Author's response to reviews

Title: Abnormal glucose tolerance and insulin resistance in polycystic ovary syndrome amongst the Taiwanese population- not correlated with insulin receptor substrate-1 Gly972Arg/Ala513Pro polymorphism

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Author's response to reviews: see over
The Answers to the Reviewer’s Reports

The 1st Reviewer’s report
Title: Abnormal glucose tolerance and insulin resistance in polycystic ovary syndrome amongst the Taiwanese population- not correlated with insulin receptor substrate-1 Gly972Arg/Ala513Pro polymorphism

Reviewer’s report:
General

重大 Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. Paper should be shortened. In the background section, the entire second paragraph should either be deleted or shortened into one or two summary sentences. The same recommendation applies to the next paragraph beginning, "Therefore, polymorphisms... This is not a review article on IRS-1 polymorphisms. You subject matter is well focused and the background should reflect this. Also, I suggest that the entire first paragraph in the "PCR and Restriction fragmented length...” section be deleted. If the reader is interested, he/she can check the Hitman reference.

Answer:
1. The second paragraph was shorten to 1/3 of the original version 368->150 words.
2. The third paragraph was shorten at the portion about IRS. Only those necessary to link the bridge between the mutation, insulin resistance, and PCOS were preserved.

Answer:
About the first paragraph in the "PCR and Restriction fragmented length.
We think that it had better been preserved because that it is too important to make mistake. If someone wants to follow the similar experiment, he had better not make mistake in his primers. We found that there was article whose primers for Codon 972 was different from others.

2. A sentence or two in the introduction explaining the genetic background of Hoklo and Hakka populations would be helpful to the non-Taiwanese readers. (Move this from the discussion.)

Answer:
Yes, we added P6 lines 13-23.

3. In the Methods section, you do not use the 1990 NIH or 2003 ASRM/ESHRE Rotterdam definition for PCOS. Since the LH/FSH ratio has been discounted as a diagnostic criterion for PCOS (because of the pulsatile secretion of gonadotropins), you will need to justify its inclusion as a diagnostic criterion. Why use a ratio of <2 rather than <3?

Answer:
We rewrite from P7 line 3 and P 14 line 11.

We plan our study before 2002, The 2003 ASRM/ESHRE Rotterdam definition of PCOS has not yet been done. Our definition is just between 1990 NIH and 2003 Rotterdam definition.: 1. chronic oligomenorrhea and/or amenorrhea must be fulfilled. 2. Two of two of the following criteria: 1) hyperandrogenism, 2) increased LH/FSH (luteinizing hormone/follicle stimulating hormone) ratio (ie >2), and 3) specific criteria for PCOS in an ultrasound scan.

Our definition is equal to the 1990 NIH definition + phenotype of “(i) oligo-ovulation (ii) increased LH/FSH >2 (iii) PCOS ultrasound scan. Therefore I replace them in the article. In our definition LH/FSH ratio > 2, which is used as criteria of PCOS before, play a role as an excluding criteria for those echographically PCO-like ovary with normal androgen level and LH/FSH<2.

Yes, the LH/FSH ratio played less role in the diagnosis of PCOS now, because of the pulsatile secretion of gonadotropins. But LH/FSH ratio can be use as excluding criteria; that is why we do not use LH/FSH ratio > 3. Those multifollicular ovaries (MFO) due to other etiology, e.g. low BMI or adolescence, will mimic the PCOS. They can be excluded in our method.

In essence, the Rotterdam 2003 expanded the NIH 1990 definition creating two new phenotypes: a) ovulatory women with polycystic ovaries and hyperandrogenism, and b) oligo-anovulatory women with polycystic ovaries, but without hyperandrogenism. Because these criteria increase the phenotypic heterogeneity of the disorder, their use will likely decrease the ability of genetic and other molecular studies to detect a common underlying abnormality with Rotterdam 2003 expanded PCOS definition. In our study
focusing on the genetic and molecular studies, we think that we had better to excluding some part of them, especially those normal ovulatory and menstruated women with polycystic ovaries and hyperandrogenism. Other article had shared the same consideration. (Ricardo Azziz, J Clin Endocrin Metab. 2006)

