Associations between tumor necrosis factor- α and lymphotoxin- α polymorphisms and idiopathic recurrent miscarriage

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Abstract

Heightened expression of tumor necrosis factor (TNF)- α and lymphotoxin- α (LT- α) was associated with pregnancy complications, including idiopathic recurrent miscarriage (RM). Whereas TNF- α and LT- α gene polymorphisms affect serum cytokine concentrations, their contribution to RM is controversial. The single nucleotide polymorphisms (SNPs) TNF- α (-238G/A, -308G/A) and LT- α (+252A/G) were investigated in 350 RM women and 200 control women. Higher frequency of the TNF- α -238A, but not the TNF- α -308A or the LT- α +252G, allele was seen in patients, with comparable frequencies of TNF- α -238G/A, TNF- α -308G/A, and LT- α +252A/G genotypes seen between both groups, except for TNF- α -238G/G, which was lower in patients. Regression analysis confirmed the association of the TNF- α -238G/A SNP with idiopathic RM, and both TNF- α -308A/TNF -238G/LT- α +252G and TNF- α -308G/TNF- α -238A/LT- α +252G haplotypes played a susceptible role in idiopathic RM. TNF- α -238G/A and -238A/A, and LT- α +252G/G genotypes were positively associated only with exclusively early RM. This supports the concept of the association of TNF- α (-238G/A) and LT- α (+252A/G) polymorphic variants in idiopathic RM.

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Introduction

Spontaneous miscarriage is a frequent common complication of pregnancy, with an estimated 1–3% of otherwise healthy women reportedly having experienced recurrent miscarriage (RM), defined as three or more consecutive pregnancy losses prior to the 20th week of gestation (Sierra & Stephenson 2006). While anatomic, autoimmune, endocrine, and chromosomal abnormalities were implicated in the etiology of RM, a significant number of RM cases remain idiopathic (Quenby *et al.* 2002). Previous studies suggested a central role of the T helper (Th)1–Th2 cytokine network in the positive or negative maintenance of pregnancy (Wegmann *et al.* 1993, Makhseed *et al.* 2001, Zhu *et al.* 2005). This was evidenced by the association of increased Th2 cytokines with successful pregnancy, while heightened Th1 activity was indicative of poor pregnancy outcome, both in experimental animals and in humans (Hill *et al.* 1995). In view of this interplay between (pro-inflammatory) Th1 and (anti-inflammatory) Th2 cells and their respective cytokines in pregnancy, dysregulated immunity stemming from altered Th1–Th2 balance was proposed as a potential mechanism underlying idiopathic RM in the face of unknown causes (Makhseed *et al.* 2001, Kwak-Kim *et al.* 2005).

Tumor necrosis factor (TNF) is a pleiotropic cytokine mainly secreted by mononuclear phagocytes, natural killer (NK) cells, and antigen-stimulated T cells (TNF- α) or lymphocytes (TNF- β or lymphotoxin- α , LT- α ; Hehlhans & Pfeffer 2005). TNF- α and LT- α exert predominantly pro-inflammatory responses, including apoptosis, in many cell types by binding two distinct cell-surface receptors (TNFR1 and TNFR2; Hehlhans & Pfeffer 2005). Due to their pro-inflammatory and pro-apoptotic capacity, TNF- α and LT- α were described to mediate several aspects of pregnancy complications, including pre-eclampsia (Anim-Nyame *et al.* 2003), miscarriage (Babbage *et al.* 2001), and RM (Rezaei & Dabbagh 2002). Several mechanisms were proposed for the pro-abortion effects of TNF- α and LT- α , including trophoblast invasion and placentation (Kwak-Kim *et al.* 2005) and induction of the expression of pro-apoptotic genes in human fetal membranes (Garcia-Lloret *et al.* 2000), which in turn accelerates membrane degradation and thus increases the susceptibility to premature rupture (Fortunato *et al.* 2001). TNF- α was also described to facilitate miscarriage indirectly by activating NK cells or macrophages (Raghupathy *et al.* 2000). Several polymorphic gene variants of TNF- α and LT- α were described, including the TNF- α -238G/A and -308G/A promoter

single nucleotide polymorphisms (SNPs), and the LT- α +252A/G SNP (Wilson *et al.* 1997, Ozaki *et al.* 2002).

