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Plasminogen activator inhibitor activity, 4G5G polymorphism of the plasminogen activator inhibitor 1 gene, and first-trimester miscarriage in women with polycystic ovary syndrome

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Abstract

We assessed whether hypofibrinolytic plasminogen activator inhibitor 1 (PAI-1 activity) showed an independent association with first-trimester miscarriage in the 430 women with polycystic ovary syndrome (PCOS) who had previous pregnancies (from a cohort of 967 women with PCOS). Prospectively, we hypothesized that Glucophage (Bristol-Myers Squibb, Princeton, NJ) promotes successful live births in women with PCOS by lowering PAI-1 activity before conception and maintaining further reductions of PAI-1 activity during the first trimester of pregnancy. We also assessed whether PAI-1 activity levels were independently related to PAI-1 genotype and to modifiable risk factors body mass index (BMI), insulin, and triglyceride. By stepwise logistic regression, with the dependent variable being previous pregnancy outcomes at 3 levels (live birth pregnancies only [n = 208]; both ≥1 live birth and ≥1 first-trimester miscarriage [n = 111]; or first-trimester miscarriages only [n = 71]) and explanatory variables PAI-1 genotype, PAI-1 activity, insulin, homeostasis model assessment of insulin resistance, BMI, and triglyceride, PAI-1 activity was positively associated with first-trimester miscarriage ($P = .004$). For each 5 IU/mL increment in PAI-1 activity, the risk being in an adverse first-trimester miscarriage category increased (odds ratio, 1.12; 95% confidence interval, 1.04-1.20). Prospectively, from pretreatment to the last preconception visit on Glucophage, in 30 women who subsequently had live births, PAI-1 activity fell 44%, but rose 19% in 23 women with first-trimester miscarriage ($P = .03$). In the 30 women with live birth pregnancies, median PAI-1 activity fell continuously from pretreatment through the first trimester (from 16.8 to 6.7 IU/mL), whereas PAI-1 activity was either unchanged or rose in women with first-trimester miscarriage. Of the 921 women with PCOS who had 4G5G data, 718 (78%) had 4G4G-4G5G genotypes vs 87 (69%) of 126 normal female controls ($\chi^2 = 4.95$, $P = .026$). The 4G allele frequency was 53% in women with PCOS vs 46% in controls ($\chi^2 = 4.3$, $P = .04$). Of the 866 women with PCOS who had PAI-1 activity data, by stepwise regression, positive independent determinants of PAI-1 activity included BMI (partial $R^2 = 10.6\%$, $P < .0001$), insulin (partial $R^2 = 2.8\%$, $P < .0001$), triglyceride (partial $R^2 = 1.1\%$, $P = .0009$), and the 4G4G-4G5G genotype (partial $R^2 = 1\%$, $P = .0011$). The PAI-1 gene 4G polymorphism is more common in women with PCOS than in normal women and, in concert with obesity, hyperinsulinemia, and hypertriglyceridemia, contributes to treatable, hypofibrinolytic, miscarriage-promoting, high PAI-1 activity. Preconception and first-trimester decrements in PAI-1 activity on Glucophage are associated with live births, whereas increments or no change in PAI-1 activity despite Glucophage appears to be associated with first-trimester miscarriage.

1. Introduction

In previous studies with small numbers of women, women with polycystic ovary syndrome (PCOS) have high miscarriage rates, primarily in the first trimester (30%-60%) [1-6]. Miscarriage in women with PCOS has been associated with the plasminogen activator inhibitor 1 (PAI-1) 4G4G genotype [2,7,8] and with high levels of the hypofibrinolytic PAI-1 gene product, PAI-1 activity [2,3,5-7]. Women with PCOS are more likely than normal women to have PAI-1 4G4G-4G5G genotypes [3,9,10], as well as higher levels of PAI-1 activity [3,11,12]. The 4G4G polymorphism of the PAI-1 gene is also an independent risk factor for miscarriage in women with repetitive pregnancy loss [13,14]. PAI-1 activity is also an independent, treatable [6,15-24] risk factor for miscarriage in women without PCOS [25,26]. In small previous studies of women with PCOS, during pregnancy, Glucophage lowers PAI-1 activity, reduces miscarriage [1,6,27], and reduces severe pregnancy complications [28].

