

Clinical Research Article

Low- and Fully N-Glycosylated Gonadotropins Circulating in Women With Polycystic Ovary Syndrome

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Abbreviations: AMS, anionic monosaccharides; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; SA, sialic acid; SU, sulfonated N-acetylgalactosamine.

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Abstract

Context: A preponderance of basic luteinizing hormone (LH) molecules having elevated bioactivity was detected in the circulation of women with polycystic ovary syndrome (PCOS). Subsequent studies have shown that LH and follicle-stimulating hormone (FSH) both circulate as glycoforms differing in number of glycans: low-N-glycosylated glycoforms, LH_{di} and FSH_{tri}, with high in vitro bioactivity, and fully glycosylated glycoforms, LH_{tri} and FSH_{tetra}, with high in vivo bioactivity.

Objective: This work aims to characterize the glycosylation patterns on circulating gonadotropin glycoforms in women with PCOS.

Methods: Serum samples, collected from 8 women with PCOS were included. The concentration, sulfonation, and sialylation of each glycoform were determined and compared with values of serum samples from healthy women: 22 women at follicular phase, 16 at midcycle, and 15 after menopause.

Results: All the women with PCOS had higher LH_{di} serum levels compared with those in the follicular-phase group. Median LH_{di} and median LH_{tri} levels were significantly elevated in PCOS women. The percentage of LH_{di} was increased from 37 to 49 and that of FSH_{tri} was decreased from 41 to 33. The LH_{di}, LH_{tri}, and FSH_{tetra} glycoforms were more sialylated and both LH glycoforms less sulfonated in women with PCOS.

Conclusion: All women with PCOS had increased serum levels of LH_{di}, compared with those in the follicular phase. The percentage of LH_{di} was increased and that of FSH_{tri} decreased in women with PCOS. The increased LH_{di} leads to maintenance of the abnormal early follicular development of the polycystic ovary, and the decreased FSH_{tri} contributes to the arrested follicle growth.

Key Words: LH glycoforms, FSH glycoforms, N-glycosylation, sulfonated N-acetylgalactosamine, sialic acid

Polycystic ovary syndrome (PCOS) is a highly prevalent disorder. This disorder accounts for 75% to 85% of anovulatory infertility [1]. Current evidence strongly indicates that the etiology of the anovulation is primarily initiated by an intrinsic ovarian failure observed already at the earliest stages of follicular development, including a disruptive activation of the primordial follicles [1, 2]. The ovarian steroid hormone feedback, including increased androgen synthesis, to the hypothalamus and pituitary gland lead, in the majority of women with PCOS, to elevated luteinizing hormone (LH) pulse frequency and elevated serum LH levels [3]. The levels of follicle-stimulating hormone (FSH) are in general within the range of that of the normal follicular phase [1]. In addition, the glycobiochemical synthesis of the gonadotropins in the pituitary gland is different in women with PCOS, leading to decreased sulfonation and increased sialylation of the circulating LH molecules [4].

LH and FSH molecules are both heterodimers consisting of an α -subunit polypeptide, which is common to LH, FSH, and thyrotropin, and of a noncovalently linked β -subunit polypeptide, which confers specificity. When α -subunits are decorated with 2 N-glycans, they can combine with β -subunits having different degrees of N-glycosylation. The LH and the FSH molecules circulate as 2 glycoforms that are termed *low-N-glycosylated*, *LH_{di}* and *FSH_{tri}*, and *fully N-glycosylated*, *LH_{tri}* and *FSH_{tetra}*, according to their total number of N-glycans per molecule [5]. Both these LH and FSH glycoforms exhibit a large heterogeneity due to variations in the decoration of the N-glycans with different number of 2 terminal anionic monosaccharides (AMS): sialic acid (SA) and sulfonated N-acetylgalactosamine (SU).

The N-glycosylation of FSH and LH β -subunit polypeptides and of the common α -subunit polypeptide occurs cotranslationally in the rough endoplasmic reticulum (ER) of the gonadotrophs in the human anterior pituitary gland. This is followed by branching of the glycans and their terminal decorations with SA and SU residues in the Golgi apparatus of these cells. The events in ER and Golgi are schematically illustrated in Figure 1; nomenclature, pathways, and design are from references [6-10].

