“LUCIAN BLAGA” UNIVERSITY FROM SIBIU

FACULTY OF MEDICINE VICTOR PAPILIAN

INSULIN RESISTANCE IN POLYCYSTIC OVARIAN SYNDROME

SUMMARY

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INTRODUCTION

Polycystic ovary syndrome is a heterogeneous disorder that combines chronic anovulation, hyperandrogenism and metabolic disorders affecting 5-10% of women of childbearing potential. Being an affection with various modes of presentation and still unknown etiology, PCOS definition and diagnostic criteria are still controversial.

The etiology of polycystic ovary syndrome is not fully understood, the syndrome resulting from the interaction of genetic factors with epigenetic and environmental factors. Susceptibility genes are numerous and each of them seems to contribute to some extent, small, but significant, to the phenotype syndrome, without being discovered a single determinant gene. In the ovarian polycystic process are involved, genetic modifications, hypothalamus and pituitary, endocrine pancreas, adrenal and of course ovaries. It is difficult to be determined the initial mechanism and how the ovary is forced to transform its cystic follicles. Under independent physiopathological aspect of the root cause of the condition, it tends to perpetuate the vicious circle to therapeutic intervention.

Research conducted in recent years consider the development of insulin resistance and hyperinsulinemia to be an important pathogenetic ring within the syndrome; some authors consider that the distinction between cystic ovaries, even in the presence of androgenization signs, and polycystic ovary syndrome or polycystic ovarian make insulin resistance development.

In light of these considerations, this paper aims to present the implications of insulin resistance in polycystic ovarian syndrome, physiopathology and genetic and endocrine - metabolic interrelationships that define the syndrome.

SPECIAL PART

Objectives

Since PCOS phenotypic changes may have genetic implications, we considered that the investigation of possible genetic mutations in the insulin resistance has a real interest, the global results are contradictory and investigations continue and the Romanian population studies are very short.

Due to the importance of association of insulin resistance and metabolic disorders in the pathogenesis of the syndrome, we chose to study two point mutations (SNP) differently

involved as a gene expression in insulin action. The option took into account the fact that in literature there is uncertain data regarding the involvement of these genes polymorphisms in diabetes and insulin resistance, and studies that suggest the involvement of genes in PCOS to other populations than the Romanian one.

For INSR were investigated multiple SNPs with conflicting results; in terms of the SNP we studied, the research seems to be just starting out, information from literature being quite limited.

For Prol2Ala polymorphism of PPARγ gene there is numerous data related to obesity, insulin resistance, diabetes mellitus and a rather small number of PCOS studies.

As a result the aim of the study was:

- Detail data on insulin resistance on a lot of PCOS patients compared to a control group by retrospective statistical analysis applied to a subset of the database Endocrine Laboratory of Molecular Genetics at the University of Medicine and Pharmacy C. Davila, Department of Endocrinology (which includes subjects previously recruited and described by polycystic ovary study group in Bucharest and Montpellier) and a PCOS group -control recruited from Tirgu Mures;
- Developing a protocol for genotyping a nucleotide polymorphism (SNP) through PCR - HRM for SNP rs2252673 that corresponds to the polymorphism of INSR gene in conducting a study of its genetic association with PCOS and respectively insulin resistance.
- Investigation of a possible association of Pro12Ala polymorphism of PPAR-γ gene with PCOS, and analysis of the relationship between polymorphism and hiperandrogenemy, namely insulin resistance study on a subset of the previous batch, represented by patients from Tirgu Mures.

1. STUDY A - rs2252673 polymorphism study of INSR gene

Materials and methods

Subjects in the study were unrelated women (n = 338), who had revised diagnostic criteria of PCOS in 2003 in Rotterdam. From the study were excluded the patients diagnosed with Cushing's syndrome, hyperprolactinemy, and androgen-secreting tumors.

