WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.041
Volume 5, Issue 4, 17-29. Research Article ISSN 2278 – 4357

THE ROLE OF ANTI-MULLERIAN HORMONE IN THE EVALUATION OF THE EFFECTIVENESS OF METFORMIN HYDROCHLORIDE THERAPY IN POLYCYSTIC OVARIAN SYNDROME

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Article Received on
26 Jan 2016,
Revised on 17 Feb 2016,
Accepted on 11 Mar 2016
DOI: 10.20959/wjpps20164-6487

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ABSTRACT

Objectives: To evaluate the role of Anti-Mullerian hormone **(**AMH**)** in the evaluation of the effectiveness of short- **(**3 months**)** and long-term **(**6 months or more**)** Metformin hydrochloride therapy in women with polycystic ovarian syndrome **(**PCOS**)**. **Material and methods:** Ninety-five women between the age of 18 and 35, who were divided into two groups: a control group of 50 healthy women (n=50) and 45 women with polycystic ovarian syndrome(n=45) (PCOS), approximately at the age of 25,09 ±1.29 and BMI 27,21 ±0,79, were examined. Serum levels of AMH, LH, FSH, Tsestorenon, Androstendion, E2, Plasma glucose and IRI were examined. The individual values of HOMA-IRI and BMI were found out. Cases diagnosed with PCOS (n=45) were treated with Metformin hydrochloride. The dose during the treatment was 3x 850 mg, a total of 2550 mg daily dose for 6 months. The index measurements were made before starting the intake of Metformin hydrochloride (T0), during the third month (T3) and the sixth month (T6) of the beginning of the treatment. **Result**s: A positive correlation between the serum values of AMH and Androstendion (r=0,490), Testosterone (r=0,427) and LH (r= 0,431) and a negative correlation between AMH, FSH (r=-0,420) and E_2 (r=-0,588) were found. A significant difference between the initial level of AMH (T0-16,09ng/ml) and the level after the third month (T3-14,29ng/ml) p>0,05 was not found. The serum level of

AMH – 3,5 ng/ml was decreased after the sixth month (T6-12,59ng/m)l compared to the first measurement (T0-16.09ng/ml) and the there was a significant difference: u=2,36, p<0,05. The difference between the measurement of BMI at the beginning of the treatment and after its end was 2,5 кg/m^2. The difference was statistically significant: u=2,21, p<0,05. The serum levels of Testosterone (0,68 ng/ml), Androstendion (0,69 ng/ml), HOMA-index (0,92) and IRI (2,92 IU/L) were decreased. **Conclusion**: This study showed the efficacy of serum AMH measurement as a prognostic biochemical marker in the follow up of metformin pro-long treatment of PCOS women.

KEYWORDS: Anti-Mullerian hormone, AMH, Metformin, Polycystic ovarian syndrome, PCOS.

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the most frequent cause of infertility, anovulation and hyperandrogenism, affecting 5–10% of women of reproductive age.[1] Anti-Mullerian hormone (AMH), also known as Mullerian-inhibiting substance, is a member of the transforming growth factor-β (TGFβ) superfamily.[2,3] The AMH is a disulfide-linked glycoprotein with a molecular weight of 140 Kda.[4] In females, the AMH is mainly secreted by the granulose cells of the ovarian early developing follicles. The expression of AMH is localized in granulose cells of the primary, pre-antral, and small antral follicles, suggesting an important role of AMH in human folliculogenesis. Since AMH is secreted exclusively in the gonads, its serum concentrations in women are thought to reflect the size of the ovarian follicle pool. Serum AMH levels are 2-3 folds increased in PCOS women,[5, 6], which is in line with the increased number of AMH-producing pre-antral and small antral follicles.[7] Increased serum AMH in women with hyperandrogenism and/or oligo-anovulation could indicate to clinicians the presence of PCOS when reliable ultrasound is not available.[8]

There are a number of researches supporting the positive correlation of androgens containing AMH in their serum.[9, 10], as well as overproduction of androgens such as intrinsic defect of theca-cells in PCOS.[11] Insulin resistance (IR) and secondary hyperinsulinemia affect approximately 65-70% of women with PCOS.[12] Many of these women are also obese, which further exacerbates their IR. The presence of hyperinsulinemia in polycystic ovarian syndrome (PCOS) was described for the first time by Burghen et al., which also describe the positive correlation between hyperandrogenism and insulin levels at the same time.[13] Androgen synthesis in insulin resistance is stimulated by the direct influence of

hyperinsulinemia on androgen metabolism. The latter is done by stimulating enzyme activity as a result of which androstenedione synthesis is increased. Insulin has another mechanism which leads to hyperandrogenemia, i.e. – inhibiting of SHBG production into liver. On the other hand, this leads to an increased concentration of free testosterone. It is proved that under the conditions of hyperinsulinemia, sensitivity to insulin is decreased, respectively to glucose utilization only in peripheral tissues, for example the muscular tissue whereas insulin has no decreasing effect in terms of the ovary level.[14]