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The DD polymorphism of the ACE gene is associated with PAI-1 activity [29,30]. Both the ACE DD and PAI-1 4G5G mutations are common in women with recurrent pregnancy loss [13]. Women homozygous for both the ACE D and PAI-1 4G alleles may benefit by low-molecular-weight heparin treatment as early as possible in the pregnancy to prevent uteroplacental microthromboses [13,14].

Beyond associations of the 4G4G PAI-1 genotype with PAI-1 activity [11,31-33], PAI-1 activity correlates positively with body mass index (BMI) [9,34], waist-to-hip ratio [35], insulin and insulin resistance [18,35-38], and triglycerides in both men and women [38-42]. Reducing hyperinsulinemia lowers PAI-1 activity [15,18]. Recognized approaches to lower PAI-1 activity include Glucophage [6,7,16-18], weight loss [16,19], and thiazolidinediones [20-24].

Increased PAI-1 activity expression is implicated in the development of atherothrombosis [43,44].

Coordinated expression of tissue plasminogen activator (TPA) and its inhibitor, PAI-1, may play an essential role in fibrinolytic activity in the early stages of placentation and separation of the placenta from maternal tissue at term [45]. Plasminogen activators and their inhibitors (PAI-1) appear to play an important role in facilitating trophoblasts invasion of the uterus and maintenance of blood fluidity within placental intervillous spaces [46].

In the current study of 967 women with PCOS, a much bigger cohort than in previous reports [1-3,6,10,11,16-18,24,27], we assessed whether PAI-1 activity had an independent association with first-trimester miscarriage in the 430 women with previous pregnancies. We hypothesized that Glucophage promotes successful live births in women with PCOS by lowering hypofibrinolytic PAI-1 activity before conception and maintaining further reductions of PAI-1 activity during the first 2 trimesters of pregnancy. We assessed whether PAI-1 activity levels were independently related to PAI-1 genotype and to modifiable risk factors (BMI, insulin, and triglyceride).

2. Materials and methods

2.1. Cases, controls

2.1.1. Cases

In the temporal order of their referral without known selection biases, we studied 967 women with PCOS who met the 2003 European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) diagnostic consensus criteria [47] (Table 1). For diagnosis of PCOS, cases had to meet 2 of the following 3 criteria [47] after exclusion of other pathologies (pituitary insufficiency, persistent hyperprolactinemia, congenital adrenal hyperplasia, etc) (Table 1):

1. oligomenorrhea or anovulation;
2. clinical and/or biochemical signs of hyperandrogenism;
3. polycystic ovaries.

Additional exclusion criteria in our study were serum creatinine level of more than 1.5 mg/dL, type 1 diabetes mellitus, and type 2 diabetes mellitus on pharmacological therapy.

In prospective studies, we compared 23 women who have PCOS with first-trimester miscarriage with 30 women who have PCOS with successful live births, all treated with diet and Glucophage (Fig. 1).

2.1.2. Controls

For case-control comparison of PAI-1 genotypes by polymerase chain reaction methods, we studied 126 healthy

Table 1
Diagnostic characteristics of the 967 patients with PCOS at pretreatment study entry

	No. of menses in previous year					All, N = 967
	0	1-3	4-6	7-10	11-12	
	n = 401 (41%)	n = 183 (19%)	n = 169 (17%)	n = 138 (14%)	n = 76 (8%)	
Ferriman-Gallwey scores ≥7	308 (83)	146 (87)	128 (84)	115 (91)	60 (83)	757 (85)
Severe acne	201 (55)	81 (50)	81 (56)	75 (61)	35 (53)	473 (55)
Clinical hyperandrogenism (FG ≥7 and/or severe acne)	347 (87)	159 (87)	146 (86)	124 (90)	70 (92)	846 (87)
Total testosterone >70 ng/dL	86 (22)	33 (18)	24 (14)	21 (15)	9 (12)	173 (18)
Free testosterone >6.8 pg/mL	40 (11)	47 (26)	30 (18)	42 (30)	17 (22)	176 (19)
Androstenedione >270 ng/dL	88 (22)	49 (27)	38 (22)	32 (23)	11 (15)	218 (23)
DHEAS >270 μg/dL	75 (19)	32 (18)	28 (17)	29 (21)	18 (24)	182 (19)
Biochemical hyperandrogenism (≥1 high androgen)	166 (41)	97 (53)	75 (44)	70 (51)	31 (41)	439 (45)
Clinical and/or biochemical hyperandrogenism	378 (94)	175 (96)	160 (95)	133 (96)	76 (100)	922 (95)
Polycystic ovaries confirmed 2003 revised criteria, 2 of the 3:	262 (65)	111 (61)	99 (59)	80 (58)	76 (100)	628[a](65)
Oligo-anovulation; clinical and/or biochemical hyperandrogenism; polycystic ovaries confirmed[a]	401 (100)	183 (100)	169 (100)	138 (100)	76 (100)	967 (100)

