

Contents lists available at ScienceDirect

Journal of Clinical & Translational Endocrinology

journal homepage: www.elsevier.com/locate/jcte

Research Paper

Normal menstrual cycle steroid hormones variation does not affect the blood levels of total adiponectin and its multimer forms



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ARTICLE INFO

Article history:

Received 23 December 2014

Received in revised form

5 March 2015

Accepted 10 March 2015

Keywords:

Adiponectin multimers

Gonadal steroids

Estradiol

Testosterone

Androgens

Menstrual cycle

ABSTRACT

Objective: Plasma total adiponectin reveals a sexual dimorphism indicating that gonadal steroids may be involved in its secretion and/or metabolism. However, results from previous reports are conflicting and data regarding the influence of ovarian steroids on adiponectin's multimer forms are scarce. The objective of the study was to assess if total adiponectin and its isoforms are affected by the changes of estradiol and progesterone during the normal menstrual cycle and the association of total adiponectin and its isoforms with the gonadal steroid levels.

Materials/methods: Quantitative determination of plasma adiponectin and its multimers was conducted in the three phases of an ovulatory cycle in 13 premenopausal women, in the follicular phase of 10 more premenopausal women, in 20 postmenopausal women and in 21 men. Moreover, serum levels of FSH, LH, prolactin, estradiol, progesterone, and testosterone, sex hormone binding globulin, glucose, and insulin were measured.

Results: The circulating levels of total adiponectin and its multimers were not affected by the normal variation of estradiol and progesterone across the ovulatory menstrual cycle. In the whole number of participants, the total adiponectin and high molecular weight adiponectin levels were significantly different between genders and associated positively with age and sex hormone binding globulin levels, and negatively with testosterone and progesterone levels and the waist/hip ratio. In the multiple logistic regression analysis, after adjustment for age, gender, and sex hormone binding globulin and progesterone levels, significant predictors of total adiponectin levels were the waist/hip ratio and testosterone levels, and of high molecular weight adiponectin the testosterone levels.

Conclusions: Normal menstrual cycle ovarian steroids are not involved directly in the regulation of secretion and/or metabolism of total adiponectin and its multimers. Testosterone seems to be responsible for the adiponectin's sexual dimorphism.

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Abbreviations: BMI, body mass index; Bio-T, bioavailable testosterone; CRP, C reactive protein; E2, estradiol; FAI, free androgen index; FSH, follicle stimulating hormone; FT, free testosterone; FT4, free thyroxine; HOMA-R, homeostasis model assessment of insulin resistance; HMW, high molecular weight; LH, luteinizing hormone; LMW, low molecular weight; MBP, mean blood pressure; MMW, mean molecular weight; PCOS, polycystic ovary syndrome; SHBG, sex hormone binding globulin; TSH, Thyroid stimulating hormone; TA, total adiponectin; TT, total testosterone; WC, waist circumference.

Funding: No funding.

Author contributions: KC recruited the subjects, obtained blood samples and collected data. SGG performed the Elisa assays. GNK designed the study, oversaw its performance and carried out the statistical analysis. All authors contributed to analyzing the data and writing the manuscript.

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Introduction

Adiponectin is produced by the adipose tissue and is secreted into the bloodstream where it accounts for up to 0.05% of total serum protein and circulates as a low molecular weight (LMW) trimer (~65 kDa), mean molecular weight (MMW) hexamer (~150 kDa) and high molecular weight (HMW) multimers (~280 and ~420 kDa) [1]. HMW isoforms appear to be the most biologically active form of adiponectin, being related to reduced abdominal fat and high basal lipid oxidation [2]. Recent studies also suggest that HMW adiponectin and the ratio of HMW to TA are associated with insulin sensitivity, antiatherogenic activities, metabolic syndrome and the prediction of cardiovascular disease [3].

Plasma adiponectin reveals a sexual dimorphism, with females having significantly higher circulating levels of TA and HMW

isoforms than males in both humans [4,5] and rodents [6–8], whereas the levels of MMW and LMW forms are comparable between sexes [7]. This sexual dimorphism leads to the hypothesis that adiponectin secretion and/or metabolism are regulated by gonadal steroids. However, previous reports, applying a diversity of study models to investigate the potential effect of sex steroids exerted on adiponectin secretion, are conflicting.

