Original article

Cerebral ultrasound abnormalities in offsprings of women with C677T homozygous mutation in the *MTHFR* gene: a prospective study

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Background: **Perinatal stroke is a common cause of neurologic disability. Being clinically under-recognized, its true incidence is not known. Maternal thrombophilia is likely to be a predisposing factor. To date, a general consensus for evaluation of babies born to mothers with genetic thrombotic predisposition is missing. This study was undertaken to assess the frequency of cerebral abnormalities in the offspring of women with homozygous C677T mutation in the *MTHFR* gene, and to seek for association with additional maternal or pregnancy risk factors.**

Methods: **Mother-infant pairs were consecutively recruited from October 2006 through February 2013. Neonates underwent a thorough physical examination at birth, and a cerebral ultrasound examination (cUS) was performed within 24 hours of their life. In neonates with major cerebral lesions, a thrombophilia panel test was obtained. Follow-up cUS was performed in babies with major or minor cerebral abnormalities.**

Results: **Ninety-one neonates (47 males) were enrolled. By cUS, abnormalities were detected in 18 (19.8%) neonates. Twelve neonates were diagnosed with a minor lesion; a major ischemic/hemorrhagic lesion was found in 6 neonates. There were a neat male preponderance and significant associations with a history of suspected miscarriage, maternal coagulation factors gene mutations, and reduced protein S or protein C activity.**

Conclusions: **Our data confirmed a high incidence of cerebral abnormalities in neonates born to women with C677T homozygous mutation in the *MTHFR* gene. cUS at birth proved to be an effective screening tool or a diagnostic test, that should be routinely performed in babies born to mothers with known thrombotic predisposition.**

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Introduction

Perinatal stroke (PS), an acute neurologic syndrome due to cerebral injury of vascular origin, is a common cause of neurologic disability. The incidence of PS is varied with a frequency ranging from 1 in 4000 live births[1] to an incidence as high as 17% in autopsy studies of term newborns.[2] The broad range of estimates relates to the definition, population enrolled, type of studies that have derived such data, and ascertainment of cases.

By convention, PS encompasses cerebrovascular events occurring between 20 weeks of fetal life and 28 postnatal days,[3] however some authors used a narrower definition (i.e. from 28 weeks of gestation to 7 or 28 days of life).[4,5] Notably, the age at stroke cannot be established with any degree of certainty and can only be conjectured to have occurred in the above mentioned time period. The major clinical-anatomic subtypes comprised: i) arterial ischemic stroke; ii) hemorrhagic stroke; and iii) cerebral sinovenous thrombosis.

Most often, symptomatology is nonspecific and neonates may remain clinically asymptomatic until several months of age, when seizures or signs of motor or cognitive impairment are first noted.[6,7] Thus, the final or presumed diagnosis of PS is often delayed.

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Remarkably, in settings with high frequency of neuroimaging, the incidence of PS has been reported to be one in 2300 live births,[8] considerably higher than the incidence in older children, estimated at 3.3 in 100 000 per year in individuals of less than 15 years old.[9] The etiological factors responsible for perinatal stroke are various and diverse for each type of vascular lesion.[10]

Even though a clear embolic source is rarely identified, thromboembolism is considered a leading cause of perinatal cerebral infarction. Among genetic predisposing conditions for spontaneous stroke, increased lipoprotein "a" [Lp(a)] levels, the coagulation factor V Leiden (mutation G1691A), the prothrombin G20210A variant, the methylenetetrahydrofolatereductase (*MTHFR*) CT677T genotype, and deficits in protein C or S, are widely described in symptomatic children and adolescents.[11,12] Conversely, the role of the mentioned prothrombotic conditions in PS is debated.