Data are expressed as number (percentage). FG indicates Ferriman-Gallwey; DHEAS, sulfate salt of dehydroepiandrosterone.
[a] Pelvic ultrasound-laparotomy result was negative in 1 woman and not done in the other 338 women.

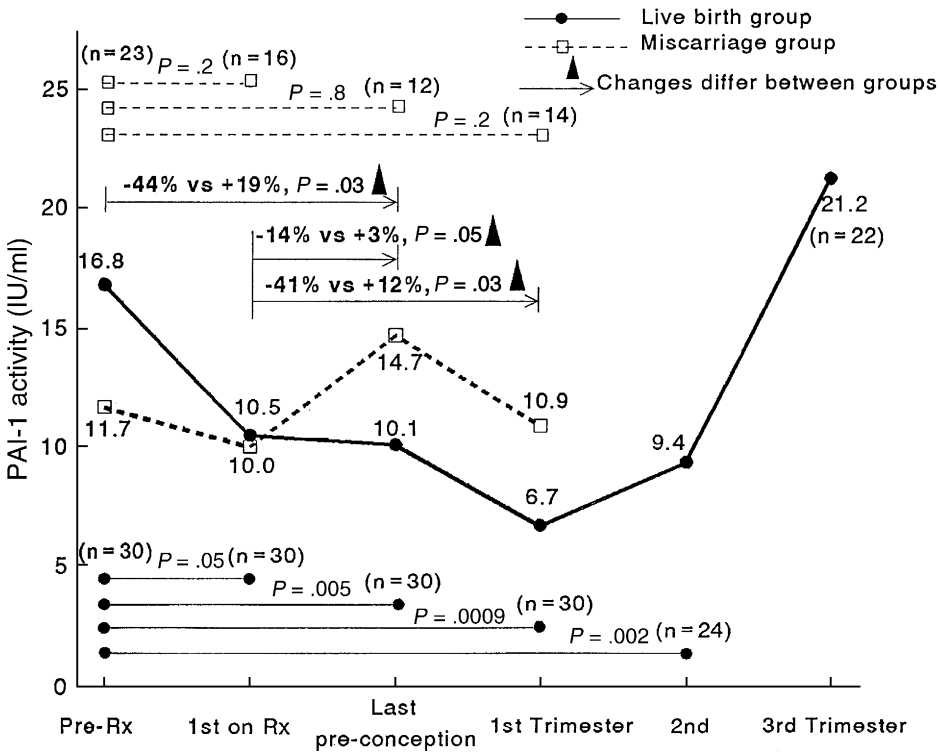

Fig. 1. Changes in plasminogen activator inhibitor activity (PAI-1 activity) from pretreatment baseline and from the first visit on metformin diet therapy to levels at the last preconception visit, and to levels during the first, second, and third trimesters of pregnancy in 30 women with PCOS having live births on Glucophage diet, and in 23 women with PCOS having first-trimester miscarriages, despite Glucophage diet. Median values are shown.

normal female controls (40 adults, 86 children) [48]. The 86 healthy girls had been sampled before same-day outpatient surgery, whereas the 40 adults included 23 healthy hospital personnel [48] and 17 healthy subjects from our outpatient clinical research center. Unlike PAI-1 activity, which is influenced by age, weight, and triglycerides [9,34-42], the PAI-1 genotype is independent of these variables [48].