4. Specifically define what the "specific criteria for PCOS on ultrasound" actually was. Did you use the Rotterdam conference suggestion?
Answer:
Yes, we added P7 lines 14-17.
Explanation:
We plan our study before 2002. The 2003 ASRM/ESHRE Rotterdam definition of echographic features of PCOS has not yet been done. BUT, The echographic features of PCOS were widely uses after several reports. We majorly followed the description and definition from the Bazot’s and S. Jonard’s articles (Pathologie fonctionnelle de l’ovaire. M Bazot et al. J Radiol 2000; 81: 1801-1818; Ultrasound examination of polycystic ovaries: is it worth counting the follicles? S. Jonard Hum Reprod. 2003 Mar;18(3):598-603.). In their description: the first important criteria is to detect the hypertrophy of the ovaries: the sum of surface (ovarian area) of two ovaries > 11 cm$^2$ (5.5cm$^2$/ovary) or ovarian volume / ovary > 11cm$^3$; the second important criteria is to detect follicle number as the presence of ≥12 follicles measuring 2–9 mm in diameter (mean of both ovaries). Therefore, we use the ultrasound definition of PCO as follows: “increased ovarian area (>5.5 cm$^2$/ovary) or volume (>11 m$^3$/ovary) and/or presence of ≥12 follicles measuring 2–9 mm in diameter (mean of both ovaries)”. Our criteria of echographic features of PCOS were very similar to the 2003 Rotterdam definition of echographic features of PCOS.

5. How did you select you (total) testosterone and SHBG discriminatory values? Are these cutoff values derived from a Taiwanese population?
Yes. Those further lower total testosterone level with lower SHBG were not well-established to diagnose the PCOS in Taiwanese population. (TC Lin, SP Weng, WS Lin, TC Kuo: The effectiveness of metformin therapy in women with polycystic ovary syndrome. Taiwanese J Obstet Gynecol 2002, 41:43-53.)

6. How many subjects did you screen to come up with you study population? Can I assume that you ruled out other causes of hyperandrogenism (i.e., 21-hydroxylase deficiency) before enrolling patients into either study group?
Yes.
We added P7 lines 17-20.
“The following diseases were excluded from our study: hyperthyroidism, hypothyroidism, congenital adrenal hyperplasia (abnormal 17-hydroxyprogesterone level), pituitary insufficiency, pituitary tumor, and pre-diagnosed known diabetes mellitus.”

7. Are the plus/minus values standard deviations or standard errors?
Answer: standard deviations

8. I would move the ranges following the mean and SD/SE values to a table. I think the mean and SD or SE surface in the text.
Yes, move the ranges out.

9. The mean BMIs and ages belong in the RESULTS.
Yes, all was deleted from the materials and methods.

9. Did you exclude known diabetics and those with other untreated endocrinopathies from the study populations? Please make this distinction in the manuscript.
Answer
known diabetics (pre-diagnosed or DM revealed by simple fasting sugar) was excluded unknown diabetics was not excluded
Yes.
We added P7 lines 17-20.
10. I assume you measured "total" testosterone. Please state this fact the first time that you mention that "testosterone" was measured. Also, please note that the laboratory values are serum levels.
   Yes.
   Yes.
   We added at P7 13 line.

11. Were all serum values drawn from the fasting state? Obviously, insulin levels were drawn fasting, but please distinguish if prolactin was drawn fasting. Were all labs drawn in the early follicular phase?
   Yes, fasting state at the morning.
   Yes, in the early follicular phase

12. Please note the glucose load for the OGTT (? 75g).
   Yes.
   We added at P8 line19.

13. After looking at Table I, it is obvious that you used more than chi-square in your statistical analysis. Table I looks like either the Student's t-test for independent samples or the Mann-Whitney test was used. Chi-square is for categorical variables. Were the continuous variables studied by a statistical test for normal distribution?
   Yes, we use Student's test. We correct it.
   Yes, they are tested with chi square distribution.

14. In the RESULTS, I recommend that you compare each insulin sensitivity index using ANCOVA (assuming your data follow a normal distribution) with BMI as the independent variable and PCOS/non-PCOS as the covariables. Insulin sensitivity is directly affected by BMI, and hence, the dependent variables(insulin sensitivity indices) should be adjusted for BMI. Show results as figures in your paper. (Note: I am not a professional statistician, so you might want to review your data with a "real" statistician.)

Answer:
These were done on my previous paper. They can be expressed as below. (TC Lin, SP Weng, WS Lin, TC Kuo: The effectiveness of metformin therapy in women with polycystic ovary syndrome. *Taiwnaese J Obstet Gynecol* 2002, 41:43-53.)

We do not show them in this article because that they were repeated and we fear that they will blur our focus of this paper.


