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ARTICLE

Association between leukaemia inhibitory factor gene polymorphism and pregnancy outcomes after assisted reproduction techniques

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Abstract Certain gene polymorphisms are associated with implantation failure and pregnancy loss. Studies of leukaemia inhibitory factor (LIF) gene polymorphisms are scarce. The LIF single nucleotide polymorphism (SNP) thymine (T)/guanine (G) (rs929271) was studied in women to determine whether an association existed with pregnancy outcomes after intracytoplasmic sperm injection (ICSI); 411 women who underwent ICSI were recruited. DNA was extracted from the peripheral blood, and the *LIF* gene SNP T/G (rs929271) was genotyped using real-time polymerase chain reaction. Participants were divided into three groups according to their LIF genotype: T/T ($n = 168$), T/G ($n = 202$) and G/G ($n = 41$). All IVF and ICSI procedures were carried out under the same clinical and laboratory conditions. The ICSI cumulative results (from fresh plus frozen cycles) of each genotype group were analysed. The G/G genotype in women was associated with a higher implantation rate (T/T: 15.9%, T/G: 16.2%, G/G: 27.0%; $P < 0.05$), ongoing pregnancy rate/patient (T/T: 31.5%, T/G: 36.1%, G/G: 53.7%; $P < 0.05$) and ongoing pregnancy rate/transfer (T/T: 18.5%, T/G: 20.2%, G/G: 36.7%; $P < 0.05$). LIF SNP T/G (rs929271) seems to be a susceptibility biomarker capable of predicting implantation efficiency and pregnancy outcomes.

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Introduction

Most women undergoing IVF and intracytoplasmic sperm injection (IVF-ICSI) can achieve embryo transfer with good-quality embryos upon routine assessment. The embryo implantation rate, however, and therefore the pregnancy rate, is consistently low despite advances in ovarian stimulation and in-vitro embryo culture and development. It is estimated that more than two-thirds of transferred embryos do not implant (CDC Centers for Disease Control and Prevention, 2012; Simon and Laufer, 2012). In fact, considering the losses during pregnancy in addition to implantation failures, only a small percentage of all women undergoing IVF-ICSI cycles have a live birth.

The successful implantation of a good-quality human embryo in a receptive endometrium requires a remarkable and complex collaboration of factors (Koot et al., 2012; Sallam, 2005), including cytokines, growth factors and hormones. Recently, the involvement of genetic factors, including polymorphisms, in the implantation process has been highlighted, especially in patients who experience repeated IVF implantation failures and recurrent spontaneous abortions. The over- or underexpression of genes that encode the proteins required for embryo implantation may be associated with these clinical issues (Madon et al., 2005; Urman et al., 2005).

Leukaemia inhibitory factor (LIF) is a multifunctional pleiotropic cytokine member of the interleukin-6 family. It was first described as a factor that inhibits the proliferation of murine myeloid leukaemic cells and induces their differentiation into macrophages (Gearing et al., 1987; Hilton et al., 1988). In humans, LIF regulates various functions and is produced by several types of cells, such as fibroblasts, osteoblasts, hepatocytes, monocytes, macrophages and T cells (Mathieu et al., 2012; Metcalf, 2003). It plays a critical role in the reproductive process and is considered essential to successful pregnancy in humans (Franasiak et al., 2014; Fritz et al., 2014; Güney et al., 2007; Kimber, 2005). A higher concentration of LIF in the follicular fluid is correlated with embryo quality, which suggests that LIF has an important role in the physiology of ovulation and early embryonic development (Arici et al., 1997). In humans, LIF controls the uterine receptivity to blastocyst implantation and trophoblastic function by promoting proliferation, invasion and differentiation (Fitzgerald et al., 2008). In the endometrium, both glandular and luminal epithelial cells express LIF; however, LIF expression is more intense in the glandular epithelium with high expression during early implantation (Aghajanova, 2004; Aghajanova et al., 2009; Hu et al., 2011; Laird et al., 1997). Unlike LIF, the expression of LIF receptor (LIFR) is greater in the endometrial luminal epithelial cells than in the glandular epithelial cells. LIF binds to the LIFR and recruits its co-receptor, interleukin 6 signal transducer (IL6ST), forming a high-affinity signalling complex (Lass et al., 2001). Following the attachment of the blastocyst to the endometrium, the trophoblast also begins expressing LIF, which may have an autocrine effect on its physiological functions (Charnock-Jones et al., 1994; Conquet and Brûlet, 1990; Kojima et al., 1995). Villous and extravillous trophoblasts express LIF and its receptor during pregnancy (Sharkey et al., 1999).

Changes in the expression of the *LIF* gene, which encodes this cytokine that is critical for implantation, have been associated with infertility. Although the importance of *LIF* gene variants in human fertility has been investigated, few studies have analysed the correlation between *LIF* gene changes and reproductive capacity. A higher prevalence of mutations near the start codon of exon 1 and in exon 3, regions that are functionally important for controlling the biological activity of LIF, was observed in infertile women and has been associated with unexplained infertility and recurrent implantation failure after IVF and embryo transfer (Giess et al., 1999; Kralickova et al., 2006; Novotny et al., 2009; Steck et al., 2004).

The single nucleotide polymorphism (SNP) thymine (T)/guanine (G) located in the untranslated region 3′ (3′ UTR) (rs929271/ c.1414T > G) is a polymorphism of the *LIF* gene that has recently been investigated. In addition to its reproductive role, LIF acts on the differentiation of neurones. This polymorphism has been positively associated with schizophrenia; in fact, the T-allele and T-carrier genotypes (T/T and T/G) of rs929271 were found to be risk factors for hebephrenic schizophrenia (Okahisa et al., 2010). During the process of reproduction, Kang et al. (2009) observed that the G allele is significantly enriched in patients under the age of 35 years but not in older patients. Furthermore, the G allele was associated with a history of fertility medication use, indicating an association between the G allele and infertility, especially in patients under the age of 35 years (Kang et al., 2009). Ucisik-Akkaya et al. (2010) reported that human embryo survival can be influenced by the genotype LIF T/G (rs929271) in a sex-dependent fashion. In contrast, Fraga et al. (2014) did not observe a correlation between LIF SNP T/G (rs929271) and recurrent pregnancy loss. In the same context, Paskulin et al. (2013) found no correlation between LIF SNP T/G (rs929271) and endometriosis or IVF failure. Furthermore, Tagliani-Ribeiro et al. (2012) analysed the correlation between genetic polymorphism and the incidence of dizygotic and monozygotic twins, and found no difference between cases and controls in the allelic or genotypic frequencies of LIF T/G (rs929271).

On the basis of the small number of published studies, and the need for a better understanding of the action of the *LIF* gene in the human reproductive process, we aimed to determine whether the LIF SNP T/G (rs929271) in women is associated with pregnancy outcomes after ICSI.

Materials and methods

Study participants

A total of 411 women with infertility issues enrolled in the ICSI programme at the Center for Human Reproduction Prof Franco Jr were included in this study. All of the women in the infertile study group met the following inclusion criteria: age 39 years or younger at the time of oocyte retrieval, a normal

karyotype for her and her partner, and no evidence of uterine defects, ultrasonographic evidence of hydrosalpinx, infections, endocrine problems, coagulation defects, and thrombophilia and autoimmune defects (including antiphospholipid antibodies).

In addition, to determine the genotype distribution in the general population, a total of 83 volunteers who had had at least two live births with no infertility treatment and no history of spontaneous abortion were included. All of the women in the control group were post-menopausal to rule out possible future infertility problems after their inclusion in the study. This inclusion criterion was based on published research (Al Sallout and Sharif, 2010; Pietrowski et al., 2005).

