Polycystic Ovary Syndrome, the G1691A Factor V Leiden Mutation, and Plasminogen Activator Inhibitor Activity: Associations With Recurrent Pregnancy Loss

Charles J. Glueck, Ping Wang, Seref Bornovali, Naila Goldenberg, and Luann Sieve

Our specific aim was to assess associations of thrombophilia, hypofibrinolysis, and polycystic ovary syndrome (PCOS) with recurrent pregnancy loss (RPL) (≥3 consecutive pregnancy losses < 20 weeks gestation). Prospective studies were performed in 33 Caucasian women referred for diagnosis and treatment of PCOS who were subsequently found to have RPL and in 16 Caucasian women referred for diagnosis and treatment of RPL, who did not have PCOS. Cases (PCOS-RPL, RPL without PCOS) were compared with controls (116 healthy Caucasian females) for the G1691A Factor V Leiden, G20210A prothrombin, C677T methylenetetrahydrofolate reductase (MTHFR), plasminogen activator inhibitor 4G/5G, and platelet glycoprotein PL A1A2 gene mutations. Cases were compared with controls (44 healthy adult Caucasian females) for serologic coagulation tests including homocysteine, proteins C, S, free S, antithrombin III, anticardiolipin antibodies IgG and IgM, dilute Russel's viper venom time, activated partial thromboplastin time, Factor VIII, Factor XI, lipoprotein (Lp)(a), and plasminogen activator inhibitor activity (PAI-Fx). The 33 Caucasian women with PCOS subsequently found to have RPL were 10% of a cohort of 322 Caucasian women who had ≥ 1 previous pregnancy and had been referred for diagnosis and therapy of PCOS over a 4.3-year period. The Factor V Leiden G1691 mutation was present in 6 of 33 women (18%) with PCOS-RPL and in 3 of 16 women with RPL without PCOS (19%) versus 2 of 116 (1.7%) female controls, Fisher's P (p_f) = .0016, p_f = .013. The 33 PCOS-RPL cases also differed from the 44 female controls for high PAI-Fx (>21.1 U/mL), 38% versus 8%, p_f = . 004. The thrombophilic G1691A Factor V Leiden mutation is associated with RPL in women with and without PCOS; hypofibrinolysis (high PAI-Fx) is also associated with RPL in women with PCOS.

RECURRENT PREGNANCY loss (RPL) has traditionally been defined by 3 or more consecutive pregnancy losses before 20 weeks gestation.[1] RPL has been estimated to occur in approximately 0.3%[2] to 1%[1] of all couples. Multiple potential etiologies for RPL have been described[1-6] including antiphospholipid antibody syndrome, thrombophilia,[7-15] parental karyotype abnormalities, uterine malformations, cervical incompetence, poorly controlled diabetes mellitus, hypothyroidism, and antithyroid antibodies. In most,[7-15] but not all,[16-22] studies, the thrombophilic G1691A Factor V Leiden mutation has been identified as an etiology for both RPL and for second and third trimester pregnancy complications.[19,23-28] Fetal carriers of the Factor V Leiden mutation are prone to miscarriage and placental infarction.[29] Preconception identification of maternal Factor V Leiden heterozygosity predicts increased fetal loss.[6,30]

The presence of the maternal G1691A Factor V Leiden mutation may be a double-edged sword.[22] Dilley et al[22] postulated that the Factor V Leiden mutation may protect against bleeding in early pregnancy. However, 2 thrombophilic mutations, G1691A Factor V Leiden and the G20210A prothrombin gene, have been implicated in very early pregnancy loss.[15] Acquired activated protein C resistance, independent of the Factor V Leiden mutation, is also a risk factor for RPL.[31] The G20210A prothrombin gene mutation has been associated with both RPL and second and third trimester pregnancy complications in most,[12,19,25,26,32] but not all,[33] studies. The thrombophilic C677T mutation of the methylenetetrahydrofolate reductase gene (MTHFR) has also been associated with RPL and second and third trimester pregnancy complications in most,[10,25,26,34,35] but not all,[12] studies. The thrombophilic antiphospholipid antibody syndrome has been associated with RPL.[36-38] Familial and acquired hypofibrinolytic disorders have also been implicated as etiologies for RPL including the 4G/5G mutation of the plasminogen activator inhibitor gene[25,39] and its gene product, plasminogen activator inhibitor activity (PAI-Fx).[40-43]

