Association Between the Glu298Asp Polymorphism in the Endothelial Constitutive Nitric Oxide Synthase Gene and Brain Infarction

Alexis Elbaz, MD, PhD; Odette Poirier, PhD; Thierry Moulin, MD; François Chédru, MD; François Cambien, MD; Pierre Amarenco, MD; on behalf of the GÉNIC Investigators*

Background and Purpose—Nitric oxide (NO) synthesized by endothelial constitutive NO synthase (ecNOS) plays a key role in vascular regulation and atherosclerosis. Little is known concerning the role of the ecNOS gene (NOS3) as a risk factor for brain infarction (BI). Our aim was to investigate the relation between the Glu298Asp polymorphism in exon 7 of NOS3 with BI and its subtypes.

Methods—Patients (n=460; cases) with BI were consecutively recruited and classified into etiological subtypes. Control subjects (n=460; controls) without a history of stroke were recruited among individuals hospitalized at the same institutions and individually matched on age, sex, and center. Genotypes of the polymorphism were determined by polymerase chain reaction.

Results—The distribution of genotypes was significantly different between cases and controls (P=0.008); the GG genotype was more frequent in cases (46.1%) than in controls (35.4%; OR, 1.56; 95% CI, 1.19 to 2.04). Among subtypes, the frequency of the GG genotype was significantly higher in cases than in controls in the lacunar subtype (OR, 2.00; 95% CI, 1.05 to 3.80); in this group, the relation between BI and LDL level was stronger among carriers of the GG genotype than among noncarriers (P for interaction, 0.05).

Conclusions—Homozygosity for the G allele of the Glu298Asp polymorphism in NOS3 was associated with BI, and especially with lacunar stroke. Our findings suggest that genetic susceptibility and LDL cholesterol have a synergistic relation. Although these findings should be replicated in a larger sample of subjects and the functionality of the Glu298Asp polymorphism has not been established, these results may help us to understand the cause of the arteriolopathy underlying lacunae and have future implications in their treatment and prevention. (Stroke. 2000;31:1634-1639.)

Key Words: brain infarction ■ nitric oxide ■ lacunar infarction ■ genetics ■ case-control studies

Nitric oxide (NO) synthesized from L-arginine by endothelial constitutive NO synthase (ecNOS) is permanently released from arterial and arteriolar endothelium. NO plays a key role in the relaxation of vascular smooth muscle cells (VSMCs); it reduces VSMC proliferation, adhesion of platelets and leukocytes, endothelial permeability, and extracellular matrix collagen synthesis.1–3 Conversely, an excess of NO may be harmful because of its oxidative role.4

In animal models, ecNOS inhibition accelerates atherosclerosis,5 whereas its administration prevents it.6 In humans with atherosclerosis, abnormalities in the endothelial NO pathway have been shown.7 There is also evidence that disturbances in the NO pathway in myocardial infarction and coronary spasm not only may be the consequence of endothelial dysfunction due to cardiovascular risk factors but also may have a genetic basis.8–12 A genetic contribution of ecNOS to plasma NO metabolite levels has been demonstrated.13,14 Several polymorphisms have been identified in the ecNOS gene (NOS3), among which is a polymorphism located in exon 7 (G894T) that modifies its coding sequence (Glu298Asp).11

There is growing evidence that genetic factors may increase the risk of brain infarction (BI).15 Our aim was to investigate the association between BI and the Glu298Asp polymorphism in NOS3 in patients with BI and matched controls. Because lacunae are associated with alterations of the arteriolar wall characterized by hypertrophy of VSMCs

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and lipid deposits, we were particularly interested in investigating the relation between the ecNOS gene and lacunae.

**Subjects and Methods**

**Cases**
Case patients (cases) were recruited consecutively among all patients admitted to 12 French neurological centers if they fulfilled the following criteria: (1) clinical symptoms suggestive of stroke, (2) no brain hemorrhage on CT scan, (3) infarct proven by MRI, (4) 18 to 85 years old, and (5) both parents of Caucasian origin. Cases were included in the week-interval after the event. Patients reporting a previous cardiovascular or cerebrovascular history were eligible.

