

# Controversial role of inhibin $\alpha$ -subunit gene in the aetiology of premature ovarian failure

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**BACKGROUND:** Premature ovarian failure (POF) is characterized by hypergonadotropic amenorrhoea before the age of 40. Inhibin  $\alpha$ -subunit (INH $\alpha$ ) gene is proposed as a candidate gene due to its role in negative feedback control of FSH. **METHODS:** Polymorphism  $-16C>T$  of INH $\alpha$  gene was studied in 61 POF patients and 82 controls above 40 years old ( $C > 40$ ). Substitution  $769G>A$  was studied in 59 POF patients, 76  $C > 40$  and 73 controls below 40 years old ( $C < 40$ ). **RESULTS:** No significant difference in risk of POF development for  $-16T$  allele was found when comparing idiopathic POF (I-POF) with  $C > 40$  (Odds ratio = 1.46; 95% confidence interval = 0.63–3.19). Implication of  $-16C>T$  polymorphism in serum inhibin levels was analysed in 46 controls, and no significant differences ( $P > 0.05$ ) were found between CC and CT + TT genotype groups when comparing either mid-follicular phase Pro- $\alpha C$  and inhibin B values or mid-luteal phase Pro- $\alpha C$  and inhibin A values. Heterozygosity for substitution  $769G>A$  was found in 1 of 59 POF woman, 2 of 76  $C > 40$  and 6 of 73  $C < 40$ . Presence of this substitution in a relevant number of control subjects is herein described for the first time. **CONCLUSION:** Our results indicate that  $-16C>T$  and  $769G>A$  variants in INH $\alpha$  gene may not be associated to POF disease.

*Key words:* inhibin  $\alpha$ -subunit gene/inhibin levels/premature ovarian failure

## Introduction

Premature ovarian failure (POF) is a syndrome clinically defined by ovarian failure before the age of 40 years. It is characterized by primary or secondary amenorrhoea, hypoestrogenism and elevated gonadotrophin serum levels (Moraes-Ruehsen and Jones, 1967). This syndrome is very heterogeneous with a multicausal pathogenesis, and any of the following—chromosomal, enzymatic, iatrogenic, autoimmune or infectious aberration—may be the cause of the disease (Hoek *et al.*, 1997). Incidence of POF among women in reproductive age is estimated in 1% (Coulam *et al.*, 1986).

Owing to frequent inheritance in families, it was suggested that POF may be a genetic disorder (Coulam *et al.*, 1983; Mattison *et al.*, 1984; Conway *et al.*, 1995). In support of this hypothesis, the FSH receptor (FSHR) has long been considered the foremost candidate gene for POF (Aittomaki *et al.*, 1995; Gromoll *et al.*, 1996; Beau *et al.*, 1998; Touraine *et al.*, 1999; Doherty *et al.*, 2002; Allen *et al.*, 2003; Meduri *et al.*, 2003). However, in a previous study, and in accordance with several other authors (Whitney *et al.*, 1995; Liu *et al.*, 1998; Conway *et al.*, 1999; de la Chesnaye *et al.*, 2001; Takakura *et al.*, 2001; Tong *et al.*, 2001), we found no mutations in exons 1–10 of

FSHR gene in 20 women with POF from among our population and postulated that FSHR isoforms might not be associated to disease risk (Sundblad *et al.*, 2004).

The glycoprotein inhibin may be a potential candidate for POF due to its role in the negative feedback control of FSH, which has a pivotal role in ovarian follicle recruitment and development during folliculogenesis (Shelling *et al.*, 2000). Dimeric inhibins are glycoproteins predominantly produced in the gonads (Burger, 1992), and are composed of  $\alpha$ - and  $\beta$ -subunits. Heterodimerization of  $\alpha$ -subunit with either form of the  $\beta$ -subunit,  $\beta_A$  and  $\beta_B$ , generates dimeric inhibin A and inhibin B, respectively. The homodimers composed of  $\beta_A$ - or  $\beta_B$ -subunits are the glycoproteins activins (Ying, 1988). It has been proposed that a large reservoir of inhibin  $\alpha$ -subunit (INH $\alpha$ ) in the ovary may be necessary to ensure dimeric inhibin formation, instead of dimerization of  $\beta$ -subunits to produce activins (Findlay *et al.*, 2001). Inhibins and activins are members of the transforming growth factor  $\beta$  superfamily. Since the development of specific assays, profiles of serum levels of inhibins throughout the menstrual cycle have duly been described. Inhibin A is primarily secreted by the mature follicle and corpus luteum; it remains low during the early and mid-follicular phase and

predominates in the luteal phase of the cycle. Conversely, inhibin B is a product of early pre-antral follicles; it presents maximal levels in the early follicular phase, falls before ovulation and remains low during the luteal phase (Groome *et al.*, 1994, 1996; Sehested *et al.*, 2000).

