A chromosome 19 locus positively influences the number of retrieved oocytes during stimulated cycles in Brazilian women

Amanda Souza Setti, Sylvia Sanches Cortezzi, Rita de Cássia S. Figueira, Ciro Dresch Martinhago, Daniela Paes de Almeida Ferreira Braga, et al.
A chromosome 19 locus positively influences the number of retrieved oocytes during stimulated cycles in Brazilian women

Amanda Souza Setti · Sylvia Sanches Cortezzi · Rita de Cássia S. Figueira · Ciro Dresch Martinhago · Daniela Paes de Almeida Ferreira Braga · Assumpio Iaconelli Jr. · Edson Borges Jr.

Received: 29 November 2011 / Accepted: 20 February 2012 / Published online: 10 March 2012
© Springer Science+Business Media, LLC 2012

Abstract

Purpose To evaluate if several genetic loci that are associated with variation in normal menopause age and early menopause can account for a poor response to controlled ovarian stimulation.

Methods A total of 71 patients age ≤35 years old who were undergoing intracytoplasmic sperm injection were genotyped for four genetic variants that are associated with normal variation in menopausal age and early menopause. The patients were divided into two groups based upon treatment response: a poor responder group (PR group, n = 21) and a normal responder group (NR group, n = 50). The genetic variants rs244715, rs9379896, rs4806660 and rs16991615 were analyzed.

Results There was no significant difference in the incidence of the genetic variants between the NR and PR group. The risk allele for the chromosome 19 variant (rs4806660) demonstrated a protective effect for a poor ovarian response. The presence of a risk allele was associated with an increased response to COS, which resulted in an elevated number of follicles (Coef: 2.54, P = 0.041) and retrieved oocytes (Coef: 1.41, P = 0.041).

Conclusions The genetic variants rs244715, rs9379896, rs4806660 and rs16991615 are not risk factors for poor ovarian response in Brazilian women. In contrast, rs4806660 is associated with higher number of follicles and retrieved oocytes. rs4806660 may be associated with an increased response to gonadotrophin stimulation in this population.

Keywords Assisted reproduction techniques · Biological markers · Controlled ovarian stimulation · Intracytoplasmic sperm injection · Single nucleotide polymorphisms

Introduction

Modern trends show that women have been postponing childbirth in recent years. This trend has consequently led to an increase in age-related infertility, which has increased the application of assisted reproductive technologies [5, 27, 58]. However, premature reduction in the ovarian follicle number has been demonstrated to significantly affect the success of in vitro fertilization (IVF), despite the age of the woman [42, 50], and is thus a challenge for these patients who undergo ART [43].

Despite recent advances in assisted conception, estimates suggest that 9–24% of patients who undergo IVF treatment have a poor ovarian response [17]. Therefore, poor ovarian
response to gonadotrophin stimulation remains a significant problem in modern fertility treatment, especially with in vitro fertilization (IVF), where a large number of oocytes is desirable.

A poor response to IVF may be an early sign of ovarian ageing and reduced ovarian reserve [11]. This phenomenon was initially described by Garcia et al. [12], who defined this condition as patients who have a peak E2 level <300 pg/mL after standard stimulation with human menopausal gonadotrophin. Poor responders have been defined based on the presence of mature oocytes [23], an elevated early follicular phase of the FSH peak [10], the number of basal antral follicles [28], the number of follicles at the end of ovarian stimulation [53], the plasma oestradiol concentration [49] or the number of oocytes retrieved [20]. A novel consensus defined poor responders as patients who have an advanced maternal age or any other risk factor for poor response in combination with a previous poor response and an abnormal ovarian reserve test prediction [11].

A large number of clinical parameters for predicting menopause age rely upon detecting peri-menopausal changes in the oocyte number and are therefore poor long-term predictors [11, 22]. Hormonal serum levels change prior to menopause, including the basal follicle-stimulating hormone (FSH), anti-Mullerian hormone (AMH) and inhibin B levels. Of these endocrine markers, AMH seems to be the best long-term predictor because AMH levels decrease approximately 10 years prior to menopause [5, 21, 37, 57]. Other additional markers of ovarian reserve are the antral follicle count (AFC) and ovarian volume [22]. Although the relationship between the test results and true ovarian reserve is unknown, these may predict in a moderate or good way quantitative parameters, but not accurately predict qualitative parameters. In conclusion, both AMH and AFC are quantitative parameters, but not accurately predict qualitative markers of ovarian reserve [6, 21]. Apart from the maternal age, endometriosis and prior pelvic surgery can also be used as predictors for a poor ovarian response [38].

