Un association of Anti-Mullerian Hormone gene (rs4807216) polymorphism in Polycystic Ovary Syndrome (PCOS) Among Iraqi Women

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Abstract
The current work aimed to assess the hormonal profile of women with PCOS, including the AMH hormone and the AMH gene (rs4807216) polymorphism. The blood samples were obtained from 60 patients with PCOS who visited Reproductive Medicine Centre of General Hospital of Kirkuk and 30 healthy women. The serum level of LH, FSH, TSH, Estrogen and AMH determined by Enzyme linked immunosorbent assay (ELISA), while Polymerase chain reaction (PCR) was used to genotype the AMH gene (rs4807216). The current results showed a significant increase in LH, TSH, Estrogen, and AMH serum levels in PCOS females as compared to the control female group at P value = 0.05. In contrast, the serum level of FSH was not significantly increased at p value = 0.05. Polymorphism rs4807216 was not significantly associated with serum AMH levels in the population study. In conclusion, Women with PCOS have high levels of LH, TSH, Estrogen, and AMH. Therefore, these hormones consider biomarkers for detecting PCOS in Iraqi females.

Introduction:
The endocrine-gynecological condition Polycystic ovarian syndrome (PCOS) affects many women in reproductive age and may be effects on the level of some sex hormones [1,2]. It is a complex disease in which genetic, endocrine, environmental, and behavioural factors all interact to produce a varied phenotype with reproductive, metabolic, and psychological features [3]. The health and quality of life of women are impacted by polycystic ovarian syndrome. The polycystic ovarian syndrome phenotypic might alter over life, necessitating a specific diagnostic approach and therapy [4]. Polycystic ovarian syndrome is a primary cause of ovulatory infertility; it is also linked to hirsutism and acne [5]. Since the polycystic ovarian syndrome criteria include typical physiological changes that take place during puberty, it
might be challenging to diagnose the condition throughout adolescence[6]. The condition progresses from a reproductive disease to a metabolic disorder as people get older. Polycystic ovarian syndrome, together with metabolic disturbances such as insulin resistance and irregularities in energy expenditure, is recognized as a key risk factor for the development of type 2 diabetes and cardiovascular disease later in life[7].

AMH gene found on chromosome 19p13.3, has five exons[8]. A member of the transforming growth factor-β (TGF-β) superfamily known as anti-Mullerian hormone (AMH) is known to originate from the granulosa cells of developing follicles (from the primary to the big antral follicle stage)[9]. AMH has been proposed as a biomarker for the detection of PCOS since serum levels of AMH correlate with the total number of antral follicles present in both ovaries[10]. The preferred ovarian reserve marker remains serum AMH. However, the lack of a worldwide standard for AMH makes comparing AMH assays difficult [11]. Aside from its diagnostic utility, the discovery of an increased serum AMH level in PCOS has raised significant pathophysiology concerns[12]. According to a fresh notion, AMH may be more important than only a measurement of ovarian follicle count in the development of PCOS as an endocrine signal[13]. The aim of this study is to assess the hormone profile and genetic polymorphism of AMH in PCOS among Iraqi females.

Materials and Methods:

Study design:
This case-control study was done at the Reproductive Medicine Center of General Hospital of Kirkuk, Iraq. Sixty (60) women with PCOS and thirty (30) normal women were enrolled in this study during the period from October 2022 until February 2023. The diagnosis of PCOS was made based on the presence of two of three Rotterdam 2003 criteria: polycystic ovaries, clinical or biochemical evidence of hyperandrogenism, oligo- or anovulation, and the exclusion of alternative etiologies[14]. The body mass index (BMI) was calculated. Hormonal examination (LH, FSH, TSH, Estrogen, and AMH), hirsutism score, and menstrual cycle disturbance were performed.

Sample collection:
Each participant had five milliliters of venous blood drawn. After 30 minutes in a gel tube and 5 minutes of centrifugation at 3000 rpm, the serum was kept at -20 C until utilized for hormonal analysis. Remaining two milliliters of whole blood was stored in an ethylene diamine tetra acetic acid (EDTA) tube, which was used to detect the AMH gene (rs4807216) polymorphism with specific primers after DNA isolation.

SNP analysis:
Using a Geneaid-provided genomic DNA purification mini kit, DNA was isolated from peripheral blood (Geneaid Biotech. Ltd, Taiwan). Two sets of primers were created specifically for allele-specific PCR. For rs4807216, the sequence of the primers was IF TTGTGTACCATCCTTTTC TCTCTTCG, IR ACAGGGAGCCCTGGGGACAT, OF
CCATCAGTGTCTCTGCTGG, and OR ACACTGTGGCTCCCTTGACTTCC. The size of products was 191 bp for G allele, 132 bp for A allele and 276 bp for outer primers.