The relative abundance in the circulation of the 2 LH and FSH glycoforms and of the spectra of molecules with different degrees of sialylation and sulfonation vary within and between individuals and are related to different clinical and physiological situations. The different forms are cleared from the circulation according to their individual clearance rate. Increased number of terminal SA residues on the glycans prolong the survival of the molecules in the circulation [11]. A mannose/sulfonated N-acetylgalactosamine-specific receptor (SU receptor) in the liver quickly removes LH molecules with 2 or more terminal SU residues from the blood circulation [12, 13]. The biological effect of such

isoform spectra will be a resultant of that of all the multiple isoforms.

The LH molecules, circulating in women with PCOS, were demonstrated to have an elevated bioactivity as measured in vitro [14, 15]. A preponderance of basic, that is, less negatively charged, LH molecules was detected in women with PCOS [16]. This combination of increased biological activity as measured in vitro and basic LH forms indicates the possibility of an excess secretion of the less negatively charged, low-N-glycosylated form, LH_{di}, in the PCOS group of women. To address this hypothesis, we have analyzed the gonadotropin glycoforms in 1 serum sample from each of 8 women with PCOS. The serum concentrations and degrees of sialylation and sulfonation of low- and fully N-glycosylated glycoforms of LH and FSH were determined as previously described [5]. The results were compared with those of serum samples obtained from 3 groups of healthy women: at the follicular phase, at midcycle, and after menopause.

Materials and Methods

Participants

One serum sample was obtained at random from each of 8 women (age range, 20-33 years) with PCOS. The samples comprised a subset from a previous study [4]. PCOS was defined according to the Rotterdam criteria [17]. The following 3 features were present in all patients: 1) amenorrhea in 2 patients and oligomenorrhea in 6 patients with 2, 4, 5, 5, 6, or 8 menstruations in the previous 12 months; 2) clinical and/or biochemical signs of hyperandrogenism; and 3) polycystic ovaries on ultrasound examination. The mean body mass index value and range for the 8 women with PCOS was 29.1 (range, 21-36). The patients had no other identifiable diseases and had normal fasting glucose levels.

Two reference groups of medical students comprised 22 women (age range, 23-39 years) at the follicular phase (day 3-10) and 16 women (age range, 22-40 years) at midcycle (serum LH levels ≥ 10 U/L). These women were included in a previously reported study of 78 healthy medical students having normal menstrual cycles [10]. The body mass index values were recorded for 7 of the women at the follicular phase with a mean and range of 22.0 (range, 20-29) and for 6 of the women at midcycle with a mean and range of 20.5 (range, 18-22). They all had normal serum testosterone levels. The donation of a serum sample was accompanied with informed consent according to the Declaration of Helsinki of Ethical Principles for Medical Research and with the approval of the ethics committee of the medical faculty of Uppsala University.