In contrast to transient parameters, genetic predictors of menopausal age are present from birth. Genetic markers may therefore provide reproductive lifespan information much earlier than traditional markers and allow women to make informed reproductive choices at earlier ages. Current estimates suggest that the heritability of menopausal age ranges from 31% to 87% [8, 33, 34, 47, 52, 55], where variability among family members is attributed to common environmental factors [33].

Numerical and structural chromosomal aberrations as well as mutations or variability in specific genes that play a role in reproductive ageing may influence reduced ovarian reserves [9]. Genetic variation occurs naturally in the human genome both as a result of errors or mistakes during cell division and DNA replication as well as due to viral and chemical mutagenesis [19]. The most common type of inter-individual genetic variation in the human genome is a single nucleotide polymorphism (SNP), which is present in at least 1% of any population, while the frequency of point mutations is less than 1% [7, 15, 19, 44].

Recent genome-wide association studies (GWAS) have successfully identified genetic loci for numerous complex traits. For example, several osteoporosis susceptibility SNPs have been described that influence the age of natural menopause in Chinese women [62], and multigenic interactions formed by three SNPs related to oestrogen signalling pathways are correlated with the normal menopause age in a Spanish population [31]. Two independent large-scale studies identified loci on chromosomes 5, 6, 19 and 20 that are associated with variation in the normal menopause age [15, 48]. Moreover, Murray et al. [35] demonstrated that these four variants increase the odds of having early menopause. However, whether these variants can account for a poor response to controlled ovarian stimulation in IVF patients is still unknown. Thus, the goal of this study was to determine if these four previously identified SNPs are correlated with a poor response in IVF patients.

Materials and methods

Patients

A total of 71 Caucasian patients undergoing intracytoplasmic sperm injection (ICSI) were included in this study. All of the patients were 35 years old or younger. The patients were divided into two groups according to their response to Controlled Ovarian Stimulation (Garcia et al.): a poor responder group (PR group, patients with number of retrieved oocytes ≤4, n=21) and a normal responder group (NR group, patients with a number of retrieved oocytes from 5 to 15, n=50). For all of the patients, we analyzed four newly identified genetic variants that have been associated with normal variation in menopausal age and early menopause: rs244715, rs9379896, rs4806660 and rs16991615 (chromosomes 5, 6, 19 and 20, respectively).

This study was approved by the local Institute review board, and written informed consent was obtained. All of the patients agreed to share the outcome of their own cycles for research purposes.

Controlled ovarian stimulation and oocyte retrieval

The patients started recFSH treatment (Gonal-F®, Serono, Geneve, Switzerland) daily from the third day of their menstrual cycles. The first ultrasound control and the E2 plasma dosage tests were performed at the seventh cycle day. Depending on the response of each patient, controlled by
ultrasound monitoring of the follicles size, the dose of recFSH was adjusted. GnRH antagonist was administered when the dominant follicle was 14 mm in mean diameter. Oocyte retrieval was performed 35 h after the administration of recombinant human chorionic gonadotrophin (rhCG, Ovidrel™, Serono, Geneve, Switzerland) through transvaginal ultrasonography.

Preparation of oocytes and morphology assessment

Retrieved oocytes were maintained in human tubal cultured medium (HTF, Irvine Scientific, Santa Ana, USA) supplemented with 10% Human Synthetic Albumin (HSA, Irvine Scientific, Santa Ana, USA), covered with mineral oil (Ovoil™, Vitrolife) and incubated for 2–3 h before cumulus cell removal. The surrounding cumulus cells were removed via incubation with a HEPES buffered-medium containing hyaluronidase (80 IU/mL, Irvine Scientific, Santa Ana, USA). The remaining cumulus cells were then mechanically removed by gentle pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, USA).