**Statistical analyses:**

GrapPad Prism 9 was used for the statistical analysis. Statistical analyses (for clinical and biochemical features) were carried out using the Student’s t-test, with differences judged significant at $P<0.05$. To evaluate the results, the Hardy-Weinberg equilibrium was used. The genotypes and allele frequencies of the rs4807216 variant were computed as percentages. The odds ratio (OR) and its 95% CI (confidence interval) were used to examine the differences between controls and patients.

**Results:**

Baseline characteristics of the PCO women are listed in Table 1. During the study period, 60 patients who met the criteria for a PCOS diagnosis were included. Among them, 13 were aged below 25 years, 42 were aged between 25-35 years, and 5 patient was over 35 years old. Family history was found in 61.7% of patients. A total of 39 out of 60 patients (65%) had menstrual irregularities as one of the presenting symptoms, whereas 49 (81.7%) presenting with hirsutism and 43 (71.7%) had acne.

Table 1: Baseline characteristics of women patients with PCOS.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients</th>
<th>Yes (%)</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below 25 years</td>
<td>13 (21.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-35 years</td>
<td>42 (70%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Over 35 years</td>
<td>5 (8.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td>37 (61.7%)</td>
<td>23 (38.3%)</td>
<td></td>
</tr>
<tr>
<td>Menstrual cycle disturbance</td>
<td>39 (65%)</td>
<td>21 (35%)</td>
<td></td>
</tr>
<tr>
<td>Hirsutism</td>
<td>49 (81.7%)</td>
<td>11(18.3%)</td>
<td></td>
</tr>
<tr>
<td>Acne</td>
<td>43 (71.7%)</td>
<td>17 (28.3%)</td>
<td></td>
</tr>
</tbody>
</table>

The mean age in the PCOS group was significantly lower than controls. According to hormonal examination, patients with PCO compared with the healthy women group had higher LH (12.703 ± 3.4 vs. 7.53 ± 2; $p<0.0001$), TSH (2.724 ± 1.03 vs. 2.038 ± 0.79; $p<0.01$), Estrogen (53.73 ± 17.08 vs. 23.04 ± 9.99; $p<0.0001$), AMH (6.782 ± 3.014 vs.1.175 ± 0.37; $p<0.0001$) are significant risk factors for developing PCOS. Whereas, there was no significant difference between FSH in the PCOS and control group (5.475 ± 1.03 vs. 5.22 ± 1.13; $p<0.303$), Table 2.
**Table 2:** Hormone level and statistical analysis between patient and healthy groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient mean ± SD</th>
<th>Healthy mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(years)</td>
<td>30.15 ± 3.93</td>
<td>32.46 ± 2.88</td>
<td>0.001**</td>
</tr>
<tr>
<td>TSH</td>
<td>2.724 ± 1.03</td>
<td>2.038 ± 0.79</td>
<td>0.01*</td>
</tr>
<tr>
<td>Estrogen</td>
<td>53.73 ± 17.08</td>
<td>23.04 ± 9.99</td>
<td>0.0001**</td>
</tr>
<tr>
<td>AMH</td>
<td>6.782 ± 3.014</td>
<td>1.175 ± 0.37</td>
<td>0.0001**</td>
</tr>
<tr>
<td>LH</td>
<td>12.703 ± 3.4</td>
<td>7.53 ± 2</td>
<td>0.0001**</td>
</tr>
<tr>
<td>FSH</td>
<td>5.475 ± 1.03</td>
<td>5.22 ± 1.13</td>
<td>0.303NS</td>
</tr>
</tbody>
</table>

** Highly significant; * Significant; NS Non significant; SD standard deviation

The T allele is the most common form of the AMH gene (rs4807216). According to current results, it is considered the most widespread allele in both female control group (0.78) and females with PCOs (0.87). The second allele of the AMH gene (rs4807216) was found to be the C allele, with a frequency of 0.13 in individuals with PCOs and a frequency of 0.22 in the female control group, Table 3.

**Table 3:** Distribution of AMH gene (rs4807216) in patients and control group with statistical analysis

<table>
<thead>
<tr>
<th>Alleles</th>
<th>PCO NO.</th>
<th>Allele frequency in PCOs</th>
<th>Healthy control no.</th>
<th>Allele frequency in Healthy control</th>
<th>Total study participants</th>
<th>OR</th>
<th>95% CI</th>
<th>χ²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (reference)</td>
<td>104</td>
<td>0.87</td>
<td>47</td>
<td>0.78</td>
<td>0.84</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>16</td>
<td>0.13</td>
<td>13</td>
<td>0.22</td>
<td>0.16</td>
<td>0.55</td>
<td>0.24</td>
<td>1.24</td>
<td>2.05</td>
</tr>
<tr>
<td>Total</td>
<td>120</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
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</tbody>
</table>

