A prospective analysis of cell-free fetal DNA concentration in maternal plasma as an indicator for adverse pregnancy outcome

Margit Bauer¹, Georg Hutterer², Martina Eder¹, Sandra Majer¹, Erik LeShane³, Kirby L. Johnson³, Inga Peter⁴, Diana W. Bianchi³ and Barbara Pertl¹*

¹Department of Obstetrics and Gynaecology, Medical University Graz, Auenbruggerplatz 14, A-8036 Graz, Austria
²Department of Urology, Medical University Graz, Auenbruggerplatz 7, A-8036 Graz, Austria
³Division of Genetics, Department of Pediatrics, Obstetrics and Gynaecology, Tufts-New England Medical Centre and Tufts University School of Medicine, Boston, MA 02111, USA
⁴Biostatistics Research Centre, Institute for Clinical Research and Health Policy Studies, Tufts-New England Medical Centre and Tufts University School of Medicine, Boston, MA 02111, USA

Objective To evaluate whether cell-free fetal (cff) DNA in maternal plasma during the second trimester is a marker for developing pregnancy-associated complications. Two PCR techniques for the detection and quantitation of fetal DNA were compared.

Methods Plasma samples were prospectively collected from 84 pregnant women carrying male fetuses before amniocentesis (14–29 weeks). We later recorded 26 pregnancies with complicated outcomes, including five cases of fetal chromosomal abnormalities. For statistical analysis, two overlapping subgroups A and B were made. Each group was separately compared for total and fetal DNA with a corresponding group considered normal using Wilcoxon rank sum test. Male fetal DNA concentration in maternal plasma was quantified using real-time quantitative polymerase chain reaction (PCR) of SRY sequences. The samples were also analyzed by quantitative fluorescent PCR (QF-PCR) using highly polymorphic short tandem repeat DNA sequences (STRs), and the percentage of relative fetal allele concentration in maternal alleles was calculated and compared to the fetal/total DNA ratio obtained by real-time PCR.

Results Quantities of total and fetal circulating DNA were significantly correlated ($r^2 = 0.44, P < 0.0001$) with a median total DNA concentration of 522 GE/mL (range 51–3047) and a median fetal DNA concentration of 8 GE/mL (range 0–879). Neither level was correlated with gestational age in pregnancies with normal ($r^2 = -0.05; P = 0.66$, and $r^2 = 0.02; P = 0.88$, respectively) and abnormal ($r^2 = 0.45; P = 0.17$, and $r^2 = 0.11; P = 0.76$, respectively) outcomes. Although both total and fetal DNA levels were always higher in women carrying pregnancies with chromosomal aberrations or having other pregnancy complications ($P$-values range from 0.028 to 0.267), these differences reached statistical significance only for total DNA levels between the group A and corresponding normal pregnancies ($P = 0.028$). The correlation between the fetal/total DNA ratio obtained by real-time PCR and the percentage of relative fetal allele concentration in maternal alleles obtained by QF-PCR was not found to be statistically significant ($r^2 = 0.04; P = 0.76$).

Conclusion Our results confirm the clinical value of fetal DNA measurement in maternal plasma during the second trimester as a supplement for the diagnosis of aneuploidies. Its use as a screening instrument for complications that develop later in pregnancy seems to be limited but needs further investigation.

Although the QF-PCR assay has the advantage of being applicable to both female and male fetuses, this approach cannot be used for quantitation of cff DNA in maternal plasma samples. Copyright © 2006 John Wiley & Sons, Ltd.

Key words: cell-free fetal DNA; maternal plasma; second trimester; pregnancy outcome; real-time PCR; fluorescent PCR

INTRODUCTION

A bidirectional transfer of cell-free fetal (cff) DNA between the fetal and maternal circulations has been demonstrated during pregnancy (Lo et al., 2000; Sekizawa et al., 2003). The presence of circulating cff DNA in maternal plasma and serum and its potential for non-invasive prenatal diagnosis was recognized for the first time in 1997 (Lo et al., 1997). The origin of cff DNA is presumably from placental cells undergoing apoptosis. Therefore, most fetal DNA circulate in membrane-bound vesicles (apoptotic bodies), which were detected by microscopic analysis in maternal plasma (Bischoff et al., 2005).