Metformin (1,1-dimethylbiguanide hydrochloride) is an oral antidiabetic drug in the biguanide class, and is approved by the US Food and Drug Administration for the treatment of type 2 diabetes mellitus.[15] Primary clinical action is to inhibit hepatic glucose production, although it also decreases intestinal glucose uptake, and increases insulin sensitivity in peripheral tissues. Metformin has antilipolytic effects, lowering circulating free fatty acid concentrations, which ultimately aids in reducing gluconeogenesis.[16] The use of metformin in PCOS is associated with increased menstrual cyclicity, improved ovulation, and a reduction in circulating androgen levels. The effect of oral antihyperglycemic medications on circulating concentrations of MIS in PCOS has not been explored, and elucidation of this effect is important for further understanding of ovarian effects of oral antihyperglycemic medication treatment.

MATERIALS AND METHODS

The current prospective comparison research was conducted in Department of Obstetrics and Gynecology at the Medical University of Plovdiv, Bulgaria and Department of Endocrinology at the Medical University of, Plovdiv, Bulgaria. Ninety-five women between the age of 18 and 35, approximately at the age of 25,09± 1.29 и BMI 27,21 ±0,79, who were divided into two groups: a control group of 50 healthy women(n=50) and 45 women with polycystic ovarian syndrome (PCOS) (n=45), were examined. The diagnosis of PCOS was based on the presence of at least 2 of the following 3 criteria: 1. oligo-ovulation and/or anovulation; 2. clinical and/or biochemical signs of hyperandrogenism; and 3. polycystic ovaries on ultrasound defined as the presence of 12, or more follicles in either ovary measuring 2-9 mm in diameter, and/or increased ovarian volume greater than 10 ml. Other causes for hyperandrogenism that mimic PCOS such as, congenital adrenal hyperplasia, Cushing syndrome, or androgen secreting tumors were excluded from this study. The tests for all laboratory indexes were done in the morning, in an extremely atraumatic way, following

the standard conditions. The serum levels of luteinising hormone (LH), follicle-stimulating hormone FSH), testosterone, androstendion, estradiol (E_2) and Anti-Mullerian hormone (AMH) were measured during the early follicular phase (day 3 – 5) of spontaneous menstrual cycle or progestin – induced uterine bleeding in oligo/ amenorrhea women. The concentrations of FSH, LH, Testosteron (T), Androstendion and E2 in the serum are fixed via hemiluminiscent analysis of automatic immunology analyser Access 2 (Beckman Coulter).

The serum concentration of AMH was fixed via uncompetitive immunochemical analysis with enzyme amplification-ELISA. Ready kits of Beckman Coulter Ins, U.S.A. were used. Plasma glucose was measured by the glucose oxidase method (Bayer Advia 1650 Chemistry System, Bayer Corp., NY; intra-assay coefficient of variance, - 2%), and insulin was measured using a competitive RIA (Coat-A-Count I; DPC). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated via the fasting concentrations of insulin and glucose by using the following formula: HOMA-IR _ fasting serum insulin (in microunits per milliliter) - fasting plasma glucose (in millimoles per liter)/22.5). *Body mass index(BMI)* was calculated by using the following equation: weight (in kilograms)/height2 (in meters). Transvaginal sonography was realized with the help of General electric 730 prof. so that the number of small follicles (<10 mm) could be found and the volume of ovaries could be estimated using the formula for ellipsoid. In order to be conducted a more detailed research of changes of clinic-laboratory indexes in women disgnosed with PCOS (n=45) , were treated with Metformin hydrochloride. The dose during the treatment was 3 x 850 mg, a total of 2550 mg daily dose for 6 months. The index measurements were made before starting the intake of Metformin hydrochloride (T0), during the third month (T3) and the sixth month (T6) of the beginning of the treatment. The statistical analysis of the data was done via Student's test, correlation and regression analysis - SPSS version 17. The results are considered to be statistically significant in P<0,05.