Despite the high rate of miscegenation in the Brazilian population, most patients and healthy study participants described their skin colour as 'white'. Written informed consent was obtained from all of the participating women. The local ethics committees approved the study on 26 October 2011 (reference number 045/11).

Genotyping

To study the SNP LIF T/G (rs929271), a sample of peripheral venous blood was collected from each participant into an EDTA-containing tube. The DNA was extracted using the QIAamp DNA Blood mini kit (Qiagen). Genotyping was carried out using real-time polymerase chain reaction (PCR) amplification with separate reactions for each sample using a Taqman SNP genotyping assay (Applied Biosystems). The PCR conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1 min.

The samples were assayed in duplicate following the manufacturer's instructions for the chosen SNP. It used a validated TaqMan® SNP Genotyping Assay (rs929271) in which the context sequence [VIC/FAM] is AACAGTGTGAACCAGC CCCCTGGAA**[G/T]**CAAGACAGAAAGGCACCCGGCCTCT. Fifteen samples of each genotype were sequenced in an automatic sequencer XL 3500 Genetic Analyzer (Applied Biosystems) to validate the genotyping results. The representative allele discrimination plot (TaqMan® Genotyper Software) of LIF T/G polymorphism is shown in **Figure 1**.

Procedures

Two ovarian stimulation protocols were used: a long gonadotrophin releasing hormone (GnRH) agonist (GnRH-a; 54.2% of cycles) protocol or a multi-dose GnRH antagonist (GnRH-ant; 45.8% of cycles) protocol. The stimulation protocol was selected at the clinician's discretion. The starting dose of FSH was based on the patient's age, anti-Müllerian hormone level and antral follicle count (Ovarian Response Prediction Index calculation) (Oliveira et al., 2012).

To induce the final oocyte maturation in both protocols (GnRH-a and GnRH-ant), 250 μg of recombinant HCG

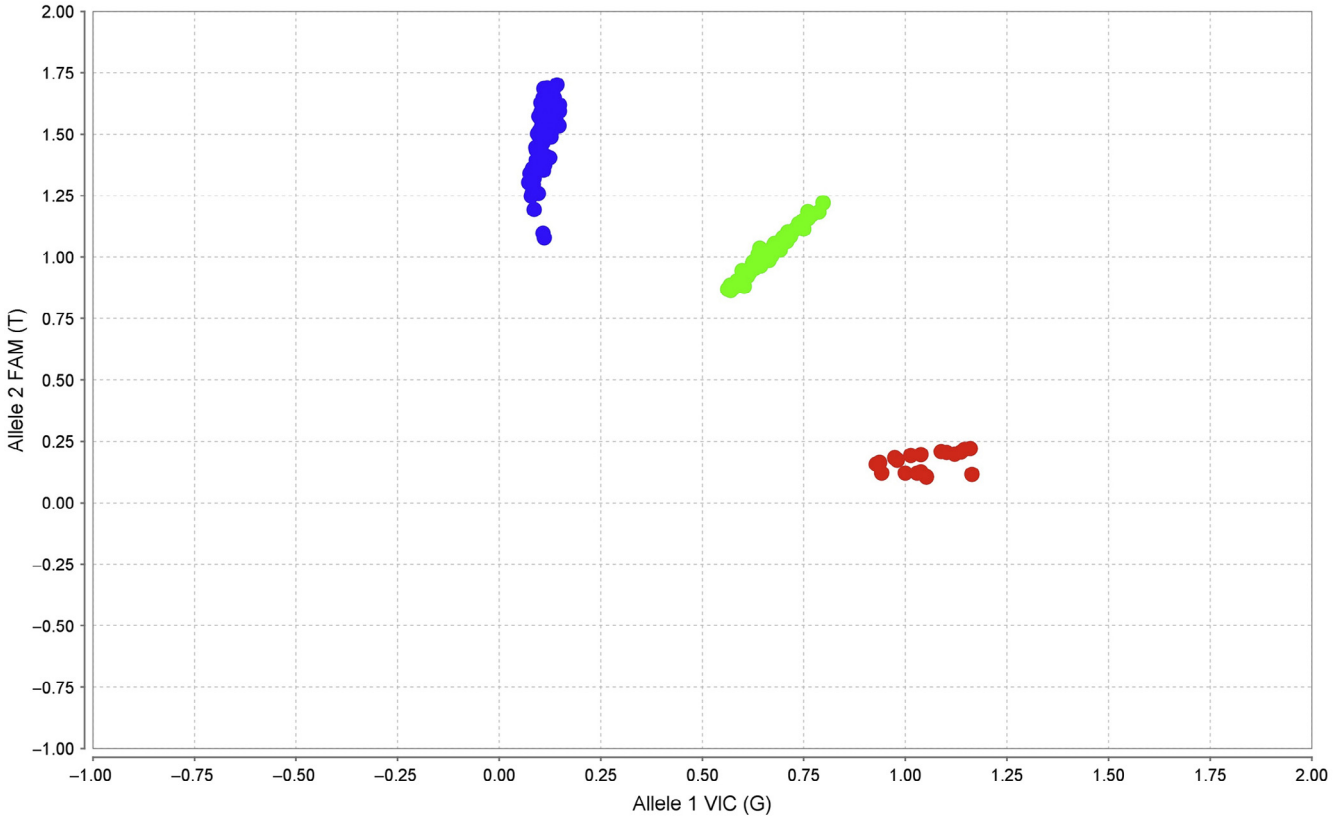

Figure 1 Representative allele discrimination plot (TaqMan® Genotyper Software) of leukaemia inhibitory factor thymine (T)/guanine (G) (rs929271) polymorphism. X axis: Allele 1 VIC, guanine (G); Y axis: Allele 2 FAM, thymine (T) – ancestral allele. Blue circle: T/T genotype; green circle: T/G genotype; red circle: G/G genotype.

(r-hCG; Ovidrel; Serono, Brazil) was administered subcutaneously when at least two follicles reached a mean diameter 17 mm or wider. The oocytes were retrieved by transvaginal aspiration under ultrasound guidance 34–36 h after the recombinant HCG injection. All metaphase II oocytes received ICSI that was carried out as previously described (Mauri et al., 2010; Oliveira et al., 2011). Oocytes were examined after 17–20 h to assess fertilization; zygotes with two distinct equal-sized pronuclei were considered normal. A total of 25–27 h after injection, on day 1 of culture, early cleavage was evaluated. Embryos, graded on day 2, were deemed top quality if there were four identical blastomeres (44 h after the sperm injection) with no fragments or multinucleation (Holte et al., 2007; Saldeen and Sundstrom, 2005). Embryos were routinely transferred after 48 h in culture, and supranumerary embryos were cryopreserved at the end of the second day. The embryos were then transferred with a Frydman catheter (Frydman® Classic Catheter 4.5 CCD Laboratoire C.C.D; Paris, France) guided by abdominal ultrasound using a 3.5-MHz convex transducer (Aloka SSD-1100; Aloka Co. Ltd, Tokyo, Japan). All of the patients received luteal phase supplementation with vaginal natural progesterone (Utrogestan®; Besins Healthcare, Sao Paulo, Brazil).

