma FFA observed in non-ovulating women results in this investigation, throughout metabolite determination in plasma, can be seen only as a speculation at the present moment.

In the current study plasma concentrations of TC, LDL-C, HDL-C and TG did not differ between non-ovulating and ovulating women. But it is worth

noting that circulating TG in non-ovulating women was slightly higher than in ovulating ones (by 15% and 24% between days 5-8 and 19-22 of the menstrual cycle, respectively).

Despite numerous studies a precise mechanism responsible for the regulation of circulating TG is yet not fully understood (26,47). However, it is well recognized that liver TG-rich lipoprotein (VLDL) production is primarily substrate driven, with the most important regulatory substrate being FFA, taken up by the liver in proportion to their delivery rate and re-estrified to TG moieties. It is worth noting also that hepatic fractional extraction of FFA is high (20-30%) markedly contributing to overall TG synthesis. In addition, under basal conditions, *de novo* FFA synthesis in the liver (DNL) accounts for about 5% of overall TG synthesis. However, circulating TG also depends on VLDL secretion from the liver, thus, any disturbances in plasma TG may be due to impaired TG synthesis and/or secretion.

It is well documented that insulin exerts an inhibitory effect on both VLDL synthesis and secretion (48). However, insulin augments *de novo* FFA synthesis due to stimulation of FFA synthase expression, thus TG-rich lipoprotein levels in plasma reflects the balance between two opposite actions of insulin in the liver.

Therefore, assuming slightly greater insulin uptake by the liver postulated earlier in non-ovulating women, and the inhibitory effect of insulin on VLDL synthesis and secretion, it seems feasible that a subtle difference in circulating TG between ovulating and non-ovulating women may be due to greater stimulation of *de novo* FFA synthesis in the latter than in the former.

In conclusion, even slightly depressed ovarian hormone secretion in regularly menstruating, but non-ovulating subjects caused significant elevation in circulating FFA accompanied by slightly lower insulin and slightly higher TG plasma levels. However, during at least 3 months of anovulation these changes did not affect TC, LDL-C and HDL-C plasma levels. Therefore elevated plasma FFA concentrations may be possibly recognized as a first sign of impaired lipid metabolism due to depressed ovary function.

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Author's contribution
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection