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Abnormal serum alanine aminotransferase levels are associated with impaired insulin sensitivity in young women with polycystic ovary syndrome

G. Targher[1], E. Solagna[1], F. Tosi[1], R. Castello[1], G. Spiazzi[1], G. Zoppini[1], M. Muggeo[1], C.P. Day[2], and P. Moghetti[1]

[1]Section of Endocrinology, Department of Biomedical and Surgical Sciences, University of Verona, Verona, Italy; [2]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, England

ABSTRACT. ***Background and aim:*** **Non-alcoholic fatty liver disease (NAFLD) and polycystic ovary syndrome (PCOS) are both associated with insulin resistance. We assessed whether NAFLD is associated with impaired insulin sensitivity in PCOS women independently of age and total adiposity.** ***Subjects and methods:*** **We enrolled 14 young PCOS women with NAFLD, 14 women with PCOS alone and 14 healthy controls, who were matched for age, body mass index, and total body fat (by bio-impedance analyzer). NAFLD was diagnosed by the surrogate measure of abnormal serum alanine aminotransferase (ALT) concentrations (defined as ALT>19 U/l) after excluding other secondary causes of liver disease (alcohol, virus, and medications). Insulin sensitivity was measured by euglycemic hyperinsulinemic clamp.** ***Results:*** **Insulin sensitivity was markedly decreased ($p<0.001$) in PCOS women with abnormal ALT levels, whereas it was similar between PCOS women with normal ALT levels and matched healthy controls (8.3±2.5 *vs* 12.1±1.7 *vs* 13.2±1.8 mg/min × kg of fat-free mass, respectively). PCOS women with abnormal ALT levels also had higher plasma triglycerides and lower HDL-cholesterol concentrations than those with PCOS alone. There was a strong inverse association between serum ALT levels and insulin sensitivity in the whole group of PCOS women ($r=-0.59$, $p=0.0013$).** ***Conclusions:*** **Abnormal serum ALT levels, as surrogate measure of NAFLD, are closely associated with impaired insulin sensitivity in young PCOS women in a manner that is independent from the contribution of age and total adiposity. Early recognition of NAFLD by radiological imaging tests in this group of young patients is warranted.**
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INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common forms of anovulatory infertility, affecting up to 10% of women of reproductive age (1-3). PCOS is strongly associated with insulin resistance and other components of the metabolic syndrome. Overall, approximately 50% to 65% of all women with PCOS have insulin resistance (4-6).

Like PCOS, non-alcoholic fatty liver disease (NAFLD), comprising a spectrum of conditions ranging from simple steatosis to steatohepatitis and cirrhosis, is now regarded as the hepatic manifestation of the metabolic syndrome, and represents the most common cause of abnormal serum alanine aminotransferase (ALT) levels in Western countries, affecting up to a third of the general population (7-9).

Considering that insulin resistance is a common feature of both PCOS (4-6) and NAFLD (7-9), it is likely that both entities may coexist within the same patient. To our knowledge, however, published data on the coexistence of PCOS and NAFLD are limited to 4 small retrospective studies (10-13).

All of these studies have consistently shown that NAFLD, as diagnosed by abnormal serum liver enzymes or liver ultrasound, is frequent in women with PCOS (10-13). Indeed, approximately 30% to 50% of PCOS patients have NAFLD, and these patients are more likely to have the metabolic syndrome than PCOS patients without NAFLD (10-13). In all of these studies, insulin resistance was estimated by fasting insulin concentrations, homeostasis model assessment or quantitative insulin sensitivity check index, which are only surrogate measures of insulin resistance (14). Additionally, none of these studies assessed whether PCOS women without NAFLD differed in their values of insulin resistance compared with non-steatotic healthy women, and whether NAFLD was associated with impaired insulin sensitivity in PCOS women independently of age and total adiposity (10-13), which are two of the strongest determinants of insulin resistance (14).

It can be argued that PCOS women with highest insulin resistance are the ones who get NAFLD. However, recent evidence suggests that NAFLD, *via* the release of pro-inflammatory mediators and intra-hepatic fat *per se* might aggravate peripheral and hepatic insulin resistance, respectively (7, 9). Thus, it remains controversial whether NAFLD may contribute to insulin resistance – frequently seen in PCOS patients – independent of any effect of age and total adiposity. Resolution of this controversy may contribute to clarify the underlying mechanisms of insulin resistance in PCOS, and may be of clinical importance in planning preventive and therapeutic strategies.

Thus, the aim of this study was to compare insulin sensitivity, as measured by euglycemic hyperinsulinemic clamp, in 3 different groups of young women, who were

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Correspondence: G. Targher, MD, Section of Endocrinology, Department of Biomedical and Surgical Sciences, University of Verona, Ospedale Civile Maggiore, Piazzale Stefani 1, 37126 Verona, Italy.

E-mail: giovanni.targher@univr.it

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matched for age, body mass index (BMI), and body fat mass, i.e., women with both PCOS and NAFLD, women with PCOS alone, and healthy controls, respectively.

MATERIALS AND METHODS

In this retrospective study, we reviewed the medical records of 64 consecutive young women with PCOS, aged between 16-35 yr and with normal glucose tolerance, who were referred to our Endocrinology Clinic for menstrual abnormalities. Several of these women have been included in a previous published study (15).

The diagnosis of PCOS was based on the presence of hyperandrogenic chronic anovulation after excluding Cushing's syndrome, late-onset 21-hydroxylase deficiency, thyroid dysfunction, hyperprolactinemia or androgen-secreting tumors (15). All of these women had chronic anovulatory oligomenorrhea or amenorrhea, clinical and/or biochemical signs of hyperandrogenism, and the large majority of them had polycystic ovaries on ultrasound. All participants met both the Rotterdam criteria and the Androgen Excess Society criteria for PCOS diagnosis (16, 17). None of the participants had other known diseases or were taking any medications.

Of the 64 women with PCOS, 43 (67.2%) subjects had complete data on serum aminotransferase levels and insulin sensitivity as measured by euglycemic hyperinsulinemic clamp. Among these 43 PCOS women, we selected on a 1:1 ratio 14 young women with NAFLD and 14 women with PCOS alone, who were matched for age, BMI, and body fat mass. Fourteen healthy women, who were matched for age, BMI, and total body fat to PCOS women, were also recruited from hospital staff members and relatives. They had regular menses, normal ovarian ultrasonography, and did not show any clinical signs of hyperandrogenism.

After excluding other common causes of chronic liver disease (i.e., alcohol abuse – defined as greater than 20 g alcohol per day – history of chronic viral hepatitis B and C, hemochromatosis and current use of potentially hepato-toxic medications) (7, 9), the diagnosis of NAFLD was based on the surrogate measure of abnormal serum ALT levels defined as ALT levels >19 U/l according to the upper laboratory ranges of normality recently proposed by Prati et al. (18). Liver ultrasound or other radiological imaging tests were not available.

Venous blood was drawn in the morning (08:00-08:30 h) after an overnight fast. Serum liver enzymes, lipids, and other biochemical blood measurements were determined by standard laboratory procedures (DAX 96, Bayer Diagnostics, Milan, Italy). Reference ranges for serum aminotransferase levels, in our laboratory, were 5-40 U/l for women. A 75-g oral glucose-tolerance test was performed in all participants. No participants had impaired glucose tolerance (i.e., 2-h glucose ≥7.8 mmol/l and <11.1 mmol/l) or diabetes (i.e., fasting glucose ≥7 mmol/l or 2-hour glucose ≥11.1 mmol/l). Serum insulin, gonadotropins, and androgens (testosterone, DHEAS, androstenedione, 17-hydroxyprogesterone at baseline and after sc administration of GnRH-agonist buserelin) were measured by commercially available radioimmunoassay kits, as described elsewhere (15, 19).