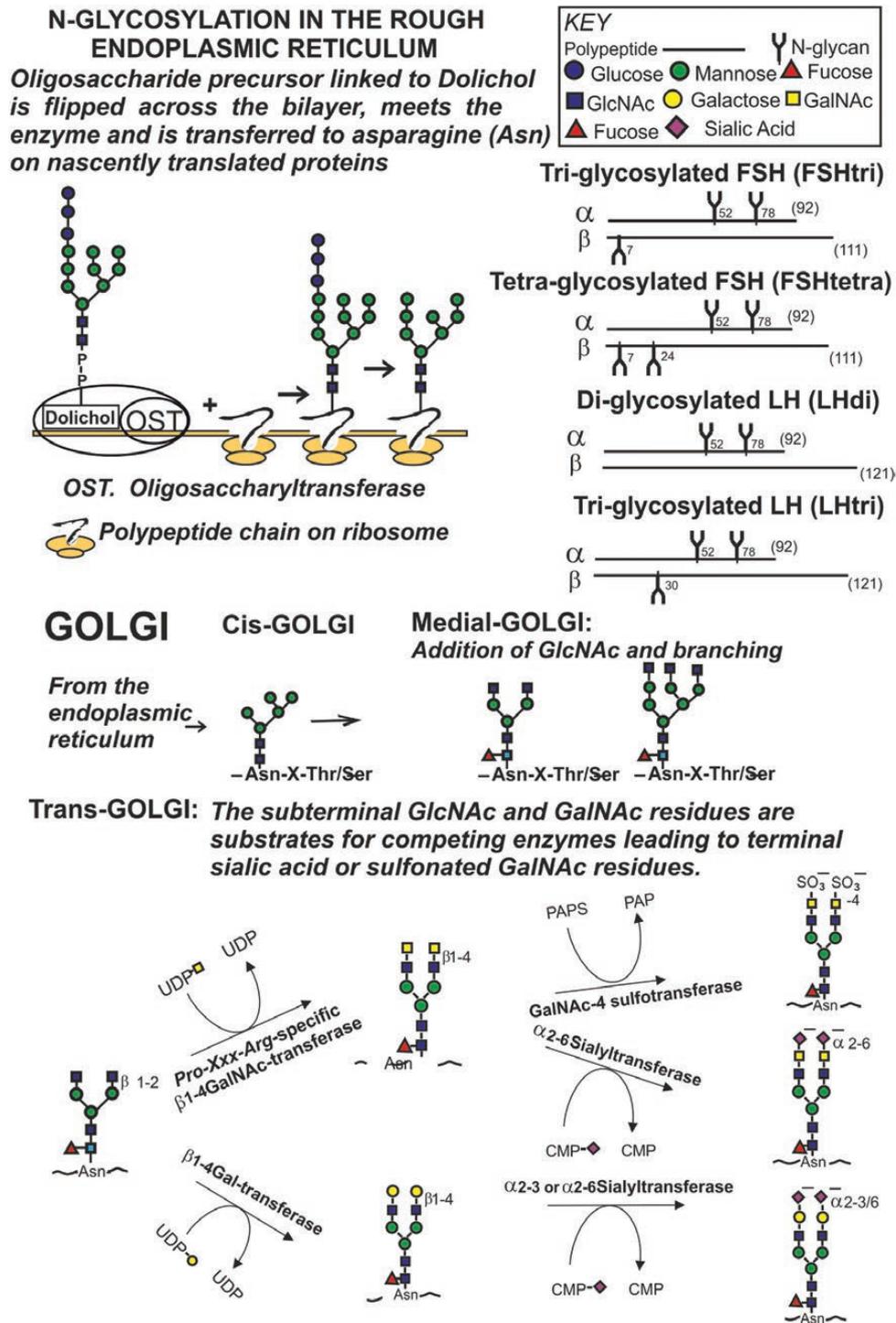


Figure 1. N-glycosylation of human FSH and LH occurs in the rough endoplasmic reticulum (ER) of the gonadotrophs in the anterior pituitary gland [6-10]. Oligosaccharide precursors linked to dolichol, a specific lipid, at the cytoplasmic side of the ER membrane are flipped across the membrane bilayer to the luminal side of the ER by use of an enzyme, a flippase. An enzymatic complex in the ER membrane, termed oligosaccharyltransferase (OST), transfers the oligosaccharide precursor to a γ amino group of asparagine (-Asn-X-Thr/Ser) on the nascently translated α -subunit polypeptides and FSH and LH β -subunit polypeptides. Fully N-glycosylated α -subunits, with 2 N-glycans, combine with FSH β -subunits with 1 or 2 N-glycans, forming FSHtri and FSHtetra, respectively, and with the LH β -subunits with none or one N-glycan, forming LHdi and LHtri molecules, respectively. The FSH and LH molecules are modified posttranslationally in the Golgi apparatus within the cell where the branching of the N-glycans occurs in the medial-Golgi. Further synthesis of the glycans and their terminal decoration with the AMS residues: sialic acid (SA) and sulfonated N-acetylgalactosamine (SU) occur in the trans-Golgi. AMS, anionic monosaccharide; CMP, cytidine 5'-monophosphate; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PAPS, 3'-phosphoadenylyl-5'-phosphosulfate; UDP, uridine diphosphate.