Oocyte morphology was assessed with an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon®, Tokyo, Japan) with a Hoffmann modulation contrast system under 400× magnification just prior to sperm injection (3–4 h after retrieval).

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed with all of the MII oocytes according to the technique described by Palermo et al. [39]. The oocytes were transferred into a micro-injection dish, which was prepared with 4-μL droplets of buffered medium (HEPES, Irvine Scientific, Santa Ana, USA), covered with mineral oil and placed on the heated stage (37.0 ± 0.5°C) of an inverted microscope. Approximately 16 h after ICSI, fertilization was confirmed by the presence of two pronuclei and extrusion of the second polar body. The embryos were maintained in a 50-μL drop of HTF medium supplemented with 10% HSA, covered with mineral oil and kept in a humidified atmosphere (6% CO₂ in air) at 37°C until transfer on day 3 of development. High-quality embryos were defined as having the following characteristics: 8–10 cells on the third day of development, less than 15% fragmentation, symmetric blastomeres and the absence of multinucleation and zona pellucida dysmorphisms.

Blood sample collection

Blood samples were collected from all of the patients prior to the beginning of infertility treatment. The blood samples were centrifuged, and the white cell buttons were removed and cryopreserved with liquid nitrogen. The cells were subsequently thawed and lysed to obtain the cellular DNA.

Genotyping

All of the samples were genotyped for the following four SNPs: rs244715, rs9379896, rs4806660 and rs16991615 on chromosomes 5, 6, 19 and 20, respectively. The genomic DNA was isolated with a QIAamp blood reagent kit (Qiagen) according to the manufacturer’s instruction. Genotype analysis was performed with a real-time PCR equipment (7500 Real Time PCR System, Applied Biosystems, Foster City, CA, USA), and TaqMan system (Applied Biosystems, Foster City, CA, USA) was used to detect the amplification product [24, 26].

Each PCR reaction was set up in a final volume of 10 μL and was comprised of 5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM of each primer, 150 nM of each TaqMan Minor Groove Binder (MGB) probe, and 2 μL of the extracted DNA. The PCR cycle consisted of an initial 2-min incubation at 50°C (to allow for AmpEraser uracil N-glycosylase cleavage of contaminant PCR products from previous reactions), denaturation at 95°C for 10 min, and amplification with denaturation (95°C, 15 s) followed by annealing (60°C, 60 s) for 45 cycles. The fluorescence was continually monitored during the annealing step for each sample. The assays were analyzed in separate reactions and in duplicate. Positive and negative controls were used in each SNP reaction.

Statistical analysis

The incidence of risk alleles was compared between the PR and NR patients. Furthermore, the influence of the individual risk allele frequency on the response to COS (total dose of FSH administered, number of follicles, number of retrieved oocytes, oocyte recovery rate and metaphase II (MI) oocyte rate) was evaluated. The results are expressed as the mean ± standard deviation (SD) for numeric continuous variables or proportions (%) for the categorical variables. The mean values were compared with Student’s t or Mann-Whitney test as appropriate, and proportions were compared with the chi-squared test. Linear regression models were used to determine the effects of the risk allele for each SNP on the variables associated with COS response. The results were expressed as regression coefficients (Coef), R² and P values. The influence of the risk allele frequency on the odds of being a case was assessed through binary logistic regression, and the results are expressed as odds ratios (OR), 95% confidence intervals (CI) and P values. P values less than 0.05 were considered to be statistically significant. The data analysis was performed...
with the Minitab (version 14) Statistical Program (Minitab Inc., USA).

**Results**

All of the individuals were successfully genotyped for all four of the SNPs. The general characteristics of the patients’ cycles and the risk allele frequency for the SNPs are shown in Table 1. None of the four variants was a determinant of the odds of being a case (Table 2).

The influence of the risk allele on the response to COS (total dose of FSH administered, number of follicles, number of retrieved oocytes, oocyte recovery rate and metaphase II (MII) oocyte rate) is shown in Table 3. Patients that possess risk allele rs244715 showed a significant protective effect related to an increased response to COS, as was demonstrated by a higher number of follicles and retrieved oocytes.