BMI was calculated by dividing weight in kilograms by height in meters squared. Blood pressure was measured with a standard mercury manometer. A tetra-polar bio-impedance analyzer (BIA-103, Akern, Florence, Italy) was used to measure body electrical resistance and to derive an estimate of total body water, total body fat and fat-free mass (FFM).

Insulin sensitivity was measured by a 2-h euglycemic hyperinsulinemic clamp technique, as previously described (19). Briefly, after overnight fasting, a primed continuous insulin infusion was started (Humulin R, Eli Lilly Co., Indianapolis, IN); this was maintained for 120 min, at a constant rate of 80 mU/m^2 × min, which makes it possible to reach steady-state plasma insulin concentrations in the high *in vivo* range. Euglycemia was maintained throughout the test duration with a variable infusion of 20% dextrose, adjusted by monitoring plasma glucose concentrations in arterialised venous blood, approximately every 10 min. We have previously shown that in non-diabetic hyperandrogenic women the endogenous glucose production is negligible at this insulin infusion rate (19). Therefore, the amount of glucose infused into each subject, namely the M-value, can be considered equivalent to the whole-body insulin-mediated glucose uptake. The M-value was calculated as the mean value of glucose infusion rate for each 10 min interval during the last 30 min of the glucose clamp. Because skeletal muscle is responsible for most insulin-mediated glucose metabolism, the M-value was expressed in mg/min × kg of FFM.

The local Ethics Committee approved the study. All participants provided their informed consent.

Statistical analysis

We calculated the power in our study. With our sample size, we had a statistical power in this study of 96% to detect a clinically significant difference in insulin sensitivity between PCOS patients with and without NAFLD at the alpha-level of 0.05. Data are expressed as means±SD unless otherwise indicated. Skewed variables (i.e., gonadotropins, androgens, triglycerides, insulin, and M-value) were logarithmically transformed to improve normality before analysis and then back-transformed to their natural units for presentation in tables and figures. Statistical analyses included one-way analysis of variance (for continuous variables) followed by the Fisher's protected least significant difference (PLSD) test for multiple comparisons between groups, chi-squared test (for categorical variables), univariate linear correlation and multivariate linear regression analysis. Non-parametric statistical tests (i.e., Mann-Whitney U test, Kruskal-Wallis test, and Spearman's rank correlation analysis) were also used, but because the results were identical to those obtained by parametric procedures, only the latter were presented. p-values <0.05 were considered statistically significant.

RESULTS

The clinical and biochemical characteristics of participants are shown in Table 1. Because of the study design, the participants were almost identical in terms of age, BMI, and total body fat. On average, PCOS women were young (mean age: 23 yr; range 16-33 yr; only 8 women had age >25 yr), lean (mean BMI: 23.7 kg/m^2; range: 19-33 kg/m^2; only 6 women had BMI between 26 and 30 kg/m^2 and 2 women had BMI >30 kg/m^2) and had normal serum liver enzymes [mean aspartate aminotransferase (AST): 21.2 U/l; range: 9-36 U/l, and mean ALT: 22.5 U/l; range: 9-53 U/l, respectively].

As shown in Table 1, PCOS women with serum ALT levels ≤19 U/l did not differ in any demographic, clinical, and laboratory characteristics compared to healthy controls, except for slightly higher plasma triglycerides. In contrast, PCOS women with abnormal ALT levels had sig-

Table 1 - *Demographic, clinical, and laboratory characteristics of the study population.*