Serum specimens from 15 apparently healthy postmenopausal women (age range, 53-78 years), residents in the Uppsala community, without a history of estrogen replacement therapy, comprised a third reference group of the study.

All serum samples were first analyzed by routine methods at the Department of Clinical Chemistry during 2000 to 2011, and the surplus of the serum samples were stored at -20°C until the LH and FSH molecules were characterized during the period 2001 to 2012 with the methods described in the following sections. This study was conducted according to the approval of the Ethics Committee of the Medical Faculty of Uppsala University (Ups 01-367, 2001-09-13, still valid) for the use of surplus clinical chemistry serum samples.

Immunoassay of Serum Follicle-Stimulating Hormone and Luteinizing Hormone

The concentrations of FSH and LH in serum samples and in separated fractions after electrophoreses were measured using time-resolved sandwich fluoroimmunoassays (Delfia, PerkinElmer-Wallac Oy) [18, 19]. The methods permitted measurements of the hormones directly in 0.075-M veronal (Sigma-Aldrich Chemie GmbH) buffer at pH 8.7 eluted from electrophoreses. All serum samples were initially tested to identify and exclude individuals with the common variant form of LH [20]. Gonadotrophin values were expressed in international units per liter (IU/L) using the International Standards for pituitary LH (80/552) and FSH (94/632) as reference standards. The detection limits were less than 0.02 IU/L serum and the interassay coefficient of variation was less than 3% for both hormones. The detection limit of the 2 hormones in fractions from electrophoresis was about 100 attogram.

Frequency of Glycoforms of Follicle-Stimulating Hormone, Luteinizing Hormone, and Anionic Monosaccharides Residues per Glycoform Molecule

All serum samples were analyzed with an electrophoresis technique using a 0.10% agarose suspension in veronal buffer at pH 8.7. The motilities were expressed in relation to that of endogenous human serum albumin. The area of eluted gonadotropin was resolved into peaks at the positions for different numbers of AMS residues per molecule. The frequencies of the 2 glycoforms of LH and of FSH in serum samples and the median numbers of AMS residues per glycoform molecule were calculated from the

distribution by electrophoresis using the FSH and LH algorithms as described previously [5].

Neuraminidase Treatment

The terminal SA residues were removed from the LH and FSH molecules in serum samples by neuraminidase treatment during 24 hours at 37°C , leaving the SU as the only AMS remaining on the molecules, as described previously [5]

Determination of Sulfonated N-Acetylgalactosamine and Sialic Acid on Glycoforms

The number of SU residues and the percentage of SU out of the AMS were determined for each glycoform of LH and FSH. The ratio of percentage SU out of the AMS per molecule on low vs fully glycosylated hormone had previously been determined in serum samples from the different groups of healthy individuals and the PCOS group, as described previously [5]. These factors were used to calculate the number of SU and SA per glycoform molecule in the serum samples [5].

Statistical Analyses

The mean values are presented with SD or, when the distribution is geometric, with the geometric mean (geomean) and the SD factor. Statistical comparisons were made by using a nonparametric Mann-Whitney test. A difference with a *P* value less than .05 was considered significant. The statistical analyses were calculated using GraphPad Prism 9 for Windows.

Results

The LH and FSH glycoform serum concentrations and degrees of sialylation and sulfonation of the group of women with PCOS were compared with corresponding values of the 3 reference groups of healthy women at the follicular phase, at midcycle, and after menopause, are presented in Table 1. The *P* values from statistical comparisons of the PCOS group vs the 3 reference groups of healthy women, using nonparametric Mann Whitney tests, also are indicated in Table 1.