Of the 71 patients, 4 (5.6%) did not possess the risk allele for rs9379896 (2 in the NR group and 2 in the PR group); 17 patients (23.9%) did not possess any risk allele for rs244715 (11 in the NR group and 6 in the PR group) and 36 patients (51.5%) did not possess any risk allele for rs4806660 (24 in the NR group and 12 in the PR group). Conversely, all of the patients and controls (100%) possessed the risk allele for rs16991615.

The homozygosity and heterozygosity incidences for the risk alleles in both groups are shown in Table 4. When the risk alleles for all four of the SNPs are combined, the number of risk alleles has no influence on the odds of being in the case group (OR: 0.65, CI: 0.40–1.04, P=0.066). None of the patients had clinical manifestations of ovarian hyper-stimulation syndrome.

**Discussion**

Although ART relies on COS for the production of a high number of oocytes, a reasonable percentage of women undergoing IVF do not respond adequately to exogenous stimulation [14, 17, 42]. The gold standard in COS is to achieve an adequate number of good quality oocytes while minimizing adverse drug response and cycle cancellation rates [27]. Poor response to COS has been associated with premature ovarian failure (POF), is an early sign of ovarian ageing [11, 36] and has a negative impact on the ART success rate [17, 42]. Because menopause drastically influences women’s fertility and health, there has been considerable interest recently regarding the mechanisms that control ovarian ageing and the timing of natural menopause [58].

Recently, four genetic loci on chromosomes 5, 6, 19 and 20 that are associated with variation in the normal menopause age and early menopause were identified in large-scale studies [15, 35, 48]. Chromosome 5 locus (rs244715) is a genetic variant at 5q35.2, in an intronic region of ZNF346 gene, with sequences of 294 amino acids (AA). The protein encoded by this gene is a zinc finger protein that preferentially binds to double-stranded RNA or RNA/DNA hybrids, rather than DNA alone, and may be involved in apoptosis. Another analyzed genetic variant (rs9379896) is at 6p24.2, in a promoter region of SYCP2L gene (synaptosomal complex protein 2-like), a nucleolar protein of 812 AA. Variation on chromosome 19 (rs4806660) is localized in an intronic region

---

**Table 1** General cycle characteristics and the risk allele frequencies

<table>
<thead>
<tr>
<th>Variable</th>
<th>PR group (n=21)</th>
<th>NR group (n=50)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (years)</td>
<td>32.4±2.6</td>
<td>32.5±2.5</td>
<td>0.1191</td>
</tr>
<tr>
<td>Dose of FSH administered (IU)</td>
<td>2206.5±700.7</td>
<td>2276.8±669.7</td>
<td>0.9059</td>
</tr>
<tr>
<td>Follicles</td>
<td>5.6±2.1</td>
<td>15.5±7.1</td>
<td>0.0002</td>
</tr>
<tr>
<td>Retrieved oocytes</td>
<td>3.1±0.8</td>
<td>10.2±3.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Oocyte recovery rate (%)</td>
<td>60.9</td>
<td>71.8</td>
<td>0.2180</td>
</tr>
<tr>
<td>MII oocyte rate (%)</td>
<td>70.2</td>
<td>74.8</td>
<td>0.5950</td>
</tr>
<tr>
<td>Risk allele frequency for rs244715 (%)</td>
<td>71.4</td>
<td>78.0</td>
<td>0.5537</td>
</tr>
<tr>
<td>Risk allele frequency for rs9379896 (%)</td>
<td>90.5</td>
<td>96.0</td>
<td>0.5764</td>
</tr>
<tr>
<td>Risk allele frequency for rs4806660 (%)</td>
<td>42.8</td>
<td>52.8</td>
<td>0.4819</td>
</tr>
<tr>
<td>Risk allele frequency for rs16991615 (%)</td>
<td>100</td>
<td>100</td>
<td>–</td>
</tr>
</tbody>
</table>

The values are the means ± standard deviation, unless otherwise noted. PR poor responder; NR normoresponder; IU international units.