Previous studies [9,10], but not all [4,11], have shown significantly higher circulating adiponectin concentrations in postmenopausal than in premenopausal and pregnant women. Specifically, serum concentrations of TA and HMW adiponectin were highest in postmenopausal women and lowest in pregnant women, whereas between the three groups MMW and LMW isoforms were comparable [10]. Existing data regarding the association between estradiol (E2) and adiponectin levels are conflicting [11,12], indicating that in women, besides E2, other factors such as age [6] and alterations in the androgen-to-estrogen ratio [5] may contribute to the mentioned differences. These might be the reason for the low adiponectin levels reported in women with polycystic ovary syndrome (PCOS) and elevated total testosterone (TT) level, although coexistent obesity and/or insulin resistance cannot be excluded [13]. Androgens also seem to influence plasma TA levels. Hypogonadal men, compared to eugonadal, have significantly higher plasma adiponectin levels, which are reduced by testosterone replacement therapy [14]. Similarly, in normal men experimental testosterone deficiency increased plasma TA levels, an effect that was suppressed when testosterone replacement therapy was also given [15]. However, supraphysiologic testosterone administration resulted in decreased plasma adiponectin levels [15].

Previous studies investigating variations of circulating adiponectin concentrations during the menstrual cycle are limited and have shown contradictory data. Moreover, only total circulating adiponectin levels were measured and the speculation that mainly the HMW isoform of adiponectin is sensitive to female sex steroids changes [12] has not been further investigated. As far as we know, there is only one study where circulating adiponectin multimer forms concentrations were measured at the early phase of the menstrual cycle [16]. Therefore, we investigated whether sex hormones affect not only plasma TA levels but also adiponectin multimer forms in all phases of a normal menstrual cycle. A normal menstrual cycle corresponds to a three-step model with low E2 levels in the follicular phase, increased E2 levels in the preovulatory phase and increased E2 plus progesterone levels in the luteal phase with the androgens remaining unchanged. Moreover, we assessed the associations between blood testosterone and estradiol with total adiponectin, HMW adiponectin and the HMW/total adiponectin ratio in healthy pre- and postmenopausal women and men.

Subjects and methods

Subjects

In the study, twenty-three premenopausal women were enrolled with mean (\pm SD) age 34.0 ± 4.7 and range 27–40 years with normal body weight (BMI 22.3 ± 2.2) and regular menstrual cycle. All women were healthy and none of them had received hormonal or any other medical treatment for at least 6 months prior to this study. Fasting blood samples were collected between 8:00 and 10:00 am. From thirteen out of the twenty-three premenopausal women blood was obtained at the three phases of a 28-days menstrual cycle, defined by the menses onset: follicular (4th–5th day), ovulatory (11th–12th day) and luteal phase (20th–21st day). From the other ten premenopausal women blood was obtained only at the follicular phase of the menstrual cycle (4th–5th day). Five of them were withdrawn from the study, in four the

studied menstrual cycle was anovulatory and in one the last time point serum samples were lost.

Blood was also obtained from twenty postmenopausal women (more than 1 year from the last menstrual period) and twenty-one men. All were healthy and none of them had received any medication during the last six months prior to this study. Postmenopausal women and men had mean age 56.0 ± 3.0 (range 51–63) and 37.0 ± 3.4 (range 32–44) years, respectively. Before blood sampling each subject underwent a thorough physical examination. Body weight and height, waist circumference (WC) and blood pressure were measured as previously described [17]. Mean arterial blood pressure (MBP) was calculated as follows: $MBP = \text{diastolic BP} + (1/3 \text{ systolic BP})$.