2.2. Study protocol

At the baseline pretreatment visit, a detailed history was taken of the number of previous pregnancies, the number of live births, the number of spontaneous miscarriages, their trimester date, and the number of elective abortions. Information was obtained regarding known causes of miscarriage (poorly controlled diabetes, thyroid disorders, connective tissue diseases, infectious diseases, drugs, alcoholism, intrauterine anatomical abnormalities, incompetent cervix, infection, etc), but not chromosomal abnormalities, which had not been studied in most abortuses. In the current study, only women with first-trimester miscarriages, free of the above known causes of miscarriage, were evaluated.

At the baseline pretreatment visit after an overnight fast, blood was drawn for measurement of insulin, glucose, triglycerides, and PAI-1 activity, and polymerase chain reaction determination of the PAI-1 genotype, as per previously published methods [6,7,9,16]. Plasminogen activator inhibitor 1 activity was measured in plasma by a chromogenic assay (Spectrolyse, Biopool International, Ventura, CA) [48,49]; the within-run coefficient of variation was 5%, and the interassay (between-day) coefficient of variation was 8%. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated, as per previously published methods [50]. Before conception, follow-up visits were scheduled every 2 months, with monthly visits during pregnancy, with all visits at the Cholesterol Center. At each preconception follow-up visit, fasting blood was drawn for measurement of insulin, glucose, sex hormones, and, in the first 3 follow-up visits, for PAI-1 activity. During pregnancy, fasting blood was drawn for estradiol, progesterone, quantitative human chorionic gonadotropin, glucose, insulin, and PAI-1 activity at each monthly visit.

At the baseline pretreatment visit, women with BMI of less than 25 or 25 or higher were respectively instructed in diets providing 8368 (2000 cal/d) or 6276 J/d (1500 cal/d), high in protein (26% of energy [calories]), and low in carbohydrate (44% of energy [calories]). Energy restrictions were dropped after conception. The targeted Glucophage dose was 2.55 g/d, 850 mg with each meal, to be continued throughout pregnancy [27,51]. In women planning to conceive, folic acid (1 mg/d) was given preconception and throughout pregnancy.

There were no selection biases in the prospective study of 30 serially studied women with live births vs 23 with first-trimester miscarriage beyond a requirement for complete data in the live birth group at pretreatment baseline, first visit on Glucophage diet, last preconception visit, and

first-trimester visits (Fig. 1). There were no differences in the obstetrical management of the 30 women with live births and the 23 with first-trimester miscarriage. There were no differences in frequency of postconception visits or the level of surveillance. At each monthly visit, weight and seated systolic and diastolic blood pressures were measured, a brief interval history was obtained, and compliance with the Glucophage regimen reviewed. Compliance with diet instruction was monitored by serial interviews with dietitians.

All 53 women had tests of thyroid function, and monthly monitoring of fasting blood glucose, insulin, C-peptide, progesterone, estradiol, human chorionic gonadotropin, total and free testosterone, and PAI-1 activity.

2.3. Statistical methods

Comparisons of the distributions of PAI-1 genotypes between cases and controls were made by χ^2 analyses.

In the PCOS cohort, stepwise regression analysis was run with PAI-1 activity as the dependent variable and explanatory variables including age, race, BMI, triglycerides, fasting serum insulin, HOMA-IR, and PAI-1 genotype (Table 2). The PAI-1 genotype was coded 3 ways: (1) the number of 4G alleles, that is, 4G4G = 2, 4G5G = 1, 5G5G = 0; (2) 4G4G = 1, 4G5G or 5G5G = 0; (3) 4G4G or 4G5G = 1, 5G5G = 0. Of the 967 women with PCOS, complete data for all dependent and explanatory variables were available for 866 (90%) (Table 2).

In those 390 women with previous pregnancies, categorized by live births only (n = 208), 1 or more live birth, and 1 or more first-trimester miscarriage (n = 111), or first-trimester miscarriages only (no live births) (n = 71) (Fig. 2), stepwise logistic regression analyses were run with the 3 levels of pregnancy outcomes as response variable. Explanatory variables included BMI, PAI-1 activity, insulin, TG, HOMA-IR, and the PAI-1 genotype coded 3 ways (as above). In the logistic regression model, data were available for 186 (89%) of 208 women with live births only, 99 (89%) of 111 with both live birth and first-trimester miscarriage, and 65 (92%) of 71 with first-trimester miscarriage only. The distributions of PAI-1 activity in these 3 pregnancy outcome groups were shown in Fig. 2, with group comparisons by Wilcoxon tests and Spearman correlations between group and PAI-1 activity.