**Table 2** The influence of the genetic variants on the odds of ovarian response to COS

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chromosome</th>
<th>Risk allele frequency</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs244715</td>
<td>5</td>
<td>74.7</td>
<td>0.68 (0.36–1.30)</td>
<td>0.242</td>
</tr>
<tr>
<td>rs9379896</td>
<td>6</td>
<td>93.2</td>
<td>0.78 (0.34–1.81)</td>
<td>0.567</td>
</tr>
<tr>
<td>rs4806660</td>
<td>19</td>
<td>47.4</td>
<td>0.81 (0.40–1.66)</td>
<td>0.566</td>
</tr>
<tr>
<td>rs16991615</td>
<td>20</td>
<td>100</td>
<td>*</td>
<td>0.088</td>
</tr>
</tbody>
</table>

SNP single nucleotide polymorphism; COS controlled ovarian stimulation; OR odds ratio; CI confidence interval

* This value could not be calculated because all of the patients possessed this risk allele.
of TMEM150B gene (transmembrane protein 150B), which encodes a protein of 233 AA. This protein belongs to the DRAM/TMEM150 family, involved in apoptosis pathway. The last analyzed variant on 20p12.3 (rs16991615) is the only SNP in an exonic region, at MCM8 gene (minichromosome maintenance complex component 8). The 840 AA protein encoded by this gene is one of the highly conserved minichromosome maintenance proteins, which binds chromatin throughout the cell cycle and is essential for DNA replication and cell proliferation [16, 54]. In this study, we sought to determine if these four loci are also associated with a poor response, embryo quality or pregnancy rate [56].

Several studies have investigated possible associations between SNPs and ovarian stimulation outcome [2, 13, 14, 27, 45, 46, 56, 59]. These studies have reported that SNPs in FSH and FSH-receptor genes may be related to adverse outcomes with COS [45, 46, 59]. Additionally, SNPs in genes that encode for other proteins that play a role in follicular development are currently under investigation. Previous studies have reported that oestrogen receptors [2], bone morphogenetic protein 15 (BMP15) [14, 32], LH hormone [3], 5,10-methylenetetrahydrofolate reductase (MTHFR) gene [51], and anti-Mullerian hormone (AMH) and its receptor AMHR2 [13] play a role in follicular development. Although a majority of these studies reported statistically significant results, these findings need to be confirmed in additional study populations.

Our data suggest that the genetic variation associated with normal menopausal age and early menopause are not significant risk factors for a poor response to COS in IVF cycles in young Brazilian patients. For all four of the SNPs that were tested, there was no evidence that the odds of being a PR case, per risk allele, was different from the odds of being a NR case. Another novel study utilised GWASs to look for SNPs in patients undergoing ICSI cycles, and these authors did not observe any correlation between the SNPs and ovarian response, embryo quality or pregnancy rate [56].

We acknowledge that this study is limited by its retrospective design and lack of sample size calculation. Nevertheless, our results indicate that the risk allele for chromosome 19 variant (rs4806660) demonstrates a protective effect for poor ovarian response. The presence of the risk allele in either in homozygous or heterozygous women was associated with an increased response to COS, which resulted in an elevated number of follicles and retrieved oocytes.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Homozygous for risk allele</th>
<th>Heterozygous for risk allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs244715</td>
<td>46.0 28.6 0.1727</td>
<td>32.0 42.8 0.3820</td>
</tr>
<tr>
<td>rs9379896</td>
<td>70.0 66.7 0.7816</td>
<td>26.0 23.8 0.8465</td>
</tr>
<tr>
<td>rs4806660</td>
<td>16.0 14.3 1.000</td>
<td>36.0 28.6 0.5949</td>
</tr>
<tr>
<td>rs16991615</td>
<td>92.0 100 0.3116</td>
<td>8.0 0.0 0.3116</td>
</tr>
</tbody>
</table>

SNP single nucleotide polymorphism; NR normoresponder group; PR poor responder group