The incidence and frequencies of RM are controlled by genetic factors, and poor pregnancy outcome was demonstrated to be associated with a predominantly Th1 cytokine profile, which was controlled to a large extent by polymorphisms in cytokine genes (Prigoshin *et al.* 2004). In view of their effect on altered regulation of TNF synthesis, secretion, and activity (Wilson *et al.* 1997, Louis *et al.* 1998), TNF- α and LT- α gene variants were implicated in the pathogenesis of RM (Reid *et al.* 2001, Daher *et al.* 2003). Conflicting associations of these gene variants with RM were presented, highlighted by the association of these polymorphisms with RM (Reid *et al.* 2001, Daher *et al.* 2003, Costeas *et al.* 2004), and susceptibility to preterm birth (Engel *et al.* 2005), while others found no association with RM and related complications (Bates *et al.* 2002, Pietrowski *et al.* 2004). In view of the critical role of TNF- α and LT- α in the maintenance of pregnancy, we investigated the contribution of the TNF- α -238G/A and -308G/A, and the LT- α +252 A/G to the pathogenesis of RM in 350 women with confirmed RM and 200 multiparous women who served as controls.

Results

Study subjects

The demographics and clinical characteristics of the study subjects are summarized in Table 1. Both patients and controls had comparable age ($P=0.27$) and similar education backgrounds ($P=0.134$) and alcohol ($P=1.00$) or tobacco ($P=0.35$) consumption. Body mass index (BMI) values were higher in patients than in controls (25.8 ± 4.0 vs 24.6 ± 3.8 , $P=0.01$); obese

women, defined as having a BMI at least equal to 30 kg/m^2 , were more frequent in patients than in controls (19.7% vs 9.5%, $P=0.002$). In addition, comparable geographical distribution was seen, with both patients and controls originating from different parts of Tunisia ($P=0.385$).

TNF- α -308G/A, TNF- α -238G/A, and LT- α +252A/G genotype analysis

The distribution of the TNF- α -238 G/A ($\chi^2=1.82$, $P=0.18$) and LT- α +252A/G ($\chi^2=0.48$, $P=0.49$), but not the TNF- α -308G/A ($\chi^2=7.67$, $P=0.006$), genotypes were within the Hardy-Weinberg equilibrium among control women. The frequency of the mutant TNF- α -308A ($P=0.388$) and LT- α +252G ($P=0.062$) alleles were similar between patients and controls, while the frequency of the TNF- α -238A allele was higher among patients ($P=0.011$; Table 2). With the exception of the TNF- α -238G/G genotype, which was lower in patients than the corresponding controls (71.1% vs 81.0%, $P=0.014$), the frequencies of the other genotypes were comparable between patients and control subjects (Table 2). Logistic regression analysis confirmed the association of the TNF- α -238G/A SNP with RM, after adjusting for the RM variables: age, tobacco consumption, oral contraceptive use, and BMI (Table 3).

TNF- α -308G/A, TNF- α -238G/A, and LT- α +252A/G haplotype analysis

Linkage disequilibrium (LD) analysis, defined by the δ (D') coefficient, revealed weak LD between TNF- α -238A and LT- α +252A patients ($P=0.056$; $\chi^2=3.65$), and between TNF- α -238A and LT- α +252G alleles among controls ($P=0.053$; $\chi^2=3.76$). Haplotype analysis was performed after controlling for some RM confounding variables. Higher association of the TNF- α -308A/TNF- α -238G/LT- α +252G ($P=0.044$) and TNF- α -308G/TNF- α -238A/LT- α +252G ($P<0.001$) haplotypes with RM was seen, thereby assigning a susceptible nature to these haplotypes (Table 4). The prevalence of the other haplotypes was comparable between cases and controls, with no protective haplotype detected (Table 4).

