Homocysteine levels in amniotic fluid
Relationship with birth-weight
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Summary
Hyperhomocysteinemia could play a similar role in the placenta to that played in adults at risk of thrombosis. Moreover, hyperhomocysteinemia in women is described to be associated with the birth of small for gestational age (SGA) newborns, although there are discrepancies on this issue. To date, there is no biochemical marker predictive of SGA in a given pregnancy. We verified the presence of a relationship between homocysteine in amniotic fluid at mid-pregnancy and birth-weight. Amniotic fluid was obtained from 459 healthy women undergoing mid-trimester amniocentesis (17.1 ± 1.2 weeks) because of maternal age. Homocysteine levels were measured in 434 (10 twin) pregnancies. In addition, femur length (FL) and biparietal diameter (BPD) were measured. Outcome of pregnancy was recorded. 233 (53.7%) foetuses were males, 201 (46.3%) females. The mean homocysteine concentration was 1.04 ± 0.72 µM, (95% C.I. 0.43–2.41). An univariate analysis showed the presence of an association with gestational age, FL, BPD. A multiple linear regression showed that homocysteine levels were significantly associated with FL (p<0.001) and BPD (p=0.011). After excluding twin pregnancies, 31 newborns (7.3%) were classified as SGA. Mean birth-weight was 2390 g in SGA, whereas it was 3360 g in 393 adequate for gestational age (AGA) newborns (p<0.001). The adjusted mean level of homocysteine was significantly lower in AGA (1.01 µM; 95%CI: 0.94–1.08) than that recorded in pregnancies resulting in a SGA (1.29 µM; 95%CI: 1.05–1.51; p=0.03). In a large setting, these data provide reference values for homocysteine in amniotic fluids. Moreover, they suggest that homocysteine levels in amniotic fluids may be higher in pregnancies with a SGA newborn.

Keywords
Homocysteine, amniotic fluid, foetal growth, prenatal diagnosis

Introduction
Homocysteine is a non-protein-forming, sulphur amino acid; its metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration (1). Because of the existence of a cellular homocysteine export mechanism (2, 3), plasma normally contains a small amount of homocysteine, averaging 10 µM. In hyperhomocysteinemia, plasma homocysteine levels are elevated and, barring impaired renal function, the occurrence of hyperhomocysteinemia indicates that homocysteine metabolism has in some way been disrupted and that the export mechanism is disposing excess homocysteine into the blood (4). This export mechanism limits intracellular toxicity, but leaves vascular tissue exposed to the possibly deleterious effects of excess homocysteine.

Determination of plasma total homocysteine (tHcy) (the sum of all forms of thiol derivatives that form homocysteine by reduction) is essential for diagnosis and follow-up of homocystinuric patients (5) and for detection of moderate hyperhomocysteinemia, a risk factor for coronary, cerebral, and peripheral vascular disease (6).

Various methods to measure tHcy have been described during the last decade (7, 8). A variety of different procedures are currently available for the determination of tHcy (6, 9, 10). The sensitivity and specificity of tandem mass spectrometry are well suited to perform high-volume analysis of tHcy (11).

It has been reported that amniotic fluid obtained from pregnancies with a foetus suffering from neural tube defects shows high homocysteine levels.

The presence of numerous infarctions in placentas from growth restricted foetuses has been described (12). A moderate hyperhomocysteinemia could play a similar role in the placenta to that played in adults who are at risk of thrombosis. In addition, it is known that hyperhomocysteinemia in women can be associ-
ated with the birth of SGA newborns (13), but there are discrepancies on this issue (14). Nevertheless, data about normal values of tHcy in amniotic fluid are sparse and inconsistent, and little data is available in Mediterranean Countries (15, 16), where dietary vitamin content differs from that in Northern Europe or North America. Moreover, to date, there is no biochemical marker considered predictive of SGA in a given pregnancy.

The aims of our study were i) to establish reference ranges for tHcy in amniotic fluid of a large sample of normal pregnancies in our geographical area, and ii) to evaluate a possible relationship between tHcy levels at mid-trimester and birth weight.