Laboratory parameters measurement

After sampling in EDTA or serum tubes, blood was centrifuged at 1465 g for 7 min and aliquots were immediately frozen at -86°C until assayed. Blood samples were analyzed for FSH, LH, E₂, TSH, FT₄, PRL, PRG, testosterone, SHBG, albumin, glucose, insulin and HbA1c by an auto analyzer (Olympus 600; Medicon, Athens, Greece) using standard techniques. Serum free testosterone (FT) and bioavailable testosterone (bio-T) were calculated from serum total testosterone, SHBG and albumin concentrations as previously described [17]. Free androgen index (FAI) was computed as the ratio of total testosterone (in nmol/L) to SHBG (in nmol/L) concentration. The homeostasis model assessment of insulin resistance (HOMA-R) index was calculated as $\text{plasma insulin (in IU/mL)} \times \text{plasma glucose (in mg/dL)}$ divided by 22.5×18 . Total adiponectin and multimers' concentration were quantified by ELISA (Bühlmann Labs, Switzerland) in the same assay. The amount of HMW, MMW and LMW adiponectin was calculated according to the manufacturer's instructions. The intra-assay CV was 5% for total adiponectin, 6% for MMW + HMW, and 5.7% for HMW adiponectin.

Written informed consent was obtained from all participants and the study protocol was reviewed and approved by the Scientific and Ethics Committee of the School of Medicine, University of Thessaly.

Statistical analysis

Results for quantitative variables are expressed as mean \pm standard deviation (SD) unless otherwise indicated. Data for qualitative variables were described as numbers and/or percentages. Student *t* test or the Mann–Whitney *U*-test was used to estimate differences between mean values, as appropriate. Comparison of frequencies was performed using χ^2 or Fisher's exact test. One way ANOVA with Bonferroni correction was used to determine trends of the repeated measures on the 13 premenopausal women during the menstrual cycle and differences across the groups. Spearman's coefficient was used to test for bivariate correlations. Multiple logistic regressions were used to examine the association between total adiponectin, HMW adiponectin or HMW/TA ratio as a dependent variable and age, gender, abdominal obesity, SHBG, progesterone, and FT or TT levels as independent variables. A probability value of $p < 0.05$ was considered statistically significant. Analyses were performed using SPSS for windows version 17.0 (SPSS Inc Chicago, IL).

Results

In the thirteen women studied at the three phases of their menstrual cycle, serum prolactin was normal (20.8 ± 8.2) and the concentrations of E₂ and P showed the typical patterns of an ovulatory cycle (Table 1). The duration of the study cycle ranged between 25 and 28 days (26.9 ± 1.3 days). In contrast to the

Table 1

Serum levels of gonadal steroids, total adiponectin and its multimers and insulin resistance index at the three phases of ovulatory cycles of 13 premenopausal women

Variable	Follicular phase	Ovulatory phase	Luteal phase	<i>p</i> value ^a
Estradiol (pg/ml)	55.6 ± 19.9	313 ± 144	162 ± 75	0.001
Progesterone (ng/ml)	0.51 ± 0.27	0.65 ± 0.40	16.9 ± 5.1	0.001
Total testosterone (ng/ml)	0.35 ± 0.21	0.39 ± 0.20	0.34 ± 0.25	0.841
Free testosterone (ng/dl)	0.47 ± 0.33	0.48 ± 0.33	0.35 ± 0.19	0.453
Bio-testosterone (ng/dl)	12.0 ± 8.3	11.9 ± 8.0	8.2 ± 5.2	0.334
Free androgen index	0.66 ± 0.61	1.08 ± 1.75	0.54 ± 0.60	0.445
Total adiponectin (µg/ml)	7.12 ± 2.09	6.99 ± 2.34	7.65 ± 2.62	0.898
HMW adiponectin (µg/ml)	4.71 ± 2.30	4.89 ± 2.79	5.82 ± 3.05	0.791
MMW adiponectin (µg/ml)	0.67 ± 0.51	0.89 ± 0.53	0.64 ± 0.21	0.633
LMW adiponectin (µg/ml)	1.74 ± 0.52	1.22 ± 0.54	1.19 ± 0.44	0.196
HMW/total adiponectin ratio	0.63 ± 0.17	0.66 ± 0.17	0.72 ± 0.16	0.681
HOMA-R	2.1 ± 2.0	1.6 ± 0.8	1.9 ± 0.7	0.664

^a *p* value across the three phases by one-way ANOVA.

significant alterations of serum E2 and P, no significant change was observed in the blood levels of total, free and bioavailable testosterone, and FAI at the three phases of the menstrual cycle (Table 1). Similarly, no significant change was detected in the blood levels of total adiponectin and its isoforms as well as the HMW/TA ratio. Insulin resistance, as indicated by the HOMA-R, was comparable at the three phases of the menstrual cycle.