As in adults and in children >6 months, the increased Lp(a) concentration is the most important risk factor.[11,13] Deficiencies in protein C or S activity, heterozygosity for factor V Leiden and prothrombin 20210, homozygosity for C677T or compound heterozygosity for the C677T/A1298C alleles in the *MTHFR* gene, the 4G polymorphism of the plasminogen activator inhibitor 1, and the presence of anti-phospholipid antibodies have also been reported.[11-16] Additionally, pregnancy, placental and/or maternal disorders might be predisposing conditions for PS.

Pregnancy is physiologically hampered by a hypercoagulable state and pregnant women often have reduced levels of protein S and elevated levels of factor V, factor VIII and fibrinogen. On the other hand, newborn infants are at risk of developing vitamin K deficiency, leading to reduced activity of vitamin K-dependent coagulation factors (i.e factor II, VII, IX, and X and the Gla-proteins C and S) and abnormal prothrombin levels, thus impairing blood clot formation. Case series and case-control studies suggest that anti-phospholipids syndrome, pre-eclampsia, diabetes, placental thrombosis or abruption, prolonged rupture of membranes, chorioamnionitis, intra-partum maternal fever, smoking during pregnancy, cocaine abuse, and history of infertility are risk conditions for perinatal stroke.[17]

Placental infarction and subsequent embolization have been proposed as the major causative events: thrombi arising in the placental veins may reach the fetal cerebral circulation through the normally patent foramen ovale and ductus arteriosus. A higher incidence of cerebro-vascular accidents has been described in infants born to mothers with genetic prothrombotic disorders, including polymorphism for the *MTHFR* gene, factor V Leiden, and prothrombin gene mutation.[4,18] Recently, we found an intriguing association between maternal homozygous C677T mutation in the *MTHFR* gene and the occurrence of major ischemic/hemorrhagic cerebral lesions in the offspring.[19]

To date, however, no studies have prospectively evaluated the incidence of major or minor sonographic cerebral abnormalities in infants born to mothers with ascertained genetic susceptibility to thrombosis. Most studies investigating PS are retrospective and limited by small size or referral bias. Additionally, while guidelines for management of pregnant women with documented thrombophilia are available,[20] a general consensus as to the basic evaluation of babies born to mothers with known thrombotic risk factors is missing.

The purpose of this study was to prospectively evaluate the incidence of major and minor cerebral abnormalities by cerebral ultrasound (cUS) in neonates born to mothers with homozygous *MTHFR* C677T mutation and to evaluate the relationship with maternal or pregnancy conditions predisposing to thrombotic events.

Methods

Study population

Eligible for the present study were neonates born to mothers with *MTHFR* C677T homozygous mutation, detected as part of thrombophilic screening tests performed in pregnant women with history of hypertension, preeclampsia, recurrent spontaneous abortions, abruptio placentae or intrauterine death, referred to the L. Sacco Hospital High-risk Pregnancy Ambulatory and Birth Center. Given the intrinsic risk of fetal neurologic impairment, exclusion criteria were: maternal substance abuse during pregnancy, multiple pregnancy, severe prematurity (gestational age <32 weeks), very low birth weight (<2SD for gestational age), congenital infection, perinatal asphyxia, chromosomal syndromes, neonatal septicemia, birth related trauma, the need for resuscitation. The study was approved by the L. Sacco Hospital Ethical Commettee. Written informed consent was obtained at birth from parents or legal guardians of the newborn.

Procedures

In the pregnant women, laboratory tests included DNA sequencing for three common gene mutations *MTHFR* C677T, the missense mutation R506Q in factor V (factor V Leiden) and prothrombin 20210G>A, detected by polymerase chain reaction and analysis of restriction

fragments.[21-23] Additionally, serum homocysteine levels, serum protein C and S activity levels and lupus anti-coagulant (LAC) antibodies were assessed. A complete pregnancy and maternal history was obtained; complications of labor and delivery were carefully observed.