It was used a control group consisting of (n = 142) women, aged between 14 and 43 years, with regular menstrual cycles, hirsutism without major problems, without hormone therapy in the last 3 months or history of infertility.

All patients signed an informed consent form. The study was approved by the U.M.F. Carol Davila Ethics Committee and U.M.F. Tirgu Mures, in accordance with the Declaration of Helsinki.

Subjects' evaluation protocol provided that:

- hirsutism to be assessed through Ferriman-Gallwey score, with an 8 points threshold value.
- all blood samples should be collected between the 3rd and 5th day of menstrual bleeding after a spontaneous or induced bleeding, or in day 7 after the progestogen-induced bleeding (follicular phase).
- blood glucose determination is made after 12 hours of fasting, total plasma testosterone, basal insulin and SHBG to be measured with the same dosage chemiluminescence kit.
- ovarian ultrasound is transvaginally performed where possible, by following the definition of consensus - Rotterdam 2003
- for determining insulin resistance of HOMA-IR and IR-HOMA2 computer formulas were used.
- other infrequent tests for measuring endocrine and metabolic parameters were used: LH, FSH, PRL, E2, lipid profile

Genotyping protocol included the following steps:

1. **DNA extract**. Genomic DNA was obtained from whole blood using DNA extraction kit Promega Wizard (Promega - Product Code A1620)
2. **DNA concentrations determination** by fluorescence - Quant-It dsDNA - Invitrogen, RotorGene 6000 nalyzer (Corbett Research) to conduct 20ng/µl dilutions, respectively 2ng/µl.
3. **Primers choice.** We designed the PCR primers (forward and reverse, with F and R suffixes), and studied the corresponding melting temperatures using Primer 3 program (http://frodo.wi.mit.edu/primer3/):

INSR : PCR pt HRM rs2252673 primers
rs2252673_F GACCCCTGGTGCCTGCTCCG
rs2252673_R aaAGGGGTTTCTGTGGCTTTTTGGTGAAGCATCTGCTCTCCAGCAC

The choice was guided by the "snap back primer" design method that I used as a genotyping principle.

4. **Amplification of target genes through PCR**. DNA amplification was performed by asymmetric PCR, using unequal quantities of F limited primers (small amount) and R in high concentrations (ratio F / R 1/15). Moreover, primer R has an aaAGGG termination that is limiting the investigated nucleotide sequence amplification chain. Being a sufficiently long oligonucleotide sequence with an area series complementary to the interest studied chain, primer R forms a "pin" loop including the SNP investigated. After PCR, two groups of products are formed: both intramolecular "hairpin" (snapback) loop - probe, as well as the area of interest - the realignment amplicon duplex between the two primers..
5. **Genotyping by High Resolution Melt (HRM) method.** HRM method analyzes double-stranded DNA samples previously amplified by PCR, by determining the melting temperature (melting) of DNA fragments obtained after PCR with fluorescent substance. HRM analysis allows distinguishing the genotypes based on different nucleotide composition, even if the products differ by a single base. In the presence of a mutation, a shape or position modification will occur in the melting curve profile, "melt" of the PCR product that is dependent on GC base content, sequence length, sequence of base pairs, the shape being different for homozygous to heterozygous. **Control experiments** aimed at verifying the results of the genotyping HRM, and were made by agarose gel electrophoresis, and by direct sequencing, samples being selected from those with HRM interpretation problems, and random to confirm the results.

Results

1.Statistical data from phenotypic aspects of the lots

Presence of significant differences was observed at the patients' age that was included in the study. Average age was higher in the control group, 28.78 ± 6.29 years, compared to the PCOS group, 24.53 ± 5.34 years.

Comparing average values for the main phenotypic parameters commonly affected in PCOS we found that the mean values for basal glucose, there are statistically significant differences for each trait.

Main clinical and laboratory control features, PCOS

All values are average ±DS.