RESULTS

The average serum concentration of AMH in PCOS (15,126ng/ml) is significantly higher than the one in the control group (3,753ng/ml). The results of serum levels of gonadotropic hormones in PCOS and the control group are shown in table 1. The average levels of LH (±SE) in PCOS are significantly higher compared to the control group (*P*<0,00001). The ovarian synthesis of E_2 in PCOS is significantly lower (*P*<0,00001). Testosterone and Androstendion values in polycystic ovarian syndrome (PCOS) (*P*<0,00001) are also

significantly higher. The difference in serum levels of FSH in the PCOS group compared to healthy control groups (P<0,00006) is of importance in statistics. IRI and LH/FSH are significantly higher in the PCOS group than in control groups. The researched variable quantity data received in the current research conform with the values of the indexes in PCOS which are popular with literature and they confirm the pathophysiological changes established in the system.

Tab. 1 ***Comparative analysis of researched PCOS indexes and the control group***

Indexes	**groups**	**Average levels**	**Difference between average levels**
AMH ng/ml	PCOS	***15,126***	***11,374****
	Cintrols	***3,753***	
FSH mIU/mL	PCOS	5,185	-0,863*
	Controls	6,047	
LH IU/mL	PCOS	10,725	5,508*
	Controls	5,218	
Testosterone ng/ml	PCOS	0,789	0,358*
	Controls	0,431	
Androstenediol ng/ml	PCOS	2,794	0,830*
	Controls	1,964	
Estradiol pmol/ L	PCOS	*198,458*	*-119,912**
	Controls	*318,370*	
Plasma glucose mmol/l	PCOS	4,634	0,045n.s
	Controls	4,589	
IRI IU/L	PCOS	7,355	1,687*
	Controls	5,667	
LH/FSH	PCOS	2,154	0,369*
	Controls	0,894	

Table 2 shows the fixed dependencies between the laboratory variables in PCOS. A positive correlation between the serum levels of AMH and Androstendion (r=0,490), Testosterone (r=0,427) и LH (r= 0,431) and a negative correlation between AMH, FSH (r=-0,420) and E_2 (r=-0,588) was noted via data processing with plural linear regression analysis. The level of blood sugar and IRI were associated in a positive way with the increased levels of androgens in PCOS cases. Correlation coefficients for Testosterone in ill people are: $r_{plasma\ gluc.}$=0,224, p=0,014; r_{IRI}=0,122, p=0,083 and for Androstendion are: $r_{plasma\ gluc.}$=0,245, p=0,007; r_{IRI}=0,182 , p=0,046. This data confirm the potential dependencies between insulin dysfunctions and androgens in the presence of PCOS. In PCOS cases there was a positive

correlation between Estradiol and FSH, r=0.444, p=0.00, i.e. the increase of the amount of Estradiol is related to the increase of the amount of FSH in PCOS. The increase of the amount of Estradiol is related to the decrease of the amount of LH and the significant relation between the two variables: r=-0.38 , p=0.00 shows this. In PCOS there was a negative correlation between LH and FSH: r=-0,42, p=0,00, which shows that when there is an increase in one of the hormones, there is a decrease in the level of other one.

Tab. 2 *Correlations between all laboratory indexes in PCOS(n=45)*

	FSH mIU/mL	**LH IU/mL**	**Testosterone ng/ml**	**Androstenedione ng/ml**	**Estradiol pmol/ L**	**Plasma gluc. mmol/l**	**IRI IU/L**
AMH ng/ml	-0,420**	,0431**	0,427	0,490	-0,588**	0,120	0,129
FSH mIU/mL	1	-0,420**	0,075	0,066	0,444**	-0,038	0,010
LH IU/mL	-0,420**	1	0,022	-0,068	-0,380**	0,029	0,048
Testosterone ng/ml	0,075	0,022	1	0,772**	0,04	0,224*	0,122
Androstenedione ng/ml	0,066	-0,068	0,772**	1	0,043	0,245**	0,182*
Estradiol pmol/ L	0,444**	-0,380**	0,04	0,043	1	-0,014	-0,121
Plasma gluc. mmol/l	-0,038	0,029	0,224*	0,245**	-0,014	1	0,368**
IRI IU/L	0,01	0,048	0,122	0,182*	-0,121	0,368**	1

The dependencies cited in the group with PCOS were not found in healthy women.

The average levels of HOMA-index between the two groups: women diagnosed with PCOS and the control group were compared. The level of HOMA-index was higher in PCOS cases than in the control group. The difference found between the relative participation of insulin resistant women in the control group and PCOS cases is statistically reliable and clearly defined: : x^2= 11,62, p=0,001 (fig. 1).