For the freezing–thawing process, an embryo freeze-thaw media kit (Irvine Scientific, USA) was used. Frozen–thawed embryo transfer was carried out after the assessment of embryo cleavage, when the division of at least one blastomere was observed after 24 h of culturing. The same protocol was used to transfer all of the frozen-thawed embryos. Oestradiol valerate (Ciclopromogyna®; Schering, SP, Brazil) was administered from day 1 to day 14 of the cycle at a daily dose of 6 mg. Progesterone (Utrogestan®; Besins Healthcare) was also introduced vaginally on day 14 at 400 mg/day as long as endometrial thickness was 6 mm or more (Oliveira et al., 1997). The embryo transfers were carried out in the same manner as in fresh cycles.

Pregnancy was diagnosed on the basis of an increase in the serum beta-HCG concentration 14 days after embryo transfer. Implantation and clinical pregnancy rates were based on the presence of a gestational sac and an image of embryonic or fetal cardiac activity on transvaginal ultrasounds 4 weeks after the transfer. The frequency of spontaneous abortion, defined as spontaneous loss of a clinical pregnancy up to 20 weeks of gestation, was based on the number of clinical pregnancies found. Ongoing pregnancy was defined when the pregnancy had completed 20 weeks or more of gestation.

End-points

The primary end-points were implantation and ongoing pregnancy rates in fresh cycles and cumulative (fresh and frozen cycles) cycles.

Statistical analysis

Hardy-Weinberg equilibrium was applied using an online calculator (http://ihg.gsf.de). The data management and univariate analysis were carried out using StatsDirect statistical software version 2.7.9 software (Cheshire, UK). The following parameters were evaluated for each LIF genotype analysed: the woman's age, cause of infertility, number of oocytes retrieved, number of oocytes in metaphase II retrieved, fertilization rate, the number of embryos transferred, the embryo implantation rate, the spontaneous abortion rate, and the ongoing pregnancy rate. The differences in the frequencies of the SNP genotypes, alleles, or both, in the infertile study and control groups were also evaluated.

To compare the means of continuous variables, the non-parametric Mann–Whitney and Kruskal–Wallis tests were used if the continuous variables were not normally distributed, and Student's *t*-test and one-way analysis of variance (ANOVA) were used if the continuous variables were normally distributed. The results are expressed as the arithmetic means ± standard deviation (SD), ranges and medians. For categorical variables, Fisher's exact test was used to check between-group associations, and the results are expressed as percentages. In addition, univariate and multivariate logistic regression analyses were used to determine the significance of the variables as determinants of implantation, spontaneous abortion and ongoing pregnancy. $P < 0.05$ was considered statistically significant.

Results

Hardy–Weinberg equilibrium

Genotype and allele distributions in both the patients and the controls conformed to expectations under Hardy–Weinberg equilibrium.

Genotype and allele distribution

The genotypic and allelic frequencies of LIF among the infertile study and control groups are presented in **Table 1**. The analysis of *LIF* gene polymorphisms showed that the heterozygous genotype (T/G) was prevalent in both groups (infertile study group: 49.1%; control group: 54.2%), with a T allele frequency of 65.5% in the infertile study group and 68.1% in

Table 1 Genotype and allele frequencies of gene leukaemia inhibitory factor thymine (T)/guanine (G) (rs929271) polymorphisms in women who underwent IVF with intracytoplasmic sperm injection and fertile controls.[a]

LIF T/G (rs929271)	*Infertile study group (n = 411)*, n %	*Control group (n = 83)*
Genotypes		
T/T	168/411 (40.9)	34/83 (41.0)
T/G	202/411 (49.1)	45/83 (54.2)
G/G	41/411 (10.0)	4/83 (4.8)
Alleles		
T	538/822 (65.5)	113/166 (68.1)
G	284/822 (34.5)	53/166 (31.9)

LIF = leukaemia inhibitory factor.
[a]No statistically significant differences were found between the two groups.

the control group. No statistically significant difference was found in the distribution of genotypes and alleles among the infertile patients and the control patients.

Demographic and ovarian stimulation cycle characteristics

Basic demographic characteristics, such as age, BMI, duration of infertility, cause of infertility and semen quality were not significantly different among the three LIF genotypes groups (T/T/, T/G and G/G) (Table 2).

Similarly, the distribution of the main characteristics of the ovarian stimulation cycle, the number of fresh embryo transfer cycles, the number of frozen–thawed embryo transfer cycles and the number of embryos transferred did not differ among the LIF groups (Table 3).

Outcomes of fresh cycles

The implantation and pregnancy rates in fresh cycles are shown in Table 4. The implantation rate was 16.8% in the homozygotic T/T group, 17.8% in the heterozygotic T/G group, and 30.1% in the homozygotic G/G group. No significant difference was found between the T/T and T/G groups ($P = 0.69$). The implantation rate in the G/G group was significantly higher than in either T/T and T/G groups ($P = 0.002$ and $P = 0.006$, respectively).

Table 2 Main characteristics of infertile women, according to their leukaemia inhibitory factor thymine (T)/guanine (G) (rs929271) genotype groups, and the controls.

Characteristics	*Women's genotypes*				
	LIF T/G (rs929271) groups				
	Infertile study group				*Control group*
	Total	T/T	T/G	G/G	Total
N	411	168	202	41	83
Age (years)	35.6 ± 4.2	35.8 ± 4.1	35.4 ± 4.3	35.3 ± 4.5	51.4 ± 9.8
BMI (kg/m^2)	24.0 ± 3.4	24.2 ± 4.0	23.8 ± 3.6	24.3 ± 3.8	29.1 ± 4.6
ORPI (AMHxAFC/age)	2.3 ± 6.8	2.1 ± 6.8	2.4 ± 6.7	2.7 ± 7.8	–
Time of infertility (years)	4.2 ± 3.1	3.9 ± 2.8	4.4 ± 3.4	4.1 ± 3.1	–
Infertility					–
Primary, *n* (%)	267 (65.0)	102 (60.7)	138, 68.3	27 (65.9)	
Secondary, *n* (%)	144 (35.0)	66 (39.3)	64 (31.7)	14 (31.1)	
Cause of infertility					–
Idiopathic	110 (26.8)	39 (23.2)	61 (30.2)	10 (24.4)	
Male	154 (37.5)	64 (38.1)	69 (34.2)	21 (51.2)	
Endometriosis	55 (13.4)	22 (13.1)	30 (14.9)	3 (7.3)	
Tuboperitoneal	51 (12.4)	26 (15.5)	21 (10.4)	4 (9.8)	
Tuboperitoneal + endometriosis	15 (3.6)	8 (4.8)	7 (3.5)	0	
Male plus tuboperitoneal	11 (2.7)	4 (2.4)	6 (3.0)	1 (2.4)	
Male plus endometriosis	14 (3.4)	4 (2.4)	8 (4.0)	2 (4.9)	
Male plus endometriosis plus tuboperitoneal	1 (0.2)	1 (0.6)	0	0	
Semen parameters					–
Total sperm count (×10^6/ml)[a]	64.7 ± 79.5	63.8 ± 59.0	66.3 ± 98.0	60.7 ± 51.8	
Motility (% spermatozoa) (rapid plus slow progression)[a]	51.0 ± 18.3	51.9 ± 15.5	51.9 ± 17.9	49.4 ± 19.3	
Normal spermatozoa (%)[b]	0.7 ± 0.9	0.8 ± 1.1	0.6 ± 0.9	0.6 ± 0.6	
Vitality (%)[a]	63.8 ± 11.9	65.1 ± 13.9	63.0 ± 15.0	64.8 ± 13.9	
DNA fragmentation (%)[c]	14.1 ± 8.0	14.4 ± 8.7	13.7 ± 7.4	14.6 ± 8.8	
Abnormal chromatin packaging/underprotamination (%)[d]	55.7 ± 16.5	53.4 ± 17.8	56.3 ± 16.3	60.3 ± 12.2	
Apoptosis (%)[e]	19.5 ± 6.5	18.9 ± 6.9	20.0 ± 6.5	18.5 ± 4.3	
Previous IVF-ICS cycles					–
Yes	203 (49.4)	75 (44.6)	104 (51.5)	17 (41.5)	
No	208 (50.6)	93 (55.4)	98 (48.5)	24 (58.5)	
Number of previous IVF-ICSI cycles	1.7 ± 2.1	1.7 ± 2.1	1.7 ± 2.2	1.3 ± 1.7	–

No statistically significant differences were found among the three leukaemia inhibitory factor genotypes groups.
BMI = body mass index; LIF = leukemia inhibitory factor; ORPI = ovarian response prediction index (Oliveira et al., 2012).
[a]According to the World Health Organization (WHO, 2010).
[b]According to motile sperm organelle morphology examination (MSOME).
[c]The percentages of DNA fragmentation by TUNEL assay.
[d]Abnormal chromatin packaging by chromomycin A3.
[e]Apoptosis by annexin-V.