	Healthy Women (no.=14)	PCOS women with ALT≤19 U/l (no.=14)	PCOS women with ALT>19 U/l (no.=14)	[1]*p*	[2]*p*
Age (yr)	26±3	24±4	23±5	ns*	ns*
Body mass index (kg/m^2)	23.3±5	23.5±3.3	24.3±3.7	ns*	ns*
Total body fat (kg)	19±5	20±5	20±6	ns*	ns*
Systolic blood pressure (mmHg)	130±3	130±10	130±18	0.86	0.90
Diastolic blood pressure (mmHg)	77±3	78±9	81±10	0.88	0.65
Fasting glucose (mmol/l)	4.9±0.4	4.6±0.5	4.9±0.5	0.69	0.45
2-h glucose (mmol/l)	4.8±0.9	5.4±0.9	6.1±0.3	0.35	0.24
Fasting insulin (mU/l)	10.7±6	10.4±4	16.4±13	0.64	0.07
Total cholesterol (mmol/l)	4.8±0.2	5.0±0.2	4.9±0.3	0.81	0.44
Triglycerides (mmol/l)	0.59±0.2	0.86±0.3	1.49±0.9	<0.05	<0.01
HDL-cholesterol (mmol/l)	-	1.48±0.3	1.25±0.3	-	<0.05
LH (IU/l)	-	10.5±7	7.6±4	-	0.24
FSH (IU/l)	-	4.9±1.0	3.9±1.2	-	0.84
Total testosterone (nmol/l)	-	3.1±0.7	2.5±0.7	-	0.78
Free testosterone (pmol/l)	-	3.7±1.4	3.5±1.2	-	0.91
Androstenedione (nmol/l)	-	10.2±3.6	12.7±4.8	-	0.34
DHEAS (mmol/l)	-	7.6±1.9	7.4±1.6	-	0.82
17-hydroxyprogesterone (nmol/l)	-	6.5±2.5	5.2±3.3	-	0.31
17-hydroxyprogesterone after GnRH-agonist stimulation (nmol/l)	-	11.1±3.4	16.1±5.7	-	<0.05
AST (U/l)	15±3	17±4	25±6	0.63	<0.001
ALT (U/l)	13±3	14±3	32±9	0.82	<0.001

Data are presented as means±SD. Differences between groups were assessed by the Fisher's protected least significant difference test (for continuous variables) and by the chi-squared test (for categorical variables). PCOS: plycystic ovary syndrome; AST: aspartate aminotransferase; ALT: alanine aminotransferase. *Matched variables. [1]*p*: values for healthy women *vs* PCOS subjects with ALT≤19 U/l; [2]*p*: values for PCOS subjects with ALT≤19 U/l *vs* PCOS subjects with ALT>19 U/l.

nificantly higher plasma triglycerides and lower HDL-cholesterol concentrations than their counterparts with normal ALT levels. The former also had a higher 17-hydroxyprogesterone response to buserelin administration, and tended to have higher fasting insulin levels, but comparable values of plasma glucose concentrations both at fasting and after oral glucose load. As expected, PCOS women with suspected NAFLD had increased serum AST and ALT levels – although the vast majority of PCOS participants had serum aminotransferase levels within the reference range (i.e., no participants had serum AST ≥40 U/l, whereas only three women had serum ALT ≥40 U/l: 46, 47 and 53 U/l, respectively). Androgens, gonadotropins, total cholesterol, and blood pressure values were not significantly different between the groups of PCOS women.

Notably, insulin sensitivity as measured by euglycemic hyperinsulinemic clamp was markedly decreased in PCOS women with abnormal ALT levels, whereas it was similar between PCOS women with normal ALT levels and matched healthy controls (Fig. 1). Almost identical results were found after adjustment for age, BMI, plasma triglycerides or 17-hydroxyprogesterone response to GnRH agonist (p=0.0021 in PCOS women with NAFLD *vs* PCOS