The monomeric inhibin form Pro- $\alpha$ C is found in the circulation at a higher concentration than that of inhibins A and B (Groome *et al.*, 1995; Bergada *et al.*, 1999). It has not yet been established whether this peptide may have a physiological role since it lacks biological action related to the regulation of FSH synthesis. On the other hand, inhibins A and B may act as endocrine factors, suppressing hypophyseal FSH biosynthesis and secretion (de Jong, 1988; de Kretser and McFarlane, 1996; Burger and Robertson, 1997), as well as intraovarian paracrine factors (Ying, 1988; Mather *et al.*, 1992; Weiss *et al.*, 1993). They contribute to the regulation of FSH secretion in the normal menstrual cycle, a process that allows the timely ovulation of a single mature follicle. Conversely, activins appear capable of direct pituitary stimulation of FSH secretion (Ying, 1988).

Several pieces of evidence suggest that the decrease in dimeric inhibin levels observed during perimenopause, and the concomitant increase in activin A, may be responsible for the high level of FSH characteristic of reproductive ageing (Klein *et al.*, 1996; Danforth *et al.*, 1998; Reame *et al.*, 1998; Santoro *et al.*, 1999). The reduction of inhibin : activin ratio observed during this period is likely due to an  $\alpha$ -subunit production deficit, which leads to the preferential formation of activin homodimers (Santoro *et al.*, 1999). Thus, it has been suggested that POF could be a consequence of mutations in the *INH $\alpha$*  gene, which may cause a decrease in the amount of bioactive inhibin, thereafter increasing the FSH concentration (Shelling *et al.*, 2000; Marozzi *et al.*, 2002).

*INH $\alpha$*  gene maps to 2q33-qter and is composed of two exons. Two polymorphic sites were identified in this gene: -16C>T in the 5'UTR and 675C>T in exon 2, the latter in complete linkage disequilibrium with the former (Montgomery *et al.*, 2000). Marozzi *et al.* (2002) studied -16C>T polymorphism and found that the derived frequency of T allele was significantly lower in POF population than in the control group. On the other hand, Shelling *et al.* (2000) identified a missense mutation (769G>A) in exon 2 of this gene, in 3 of 43 women with POF. This substitution was subsequently described in 7 of 157 Italian women with POF (Marozzi *et al.*, 2002) suggesting an association between the 769G>A variant and the development of POF.

The purpose of the present work was to study *INH $\alpha$*  gene as a putative candidate gene for POF in a cohort of patients from Argentina.

## Materials and methods

### Subjects

The population studied comprised 61 patients with POF, all with normal 46,XX karyotype. Among these patients, 18 presented POF in association with autoimmune diseases (AAD-POF patients). Fifteen of the AAD-POF patients presented autoimmune thyroiditis, two of them associated to vitiligo, two patients presented systemic lupus erythematosus and one presented myasthenia gravis. The remaining 43 POF patients were considered idiopathic (I-POF patients) because

they did not show any other POF-related condition (i.e. ovarian surgery, previous chemo- or radiotherapy or metabolic disorders such as galactosemia). Patients presenting antibodies directed to FSHR, determined by radioreceptor assay (Chiauzzi *et al.*, 2004), were excluded from the present work. Sixteen patients had a family history of premature menopause, whereas the remaining 45 were classified as having sporadic POF. Patients had been characterized as POF due to amenorrhoea for over a year starting before the age of 40 (range, 15–39 years old) and serum FSH level above 40 mIU/ml (normal follicular phase levels, 2–9 mIU/ml) in two consecutive determinations. Nine patients presented primary amenorrhoea and 52 secondary amenorrhoea. Plasma 17 $\beta$ -estradiol levels were below 15 pg/ml (normal follicular phase levels, 20–120 pg/ml).