Cff DNA has been detected in the maternal circulation as early as 32 days of gestation, and increases by 21% per week in the first trimester (Wataganara et al., 2004a). A calculation of the cff DNA concentration in maternal plasma revealed a mean of 3.4% and 6.2% of the total plasma DNA in early and in late pregnancy,
respectively (Lo et al., 1998). The biologic significance of fetal DNA release into maternal plasma is a topic of ongoing investigations, and its use for clinical applications is currently being evaluated (Rijnders et al., 2004). Fetomaternal transfusion of cells and DNA is a potential indicator for placental health, and increased levels of fetal cells and DNA in maternal circulation are regarded as a breakdown of the placental barrier and linked to pregnancy-associated complications (Holzgreve et al., 1998).

Recent data have indicated an increase of circulating cfDNA in maternal plasma under certain pathologic conditions, such as certain chromosomal aneuploidies (Lo et al., 1999a; Zhong et al., 2000; Farina et al., 2003), preeclampsia (Lo et al., 1999b; Zhong et al., 2001; Lau et al., 2002), preterm labour (Leung et al., 1998; Farina et al., 2005), and invasive placenta (Sekizawa et al., 2002). In preeclampsia, the levels of cfDNA are elevated even before the onset of disease and provide a potential prediction marker (Leung et al., 2001; Zhong et al., 2001; Farina et al., 2004a).

The aim of our prospective study was first to investigate the clinical value of cfDNA measurement in maternal plasma during the second trimester as an indicator for the progression to adverse pregnancy outcome. Secondly, we wanted to compare real-time quantitative polymerase chain reaction (PCR) for the SRY gene and fluorescent PCR of STRs for the detection and quantitation of fetal DNA in maternal plasma since the currently used method for DNA quantitation can only be applied to male fetuses.

MATERIALS AND METHODS

Patients

Eighty-four healthy pregnant women carrying male fetuses and attending the Prenatal Diagnosis Unit at the Department of Obstetrics and Gynaecology, Medical University Graz were recruited with informed consent. They underwent amniocentesis for increased risk of chromosomal aneuploidies at a mean gestational age of 15 weeks (14–29 weeks). Amniocentesis was performed at a gestational age of 14 weeks in 4 cases, at 15 weeks in 35 cases, at 16 weeks in 25 cases, at 17 weeks in 9 cases, and one case each at 18, 19, 20, and 29 weeks. The gestational age of the aneuploid fetuses at amniocentesis was 15 and 16 weeks, respectively. In six cases, including one aneuploid fetus, gestational age at amniocentesis was not available. In one case, amniocentesis was performed at 29 weeks owing to severe intrauterine growth restriction (IUGR) and to plan the obstetric management. During the follow-up period, all pregnancy-associated complications were recorded. Fifty-eight of 84 pregnant women had a normal pregnancy and delivery of a healthy boy at term. In our series, five pregnancies were complicated by fetal chromosomal abnormalities (two cases with trisomy 21, one case each of triploidy, 47,XXX, and DiGeorge syndrome). Seven women with preterm labour and preterm deliveries at gestational ages between 32 and 37 weeks, five cases with preterm premature rupture of membranes (PPROM), one case of hemolysis, elevated liver enzymes low platelet count (HELLP) syndrome, three preexisting hypertonus, one intrauterine fetal death at 25 weeks of gestation, one severe intrauterine fetal growth restriction, and three cases of gestational diabetes were recorded. Our protocol was approved by the ethical committee of the medical university.

Samples and DNA extraction

Venous blood samples, 10 mL on average, were collected into tubes containing EDTA before amniocentesis. Plasma was obtained by centrifugation at 2000 g for 10 min. The samples were recentrifuged at 13 000 g, the supernatants were collected into fresh tubes and stored at −20°C until further processing. For analysis, DNA was extracted from plasma samples using the QIAamp Blood kit (Qiagen, Valencia, Calif) according to the blood and body fluid protocol. The DNA preparations were eluted in 50 μL of elution buffer.