Distribution of TNF- α -308G/A, TNF- α -238G/A, and LT- α +252A/G genotypes

Table 5 describes the associations between the TNF- α and LT- α gene variants and the pregnancy losses, according to the timing-related categorization. Increased prevalence of TNF- α -238 G/A and A/A, together with the LT- α +252G/G genotypes, were seen in exclusively early RM ($P<0.05$). Comparable frequencies of the TNF- α

Table 1 Demographics of recurrent miscarriage cases and controls.

	Cases ^a	Controls ^a	P^b
Age	28.9 $\pm$ 5.9	28.4 $\pm$ 3.8	0.27
Education			
Intermediate	112 (32.0) ^c	70 (35.0)	0.134
Secondary	145 (41.4)	92 (46.0)	
University	93 (26.6)	38 (19.0)	
Smokers	23 (6.6)	9 (4.5)	0.35
Alcohol	10 (2.9)	5 (2.5)	1.00
Body mass index (BMI, kg/m ²)	25.8 $\pm$ 4.0	24.6 $\pm$ 3.8	0.01
Oral contraceptive	93 (26.6)	46 (23.0)	0.42
Number of pregnancies	4.1 $\pm$ 1.4	3.8 $\pm$ 1.4	0.02
Number of children	0.51 $\pm$ 0.72	3.8 $\pm$ 1.4	<0.001
Abortions	3.6 $\pm$ 1.1	0.0 $\pm$ 0.0	<0.001
Pregnancy loss			
Early loss	71 (20.3)	0.0 $\pm$ 0.0	
Late loss	79 (22.6)	0.0 $\pm$ 0.0	
Early-late loss	200 (57.1)	0.0 $\pm$ 0.0	

^aA total of 350 patients and 200 controls were included. ^bStudent's *t*-test for continuous variables and Pearson's χ^2 test for categorical variables. ^cPercentage of total within each group/subgroup.

Table 2 Allele and genotype frequencies of tumor necrosis factor- α (TNF- α) and lymphotoxin- α (LT- α) polymorphisms.

SNP	Genotypes			Alleles	
	G/G	G/A	A/A	G	A
TNF- α –308G/A					
Cases ^a	305 (87.1) ^b	31 (8.9)	14 (4.0)	0.92 $\pm$ 0.01 ^c	0.08 $\pm$ 0.01
Controls ^a	168 (84.0)	27 (13.5)	5 (2.5)	0.91 $\pm$ 0.01	0.09 $\pm$ 0.01
P	0.371	0.119	0.494	0.354	0.388
TNF- α –238G/A					
Cases	249 (71.1)	82 (23.4)	19 (5.4)	0.83 $\pm$ 0.01	0.17 $\pm$ 0.01
Controls	162 (81.0)	34 (17.0)	4 (2.0)	0.90 $\pm$ 0.02	0.11 $\pm$ 0.02
P	0.014	0.095	0.087	0.053	0.011
LT- α +252A/G					
Cases	259 (74.0)	75 (21.4)	16 (4.6)	0.85 $\pm$ 0.01	0.15 $\pm$ 0.01
Controls	162 (81.0)	35 (17.5)	3 (1.5)	0.90 $\pm$ 0.02	0.10 $\pm$ 0.02
P	0.079	0.319	0.098	0.058	0.062

^aStudy subjects comprised 350 RM cases and 200 control subjects. ^bPercentage of total. ^cAllele frequency $\pm$ s.e.m.

–308G/A, TNF- α –238G/A, and LT- α +252A/G genotypes were seen between exclusively late and combined early–late RM cases and control subjects, thereby restricting the contribution of the TNF- α and LT- α gene variants to exclusively early RM.