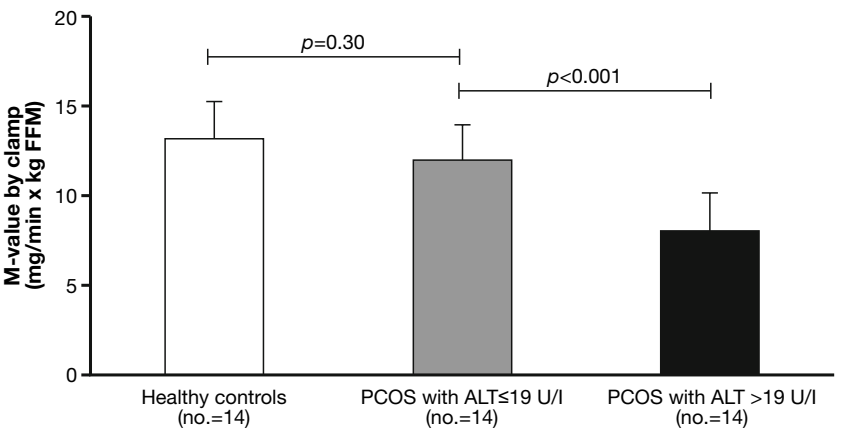

Fig. 1 - *Insulin sensitivity (M-value) as measured by euglycemic hyperinsulinemic clamp in healthy controls and in women with polycystic ovary syndrome (PCOS) grouped by serum alanine aminotransferase (ALT) levels. The 3 groups of participants were matched for age, body mass index, and total body fat. FFM: fat-free mass.*

alone). Results remained unchanged even when overweight or obese PCOS women (no.=4 for each subgroup) were excluded from analysis. Also in this case, PCOS women with abnormal ALT levels (no.=10) had greater insulin resistance than those (no.=10) with normal ALT levels (9.0±3.1 *vs* 12.8±2.3 mg/min × kg of FFM, respectively; p=0.007).

In the whole group of PCOS women, serum ALT levels were positively associated (p<0.05-0.01) with BMI (r=0.38), triglycerides (r=0.39), fasting insulin (r=0.41), and negatively with HDL-cholesterol (r=–0.57). Serum ALT levels did not significantly correlate to blood pressure, plasma glucose, gonadotropins or androgens, except for a positive correlation to serum 17-hydroxyprogesterone after buserelin administration (r=0.46, p=0.013).

Notably, as shown in Figure 2, there was a strong, inverse, association between serum ALT levels and insulin sensitivity in the whole group of PCOS women. The adjustment for age, BMI, and plasma triglycerides did not materially alter this association (standardized beta coefficient =–0.63, p=0.0015).

DISCUSSION

This study has shown, for the first time, that whole-body insulin sensitivity, as measured by euglycemic hyperinsulinemic clamp, was markedly decreased in young PCOS women with suspected NAFLD (as ascertained by the surrogate measure of abnormal serum ALT levels after excluding other secondary causes of chronic liver disease, i.e., alcohol abuse, viral hepatitis, and use of hepato-toxic medications) (7-9), whereas it was essentially superimposable between women with PCOS alone and healthy controls, who were matched for age, BMI, and body fat mass. Thus, our findings suggest that NAFLD might help to explain why some, but not all, patients with PCOS have insulin resistance. Obviously, we cannot exclude *a priori* that genetic variability or other unmeasured factors, which can co-segregate with abnormal serum ALT levels, may play a role in explaining our results.

In our study, we also found that the 17-hydroxyprogesterone response to GnRH-agonist administration was greater in PCOS women with NAFLD than in those with PCOS alone, and it was positively associated with serum ALT levels in the whole group of PCOS women. Consistent with previous data, suggesting that the increased response of 17-hydroxyprogesterone to buserelin, found in a subset of PCOS women, could be due to an excessive stimulation by insulin of the CYP17 enzyme pathway in the ovary (20), we hypothesize that our finding may likely reflect the strong inverse association of insulin sensitivity with both 17-hydroxyprogesterone response to buserelin (r=–0.57; p<0.01) and serum ALT levels (r=–0.59; p=0.0013) in these women. This hypothesis is also consistent with our previous findings that insulin infusion stimulates the CYP17 pathway in the adrenal gland (21), and that 17-hydroxyprogesterone response to buserelin clusters with the typical components of the insulin resistance syndrome (22). Thus, 17-hydroxyprogesterone response to GnRH-agonist administration and serum ALT could be two different biochemical markers of the same abnormality, i.e., the altered insulin sensitivity in PCOS women.