At birth, all infants underwent a thorough physical examination; anthropometrics and apgar scores were recorded. Within 24 hours after birth, a transfontanellar cUS was carried out, using a multifrequency 7.5 MHz transducer (US LogiQ P5, General Electric); seven coronal and five sagittal scans through the anterior fontanel were obtained. The major cerebral lesions were considered as follows: i) intra-ventricular hemorrhage (IVH) grade 2 or more severe; ii) post-hemorrhagic hydrocephalus; iii) ventriculomegaly; and iv) periventricular leukomalacia. Minor abnormalities such as IVH grade 1, mild ventricular asymmetry, choroid plexus or subependimal cysts were carefully sought. A scoring system for severity was adopted. Peri- and intra-ventricular hemorrhages were classified according to Volpe[24] and periventricular leukomalacia according to de Vries et al.[25]

Subsequently, infants in whom a minor cerebral lesion was detected, underwent a neurological examination, and a follow-up cUS was performed at 1-2 months of life; in infants with major cerebral lesions, a brain magnetic resonance imaging (MRI) was performed, together with repeated neurological evaluations and cUS follow-up.

In symptomatic neonates and in those with major cerebral lesions, blood was drawn for further study. Serum homocysteine, protein C and protein S activity were assessed; DNA testing for *MTHFR* C677T mutation, factor V Leiden and prothrombin G20210A variant was also performed. To avoid unnecessary risks related to the blood draw and given the cost of the genetic testing, no additional laboratory studies were carried out in asymptomatic babies with no evidence of cerebral abnormalities.

Statistical analysis

Collected data were recorded in matrix Excel 2007 (Microsoft) and analyzed with the package SPSS 20.0 for Windows. Descriptive statistics were calculated for continuous variables (mean and standard deviation) and for categorical variables (percentage frequency) both for the whole cohort and considering separately the three groups defined as: normal neonates (normal cUS), neonates with mild cerebral ultrasound abnormalities (minor cUS abnormalities), and neonates with severe cerebral ultrasound abnormalities (major cUS abnormalities).

To make comparisons, Student's *t* test and Fisher's exact test were used respectively for continuous variables and nominal variables. *P* values were calculated with two-tailed test. To express the strength of association between the results observed on ultrasound brain and nominal variables considered, odds ratios (OR) were calculated. A *P* value <0.05 was considered statistically significant.

Results

From October 2006 through February 2013, 93 mother-infant pairs were consecutively recruited. One male neonate was excluded because of maternal seroconversion for toxoplasma gondii in the late third trimester, and one female neonate was excluded because of severe prematurity. Thus, in 91 neonates (47 males, 51%) enrolled, 84 were delivered at term and 7 were pre-term (32 weeks<gestational age<37 weeks). Because of some missed data, statistical analysis was performed on 88 subjects.

Infant, maternal and gestation characteristics are shown in Table 1. Fifty-one (58%) neonates were vaginally delivered. Their gestational age ranged from 225 to 292 days (mean 271 days), and birth weights ranged from 1855 to 4500 g (mean=3166 g). Birth weight was appropriate for gestational age in 82/88 neonates (93.2%), while 6 neonates (6.8%) were small for gestational age (SGA) being their birth weight less than 10th percentile for their estimated gestational age birth weight. In our cohort, no neonates were large for gestational age with birth weight >90th percentile.

With regard to treatment, during pregnancy all the enrolled women were on folic acid supplementation and 47 were also receiving antithrombotic prophylaxis: 32 women (35%) were treated with low-dose aspirin (ASA) 50 mg/day, 13 (13%) with low-molecular weight heparin (LMWH) 4000 IU once a day, and 6 (6.5%) with combined ASA and LMWH.

Cerebral abnormalities were detected by using cUS in 18 of the 91 (19.8%) infants, of whom 12 were diagnosed with a minor lesion but the remaining 6 with a major ischemic/hemorrhagic lesion. Notably, only 2 babies diagnosed with a major cerebral lesion developed symptoms within 48 hours of life. No infants with minor cerebral abnormalities were found to be symptomatic. In the 12 infants with minor cerebral abnormalities, 6 showed mild ventricular asymmetry, 5 choroid plexus cysts and 1 IVH grade 1.