	Controle	**PCOS**	P Value
BMI (kg/m^2)	23.04±4.29	28.02±7.17	<0.0001
WHR ratio	0.75±0.06	0.83±0.09	<0.0001
Basal insulin (μIU/ml)	8.86±9.38	18.60±19.92	<0.0001
Basal glucose (mg/dl)	87.39±11.44	88.23±22.85	0.704
HOMA	2.04±2.28	4.11±4.61	<0.0001
HOMA2	1.15±0.92	2.17±1.51	<0.0001
FAI	2.66±2.26	8.58±8.12	<0.0001
SHBG	107.22±103.62	53.32±50.75	<0.0001
Hirsutism scor Ferriman	2.11±1.8	7.95±5.01	<0.0001

PCOS is significantly statistically associated with an increase of the body weight, obesity is more common than overweight (p <0.0001, Chi square test)

An BMI > 25kg / m² is present in 20% of women belonging to the control group, whereas in women with PCOS the value is higher than 61.6%, the percentages are close and in central obesity 16.80%, respectively 62, 50%. Moreover, PCOS is significantly statistically associated with an increase in body weight, obesity being more common than overweight (p <0.0001)

Laboratory data for basal glucose and insulinemy were available in 437 patients, representing 91.05% of the total sample. Calculation of HOMA-IR and IR-HOMA2 was performed on the same data.

Mean a jeun glucose values in patients from the PCOS group do not differ significantly from the average glucose control group, p = ns. Mean values of the a jeun insulin dosage are higher in PCOS group compared to control group. The difference is significant only statistically (p <0.0001).

In this study we defined insulin resistance according to the HOMA-IR insulin resistance index. This has significantly higher mean values in PCOS group compared to the control group.

For obesity interrelation - insulin resistance we compared HOMA IR and BMI to the whole study group; there is a positive correlation between the two parameters, insulin resistance increasing proportionally with weight mass index.

Comparing analyzing insulin resistance with SHBG as an expression of bioavailable testosterone we observe a negative correlation between the two parameters, decreased SHBG being accompanied by a decrease in insulin sensitivity.

Between the degree of insulin resistance and hyperandrogenism there is a positive correlation; decrease insulin sensitivity changes proportionally with increasing free androgen index.

If we analyze clinical parameters related to hyperandrogenism, we observe, as expected, that hirsutism (estimated at 92.5% of patients) does not appear (only sporadically) to control group, instead it frequently appears (62.2%) to PCOS patients.

Paradoxically, the presence of hirsutism appears not to correlate with total testosterone value, the correlation being statistically insignificant ($p = 0.403$). Instead, for the analysis of hirsutism according to FAI (free androgen index), there is a statistically significant correlation with the Ferriman score value to PCOS patients ($p = 0.005$).

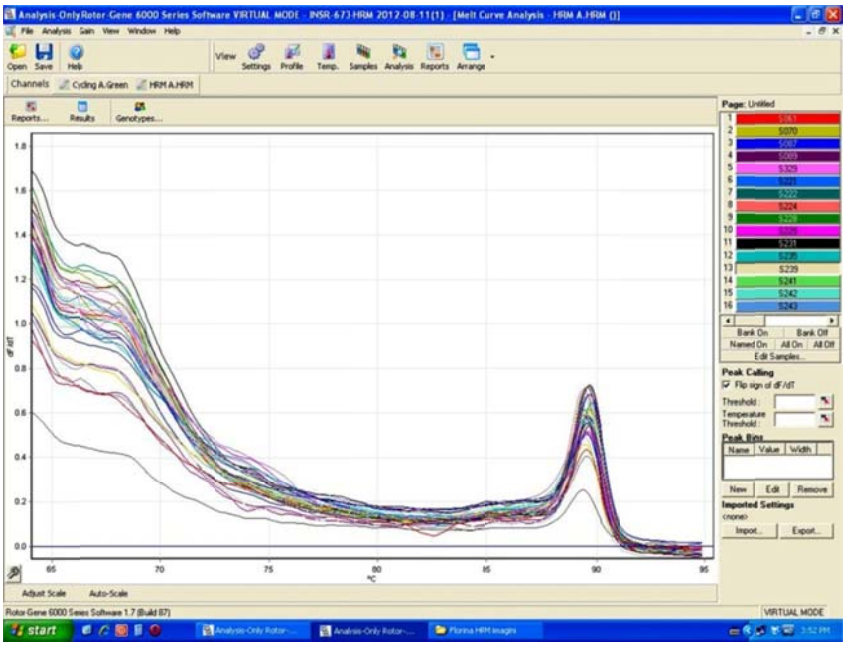
2.rs 2252673 INSR polymorphism genotyping - results