Fluorescent PCR of STRs

Highly polymorphic short tandem repeat sequences (STRs) were chosen for detection of fetal DNA in maternal plasma. D21S11, D21S1411, and D21S1412 are present on chromosome 21. D18S536, D18S535, and DNA sequences from the myelin basic protein (MBP) gene are located on chromosome 18. D13S631 and D13S634 are specific for chromosome 13. The primers for the repeats of MBP gene amplify two STR loci (locus A and locus B) simultaneously. The STR markers were composed into three sets as described by Pertl et al. (2000).

The forward oligonucleotide primers for D21S11, D12S1412, D13S634, MBP, D18S535, and the AMXY locus were 5’e-end-labelled with 5-carboxy-fluorescein (blue), while the forward primers D21S1411 and D13S631 were labelled with 2’,7’-di-methoxyloxy 4’,5’-dichloro-6-carboxy-fluorescein (green).

PCR amplification was performed in a total volume of 25 μL, containing extracted genomic DNA (5 μL), 200 μM dNTPs, 5–20 pmoles of each primer, 1X Taq polymerase buffer (3 mM MgCl2), and 1.5 U Taq polymerase (both Promega, Madison, WI, USA). After initial denaturation at 94°C for 5 min, hot start PCR was done for 25 cycles at 94°C for 48 s, 60°C for 48 s, and 72°C for 1 min. Final extension was at 72°C for 5 min. The PCR products (3 μL) were mixed with 2.6 μL gel loading buffer and 0.4 μL Genescan-500 Rox (Applied Biosystems Inc, Warrington, UK) containing the reference molecular-size standard. Electrophoresis was performed on a 373 DNA sequencer (Applied Biosystems Inc., Warrington, UK) using a 6% denaturing polyacrylamide gel. The amplification products were analyzed and their relative fluorescent intensities calculated using Genescan 672 software (Applied Biosystems, Inc., Warrington, UK) (Pertl and Adinolfi, 1998; Pertl et al., 1999).
The percentage of fetal DNA was calculated by dividing the peak area of the fetal allele by the peak area of both maternal alleles and multiplying by 100.

**TaqMan PCR analysis**

Real-time quantitative PCR analysis was performed in Boston using a sequence detector (no. 7700; Perkin-Elmer Applied Biosystems, Foster City, Calif) with the TaqMan 5′nuclease assay. Amplification primers and fluorescent probes, designed to detect the SRY gene on the Y chromosome, were used for detecting fetal DNA in maternal plasma. As a control for the amplification of total (maternal plus fetal) plasma DNA, all samples were subjected to a TaqMan assay for the β-globin gene on chromosome 11. Each primer set used in the present study is described by Lo et al. (1998).

**Statistical analysis**

Descriptive statistics including mean, standard deviation, median, 25th and 75th percentiles, minimum, and maximum, were generated for gestational age and total and fetal DNA in maternal plasma. Two overlapping subgroups, A and B, were made for statistical analysis according to pregnancy-associated complications. Group A included chromosomal aberrations, growth restriction, HELLP syndrome, intrauterine death, gestational diabetes, and preterm delivery and group B comprised all complications we recorded (including PPROM, hypertension, preterm labour and delivery). Each group was separately compared for total and fetal DNA with a corresponding normal using Wilcoxon rank sum test. Spearman correlation analysis was performed to estimate the correlation between total and fetal DNA levels, as well as between the ratio of fetal/total real-time PCR results and the percentage calculated from the STR analysis in cases and controls separately. All statistical analyses were performed using SAS/STAT software (SAS Institute, Inc., Cary, NC). Statistical significance was assigned where p-value was less than 0.05.

**RESULTS**

Fetal derived Y-chromosome sequences were detected in 80/84 (95%) samples using real-time PCR. Quantities of total and fetal circulating DNA were significantly correlated (Figure 1) \( r^2 = 0.44, P < 0.0001 \) with an overall median total DNA concentration of 522 GE/mL (range 51–3047) and a median fetal DNA concentration of 8 GE/mL (range 0–879). Pregnancies with normal outcome had a median concentration of total and cff DNA in maternal plasma of 483 GE/mL (range 150–1416) and of 8 GE/mL (range 0–27), respectively.