Eighty-three women above the age of 40 ( $C > 40$ ) with normal menstrual history and without premature menopause were also included in this study as controls. All but one were of proven fertility, and only four were below 45 years of age. For the study of the implication of -16C>T polymorphism in serum inhibin levels, 46 controls were selected according to the following criteria: (i) age below 40; (ii) regular menses (duration, 25–35 days); (iii) no family history of premature or early menopause; (iv) no family history of autoimmune disorders; and (v) no consumption of oral contraceptives or other hormonal medications at the time of inclusion in the study. Weight and height of each subject was registered, and body mass index was calculated according to the formula: weight/(height)<sup>2</sup>. Twenty-nine per cent of these women were of proven fertility. Two blood samples were taken from each volunteer, the first between days 7 and 10 (mid-follicular phase) and the second between days 21 and 24 (mid-luteal phase) of the menstrual cycle. The exact day of blood extraction at mid-follicular and mid-luteal phases was determined for each subject—among the referred days—according to the duration of her own menstrual cycle. From each blood sample, DNA was isolated as described below and serum was used for hormone determinations. FSH, estradiol, inhibin B and Pro- $\alpha$ C values were measured on day 7–10 serum samples (mid-follicular phase), whereas progesterone, inhibin A and Pro- $\alpha$ C levels were determined on day 21–24 serum samples (mid-luteal phase).

In addition, 73 normally menstruating women below the age of 40 ( $C < 40$ ), without family history of premature or early menopause and with no history of autoimmune disorders, were also included for 769G>A study. Thirty-three of them were of proven fertility.

The protocol was approved by the Institutional Review Board of the Instituto de Biología y Medicina Experimental. Informed consent was obtained from all patients and controls.

### DNA analysis

DNA was isolated from peripheral blood leukocytes by standard methods. Two regions of *INH $\alpha$* -subunit gene (NM\_002191) were amplified from genomic DNA by PCR with specific oligonucleotide primers. The first region of 240 bp (fragment A), which includes 140 bp of 5'UTR and 100 bp of exon 1, was amplified by primers AF (5'-GACTGGGGGAAGACTGGATGA-3') and AR (5'-TCACCTTGGCCAGAACAAGT-3'). The second region of 396 bp (fragment B), which comprises part of exon 2, was amplified by primers BF (5'-AGCAGCCTCCAATAGCTCTG-3') and BR (5'-AGCTCCTGGAAGGAGATGTTTC-3'). Genomic DNA (200ng) was amplified in a 50 ml volume reaction containing 1  $\times$  PCR buffer (Invitrogen, Carlsbad, CA, USA), 2mM MgCl<sub>2</sub> (Invitrogen), 2.5% DMSO (Merck KgaA, Darmstadt, Germany), 0.2 mM of each dNTP (Promega, Madison, WI, USA), 0.5 mM of each specific primer and 1.5U of *Taq* DNA polymerase (Invitrogen). After an initial denaturation at 90°C for 1 min, the samples underwent 35 cycles of amplification (94°C denaturation for 40 sec, 57°C annealing for 30 sec, 72°C extension for 1 min); the last cycle was followed by 7 min extension at 72°C.

Polymorphism  $-16C>T$  in 5'UTR was screened in the samples by restriction enzyme analysis using SpeI (New England Biolabs, Ipswich, MA, USA). Briefly, fragment A was amplified by PCR and 3  $\mu$ l of purified PCR product were digested overnight at 37°C with 5 U of SpeI, electrophoresed on 8% polyacrylamide gels, stained with ethidium bromide and photographed. Presence of the 240 bp fragment indicated a homozygous variant for C, whereas presence of two fragments of 120 bp corresponded to homozygous variant T.

Substitution 769G>A of exon 2 was analysed by digestion of fragment B with two different restriction enzymes. Two microlitres of purified PCR product were digested overnight at 37°C with 5 U of BsrFI and analysed as described above. The restriction site that renders two fragments of 340 and 56 bp is abolished in the mutated allele. In addition, 3  $\mu$ l of purified PCR product were digested overnight at 37°C with 3 U of Fnu4HI, electrophoresed on 15% polyacrylamide gels, silver stained and dried. The 396 bp fragment renders four fragments of 153, 107, 51 and 25 bp—among others of lower molecular weight—in the wild-type allele, whereas the allele with substitution 769G>A renders four fragments of 153, 107, 76 and 51 bp, among others of lower molecular weight. In addition, all heterozygous samples—together with DNA from two control individuals—were further analysed by direct sequencing. New PCR products were purified by GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Cardiff, Wales, UK), following the Big Dye terminator sequencing protocol (Applied Biosystems, Foster City, CA, USA) and analysed using ABI prism DNA analyser.

#### Hormone measurements in serum

Follicular phase serum FSH was measured by enzymeimmunoassay (EIA) using a FSH EIA kit (Immunometrics, London, UK). Intra- and inter-assay coefficients of variance (CV) were 5.3 and 19.4, 3.9 and 11.1, and 3.6 and 12.6%, for low, medium and high pools, respectively. Assay sensitivity was 0.5 mIU/ml (second IRP 78/549).