The observed cases of major cerebral lesions included: 2 cases with ischemic stroke, of whom one presenting with myoclonic seizures; 2 cases with intraventricular hemorrhage, of whom one developed generalized seizures;

Table 1. Perinatal and maternal risk factors for the cases and the 70 babies with no sonographic alterations

Variables	Total	Normal cUS	Minor cUS abnormalities	Major cUS abnormalities
N	88	70	12	6
Newborn factors				
Gender (female), *n* (%)	41 (47)	37 (53)	4 (33)	0 (0)
Gestational age (d), mean (SD)	271 (11)	272 (10.2)	272 (11.9)	261 (13.8)
Birth weight (g), mean (SD)	3166 (448)	3159 (440)	3290 (474)	2938 (576)
SGA, *n* (%)	6 (7)	4 (5)	1 (8)	1 (17)
Apgar at 1 min <7, *n* (%)	3 (3)	1 (1)	1 (8)	1 (17)
Apgar at 5 min <7, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)
pH, mean (SD)	7.32 (0.08)	7.32 (0.08)	7.31 (0.05)	7.31 (0.12)
Maternal factors				
Age (y), mean (SD)	33.4 (4.95)	33.3 (4.35)	34.8 (7.79)	32.0 (5.21)
Factor V Leiden, *n* (%)	9 (10)	6 (9)	0 (0)	3 (50)
Prothrombin 20210G>A mutation, *n* (%)	6 (7)	4 (6)	0 (0)	2 (33)
Low Protein C activity,* *n* (%)	12 (14)	7 (10)	0 (0)	5 (83)
Low Protein S activity,† *n* (%)	19 (22)	11 (16)	3 (25)	5 (83)
Serum homocysteine (μmol/L),‡ mean (SD)	7.33 (1.57)	8.25 (6.23)	7.05 (3.26)	5.43 (1.97)
LAC Ab+, *n* (%)	1 (1)	1 (1)	0 (0)	0 (0)
Pregnancy factors				
Vaginal delivery, *n* (%)	49 (57)	40 (59)	6 (50)	3 (50)
Anti-thromboticprophylaxis low dose ASA, *n* (%)	37 (42)	27 (39)	5 (42)	5 (83)
LMWH, *n* (%)	18 (20)	15 (21)	2 (17)	1 (17)
Vitamin B12 supplementation, *n* (%)	5 (6)	4 (6)	1 (8)	0 (0)
Polyabortivity, *n* (%)	40 (46)	30 (43)	6 (50)	4 (67)
History of endo-uterine death, *n* (%)	5 (6)	5 (7)	0 (0)	0 (0)
Suspected miscarriage, *n* (%)	6 (7)	4 (6)	0 (0)	2 (33)
Hypertension or pre-eclampsia, *n* (%)	4 (4)	4 (6)	0 (0)	0 (0)
Smoking, *n* (%)	2 (2)	0 (0)	1 (8)	1 (17)

*: adult normal protein C activity: 70%-138%; †: adult normal protein S activity: 65%-120%; ‡: adult normal serum homocysteine: 5-12 μmol/L. SGA: small for gestational age; LAC Ab: lupus anticoagulant antibodies; ASA: acetyl salicylic acid; LMWH: low molecular weight heparin; cUS: cerebral ultrasound.

one case with severe ventricular enlargement; and one case with periventricular leukomalacia evolving into cystic lesions.

Perinatal and maternal risk factors for the neonates with and without cUS alterations were compared (Table 1). There were no significant differences between these neonates with respect to maternal age, mode of delivery, Apgar score at 1 or 5 minutes, umbilical cord blood pH, birth weight, gestational age, LAC antibodies, history of abortion or intra-uterine fetal death, hypertension or pre-eclampsia during pregnancy, use of anti-thrombotic prophylaxis, smoking, and maternal serum homocysteine levels.