According to the genetic database on the rs2252673 SNP, wild-type allele contains cytosine, and mutant allele (rare) contains guanine. (UCSC Genome Browser).

SNP rs2252673 is a site class III SNP (changed bases are G / C) and is more difficult to be HRM genotyped, due to small Tm differences between alleles (the typical shift Tm is small, 0,2-0,5^{o}C.

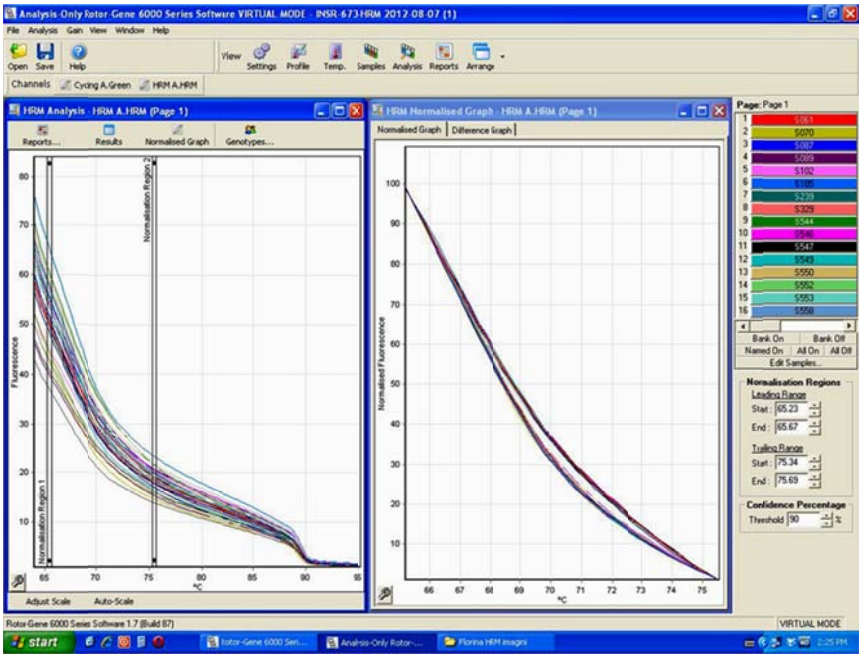
Of the total of 480 DNA samples introduced into the reaction, the overwhelming majority had an interpretable behavior for HRM analysis; 9 samples had amplifying problems to PCR reaction, being uninterpretable to their subsequent reintroduction in experiments, were removed from the study.

The images obtained and HRM and melting analyze are shown in the following figure:



The melting curves aspect individualize two melting curves, determined by different Tm of PCR obtained products (probe and amplicon)

Reference allele of this SNP is considered on the antisense strand, the gene being also antisense transcribed. Based on allele frequency and knowing the primers design, we determined that the right cluster HRM analysis corresponds with the common genotype, C / C genotype, and cluster left genotype C / G.

