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Immuno-Molecular Etiology of Recurrent Pregnancy Loss and the Anthropological Perspective

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ABSTRACT It is a well-known fact that pro-inflammatory cytokines exert an adverse effect on conceptus and result in pregnancy failure and there are mutations reported in certain genes regulating the production of these Th-1 type cytokines. In view of the cytokine gene polymorphisms known to cause high and low production of various pro-inflammatory and anti-inflammatory cytokines, a number of studies have been performed to reveal the association between these polymorphisms and recurrent pregnancy loss, but surprisingly contradictory results are reported which may be attributed to inclusion of heterogeneous samples resulting in false positive or false negative results. In the present paper, an attempt has been made to review the previous studies of association between two molecular genetic markers, TNF-α (-308 G/A) and IFN- γ (+874 A/T), and recurrent pregnancy loss.

One aspect of population genetics and molecular anthropology is to study the Darwinian fitness of the population which in turn is related to its reproductive fitness. The reproductive fitness of a population is explained by viability and regeneration.

The implanting embryo inherits its antigens from both father and mother and is thus half-foreign to mother's body. In other words, embryo acts as an allograft to the mother's body and yet unlike a mismatched organ transplant it is not normally rejected by the mother's immune system, and survives normally in the mother's womb during the entire gestational period in the cases of normal successful pregnancies.

This fact has provoked the researchers to study (i) how mother's immune system reacts to the implanting embryo? That is, does the mother's immune system recognize the implanting embryo as foreign? And if it does then (ii) how the implanting embryo protects itself from the mother's immune system to survive successfully? Or in other words, how the implanting embryo suppresses the mother's immune system so that it is not treated as foreign and thus rejected by the mother's body?

It has been proposed by various researchers that in order to prevent the rejection of implanting embryo, mother's immune system gets modulated in such a way that it helps fetal allograft to survive. A number of biological pathways have been revealed which are involved in this modulation. Some of those are listed below.

1. Trophoblasts, decidual cells and cells of lymphnodes draining the uterus at the time of implantation, suppress the mother's immune responses (Clarke et al. 1984; Bobe et al. 1986).
2. There is a lack of strong maternal cell mediated anti-fetal immunity and a dominant humoral response (Mosmann and Coffmann 1989; Wegmann et al. 1993; Romagnani 1994; Mosmann and Sad 1996; Voison and Raghupathy 1995).
3. Semen contains TGF-β, which helps maternal immune system to tolerate molecular signatures by altering the production of inflammatory cytokines (Tremellen et al. 2000).
4. HLA released from the trophoblasts into mother's blood stream seems to protect them from the attack. Soluble HLA-G makes certain type of T-cells which attack fetal cells bearing father's antigens to commit suicide (Fournel et al. 2000).
5. CRH secreted by both implanting embryo and the lining of uterus stimulate trophoblasts to produce Fas ligand which binds to cell surface receptor that triggers cell death of mother's T cells (Makrigiannakis 2001).
6. Previous exposure to a fetus carrying a

particular suite of paternal genes makes the immune system more likely to bear first born's subsequent siblings (Pearson 2002).

7. NK cells flooding the uterus at the time of implantation carry receptors that interact with HLA-C and HLA-E on surface of trophoblasts, triggering the production of particular cytokines that help trophoblasts to invade or limit the extent to which it invades (Mofett-King 2002).
8. Anti-inflammatory cytokines dominate during pregnancy and act antagonistically to pro-inflammatory cytokines and promote embryonic development and placentation (Raghupathy 1997).

However, in cases of pregnancy loss, dysregulation of the mother's immune system could be responsible for the failure where the implanting embryo is recognized as foreign and is thus rejected by the mother's immune system, resulting in spontaneous abortion.

WHO defines the spontaneous abortion as "the expulsion or extraction of a fetus weighing 500 g or less (approximately equal to 20-22 weeks of gestation) or another product of gestation of any weight and specifically designated irrespective of gestational age whether or not there is evidence of life" (WHO 1976). Most of the spontaneous abortions or miscarriages occur in the first trimester and affect about 15% of all recognized pregnancies (Daher et al. 2003)

Recurrent pregnancy loss (RPL) is defined as "the occurrence of three or more clinically detectable pregnancy losses (Stirrat 1990), which usually occur before 20 weeks of gestation and affects about 0.34% of women who conceive (Christiansen et al. 2005). The women suffering from RPL are the women who show a history of three or more sequential first trimester pregnancy losses without any live issue in between or there after.