Risk factors for pregnancy loss

We examined the relationship between the stage of pregnancy loss and the frequency of TNF- α and LT- α gene variants by performing three logistic regression analysis models, with the dependent variables being exclusively early, exclusively late, and combined early–late RM, and the independent potentially confounding variables being age, tobacco consumption, oral contraceptive use, BMI, and the TNF- α –308G/A, TNF- α –238G/A, and LT- α +252A/G SNPs (Table 6). The results of the corresponding regression analysis model are shown in Table 6. BMI constituted an independent risk factor for all stages of RM. For early RM, TNF- α –238G/A (odds ratios; OR=2.02, 95% confidence intervals; 95% CI=1.23–3.31) and LT- α +252A/G (OR=1.67, 95% CI=1.01–2.76) were the only inherited variables selected using this technique. For exclusively late RM, oral contraceptive use was the selected variable (OR=1.83, 95% CI=1.01–3.32), while for combined early–late RM, smoking was the selected variable (OR=2.70, 95% CI=1.05–6.93). Adjusting for the variables selected, TNF- α –308G/A SNP was not associated with any of the stages of idiopathic RM.

Discussion

Several mechanisms were previously described for the pathogenesis of RM, including chromosomal anomalies, hormonal problems, uterine abnormalities, infections, and autoimmune disorders. Since these mechanisms account for the etiology of less than half of the RM cases

at the best estimate (Quenby *et al.* 2002), dysregulated immunity was demonstrated as the underlying cause of a proportion of idiopathic RM cases, in particular altered Th1–Th2 cytokine balance (Hill *et al.* 1995, Makhseed *et al.* 2001, Zhu *et al.* 2005), in which Th1-mediated immunity may facilitate pregnancy failure (Jenkins *et al.* 2000), while Th2 cytokines (interleukin (IL)-4, IL-10) promote successful pregnancy (Jenkins *et al.* 2000, Daher *et al.* 2003). As TNF- α and LT- α levels are under genetic control (Wilson *et al.* 1997), we investigated in this case-control study TNF- α and LT- α gene polymorphisms in women with idiopathic RM, specifically with regard to the stage of the losses. Our results demonstrated the TNF- α –238G/A and LT- α +252A/G, but not the TNF- α –308G/A, SNPs were independently associated with the risk of early, but not late or combined early–late RM.

RM is a multifactorial condition, and both acquired and inherited risk factors reportedly contributed to its pathogenesis. Cases were included if they had three or more RM of unknown etiology with the same partner, without any known personal or family history of venous and arterial thromboembolic events. As diabetes was suggested to be a potential complication of RM (Temple *et al.* 2002), all cases and control subjects were normoglycemic, evidenced by glycated hemoglobin (HbA1c) values $\leq$ 6.00. It was shown by several

Table 3 Predictors of recurrent miscarriage.

Factor	P	OR	95% CI
Age	0.360	1.02	0.98–1.05
Smoker	0.298	0.65	0.29–1.46
Oral contraceptive	0.264	0.79	0.52–1.20
BMI	0.001	0.85	0.78–0.93
TNF- α –308G/A ^a	0.210	1.38	0.83–2.28
TNF- α –238G/A ^a	0.012	1.73	1.13–2.66
LT- α +252A/G	0.059	1.52	0.98–2.34

^aHomozygote + heterozygote carriers.

Table 4 Matched odds ratios for recurrent miscarriage (RM) for tumor necrosis factor- α (TNF- α)-lymphotoxin- α (LT- α) haplotypes.

Haplotype (–308/–238/+252)	Patients ^a	Controls ^a	P ^b	OR (95% CI)
G G A	0.663 ± 0.020 ^c	0.720 ± 0.011	0.197	0.76 (0.53–1.12)
G G G	0.102 ± 0.013	0.095 ± 0.026	0.416	1.04 (0.94–1.15)
G A A	0.117 ± 0.016	0.089 ± 0.027	0.077	1.08 (0.99–1.17)
A G A	0.050 ± 0.012	0.077 ± 0.024	0.378	0.94 (0.83–1.07)
A A A	0.017 ± 0.010	0.012 ± 0.011	0.517	1.07 (0.88–1.29)
A G G	0.014 ± 0.013	0.004 ± 0.008	0.044	1.23 (1.01–1.50)
G A G	0.034 ± 0.012	0.004 ± 0.006	<0.001	1.27 (1.15–1.39)