Women with polycystic ovary syndrome (PCOS) have a high frequency of first trimester spontaneous abortion (SAB),[42-48] ranging from 73%[43] to 62%,[44] 42%,[46] 35%,[47] and 25%[48] of pregnancies. Metformin lowers the rate of first trimester SAB in PCOS.[43-46] In the largest PCOS-pregnancy study to date (72 women, 84 fetuses),[44] metformin during pregnancy safely reduced first trimester SAB from 62% to 26%, $P < .0001$. On metformin, reductions in serum insulin and PAI-Fx, an independent significant determinant of SAB,[42] are correlated.[43] To date, however, no placebo-controlled, blinded trials of metformin in prevention of SAB in PCOS have been published.

Hyperinsulinemia is an independent, significant risk factor for RPL in PCOS.[44] In 72 women with PCOS, pretreatment fasting serum insulin was a significant explanatory variable for total (previous and current) first trimester SAB, odds ratio 1.32 (for each 5 μU/mL increase in insulin), 95% confidence interval (CI) 1.09 to 1.60, $P = .0005$.[44] Craig et al[49] have reported that women with RPL have a significantly increased prevalence of insulin resistance when compared with matched fertile controls. They speculated[49] that the insulin-RPL association was mediated[42] through hypofibrinolytic high PAI-Fx, an independent determinant of SAB.

From the Cholesterol Center, Jewish Hospital, Cincinnati, OH.
Submitted March 27, 2003; accepted June 24, 2003.
Address reprint requests to Charles J. Glueck, MD, Cholesterol Center, Jewish Hospital, 3200 Burnet Ave, Cincinnati OH, 45229.

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Table 1. Characteristics of Women With RPL and PCOS, RPL Without PCOS, Adult Female Controls for Serologic Tests, and Female Controls for PCR Tests

	Group I RPL With PCOS	Group II RPL Without PCOS	Group III Serologic Controls (adult females)	Group IV PCR Controls (44 adult females, 72 female children)	Group Comparisons
No.	33	16	44	116	
Age (mean ± SD)	37.2 ± 8.1	38.9 ± 10.9	54.2 ± 18.6		I *v* III, $P = .0002$; II *v* III, $P = .011$
BMI (kg/m^2) (mean ± SD)	36.8 ± 9.1	34.9 ± 11.9	27.1 ± 5.1		I *v* III, $P = .0002$; II *v* III, NS
	Median 35.7	Median 32.8	Median 26.9		
Previous pregnancy loss	145/187 (78%)	73/96 (76%)			
PAI-Fx >21.1 U/mL	12/32 (38%)	1/16 (6%)	3/37 (8%)		I *v* III, $p_f = .004$; II *v* III, NS
G1691A V Leiden heterozygosity	6 (18.2%)	3 (19%)		2 (1.7%)	I *v* IV, $p_f = .0016$; II *v* IV, $p_f = .013$

Abbreviations: PAI-Fx, plasminogen activator inhibitor activity; p_f, Fisher's *P*; NS, not significant.

PAI-Fx rises with increasing levels of serum insulin and decreases when insulin is reduced by metformin.[42,43,50,51]

In women with recurrent miscarriage, screening reveals a higher than normal incidence of polycystic ovaries.[52] Hence, a high level of fetal loss is characteristic not only of women with RPL with thrombophilia,[7-15,19,23-28,31,32,34-39] and/or hypofibrinolysis,[25,39-43] but also of women with PCOS.[42-48]

Blumenfeld and Brenner[53] have proposed that placental thrombosis may be the final common pathophysiologic pathway for RPL. Prophylactic therapy with low molecular weight heparin[54-59] or unfractionated heparin[60] in women with heritable and acquired thrombophilia reduces pregnancy wastage compared with their previous pregnancies without thromboprophylaxis. To date, however, no placebo-controlled, blinded trials of low molecular weight heparin in prevention of RPL have been published. The only controlled clinical trials involving heparin and RPL have been performed in women with antiphospholipid antibody syndrome.[17,61]

Because thrombophilia, hypofibrinolysis, and PCOS are all associated with RPL, our specific aim was to evaluate their associations with RPL in 33 Caucasian women referred for diagnosis and treatment of PCOS, who were subsequently found to have a history of RPL, and in 16 Caucasian women referred for diagnosis and treatment of RPL, who did not have PCOS.