Neither level was correlated with gestational age in pregnancies with normal \( r^2 = -0.05; P = 0.66 \), and \( r^2 = 0.02; P = 0.88 \), respectively or abnormal \( r^2 = 0.45; P = 0.17 \), and \( r^2 = 0.11; P = 0.76 \), respectively.

Two pregnancies that were complicated with triploidy and trisomy 21 exhibited particularly high concentrations of cff DNA in maternal plasma with 141 and 879 GE/mL, respectively. Although both total and fetal DNA levels were always higher in women carrying pregnancies with chromosomal aberrations or having other pregnancy complications \( P \)-values range from 0.028 to 0.267), these differences reached statistical significance only for total DNA levels between the group A and corresponding normal pregnancies \( P = 0.028 \) (Table 1). The significant differences detected in total DNA levels between normal and abnormal pregnancies are driven by the aneuploid pregnancies and excluding this group resulted in nonsignificant differences between the groups. The statistical analysis for group B showed no difference for both total and fetal DNA \( P = 0.097 \) and \( P = 0.267 \), respectively (Table 1). Figure 2 shows the log-transformed cff DNA levels in normal pregnancies compared to all complicated ones.

Fetal specific alleles were amplified in 72/84 (86%) of maternal plasma samples using fluorescent PCR amplification of short tandem repeat sequences (STRs). Fetal

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**Figure 1**—Correlation between total and fetal DNA levels in maternal plasma (Spearman: \( r^2 = 0.44; P < 0.0001 \)).

**Figure 2**—Levels of fetal DNA concentrations in maternal plasma in normal (1) and complicated (2) pregnancies (logarithmic scale).
Table 1—Total and fetal DNA concentrations in complicated and normal pregnancies

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>N</th>
<th>Gestational age</th>
<th>Total DNA, Median (25th–75th percentile)</th>
<th>Fetal DNA, Median (25th–75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisomy 21a</td>
<td>2</td>
<td>16.0</td>
<td>1011 (838–1185)</td>
<td>452 (25–879)</td>
</tr>
<tr>
<td>Triploidy</td>
<td>1</td>
<td>15.0</td>
<td>3047</td>
<td>141</td>
</tr>
<tr>
<td>XXY</td>
<td>1</td>
<td>15.0</td>
<td>205</td>
<td>0</td>
</tr>
<tr>
<td>DiGeorge syndrome</td>
<td>1</td>
<td>15.0</td>
<td>588</td>
<td>5</td>
</tr>
<tr>
<td>PPROM</td>
<td>5</td>
<td>15.8 ± 1.9</td>
<td>570 (486–1048)</td>
<td>5 (5–7)</td>
</tr>
<tr>
<td>Spontaneous preterm labour and delivery</td>
<td>7</td>
<td>15.7 ± 0.5</td>
<td>738 (525–860)</td>
<td>11 (9–17)</td>
</tr>
<tr>
<td>Fetal death at 25 weeks</td>
<td>1</td>
<td>17.0</td>
<td>950</td>
<td>6</td>
</tr>
<tr>
<td>HELLP syndrome</td>
<td>1</td>
<td>17.0</td>
<td>926</td>
<td>0</td>
</tr>
<tr>
<td>Growth restriction</td>
<td>1</td>
<td>29.0</td>
<td>1244</td>
<td>27</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>3</td>
<td>15.5 ± 0.7</td>
<td>570 (549–649)</td>
<td>15 (3–16)</td>
</tr>
<tr>
<td>Hypertonus</td>
<td>3</td>
<td>15.5 ± 0.7</td>
<td>457 (51–689)</td>
<td>2 (0–8)</td>
</tr>
<tr>
<td>Normal</td>
<td>58</td>
<td>15.6 ± 1.1</td>
<td>483 (371–682)</td>
<td>8 (5–12)</td>
</tr>
<tr>
<td>Group A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>17.1 ± 4.0</td>
<td>838 (649–950)</td>
<td>16.5 (7.5–26)</td>
</tr>
<tr>
<td>Group B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26</td>
<td>16.3 ± 2.9</td>
<td>679.5 (525–926)</td>
<td>8 (5–16)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Gestational age was available for one individual only.
<sup>b</sup> <i>p</i>-value for comparison with the normal pregnancy group is 0.028 for total DNA and 0.097 for fetal DNA.
<sup>c</sup> <i>p</i>-value for comparison with the normal pregnancy group is 0.207 for total DNA and 0.267 for fetal DNA.