**Controls**
Control subjects (controls) without a history of stroke were recruited among individuals hospitalized at the same institutions for any reason other than neurological diseases; these consisted of orthopedic (46%), ophthalmological (12%), rheumatological (11%), surgical (6%), and other (25%) causes. One control was matched by sex, age (±5 years), and center to each case. Subjects reporting a positive cardiovascular history other than stroke were eligible. Their parents had to be of Caucasian origin.

**Data Collection and Risk Factor Definition**
Information on demographic characteristics and risk factors was collected by use of a structured questionnaire. Hypertension was defined as a history of treated hypertension. Smoking history was coded as never, ex, and current smoker. Subjects were classified as diabetic when they were being treated for insulin-dependent or non–insulin-dependent diabetes. Use of lipid-lowering drugs was assessed. History of myocardial infarction, angiplasty, coronary artery bypass surgery, or lower-limb arterial disease was recorded; a positive cardiovascular history was defined as the presence of any of these diseases. A history of stroke or transient ischemic attacks was obtained in cases.

**Investigations**
ECGs and extracranial duplex and transcranial Doppler echocardiographies were performed on all cases and controls. The presence of plaques, arterial stenoses, and occlusions was assessed. Two-dimensional echocardiography results were available for 464 patients (91%), and transesophageal echocardiography was performed in 358 (77%). Radiographs or magnetic resonance cerebral angiograms were performed on 208 patients (41%). Blood was drawn in the morning from fasting subjects for DNA extraction and lipid profile (LDL) were adjusted for use of lipid-lowering therapy; because LDL were multiplied by the relation between the ecNOS gene and lacunae.

**Glu298Asp Polymorphism in the ecNOS Gene**
The missense Glu298Asp variant results from a G→T substitution, which leads to the replacement of glutamic acid by aspartic acid at position 298. The genotyping protocol is available on the Internet site http://genecanvas.idf.inserm.fr/.

**Data Analysis**
Allelic frequencies were calculated by gene counting. Hardy-Weinberg equilibrium was tested with the x² statistic. To compare the overall distribution of the genotypes of the polymorphism between cases and controls, we used the −2 log-likelihood statistic of a conditional logistic regression model. Odds ratios (ORs) associated with the GT and GG genotypes, considering the TT genotype as the reference group, were computed by conditional logistic regression. No excess risk of BI was associated with the heterozygous GT genotype in the whole sample or in any subtype.

ORs were therefore computed assuming a recessive model, by comparing the frequency of the GG genotype in cases and controls with the frequency of GT+TT genotypes pooled together; the statistical validity of this approach was tested with a collapsibility test.17 Our analyses concerned the whole study group and were subsequently stratified according to the main BI subtypes (atherothrombotic, lacunar, cardioembolic strokes, and strokes of unknown cause); in each stratum, cases were compared with matched controls by conditional logistic regression. Analyses concerning strokes of undetermined cause are not reported, because this is by definition a highly heterogeneous group. We also report analyses restricted to cases and matched controls both free of previous cardiovascular or cerebrovascular history.

Multiplicative first-order terms were introduced into the models to test for an interaction between the GG genotype and LDL level (defined as a continuous variable).18 ORs according to tertiles of the distribution, a logarithmic transformation of the blood sample delay was used. The research protocol was approved by the ethics committee of Hôpital Cochin, and all subjects signed informed consent forms.

**Results**
Among the 510 cases and 510 controls included in the study, DNA could be extracted and amplified for 474 cases (92.9%) and 491 controls (96.3%), corresponding to 460 matched pairs. The characteristics of the study subjects are shown in Table 1; the distribution of BI subtypes is shown in Table 2. Distributions of genotypes of the Glu298Asp polymorphism in cases and controls and allele frequencies are shown in Table 3, overall and according to subtypes. Among controls, there were no differences in the frequency of genotypes according to the main hospitalization departments (P=0.7, data not shown). The distribution of genotypes was

“Other etiologies” were rare causes, such as polycythemia vera, cerebral arteritis, or thrombocytopenia.

“Undetermined cause” was when ≥2 etiologies as defined above coexisted in the same individual.

“Unknown cause” was when no etiology was identified. Patients with an isolated elevation of antiphospholipid antibodies, patent foramen ovale, atrial septal aneurysm, valvular strands, mitral valve prolapse, or mitral annulus calcifications belonged to this group.

