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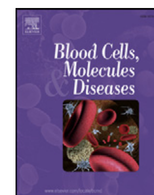

Inherited and acquired thrombophilia in Indian women experiencing unexplained recurrent pregnancy loss☆

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ABSTRACT

The most frequently hypothesized cause of unexplained recurrent pregnancy loss (RPL) refers to a defective maternal haemostatic response leading to uteroplacental thrombosis. Approximately 20% women suffering from pregnancy loss (PL) are associated with autoimmune disorders and more than 50% remain idiopathic after common traditional investigations. The present study aims to investigate the prevalence of different genetic and acquired thrombophilia markers in a large series of Indian women with RPL. Such studies will help analyze the markers which pose maximum risk and help in the appropriate treatment in subsequent pregnancies.

The study comprised of 587 women with no apparent etiological causes of RPL and 115 healthy women controls. p values were calculated with two tailed Fisher's exact test; statistical significance was assumed at $p < 0.05$, 95% confidence interval. Relative risks were also calculated.

Among genetic thrombophilia, the risk of PL was highest with protein S deficiency (16%, $p = 0.006$) followed by plasminogen activator inhibitor-1 4G/4G (23%, $p = 0.007$) polymorphism. Among acquired markers, the risk of PL was the highest in women with anti-cardiolipin antibodies (24%, $p = 0.0001$), followed by anti-annexin V antibodies (23%, $p = 0.0009$) and lupus anticoagulants (8%, $p = 0.02$).

Thrombophilia, inherited and acquired, is an important contributing factor in unexplained RPL and should be screened in the order of its prevalence.

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1. Introduction

Pregnancy loss (PL) affects up to 15% of reproducing couples and recurs in 2% to 3%. It is a common occurrence and may occur due to multiple reasons; the common causes include anatomical defects, chromosomal aberrations, endocrine factors, infections and other immunological factors. Diagnosis of recurrent pregnancy loss (RPL) takes a toll on the couple, both emotionally and financially, despite a wide range of investigations; the cause remains unknown for more than 50% of cases [1]. A defective maternal hemostatic response leading to uteroplacental thrombosis along with hypoxia has been hypothesized to subsequently lead to adverse pregnancy outcomes like PL, placental abruption, intrauterine growth restriction or death and pre eclampsia. This may include thrombosis in decidual vessels, impairment of trophoblast invasion, villitis and placental microthrombi [2]. Thrombophilia, both genetic and acquired have been described as risk factors for increasing susceptibility to adverse pregnancy outcome. Approximately 20% of RPL is associated with autoimmune disorders and more than 50% of cases remain idiopathic after common traditional investigations [3].

Hereditary thrombophilia is defined as a genetically determined increased risk of thrombosis. Interaction among the genetic defects and the acquired predisposing factors is the common cause of thrombosis. Antiphospholipid antibodies (APLAs) are a heterogeneous group of immunoglobulins which target a diversity of phospholipids, phospholipid-proteins and phospholipid binding proteins, which prevail as cell membrane components. It is an autoimmune hypercoagulable disorder which provokes inflammatory immune responses and recurrent arterial and venous thrombosis. Thrombosis of placental tissue, inhibition of syncytium-trophoblast differentiation, complement activation, placental/embryo apoptosis, directly acting on trophoblast cell surface anticoagulant are a few theories regarding the mechanism of APLA mediated adverse pregnancy events [4,5]. The three most commonly tested APLAs in clinical practice include detection of a lupus anticoagulant (LA), and anti-cardiolipin antibodies (ACLA) and anti-β_2-glycoprotein 1 (Aβ_2GP1) [6]. APLAs against other antigenic targets such as annexin V, prothrombin, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are also found to be associated with thrombosis and pregnancy complications [7]. Many studies have emphasized the significance of APLAs as potential causative factors for RPL and it is found that the prevalence in women with RPL of APLAs varies from

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4.2 to 42% [17]. This difference may be due to ethnicity, sample size, no. of different antibodies tested, and variability in laboratory testing.

There are limited reports from India on the prevalence of different inherited as well as acquired thrombophilia markers in women with RPL. Such studies will help to analyze the markers which pose the maximum risk in the Indian population and eventually help in carrying out these investigations in a more strategized manner so as to help the couples financially. The present study thus aims to investigate the prevalence of different inherited thrombophilia markers like protein C (PC), protein S (PS), antithrombin (AT) deficiency, Factor V Leiden mutation (FVL), endothelial protein C receptor (EPCR) 23 bp insertion, Methylenetetrahydrofolate reductase (MTHFR C677T) polymorphism, plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism and acquired markers like LA, ACLA, anti-β_2-glycoprotein 1 (Aβ_2GP1) and anti-annexin V (AAnnVA) antibodies in women with unexplained RPL.