<table>
<thead>
<tr>
<th>Pearson r Significance</th>
<th>Testosterone</th>
<th>Insulin</th>
<th>Body weight</th>
<th>BMI</th>
<th>Fasting blood sugar</th>
<th>Glucose/insulin</th>
<th>LH/FSH</th>
<th>LH</th>
<th>PRL</th>
<th>Acne</th>
<th>Hirsutism</th>
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</thead>
<tbody>
<tr>
<td>Testosterone</td>
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<tr>
<td>Insulin</td>
<td>0.372*</td>
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<tr>
<td>Body weight</td>
<td>0.304*</td>
<td>0.457**</td>
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<tr>
<td>BMI</td>
<td>0.341</td>
<td>0.438*</td>
<td>0.948**</td>
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<tr>
<td>Fasting blood sugar</td>
<td>0.334</td>
<td>0.192</td>
<td>0.547**</td>
<td>0.401*</td>
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<tr>
<td>Glucose/Insulin</td>
<td>-0.140</td>
<td>-0.718**</td>
<td>-0.316</td>
<td>-0.384*</td>
<td>0.150</td>
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<tr>
<td>LH/FSH</td>
<td>0.119</td>
<td>-0.001</td>
<td>-0.100</td>
<td>-0.063</td>
<td>-0.127</td>
<td>0.026</td>
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<td></td>
<td>0.234</td>
<td>0.171</td>
<td>-0.014</td>
<td>-0.005</td>
<td>0.195</td>
<td>-0.107</td>
<td>0.587**</td>
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<td>PRL</td>
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<td>0.030</td>
<td>-0.246</td>
<td>-0.255</td>
<td>-0.239</td>
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<td>0.348</td>
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<td></td>
<td>Acne</td>
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<td></td>
<td>0.189</td>
<td>-0.015</td>
<td>-0.201</td>
<td>-0.128</td>
<td>-0.323</td>
<td>-0.101</td>
<td>-0.069</td>
<td>0.083</td>
<td>-0.090</td>
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<td></td>
<td>Hirsutism</td>
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<tr>
<td></td>
<td>0.373*</td>
<td>0.317</td>
<td>0.305*</td>
<td>0.339*</td>
<td>0.012</td>
<td>-0.234</td>
<td>-0.132</td>
<td>-0.112</td>
<td>0.121</td>
<td>0.571**</td>
<td></td>
</tr>
</tbody>
</table>

**: p<0.01; *: p<0.05

15. I feel that the percentage of subjects above and below the discretionary values is extraneous information, and I would delete it. What I believe you want to relate the reader is the comparison between the two study groups. If you want to show the linear distribution of the results, this can be done by a linear regression graph (if ANCOVA figures are used, that will accomplish the same thing.)
Answer:
We show them because that they were cut-off values of insulin resistance reported by other study group. The cut-off values of insulin resistance were controversial and not yet definitely defined. We show them with AGT to compare and demonstrate their relationship with PCOS and their prevalence in PCOS.

16. The incidence of AGT seems high in your patient population. Please address this issue in your DISCUSSION section.
Answer:
Yes.
We added (infertility/endocrine-> infertility/endocrine/obesity) at P7 line 1 and P 14 line 22.
We add P14 line 21 and P 15 line 5.
The incidence of AGT seems high in my patient population. I think that it is because my subjects have higher body weight and lower SHBG. In some articles there is similar incidence of AGT. If your PCOS group were collected from infertile cases, the incidence will be lower. We have a special clinic only for PCOS and Endocrine problems. The patients came for the reasons of infertility, irregular menstruation, and obesity. Also another reason is that I do not include those multifollicular ovaries (MFO), mimicking the PCOS, due to other etiology, e.g. low BMI or adolescence. They can be excluded in our including criteria. But they may be included in 2003 ASRM/ESHRE Rotterdam definition of PCOS.

17. On page 11, delete the sentence "We first analyzed...above." It is repetitive. The same suggestion in the next paragraph for "We further investigated...section."
Yes, they are deleted.

18. Because of your relatively small study population, do you think that any of your results could be explained by a beta-error? Please comment on this. Also, could your small population explain that absence of finding an IRS-1 mutation?
Yes, Beta-error is possible for complete absent of polymorphisms. But at least, it is rare and less frequent in the incidence of 972/513 polymorphisms. That is why we say “comparing....to 513[32]” and say “we cannot resoundingly declare a similar and universal absence of codon 972/513 polymorphisms”. We will say that it is rare and less than other population.
We rewrite from P18 line 4 to P14 line 7 (as below).