Table 3 Ovarian stimulation cycle characteristics of the study population according to leukaemia inhibitory factor thymine (T)/guanine (G) (rs929271) genotype groups.[a]

Cycle	*Women's genotypes*			
Characteristics	*LIF T/G (rs929271) groups*			
	Total	T/T	T/G	G/G
Number	411	168	202	41
Long GnRH agonist protocol, *n* (%)	231 (56.2)	91 (54.2)	118 (58.4)	22 (53.7)
Multi-dose GnRH antagonist protocol, *n* (%)	180 (43.8)	77 (45.8)	84 (41.6)	19 (46.3)
Duration of r-FSH/rLH therapy (days)	10.3 ± 2.4	10.3 ± 2.5	10.3 ± 2.2	10.6 ± 2.8
FSH doses (IU)/cycle	2195 ± 905	2200 ± 941	2256 ± 908	1901 ± 676
LH dose (IU)/cycle	1063 ± 377	1065 ± 416	1064 ± 352	1040 ± 337
Oocytes/cycle (*n*)				
Total	8.3 ± 4.9	8.5 ± 4.9	8.0 ± 5.1	9.0 ± 4.4
MII	6.3 ± 4.2	6.5 ± 4.1	6.0 ± 4.2	7.0 ± 3.7
Top-quality embryos, *n* (%)	989/1503 (65.8)	411/630 (65.2)	492/747 (65.9)	86/126 (68.3)
Transfers (*n*)				
Fresh embryo transfer	1.4 ± 0.8	1.3 ± 0.8	1.4 ± 0.9	1.2 ± 0.5
Frozen–thawed embryo transfer	0.4 ± 0.6	0.4 ± 0.6	0.4 ± 0.6	0.3 ± 0.4
Cumulative (fresh plus frozen)	1.7 ± 1.0	1.7 ± 1.0	1.7 ± 1.0	1.6 ± 0.6
Total embryos transferred (*n*)				
Fresh	3.0 ± 2.2	3.1 ± 2.3	3.0 ± 2.1	2.5 ± 1.7
Frozen	0.7 ± 1.2	0.7 ± 1.2	0.7 ± 1.3	0.5 ± 0.9
Fresh plus frozen	3.7 ± 2.5	3.7 ± 2.6	3.7 ± 2.6	3.1 ± 1.7

GnRH = gonadotrophin-releasing hormone; LIF = leukemia inhibitory factor; MII = second metaphase; r-FSH = recombinent FSH; rLH = recombinent LH.
[a]No statistically significant differences were found among the three LIF genotypes groups.

Table 4 Relationship between women's leukaemia inhibitory factor T/G (rs929271) polymorphisms and implantation and pregnancy rates after fresh intracytoplasmic sperm injection cycles.[a]

Clinical outcomes of gresh cycles	*Women's genotypes LIF T/G (rs929271) groups*				
	Total	T/T	T/G	G/G	*P*
Number	411	168	202	41	
Implantation rate	225/1223 (18.4)	87/519 (16.8)[a]	107/601 (17.8)[b]	31/103 (30.1)[a,b]	0.002[a] 0.006[b]
Spontaneous abortion rate/clinical pregnancy, *n* (%)	51/178 (28.7)	26/70 (37.1)	20/84 (23.8)	5/24 (20.8)	NS
Ongoing pregnancy rate/transfer, *n* (%)	127/560 (22.7)	44/225 (19.6)[a]	64/286 (22.4)[b]	19/49 (38.8)[a,b]	0.007[a] 0.01[b]
Ongoing pregnancy rate/patient, *n* (%)	27/411 (30.9)	44/168 (26.2)[a]	64/202 (31.7)	19/41 (46.3)[a]	0.02 [a]

LIF = leukaemia inhibitory factor; NS = non-significant.
[a]Values within rows with the same superscript letter were significantly different.

The spontaneous abortion rate was 37.1% in the homozygotic T/T group, 23.8% in the heterozygotic T/G group, and 20.8% in the homozygotic G/G group. No significant difference was found among the three LIF genotype groups.

The ongoing pregnancy rate per transfer was 19.6% in the homozygotic T/T group, 22.4% in the heterozygotic T/G group and 38.8% in the homozygotic G/G group. No significant difference was found between the T/T and T/G groups ($P = 0.44$). The ongoing pregnancy rate per transfer in the G/G group was significantly higher than in the either T/T and T/G groups ($P = 0.007$ and $P = 0.01$), respectively.

The ongoing pregnancy rate per patient was 26.2% in the homozygotic T/T group, 31.7% in the heterozygotic T/G group and 46.3% in the homozygotic G/G group. No significant difference was found between the T/T and T/G groups ($P = 0.24$) and between the T/G and G/G groups ($P = 0.10$). The ongoing pregnancy rate per patient in the G/G group, however, was significantly higher than in the T/T group ($P = 0.02$).

Regression analysis of fresh cycles

Univariate and multivariate logistic regression analysis, effects of variables to predict embryo implantation and ongoing clinical pregnancy ICSI cycles are shown in **Table 5**. Univariate logistic regression analysis revealed that LIF GG genotype ($P = 0.02$) was a factor that significantly predicted embryo implantation. As expected, women's age ($P = 0.002$), number of oocytes retrieved ($P = 0.0004$), number of oocytes in metaphase II retrieved ($P = 0.001$), and percentage of top-quality

Table 5 Univariate and multivariate logistic regression analysis of different variables to predict implantation, spontaneous abortion and ongoing pregnancy after fresh intracytoplasmic sperm injection cycles.