The LH_{di}, LH_{tri}, and LH_{total} mean concentrations of the PCOS group were significantly higher than those of the follicular-phase group. The distributions of the individual LH_{di} and LH_{tri} values of the 2 groups of women in relation to age are shown in Figure 2. All women with PCOS had higher LH_{di} serum levels compared with those of the

Table 1. Properties of circulating low- and fully N-glycosylated luteinizing hormone and follicle-stimulating hormone glycoforms in women with polycystic ovary syndrome compared with those in healthy women at follicular phase, at midcycle, and after menopause

	Women with PCOS	Healthy women		
		Follicular phase	Midcycle	Postmenopausal
No. of women	8	22	16	15
Age, mean; range, y	27; 20-33	28; 23-39	28; 22-40	66; 53-78
	Geometric mean	Geometric mean	Geometric mean	Geometric mean
	SD factor	SD factor	SD factor	SD factor
Serum concentration, IU/L				
LHdi, conc.	6.91; 1.49	1.34; 1.56 ^a	12.5; 1.94 ^d	1.32; 1.70 ^a
LHtri, conc.	7.24; 1.40	2.38; 1.41 ^a	7.51; 1.96 ^{NS}	21.5; 1.38 ^a
FSHtri, conc.	1.79; 1.38	2.09; 1.36 ^{NS}	4.07; 1.56 ^b	5.42; 1.49 ^a
FSHtetra, conc.	3.78; 1.23	3.04; 1.33 ^{NS}	2.68; 1.63 ^{NS}	50.6; 1.28 ^a
LHtotal, conc.	14.6; 1.31	3.83; 1.33 ^a	20.8; 1.80 ^{NS}	22.9; 1.40 ^c
FSHtotal, conc.	5.68; 1.15	5.17; 1.31 ^{NS}	7.04; 1.42 ^d	56.1; 1.29 ^a
Percentage of low and fully N-glycosylated LH and FSH				
	Mean; SD	Mean; SD	Mean; SD	Mean; SD
Percentage of LHdi	48.6; 12.5	37.0; 11.3 ^d	61.8; 13.9 ^{NS}	6.04; 1.98 ^a
Percentage of LHtri ^c	51.4; 12.5	63.0; 11.3 ^d	38.2; 13.9 ^{NS}	94.0; 1.98 ^a
Percentage of FSHtri	32.6; 10.4	40.9; 6.19 ^c	59.5; 14.0 ^b	9.92; 2.48 ^a
Percentage of FSHtetra ^e	67.4; 10.4	59.1; 6.19 ^c	40.5; 14.0 ^b	90.1; 2.48 ^a
Degrees of sialylation and sulfonation. No. of SA and SU residues per molecule				
	Mean; SD	Mean; SD	Mean; SD	Mean; SD
SA on LHdi	1.70; 0.16	1.31; 0.13 ^a	1.68; 0.16 ^{NS}	1.39; 0.12 ^b
SA on LHtri	2.85; 0.20	2.27; 0.16 ^a	2.77; 0.20 ^{NS}	2.71; 0.16 ^{NS}
SU on LHdi	0.73; 0.16	1.13; 0.13 ^a	0.85; 0.17 ^{NS}	0.77; 0.11 ^{NS}
SU on LHtri	0.85; 0.19	1.33; 0.15 ^a	1.01; 0.19 ^{NS}	1.01; 0.15 ^d
SA on FSHtri	5.64; 0.07	5.57; 0.09 ^{NS}	5.68; 0.09 ^{NS}	5.48; 0.12 ^c
SA on FSHtetra	7.16; 0.05	7.05; 0.08 ^b	7.18; 0.10 ^{NS}	7.28; 0.10 ^c
SU on FSHtri	0.18; 0.05	0.32; 0.09 ^b	0.28; 0.06 ^b	0.23; 0.10 ^{NS}
SU on FSHtetra	0.18; 0.05	0.29; 0.08 ^b	0.26; 0.06 ^c	0.21; 0.09 ^{NS}

Statistical comparisons with the PCOS group using nonparametric Mann-Whitney tests.

Abbreviations: conc., concentration; FSH, follicle-stimulating hormone; LH, luteinizing hormone; NS, not significant ($P > .05$); PCOS, polycystic ovary syndrome; SA, sialic acid; SU, sulfonated N-acetylgalactosamine.

^a P less than .0001.

^b P less than .001.

^c P less than .01.

^d P less than .05.