The causes of the recurrent pregnancy losses are mainly categorized into fetal and maternal causes. Fetal causes are the one which arise if there is something wrong with the pregnancy i.e. chromosomal abnormality in the fetus itself or some congenital malformations. The maternal causes arise if there is problem within the uterine environment that does not allow the fetus to grow properly and is thus aborted. Meka and Reddy (2006) described various genetic (single gene mutations, polygenic and cytogenetic factors) and non-genetic (congenital uterine abnormalities, infections, hormonal imbalances, nutritional deficiencies and psychological factors) causes, which are described briefly in the following.

1. ***Uterine Anatomical Defects:*** Uterine septum, uterine leiomyomata and weak cervix .are the main causes of pregnancy loss. An estimated 10 - 15% of couples with recurrent pregnancy loss have an anatomic abnormality of the uterus as the primary reason mainly because of the incompetent cervix which results in the mid trimester loss of pregnancy.
2. ***Uterine Infections****:* Numerous organisms have been implicated in the sporadic cause of miscarriage, but common microbial causes generally have not been confirmed. The major organisms which can lead to infrequent RPL are *Listeria Monocytogenes, Toxoplasma Gondii, Rubella, Herpes simplex, Measles, Cytomegalovirus and Coxsackievirus.* Few studies have been done to determine the extent of maternal infection w.r.t. *Toxoplasma Gondiiin* reproductive disorders in India (Oumachigui et al. 1980; Pal and Aggarwal 1979).
3. ***Chromosomal Aberrations****:* Majority of first trimester (50%) pregnancy losses occur due to chromosomal abnormalities. Fetuses carrying chromosomal abnormalities are expelled naturally in the early stages of pregnancy as it is not viable. In addition, balanced translocations in couples which otherwise do not have lethal effect on the individual but result in the chromosomal abnormalities in the fetus at the time of formation of daughter cells, also result in the miscarriage at the early stages of pregnancy.
4. ***Hormonal Disorders:*** These constitute about (10 - 20) % of causes of RPL. Thyroid, diabetes mellitus, inadequate progesterone, insufficient luteal progesterone and increased androgens due to polycystic ovary syndrome either result in infertility or early pregnancy loss.
5. ***Haematological Problems:*** It includes the thrombophilia and hemorrhage occurring in the placenta leading to either miscarriage or initiating abruption or restricted blood flow finally leading to fetal death. Kumar et al. (2003) showed an association of MTHFR gene mutation in women with unexplained RPL. Mukhopadhyay (2006) also reported a statistically significant positive association

between inherited thrombophilia w.r.t. MTHFR C677T and Factor II G20210A gene mutations and RPL and a trend towards a positive association between Factor V Leiden and RPL.

6. ***Immunological Factors:*** They are the most poorly understood causes. They are mainly categorized into autoimmune disorders, where mother's immune system attacks her own organs and tissues eg. Anti-phospholipid syndrome resulting in RPL, fetal death and thrombosis (Wilson et al. 1999) and alloimmune disorders, where the mother's immune system attacks tissues considered to be foreign eg. Immunologically mediated abortions. Meka nd Reddy (2006) described the role of human leukocyte antigens (HLA) in pregnancy loss. Excess sharing of HLA between spouses has been considered by some to be a mechanism leading to maternal hyporesponsiveness to paternal antigens encountered in pregnancy and therefore subsequent miscarriage (Beer et al. 1981).
7. ***Other Factors:*** These include ovarian age, life style factors and psychological factors are known to be associated with termination of pregnancy but they being the cause of recurrent pregnancy loss are not well established.

The present paper attempts to review 'immunologically mediated pregnancy loss' which may explain the otherwise unexplained habitual pregnancy losses.

Immunologically Mediated Recurrent Pregnancy Loss (IMRPL)

Although several etiological factors have been well established, still about 50% cases of recurrent pregnancy losses remain unexplained (Daher et al. 2003) which might be explained by the immunological factors (Shormilla and Knapp 2000). It has been suggested that in some women this may be due to an exaggerated maternal immune response to the fetus (Babbage et al. 2001). As described earlier normal pregnancy is associated with modulation of the mother's immune system in order to accept the conceptus, which go against the laws of immunology whereas in case of spontaneous abortion, the laws of immunology appear dominant over that of obstetrics.