Mid-follicular phase serum estradiol was measured by radioimmunoassay using a COAT-A-COUNT Estradiol kit (Diagnostic Products Corporation, Los Angeles, CA, USA). Intra- and inter-assay CV were 7.0 and 8.1, 4.3 and 6.8, and 4.0 and 4.2% for low, medium and high pools, respectively. Assay sensitivity was 8 pg/ml.

Mid-luteal phase serum progesterone was measured by radioimmunoassay using a COAT-A-COUNT Progesterone kit (Diagnostic Products Corporation). Intra- and inter-assay CV were 8.8 and 9.7, 4.0 and 5.7, and 2.7 and 3.9% for low, medium and high pools, respectively. Assay sensitivity was 0.02 ng/ml.

Serum inhibin A, B and Pro- $\alpha$ C were measured using a two-site enzyme-linked immunosorbent assay (Oxford Bio-Innovation, Oxon, England) specific for each peptide as previously described (Groome *et al.*, 1994, 1995, 1996). Recombinant inhibins A and B (Genentech, San Francisco, CA, USA) and a partially purified (>75% purity) Pro- $\alpha$ C preparation were used as standards. Assay sensitivity was 7 pg/ml for inhibin A, 15 pg/ml for inhibin B and 2 pg/ml for Pro- $\alpha$ C. Intra- and inter-assay CV were <10% for the three assays.

All samples from an individual woman were measured in duplicate in the same assay run.

#### Statistical analysis

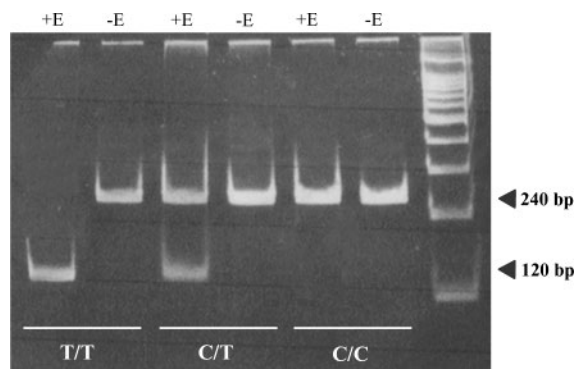
Odds ratio (OR) and 95% confidence interval have been calculated to estimate the relative risk of T allele and the different genotype combinations. Unpaired *t*-test was used to compare hormonal values in different genotype control groups. Mann-Whitney *U*-test was used when appropriate.

## Results

A mutation study of the INH $\alpha$  gene was carried out in POF patients and control subjects.

Our analysis revealed the presence of polymorphism  $-16C>T$  already reported (Montgomery *et al.*, 2000). The PCR product (fragment A) comprising nucleotide  $-16$  was digested with SpeI (Figure 1). Allelic frequencies and genotypes found in the 61 POF patients and in 82 control individuals above 40 years of age ( $C > 40$ ) are summarized in Table I. The T variant was considered the putative genetic risk factor for OR calculation. We were unable to demonstrate an association between this variant and the risk of being affected with I-POF (Table I).

In addition, the implication of the single nucleotide polymorphism in serum inhibin levels was analysed in subjects not affected by the disease. We evaluated inhibin A, inhibin B and Pro- $\alpha$ C peptide levels and correlated these data to the genotype of  $-16C>T$  polymorphism in 46 cycling women below 40 years of age. Given that T allele was considered the putative genetic risk variant, samples were divided into two groups: (i) CC genotype ( $n = 34$ ) and (ii) CT + TT genotypes ( $n = 12$ ).



**Figure 1.** Analysis of polymorphism  $-16C>T$  of 5'UTR by restriction enzyme digestion. Fragment A was digested with 5 U of SpeI. Presence of the 240 bp fragment indicated a homozygous variant for C, whereas presence of two fragments of 120 bp corresponded to homozygous variant T. (+E), PCR product incubated overnight with restriction enzyme; (-E), PCR product incubated overnight without restriction enzyme.

**Table I.** Genotypes and allelic frequencies of polymorphism  $-16C>T$  of 5'UTR observed in controls and patients

Genotype	C > 40 (n/total <sup>a</sup> )	I-POF (n/total)	AAD-POF (n/total)
A. Genotypes counts <sup>b</sup>			
C/C	49/82	22/43	14/18
C/T	29/82	18/43	4/18
T/T	4/82	3/43	0/18
B. Allelic frequencies <sup>c</sup>			
C	0.774 (127/164)	0.721 (62/86)	0.889 (32/36)
T	0.226 (37/164)	0.279 (24/86)	0.111 (4/36)

<sup>a</sup>Genotype of C<sub>124</sub> could not be determined.