Minor or major cerebral abnormalities were found in 12 of 18 male neonates (male/female ratio: 3.25); remarkably, all 6 neonates with major cerebral lesions were males.

Compared with mothers of neonates with normal cUS, mothers of neonates with minor or major cerebral lesions were more likely to be carriers of the factor V Leiden, prothrombin 20210G>A mutation, or to have reduced protein C or S activity. Additionally, mothers of these neonates were more likely to have experienced warning signs or symptoms for miscarriage, supporting the hypothesized association between maternal *MTHFR* mutation and hypercoagulable status.

In the subgroup of 6 neonates with major cerebral lesions, 3 neonates were homozygous for the C677T *MTHFR* mutation (case 3, 14, 39) and 1 homozygous for the factor V Leiden (case 39), but 3 heterozygous for the same factor (case 3, 6, 14) and 1 for the prothrombin 20210G>A (case 10). Serum homocysteine levels were above the normal limit in 1 neonate (case 39). Protein C and S activity was reduced in 5 of 6 neonates. The characteristics of the 6 neonates with a major cerebral lesion and the distribution of coagulation factors are shown in Table 2.

Follow-up cUS documented post-hemorrhagic triventricular hydrocephalus in both neonates with intraventricular hemorrhage, whereas the brain injury did not evolve in any neonate diagnosed with ischemic stroke or severe ventricular enlargement.

Discussion

The actual incidence of perinatal stroke is likely underestimated. Since newborns often present with subtle symptomatology, who might be missed. Thus, undiagnosed neonatal cerebral infarctions may be responsible for cerebral palsy or neurologic impairment in neonates with an unremarkable perinatal history.

In the pathogenesis of PS, complex and multifactorial

contributions of maternal, pregnancy and neonatal conditions predisposing to clot formation have been identified. As recently described, genetic susceptibility seems to play an important role.[26] Maternal genetic thrombophilia has been widely investigated in its association with various pregnancy complications,[27-31] but its role in PS is not clear.

The present study aimed to assess the extent to which a specific clotting factor abnormality in the mother (the homozygous C677T mutation in the *MTHFR* gene) may contribute to the occurrence of major or minor cerebrovascular lesions in the offspring. As important triggering factors for ischemic stroke in neonates, maternal substance abuse during pregnancy, multiple pregnancy, severe prematurity, very low birth weight, congenital infections, perinatal asphyxia, chromosomal syndromes, neonatal septicemia, birth related trauma and the need for resuscitation were exclusion criteria.[14]

In our cohort, the incidences of cerebral abnormalities and major cerebrovascular lesions detected by cUS in the first 24 hours of life were 19.8% and 6.6%, respectively. Compared to retrospective data from healthy full-term neonates[32] and prospective data from clinically unselected newborns,[29,30] the incidence of cerebral abnormalities was remarkably higher in our cohort. Also the incidence of major ischemic/hemorrhagic lesions was higher than that reported in perinatal or presumed PS affected infants and children,[3,6] supporting the hypothesis that maternal polymorphisms for clotting factors may represent a prothrombotic condition for the offspring.[19]

The second objective of our study was to determine if the identified ultrasound abnormalities might be related to neonatal, maternal or pregnancy predisposing conditions additional to the maternal C677T-*MTHFR* homozygous genotype.

In line with previous reports in older children,[33] we documented a neat male preponderance (male/female ratio: 3.25); the reason of this finding is still unclear. Differently from the study by Chabrier et al,[34] none of the infants in our cohort were large for gestational age. However, being larger than females, male infants may be more likely to experience difficulties during delivery. Differences in response to hypoxia-ischemia and cell death pathways between males and females may be relevant.[35] Moreover, protective effects of estrogens in female infants should be taken into account.

In our cohort, no other neonatal factors were found to be associated with cerebral abnormalities. Notably, most of the neonates with major cerebral lesions had one or more genetic mutation additional to the inherited C677T-*MTHFR* etero- or homozygous genotype. However, the small number of cases and the lack of data in neonates with minor or no cUS abnormalities precluded meaningful subgroup analyses. Also, 4 of 6 neonates with major cerebral lesions had low protein C or protein S activity levels. This finding is of not sure interpretation, given the lack of standardized normal values in neonates.