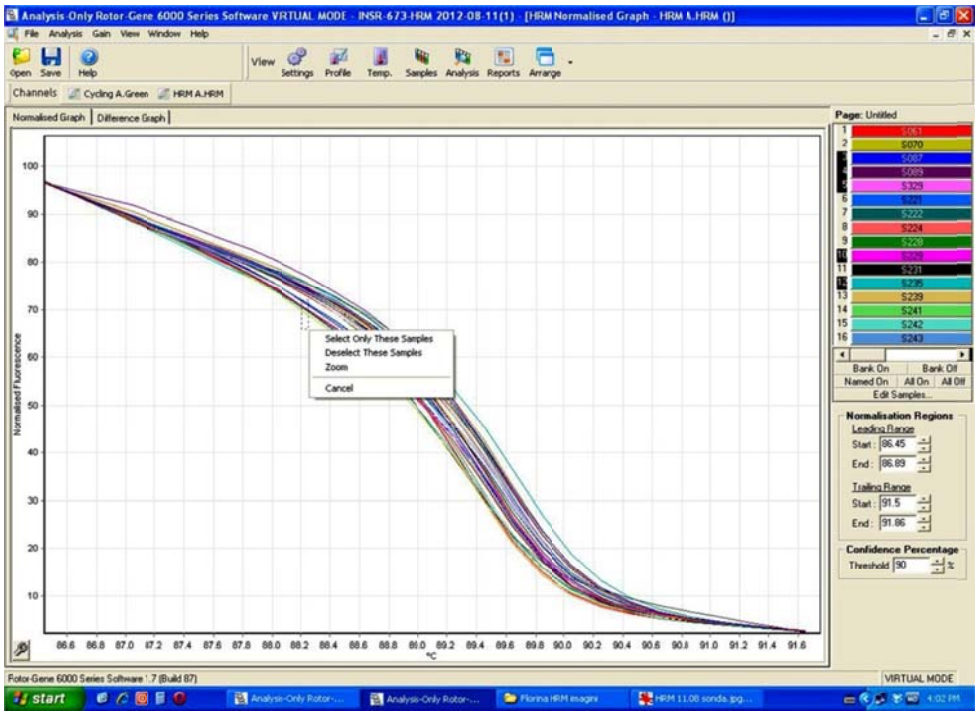


HRM probe

DNA samples that did not have an appropriate graphic behavior to the two types of clusters were followed by amplicon.

As neither here could be included they were eliminated from the analysis

On the amplicon, at a higher melting temperature, the curves are interpreted as follows: the right cluster corresponds to the homozygous and the left are the heterozygote.



The aspect of HRM amplicon curve analysis

To verify the results, the comparative method was applied to samples following their framing probe, respectively amplicon; the overlap degree was significant, the remaining evidence with uncertain outcome being subsequently sequenced.

Parallel interpretations were made on clusters for the two individual genotypes.

Statistical analisys of SNP rs2252673 association with PCOS

Testing statistical association between INSR genotypes and the PCOS diagnosis (vs. control) showed the lack of frequency significant differences for the two identified genotypes, and association with certain phenotypic aspects.

Interpretation of the degree of obesity according to obtained genotypes revealed no statistically significant association with them; obesity (BMI ≥ 30) is present in similar proportions in both homozygous and heterozygous. (p = 1)

Regarding insulin resistance, there was no statistically significant association between decreased insulin sensitivity and INSR genotypes (homozygous, heterozygous). Parameters that show impaired insulin sensitivity even if they correlate linearly, HOMA IR and HOMA 2IR present different association indices. (p = 0.836 for HOMA IR and 0.468 HOMA 2IR, valid indexes for the whole lot)

Concerning the possible association between genotypes and hyperandrogenism, the results demonstrated no statistically significant difference in genotype distribution according to the FAI or the control group (p = 1) and in the PCOS group (p = 0.184)

2. STUDY B - Pro12Ala polymorphism study of PPAR-γ gene

Materials and methods

We conducted a case-control study consisting of 89 Romanian women, of which 47 with PCOS and 42 controls with a mean age of 25.10 ± 5.57. Patients were recruited through the specialized Tg Mures County Hospital ambulatory and the Department of Endocrinology Tg Mures, between 2009 and 2011.