Table 2—Relative maternal/fetal fluorescent intensity ratios of all markers used

<table>
<thead>
<tr>
<th>STR marker</th>
<th>Mean %</th>
<th>Range %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBPA</td>
<td>0.4–23</td>
<td></td>
</tr>
<tr>
<td>MBPB</td>
<td>0.4–75</td>
<td></td>
</tr>
<tr>
<td>D13S631</td>
<td>0.8–9</td>
<td></td>
</tr>
<tr>
<td>D18S535</td>
<td>0.2–25</td>
<td></td>
</tr>
<tr>
<td>D21S11</td>
<td>1–17</td>
<td></td>
</tr>
<tr>
<td>D21S1411</td>
<td>0.1–47</td>
<td></td>
</tr>
<tr>
<td>D21S1412</td>
<td>0.01–13</td>
<td></td>
</tr>
<tr>
<td>D18S386</td>
<td>0.5–13</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The aim of our prospective study was to evaluate the potential clinical application of fetal DNA quantitation during the second trimester as a predictive marker for adverse pregnancy outcome. A significant increase in circulating fetal DNA concentrations in the plasma of second-trimester pregnant women would be a beneficial screening marker for the development of complications in pregnancy.

Our results show similarities with some studies but also discrepancies to others. We found, for normal pregnancies, a median concentration of total and cff DNA in maternal plasma of 483 GE/mL (range 150–1416 GE/mL) and 8 GE/mL (range of 0–27 GE/mL), respectively.

Total and fetal DNA levels were significantly correlated, but neither level was correlated with gestational age, a finding that contradicts previous studies. However, in contrast to our data, a correlation between cff DNA and gestational age has been reported for the first (Farina et al., 2002; Wataganara et al., 2004a) and third trimester (Lo et al., 1998). Recent studies aiming at establishing the normality of cff DNA in maternal plasma in early pregnancy, which is mandatory for screening purposes, revealed median values of 25.4 GE/mL (Lo et al., 1998) and 23.3 GE/mL (Farina et al., 2002). Presumably, variations in fetal DNA concentrations depend on placental parameters, but the variables affecting fetal DNA release into maternal circulation are not exactly known at present. There is some evidence that apoptotic processes in the placenta are the source for cff DNA, since cff DNA is stored in apoptotic bodies (Bischoff et al., 2005). Wataganara et al. (2005) investigated the influence of placental volume measured by...
three-dimensional sonography on plasma cff DNA levels, but found no correlation.

We identified two plasma samples with an 18- and 93-fold increase in the median cff DNA concentration of 8 GE/mL, originating from 2/3 (67%) women carrying fetuses affected by aneuploidies. Thus, we could predict 1/2 (50%) pregnancies affected by trisomy 21 and the one affected by triploidy. This is in accordance with other authors who identified high concentrations of cff DNA in pregnancies complicated by trisomy 21 (Lo et al., 1999b; Zhong et al., 2000; Farina et al., 2003), but the results showed a considerable degree of overlap with euploid pregnancies. No significant difference in the cff DNA concentration between euploid and aneuploid pregnancies was found by Ohashi et al. (2001) and Spencer et al. (2003), which may indicate the existence of a subgroup of trisomy 21 pregnancies with low cff DNA levels. This might be why we missed one of our trisomy 21 cases. As we and Zhong et al. (2000) could show, there is also evidence that cff DNA is increased in pregnancies with 69 XXY fetuses. Cff DNA was within normal range for the pregnancy with the 47 XXY fetus, which validated the result of Zhong et al. (2000) who investigated plasma of two Klinefelter pregnancies, which also showed no significant elevation of DNA levels. No data for cff DNA levels are available for pregnancies with DiGeorge fetuses, but since there is no evidence for placental abnormalities, elevated cff DNA levels might not be expected.

Elevated levels of cff DNA have been reported in a number of pregnancy related complications associated with placental pathology such as preeclampsia (Lo et al., 1999a; Zhong et al., 2001; Lau et al., 2002; Farina et al., 2004a), HELLP syndrome (Swinkels et al., 2002), IUGR (Caramelli et al., 2003), and preterm labour (Leung et al., 1998; Farina et al., 2005).