Table 2
Significant independent determinants of PAI-1 activity in 866 women with PCOS

Variables	Coefficient sign	Partial R^2 (%)	Model R^2 (%)	P
BMI	+	10.6	10.6	<.0001
Insulin	+	2.8	13.4	<.0001
Triglyceride	+	1.1	14.5	.0009
4G4G or 4G5G = 1, 5G5G = 0	+	1.0	15.5	.0011
Age	−	0.5	16.0	.025

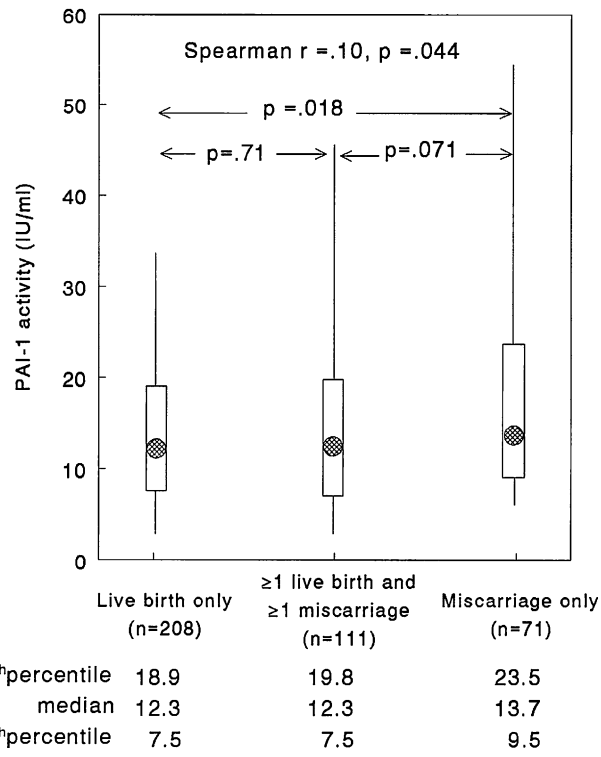

Fig. 2. Median, 25th to 75th percentile range, and 5th and 95th percentiles of PAI-1 activity in 390 women with PCOS categorized into 3 groups: live birth only (n = 208), 1 or more live birth and 1 or more first-trimester miscarriage (n = 111), and first-trimester miscarriage only (n = 71).

The distributions of the 4G4G, 4G5G, and 5G5G genotypes in PCOS and control females were tested for Hardy-Weinberg equilibrium using χ^2 test vs the expected distribution pattern ($p^2 + 2pq + q^2$).

The changes in PAI-1 activity from pretreatment baseline, and from the first visit on therapy to the last levels of preconception, to levels during the first and second trimesters were tested using paired Wilcoxon tests (Fig. 1). Median PAI-1 activity values are displayed for the first, second, and third trimesters in Fig. 1, using all available values within the respective trimesters.

To compare the changes in PAI-1 activity on Glucophage between live birth and miscarriage groups (Fig. 1), Wilcoxon tests were used.

All statistical analyses were done using SAS for Windows 9.1.3 (SAS Institute, Cary, NC).

3. Results

3.1. Universe of cases with PCOS

Over a 9.5-year period (July 1995–January 2005), of 1175 women consecutively referred for diagnosis and therapy for PCOS, 967 met the 2003 ESHRE/ASRM criteria [47] for diagnosis of PCOS (Table 1). Of the 967 women, 92% were oligomenorrheic, 87% had clinical hyperandrogenism, 45% biochemical hyperandrogenism,

and 95% clinical and/or biochemical hyperandrogenism (Table 1).

3.2. Plasminogen activator inhibitor 1 genotypes

Of the 921 women with PCOS who had 4G5G data, 718 (78%) had 4G4G-4G5G genotypes vs 87 (69%) of 126 normal female controls ($\chi^2 = 4.95$, $P = .026$). The 4G allele frequency was 53% in women with PCOS vs 46% in controls ($\chi^2 = 4.30$, $P = .038$). The PAI-1 genotype distributions in both women with PCOS and normal female controls were in Hardy-Weinberg equilibrium.