2. Materials and methods

2.1. Patient & controls

643 women suffering from RPL, attending the Outpatient Department of Obstetrics and Gynecology of King Edward's Memorial Hospital, Wadia Maternity Hospital as well as other hospitals, were referred for thrombophilia work up to the Department of Haemostasis and Thrombosis at National Institute of Immunohaematology, Mumbai, between February 2009 and July 2013. After excluding 56 women, 587 women who had no apparent or presumptive etiological causes of RPL i.e. chromosomal aberrations, glucose tolerance test, fasting blood glucose test, anatomic abnormality, intrauterine adhesions, cervical incompetence, hormonal disorders, infections were included in the present study. RPL was defined as 2 or more PL, wherein the pregnancy was documented by an ultrasonography or a histopathological test occurring i) at or before the 10th week of gestation-early group ii) beyond the 10th week of gestation with or without growth retardation-late group and iii) women with both early and late PL [8]. A flow diagram explaining the patient selection is shown in Fig. 1.

The control group comprised of 115 age matched women having at least one live birth with no history of PL, concurrent, autoimmune or infectious disease. They were not on any medication and were not pregnant at the time of blood collection.

None of the patients or controls had any other serious medical comorbidities like renal disease, systemic lupus erythematosus etc.

2.2. Ethics approval

The study was approved by the Institutional Ethics Review Board i.e. “Institutional Committee for Research on Human Subjects, National Institute of Immunohaematology (ICMR)” and a written informed consent was obtained from all participants. All investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.3. Blood sampling

Blood samples were collected at least 4 months (4–24 months) after their last PL or delivery for patients and controls, respectively, as haemostatic changes noted during pregnancy normalizes after delivery within 4 to 6 weeks. Blood samples were immediately mixed gently with one tenth volume of 0.129 M sodium citrate and then centrifuged at 1500 *g* for 15 min at room temperature twice so as to obtain platelet poor plasma. Plasma was stored at −80 °C until use and whole blood was kept for DNA extraction. The LA detection was always performed on fresh plasma. 2 ml of blood was also collected in EDTA bulbs for complete blood count.

2.4. Detection of different inherited thrombophilia markers

1. *Protein C and Protein S antigen levels*: PC and free PS antigen levels in the patients' plasma were measured by enzyme linked Immunosorbent assay (ELISA) using commercial kits (Diagnostica Stago, Asnieres, France).
2. *Antithrombin levels*: AT levels were detected by the chromogenic substrate method in fully automated coagulometer using commercial kits (Diagnostica Stago, Asnieres, France).

The laboratory normal range for PC, PS and AT levels is 70–140%.

3. *Factor V Leiden mutation*: FVL was done by direct DNA sequencing.
4. *MTHFR C677T mutation*: This was done by PCR using specific primers followed by digestion with restriction enzyme *HinfI* and visualization in a 10% polyacrylamide gel [9]
5. *EPCR 23 bp insertion mutation*: This was done by PCR using specific primers followed by direct analysis in a 10% PAGE [10].
6. *PAI 1 4G/4GPolymorphism*: This was detected by allele specific PCR followed by analysis in a 2% agarose gel [11].

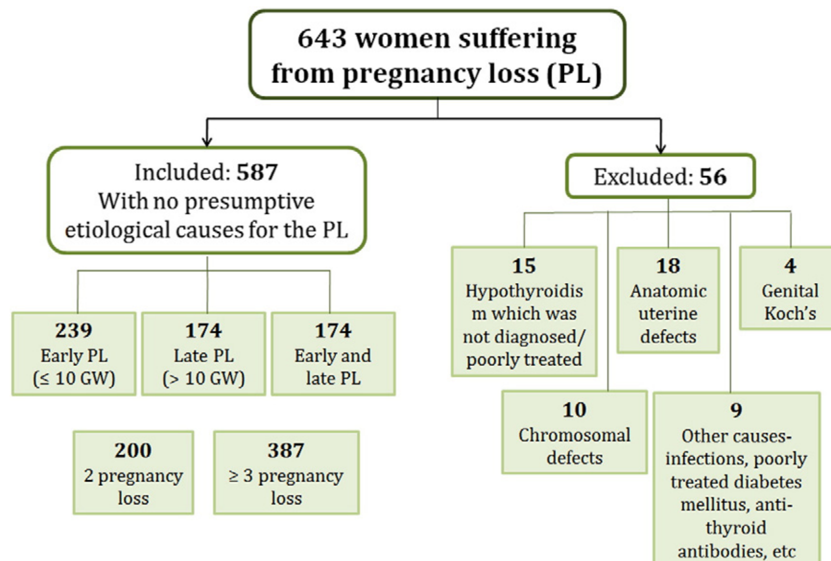

Fig. 1. Patient selection. 643 women were referred for thrombophilia workup, out of which 587 were included and 56 women were excluded for having known presumptive causes of PL like poorly treated hypothyroidism and diabetes mellitus, uterine anatomic defects, chromosomal defects etc.

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