It has been reported that cytokines play an important role in success and failure of pregnancy (Raghupathy 1997). Cytokines are immunomodulatory proteins representing a group of proteins and peptides that are used in organisms as signaling compounds allowing communication between the cells. They are particularly important in both innate and adaptive immune responses. Due to their central role in the immune system, cytokines are involved in a variety of immunological, inflammatory and infectious diseases. However, not all their functions are limited to the immune system, as they are involved in several developmental processes during embryogenesis. They are produced by a variety of cells (both haemopoietic and non- haemopoietic) and the same cytokine is even produced by different types of cells at the same time in response to any foreign particle eg. IFN family cytokines are produced by Th-1 cells, NK cells and macrophages at the same time in response to any viral infected cell. They have effects on both nearby cells and throughout the organisms and sometimes these effects are strongly dependent on the presence of other chemicals and cytokines.

Although various classifications for cytokines have been suggested on the basis of their mode of action, structure, receptors, etc. but depending on their inflammatory reactions, they are broadly categorized into pro-inflammatory and anti-inflammatory cytokines which are produced by Th-1 and Th-2 cells, respectively. Helper T cells are so called as they help in stimulating cellular immunity and inflammation and also in stimulating B cells to produce various antibodies. Two functionally distinct subsets of helper T cells secrete cytokines which promote these different activities. Th-1 cells produce pro-inflammatory cytokines namely TNF-α, TNF-β, IFN-γ, IL-1, IL-2, etc. which activate Tc and macrophages to stimulate cellular immunity and inflammation, whereas Th-2 cells produce anti-inflammatory cytokines namely IL-4, IL-5, IL-6,

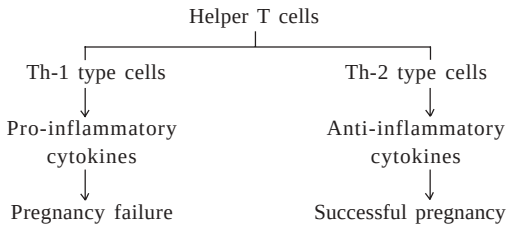

Fig. 1. Schematic representation of the effects of Th-1 and Th-2 type cytokines in pregnancy.

IL-10, IL-12, etc. which stimulate antibody production by B cells and act antagonistically to Th-1 type cytokines.

The role of pro-inflammatory and anti-inflammatory cytokines as depicted in Figure1 is described below.

Pro-inflammatory Cytokines

Th-1 cells are involved in cell-mediated inflammation and produce pro-inflammatory cytokines which inhibit trophoblast growth and differentiation. Some of the first studies on RPL associated abnormal immune reactivity in the context of Th1-Th2 paradigm demonstrates *in vitro* that trophoblast antigens activate lymphocytes of RPL susceptible women to produce embryotoxic cytokines i.e. TNF-α, IFN-γ and IL-2 (Yamada et al. 1994; Hill et al. 1995; Hill, 1995). TNF-α and IFN-γ inhibit embryonic and fetal development as well as the proliferation of human trophoblast lines (Haimovici et al. 1991) as both these cytokines are cytotoxic to embryonic fibroblast like cells (Suffys et al. 1989). It is also reported that IL-2, TNF-α and IFN-γ together terminate normal pregnancy when injected (Chaouat et al. 1990). Also, IFN-α inhibits secretion of GM-CSF from uterine epithelium necessary for successful pregnancy (Robertson et al. 1994). TNF-α is also known to cause fetal expulsion due to uterine contraction or may even cause necrosis of implanted embryo or it could act by thrombosing the blood supply to conceptus (Raghupathy 1997).

It is also well known that Th-1 type cytokines induce programmed cell death (apoptosis), the effect of which could comprise the trophoblast barriers separating the semiallogenic fetus from the mother's immune system, leading to fetal rejection or abortion. Th-1 type cytokines may also act by inducing the development of NK, LAK and CTL cells that cause fetal death, as they are capable of killing trophoblasts (Drake and Head, 1989). TNF-α is also reported to act along with the hormones and causes thromboses in the placenta during pregnancy resulting in miscarriage and its production is enhanced at the onset of labor and spontaneous abortion (Daher et al. 1999).