Four recent retrospective studies assessed the frequency of NAFLD in PCOS women by using serum liver enzymes or liver ultrasound. Schwimmer et al. found that 30% (21 of 70) of young PCOS women had suspected NAFLD as diagnosed by abnormal serum ALT levels (10). Setji et al. found that 15% (29 of 200) of PCOS women had elevated ALT levels, and that all of 6 women with persistently elevated ALT levels, who underwent liver biopsy, had a severe form of NAFLD (11). Finally, Cerda et al. (12) and Gambarin-Gelwan et al. (13) found that in PCOS women the prevalence of ultrasound-diagnosed NAFLD was 41% (17 of 41 patients) and 55% (48 of 88

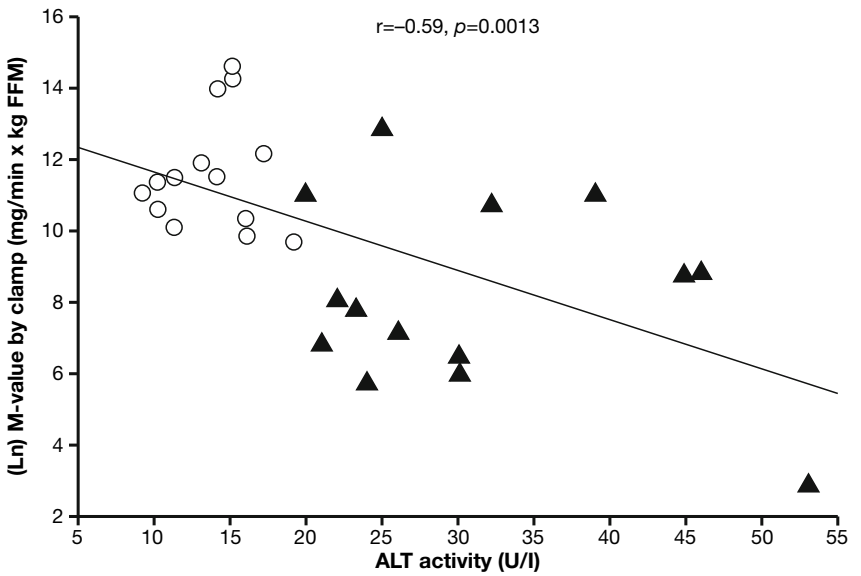

Fig. 2 - *Association between serum alanine aminotransferase (ALT) levels and insulin sensitivity (M-value) as measured by euglycemic hyperinsulinemic clamp in polycistic ovary syndrome (PCOS) women with ALT≤19 U/l (no.=14, circles) and those with ALT>19 U/l (no.=14, triangles).*

patients), respectively. Notably, in all of these studies, PCOS patients with NAFLD had higher BMI, triglycerides, and lower HDL-cholesterol concentrations than PCOS patients without NAFLD.

Overall, the evidence from these studies indicates that NAFLD is a common condition in young PCOS women, affecting up to nearly half of these patients, and that PCOS women with NAFLD are more likely to have the metabolic syndrome than their counterparts without NAFLD (10-13). This strongly suggests that PCOS women should be routinely screened for NAFLD – using radiological imaging tests – given the potentially progressive nature of the liver disease, and that an early introduction of lifestyle changes (moderate exercise and gradual weight reduction) and specific drug treatments (e.g., metformin and pioglitazone) may possibly improve NAFLD (7).