Among the maternal factors considered in the analysis, the following were found to be associated with cerebral abnormalities in the offspring: factor V Leiden carriage (P=0.0102), prothrombin gene G20210A variant (P=0.0363), reduced protein S or protein C activity (P=0.0040 and P=0.0011, respectively). No correlations were found between neonatal cerebral abnormalities and maternal anti-thrombotic prophylaxis.

The lack of association with maternal serum homocysteine level was of particular interest. It is known that the C677T *MTHFR* genotype is associated with reduced enzymatic activity and mild increase in blood homocysteine concentrations, particularly in subjects with lower blood folic acid levels.[36] In our cohort, however, no significant difference was found in maternal homocysteine levels, thus additional pathogenetic mechanisms might be responsible for the increased thrombotic risk in the offspring. On the other hand, a statistically significant association was found

Table 2. Characteristics of the 6 neonates with a major cerebral lesion and distribution of coagulation factors mutations

Factors	Case 3	Case 6	Case 10	Case 14	Case 23	Case 39
Maternal thrombophilic factors						
Factor V Leiden	+/-	+/+	-/-	+/-	-/-	+/-
Prothrombin 20210G>A	-/-	-/-	+/-	+/-	-/-	-/-
Protein C activity %*	46	60	138	66	119	55
Protein S activity %†	63	19	65	55	72	71
Serum homocysteine, μmol/L‡	5.70	4.80	7.20	8.10	3.9	2.9
Newborn thrombophilic factors						
C677T *MTHFR*	+/-	+/-	+/+	+/+	+/-	+/+
Factor V Leiden	+/-	+/-	-/-	+/-	-/-	+/+
Prothrombin 20210G>A	-/-	-/-	+/-	-/-	-/-	-/-
Serum homocysteine, μmol/L‡	4.0	4.5	6.0	5.9	6.4	14.7
% protein C activity	12	-	27	20	23	55
% protein S activity	10	-	25	60	49	71

*: adult normal protein C activity: 70%-138%; †: adult normal protein S activity: 65%-120%; ‡: adult normal serum homocysteine: 5-12 μmol/L. "-": missing; "+/+": homozygous mutation; "+/-": heterozygous mutation; "-/-": absence of mutation.

between the occurrence of cerebral lesion and reported signs or symptoms suspicious for miscarriage, supporting the hypothesis that factors responsible for PS might eventually cause spontaneous abortion.

Major limitations of this study include the absence of control subjects, the lack of histopathological examination of the placentas, and the incomplete prothrombotic risk assessment in the offspring, not comprising serum Lp(a) and blood folic acid levels or other clotting factors polymorphisms. Additionally, despite the quite large population enrolled, the number of neonates with major cerebrovascular lesions was relatively small. Conversely, the main strength of our investigation was its prospective design, with consecutive recruitment and no ethnic restriction, thus avoiding bias of population selection. Also, cUS scans were performed by a single trained operator with the same ultrasound machine, avoiding inter-operator biases.

In conclusion, our data confirmed a high incidence of cUS abnormalities in neonates born to mothers with *MTHFR* C677T homozygous mutation. Among additional maternal and pregnancy putative predisposing conditions, a statistically significant association was found solely with other maternal polymorphisms for clotting factors and suspected miscarriage during the current pregnancy. cUS at birth proved to be a safe and effective screening tool and a diagnostic test in the offspring of women with known predisposition for thrombosis.

Although this is an important first look at genetic maternal thrombophilia as a predisposing condition for the occurrence of cerebral lesions in the offspring, larger prospective studies comparing healthy neonates to those born to mothers with genetic thrombotic risk are needed to establish the maternal pro-thrombotic state as a causative factor in neonatal stroke and central nervous system vascular damage.

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