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TABLE 1. General Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=460)</th>
<th>Controls (n=460)</th>
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</thead>
<tbody>
<tr>
<td>Age, y (median; range)</td>
<td>69; 20–85</td>
<td>68; 20–89</td>
</tr>
<tr>
<td>Male sex, % (n)</td>
<td>61.1 (281/460)</td>
<td>61.1 (281/460)</td>
</tr>
<tr>
<td>History of hypertension, % (n)</td>
<td>52.2 (239/458)</td>
<td>36.1 (165/457)*</td>
</tr>
<tr>
<td>History of diabetes mellitus, % (n)</td>
<td>19.0 (87/458)</td>
<td>11.3 (52/460)*</td>
</tr>
<tr>
<td>Total cholesterol, g/L (SD)</td>
<td>2.01 (0.43)</td>
<td>1.82 (0.43)*</td>
</tr>
<tr>
<td>LDL cholesterol, g/L (SD)</td>
<td>1.25 (0.35)</td>
<td>1.11 (0.35)*</td>
</tr>
<tr>
<td>Current smoking, % (n)</td>
<td>28.8 (132/459)</td>
<td>20.4 (94/460)†</td>
</tr>
<tr>
<td>Cardiovascular history, % (n)</td>
<td>21.5 (98/456)</td>
<td>11.6 (53/457)*</td>
</tr>
<tr>
<td>Stroke history, % (n)</td>
<td>20.9 (96/459) . . . .</td>
<td></td>
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Proportions were compared by \( \chi^2 \) analysis. Means were compared by Student's t test; medians were compared by Wilcoxon test. *P<0.001. †P<0.01.

...significantly different between cases and controls (P=0.008). A significant difference (P<0.03) in the distributions of genotypes was also observed when analyses were restricted to pairs of cases and matched controls both free of previous cardiovascular and cerebrovascular history (cases: 50.0% GG, 40.1% GT, 9.9% TT; controls: 36.0% GG, 50.8% GT, 13.2% TT).

In the whole study group and in each subtype, the GG genotype and the G allele were more frequent in cases than in controls. Considering TT carriers as the reference group, ORs for BI were 1.57 (95% CI, 1.00 to 2.50) for GG carriers and 0.90 (95% CI, 0.57 to 1.41) for GT carriers. Analyses restricted to cases and matched controls both free of cardiovascular and cerebrovascular history yielded similar results (GG: OR, 1.85; 95% CI, 1.04 to 3.29; GT: OR, 1.08; 95% CI, 0.61 to 1.90).

Because GT carriers were not at increased risk of BI (collapsibility test, P=0.90), ORs associated with the GG genotype were therefore computed with GT+TT carriers considered as the reference group (Table 3). We found a significant overall association between BI and the GG genotype (P<0.001); according to subtypes, this association was significant in lacunar strokes (P=0.02), and a borderline association was found in strokes of unknown cause (P=0.08).

Analyses adjusted for cardiovascular history or analyses restricted to cases and controls free of cardiovascular or cerebrovascular history yielded similar results, except for strokes of unknown cause, in which the association reached significance. When analyses were restricted to 274 subjects with known stroke etiology and matched controls, we obtained similar findings (OR, 1.57; 95% CI, 1.10 to 2.23).

The relations between several variables and the genotype were studied separately in cases and controls. There were no significant associations between the genotype and hypertension, smoking, diabetes, or total/LDL cholesterol levels either in cases or in controls (data not shown). After adjustment for these risk factors, the association with the ecNOS polymorphism was not modified overall (OR, 1.45; 95% CI, 1.06 to 1.98), in lacunar strokes (OR, 2.14; 95% CI, 1.00 to 4.82), or in strokes of unknown cause (OR, 1.67; 95% CI, 0.86 to 3.22), whereas it decreased in atherothrombotic strokes (OR, 1.01; 95% CI, 0.49 to 2.06).