Anti-inflammatory Cytokines

Th-2 cells encourage antibody production and produce anti-inflammatory cytokines that promote embryonic development and placentation. For instance, IL-4, IL-6 and IL-10 are propitious to the success of pregnancy and deficiency of these cytokines leads to poor placentation, subnormal growth and even sometimes fetal death (Clark and Chaouat 1989). These anti-inflammatory cytokines are also known to control the action of Th-1 dependent cytokines as they act antagonistically on Th-1 cells (Wegmann et al. 1993; Romagnani 1994; Raghupathy 1997) which otherwise might attack fetus or the trophoblasts in general.

Th-1 Bias in Pregnancy Failure

Peripheral blood mononuclear cells (PBMCs) from a significant number of women with a history of RPL showed a greater cell proliferation and produced soluble factors that were toxic to mouse embryos and human trophoblast lines when stimulated *in vitro* with trophoblast antigen extracts (Yamada et al. 1994; Hill et al. 1995). Out of 244 women with unexplained RPL, 160 were shown to have PBMCs that responded *in vitro* to trophoblast antigens by producing embryotoxic activity and high levels of pro-inflammatory cytokines but a very low level of anti-inflammatory cytokines. Conversely, women who were not prone to RPL responded without production of Th-1 type cytokines but had IL-10 (Th-2 type cytokine) activity (Hill et al. 1995).

It has been investigated by enzyme linked immuno sorbent assay (ELISA) testing that there is increased production of pro-inflammatory cytokines (Th-1 type) and reduced production of anti-inflammatory cytokines (Th-2 type) in women with recurrent pregnancy losses suggesting Th-1 bias in pregnancy failure and Th-2 bias in successful pregnancy (Wegmann et al. 1993; Tangri and Raghupathy 1993; Tangri et al. 1994; Yamada et al. 1994; Hill et al. 1995; Hill 1995) suggesting that these may be an etiological factor in recurrent miscarriages (Jenkins et al. 2000; Raghupathy et al. 2000; Mueller-Ekharat et al. 1994).

It was also shown by dot-blot and northern hybridization techniques that the expression of TNF-α, IFN-γ and IL-2 is significantly greater in placentas of abortion prone pregnancies compared with those of normal pregnancies (Tangri and Raghupathy 1993). Tangri and his colleagues observed using Elisa and bioassay

that a significantly greater production of TNF-α, IFN-γ and IL-2 in mixed lymphocyte placental reaction (MLPR) supernatants in abortion prone mating combinations compared to normal combinations (Tangri et al. 1994) where IFN-γ was produced at 55-fold greater concentration, TNF-α at 10-fold greater concentration and IL-2 at 25-fold greater concentration. These studies suggest that a Th-2 bias is necessarily maintained in case of normal pregnancy to act anta-gonistically to Th-1 cytokines whereas reproductive failure is characterized by Th-1 bias.

Th-2 to Th-1 switch

Various causes have been suggested for the possible mechanisms that shift Th-2 to Th-1 dominant environment in pregnancy failure. Hill and his colleagues suggest that women with RPL may have a fundamental aberration in the regulation of immune responses that skews the pattern from Th-2 type to Th-1 type cytokines in reproductive failure (Hill et al. 1995; Hill 1995).

The Th-2 to Th-1 shift in pregnancies may be due to one or more factors. The deficiency of some putative immunomodulatory molecules like PIBF, placental factors, IL-10 or TGF-β2 may be responsible. It is also likely that a balance between IL-12 (favouring Th-1 response) and IL-4 (favouring Th-2 response) determines the eventual outcome of the Th-1 – Th-2 dichotomy during an immune response (Trinchieri 1993).

Infection during pregnancy, particularly by intracellular parasites, may well be an important factor that drives the response in a certain direction. Th-1 type cells induced by the infection may traverse the fetal interface or may produce cytokines that affect the trophoblasts (Krishnan et al. 1996). It is also assumed that a previous abortion due to some other cause may prime the mother for subsequent Th-1 biased responses (Hill 1995). Infections with agents such as *Toxoplasma Gondii* and CMV that lead to predominantly cellular immune responses and the production of pro-inflammatory cytokines have been associated with recurrent miscarriages (Stary-Pederson and Stray-Pederson 1984; Lim et al. 1996). Such infections may prime the mother to produce pro-inflammatory responses in subsequent pregnancy (Hill 1995).