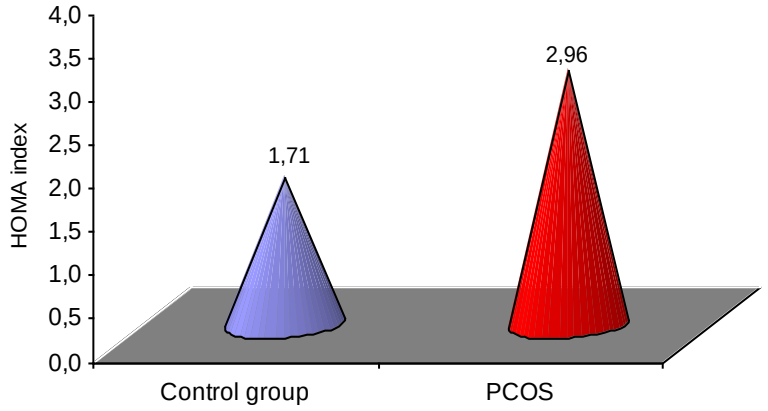

Fig. 1 Intergroup comparison of HOMA-index

It was noted that the risk for PCOS cases for insulin resistance compared to the same risk in the control group had increased approximately five times: OR = 4,55 (95% CI 1,59 – 11,54, p = 0,001).

In table 3 you can see the changes in the analysed indexes in PCOS cases after 6-month monotherapy with Metformin hydrochloride of 2550 mg daily dose. A significant difference between the initial level of AMH (16,09ng/ml) and the one after the third month (T3-14,29ng/ml): p>0,05 was not found. A decrease in the serum level of AMH - 3,5 ng/ml after the sixth month (T6-12,59ng/ml) compared to the initial measurement was noted and the difference was significant: u=2,36, p<0,05. There was a tendency of decrease in BMI in every measurement during the treatment. The difference between the measurement of BMI at the beginning of the treatment and after its end is 2,5 kg/m^2. The difference is statistically significant: u=2,21, p<0,05. A statistically significant decrease in the serum levels of Testosterone (0,68 ng/ml), Androstendion (0,69 ng/ml), HOMA-index (0,92) and IRI (2,92 IU/L) was found fron the initial measurement (T0) to the last one (T6).

After a 6-month Metformin hydrochloride treatment, the number of menstrual cycles in these cases grew bigger and the increase was statistically significant compared to the number of cycles after the third month (p<0,05). The number of menstrual cycles after the third month was about 2,5 and after a six-month treatment it was a little fewer than 5.

Tab. 3 *PCOS* Indexes during the course of Metformin treatment

Indexes	Number	Outgoing values T 0		3 months later T 3		6 months later T6	
		Average Levels	SE	Average Levels	SE	Average Levels	SE
AMH ng/ml	50	*16,09*	1,02	*14, 29*	1,13	*12,59*	1,07
BMI kg/m^2	50	27, 21	0,79	26, 11	0, 67	24,71	0,82
Testosterone ng/ml	50	1,77	0,15	1,49	0,26	1,09	0,37
Androstenedioneng/ml	50	2,78	0,25	2,70	0,28	2,09	0,43
HOMA index	50	3,07	0,37	2,61	0,35	2,15	0,47
IRI IU/L	50	13,90	1,46	12,20	1,08	10,98	1,20
Number of menstrual cycle	50			2,56	0,15	4,78	0,24

After a six-month Metformin hydrochloride treatment, an ultrasound test of insulin resistant cases with PCOS was done. It was noted that the number of follicles had not changed significantly compared to their number during the first examination (u=1,02, p>0,05).

DISCUSSION

Patients with PCOS have a number of metabolic and endocrine disturbances, which play an active role in the pathogenesis of the syndrome. The results of our study show signifi cant diff erences in the hormonal profi le of the patients with PCOS compared to the healthy subjects as well as in the women with PCOS before and after metformin therapy. In accordance with previous studies, we found that women with PCOS have signifi cantly higher serum AMH levels in comparison with the healthy subjects.[17, 18, 19] The positive relationship between serum AMH and androgen levels, also detected in the present study, seems to be a specific occurrence in PCOS.[17,20] In this respect, in addition to inhibiting the sensitivity of granulosa cells to FSH, AMH also has a negative effect on aromatase activity[10], contributing to the onset and maintenance of intrafollicular hyperandrogenism.[21] There may be an increase in the number of follicles in the early stage of development, which present a large number of androgen receptors. AMH may thus contribute to intrafollicular hyperandrogenism as a local gonadal factor. Extra-ovarian and genetic factors are also involved in this process, particularly important among them LH and hyperinsulinemia.