Variable	*Implantation*			*Spontaneous abortion*			*Ongoing pregnancy*		
	OR	*95% CI*	P	*OR*	*95% CI*	P	*OR*	*95% CI*	P
Univariate analysis									
LIF Genotypes (rs929271)									
T/T	0.87	0.58 to 1.31	NS	1.88	0.96 to 3.67	NS	0.68	0.44 to 1.06	NS
T/G	0.85	0.57 to 1.27	NS	0.67	0.34 to 1.32	NS	1.06	0.69 to 1.61	NS
G/G	2.15	1.10 to 4.19	0.02	0.59	0.20 to 1.69	NS	2.12	1.09 to 4.10	0.02
Age (years)	0.93	0.88 to 0.97	0.002	1.21	1.10 to 1.32	0.0001	0.87	0.82 to 0.92	0.0001
BMI (Kg/m^2)	0.96	0.91 to 1.01	NS	1.07	0.98 to 1.18	NS	0.95	0.90 to 1.01	NS
ORPI (AMHxAFC/age)	1.03	0.99 to 1.07	NS	0.95	0.88 to 1.03	NS	1.03	0.99 to 1.07	NS
Time of infertility (years)	0.97	0.90 to 1.03	NS	0.99	0.86 to 1.13	NS	0.92	0.86 to 1.53	NS
Infertility: primary/secondary	0.71	0.46 to 1.10	NS	1.70	0.83 to 3.46	NS	0.60	0.77 to 2.02	NS
Cause of infertility									
Idiopathic	1.00	0.64 to 1.56	NS	1.42	0.69 to 2.94	NS	0.84	0.51 to 1.36	NS
Male	1.016	0.68 to 1.51	NS	0.86	0.44 to 1.69	NS	1.11	0.72 to 1.70	NS
Endometriosis	1.56	0.96 to 2.56	NS	1.11	0.52 to 2.37	NS	1.45	0.87 to 2.40	NS
Tuboperitoneal	0.73	0.43 to 1.22	NS	0.77	0.30 to 1.95	NS	0.78	0.45 to 1.37	NS
Semen parameters									
Total sperm count (×10^6/ml)[a]	1.00	0.99 to 1.01	NS	1.00	0.99 to 1.01	NS	1.00	0.99 to 1.01	NS
Motility (% spermatozoa) (rapid and slow progression)[a]	1.00	0.98 to 1.01	NS	1.04	0.98 to 1.06	NS	0.99	0.97 to 1.01	NS
Normal spermatozoa (%)[b]	1.09	0.87 to 1.35	NS	1.41	0.87 to 1.96	NS	0.89	0.70 to 1.14	NS
Vitality(%)[a]	1.00	0.98 to 1.01	NS	1.02	0.99 to 1.04	NS	0.99	0.98 to 1.01	NS
DNA fragmentation (%)[c]	1.00	0.97 to 10.4	NS	1.01	0.97 to 1.06	NS	0.98	0.94 to 1.02	NS
Abnormal chromatin packaging/ underprotamination[d] (%)	1.00	0.98 to 1.01	NS	0.99	0.97 to 1.02	NS	1.00	0.98 to 1.02	NS
Apoptosis (%)[e]	1.00	0.97 to 1.03	NS	0.96	0.90 to 1.03	NS	1.00	0.97 to 1.04	NS
Previous IVF-ICSI cycles	0.73	0.50 to 1.1	NS	1.23	0.63 to 2.41	NS	1.03	0.83 to 1.42	NS
Number of previous IVF-ICSI cycles	0.96	0.87 to 1.06	NS	1.15	0.81 to 1.34	NS	0.99	0.80 to 1.05	NS
Ovarian stimulation protocol	0.89	0.59 to 1.33	NS	0.63	0.32 to 1.26	NS	1.03	0.67 to 1.6	NS
Duration of r-FSH/rLH therapy (days)	1.00	0.99 to 1.2	NS	0.99	0.86 to 1.13	NS	1.06	0.98 to 1.2	NS
FSH doses (IU)/cycle	1.00	0.99 to 1.01	NS	1.00	0.97 to 1.05	NS	1.00	0.98 to 1.02	NS
LH dose (IU)/cycle	1.00	0.99 to 1.01	NS	1.00	0.98 to 1.01	NS	1.00	0.99 to 1.01	NS
Oocytes/cycle (*n*)									
Total	1.06	1.01 to 1.11	0.004	0.92	0.85 to 1.05	NS	1.10	1.03 to 1.13	0.0007
MII	1.10	1.05 to 1.16	0.001	0.92	0.84 to 1.02	NS	1.10	1.05 to 1.16	0.0001
Top-quality embryos (%)	1.02	1.01 to 1.03	0.0001	0.99	0.98 to 1.01	NS	1.02	1.01 to 1.03	0.0001
Transfers (*n*)	1.15	0.89 to 1.5	NS	1.66	0.87 to 4.8	NS	1.71	0.89 to 2.07	NS
Total embryos transferred (*n*)	1.11	0.81 to 1.22	NS	1.53	0.89 to 1.93	NS	0.92	0.82 to 1.02	NS
Multivariate analysis									
LIF genotypes (rs929271)				–	–	–			
G/G	2.21	1.07 to 4.5	0.03				2.90	1.09 to 3.93	0.03
Age (years)	0.92	0.87 to 0.97	0.004	–	–	–	0.90	0.84 to 0.95	0.007
Oocytes/cycle (*n*)				–	–	–			
Total	0.91	0.82 to 1.01	0.09				0.98	0.87 to 1.10	NS
MII	1.14	1.02 to 1.03	0.02				1.09	0.95 to 1.24	NS
Top-quality embryos (%)	1.02	1.01 to 1.03	<0.0001	–	–	–	1.02	1.01 to 1.03	0.001

BMI = body mass index; ICSI = racytoplasmic sperm injection; MII = second metaphase; NS = non-significant; ORPI = ovarian response prediction index (Oliveira et al., 2012); r-FSH = combinent FSH; rLH = combinent LH.

[a]According to the World Health Organization (2010).

[b]According to motile sperm organelle morphology examination (MSOME).

[c]The percentages of DNA fragmentation by TUNEL assay.

[d]Abnormal chromatin packaging by chromomycin A3.

[e]Apoptosis by annexin-V.

Table 6 Logistic regression analysis among three leukaemia inhibitory factor SNP T/G (rs929271) genotypes and the prediction of embryo implantation, spontaneous abortion and ongoing pregnancy after fresh ICSI cycles.

LIF genotypes (rs929271)	*Implantation*			*Spontaneous abortion*			*Ongoing pregnancy*		
	OR	*95% CI*	P	*OR*	*95% CI*	P	*OR*	*95% CI*	P
GG	*Reference*			*Reference*			*Reference*		
TG	2.15	1.08 to 4.31	0.03	0.79	0.26 to 2.39	NS	1.89	0.95 to 3.77	NS
TT	2.15	1.06 to 4.35	0.03	0.44	0.14 to 1.33	NS	2.44	1.20 to 4.97	0.01

ICSI = intracytoplasmic sperm injection; LIF = leukaemia inhibitory factor; NS = non-significant; SNP = single nucleotide polymorphism.

Table 7 Relationship between women's leukaemia inhibitory factor T/G (rs929271) polymorphisms and implantation and pregnancy rates after intracytoplasmic sperm injection in cumulative (fresh plus frozen–thawed) cycles.[a]

Clinical outcomes cumulative (fresh plus frozen–thawed) cycles	*Women's genotypes LIF T/G (rs929271) groups*				
	Total n *(%)*	*T/T* n *(%)*	*T/G* n *(%)*	*G/G* n *(%)*	P
Implantation rate	255/1503 (17.0)	100/630 (15.9)[a]	121/747 (16.2)[b]	34/126 (27.0)[a,b]	0.004[a] 0.005[b]
Spontaneous abortion rate	58/206 (28.2)	30/83 (36.1)	23/96 (24.0)	5/27 (18.5)	NS
Ongoing pregnancy rate/transfer	148/709 (20.9)	53/287 (18.5)[a]	73/362 (20.2)[b]	22/60 (36.7)[a,b]	0.003[a] 0.007[b]
Ongoing pregnancy rate/patient	148/411 (36.0)	53/168 (31.5)[a]	73/202 (36.1)	22/41 (53.7)[a]	0.01[a]

LIF = leukemia inhibitory factor; NS = non-significant.
[a]Values within rows with the same superscript letter were significantly different.

embryo ($P = 0.0001$) were also factors that significantly predicted implantation. With multivariate analysis, LIF GG genotype remained as a significant independent factor to predict embryo implantation ($P = 0.03$).