Mean [SD] LDL cholesterol level was higher in lacunar cases (1.33 g/L [0.37 g/L]) than in matched controls (1.08 g/L [0.34 g/L]; P<0.001). Our data are in favor of an interaction between the GG genotype and LDL cholesterol level among lacunar strokes (Table 4). The trend in BI risk associated with LDL level was stronger among carriers of the GG genotype (OR associated with an increase of 1 SD in LDL level, 3.75; 95% CI, 1.73 to 8.14) than among noncarriers (OR, 1.57; 95% CI, 1.03 to 2.40; test for interaction: P=0.05). When analyses were restricted to lacunar cases and controls free of previous cardiovascular and cerebrovascular history, the interaction remained significant (P=0.03). Our data may also suggest a trend toward an interaction (P=0.10) between the GG genotype and current smoking among lacunar strokes; ORs (95% CI) were 1.41 (0.49 to 4.09) for smoking only, 1.57 (0.77 to 3.20) for the GG genotype only, and 14.50 (1.71 to 123.39) for exposure to both risk factors. No interaction between the ecNOS polymorphism and LDL cholesterol or smoking was found in the whole study group (P=0.30 and P=0.50, respectively) or in other subtypes.

Discussion

Our findings suggest that homozygosity for the G allele of the Glu298Asp polymorphism in the ecNOS gene is associated with BI, and in particular with lacunar stroke. Our results also suggest a gene-environment interaction model in which genetic susceptibility and LDL cholesterol have a synergistic relation. Recently, Philip et al20 showed that enhanced vascular responsiveness was associated with the Asp allele of the polymorphism; the authors concluded that although this finding does not strictly prove the functionality of the polymorphism, subjects carrying this allele may have a lower production of NO. Alternatively, this association may reflect linkage disequilibrium between this polymorphism and another functional variant.

The main strengths of this study are the inclusion of a large sample of BI patients and the careful categorization of cases into subtypes. Indeed, BI can result from very different causes. If genetic susceptibility factors do play a role, their effect, or at least their strength, may be different from one cause to another; thus, genetic studies of BI should allow analyses according to subtypes.

We found a significant association between the polymorphism and BI. Among subtypes, this relation was significant in lacunar strokes. Although there is still much contro-

TABLE 2. Brain Infarction Subtypes

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>n (%)</th>
<th>Age, y (median; range)</th>
<th>Male sex, %</th>
</tr>
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<tbody>
<tr>
<td>Atherothrombotic</td>
<td>106 (23.0)</td>
<td>65; 41–85</td>
<td>83.0</td>
</tr>
<tr>
<td>Lacunar</td>
<td>95 (20.7)</td>
<td>70; 25–85</td>
<td>61.1</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>73 (15.9)</td>
<td>72; 37–85</td>
<td>42.5</td>
</tr>
<tr>
<td>Undetermined cause</td>
<td>59 (12.8)</td>
<td>73; 24–83</td>
<td>66.1</td>
</tr>
<tr>
<td>Dissection</td>
<td>11 (2.4)</td>
<td>40; 30–57</td>
<td>54.5</td>
</tr>
<tr>
<td>Rare cause</td>
<td>9 (2.0)</td>
<td>53; 25–74</td>
<td>22.2</td>
</tr>
<tr>
<td>Unknown cause</td>
<td>107 (23.3)</td>
<td>65; 20–85</td>
<td>53.3</td>
</tr>
</tbody>
</table>
versy concerning their cause, lacunae are believed to result from the occlusion of small penetrating arteries as a consequence of lipohyalinosis, which is associated with wall thickening, lipid deposition, VSMC degeneration, deposition of extracellular matrix collagen, and enhanced vascular permeability with extravasation of plasma proteins. The mutant allele of the T(2786)C polymorphism in the promoter of the ecNOS gene (which is associated with a reduced promoter activity and endothelial synthesis of NO) and the Asp allele of the Glu298Asp polymorphism predispose to coronary spasm in the Japanese population. Conversely, myocardial infarction and atherosclerotic coronary artery disease severity are associated with the a allele of the 27-bp repeat polymorphism in intron 4 of the ecNOS gene. Using complex segregation analysis, Wang et al showed that homozygotes for this allele had significantly higher levels of circulating NO; this finding was confirmed in a sample of unrelated individuals. Although the hemodynamic consequences of NO deficit are likely to be responsible for the association between the ecNOS gene and coronary spasm, an explanation for the association between myocardial infarction and the ecNOS gene is likely to be more complex. Adachi and Wang hypothesized that the association between the a allele of the intron 4 polymorphism and myocardial infarction may be explained by the oxidative role of NO. NO reacts with superoxide to generate peroxynitrite, a powerful oxidant known to be highly cytotoxic. This may provide a clue to understanding the association we have observed. In addition, peroxynitrite increases lipid oxidation, and oxidized LDL plays a central role in the oxidative theory of atherosclerosis. In this study, there was an association between lacunar strokes and LDL cholesterol level, and our findings suggested that the polymorphism and LDL cholesterol may interact in determining the risk of BI. Lüscher and NoIl recently hypothesized that carriers of ecNOS mutations with hyperlipidemia may show more pronounced vascular alterations than those without. The interaction between LDL cholesterol and the polymorphism was tested in view of the complex relations between lipids and NO reviewed in detail by Wever et al. Evidence for gene-environment interaction was restricted to the lacunar group and was based on a small number of subjects. Further studies on the relation between lacunar strokes, oxidized LDL, and NO and based on larger samples are needed. Wang et al