^aStudy subjects comprised 350 patients and 200 control subjects. ^bPearson's χ^2 test. ^cHaplotype frequency $\pm$ S.E.M.

groups that the classic TORCH agents did not impart significant risk of RM (Paukku *et al.* 1999, Kavalier 2005), supported by the recent recommendation of The Royal College to abandon TORCH screening in the routine RM workout (Kavalier 2005). In spite of this we included only those negative for the standard TORCH agents, thereby ruling out any possible contribution of bacterial, viral, and toxoplasma infections to the pregnancy outcomes.

Our results were reminiscent of a previous study, in which the TNF- α –238G/A, but not the –308G/A, SNP were associated with RM (Bertolaccini *et al.* 2001). This lack of association of the TNF- α –308G/A SNP with increased RM risk was also reported for Caucasian (Baxter *et al.* 2001, Bertolaccini *et al.* 2001, Pietrowski *et al.* 2004, Prigoshin *et al.* 2004) and Iranian women (Kamali-Sarvestani *et al.* 2005), and in apparent partial disagreement with the study of Costeas *et al.* (2004). A recent meta-analysis (Daher *et al.* 2003) confirmed the lack of association of the TNF- α –308G/A SNP with RM, and suggested instead that interferon- γ (874T/T) and IL-10 (–1082G/G) gene variants represented stronger RM inherited risk factors. While the differences in ethnic background between the study groups, in the selection of RM cases, and in the sample size cannot be overlooked, it should also be noted that the differences in cytokine production induced by gene polymorphisms is not always indicative of immune/inflammatory events that occur at the local maternofetal interface during pregnancies/miscarriage, as was suggested (Babbage *et al.* 2001), since the cytokines (which are generally short acting) exert their effects both in a paracrine and autocrine manner.

Increased prevalence of two of the TNF- α –308G/A/TNF- α –238G/A/LT- α +252A/G haplotypes, –308A/–238G/+252G and –308G/–238A/+252G (out of the possible maximum of eight haplotypes), was seen among RM cases, after controlling for potentially confounding variables. This was in apparent disagreement with the study of Baxter *et al.* (2001) who failed to find an association between RM and TNF- α -LT- α haplotypes. The differences may be reconciled by ethnic differences, patient selection, and the low number of subjects included in their study (76 cases and 69 controls, compared with the 350 RM cases and 200 controls analyzed here), which may

have missed the detection of susceptible (and possibly protective) haplotypes.

A dominant role of the innate, rather than the adaptive, immune system in RM was proposed previously (Sacks *et al.* 1999, Borzychowski *et al.* 2005), highlighted by defective HLA expression, and the detection of large granular lymphocytes and monocytes cellular infiltrates in RM (Sacks *et al.* 1999, Babbage *et al.* 2001). Altered immunity in RM was dominated by the Th1/Th2 hypothesis, which proposes that the fetus escapes maternal-derived T-cell responses through skewing the Th0 differentiation toward Th2 pathway (Saito *et al.* 1999), which in turn dampens (harmful) Th1 immunity (Wegmann *et al.* 1993, Makhseed *et al.* 2001, Zhu *et al.* 2005). We propose here that normal pregnancy, and hence RM, should be viewed as inflammation state, in addition to the widely adopted altered Th1–Th2 (Saito *et al.* 1999), in which pro-inflammatory mediators and cells act locally at the implantation site, and also more systemically by amplifying the immune/inflammatory responses at the fetomaternal junction (Sargent *et al.* 2006). Insofar as TNF activates NK cells, which subsequently damage the placenta (Raghupathy 1997), it was interesting to find that high NK cell activities were seen prior to conception

Table 5 Association of tumor necrosis factor- α (TNF- α)-lymphotoxin- α (LT- α) gene variants with the stage of pregnancy loss.