Patients' inclusion criteria and the assessing protocol were similar to those with Study A.

Genotyping protocol included the following steps:

1. **DNA extraction** was made from whole blood with the ZymoBead ™ Genomic DNA Extraction Kit:

2. Choice primers. The selected primers (Fermentas) present the following nucleotide sequence:

5'-GCCAATTCAAGCCCAGTC -3 '

5'-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG -3 '

2.Amplification and genotyping by PCR - RFLP (fragment length polymorphism)

1. Amplifying by PCR the genomic fragment that contains the mutation or the studied polymorphisms
2. Digesting the amplification products with the appropriate restriction enzyme for 2 hours at 60 ° C [5U Bsh1236I (BstUI) (Fermentas ®, Lithuania)
3. Agarose gel electrophoresis of restriction fragments
4. Interpreting the agarose electrophoresis gel in UV light using the fotodocumenting system.

Statistical analysis was performed using EpiInfo for Windows. "T" test was used to compare averages and F test for assessing the differences between variables. Data normality was checked by applying Kolmogorov-Smirnov test. For all analysis we used $\alpha = 0.05$; all p-value less than α were considered statistically significant. Binary variables were analyzed with Fisher's exact test.

The experimental protocol was approved by the University's Research and Ethics Commission, and the consent form was signed by each patient.

Results

1. Phenotypic characterization of control and PCOS subjects

There were statistically analyzed the most important clinical aspects of the syndrome and insulin resistance and hyperandrogenism defining parameters.

Main clinical and laboratory features to control, PCOS.

All values are mean ±DS.

	Control n=42	**PCOS n=47**	P Value
Age	28.64±4.34	25.06±5.52	0.001
BMI (kg/m^2)	22.74±1.87	28.70±6.43	<0.0001
WHR ratio	0.71±0.03	0.84±0.11	<0.0001
Basal Insulin (μIU/ml)	9.25±7.18	15.03±12.10	0.008
Basal Glucose (mg/dl)	90.46±8.23	92.83±7.96	0.171
HOMA	2.04±1.54	3.43±2.69	0.004
HOMA2	1.18±0.88	1.99±1.38	0.002
Total Testosterone (ng/ml)	0.48±0.2	0.071±0.31	<0.0001
SHBG	76.02±41.79	30.97±14.45	<0.0001
Ferriman Hirsutism scor	0.90±2.26	11.06±3.69	<0.0001

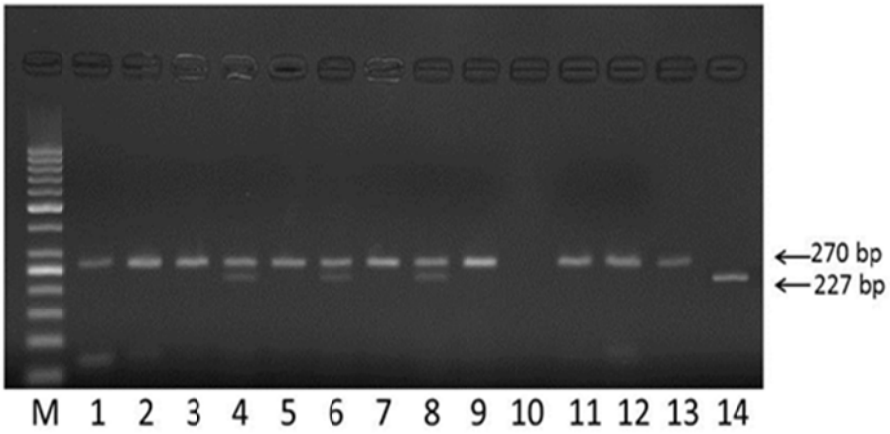
In terms of clinical aspects, statistically significant differences were detected on obesity, disposal and hiperandogenism signs between the two studied groups. Women from PCOS group had higher BMI ($p < 0.0001$), incidence or central obesity degree is higher ($p < 0.0001$), and are more hirsute; the findings are consistent with the phenotypic characteristics of PCOS.