MATERIALS AND METHODS

Study Design and Subjects

This study followed a protocol approved by the Jewish Hospital Institutional Review Board, with written informed consent. The current report was a prospective consecutive case series of 33 women referred for the diagnosis and therapy of PCOS, and subsequently found to have a history of RPL, and 16 women referred for the diagnosis and therapy of RPL, without known PCOS, all free of exclusionary criteria, summarized below (Table 1). The 33 Caucasian women with PCOS came from a cohort of 322 Caucasian women who had one or more pregnancies and who were consecutively referred from January 16, 1998 to May 20, 2002 for a study of efficacy and safety of metformin therapy in PCOS.

To enter the current study, women had to be free of anatomic etiologies for RPL as determined by their referring obstetricians-gynecologists including: uterine structural abnormalities (assessed by sonogram, hysterosalpingogram) and cervical incompetence. Further, any women with a history of poorly controlled diabetes, hypothyroidism, and previously known antiphospholipid antibody syndrome[17,36-38,55,61] were excluded.

No exclusions were made for parental karyotyping abnormalities,[1,2] most of which had never been analyzed after previous pregnancy losses, and which are estimated to occur in <5% of women with RPL.[60]

All coagulation measures were made in the nonpregnant state in both cases and controls, with the women not taking hormones, corticosteroids, or anticoagulants that could affect serologic measures of coagulation. Fasting blood was obtained for polymerase chain reaction (PCR) and serologic measures of coagulation in the early morning with subjects in the seated position, using previously published methodologies.[25,26,39,42,62-68]

Clothed weight (without shoes or over-clothes) and height were measured at study entry. Reproductive history, diagnosis of previous gestational diabetes, and menstrual status in the previous year were recorded. At entry, after an overnight fast, in the women with PCOS, systolic and diastolic blood pressure, glucose, hemoglobin A_{1C}, blood urea nitrogen, creatinine, lipid profile, endocrine status (estradiol, progesterone, luteinizing hormone [LH], follicle-stimulating hormone [FSH], androstenedione, dehydroepiandrosterone sulfate [DHEAS], testosterone, sex hormone-binding globulin), serum insulin, and lactic acid were measured, as previously described.[43-45] At entry, 17-OH progesterone, prolactin, thyroxine (T_4), thyrotropin (TSH), and cortisol were measured to rule out diseases that can mimic PCOS or affect its presentation.[43-45]

Diagnosis of PCOS

The diagnosis of PCOS[43-46] was based on 1990 National Institutes of Health criteria,[69] which includes oligo-amenorrhea, biochemical or clinical evidence of hyperandrogenism, and exclusion of other disorders, which could mimic PCOS including virilizing tumors, Cushing's syndrome, adult onset congenital adrenal hyperplasia, and persistent prolactinemia.[43-45,50,51] We defined oligomenorrhea by ≤ 9 menses per year, amenorrhea by no menstrual periods for 1 year. Clinical hyperandrogenism was defined by hirsutism using the original (11 body areas measured) Ferriman-Gallwey[70] Score ≥ 7, and/or severe persistent acne. Biochemical hyperandrogenism was defined by elevations of total or free testosterone, androstenedione, or DHEAS.[43-45] Polycystic ovaries were identified anatomically by the presence of ≥ 10 subcapsular follicles 2 to 8 mm in diameter by pelvic ultrasound or laparoscopy.[50,51] The presence of acanthosis nigricans was recorded, but was not among the diagnostic criteria required for study entry.