SNP	Controls ^a None	Patients ^a		
		Early	Late	Early–late
TNF- α –308G/A				
G/G	68 (84.0) ^b	140 (87.5)	70 (90.9)	95 (84.1)
G/A	27 (13.5)	14 (8.8)	5 (6.5)	12 (10.6)
A/A	5 (2.5)	6 (3.8)	2 (2.6)	6 (5.3)
TNF- α –238G/A				
G/G	162 (81.0)	107 (66.9)	59 (76.6)	83 (73.5)
G/A	34 (17.0)	41 (25.6)*	15 (19.5)	26 (23.0)
A/A	4 (2.0)	12 (7.5)*	3 (3.9)	4 (3.5)
LT- α +252A/G				
A/A	162 (81.0)	114 (71.3)	60 (77.9)	85 (75.2)
A/G	35 (17.5)	36 (22.5)	15 (19.5)	24 (21.2)
G/G	3 (1.5)	10 (6.3)*	2 (2.6)	4 (3.5)

*P<0.05 versus corresponding control (Fisher's exact test).

^aA total of 350 patients and 200 controls were included. ^bPercentage of subjects within each category.

Table 6 Predictors of stages of pregnancy loss.

Factor	Early		Late		Early + late	
	OR	95% CI	OR	95% CI	OR	95% CI
Age	0.99	0.95–1.03	1.00	0.95–1.05	0.97	0.93–1.01
Smoker	1.29	0.48–3.47	0.91	0.23–3.61	2.70	1.05–6.93
Oral contraceptive	1.10	0.66–1.83	1.83	1.01–3.32	1.24	0.71–2.17
BMI	0.93	0.88–0.98	0.91	0.85–0.98	0.92	0.87–0.98
TNF- α –308G/A ^a	0.78	0.42–1.45	0.52	0.22–1.26	0.94	0.49–1.80
TNF- α –238G/A ^a	2.02	1.23–3.31	1.43	0.74–2.77	1.55	0.88–2.74
LT- α +252A/G ^a	1.67	1.01–2.76	1.30	0.67–2.54	1.56	0.88–2.78

^aHomozygote + heterozygote carriers.

(Aoki *et al.* 1995), and early in gestation (Yamada *et al.* 2001), and that normal pregnancy was not the result of altered Th1/Th2 or Tc1/Tc2 cell ratios, but rather decreased NK1/NK2 and NKT1/NKT2 cell ratios (Borzychowski *et al.* 2005).

While this study clearly demonstrated an association between TNF- α –238G/A and LT- α +252A/G polymorphisms and idiopathic RM, several limitations to these findings are noteworthy. With regard to possible functional role of TNF- α and LT- α polymorphisms, varied cytokine gene polymorphism and cytokine production was noted between patient groups and healthy subjects (Koss *et al.* 2000), thereby necessitating the assessment of the relationship between gene polymorphisms and TNF- α –LT- α production in RM. In addition, the association of other genetic polymorphisms with RM, through LD with TNF- α –LT- α polymorphisms, should not be overlooked. Further study aiming at addressing these two points is required to characterize more precisely the role of the TNF system in RM.

Materials and Methods

Study subjects

This was a retrospective case–control study, performed at the Maternity Center of Hôpital Farhat Hached of Sousse, Tunisia. Data collection procedures were the same for patients and control subjects. Cases comprised 350 fertile women with a history of three to six unexplained consecutive pregnancy losses with the same partner, which had occurred by clinical definition from the beginning of pregnancy to the end of the 30th week of gestation. Patients were referred from local hospitals and/or gynecology consultants to the Department of Obstetrics and Gynaecology at CHU Farhat Hached (Sousse, central Tunisia), for RM workup. The gestational age at the time of spontaneous abortion was calculated on a clinical basis and was defined as the time between the date of the first day of the last normal menstrual period and the date of the first clinical symptom related to pregnancy loss. Exclusion criteria included preclinical miscarriages, anatomical abnormalities, endocrine disorders including diabetes mellitus (HbA1c levels <6.00), autoimmune disease, arterial hypertension, liver function abnormalities, drug abuse, previously known systemic disease, and personal and/or a family history of thromboembolism.