| TABLE 3. Distribution of ecNOS Glu298Asp Genotypes in Cases and Controls |
|-----------------|------|-----|--------|-------|-------|-------|
|                  | GG   | GT  | TT     | G,   | OR (95% CI)* | OR (95% CI)† |
|                  |     |     |        | %    |       |       |
| Cases            | 212 (46.1) | 187 (40.7) | 61 (13.3) | 66.4  |
| Atherothrombotic | 49 (46.2)  | 41 (38.7)  | 16 (15.1) | 65.6  |
| Lacunar          | 32 (33.7)  | 46 (48.4)  | 17 (17.9) | 57.9  |
| Cardioembolic    | 35 (48.0)  | 30 (41.1)  | 8 (11.0)  | 68.5  |
| Unknown cause    | 37 (34.6)  | 58 (54.2)  | 12 (11.2) | 61.7  |

Values are n (%).

*By conditional logistic regression. ORs are based on the comparison of GG frequency with the frequency of GT and TT pooled together.
†Adjustment for cardiovascular history.
‡Number of matched pairs.
§Hardy-Weinberg equilibrium: P=0.25.

| TABLE 4. LDL Cholesterol Level, the Glu298Asp Polymorphism in the ecNOS Gene, and Lacunar Stroke |
|-----------------|------|-----|-------|-------|-------|
|                  | GG   | LDL | Controls, n | Cases, n | OR (95% CI)* |
|                  |     |     |     |      |       |
| No               | 0–1.02 | 15 | 15 | 2.77 (0.67–11.51) |
| Yes              | 0–1.02 | 16 | 7  | 1.60 (0.34–6.93)  |
| Yes              | >1.32 | 23 | 17 | 3.74 (1.20–11.65) |

*By conditional logistic regression, adjusted for cardiovascular history, lipid-lowering drug use, and blood sample delay.†Cutoffs: 33rd and 66th percentiles of the LDL distribution.
reported that smoking and the intron 4 polymorphism may interact in determining coronary heart disease severity; although our data may suggest a trend toward an interaction among lacunar strokes, it was not statistically significant at the traditional \( P=0.05 \) level.

Two studies did not find a significant association between BI and the Glu298Asp polymorphism.\(^{20,29}\) However, their results are interesting to discuss in view of our own results. Markus et al.\(^{28}\) found that the frequency of the \( GG \) genotype (\( nn \) in their study) was higher in 75 lacunar cases (42.7\%) than in other subtypes (\( nn \) other subtypes, \( n=286: \) 33.2\%; \( P=0.13 \); in the study by MacLeod et al.\(^{29}\) the \( G \) allele was also more frequent in cases (69.7\%) than in controls (65.0\%), but this difference was significant only at the \( P=0.08 \) level. There are some methodological differences between these studies and ours. First, our study included the largest number of cases, especially of lacunar stroke, yielding increased statistical power compared with the other studies, and analyses restricted to cases and controls free of cardiovascular history were performed. Second, we included cases with infarcts proven by MRI for whom we used a systematic and complete diagnostic workflow; analyses according to subtypes were not reported in one study.\(^{29}\)