3.3. Determinants of PAI-1 activity in women with PCOS

By stepwise regression, positive independent determinants of PAI-1 activity (Table 2) included BMI (partial $R^2 = 10.6\%$, $P < .0001$), serum insulin (partial $R^2 = 2.8\%$, $P < .0001$), triglyceride (partial $R^2 = 1.1\%$ $P = .0009$), and the 4G4G-4G5G genotype (partial $R^2 = 1\%$, $P = .0011$); PAI-1 activity was inversely associated with age (partial $R^2 = 0.5\%$, $P = .025$).

3.4. Correlates of first-trimester miscarriage

Women with first-trimester miscarriage only had higher median PAI-1 activity than women with live births only (13.7 vs 12.3 IU/mL, $P = .012$), and the distribution of PAI-1 activity was shifted toward higher values (Fig. 2). Women with 1 or more live birth and 1 or more first-trimester miscarriage had intermediate PAI-1 activity levels (Fig. 2). There was a correlation between group and PAI-1 activity levels ($r = 0.10$, $P = .044$), with higher levels in women with first-trimester miscarriages only (Fig. 2).

Median PAI-1 activity did not differ between women miscarrying before 10 weeks (13.3 IU/mL) and those miscarrying 10 or more but less than 14 weeks (12.8 IU/mL) later in the first trimester ($P = .57$).

By stepwise logistic regression with the dependent variable being 3 levels of previous pregnancy outcomes (live birth pregnancies only [n = 208], both ≥ 1 live birth and ≥ 1 first-trimester miscarriage [n = 111], and first-trimester miscarriages only [n = 71]) and explanatory variables including PAI-1 genotype, PAI-1 activity, insulin, HOMA-IR, BMI, and triglyceride, PAI-1 activity was positively associated with miscarriage ($P = .004$). For each 5 IU/mL increment in PAI-1 activity, the risk being in an adverse first-trimester miscarriage category increased (odds ratio, 1.12; 95% confidence interval, 1.04-1.20).

3.5. Changes in PAI-1 activity on Glucophage diet from pretreatment baseline through pregnancy: relationship to first-trimester miscarriage

On Glucophage, 158 women with PCOS conceived, and as of March 28, 2005, 131 had live births and 23 (15%) had first-trimester miscarriage. In 30 of the 131 women having live births, complete PAI-1 activity measures were available at pretreatment baseline, at the first visit on Glucophage diet, at the last preconception visit, and during the first-trimester visits (Fig. 1).

There were no differences between the 30 women with live births and the 23 with first-trimester miscarriage in obstetrical management, PCOS management, or type of surveillance.

Comparing the women with live births with those having miscarriage in Fig. 1, at initial pretreatment, we found that age (median, 30 vs 31; $P = .23$), BMI (33.8 vs 34.8, $P = .72$), and race (93% vs 100% white, Fisher $P = 1.0$) did not differ. There were no differences ($P > .05$) between the miscarriage and live birth groups at pretreatment baseline in PAI-1 activity, insulin, and HOMA-IR. The duration from the initial pretreatment visit to conception was longer in women with live births (14.6 vs 7.4 months, $P = .0004$). On Glucophage, fasting serum insulin decreased more from baseline to the first-trimester in women in the live birth group (50% vs 19%, $P = .042$).

In the 30 women with live births, median PAI-1 activity fell 44% ($P = .005$) from pretreatment baseline to the last prepregnancy measure vs an increment of 19% ($P = .8$) in the 23 women with first-trimester miscarriage (12 pairs available). These changes in PAI-1 activity differed between groups ($P = .03$; Fig. 1). For the 30 women with live births, at the first follow-up visit on Glucophage diet (2 months after baseline), at their last preconception visit, and during the first trimester, PAI-1 activity was lower on treatment than at pretreatment baseline ($P \leq .05$) (Fig. 1). In contrast, over the same time frame, in the 23 women of the miscarriage group, there were no significant decrements ($P \geq .2$) in PAI-1 activity on treatment. From the levels of PAI-1 activity at the first treatment visit to the end of the first trimester, PAI-1 activity fell 41% in the 30 women with live birth pregnancies, but rose 12% in the women with miscarriage (9 pairs available) ($P = .03$; Fig. 1).