AMH gene (rs4807216) has three possible genotypes, only two genotypes were detected in the PCOS patients (TT and TC) while three genotypes were detected in the females of control groups (TT, TC and CC). The reference genotype was the TT, which had 44 and 18 occurrences in the PCOs women and women healthy groups, respectively. Additionally, after analyzing various models of inheritance, it was determined that no specific genotypes were linked to an increased risk of PCO, Table 4.
Table 4: Distribution of genotypes of AMH gene (rs4807216) in patients and control group with statistical analysis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Study participants</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCOS No.</td>
<td>Healthy control No.</td>
<td>OR</td>
<td>95% CI Lower</td>
<td>95% CI Upper</td>
<td>χ²</td>
<td>p-value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT (reference)</td>
<td>44</td>
<td>18</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TC</td>
<td>16</td>
<td>11</td>
<td>0.59</td>
<td>0.23</td>
<td>1.52</td>
<td>1.17</td>
<td>1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0</td>
<td>1</td>
<td>0.13</td>
<td>0.005</td>
<td>3.56</td>
<td>2.35</td>
<td>2.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group of genotypes and models of inheritances</td>
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<td></td>
<td></td>
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<tr>
<td>Co dominant model</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC vs. TT</td>
<td>0 vs. 44</td>
<td>1 vs. 18</td>
<td>7.21</td>
<td>0.28</td>
<td>18.54</td>
<td>2.35</td>
<td>2.35</td>
<td>P = 0.23</td>
<td></td>
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<tr>
<td>Dominant model</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>TT vs. TC + CC</td>
<td>44 vs. 16</td>
<td>18 vs. 12</td>
<td>7.21</td>
<td>0.45</td>
<td>1.37</td>
<td>1.65</td>
<td>1.65</td>
<td>P = 0.2</td>
<td></td>
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<tr>
<td>Recessive model</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT + TC vs. CC</td>
<td>60 vs. 0</td>
<td>30 vs. 0</td>
<td>0.5</td>
<td>0.009</td>
<td>26.02</td>
<td>26.02</td>
<td>26.02</td>
<td>P = 0.73</td>
<td></td>
</tr>
<tr>
<td>Over-dominant model</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT + CC vs. TC</td>
<td>44 vs 16</td>
<td>19 vs. 11</td>
<td>0.62</td>
<td>0.24</td>
<td>1.6</td>
<td>0.95</td>
<td>0.95</td>
<td>P = 0.33</td>
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</tbody>
</table>

In patients groups, there did not appear to be any significant difference in the AMH level between TT and TC genotypes (6.95 ± 3.26 vs. 6.07 ± 2.80; p < 0.34), also in the control group, there doesn’t seem to be a significant difference in AMH levels among TT, TC and CC genotypes (1.19 ± 0.47, 1.13 ± 0.16 vs. 1.12 ± 0.00; p < 0.91), Figure 1.

Fig. 1 Serum level of AMH among control and patients according to rs4807216 polymorphism of AMH gene

Discussion:
PCOS affects 6-26% of women of reproductive age worldwide, according to estimates[15]. The majority of PCOS cases are discovered between the ages of 20 and 30, while it can occur at any age after menarche[16]. In the year 2019, there were a total of 6,647,566
reported cases of PCOS in the MENA region. This means that there were 2079.7 cases per 100,000 women, after an age-standardized point. This is a significant increase of 37.9% since 1990[17]. According to previous studies that agree with current data, the cause of PCOS is unknown but women with a family medical history are at higher risk[18,19]. When compared to other studies, it is clear that common clinical manifestations in PCOS patients include menstrual cycle disturbance, hirsutism, and acne[20,21].

The mean age of PCOS patients in this study was substantially lower than that of controls (p = 0.005). This finding is consistent with previous research, which found that the proportion of women with PCO declined with age[22,23]. This can be caused by a decline in the number of antral follicles that occurs in normal women during their reproductive years, a phenomena that also applies to PCOS sufferers [24]. Another study found that half of women diagnosed with PCOS at the age of 30 no longer have these characteristics 8 years later[25].

LH and TSH levels increased significantly in our study of hormonal profile analysis of PCOS patients compared to the control group, whereas FSH levels did not differ significantly between cases and controls. The relationship between PCOS and thyroid hormones is still a topic of debate. Interestingly in previous research, TSH level analysis revealed a significant increase in correlation with PCOs women, while no significant increase in FSH and LH was detected[26]. At LH and FSH levels these results support evidence from previous studies[27]. Also this finding is contrary to previous studies which have suggested that no significant increase in TSH was detected[28]. Women who have PCOS may experience estrogen dominance, often known as high amounts of estrogen. This disorder, which is a hormonal imbalance, can lead to irregular menstrual cycles, unwelcome hair growth, and acne[29].