Regarding metabolic status a jeun glucose values (p = 0.171) are not statistically significantly different as environments in the two groups; however, basal insulin (p = 0.008) and the parameters that define insulin resistance, HOMA IR and HOMA 2IR are significantly higher in patients with PCOS (p <0.005).

2. PPAR-γ genotype analysis and association with PCOS

Product analysis by PCR by agarose gel migration and UV light to highlight the three individual genotypes:

- A single 270 bp product fragment (base pairs) - CC homozygous wild type (genotype Pro12Pro)
- Two 227 and 43 bp fragments - homozygous GG (genotype Ala12Ala)
- Three 270, 227 and 43 bp fragments - heterozygous CG (genotype Pro12Ala)



Analysis of PPAR-γ gene polimorphism

(peroxisome proliferator-activated receptor γ) PCR-RFLP. M: marker molecular 50 bp ADN, line 10: negative control ; lines 1–3, 5, 7, 9, 11-13: homozygous CC (Pro12Pro), lines 4, 6, 8: heterozygous CG (Pro12Ala), line 14: homozygous GG (Ala12Ala). 43-bp DNA fragments cannot be seen in the image

Distribution of Pro12Ala polymorphism of PPAR-γ gene was in Hardy Weinberg equilibrium in both groups Ala allele incidence was 10.68% (12 patients) in the PCOS group and 9.79% (11 patients) in the control group. One homozygous patient for the Ala allele was diagnosed in the control group.

In PCOS group the ponderal index, central obesity and hirsutism are comparable between subjects with Pro / Pro genotype and with X / Ala (p = ns) genotype.

Glucose metabolism assessed by insulin, insulin resistance degree and basal glucose and defining insulin resistance parameters HOMA IR and HOMA 2IR do not show significant changes associated with the two genotypes (p = ns).

The androgenic profile (total testosterone, SHBG and FAI) has no statistically significant differences associated with Ala allele (p = ns), too.

CONCLUSIONS

1. PCOS is a heterogeneous disorder of uncertain etiology involving a possible affected genetic land, with multiple minimum modified genes whose clinical expression by interaction with environmental factors may lead to phenotypic features of the syndrome.
2. Phenotypic appearance essentially characterized by hyperandrogenism associates in high percentage obesity and alterations in insulin sensitivity ranging from insulin resistance to type 2 diabetes. Evaluation of metabolic alterations and their long-term impact in terms of possible complications is an extremely important aspect of the syndrome and is still a challenge in terms of research. The ethnic substrate, genetic background, personal history and collateral family history and the degree of obesity should be taken into consideration as they can aggravate or even activate the trigger glucose metabolic disorders in women with PCOS.
3. Clinical judgment becomes even more important as the currently available therapeutic intervention is not able to cover all sides to balance pathological condition. More therapeutic tools, although some are clearly beneficial for certain abnormalities may act by exacerbating others.
4. The study included a total of 338 cases with PCOS pathology and 142 control cases (STUDY A); of the entire group was selected a subset of 89 patients (STUDY B) which was further investigated for another possible genetic association.
5. Patients were clinically and paraclinically investigated being monitored certain parameters: body mass index (BMI), the ratio waist / hip (WHR), the degree of insulin resistance (HOMA-IR, HOMA2-IR), free androgen index (FAI).
6. For the entire group (STUDY A) was optimized and applied a genotyping protocol for a SNP of INSR rs2252673 gene, followed by a study of its association with PCOS.
7. Subgroup of STUDY B was genotyped and investigated for association with Pro12Ala polymorphism of PPARγ gene with PCOS.
8. Impaired insulin sensitivity, although present in both groups has a higher incidence in

women with PCOS (58.75%) compared with controls (21.11%), with mean values of HOMA-IR and HOMA2-IR indexes, statistically significantly altered p <0.0001 (STUDY A). For STUDY B the results were comparable, insulin resistance results incidence being 55.3% in women with PCOS, 14.3% in the control group, with significantly higher mean values for the group case by assessing the two indices, HOMA IR and HOMA 2IR (p <0.005).