4. Discussion

In the current study, the 4G allele of the PAI-1 gene was more common in women with PCOS than in normal women in agreement with Diamanti-Kandarakis et al [10] and contributed to hypofibrinolytic miscarriage-promoting PAI-1 activity levels in concert with obesity, hyperinsulinemia, and hypertriglyceridemia. In much smaller cohorts of women with PCOS, we have previously shown that the 4G4G PAI-1 mutation [8] was an independent determinant of serious pregnancy complications and that PAI-1 activity was an independent determinant of miscarriage [2,3,6,7] and recurrent pregnancy loss [11]. Similarly, Gris et al [25,26] have reported that PAI-1 activity is associated with recurrent pregnancy loss in cohorts not identified as having PCOS. Antiphospholipid antibodies, known to be associated with recurrent pregnancy loss, may exert some of their effect through increasing PAI-1 activity [52].

The current study revealed that PAI-1 activity was positively associated with first-trimester miscarriage in

women with PCOS ($P = .004$) and also showed that there were several modifiable positive independent determinants of PAI-1 activity, including BMI (partial $R^2 = 10.6\%$, $P < .0001$), serum insulin (partial $R^2 = 2.8\%$, $P < .0001$), and triglyceride (partial $R^2 = 1.1\%$, $P = .0009$).

We hypothesize that previously reported significant reductions of first-trimester miscarriage in women with PCOS on Glucophage [1,6] are mediated through Glucophage diet's reduction of PAI-1 activity [6,16-18,51], with concurrent reductions in the major determinants of PAI-1 activity, BMI, insulin, and triglycerides [5,16-18,51]. In the current study, in the 30 women who subsequently had live births, median PAI-1 activity fell continuously from pretreatment through the first trimester (from 16.8 to 6.7 U/mL), whereas PAI-1 activity was either unchanged or rose in the 23 women who sustained first-trimester miscarriage. Failure of PAI-1 activity to fall on Glucophage diet appears to be associated with first-trimester miscarriage in women with PCOS and should, we think, redouble efforts to lower PAI-1 activity with better attention to diet and, where possible, increased doses of Glucophage in an attempt to forestall miscarriage.

During normal pregnancy, maternal hemostasis alters to protect against bleeding with a rise in plasma clotting factor levels and increased inhibition of fibrinolysis [53]. Thus, a hypofibrinolytic and hypercoagulable state may be established in the placenta during pregnancy [53]. However, little infarction is present in the normal placenta, with the placenta maintaining fibrinolytic activity despite the hypercoagulable state [54]. Suppression of fibrinolytic activity plays an important role in the prevention of hemorrhage during pregnancy and labor [53,54]. Tissue plasminogen activator and its inhibitor, PAI-1 activity, may also play a key role in fibrinolytic activity in the early stages of placentation [45]. Plasminogen activators and their inhibitors (PAI-1) appear to play an important role in maintenance of blood fluidity within placental intervillous spaces [46]. We hypothesize that the association of PAI-1 activity with miscarriage in women with PCOS, as in the current and previous [6] studies, and in women with recurrent pregnancy loss [25,26], reflects an imbalance [46] between TPA and PAI-1 activity. We speculate that the TPA–PAI-1 activity balance can be restored by Glucophage's lowering of PAI-1 activity [6,7,16-18,51], probably accounting for Glucophage-associated diminution of miscarriage [1,6] and reduction in complications of pregnancy [28] in women with PCOS. In the current study, in women with PCOS, on Glucophage, major reductions in PAI-1 activity from pretreatment through conception and into the second trimester were associated with live births, whereas women whose PAI-1 activity did not fall on Glucophage appear to be more likely to miscarry. Speculatively, failure to reduce PAI-1 activity during pregnancy in women with PCOS tips the fibrinolysis-antifibrinolysis balance [53] toward thrombosis, leading to placental insufficiency.

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This study was carried out following a protocol approved by the Jewish Hospital IRB with signed informed consent.

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