**Patients and methods**

**Patients**

Amniotic fluid was obtained from 459 healthy females undergoing mid-trimester amniocentesis because of maternal age. Amniotic fluid was drawn at a gestational age of 17.1 ± 1.2 weeks (mean ± SD). Ten were twin, 449 singleton pregnancies. Overall, 25 chromosomal abnormalities were recorded in singleton pregnancies [8 cases of 21 trisomy, 4 Turner syndromes, 3 chromosome inversions, 3 translocations (2 Robertsonian and 1 reciprocal), 2 cases of Klinefelter syndrome, 1 tetraploidy, 1 case of 18 trisomy, 1 X ring chromosome, 1 case of 47,XXX karyotype, 1 case of 47,XYY karyotype] and were excluded. In total, 434 pregnancies were considered. Transabdominal sonography at 21 weeks excluded the presence of foetal anomaly. Samples of amniotic fluid were taken from each mother, placed on ice, centrifuged at 4°C, 3,000 rpm within 1 h and the supernatant was collected and stored at −80°C until testing. Most women (n=402, 87.6%) were being supplemented with folic acid at a dosage of 1–10 mg/day. Of these, 395 (86.1%) did not smoke and 12 (2.6%) smoked 1–10 cigarettes/day (Table 1). None of the women suffered from chronic or pre-existing disorders, such as diabetes, hypertension, thyroid disorders or renal insufficiency.

FL and BPD were measured at the time of mid-trimester amniocentesis to verify the correspondence of foetal biometry and gestational age on the basis of last menstrual period. Birthweight, weeks of gestational age and gender of newborns were recorded at birth.

The study was carried out after approval of the Institutional Review Board.

**Methods**

An amniotic fluid sample (100 µl) was mixed with 20 µl of internal standard solution (2 nM homocysteine-d8). When reduced, homocysteine-d8 yields homocysteine-d4 (Hcy-d4) at double the initial concentration. Complete reduction of disulphides was accomplished by the addition of 20 µl of 500 mM dithiothreitol, which was allowed to react at room temperature for 15 min. Proteins were precipitated by the addition of 200 µl of 1 ml/l formic acid and 0.5 ml/l trifluoroacetic acid in acetonitrile. After 1 min centrifugation at 13,400 x g, 100 ml of the clear supernatant was transferred to an autosampler vial.

A bench top triple quadrupole mass spectrometer API 3000 (Applera) operated in ion evaporation mode with the TurbolonSpray ionization probe source (operated at 5,600 V) was used. Peripherals included an Integral 100Q (Applera) pump and an autosampler. To enhance the stability of the signal, separation of tHcy and Hcy-d4 from the bulk of the specimen matrix was achieved by using a short column (LC-CN, 3 cm 3 4.6 mm; Supelco). Autosampler injections of 1 ml (corresponding to 0.3 ml of the original sample) were made using a mobile phase composed of acetonitrile in 1 ml/l aqueous formic acid (600 ml/l acetonitrile=400 ml/l aqueous formic acid) at a flow rate of 1.0 ml/min. The column was directly connected to the TurbolonSpray ionization probe operating with the turbo gas on (6 l/min; sensor temperature, 250°C) with the LC column effluent flow splitting set at 1:5. The retention times of tHcy and Hcy-d4 were between 1.0 and 1.5 min in a 2.5-min chromatographic analysis. Total instrument acquisition cycle time was 3 min per sample.

**Statistical analysis**

All the analyses were performed according to the Statistical Package for Social Science (SPSS 6.1 for Macintosh). The significance of differences in means was evaluated by non-parametric test.

Data were analysed by means of Spearman coefficient of correlation, in which a possible association between homocysteine levels and maternal age, gestational age based on the menstrual history, FL, and BPD was tested. Each association was corrected for the presence of potentially confounding variables (maternal age, gestational age, gender, FL, BPD) by means of a multiple linear regression model. General factorial ANOVA models, adjusted for the same variables were used to obtain adjusted means of homocysteine according to whether babies were AGA or SGA at birth. SGA newborns were defined according to Battaglia (17) as those with a birth-weight under the tenth percentile.

Two-sided p-values <0.05 were considered statistically significant.

**Results**

During the enrolment period, a total of 424 women attending the Clinic were considered eligible for the study. Of them, 10 had twin pregnancies (11 females, 9 males), and all were dizygotic. In 233 (53.7%) cases, foetuses were males, and in 201 (46.3%) females. Mean age (± SD) of women included in the study was 37.1 ± 3.6 years. Overall, tHcy levels in amniotic fluid were 1.04 ± 0.72 µM, (95% C.I. 0.43–2.41). In amniotic fluids of male foetuses, the value was 1.0 ± 0.6 µM, while it was 1.1 ± 0.8 µM in
amniotic fluids from female foetuses; this difference was not statistically significant (p=0.37). tHcy levels in amniotic fluids were not significantly related to maternal age (Spearman correlation, p=0.73). As far as the twin pregnancies are concerned, amniotic fluid was obtained from 20 amniotic sacs, in which tHcy levels were 1.09 ± 0.30 μM (95% CI. 0.50–1.49). This value was not significantly different when compared to that obtained in the group with singleton pregnancies. Women supplemented with folic acid did not show significantly different levels of tHcy levels (1.06 ± 0.83 μM vs 1.03 ± 0.91 μM, respectively). Similarly, tHcy levels in smokers did not significantly differ from those recorded in non-smokers (data not shown).