Homeostasis model assessment for insulin resistance (HOMA IR)[71] and hyperinsulinemia[43-45,50,51] were not required criteria for

entry into the study. Three women with well-controlled type 2 diabetes, already receiving metformin at study entry, participated in the study. Comparable to our previous reports,[43-45] exclusion criteria included serum creatinine >1.5 mg/dL, other virilizing endocrinopathies (as above), pituitary insufficiency, or type 1 diabetes. Women taking valproic acid (Depakote, Abbott Laboratories, Chicago, IL), androgens, estrogen-progestins, estrogen-androgens, or drugs known to effect endogenous sex hormones or lipoprotein metabolism in the 2 months preceding the study were also excluded.

Normal Controls

For comparisons of mutant or polymorphic genes that have been associated with thrombophilia or hypofibrinolysis, we used 116 Caucasian females (21 healthy adult hospital personnel, 23 healthy adults, and 72 healthy female children[65] sampled before same day orthopedic surgery), Table 1. There were no differences ($p_f \geq 0.1$) between the 72 female children and the 44 adult females for any of the thrombophilic or hypofibrinolytic gene mutations.

None of the 44 adult females had experienced RPL. Most of the normal female controls were studied contemporaneously with the cases.

For comparisons of non-PCR measures of coagulation, we used the 44 Caucasian adult women (as above) who were not pregnant and not taking estrogens or oral contraceptives at the time of blood sampling (Table 1). None of the controls were taking drugs that might affect serologic coagulation measures. We were unable to match body mass index (BMI) in the controls to women with PCOS, later found to have RPL, because of obesity in 45% and severe obesity in 30% of the women with PCOS.

Fifty-four normal Caucasian female controls not receiving oral contraceptives or hormone replacement therapy were used for comparison of activated partial thromboplastin time (APTT) and dilute Russel's viper venom time (DRVVT).

Blood Sampling and Plasma Preparation

Fasting blood was drawn from 8:30 to 10 AM from seated patients. Blood was collected in 3.2% buffered sodium citrate. The samples were immediately centrifuged at 2,600 × *g* for 15 minutes to obtain platelet-poor plasma. Blood for PCR analysis was drawn in EDTA and the DNA extracted for subsequent analysis.

Coagulation Assays

PCR assays for 5 gene mutations were performed as previously described.[25, 26,62-65,67,68] The mutations studied included the G1691A Leiden mutation in the Factor V gene, G20210A mutation of the prothrombin gene, C677T mutation of the MTHFR gene, 4G/5G polymorphism of the PAI-1 gene promoter, and the platelet glycoprotein IIb/IIIa mutation (PL A1/A2, thymine to cytosine substitution at position 196).

The non-PCR coagulation tests in plasma and serum were performed following previously published methodology.[66-68] The following tests were performed in plasma: DRVVT, APTT, Factor VIII, PAI-Fx, protein C antigenic, protein S total (antigenic), protein S free (antigenic), and antithrombin III (functional). The following tests were performed in serum: anticardiolipin antibodies, homocysteine, and lipoprotein (Lp)(a).

Statistical Methods

Comparisons between patients and controls for numerical measures were made by Wilcoxon tests. Categorical comparisons were made using χ^2 analyses or Fisher's exact tests.[72] Power and sample size calculations were performed as per Zelterman.[73] These analyses calculated the chance (power) to declare the case-control difference at the $P < .05$ level, for a given sample size, under the assumption that true differences existed between cases and controls, as observed from our data.

RESULTS

Women With PCOS and RPL

The 33 Caucasian women with PCOS, subsequently found to have RPL, came from a larger cohort of 322 white women with PCOS with ≥1 previous pregnancy referred from January 16, 1998 to May 20, 2002 for an assessment of efficacy and safety of metformin therapy. Thus, 10% of our cohort of women, referred only for diagnosis and treatment of PCOS, were found to have RPL.