Additional exclusion criteria included abnormal thyroid function, thyroid antibodies (antithyroglobulin and antithyroid peroxidase antibodies) identified even in the absence of abnormal thyroid function, hyperprolactinemia prior to luteal phase defects (a normal luteal phase of at least 12 days and a plasma progesterone level above 25 ng/ml), erythroblastosis fetalis (Rh disease), immune thrombocytopenic purpura, and fetomaternal alloimmune thrombocytopenia. Chromosomal aberrations and Rh incompatibility were ruled out before inclusion in the study. In addition, all subjects included in the study were negative for the TORCH agents, *Toxoplasma gondii*, rubella, cytomegalovirus (CMV), herpes simplex viruses (HSV-1 and HSV-2), varicella zoster virus (VZV), and human immunodeficiency viruses (HIV-1 and HIV-2), by indirect ELISA. Transvaginal ultrasound examination was done to confirm spontaneous miscarriage (no heartbeat detection).

Pregnancy losses were classified as early (5–10 weeks) and late (11–30 weeks; Table 1). Control subjects comprised 200 healthy women (with a combined total of 1223 successful pregnancies) who were examined in the Outpatient Department of Gynaecology and Obstetrics of the University Hospital of Sousse, as a routine checkup following uncomplicated pregnancy. Controls were matched with patients according to a number of risk factors (smoking, alcohol consumption, oral contraceptive use); age distribution was comparable between RM cases (mean age 28.9 ± 5.9 years) and normal fertile controls (mean age 28.4 ± 3.8 years; Table 1). Patients' and controls' venous blood was collected from the study participants in EDTA tubes (for DNA extraction), and all subjects were required to sign an informed consent prior to entering the study, which was conducted after all institutional ethics requirements were met.

TNF- α and LT- α gene polymorphisms

Total genomic DNA was isolated from leukocyte-rich interphase layer of EDTA anticoagulated blood by the phenol–chloroform method, was dissolved in nuclease-free water, and stored at 4 °C pending assay. TNF- α –308G/A and –238 G/A and LT- α +252A/G gene polymorphism were determined by PCR restriction fragment length polymorphism analysis as described previously, using the following primers: TNF- α –308G/A: sense, 5'-GAG GCA ATA GGT TTT GAG GGC CAT-3' and anti-sense, 5'-GGG ACA CAC AAG CAT CAA G-3'; TNF- α –238G/A: sense, 5'-AAA CAG ACC ACA GAC CTG GTC-3' and anti-sense, 5'-CTC ACA CTC CCC ATC CTC CCG GAT C-3'; LT- α +252A/G: sense, 5'-CTC CTG CAC CTG CTG CCT GGA TC and anti-sense,

5'-GAA GAG ACG TTC AGG TGG TGT CAT. Genotype determination was made following digestion with specific restriction endonuclease (BamHI for TNF- α -238G/A and NcoI for TNF- α -308G/A and LT- α +252A/G).

Statistical analysis

Statistical analysis was performed on SPSS v. 13.0 statistics software (SPSS Inc., Chicago, IL, USA). Quantitative data were presented by their mean ($\pm$ s.d). Pearson's χ^2 and Fisher's exact tests were used to compare the allele and genotype frequencies. LD analysis, the non-random association between two loci, was calculated using the HLAStat 2000 software. Haplotype frequency was determined using the H-Plus haplotype estimation software (<http://cougar.fhcr.org/hplus/>). Logistic regression analysis, performed in a monovariate then multivariate way, taking the control women as the reference group, was performed in order to determine the crude OR and 95% CI associated with the risk of recurrent idiopathic pregnancy loss. Statistical significance was set at $P < 0.05$.

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