An univariate analysis showed the presence of an association with gestational age, FL and BPD (Table 2). After excluding twin pregnancies, 31 newborns (7.3%) were classified as SGA. In this setting, the mean birth-weight was 2390 g, whereas it was 3360 g in 393 AGA newborns (p<0.001; Mann-Whitney U-test). Mean (±SD) homocysteine levels measured in the amniotic fluids were higher in the SGA group [1.31 μM (1.64)] than those detected in pregnancies resulting in an AGA newborn [1.02 mM (0.55); p=0.027; Mann-Whitney U-test]. Each association was corrected for the presence of potentially confounding variables (maternal age, gestational age, gender, FL, BPD, AGA/SGA status) by means of a multiple linear regression model. Homocysteine levels in amniotic fluid resulted to be significantly associated with FL (r²=0.106, p<0.001), BPD (r² = 0.014, p=0.018), and AGA/SGA status (r²=0.012, p=0.029) (Table 2).

A general factorial ANOVA model, which included the same variables, was performed to obtain adjusted means of homocysteine according to AGA or SGA status. The adjusted mean level of homocysteine detected in the amniotic fluid was significantly lower in AGA (1.01 μM; 95%CI: 0.94–1.08) than that measured in pregnancies that resulted in a SGA newborn (1.29 μM; 95%CI: 1.05–1.51; p=0.03).

**Discussion**

The aminoacids pool in the amniotic fluid mainly derives from maternal and placental metabolism, although the foetus contributes to this pool through transepidermal and urinary losses (18). Many studies were carried out in amniotic fluids (16, 18, 19) and the profile of aminoacids has been shown to be very similar to that found in maternal blood: foetal contribution is limited in normal conditions, while in the presence of a metabolic disorder amino acid levels in amniotic fluids are abnormal (20). It was previously reported that tHcy plasma levels in non-pregnant women are significantly different from those recorded in pregnant women (19). Our report shows that mean tHcy values in amniotic fluid are usually much lower than those observed in young adults (21), being 1.05 ± 0.7 μM. These values are very similar to those reported in previous studies (22, 23): Wenstrom, et al. measured tHcy levels in amniotic fluid in different settings, and found in two different groups of controls values of 1.0 ± 0.7 and 1.0 ± 0.63 μM, respectively. Moreover, our data confirm and extend those obtained in a lower number of pregnancies (n=23) in a group of women from a different ethnic background (Northern Europe) (15).

The identified causes of foetal growth restriction are of maternal (i.e. hypertensive disorders, prolonged pregnancy, maternal diseases) or foetal (malformations, transplacental infections, multiple pregnancy) origin, although in about 20% of cases there is an apparent absence of underlying pathologic conditions. We hypothesized that an elevated homocysteine level in amniotic fluid could play a similar role to that played in atherothrombosis of adults, and that this marker of “vascular” disease could be implied in the pathogenesis of SGA. The lack of a difference in amniotic fluid levels of tHcy between folate supplemented and non-supplemented women could be due to restricted power of the sub-analysis, or could indicate that folic acid supplementation is not enough to prevent SGA.

Our data show an inverse relationship between tHcy levels in amniotic fluid in mid-pregnancy and birthweight. Other authors (14) did not find a relationship with tHcy values in maternal plasma in women who developed preeclampsia and had a growth restricted foetus. It might be that the foetal and not the maternal compartment shows significant differences of tHcy levels predicting a lower birthweight.

Moreover, our data have provided valuable reference values for vitamins and tHcy before, during, and after pregnancy so as to contribute to a better diagnosis of maternal deficiencies and to further study the relationship between maternal vitamin status and adverse course and outcome of pregnancy.

A study carried out in France (16) measured the concentration of homocysteine and some aminoacids in amniotic fluid in order to use them as reference values for prenatal diagnosis of aminoacidopathies. However, that study considered a small sample size of pregnancies between the 10th and 32nd week, and differences between groups were not statistically significant.

We considered a larger sample size and a homogeneous group of women undergoing mid-trimester amniocentesis because of maternal age. In addition, our study shows an association between homocysteine levels in midpregnancy values and ultrasonographic parameters of foetal growth. More interestingly, homocysteine values at this time can predict the birth of a SGA.

Our suggestion is that homocysteine may be an important marker in human foetus growth.

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**Table 2: Relationship between homocysteine levels in amniotic fluid and clinical and ultrasonographic variables. Multiple linear regression.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>r²</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>FL</td>
<td>0.106</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BPD</td>
<td>0.014</td>
<td>0.018</td>
</tr>
<tr>
<td>AGA/SGA status</td>
<td>0.012</td>
<td>0.029</td>
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References