Table 2 shows the anthropometric characteristics, biochemical variables, and hormone and adiponectin levels of 23 premenopausal women studied in the early follicular phase, 20 postmenopausal women and 21 men. Postmenopausal women were older compared to premenopausal women who had comparable age to the men. BMI and WC were normal in premenopausal women and men, whereas postmenopausal women were overweight with central adiposity. Mean blood pressure was significantly higher in men compared to both groups of women. All three groups had normal mean levels of CRP and were euthyroid. Blood levels of FSH, LH and prolactin were different between groups, as expected (data not shown). SHBG levels were significantly lower in men compared to both groups of women, who had comparable levels. Insulin levels and insulin resistance index were comparable between the three groups. Total, free and bioavailable testosterone levels as well as FAI (data not shown) were significantly higher in men, as expected, compared to pre- and postmenopausal women,

who had comparable androgen levels. E2 levels were comparable in men and postmenopausal women but significantly lower compared to premenopausal women levels.

Total, HMW and MMW adiponectin levels were comparable between pre- and postmenopausal women but significantly lower in men (Table 2, Figure 1), whereas no difference was found in the LMW adiponectin levels or in the HMW/TA ratio between the studied groups. Similarly, the percent distribution of HMW, MMW and LMW adiponectin isoforms did not show any significant difference between the three groups.

In the group of women studied in the three phases of the menstrual cycle, no correlation was found between adiponectin or its isoforms levels and sex steroids, insulin and glucose levels or body composition and HOMA-R. By contrast, in the whole number of participants, serum levels of TA and HMW adiponectin were correlated positively with age and SHBG levels and negatively with androgen levels, progesterone levels, and W/H ratio. In the multiple logistic regression analysis, after adjustment for age, gender, and SHBG and progesterone levels, significant predictors for the blood TA, were the waist/Hip ratio and androgens (*p* = 0.016). W/H ratio and FT levels account for 59.5% of the variation in TA levels, with the former predicting 52.1% of the variation. When HMW adiponectin was used as the outcome variable, androgens were the only predictors (*p* = 0.001), accounting for 31.1% of the variation in HMW adiponectin levels. HMW/TA ratio was not predicted by any of the variables included in the model.

Discussion

The sexual dimorphism in plasma adiponectin levels in both humans [4,5] and animals [6–8] obviously raises the question as to whether gonadal steroids are involved in this issue. *In vitro* and experimental data demonstrate that both androgens and estrogens exert an influence on circulating adiponectin levels [4,6,18]. Moreover, a recent *in vitro* study has shown that progesterone stimulates the secretion of adiponectin from 3T3-L1 adipocytes [18]. Our study is the first one examining the effect of estrogens and of estrogens plus progesterone on the levels of HMW, MMW and LMW isoforms of adiponectin throughout a normal menstrual cycle. Our results demonstrate that during the three phases of an ovulatory menstrual cycle, the mentioned sex steroids variation does not affect the circulating levels of total adiponectin and its multimer forms.

Table 2

Clinical and laboratory characteristics in premenopausal women (follicular phase), postmenopausal women, and men