In our series, cff DNA determined in the second trimester did not provide a prediction marker for one case of HELLP syndrome, which developed at 28 weeks of gestation, or for the seven pregnancies that subsequently had preterm labour and preterm delivery, or for the pregnancies complicated by gestational diabetes. Since fetal DNA concentrations in maternal plasma increased before the development of disease, some authors have proposed its use as a screening marker for preeclampsia (Lo et al., 2003; Zhong et al., 2002; Farina et al., 2004a). It was also shown that pregnant women who subsequently developed preeclampsia had elevated total DNA levels measured as beta-globin concentration during the second trimester (Farina et al., 2004b). Preeclampsia exhibits a two-stage elevation of cff DNA with an initial increase between 17 and 28 weeks of gestation. The level of cff DNA elevation correlated with severity, early onset of disease, and fetal growth restriction (Levine et al., 2004). The fact that we found no cff DNA elevation in the plasma of the woman who developed HELLP syndrome may be related to the gestational age of 17 weeks when the blood was taken. It is also worth emphasizing that all studies showed an overlap of preeclamptic and uncomplicated pregnancies. However, since there was only one case of HELLP syndrome in our study, no conclusions can be drawn from this result.

Recently, corticotropin-releasing hormone (CRH) mRNA in maternal plasma was introduced as a new molecular marker for preeclampsia (Ng et al., 2003a; Farina et al., 2004c). Ng et al. (2003a) demonstrated a tenfold increase of maternal plasma CRH mRNA in preeclampsia, compared with nonpreeclamptic controls matched for gestational age, which was twice (Lo et al., 1999b) and four times (Farina et al., 2004c) the circulating fetal DNA detected.

Preterm labour and preterm delivery as well as PPROM are problems that occur later and are mostly triggered by infection; therefore, it is unlikely that cff DNA may predict this event in asymptomatic pregnant women. High concentrations of cff DNA in maternal serum were reported in women with an increased risk of spontaneous preterm delivery due to preterm labour or preterm premature rupture of the membranes (Farina et al., 2005). In contrast to our study, the women tested already had an obstetric complication that was responsible for preterm delivery. The cff DNA status in pregnancies complicated with gestational diabetes was also unchanged. A high percentage of women with gestational diabetes show an increased body mass index (BMI), a finding that was shown to be inversely correlated to second-trimester cff DNA levels (Wataganara et al., 2004b).

In the second part of our study, we compared the results obtained by TaqMan PCR and fluorescent PCR of STRs to quantify cff DNA in maternal plasma. The major drawback of TaqMan PCR is the fact that the method can be used only for male fetuses, which limits its application for screening policies. Fluorescent PCR of STRs identifies the paternally inherited alleles of fetal DNA, which allows detection of cff DNA in a high percentage of cases independent of fetal gender. Comparison of the fetal/total DNA concentration obtained by TaqMan PCR analysis with the relative fetal–maternal fluorescent intensities revealed no correlation; therefore, this system cannot be used to determine absolute concentrations. Sekizawa et al. (2003) successfully used fluorescent PCR to determine the relative concentration of fetal DNA in maternal plasma and maternal DNA in umbilical plasma. However, in contrast to our approach a comparison to TaqMan PCR was not performed.

Our results confirm the clinical value of fetal DNA measurement in maternal plasma during the second trimester as a possible supplement for the diagnosis of chromosomal aberrations. However, at present its clinical use as a screening instrument is limited by the fact that (1) there is an overlap in cff DNA concentrations between complicated and normal pregnancies, (2) variables affecting fetal DNA release into the maternal circulation are not known in detail and that (3) the currently available method is only applicable to male fetuses. We could demonstrate that fluorescent PCR of STRs is a useful tool to determine relative concentrations of cff DNA of both male and female fetuses, but it is inappropriate for quantification purposes. A future perspective in this field offers the detection of mRNA.
in maternal plasma, which allows gender- and polymorphism independent screening analysis (Poon et al., 2000; Ng et al., 2003b).

REFERENCES