<sup>b</sup>OR<sub>CC versus (CT + TT); I-POF versus C<sub>>40</sub></sub> = 1.46; CI 95% = 0.63–3.19.

<sup>c</sup>OR<sub>T versus C; I-POF versus C<sub>>40</sub></sub> = 1.33; CI 95% = 0.70–2.51.

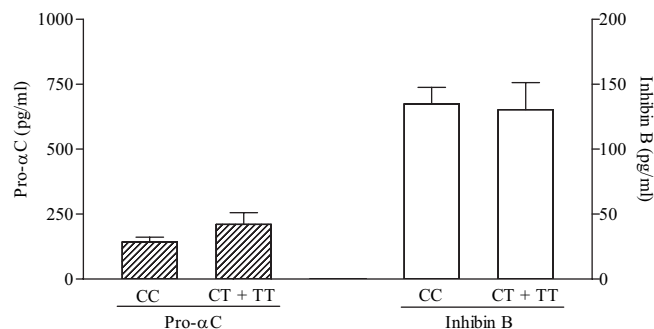
AAD-POF, Premature ovarian failure in association with autoimmune disease; I-POF, idiopathic premature ovarian failure.

**Table II.** Clinical characteristics and hormonal profile of control subjects studied to analyse the implication of  $-16C>T$  polymorphism in serum inhibin levels

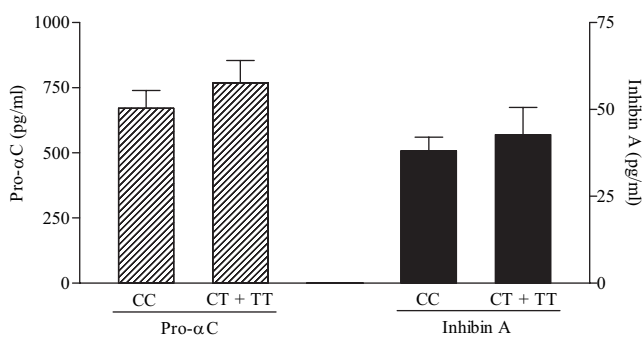
Genotype	n	Age	Cycle length (days)	BMI	FSH (mIU/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
CC	34	29.3 $\pm$ 0.7 (25.0–39.0)	28.2 $\pm$ 0.3 (25.0–32.0)	21.1 $\pm$ 0.5 (17.1–30.2)	4.7 $\pm$ 0.2 (1.6–7.4)	70.1 $\pm$ 6.7 (27.0–176.0)	10.8 $\pm$ 0.8 (4.4–18.6)
CT + TT	12	29.3 $\pm$ 0.6 (27.0–32.0)	27.9 $\pm$ 0.6 (26.0–30.0)	20.4 $\pm$ 0.5 (17.0–23.2)	4.4 $\pm$ 0.4 (2.9–6.7)	91.8 $\pm$ 18.5 (32.0–235.0)	11.2 $\pm$ 1.4 (4.5–20.0)

Data are expressed as mean  $\pm$  SEM (range).

**A. Follicular phase**



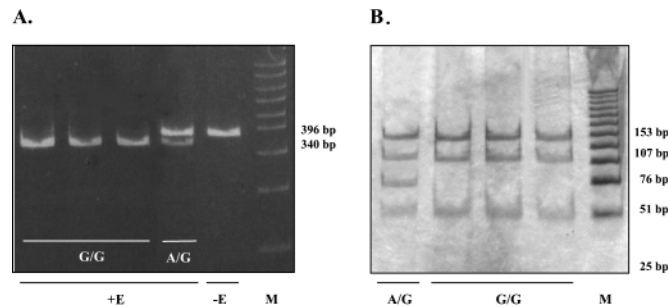
**B. Luteal phase**



**Figure 2.** Implication of  $-16C>T$  polymorphism genotypes in serum inhibin levels. Serum inhibin A, inhibin B and Pro- $\alpha$ C levels were evaluated in 46 control subjects (CC genotype,  $n = 34$ ; CT + TT genotypes,  $n = 12$ ). Data are expressed as mean  $\pm$  SEM.

Clinical characteristics and hormonal profile of control subjects from both groups are summarized in Table II. Figure 2 shows serum inhibin levels (mean  $\pm$  SEM in pg/ml) determined in the different genotype groups. No significant differences ( $P > 0.05$ ) were found between both groups in mid-follicular phase Pro- $\alpha$ C or inhibin B levels (CC: 146.30  $\pm$  18.80 versus CT + TT: 210.55  $\pm$  44.63 and CC: 134.90  $\pm$  12.63 versus CT + TT: 130.44  $\pm$  20.85, respectively) and mid-luteal phase Pro- $\alpha$ C and inhibin A levels (CC: 671.01  $\pm$  68.61 versus CT + TT: 768.06  $\pm$  86.44 and CC: 38.09  $\pm$  3.92 versus CT + TT: 42.73  $\pm$  7.86, respectively).