The present study demonstrated a significant, important and positive correlation between LH and AMH. Some authors suggest that the action of LH is inadequate in patients with POCS, not only in the later phases of follicular development, but also in the early phases.[22] Since the action of AMH on the stages of early follicular development occurs independently of the hypothalamus–pituitary–ovary axis, the correlation between the levels of LH and AMH suggests a possible synergistic action of these hormones on the dysfunction of follicular recruitment and development in PCOS. Insulin can increase androgen production in several ways. It interacts with both its own receptor[25], and insulin growth factor receptor type I (IGF-I) in the ovary[26], leading to stimulation of the ovarian cytochrome P450c17.[14]

There is also evidence in PCOS that insulin can impair adrenal androgen production, increasing androgen production.[27] Insulin directly decreases serum SHBG concentration, which increases the levels of free testosterone, the active fraction of this androgen.[14] In addition, insulin appears to increase the sensitivity of pituitary gonadotropes to gonadotropin-releasing hormone (GnRH) action, improving the ovarian response to gonadotropins. These findings, and the clinical evidence of insulin resistance and hyperinsulinemia in PCOS patients, suggest that insulin plays a critical role in PCOS.

Very few studies with mixed results are available on the possible eff ects of metformin on ovarian morphology and serum AMH levels in women with PCOS.[28, 29] Thus, it was of interest to us whether the improvement of hyper-androgenism by metformin can be associated with changes in the levels of serum AMH. Our study patients received 2550 mg of metformin per day continuously for 6 months. It has been found that metformin treatment of PCOS patients results in significant reduction in circulating AMH. Suppression of AMH occurs only after protracted treatment (after 4 months). The results of the present study are consistent with previous studies, in which the treatment with insulin sensitizer metformin resulted in the reduction of AMH levels.[29, 30]

The AMH levels are significantly elevated in women with PCOS, and they may serve as a marker for evaluation of treatment efficacy with metformin. However, other studies did not find significant changes in serum AMH after metformin treatment in PCOS[31, 32], which may be attributed to a low dose (twice 850 mg/day for 6 months). It has been shown that 6 months of androgen suppression by metformin treatment failed to influence circulating AMH levels.[33] Also, it has been found that despite the improvement of metabolic parameters and the reduction of androgen levels, AMH levels did not change after metformin treatment, and the dose and possibly the time of use, of metformin are factors associated with the reduction of AMH levels.[34] The reason for the reduction in AMH concentrations after metformin remains controversial. In a prospective study,[35] metformin acutely improved IR indexes and restored ovarian morphology. Metformin seems to suppress the hepatic gluconeogenesis. Also, metformin improves the peripheral resistance to insulin, increase the consumption of glucose in skeletal muscles, and decrease the intestinal glucose absorption. Metformin enhances insulin action at cell levels by enhancing the caption of glucose in adipose and muscular cells, and by increasing the ligation to the insulin receptors. Also, the beneficial role of metformin is due to: increase in the hepatic production of SHBG, thus lowering the circulating free testosterone, decrease the adrenal androgen production, decrease androgen production in the ovary, normalize LH and slightly increase FSH levels, and decrease the insulin concentrations. Moreover, metformin has been demonstrated to induce regular menstrual cycles, increase ovulation, ameliorate hirsutism, and produce a slight weight loss.[30] Our study demonstrated that nonobese women with PCOS respond better than obese women to metformin treatment at a dosage of 2,500mg/day for the first 3 months. Nonobese women showed a statistically significant decrease in serum androgens level, fasting insulin level, and area under the curve of insulin, and also an improvement in menstrual cyclicity.

For the 10% to 30% of women with PCOS who are nonobese, weight loss is not a treatment option.[18] Our study demonstrates that nonobese PCOS women may benefit from metformin as a first-line therapy. Metformin may be a therapeutic option for PCOS based on our data showing improvement in laboratory and clinical parameters.

CONCLUSION

Serum AMH is a useful prognostic biochemical marker for Metformin treatment in PCOS women. Metformin is able to decrease AMH in PCOS, presumably by an insulin-dependent mechanism of action. Maybe, the dose and possibly the time of the metformin use are factors associated with the reduction of AMH levels. Our data are to be considered preliminary, and further studies are needed to clarify whether the decrement of AMH obtained with metformin could or could not be considered a marker of the efficacy of the drug on the ovarian function.

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