Mean ± SD age in the cohort of 33 women with PCOS and RPL was 37 ± 8; range, 25 to 61 years (Table 1). They were obese, with median weight 95.5 kg and median BMI 35.7 (Table 1). Of the 33 women, only 2 (6%) had normal BMI (≤25), 6 (18%) had BMI >25 but <30 (overweight), 15 (45%) had BMI ≥30 but <40 (obese), and 10 (30%) had BMI ≥40 (extremely obese).[74]

Of the 33 women with PCOS and RPL, at pretreatment baseline, 16 (48%) were amenorrheic, and 16 (48%) were oligomenorrheic: 7 women had 1 to 3 menses/year, 5 had 4 to 6, 4 had 7 to 9, only 1 had ≥10 menses/year. All 33 women had documented clinical and/or biochemical hyperandrogenism. The Ferriman-Gallwey score[70] was ≥7 in 29 (97% of 30 women measured). Thirteen women (41%) had severe clinical acne, and 10 (30%) had ≥1 elevated serum androgen level. Median fasting serum insulin was 18 μU/mL, interquartile range 14 to 28 μU/mL; 17 (52%) of the women had fasting hyperinsulinemia (insulin ≥17 μU/mL). Insulin resistance (≥ the 90th percentile for 161 healthy normal female controls [≥5.54]) was present in 12 (36%) of the women. Beta-cell function (insulin secretion) was high (≥ 361, the 90th percentile for 161 healthy normal female controls) in 8 (24%) of the women.

No women had congenital adrenal hyperplasia (normal 17-OH progesterone); 1 had transient hyperprolactinemia.

The 33 women with PCOS had 187 previous pregnancies, with 42 live births (22%), Table 1. The median number of previous consecutive pregnancy losses was 3.

Of the 33 women with PCOS and RPL, 3 had well-controlled, preconception type 2 diabetes mellitus.

By selection, none of the 33 women with PCOS and RPL or the 16 with RPL without PCOS had uterine structural abnormalities or cervical incompetence, poorly controlled diabetes, hypothyroidism, or previously known antiphospholipid antibody syndrome.

Women With RPL Without PCOS

The 16 women referred because of RPL had 96 previous pregnancies, 20 live births (21%) and 73 SABs (76%), Table 1. The median number of previous consecutive pregnancy losses was 4. None had type 2 diabetes.

Controls

The 44 control women were older (54 ± 19 years) than the 33 women with PCOS and RPL (37 ± 8), $P = .0002$, or the

16 women with RPL without PCOS (39 ± 11), $P = .011$, Table 1.

Coagulation Disorders

Heterozygosity for the G1691A Factor V Leiden mutation occurred in 6 of 33 women with PCOS-RPL (18.2%) versus 2 of 116 controls (1.7%), $\chi^2 = 13.7$, $P = .0002$, Fisher's $P = .0016$, Table 1. Our data had power >95% to declare the difference between the Factor V mutation rates of cases versus controls significant at $P < .05$. The 33 women with PCOS-RPL did not differ from controls for any of the other PCR cDNA measures, $P > .05$.

Heterozygosity for the G1691A Factor V Leiden mutation occurred in 3 of 16 women with RPL without PCOS (19%) versus 2 of 116 controls (1.7%), $\chi^2 = 11.2$, $P = .0008$, Fisher's $P = .013$, Table 1. Our data had power >91% to declare the difference between the Factor V mutation rates of cases versus controls significant at $P < .05$. The 16 women with RPL without PCOS did not differ from controls for any of the other PCR cDNA measures, $P > .05$.

The 33 PCOS-RPL cases also differed from the 44 female controls for high (>21.1 U/mL) PAI-Fx, 38% versus 8%, $p_f = .004$, Table 1. They did not differ from controls for any other serologic tests of coagulation, $P > .05$.

The 16 women with RPL without PCOS did not differ from controls for any serologic tests of coagulation.

DISCUSSION

Without metformin, women with PCOS have an increased frequency of first trimester SAB, ranging from 25%[48] to 35%,[47] 39%,[43] 42%,[46] 44%,[42] 62%,[44] and 73%.[43] Within this framework of a high level of fetal loss in women with PCOS, it was not surprising that RPL had occurred in 10% of a cohort of 322 Caucasian women with PCOS who had ≥ 1 previous pregnancy, referred over a 4.3-year period for an assessment of efficacy and safety of metformin. This 10% incidence of RPL in women who were referred solely because of PCOS is much higher than estimates of 0.3%[2] to 1%[1] of all couples. We speculate that our cohort of 322 Caucasian women with PCOS who had ≥ 1 previous pregnancy is reflective of a general "PCOS universe," being reasonably similar in age and BMI to other reported PCOS cohorts[46-48,50,51] and diagnosed following widely applied National Institutes of Health critera.[69]

Because metformin sharply reduces SAB in women with PCOS,[43-46] understanding of any concurrent roles of thrombophilia, hypofibrinolysis, and PCOS in women with RPL might, speculatively, be useful in setting the groundwork for combined Enoxaparin[54-59] metformin[43-46] therapy in pregnant women with PCOS-RPL and the G1691A Factor V Leiden mutation.