Substitution 769G>A of exon 2 was analysed by restriction enzyme digestions in 59 of 61 patients and in 76 of 83 control women above 40 years. PCR product comprising nucleotide 769, fragment B, was digested with BsrFI (Figure 3A) and/or with Fnu4HI (Figure 3B). Two of 76 controls and 1 of 59 POF patients, an AAD-POF woman with associated autoimmune thyroiditis, were heterozygous for this mutation (Table III). In addition, 73 cycling women below 40 years of age were also



**Figure 3.** Analysis of substitution 769G>A of exon 2 by restriction enzyme digestions. (A) Fragment B digested with BsrFI: PCR product of 396 bp rendered two fragments of 340 and 56 bp in the wild-type allele (G) and remained uncleaved in the mutated allele (A). (+E), PCR product incubated overnight with restriction enzyme; (–E), PCR product incubated overnight without restriction enzyme. (B) Fragment B digested with Fnu4HI: the 396 bp fragment rendered four fragments of 153, 107, 51 and 25 bp—among others of lower molecular weight—in the wild-type allele, whereas the allele with substitution 769G>A rendered four fragments of 153, 107, 76 and 51 bp, among others of lower molecular weight.

**Table III.** Genotypes of substitution 769G>A observed in controls and patients

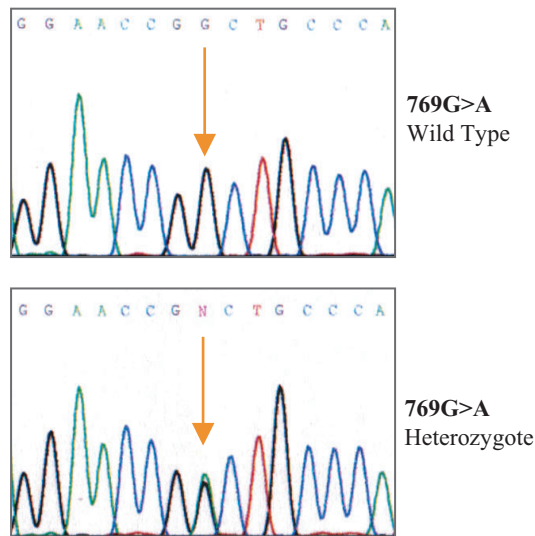
Genotype	C < 40 (n/total)	C > 40 (n/total)	I-POF (n/total)	AAD-POF (n/total)
G/G	67/73	74/76	42/42	16/17
G/A	6/73	2/76	0/42	1/17
A/A	0/73	0/76	0/42	0/17

AAD-POF, Premature ovarian failure in association with autoimmune disease; I-POF, Idiopathic premature ovarian failure.

evaluated for the presence of this mutation. We found six of these women heterozygous for 769G>A substitution (Table III). The presence of this variant was further confirmed in all heterozygous samples by direct sequencing (Figure 4). Characteristics of individuals carrying the INH $\alpha$  769G>A variant are summarized in Table IV. Serum FSH values of four of six heterozygous 769G>A women below the age of 40 were measured, and all of them were in the normal range (2–9 mIU/ml).

**Discussion**

During menopausal transition, the increase in FSH secretion coincides with an increment in follicular depletion rate (Richardson *et al.*, 1987). A decline in serum inhibin concentration has also been shown to occur when the ovarian follicular reservoir begins to subside (MacNaughton *et al.*, 1992), being almost undetectable in post-menopausal women (Buckler *et al.*, 1991).



**Figure 4.** Electropherogram showing a heterozygous 769G>A transition in INH $\alpha$  gene compared with the wild-type sequence. Arrows indicate the G to A change in the heterozygous sample and the corresponding nucleotide in the wild-type sequence.

**Table IV.** Clinical details of the heterozygous subjects for substitution 769G>A

Subject ID	Classification	Age	Phenotype	FSH levels (mIU/ml)
P39	AAD-POF	30	1° amenorrhea	77
C15	C < 40	29	Regular menses (1 child/pregnant)	ND
C16	C < 40	31	Regular menses (2 children/pregnant)	ND
C27	C < 40	27	Regular menses	6 <sup>a</sup>
C32	C < 40	27	Regular menses	4 <sup>a</sup>
C36	C < 40	34	Regular menses (1 child)	5.3 <sup>a</sup>
C49	C < 40	28	Regular menses	4.3 <sup>a</sup>
C102	C > 40	43	Regular menses (2 children)	NA
C118	C > 40	54	Menopause at 48 (5 children)	ND

<sup>a</sup>Measured at follicular phase.