^eDegree of N-glycosylation expressed as percentage of fully N-glycosylated glycoforms, percentage of LHtri and of FSHtetra.

follicular-phase group. There was no significant correlation between LHdi or LHtri values and the ages of the women.

The FSHtri, FSHtetra, and FSHtotal concentrations of the PCOS group were not significantly different from those of the follicular-phase group. The frequency of the low-N-glycosylated LHdi glycoform in the PCOS group was 49% compared to 37% in the follicular-phase group. The frequency of the low-N-glycosylated FSHtri glycoforms in PCOS was 33% vs 41% during the follicular phase.

Women with PCOS had a significantly increased number of SA residues and decreased number of SU residues per molecule both on the LHdi and LHtri

glycoforms compared with women at the follicular phase. The sialylation of FSHtri was not significantly different, whereas that of FSHtetra was increased compared with the follicular-phase values.

The mean values of degree of sialylation and sulfonation of the LH glycoforms and of sialylation of the FSH glycoforms of the PCOS group were similar to those of the midcycle group. The PCOS group had a similar mean degree of N-glycosylation of LH as the midcycle group, whereas that of FSH was significantly higher.

The values for different glycobiological properties of LH and FSH in the group of postmenopausal women are included in Table 1, as they represent the basal synthesis

and secretion of gonadotropins, when the feedback of gonadal steroids is very low. The mean degree of fully N-glycosylated forms for the postmenopausal women was 94% for LH and 90% for FSH compared to 51% for LH and 67% for FSH for the PCOS women.

Discussion

The present study is the first report on the serum levels and properties of the different glycoforms of LH and FSH in patients with PCOS. The major findings concern significant deviations of the low-N-glycosylated glycoforms LHdi and FSHtri, as compared with a follicular-phase group. The PCOS group had 5.2 times elevated mean concentration of the LHdi glycoform, and an elevated mean percentage of LHdi, which was 49 vs 37 for the follicular-phase group. The mean percentage of the FSHtri glycoform of the PCOS group was 33, as compared with the follicular-phase value of 41.

We have previously reported that in women with PCOS, the serum LH molecules were more sialylated and less sulfonated than those circulating during the follicular phase of the normal cycle [4]. The present study confirms these observations and shows, in addition, that it concerns both LH glycoforms, LHdi and LHtri. The increased degree of sialylation and decreased degree of sulfonation of the molecules lead to prolonged half-lives of the LH glycoforms in the circulation and contribute to the elevated blood levels of both LH glycoforms in women with PCOS.

The biological effects of the low- and fully glycosylated FSH and LH molecules have been extensively studied [21-31]. The results permit the following conclusions: A) Low-N-glycosylated forms have a high biopotency in all in vitro bioassays and in receptor binding assays. B) These molecules disappear fast from the circulation, which leads to low activity in bioassays conducted in vivo. C) Fully

N-glycosylated forms have a high activity in bioassays conducted in vivo. D) These molecules have a low or delayed biopotency in bioassays conducted in vitro and in receptor binding assays.

A glycan on the fully N-glycosylated glycoforms, at the amino acid position 30 on the β -subunit of the LH molecule and at position 24 on the β -subunit of the FSH molecule, prevents a rapid hormonal effect of these glycoforms at the receptor of the target cells. Several in vitro studies indicate a delayed response to these fully N-glycosylated glycoforms [32, 33]. This suggests that, after an initial binding to the receptor, the biopotency of the glycoform increases because of conformational molecular changes occurring on the glycoform itself or on the receptor. These conformational changes of the glycoform in vivo may include removal of terminal AMS residues on the interfering glycan or even of the whole glycan.

The low-N-glycosylated forms, LHdi and FSHtri, which thus exhibit the combination of higher in vitro bioactivity and shorter half-lives in the circulation, play major roles for the control of oocyte maturation and ovulation during the normal menstrual cycle [10]. In the present study, all women with PCOS had elevated serum concentrations of LHdi as compared with follicular-phase levels (see Figure 2). The gonadotropins are secreted in a pulsatile manner, and the combination of high bioactivity and short half-lives of these LHdi molecules will contribute to an efficient pulsatile ovarian stimulation including maintenance of the receptor synthesis in the ovary. In the polycystic ovary, these LHdi glycoforms will stimulate excess androgen production by the theca cells and contribute to the maintenance of the abnormal dynamic of the early follicular development. The decreased frequency of the biologically active low-N-glycosylated FSH molecules, FSHtri, may also contribute to the arrested follicle growth.