Further, the production of pro-inflammatory and anti-inflammatory cytokines is partly under genetic control. Genetic polymorphisms associated with high and low production of a number of cytokines including TNF-α, IFN-γ and IL-10 have been found (Wilson et al. 1992; Wilson et al. 1997; Turner et al. 1997; Pravica et al. 1999 and Bidwell et al. 2001). In present paper emphasis is laid on TNF-alpha and IFN-gamma gene polymorphisms as these two cytokines have the most adverse effects on the implanting embryo.

TNF (tumour necrosis factor) is a family of cytokines that shares a cysteine rich common extracellular binding domain and includes several other non-cytokine ligands like CD40, CD27 and CD30 besides the ligands on which the family is so named. These are also referred to a group of cytokines which are capable of causing apoptosis. TNF-α is the most well known member of this class and sometimes the term TNF is used to refer to this specific form. The chromosomal location of the gene controlling the secretion of TNF-α is 6p21.3 (Fig. 2). A number of polymorphisms have been reported in this gene like (-238), (-308) and (-863) and those which are present in the promoter region especially (-308 G/A) are known to cause an altered promoter activity and thus resulting in an increased production of TNF-α cytokine in blood among humans (Wilson et al. 1997).

IFN (interferon) is a subfamily of four α helix bundle family of cytokines which have three

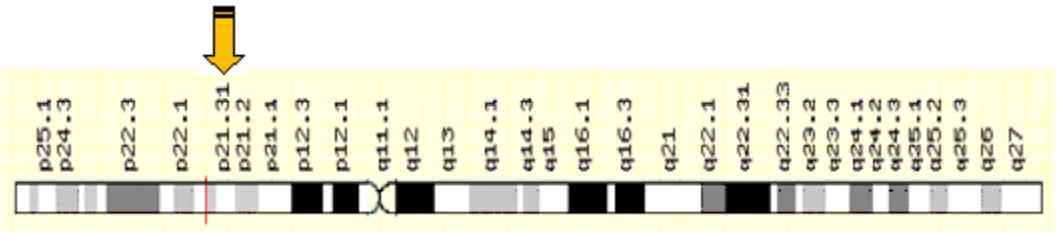

Fig. 2. Location of TNF-alpha on chromosome 6.
(Entrez Gene cytogenetic band: 6p21.3, Ensembl cytogenetic band: 6p21.33)

dimensional structures with four bundles of α helices. Interferon family cytokines are known to inhibit virus replication in the infected cells and IFN-γ in addition also stimulates antigen presenting cell in the MHC expression. The chromosomal location of the gene regulating the secretion of IFN-γ is 12q14 (Fig. 3). Again a large number of polymorphisms are reported in this gene but specifically (+874 A/T) is known to cause an overexpression of the gene and thus resulting in an increased production of IFN- γ cytokine (Pravica et al. 1999). A number of studies have been performed regarding the association of the above two mentioned polymorphisms with various inflammatory diseases like renal disorders, leishmaniasis, arthritis and even recurrent pregnancy losses.

Cytokine Gene Polymorphisms and RPL

In view of the cytokine gene polymorphisms known to cause elevated levels of pro-inflammatory cytokines and thus RPL, it was suspected that women carrying these polymorphisms might be genetically predisposed to developing habitual abortions. Few studies have been performed to investigate the association between recurrent pregnancy loss and the above described cytokine gene polymorphisms i.e. TNF-α (-308 G/A, -238 G/A), IFN-γ (+874 A/T) etc. For example, Babbage et al. (2001) performed a case – control study in which 43 Caucasian women suffering from RPL attending a particular hospital in UK and 73 Caucasian ward staff women as controls were included. Both the groups (cases and controls) were screened for TNF-α (-308 G/A) and IFN-γ (+874 A/T) along with some other markers and they reported no association between RPL and any of the two molecular markers. Similarly, Baxter et al. (2001) studied 145 British Caucasian couples, 76 case and 69 control couples, for TNF-α (-308 G/A) in addition to some more cytokine gene polymorphisms. This study hypothesized that elevated maternal and fetal levels of TNF and TNF (-308) polymorphism are associated with premature membrane rupture and preterm delivery (Roberts et al. 1999; Ferriman et al. 2000) but found no association between RPL and variant allele of TNF-α. Reid et al. (2001) assessed the carriage of rarer alleles of TNF-α*2 and IL-1β*2 among women with RPL and observed an increased incidence in the carriage of TNF-α*2 more pronounced in the women with two or more sequential miscarriages as compared to the normal women.