AAD-POF, POF in association with autoimmune disease.

NA, data not available; ND, FSH levels not determined due to subject's hormonal condition.

Indeed, it was suggested that this decrease in inhibin A and B tones may be responsible for the high level of FSH, characteristic of reproductive ageing (Klein *et al.*, 1996; Reame *et al.*, 1998; Santoro *et al.*, 1999). Studies on hormonal patterns of POF patients have implicated inhibin as being involved in the disease mechanism. A defect in inhibin secretion has been reported to be involved in POF and natural menopause (Pampfer and Thomas, 1989). In addition, Petraglia *et al.* (1998) found that serum inhibin A and B levels in POF women were similar to those observed in post-menopausal women.

Considering that reduction of the inhibin : activin ratio observed during menopause could probably be due to a deficit of the INH $\alpha$  production (Santoro *et al.*, 1999), POF condition

could be thought of as a consequence of mutations in the INH $\alpha$  gene, which may cause a decrease in the amount of bioactive inhibin, and consequently, an increase in FSH concentration. Two previous studies have suggested a promising involvement of the INH $\alpha$  gene in the aetiology of POF (Shelling *et al.*, 2000; Marozzi *et al.*, 2002).

Two polymorphic sites were identified in this gene: -16C>T in 5'UTR and 675C>T in exon 2, the latter in complete linkage disequilibrium with the former (Montgomery *et al.*, 2000). Marozzi *et al.*, (2002) studied -16C>T polymorphism and found that the derived frequency of the T allele was significantly lower in POF population than in the control group. In the present work, we were unable to demonstrate any association between the T variant and the risk of being affected with POF. On the other hand, we measured serum monomeric and dimeric levels of inhibins in control cycling women. Inhibin levels were found to be similar in women with different polymorphism genotypes, all of them in the normal range (Groome *et al.*, 1995; Sehested *et al.*, 2000), suggesting that ovarian production of inhibins may not be affected by -16C>T allelic variant adding support to the concept that the 5'UTR allelic variant might not be involved in abnormal ovarian function.

Presence of the 769G>A substitution in exon 2 of INH $\alpha$  gene was described for the first time by Shelling *et al.* (2000) in 3 of 43 women with POF compared to 1 of 150 controls. Later on, Marozzi *et al.* (2002) reported that 769G>A transition was significantly more frequent in POF patients (7 of 157) than in control women (0 of 100). The substitution has been hypothesized to impair the binding of inhibin to its putative receptor (Shelling *et al.*, 2000). Recently, this mutation has also been found in 9 of 80 POF patients and 0 of 100 control subjects from India (Dixit *et al.*, 2004). However, it was found neither in 84 POF patients nor in 100 control subjects from Korea (Jeong *et al.*, 2004). Moreover, the functional significance of the amino acid variant at codon 257 is still unknown, given that no functional studies have been performed to date. In the present study, we investigated the presence of substitution 769G>A in exon 2 of INH $\alpha$  gene in order to determine if it could contribute to explain the pathophysiology of POF patients from among our population. In accordance with a study in Korean POF patients (Jeong *et al.*, 2004), we were unable to demonstrate a significant association between this mutation and ovarian failure. We observed only one patient, an AAD-POF woman, and 2 of 76 control women above 40 years old with the mutation described by Shelling *et al.* (2000).

It is noteworthy that some isolated cases of 769G>A substitutions were previously described in normal women. Studying 150 DNA samples from the general population—males and females of various ages—as controls, Shelling *et al.* (2000) found a normally menstruating 26-year-old female heterozygous for this substitution. In addition, the authors also reported that a patient's mother was found to be a carrier of the variant and had undergone normal menopause at 55 years. Another young 26-year-old woman, with regular menses but a carrier of 769G>A substitution, was described by Marozzi *et al.* (2002). Even though occurrence of mutation without POF manifestation was explained by these authors through incomplete penetrance, these findings could imply that this