From a mechanistic perspective, PCOS women with highest insulin resistance could be the ones who get NAFLD. It is known that insulin resistance plays a pathogenetic role in the development of NAFLD (7-9). However, NAFLD, especially in its necro-inflammatory form (steatohepatitis), may aggravate whole-body insulin resistance independently of obesity (7-9). This conclusion is further supported by the strong, positive, association between liver fat content and direct measures of hepatic and peripheral insulin resistance (9, 23), and it is also corroborated by prospective studies demonstrating that raised serum liver enzymes predict new-onset Type 2 diabetes and insulin resistance independently of obesity (9, 24). In particular, there is now growing evidence suggesting that NAFLD *per se* may aggravate insulin resistance through the release of pro-inflammatory mediators, including C-reactive protein, interleukin-6 and other inflammatory cytokines (9, 25-27). The systemic release of pro-inflammatory/pro-coagulant mediators from the steatotic liver is also one of the major underlying mechanisms by which NAFLD can predict the future risk of major cardiovascular events (28-31).

The major limitations of the study derive from its retrospective and cross-sectional design. Since complete laboratory data were not available on all of the patients, the magnitude of potential relationships between insulin sensitivity and serum ALT levels may have been blunted. Moreover, the cross-sectional design of our study precludes the establishment of causal or temporal relationships between NAFLD and insulin sensitivity in PCOS patients. Prospective studies will be required to resolve these issues. Another important limitation of the study is that the diagnosis of NAFLD was based on the surrogate measure of abnormal serum ALT levels after excluding other secondary causes of chronic liver disease, but was not confirmed by radiological imaging tests or liver biopsy. It is known that none of the radiological features can distinguish between non-alcoholic steatohepatitis and other forms of NAFLD, and that only liver biopsy can assess the severity of damage and the prognosis (7-9). However, we believe that liver biopsy would have been unethical to perform in these young PCOS women since they had serum liver enzymes within the reference range (i.e., only 3 subjects had ALT≥40 U/l). Although serum ALT levels are commonly used to screen for NAFLD in clinical practice (7-9), serum ALT levels even within the reference range that is normally used in most laboratories, do not definitely exclude the presence of underlying NAFLD. For that reason, we used a very conservative cut-off of abnormal serum ALT levels (i.e., ALT>19 U/l), thus improving the sensitivity of the serum ALT measurement in diagnosing NAFLD. Indeed, in the study by Prati et al., the adoption of this lower cut-off of normality for serum ALT values had a sensitivity of 76% and a specificity of ~90% in identifying participants with liver injury as diagnosed by liver biopsy (18). Although some non-differential misclassification of NAFLD on the basis of this more conservative cut-off is likely (i.e., some of PCOS women with serum ALT ≤19 U/l could have underlying NAFLD), this limitation would serve to attenuate the magnitude of our effect measures toward null. Thus, our results can probably be considered as conservative estimates of the relationship between NAFLD and insulin sensitivity. Finally, whether these observations can also be extended to obese PCOS women remains to be determined.

Despite these limitations, this study has several strengths. First, we measured whole-body insulin sensitivity by the euglycemic hyperinsulinemic clamp, which is the gold standard for assessing insulin sensitivity (14). Second, we carefully selected a group of young PCOS women with and without suspected NAFLD, who were matched for age and total adiposity, in order to exclude the influence of two of the strongest determinants of insulin sensitivity. Third, we used standard clinical criteria and uniform laboratory methods for diagnosing PCOS. Finally, none of participants had hypertension, dyslipidemia, glucose intolerance or were taking any medications known to adversely affect both insulin sensitivity and serum liver enzymes.

In conclusion, our findings suggest that abnormal serum ALT levels, as a surrogate measure of NAFLD, are strongly associated with impaired insulin sensitivity in young PCOS women. Moreover, PCOS women with ALT levels ≤19 U/l did not differ in insulin sensitivity compared with age-, BMI-, and total body fat-matched healthy controls. Future controlled clinical trials prospectively evaluating NAFLD by radiological imaging tests in young PCOS women are needed.

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