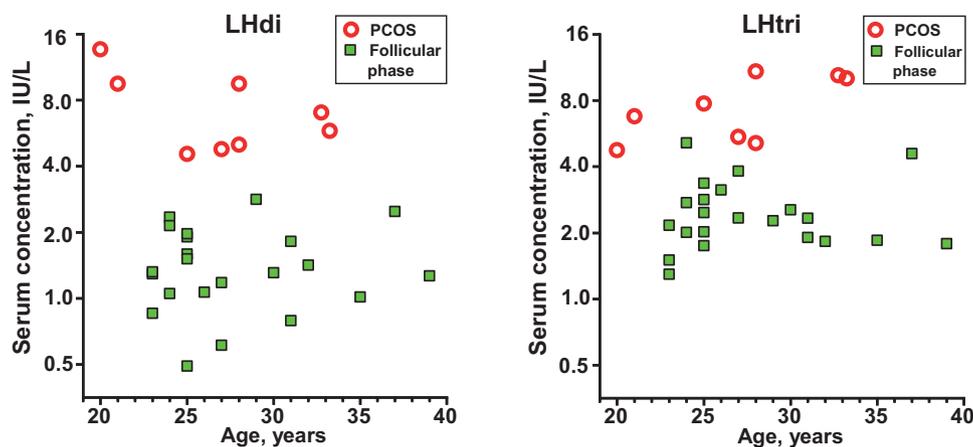


Figure 2. Concentrations of low-N-glycosylated luteinizing hormone (LH) molecules, (left panel) LHdi, and of fully N-glycosylated LH molecules (right panel) LHtri in serum samples of 8 women with polycystic ovary syndrome (PCOS) compared with those at follicular phase, days 3 to 10, of 22 healthy women with normal menstrual cycles. The values are presented in relation to age of the women.

Progestins of the levonorgestrel family, commonly used for contraception in women, have a weak androgen effect [34]. We observed that the glycosylation and glycan modifications of the circulating LH and FSH molecules were of a similar nature in women using such progestins as those found in the present study for women with PCOS [35]. The progestin-treated women had a significantly increased LHdi serum concentration. The mean ratio of the low-N-glycosylated LH and FSH glycoforms, LHdi/FSHtri, was significantly elevated during progestin treatment compared with that of the follicular phase [35]. Furthermore, progestin treatment induced a significantly increased sialylation of LHdi, LHtri, and FSHtetra and decreased sulfonation of LHdi and LHtri that was similar to the findings for the group of women with PCOS. The sialylation of the low-N-glycosylated FSH glycoform, FSHtri, was not affected in either of the 2 studies. The PCOS group of women had more pronounced changes of the glycosylation and glycan properties than the progestin-treated group of women. However, it is noteworthy that there is an increased secretion of ovarian androgens in women with PCOS and that the progestin used for contraception in women has a weak androgen effect. Both groups of women had modifications of their circulating LH and FSH molecules in the same direction for each of the previously presented 7 variables of gonadotropin glycochemistry.

In conclusion, all women with PCOS had increased serum levels of LHdi compared with follicular-phase values. The percentage of LHdi was increased and that of FSHtri decreased in women with PCOS. Elevated levels of the less acidic LHdi glycoforms in the PCOS group explain previous observations of basic LH forms with increased in vitro bioactivity in women with PCOS. In the polycystic ovary, these LHdi glycoforms stimulate excess androgen production by the theca cells and contribute to the maintenance of the abnormal dynamic of the early follicular development. The decreased frequency of the biologically active low-N-glycosylated FSH molecules, FSHtri, may also contribute to arrested follicle growth.

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Additional Information

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Disclosures: The authors have nothing to disclose.

Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in “References.”

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