Variable	Premenopausal women	Postmenopausal women	Men	<i>p</i> value ^a
N	23	20	21	
Age (range) (years)	34 (24–41)	56 (51–63)	37 (32–44)	
BMI (kg/m ²)	22.3 ± 2.15	27.3 ± 5.1	24.9 ± 1.82	0.001
Waist circumference (cm)	78.4 ± 6.2	89.3 ± 10.2	94.6 ± 5.7	0.001
Smoking (yes/no)	5/18	4/16	9/12	NS
CRP (mg/dl)	0.30 ± 0.3	0.51 ± 0.5	0.25 ± 0.2	0.074
SHBG (nmol/L)	62.4 ± 26.5	64.3 ± 27.2	29.2 ± 11.5	0.003
HOMA-R	1.9 ± 1.6	2.1 ± 1.3	2.2 ± 1.0	0.820
Total testosterone (ng/ml)	0.36 ± 0.16	0.27 ± 0.17	5.4 ± 1.7	0.001
Free testosterone (ng/dl)	0.47 ± 0.31	0.40 ± 0.28	11.7 ± 3.4	0.001
Bio-testosterone (ng/dl)	12.2 ± 7.8	10.1 ± 7.1	308 ± 83	0.001
Estradiol (pg/ml)	56.7 ± 29.7	11.7 ± 10.6	24.9 ± 9.6	0.001
Total adiponectin (µg/ml)	7.09 ± 1.87	6.78 ± 3.32	2.59 ± 0.81	0.001
HMW adiponectin (µg/ml)	4.74 ± 2.06	4.22 ± 2.54	1.48 ± 0.85	0.001
MMW adiponectin (µg/ml)	0.71 ± 0.46	1.11 ± 0.88	0.56 ± 0.26	0.027
LMW adiponectin (µg/ml)	1.65 ± 0.51	1.45 ± 1.90	0.55 ± 0.32	0.056
HMW/total adiponectin ratio	0.64 ± 0.15	0.61 ± 0.19	0.54 ± 0.18	0.345
HMW adiponectin (%)	63.5 ± 15.3	61.3 ± 19.1	54.0 ± 17.7	0.346
MMW adiponectin (%)	11.2 ± 8.2	17.8 ± 12.1	23.6 ± 12.7	0.071
LMW adiponectin (%)	25.2 ± 11.1	20.9 ± 15.1	22.4 ± 11.9	0.779

^a *p* value across the three groups by one-way ANOVA.

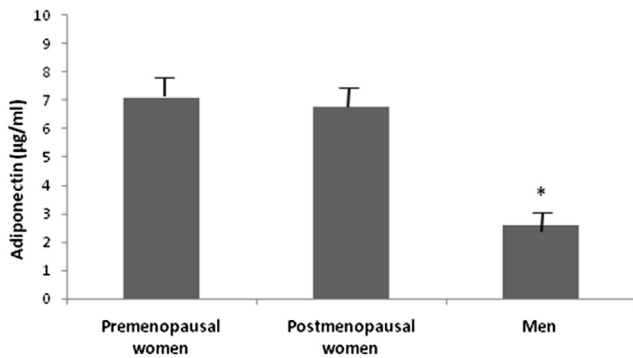


Fig. 1. Mean (\pm SEM) serum total adiponectin levels in the follicular phase of 23 premenopausal women, postmenopausal women and men * $P < 0.01$ compared to pre- and postmenopausal women by one-way ANOVA analysis.

The results of our study are in line with previously published data [18–23], showing that normal changes of estrogen and progesterone during a normal cycle do not affect total adiponectin levels. The effect of E2 administered in ovariectomized premenopausal women on total adiponectin was similar [11]. Moreover, in a recent in vitro study [24] in human fat cells, where adiponectin is mainly synthesized, increasing concentrations of estradiol did not change either adiponectin mRNA expression and secretion or intracellular protein expression of HMW, MMW and LMW adiponectin isoforms. However, the absence of a suppressive effect of estrogens on TA and its multimer levels in our case, even though a significant increase of E2 alone or combined with progesterone was evident, is in contrast with published results indicating a suppressive effect of estrogens on adiponectin production in mice and in 3T3-L1 adipocytes [6,18], and an independent negative association of E2 with TA levels in a group of 121 pre- and postmenopausal women without a history of diabetes [25]. The comparable levels of TA and its multimers that we found in pre- and post-menopausal women are incompatible with the suppressive effect of estrogens, despite the fact that a significant difference in E2 levels was present. The fact that treatment of postmenopausal women with E2 resulted in an increase, no change or a decrease in TA [11,12,26] could probably be explained by differences in the design of each study and the presence of a number of adiponectin confounding factors such as age [9], androgens [14,15], prolactin [6], HOMA-R [27], body weight [6], physical activity [23,28], and food consumption [6], all of which were stable in our study. The negative association between the HMW and MMW of adiponectin forms and E2 or progesterone levels reported in a prior study [10] is also in discordance with our results and may possibly be explained by the inclusion in the study of pregnant women with higher and long-lasting estrogen and progesterone levels in this study.