In the largest PCOS-pregnancy study to date,[44] without metformin,[40] women with PCOS had 100 previous pregnancies (100 fetuses), with 34 (34%) live births and 62 (62%) first trimester SABs. In these 40 women's current pregnancies on metformin (46 pregnancies, 47 fetuses), there have been 33 live births (70%), 2 pregnancies ongoing ≥ 13 weeks (4%), and 12 SAB (26%), $P < .0001$.[44] Metformin's miscarriage-sparing effect in women with PCOS[43-46] may, in part, be mediated through its reduction of PAI-Fx,[42,43] an independent risk factor for miscarriage by virtue of inadequate thrombolysis of placental thrombi. Metformin's ability to lower PAI-Fx and to reduce SAB[43-46] is, speculatively, further mediated through reduction in fasting serum insulin[44,49] and insulin resistance, as reported in women with PCOS before pregnancy[50,51] and in women with PCOS who continue metformin through pregnancy.[43,44]

The majority,[7-15,25,26] but not all,[16-22] studies of the G1691A Leiden mutation of the Factor V gene have shown it to be more common in women with RPL than in matched controls. Thus, the current study's finding of the V Leiden mutation in 18% of women with PCOS-RPL and in 19% of women with RPL without PCOS versus 1.7% in controls is consistent with most[7-15,25,26] published studies. Our finding of an increased incidence of the V Leiden mutation in women with PCOS and RPL probably does not reflect their conjoined inheritance.[75,76] Women with PCOS are not more likely than normal women to have resistance to activated protein C.[75] Tsanadis et al[76] also did not find a significant enrichment with thrombophilia in 30 women with PCOS compared with normal controls. The Factor V Leiden mutation enrichment in PCOS-RPL probably reflects Factor V Leiden mutation's contribution to RPL[56] and to severe second and third trimester complications of pregnancy,[25,26] not a coinheritance with PCOS.[75,76]

Hypofibrinolysis, associated with the 4G/4G polymorphism of the PAI-1 gene,[25,39] has been associated with major second and third trimester pregnancy complications. High levels of PAI-Fx,[40-43] the PAI-1 gene product, have been associated with SAB. PCOS is associated with insulin induced, metformin-reversible, high PAI-Fx.[43,50,51,77] The current study's observation of higher PAI-Fx in the 33 women with PCOS-RPL, 38% versus 8% in controls, is consistent with our earlier reports.[42,43] Although no statistically significant V Leiden*PAI-1 gene mutation interaction term could be demonstrated in the current study, a G1691A Factor V Leiden*4G/4G PAI-1 gene interaction has previously been associated with major second and third trimester complications of pregnancy.[25]

Placental thrombosis may be a major final common pathway in repeated pregnancy wastage.[23,53,54] In women with heritable or acquired coagulation disorders, thromboprophylaxis with low molecular weight heparin[54,56,78] or unfractionated heparin[60] reduces pregnancy wastage versus antecedent pregnancies without heparin therapy.

We believe that RPL in women with and without PCOS should trigger assessment of thrombophilia and/or hypofibrinolysis, and consideration for thromboprophylaxis with heparin[54,56,60,78] in the presence of major gene thrombophilias[7-15,19,25,26,31,34,35] (G1691 Factor V Leiden, G20210A prothrombin, C677T MTHFR), particularly with concurrent 4G4G PAI-1 gene mutations.[25,39] In women with PCOS-RPL, although PCOS is a metformin-treatable risk factor for RPL,[42-44,46-48] the G1691A Factor V Leiden mutation should also be assessed as a concurrent, lovenox-treatable risk factor for RPL.

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