The polymorphism was not associated with atherothrombotic stroke, the presence of plaques at the carotid bifurcation, or intima-media thickness (data not shown). \( GG \) frequency was slightly higher in cases with atherothrombotic stroke than in matched controls, without reaching statistical significance. If we used a different cutoff for carotid stenosis (>70\%) to define atherothrombotic stroke, similar results were observed (OR, 1.46; 95\% CI, 0.77 to 2.77). We cannot rule out that the sample size of this subtype provided insufficient power to detect a modest effect of eNOS, in particular compared with the effect of other risk factors. However, when the latter were introduced in multivariate models, the strength of the association was reduced, suggesting that the polymorphism was not an independent risk factor.

In patients with BI of unknown cause, we found a significant association for those free of previous vascular history. Nevertheless, because this is likely to represent a heterogeneous group of patients, further speculations about this association are not warranted.

Allelic association studies may suffer from survival bias if they include prevalent cases and if survival is related to the gene under investigation. We included cases in the week after the event. Early case fatality rates in BI are rather low, in particular compared with the effect of other risk factors.

To study BI genetic susceptibility, other study designs may be considered. Sib-pair analyses can detect linkage and provide stronger evidence in support of causality; their feasibility is difficult to evaluate in the context of BI, because there is little knowledge about recurrence risks of BI among siblings. A recent study showed no significant association between stroke (ischemic and hemorrhagic combined) in probands and their siblings; however, the number of probands with stroke who had an affected sibling was small, which led to lack of statistical power.\(^{31}\) Family-based tests that use information from parents (eg, transmission disequilibrium test) can detect linkage in the presence of association and are not prone to population stratification bias.\(^{32,33}\) They are likely to be difficult to use in this context, however, because BI affects primarily older individuals whose parents are often deceased. A sib-transmission/disequilibrium test,\(^{33}\) which uses information from affected and unaffected sibs, has been proposed for late-onset diseases; it is less powerful, however, than the transmission/disequilibrium test or case-control studies.

In conclusion, we have identified an association between BI, and in particular lacunar stroke, and the Glu298Asp polymorphism in the \( eNOS \) gene. Our data are consistent with a gene-environment interaction model in which genetic susceptibility and LDL cholesterol have a synergistic relation. These results should be replicated in a larger sample of lacunar strokes, and the functionality of the Glu298Asp polymorphism should be investigated. Further studies focused on the metabolism of NO in lacunae may help us to understand the cause of the arteriolopathy underlying lacunae and may open future perspectives for lacunar stroke treatment and prevention. Finally, because stroke is likely to be a multifactorial disease, several genes with weak or moderate effects are likely to be involved, and other candidate genes should also be investigated.

**Appendix**

**Committees**

Scientific Committee: Annick Alpérovitch, Pierre Amarenco, Marie-Germaine Bousser, Éric Bruckert, François Cambien, Loïc Capron, François Chédru, Alexis Elbaz, Marc Hommel, Didier Leys, Pierre-Ploun, Alain Rosa, Gérard Tobelem, Frank M. Yatsu.

Executive Committee: Pierre Amarenco (Chairman and Principal Investigator), François Chédru, Ariel Cohen, Alexis Elbaz, Marc Hommel, Didier Leys, Alain Rosa, Pierre-Jean Touboul.

**Participating Institutions and Investigators**

The following Institutions and Investigators participated in the GENIC study (Étude du profil Génétique de l’Infarctus Cérébral). The number of patients and controls included are given in parentheses.

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Besançon, Jean Minjouz Hospital, Center Hospitalier et Universitaire de Besançon (100): Fabrice Vuiller, Marie-Hélène Sniadlo, Laurent Tatu, Thierry Moulin. Ultrasoundography: Jean-Pierre Weisert, Françoise Cattain.

Meaux, Center Hospitalier Général (90): François Chédru, Alain Amérit, Jean-François Lefort, Chantal Kaci, François Thuillier. Ultrasoundography: Laurent Marcy, Philippe Chantecau.


Grenoble, Center Hospitalier et Universitaire de Grenoble (84): Assia Jaillard, Marc Hommel, Bernard Bertrand, Patrick Carpentier. Ultrasoundography: Jean-Luc Bossou.


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References

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