In both humans [4–6] and rodents [7,8], blood TA levels were found lower in males compared to BMI and age-matched females and this has been proposed as being due to the effect of male sex hormones. In line with these data we found that TA levels were significantly lower in men compared to pre- and postmenopausal women. Moreover, in the multiple regression analysis of all three groups together, W/H ratio and testosterone levels were independent negative predictors of TA levels. When HMW adiponectin was used as the dependent variable, androgens were the only predictors.

Our results are in accordance with published data suggesting an inhibitory effect of androgens on circulating total adiponectin concentrations. Castration in male mice and gonadectomy in rats of both sexes was followed by an increase in plasma adiponectin levels that was reversed when testosterone was administered [4,8], ablating the difference in TA levels between males and females [8]

while maintaining HMW adiponectin levels [8]. The inhibitory effect of androgens on the levels of total adiponectin was equal using testosterone enanthate and a non-aromatizable androgen, indicating that estrogens are not involved in this effect [8]. Similarly, in cultured 3T3-L1 adipocytes, both testosterone and dihydrotestosterone reduced the secretion of adiponectin in the culture media [4], indicating a negative regulation on adipocytes that was also observed in humans. Notably, the experimentally-induced testosterone deficiency in normal men was followed within days by a significant increase in TA levels, an effect that was prevented by testosterone replacement without changing the BMI [15], while female-to-male transsexuals experienced a reduction in adiponectin following testosterone administration [29]. Moreover, in boys during pubertal development an androgens-associated progressive decline of adiponectin has been demonstrated, resulting in lower levels compared to girls after the completion of puberty [30].

Studies in adults [25,31] and adolescents [30] show that adiponectin decreases with increasing degree of obesity and the reduction appears to precede the actual development of insulin resistance, metabolic derangements and diabetes [31]. Specifically, in epidemiologic studies central fat mass is more closely associated with the development of insulin resistance and cardiovascular disease [32]. W/H ratio is a reliable index of central obesity and here we show that W/H ratio is an independent negative predictor of total adiponectin, in accordance with a number of published data [12,25], but not all [8,33]. The administered testosterone-induced reduction of visceral and total-body fat mass in young [29] and older men [34] and of circulating adiponectin concentrations in hypogonadal [7,14,35] and eugonadal [15] men may indicate an influence of testosterone on the secretion and/or metabolism of adiponectin in a way that seems to be directionally concurrent to adiposity.

Our finding that the concentration of adiponectin HMW form is lower in men compared to pre- and postmenopausal women is consistent with previous reports [4,7], suggesting a sexual dimorphism of adiponectin expression. It has been reported that testosterone selectively impedes the secretion of HMW adiponectin [7], and this action is mainly considered responsible for the sexual dimorphism of adiponectin in both human and rodents and could in part explain why men have a higher risk of insulin resistance and are less sensitive to pioglitazone.

Whether sex steroids have a direct effect on adiponectin expression, cellular trafficking and/or metabolism by adipocytes is not known. Recently, published data have shown that estrogens and androgens significantly increase and decrease, respectively, peroxisome proliferator-activated receptor γ (PPAR γ) protein expression in 3T3-L1 mature adipocytes only after long-term exposure [36]. This information, in conjunction with the observation that PPAR γ agonists increase HMW oligomers from adipocytes by regulating a pair of molecular chaperons in the endoplasmic reticulum [37], could indicate that gonadal steroids may function as indirect opposite regulators of HMW oligomer secretion influenced by intracellular PPAR γ agonists or other factors. It has been also proposed that adiponectin is secreted from adipocytes through two distinct secretory pathways, a constitutive and a regulated one [38]. The involvement of Rho-kinase in the estradiol-induced inhibition of total adiponectin secretion has also been suggested, in vitro, in differentiating 3T3-L1 adipocytes but not in the progesterone-induced stimulation of adiponectin secretion [18]. However, these results must be carefully assessed due to the fact that higher concentrations of estradiol and progesterone were used compared to respective blood levels in menstruating women. The comparable levels of HMW multimers in the three phases of normal menstrual cycles, as demonstrated in our study, may indicate the need for more ovulating women recruitment and longer time exposure to

estrogens. More research is needed to elucidate the mechanisms of the influence of sex steroids on adiponectin secretion from adipose tissue.

Conflict of interest

The authors declare they have no conflicts of interest.

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