substitution is also present in control subjects, becoming a putative polymorphism with no clinical consequences. In view of these considerations, we therefore included a group of 73 normal cycling women below 40 years of age in the analysis, and found this substitution in six of them. Presence of this variant in a relevant number of control subjects is herein for the first time described. Even though the possibility of POF development cannot be ruled out in these control subjects, the following should be considered: (i) 769G>A substitution frequency (8.2%) observed in this group is definitively above POF incidence, estimated in 1% of women of reproductive age; and (ii) if these heterozygous 769G>A control women were to develop POF, a similar frequency might be expected to be found in POF patients. It is interesting to note that Shelling *et al.* (2000) and Dixit *et al.* (2004) found that all POF patients with 769G>A substitution developed POF before the age of 25. The authors therefore suggested that this substitution could be associated to a relatively severe early onset of POF. If so, the possibility of POF development in our control heterozygous women between the ages of 27 and 34 (Table IV) might be even lower. Nevertheless, considering that Marozzi *et al.* (2002) found that the mean age of menopause onset of the seven patients carrying 769G>A substitution was  $35.6 \pm 3.7$  years, an appropriate follow-up of our heterozygous control women below 40 should be carried out.

It has been hypothesized that this mutation may be sufficient to impair binding affinity of inhibin to its putative receptor, leading to a subsequent inability to regulate the FSH level by negative feedback (Shelling *et al.*, 2000). Consequently, it might be plausible to find relative higher levels of FSH in control women found to be heterozygous, as compared with subjects without such substitution. However, in the present study, heterozygous 769G>A women below 40 showed normal FSH values. On the other hand, the only patient identified to date in the literature to be homozygous for 769G>A substitution did not present a particular severe phenotype, with FSH levels similar to those presented by other POF patients with a wild-type genotype (Dixit *et al.*, 2004). These two observations, together with the fact that in our study 769G>A frequency in POF patients was similar to—or even lower than—that found in control women, may suggest that this substitution in  $\text{INH}\alpha$  gene might not be involved in the aetiology of POF. Nevertheless, in order to better define the role of 769G>A in POF development, the number of patients and controls included in the analysis should be increased.

It has long been recognized that POF could be associated with nearly all organ-specific autoimmune diseases as well as with some nonorgan-specific disorders (de Moraes *et al.*, 1972; Coulam, 1983; La Barbera *et al.*, 1988; Betterle *et al.*, 1993; Hoek *et al.*, 1997; Forges *et al.*, 2004). The frequent relationship between POF, Hashimoto's thyroiditis, Addison's disease and other autoimmune diseases suggests that all of them are different manifestations of a general immunologic disorder, and might imply an autoimmune mechanism acting as the aetiological agent in these POF patients (Coulam, 1983; La Barbera *et al.*, 1988). Indeed, patients with a record of autoimmune diseases are usually excluded when I-POF is the target of study. Nevertheless, considering that the exact role of autoimmunity in the pathophysiology of this disease still remains controversial (Hoek *et al.*, 1997;

Forges *et al.*, 2004), in this work patients with autoimmune-associated diseases were analysed in a separate group. Surprisingly, the only POF patient found to be heterozygous for 769G>A substitution belongs to this AAD-POF group. This outcome might question the role of  $\text{INH}\alpha$  gene in POF aetiology, reinforcing our findings in controls. Alternatively, it could add further support to the concept of the multifactorial nature of the disorder, if a role of  $\text{INH}\alpha$  gene was ultimately confirmed.

It has been proposed that changes in inhibin secretion due to alterations in  $\text{INH}\alpha$  gene could be responsible for the increase in FSH levels found in POF patients (Shelling *et al.*, 2000; Marozzi *et al.*, 2002), thus becoming the cause of the disease mechanism. Alternatively, the reduction in inhibin tones could be a natural consequence of the reduction in the number of follicles in these patients' failing ovaries, like in physiological menopause. Considering our results, low inhibin tones might be thought of as a consequence of premature follicular depletion, or alternatively, the decreased inhibin levels could indeed be responsible for high FSH levels characteristic of POF patients—becoming the cause of the disease mechanism—deriving from some alteration(s) in the regulation mechanism(s) of inhibin secretion, instead of from defects in the  $\text{INH}\alpha$  gene itself.

In conclusion, in contrast to previous studies (Shelling *et al.*, 2000; Marozzi *et al.*, 2002; Dixit *et al.*, 2004) and in accordance to Jeong *et al.* (2004), our results might indicate that -16C>T and 769G>A variants in  $\text{INH}\alpha$  gene may not be associated with POF disease. Other studies on the general population, as well as more detailed functional studies, are required to elucidate if substitution 769G>A is a 'normal' variant of  $\text{INH}\alpha$  gene. In addition, further investigations into a larger number of POF patients from different populations should be conducted, in order to define the role of  $\text{INH}\alpha$  gene in the aetiology of POF.

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