---

Table 3 The influence of the risk alleles on COS response

<table>
<thead>
<tr>
<th>Variable</th>
<th>rs244715</th>
<th>rs9379896</th>
<th>rs4806660</th>
<th>rs16991615</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coef</td>
<td>R² (%)</td>
<td>P</td>
<td>Coef</td>
<td>R² (%)</td>
</tr>
<tr>
<td>FSH</td>
<td>−76.8</td>
<td>0.8</td>
<td>0.458</td>
<td>137.0</td>
</tr>
<tr>
<td>Nº follicles</td>
<td>−0.09</td>
<td>0.0</td>
<td>0.936</td>
<td>1.02</td>
</tr>
<tr>
<td>Nº oocytes</td>
<td>−0.258</td>
<td>0.2</td>
<td>0.692</td>
<td>0.324</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>−1.95</td>
<td>0.5</td>
<td>0.561</td>
<td>−2.26</td>
</tr>
<tr>
<td>MII oocyte (%)</td>
<td>0.594</td>
<td>0.1</td>
<td>0.852</td>
<td>4.16</td>
</tr>
</tbody>
</table>

COS controlled ovarian stimulation; Coef coefficient; R² coefficient of determination; P P value; MII metaphase II

Table 4 The homozgyosity and heterozygosity incidences of the risk alleles in the NR and PR groups

<table>
<thead>
<tr>
<th>SNP</th>
<th>NR (%)</th>
<th>PR (%)</th>
<th>P value</th>
<th>NR (%)</th>
<th>PR (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs244715</td>
<td>46.0</td>
<td>28.6</td>
<td>0.1727</td>
<td>32.0</td>
<td>42.8</td>
<td>0.3820</td>
</tr>
<tr>
<td>rs9379896</td>
<td>70.0</td>
<td>66.7</td>
<td>0.7816</td>
<td>26.0</td>
<td>23.8</td>
<td>0.8465</td>
</tr>
<tr>
<td>rs4806660</td>
<td>16.0</td>
<td>14.3</td>
<td>1.000</td>
<td>36.0</td>
<td>28.6</td>
<td>0.5949</td>
</tr>
<tr>
<td>rs16991615</td>
<td>92.0</td>
<td>100</td>
<td>0.3116</td>
<td>8.0</td>
<td>0.0</td>
<td>0.3116</td>
</tr>
</tbody>
</table>
Because inherited variations in the DNA sequence between individuals can have a major impact on drug response, we hypothesise that rs4806660 is associated with an increased response to gonadotrophin stimulation in Brazilian patients undergoing ART [19, 44]. Several different genetic studies regarding menopausal age have had difficulty identifying genetic loci that are associated with this complex trait. When the different types of studies are compared, there is almost no overlap in the genes that are associated with menopausal age [46, 58]. Single or combined SNPs, nevertheless, are able to modify endocrine feedback systems and hormone function, thereby establishing inter-individual reproductive performance that can range from high rates of fertility to infertility [45]. However, it is currently unclear how SNPs alone determine the susceptibility of an individual to complex diseases or to adverse drug interactions as most common traits and phenotypes result from long-term interactions between genetic and environmental factors [19, 44].

Another reason that the identified genes may differ from study to study may be related to differences in ethnicity due to genetic heterogeneity. Ethnic-specific variation in the distribution of polymorphisms has been previously demonstrated [1, 4, 14, 46], and the difficulty in correlating any genetic variability with ethnicity has been previously discussed [40, 61]. Studies in Brazilian populations are even more complicated because the population is miscegenated [41]. Nevertheless, the identification of risk alleles in patients previous to COS may help predict poor or normal ovarian response and prevent ovarian hyperstimulation syndrome. The genetic variants rs244715, rs9379896, rs4806660 and rs16991615 are not risk factors for a poor ovarian response in Caucasian Brazilian women undergoing ART. Instead, rs4806660 is associated with a higher number of follicles and retrieved oocytes. We hypothesise that the genetic variant rs4806660 is associated with an increased response to gonadotrophin stimulation in this population.

References

10. Esposito MA, Coutifaris C, Barnhart KT. A moderately elevated day 3 FSH concentration has limited predictive value, especially in younger women. Hum Reprod. 2002;17:118–23.


43. University S. Stanford On Line Universal resource for Clones and ESTs (SOURCE); 2000.