19. In the CONCLUSION, delete part of the first sentence (from "Comparing...to 513[32]. It is unnecessarily repetitive.
Yes, it is deleted and revised as below. (from P18 line 4 to P14 line 7)
Considering the statistical analysis on this scale of PCOS-affected subjects, being high risk of these polymorphisms, we cannot resoundingly declare a universal absence of codon 972/513 polymorphisms for Taiwanese PCOS women according to our study of 92 Taiwanese subjects. For this present study, however, we can conclude that no such polymorphism was found for all study subjects and that these polymorphisms
were rare and less than other ethnic population.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1. "The level of insulin resistance" is better characterized as "insulin sensitivity indices"
2. Put the formulae for the glucose/insulin ratio and HOMA-IR as footnotes in Table I.
   Answer: Yes.
3. In Table I, if the P value is <0.05, please state the precise number (i.e., <0.01, 0.0055, etc.)

The 2nd Reviewer’s report

Reviewer’s report:
General Manuscript is of interest for groups working on the same field. In a sense, this is a negative result; the polymorphisms you tried to detect, could not be found in your population. You could try PPARgamma2Pro12Ala and PGC-1alphaGly482Ser, instead.
Thank you for your kindly suggestion, we will arrange them in our trial test.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Minor revisions needed are: use of abbreviations is not precise: once you give a term in a whole text, and then give an abbreviation, stick to the abbreviation. One time the abbreviation NIDDM was given first and the whole text in parenthesis, it should be opposite. HOMA-IR was given a couple of times until the whole text for it was given, AGT was given many times after the abbreviation was defined etc. Is your definition of PCOS supported by any international consensus, if yes, give a reference.
Answer:
Yes.
HOMA-IR was given at page 10 for its first appearance.
Opposite abbreviation of NIDDM -> reversed
AGT:
Abstract' result: abnormal glucose tolerance -> AGT
Page 10 : Repeated given of AGT -> deleted
About the definition of PCOS:
Answer:
We rewrite from P7 line 3 and P 14 line 11.

We plan our study before 2002, The 2003 ASRM/ESHRE Rotterdam definition of PCOS has not yet been done. Our definition is just between 1990 NIH and 2003 Rotterdam definition.
1. chronic oligomenorrhea and/or amenorrhea must be fulfilled.
2. Two of two of the following criteria: 1) hyperandrogenism, 2) increased LH/FSH (luteinizing hormone/follicle stimulating hormone) ratio (ie >2), and 3) specific criteria for PCOS in an ultrasound scan.
    Our definition is equal to the 1990 NIH definition + phenotype of “(i) oligo-ovulation (ii) increased LH/FSH >2 (iii) PCOS ultrasound scan. Therefore I replace them in the article.
In our definition LH/FSH ratio > 2, which is used as criteria of PCOS before, play a role as an excluding criteria for those echographically PCO-like ovary with normal androgen level and LH/FSH<2,

In essence, the Rotterdam 2003 expanded the NIH 1990 definition creating two new phenotypes: a) ovulatory women with polycystic ovaries and hyperandrogenism, and b) oligo-anovulatory women with polycystic ovaries, but without hyperandrogenism. Because these criteria increase the phenotypic heterogeneity of the disorder, their use will likely decrease the ability of genetic and other molecular studies to detect a common underlying abnormality with Rotterdam 2003 expanded PCOS definition. In our study focusing on the genetic and molecular studies, we think that we had better to excluding some part of them, especially those normal ovulatory and menstruated women with polycystic ovaries and hyperandrogenism.
Other article had shared the same consideration . (Ricardo Azziz, J Clin Endocrin Metab. 2006)

Results: you give some of the results three times: characteristics in the Subjects section, in the Results section and in the Table 1. One time would be sufficient, in the results section they are not needed, Give them in the subject section and refer to Table 1. Statistics: you write that you were using x^2-test. Did you mention Student's t-test which you certainly have used. Correct term is "acanthosis nigricans". Language polishing is required: abstract 2nd line: "was", shoud be "are"; 6th line: should be "We also tried."

Answer:
1. Repeated results in Subject section and Result section-> the results in Subject section were deleted
2. Statistics: Yes, it is Student’s t-test. It is corrected.
3. Acanthosis nigricans: corrected in Table 1 and page 10
4. abstract 2nd line: "was", shoud be "are"; -> corrected
   6th line: should be "We also tried." -> corrected

Revision made by author without reviewer’s suggestion:
We also make some change in the table 1. We add the AGT row below the IGT row.

| AGT(%) | 46.8% | 6.25% | <0.05 |

Thank you for your suggestion

Regard,

Dr. Lin, Ta-Chin M.D. Ph.D.