Univariate logistic regression analysis did not demonstrate a relationship between LIF SNP T/G (rs929271) genotypes and spontaneous abortion. Only women's age ($P = 0.0001$) was a factor that significantly predicted spontaneous abortion.

Univariate logistic regression analysis revealed that LIF GG genotype ($P = 0.02$) was a factor that significantly predicted ongoing pregnancy. As expected, women's age ($P = 0.0001$), number of oocytes retrieved ($P = 0.0007$), number of oocytes in metaphase II retrieved ($P = 0.0001$) and percentage of top-quality embryos ($P = 0.0001$) were also factors that significantly predicted implantation. With multivariate analysis, LIF GG genotype remains as a significant independent factor to predict ongoing pregnancy ($P = 0.03$).

Logistic regression analysis among three LIF SNP T/G (rs929271) genotypes and the prediction of embryo implantation, spontaneous abortion and ongoing pregnancy in ICSI cycles, using GG genotype as a reference is shown in **Table 6**. Women with GG genotype presented 2.2 times more chance of occurrence of embryo implanting than women with TG genotype. Similarly, women with GG genotype also presented 2.2 times more chance of occurrence of embryo implanting than women with TT genotype. Regarding the occurrence of spontaneous abortion, no significant difference among the three genotypes was found. On the other hand, women with genotype GG were twice as likely to have an ongoing pregnancy compared with women with TT genotype. No significant difference, however, was found between the genotypes GG and TG in pregnancy.

Outcomes in cumulative cycles

Cumulative (fresh plus frozen–thawed) cycle outcomes are shown in **Table 7**. The implantation rate was 15.9% in the homozygotic T/T group, 16.2% in the heterozygotic T/G group and 27.0% in the homozygotic G/G group. As for the fresh cycles, no significant difference was found between the T/T and T/G groups ($P = 0.88$), but the implantation rate in the G/G group was significantly higher than in either the T/T or T/G group ($P = 0.004$ and $P = 0.005$, respectively).

The spontaneous abortion rate was 36.1% in the homozygotic T/T group, 24.0% in the heterozygotic T/G group, and 18.5% in the homozygotic G/G group. No significant difference was found among the three LIF genotype groups.

The ongoing pregnancy rate per transfer was 18.5% in the homozygotic T/T group, 20.2% in the heterozygotic T/G group, and 36.7% in the homozygotic G/G group. No significant difference was found between the T/T and T/G groups ($P = 0.61$). The ongoing pregnancy rate per transfer in the G/G group was significantly higher than in either the T/T ($P = 0.003$) or the T/G group ($P = 0.007$). The ongoing pregnancy rate per patient was 31.5% in the homozygotic T/T group, 36.1% in the heterozygotic T/G group, and 53.7% in the homozygotic G/G group. No significant difference was found between the T/T and T/G groups ($P = 0.37$) and between the T/G and G/G

groups ($P = 0.05$). The ongoing pregnancy rate per patient in the G/G group was significantly higher than in the T/T group ($P = 0.01$).

Regression analysis of cumulative cycles

Univariate and multivariate logistic regression analysis and effects of variables to predict embryo implantation and ongoing pregnancy in cumulative ICSI cycles are shown in **Table 8**. Univariate logistic regression analysis revealed that LIF GG genotype ($P = 0.01$) was a factor that significantly predicted embryo implantation. As expected, women's age ($P = 0.0002$), number of oocytes retrieved ($P = 0.0001$), number of oocytes in metaphase II retrieved ($P = 0.0001$), and percentage of top-quality embryo ($P = 0.0001$) were also factors that significantly predicted implantation. With multivariate analysis, LIF GG genotype remained as a significant independent factor to predict embryo implantation ($P = 0.02$).

Univariate logistic regression analysis did not demonstrate a relationship between LIF SNP T/G (rs929271) genotypes and spontaneous abortion. Only women's age ($P = 0.0001$) was a factor that significantly predicted spontaneous abortion.

Univariate logistic regression analysis revealed that LIF GG genotype ($P = 0.01$) was a factor that significantly predicted ongoing pregnancy. As expected, women's age ($P = 0.0001$), number of oocytes retrieved ($P = 0.0001$), number of oocytes in metaphase II retrieved ($P = 0.0001$), and percentage of top-quality embryo ($P = 0.0001$) were also factors that significantly predicted ongoing pregnancy. With multivariate analysis, LIF GG genotype remains as a significant independent factor to predict ongoing pregnancy ($P = 0.02$).

Logistic regression analysis among three LIF SNP T/G (rs929271) genotypes and the prediction of embryo implantation and ongoing pregnancy in cumulative ICSI cycles, using GG genotype as a reference are shown in **Table 9**. Women with GG genotype presented 2.4 times more chance of occurrence of embryo implanting than women with TG genotype. Similarly, women with GG genotype also presented 2.3 times more chance of occurrence of embryo implanting than women with TT genotype. Regarding the occurrence of spontaneous abortion, no significant difference was found among the three genotypes. On the other hand, women with genotype GG presented 2.1 and 2.6 times more likely to become pregnant compared with women with TG and TT genotypes, respectively.

Discussion

LIF is a pluripotent cytokine that plays a role in embryo implantation. Reduced production of this cytokine is common in the uterine microenvironment of infertile women. Because no correlation was observed between the LIF concentrations in uterine flushing and in serum, measurements of LIF in blood samples do not seem to be applicable to these analyses. Conversely, LIF measurement in uterine flushing can be a useful diagnostic tool; however, it is a laborious procedure and is difficult to use for predicting implantation (Mikolajczyk et al., 2003). Genetic control could be an option for indirect assessment, but the association between genetic background and female fertility is still largely unknown.

The results obtained in the present study demonstrate improved implantation and ongoing pregnancy rates after ICSI treatment in infertile homozygous G/G women. In other words, these results indicate that, although the T allele is more common, it seems to be associated with worse outcomes. Other investigators analysed different SNPs in the *LIF* gene and also reported their influence on IVF treatment outcomes. Novotny et al. (2009) analysed 15 infertile women with the G to A transition at position 3400 and observed that in mutation-positive women, idiopathic infertility and endometriosis have a negative effect on the outcome of IVF treatment. In their study of sequence mutations in *LIF* genes among pregnant and non-pregnant women who underwent IVF, Sabry et al. (2014) detected a DNA sequence transition from C (in IVF positive pregnancy cases) to T (in IVF negative pregnancy cases) at nucleotide 351 (NT_011520.12). To the best of our knowledge, this present study is the first to relate the LIF SNP T/G (rs929271) with clinical outcomes after IVF treatment; therefore, a comparison of results is difficult.

Kang et al. (2009) observed that the G allele is significantly enriched in patients aged less than 35 years, but not in older patients. This higher prevalence of the G allele among young women could influence pregnancy outcomes. The data obtained in the present study, however, do not support this assumption; instead, they show a similar distribution of genotypes and alleles in the infertile study group and the control group, even when only the patients of infertile study group aged younger than 35 years ($n = 161$) were considered (TT: 37.3%, T/G: 51.5%, G/G: 11.2; T: 63%, G: 37%). Population characteristics and the sample size might explain these differences. Fraga et al. (2014) and Paskulin et al. (2013) also analysed the Brazilian population and demonstrated distributions of LIF (rs929271) polymorphism genotypes or alleles that were similar to the distributions observed in the current study. Paskulin et al. (2013) also found no difference in the distribution of LIF (rs929271) polymorphism genotypes or alleles between fertile and infertile women, which is similar to our findings. Despite a significantly higher embryo implantation rate among homozygous G/G women in our study, no differences were observed in twinning rates among the three genotypes of LIF (rs929271) polymorphism in the infertile population (unpublished observation), which is in agreement with the finding obtained by Tagliani-Ribeiro et al. (2012) for the general population.