Recent studies have shown a variety of relationships between elevated plasma levels of anti-Mullerian hormone (AMH) and various clinical characteristics of PCOS[30]. The antral follicle count (AFC) on ultrasound is one of the diagnostic criteria according to the Rotterdam Criteria. The number of follicles observed in ultrasonography grows as ultrasonography technology advances and device accuracy rises, however this growth is still reliant on the particular equipment. Small antral follicles, which are precisely the ones observed on ultrasound, produce serum AMH[31].AMH stimulated hypothalamic gonadotropin-releasing hormone (GnRh) neurons, which express AMH receptors, in both mice and humans in vivo[32]. AMH, on the other hand, is known to suppress follicle-stimulating hormone (FSH) and raise LH concentration and LH: FSH ratio are considered markers of PCOS [33]. These studies' findings suggested that AMH levels may rise prior to LH elevation and FSH suppression in women at risk of developing PCOS[34]. In addition to raise LH hormone in patients with PCOS serum level of prolactin hormone showed depending on the past studies[35].

Regarding to the AMH gene (rs4807216) frequency among studied group, these results indicate no significant association between the genotypic distribution of the AMH gene variant (rs4807216) and the occurrence of PCOS under any of the tested inheritance models. Contrary to our findings, several genome-wide association studies (GWAS) and exome-sequencing studies have identified different variants in the AMH gene as potential predictors of PCOS, as reported by[36]. The complex genetic roots of PCOS are emphasized by this difference, which stresses the need for more research in diverse populations and under
varying circumstances. To the best of our knowledge, this is the first study conducted in Iraq to investigate the association between AMH gene (rs4807216) polymorphisms and the occurrence of PCOS. These results add to the broader international body of research, contributing unique data from a previously under-studied population.

To date, numerous genetic loci and individual genes have been linked to this condition. The advent of genome-wide association studies (GWAS) and other genetic investigations has changed the landscape of detecting and treating this reproductive and metabolic disorder known as PCOS[37].

Regarding to the impact of AMH (rs4807216) polymorphism on AMH level, results indicates no significant relationship between AMH gene polymorphisms and serum AMH levels in women with PCOS. Similar to our study's aim, previous research has also examined the relationship between AMH gene polymorphisms and serum AMH levels in women with PCOS. For example, a study conducted by[38]. A novel AMH promoter polymorphism, rs10406324, was linked to reduced serum AMH levels in PCOS patients, implying a function in AMH gene regulation.

**Conclusion:**

Women with PCOS have high levels of level of LH, TSH, Estrogen, and AMH. Therefore these hormones consider biomarkers for detecting PCOS in Iraqi females. Polymorphism rs4807216 was not significantly associated with serum AMH levels in the population study. AMH level not significantly associated with genotypes of AMH rs4807216 gene.

**Limitation of the study:**

Our study has several limitations, and we advise caution in the interpretation of the findings. First, the sample size was small. Second, study just included two SNP.

**Acknowledgments:**

We are grateful to all participants for generously participating in this study.

**References:**


دور تعدد أشكال الجين المضاد للهرمون المولري (rs4807216) في متلازمة المبيض المتعدد الكيسات (PCOS) بين النساء العراقيات

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الخلاصة:

نهدف العمل الحالي إلى تقييم الوضع الهرموني للأفراد المصابين بمتلازمة تكيس المبايض، بما في ذلك هرمون AMH وتنوع أشكال جين AMH (rs4807216). في البداية، تم الحصول على عينة الدم من 60 مريضة أفرادًا الذين زاروا مركز الطلب التناسلي في مستشفى كركوك العام و30 امرأة سليمة. تم تحديد مستويات الهرمونات: LH، FSH، TSH، الاستروجين، والإستروجين بواسطة محاذاة الامتصاص المناعي المرتبط بالإنزيم (ELISA)، وتم استخدام تفاعل البوليميراز المتسلسل (PCR) في التنميط الجيني لجين AMH (rs4807216). أظهرت النتائج الحالية زيادة معنوية في مستويات LH، TSH، الاستروجين، والإستروجين، وAMH في إناث متلازمة PCOS، مقابلًا السكان الصلبة عند قيمة P = 0.05. في المقابل، لم يرتفع مستوى FSH في الدم بشكل معنوي عند قيمة P = 0.05، ولم يتغير عدد الأشكال rs4807216 بشكل كبير في الدراسات السكانية. في الختام، النساء المصابات بمتلازمة تكيس المبايض لديهن مستويات عالية من مستوى LH، FSH، TSH، الاستروجين، والإستروجين، وAMH. لذا تعتبر هذه الهرمونات مؤشرات حيوية للكشف عن متلازمة تكيس المبايض لدى الإناث العراقيات.

الكلمات المفتاحية:
متلازمة المبيض المتعدد الكيسات، متلازمة تكيس المبايض، الهرمون المضاد لمولري، هرمون AMH، تعدد الأشكال rs4807216.

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