9. Patients with PCOS are overweight and most of them are obese, more than women in the control group. ABMI > 25kg / m² is present in 20% of women belonging to the control group, whereas in women with PCOS the value is higher than 61.6%, the percentages being close in central obesity 16.80%, too, respectively 62, 50%. Moreover, PCOS is associated with a statistically significant increase in body weight, obesity being more common than overweight (p <0.0001), data available for (STUDY A). Behavior is similar for patients in STUDY B where PCOS patients had higher BMI (p <0.0001), and the incidence or degree of central obesity is higher (p <0.0001).
10. General and abdominal obesity present an association relationship (<0.0001) and a positive linear correlation with insulin resistance (<0.0001) in PCOS, conclusion that applies to both studies. Patients with changes in glucose metabolism have a higher BMI (overweight, obese) and a ratio waist / hip over 0.8
11. Insulin resistance characterized by HOMA IR and HOMA 2IR shows a statistically significant positive correlation with FAI, hirsutism and menstrual disorders, and a negative one with SHBG (p <0.005). In other words, patients with impaired insulin sensitivity have a greater clinical and paraclinical hyperandrogenism, and frequently present oligomenorrhea or amenorrhea. Further on, decrease of SHBG concentration correlates with increased insulin resistance.
12. For STUDY A, by optimization of the HRM genotyping protocol we processed 480 cases, 471 samples were classified into one of the two variants identified in HRM analysis: genotype 1-wilde type homozygous and genotype 2- heterozygous (with a insignificant mutant homozygous percent less than 1%).
13. For STUDY B, the genotyping of Pro12Ala polymorphism of PPARγ gene was adapted a method that uses enzymatic digestion and DNA electrophoresis, identifying genotypes and allelic variants.
14. Statistical analysis of the association of PCOS with INSR suggests that the gene by rs 2252673 does not significantly interfere with the risk of developing PCOS in Romanian population, with no significant differences between frequency of genotypes in terms of

diagnosis. Similarly, the genotype seems not to influence insulin resistance, obesity or the degree of hyperandrogenism.

15. The results of statistical analyzes of the association study between Pro12Ala polymorphism and PCOS do not support its significant involvement in the diagnosis of the syndrome; the frequency of genotypes and alleles was proportionally balanced distributed both in PCOS group and in the controls; behavior was similar in relation to obesity, insulin resistance and hyperandrogenism.
16. Regarding the study's integration in literature we can say that the results of Pro12Ala polymorphism of the PPARγ gene association that are described in publications are contradictory; most of the published work is consistent with the results obtained in this thesis, although there are reported studies in which the association was significant.
17. INSR association through rs2252673 with PCOS was investigated in only one study until now, the study concluding that the gene is involved in the occurrence and development of PCOS.
18. The results should be interpreted as preliminary, in light of the limited statistical power of the study and open the way for a larger study with adequate power to understand the gene association study with PCOS.
19. From a theoretical perspective this study intends to open a further research on etiopathogenesis syndrome, especially on potential genetic implications.
20. From a practical standpoint, having in view the development of a new genotyping algorithm by HRM through STUDY A, we consider that we started implementing a new genotyping protocol with a shorter working time, low cost and low DNA consumption, which can show the usefulness especially for genotyping large batches of subjects. In the same spirit, the method used for genotyping Pro12Ala polymorphism of PPARγ gene, can be applied to larger groups of cases with higher statistical power.

Key words:ovary polycystic syndrome (PCOS), insulin resitance, INSR (insulin receptor gene), PPARγ (peroxisome proliferator activated receptor gamma)