Our understanding of the clinical implications of the LIF SNP (rs929271) includes the current knowledge about gene expression. For a given gene, untranslated regions, including 5' and 3' UTRs and introns, are the main regions involved in the regulation of gene expression (Barrett et al., 2012, 2013). Variations within these non-coding sequences produce phenotypic variation among individuals (Mattick, 2001). In molecular genetics, a UTR refers to either of two sections, one on each side of a gene that encodes a protein: the 5′ side (the 5′ UTR or leader sequence) or the 3′ side (the 3′ UTR) located downstream from the protein-coding sequence. Similar to the 5′ region, the 3′ UTR (the site of the SNP (rs929271) in the *LIF* gene) is transcribed into mRNA but not translated into protein. These segments contain DNA elements involved in regulating gene expression through transcript cleavage, stability and polyadenylation, translation and

Table 8 Univariate and multivariate logistic regression analysis of different variables to predict implantation, spontaneous abortion and ongoing pregnancy after cumulative (fresh plus frozen-thawed) intracytoplasmic sperm injection cycles.

Variable	*Implantation*			*Spontaneous abortion*			*Ongoing pregnancy*		
	OR	*95% CI*	P	*OR*	*95% CI*	P	*OR*	*95% CI*	P
Univariate analysis									
LIF genotypes (rs929271)									
T/T	0.90	0.61 to 1.34	NS	1.03	0.95 to 3.08	NS	0.71	0.46to 1.07	NS
T/G	0.81	0.54t o 1.19	NS	0.66	0.35 to 1.25	NS	0.96	0.67 to 1.51	NS
G/G	2.40	1.21 to 4.71	0.01	0.50	0.18 to 1.41	NS	2.29	1.19 to 4.40	0.01
Age (years)	0.91	0.87 to 0.95	0.0002	1.22	1.11 to 1.33	0.0001	0.85	0.81 to 0.90	0.0001
BMI (kg/m^2)	0.95	0.90 to 1.01	NS	1.05	0.96 to 1.15	NS	0.95	0.91 to 1.01	NS
ORPI (AMH × AFC/age)	1.03	0.99 to 1.07	NS	0.95	0.88 to 1.03	NS	1.03	0.99 to 1.1	NS
Time of infertility (years)	1.09	0.95 to 1.19	NS	0.98	0.85 to 1.11	NS	1.08	0.99 to 1.18	NS
Infertility primary/secondary	0.72	0.47 to 1.10	NS	1.80	0.92 to 3.50	NS	0.56	0.36 to 1.19	NS
Cause of infertility									
Idiopathic	1.08	0.69 to 1.67	NS	1.54	0.78 to 3.02	NS	0.79	0.50 to 1.27	NS
Male	0.99	0.67 to 1.47	NS	0.77	0.40 to 1.45	NS	1.19	0.79 to 1.78	NS
Endometriosis	1.29	0.80 to 2.08	NS	1.17	0.56 to 2.43	NS	1.27	0.78 to 2.08	NS
Tuboperitoneal	0.80	0.48 to 1.32	NS	0.74	0.31 to 1.75	NS	0.88	0.52 to 1.49	NS
Semen parameters									
Total sperm count ($\times 10^6$/ml)[a]	1.00	0.99 to 1.01	NS	1.00	0.99 to 1.01	NS	1.00	0.99 to 1.01	NS
Motility (% spermatozoa) (rapid plus slow progression)[a]	1.00	0.98 to 1.01	NS	1.02	0.97 to 1.04	NS	0.99	0.97 to 1.01	NS
Normal spermatozoa (%)[b]	1.08	0.90 to 1.31	NS	1.30	0.96 to 1.73	NS	0.93	0.76 to 1.14	NS
Vitality(%)[a]	1.00	0.99 to 1.01	NS	1.01	0.99 to 1.04	NS	0.99	0.98 to 1.01	NS
DNA fragmentation (%)[c]	1.02	0.99 to 1.04	NS	1.02	0.97 to 1.06	NS	1.00	0.97 to 1.03	NS
Abnormal chromatin packaging/ underprotamination (%)[d]	1.00	0.98 to 1.02	NS	0.99	0.97 to 1.02	NS	1.00	0.99 to 1.02	NS
Apoptosis (%)[e]	1.00	0.97 to 1.03	NS	0.98	0.92 to 1.04	NS	1.00	0.97 to 1.04	NS
Previous IVF-ICSI cycles	0.88	0.59 to 1.30	NS	1.76	0.93 to 3.31	NS	0.71	0.47 to 1.07	NS
Number of previous IV-ICSI cycles	0.98	0.89 to 1.07	NS	1.14	0.95 to 1.31	NS	0.95	0.83 to 1.02	NS
Ovarian stimulation protocol	0.96	0.64 to 1.41	NS	0.78	0.41 to 1.48	NS	0.95	0.64 to 1.41	NS
Duration of r-FSH/rLH therapy (days)	1.09	0.91 to 1.19	NS	0.98	0.85 to 1.11	NS	1.08	0.99 to 1.18	NS
FSH doses (IU)/cycle	1.00	0.99 to 1.01	NS	1.00	0.99 to 1.01	NS	1.00	0.99 to 1.01	NS
LH dose (IU)/cycle	1.00	0.99 to 1.01	NS	0.99	0.97 to 1.03	NS	1.00	0.99 to 1.01	NS
Oocytes/cycle (*n*)									
Total	1.10	1.05 to 1.14	0.000	0.93	0.86 to 1.10	NS	1.11	1.06 to 1.16	0.0001
MII	1.14	1.09 to 1.21	0.0001	0.92	0.84 to 1.07	NS	1.15	1.09 to 1.22	0.0001
Top-quality embryos (%)	1.02	1.01 to 1.03	0.0001	0.99	0.98 to 101	NS	1.02	1.01 to 1.03	0.0001
Transfers (*n*)									
Fresh embryo transfer	1.00	0.75 to 1.33	NS	1.55	0.83 to 2.21	NS	0.75	0.54 to 1.03	NS
Frozen/thawed embryo transfer	1.14	0.80 to 1.60	NS	1.50	0.92 to 2.43	NS	0.96	0.67 to 1.38	NS
Cumulative (fresh plus frozen)	1.05	0.84 to 1.30	NS	1.51	0.89 to 2.29	NS	0.83	0.65 to 1.07	NS
Total embryos transferred (*n*)									
Fresh	1.03	0.93 to 1.15	NS	1.49	0.91 to 1.82	NS	0.91	0.81 to 1.02	NS
Frozen	1.05	0.89 to 1.24	NS	1.18	0.93 to 1.49	NS	0.99	0.83 to 1.18	NS
Fresh plus frozen	1.04	0.95 to 1.14	NS	1.35	0.92 to 1.58	NS	0.93	0.84 to 1.03	NS
Multivariate analysis									
LIF genotypes (rs929271)									
G/G	2.05	1.20 to 4.19	0.02				2.05	1.10 to 4.16	0.02
Age (years)	0.94	0.89 to 0.99	0.02				0.87	0.83 to 0.92	0.0001
Oocytes/cycle (*n*)									
Total	0.95	0.87 to 1.05	NS				0.97	0.88 to 1.07	NS
MII	1.16	1.03 to 1.30	0.009				1.14	1.01 to 1.29	0.02
Top-quality embryos (%)	1.02	1.01 to 1.03	0.0002				1.02	1.01 to 1.03	0.002

BMI = body mass index; ICSI = intracytoplasmic sperm injection; LIF = leukaemia inhibitory factor; MII = second metaphase; NS = non-significant; ORPI = ovarian response prediction index (Oliveira et al., 2012).