Daher et al. (2003) screened 48 Brazilian Caucasian women with RPL and 108 healthy Brazilian Caucasian individuals (82 females and 26 males) for TNF-α (-308 G/A) and IFN-γ (+874 A/T) along with IL-10 (-1082 A/T) and also performed a meta-analysis including their own data and all the previous studies mentioned above. The results showed statistically higher frequencies of IFN-γ genotype TT (+874) i.e. p=0.04, OR=1.92 as well as a trend towards increased frequencies of A/A and A/G (-308) TNF-α genotypes when compared to general population i.e. p=0.18 and OR=1.31. Pietrowski et al. (2004) performed an association based case-control study on white Caucasian women, including 168 in the study group and 212 in the control group and concluded that TNF-α (-308) polymorphism and resting blood TNF-α levels do not correlate with the propensity to RPL in Caucasian women.

Prigoshin et al. (2004) studied TNF-α (-308) and IFN-γ (+874) polymorphisms along with other pro-inflammatory and anti-inflammatory cytokine gene polymorphisms in Caucasian Argentine women 41 with RPL and 54 control, and showed a significant association between

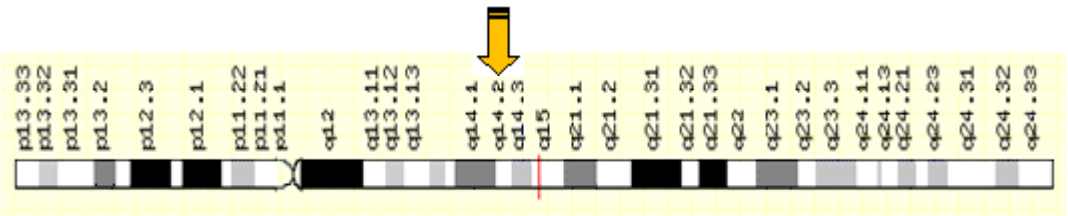

Fig. 3. Location of IFN-gamma on chromosome 12
(Entrez Gene cytogenetic band: 12q14, Ensembl cytogenetic band: 12q15)

RPL versus controls concerning IFN-γ (+874 A/T) where TA genotype was found to be more in the patient group (65% versus 35.8%, p=0.01). However, no association was found between RPL versus controls concerning the TNF-α (-308) polymorphism. Kamali-Sarvestani et al. (2005) performed case-control study on Iranian women with reference to above two polymorphisms along with other Th-1 and Th-2 cytokine gene polymorphisms in which 139 women in the study group and 143 women in the control group were investigated and it was concluded that there is no association between the selected molecular markers and the manifestation of RPL.

SUGGESTED FUTURE LINE OF ACTION

The effects of increased production of pro-inflammatory cytokines on pregnancy failure have already been described (i.e. Th-1 bias in pregnancy failure) and the association between genetic polymorphisms causing elevated production of these cytokines is also well established (i.e. Th-2 to Th-1 switch). Therefore, association between these cytokine gene polymorphisms and recurrent pregnancy loss is also expected but surprisingly, the results are contradictory.

The possible reason for this dualism could be the inclusion of heterogeneous samples as most of these studies are hospital based which can result in false positive or false negative results. Moreover, association between the selected markers and the manifestation of the disease (i.e. RPL) is also population specific. The selected molecular markers i.e. TNF-α (-308) and IFN-γ (+874) are the candidate genes for the recurrent pregnancy loss and act as one of the possible causes of the disease along with the other genes in the region which are also influenced by the environmental causes. The extent of the manifestation of the disease vary in different ethnic groups as they are largely influenced by the mating patterns, surrounding genetic environment, life style factors and other environmental factors which are population specific.

Thus, there is a need to screen the different ethnic groups using TNF-α (-308) and IFN-γ (+874) markers by taking homogeneous samples for each of them to understand the extent of association between RPL and these molecular markers in different populations which would further help in bringing out the community specific associations leading to community / individual specific pharmacological approaches. This would help in the more efficient management of immunologically mediated recurrent abortions at the population / individual level.

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