[a]According to the World Health Organization (WHO, 2010).

[b]According to motile sperm organelle morphology examination (MSOME).

[c]The percentages of DNA fragmentation by TUNEL assay.

[d]Abnormal chromatin packaging by chromomycin A3.

[e]Apoptosis by annexin-V.

Table 9 Logistic regression analysis among three leukaemia inhibitory factor single nucleotide polymorphism T/G (rs929271) genotypes and the prediction of embryo implantation, spontaneous abortion and ongoing pregnancy after cumulative (fresh plus frozen–thawed) intracytoplamsic sperm injection cycles.

LIF genotypes (rs929271)	*Implantation*			*Spontaneous abortion*			*Ongoing pregnancy*		
	OR	*95% CI*	P	*OR*	*95% CI*	P	*OR*	*95% CI*	P
GG	Reference			Reference			Reference		
TG	2.44	1.20 to 5.00	0.01	0.69	0.23 to 2.05	NS	2.08	1.06 to 4.17	0.03
TT	2.33	1.15 to 4.76	0.02	0.36	0.12 to 1.07	NS	2.56	1.28 to 5.26	0.007

LIF = leukaemia inhibitory factor; NS = non-significant.

mRNA localisation and in the interaction with new types of RNA, e.g., microRNA (Barrett et al., 2012, 2013). The 3′ UTR is a versatile region that is enriched by regulatory elements and is vital for the correct spatial, temporal expression of genes, or both. It has been claimed that the LIF SNP (rs929271), located in the 3′ UTR region of the *LIF* gene, reduces the stability of the mRNA (Fraga et al., 2014). This claim, however, seems to be based only on the location of the polymorphism. To the best of our knowledge, no specific evaluation has been made of the effect of the SNP T/G (rs929271) on either RNA or on the LIF level and activity. Studies addressing regulatory functions are needed to reveal the potential of the LIF SNP T/G (rs929271).

The TP53 gene plays a critical role in regulating maternal reproduction and blastocyst implantation and acts to regulate apoptosis and angiogenesis (Hu et al., 2009). This function is mediated by genes involved in the TP53 pathway, including the *LIF* gene (Hu et al., 2008, 2009; Kang et al., 2009), which regulate their basal and inducible transcription through direct sequence-specific DNA binding and transcriptional activation (Kang et al., 2009). Changes in the *TP53* gene alter the activity of the *TP53*-regulated genes, which can change the levels or the activity of the final proteins. A TP53 SNP in codon 72 (rs1042522) leads to a change from arginine (Arg) to proline (Pro). Compared with the Pro allele, the Arg allele exhibits greater transcriptional activity towards a subset of *TP53* target genes, including *LIF*. In fact, the Arg allele seems to induce the expression of LIF more effectively. Studies have shown that the expression of LIF is two times greater in cells that express the Arg allele compared with cells that express the Pro allele, with a potential effect on embryo implantation (Feng et al., 2011; Jeong et al., 2010; Kang et al., 2009). Therefore, TP53 efficiency directly affects implantation and fertility, and some studies have linked the Pro allele with infertility resulting from implantation failure (Feng et al., 2011; Kay et al., 2006; Tagliani-Ribeiro et al., 2012). Considering the potential influence of the SNP at codon 72 Arg/Pro of the *TP53* gene (rs1042522) on the *LIF* gene and embryo implantation, we could suggest that the results found in our study for the LIF SNP T/G (rs929271) were actually caused by variations in the *TP53* gene. In our study, however, all of the patients were also genotyped for the SNP at codon 72 Arg/Pro of the *TP53* gene (rs1042522), and we found no relationship with implantation, pregnancy rates, or both. In addition, the distribution of genotypes of the SNP at codon 72 of the *TP53* gene (rs1042522) was similar among the three genotypes of LIF SNP T/G (rs929271). These results indicate that LIF SNP T/G (rs929271) seems to influence clinical outcomes after IVF treatment independent of the *TP53* gene, at least for the SNP at codon 73 Arg/Pro. The population characteristics, however, and sample size in our study may have influenced the results.

The significant relationship between the LIF SNP (rs929271), pregnancy and implantation rates after IVF treatment is the primary strength of this study. The genetic influence, however, requires deeper consideration. The complexity of higher organisms results from the high number and complexity of their regulation pathways (Levine and Tjian, 2003). Indeed, other polymorphisms in the *LIF* gene or different genes have been associated with infertility and repeated implantation or pregnancy failure. In their screening of mutations of the *LIF* gene in infertile women, Giess et al. (1999) identified point mutations in exon 1 (Position 715 Transition C to A) and two mutations in exon 3 (Position 3400 Transition G to A; Position 3424 Transition G to A) that correspond to regions of the LIF protein that are important for interaction with the LIF receptor. In another patient screening study, Steck et al. (2004) reported a case of recurrent IVF failure associated with *LIF* gene polymorphism G > T transversion at base number 3453 in exon 3 that did not alter protein conformation; furthermore, in patients with unexplained infertility, they found one mutation (G > A transversion at position 3400 in exon 3) that led to a change of valine to methionine at position 64 of the mature LIF protein and one polymorphism (G > A transition at position 3441 in exon 3) that did not alter the protein structure. Kralickova et al. (2006) investigated the prevalence of *LIF* gene mutations in a population of infertile women and found a significantly higher frequency of the functionally relevant mutation (the G to A transitions at the position 3400). Novotny et al. (2009) (G > A transition at position 3400) and Sabry et al. (2014) (C > T at nucleotide 351) also observed the effect of LIF mutations on the outcome of IVF treatment.

In addition to the SNPs at codon 72 of the TP53 gene, studies of the TP53 signalling pathway have suggested associations between pregnancy and implantation, and polymorphisms of the genes *MDM2* (rs2279744), *MDM4 (*rs1563828) and *USP7* rs1529916 (Kang et al., 2009; Feng et al., 2011; Tagliani-Ribeiro et al., 2012; Fraga et al., 2014). Polymorphisms of the genes *VEGF* (-1154 G > A; +405G > C) (Boudjenah et al., 2014; Goodman et al., 2008), *TNF*α (-308A>G) (Boudjenah et al., 2014), *PAI-1* 4G/5G (Khosravi et al., 2014) and *ESR1* (A > G rs9340799) (Paskulin et al., 2013) have also been implicated. Therefore, considering the myriad of polymorphisms and genes that are at least potentially involved in embryo implantation, the associations among different

genes must be analysed before these findings can be translated to clinically relevant settings. Considering a single genomic polymorphism is a very limited approach, efforts to outline a 'genetic profile' associated with clinical results are the best way to determine the correct gene influence. Therefore, studies that include a large sample, consider the various polymorphisms within a gene and among different genes, and include different ethnic groups are still needed.

In conclusion, our results reveal a potential novel genetic biomarker for predicting implantation and pregnancy after ICSI. Our results demonstrate an around a two-fold increased chance of embryo implantation and ongoing pregnancy after ICSI in infertile women with the LIF (rs929271) GG genotype. The significance of only one SNP, however, is limited. Further studies exploring the association of different genes and their polymorphisms are needed and will help clarify the influence of genetic variants on the clinic outcomes of treatment for infertility. The development of a prognostic genetic profile will make it possible to determine different polymorphisms that have potential benefits for clinical practice.

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