Interferon Beta Modulates Endothelial Damage in Patients with Cardiac Persistence of Human Parvovirus B19 Infection

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Background. In a phase 1 study, we investigated whether interferon beta reduced endothelial damage in patients with cardiac persistence of human parvovirus B19 (B19V) infection.

Methods and results. In vitro, B19V infected cultivated endothelial cells (ECs), which led to a reduction in their viability ($P = .007$). Interferon beta suppressed B19V replication by 63% ($P = .008$) in ECs and increased their viability ($P = .021$). Circulating mature apoptotic ECs (CMAECs [CD45−/H11002 CD146+ cells expressing von Willebrand factor and annexin V]) and circulating progenitor cells (CPCs [CD34+KDR+ cells]) were quantified by flow cytometry in 9 symptomatic patients with cardiac B19V infection before and after 6 months of interferon beta therapy (16 MU) and were compared to levels in 9 healthy control subjects. Endothelial dysfunction was measured using flow-mediated dilatation of the forearm. Patients with B19V persistence had significantly higher levels of CMAECs than did control subjects, which normalized after treatment (mean ± standard deviation, vs $P = .004$). Similar improvement was shown for flow-mediated dilatation ($P = .04$) in the treatment group only ($P = .017$ for the comparison with untreated patients with B19V persistence [$n = 5$]). There were significantly higher numbers of CPCs in patients with B19V persistence before therapy (mean ± standard deviation, $P = .02$) than in control subjects, which normalized after treatment ($P = .03$).

Conclusion. Thus, we present (for the first time, to our knowledge) a modulation of virally induced chronic endothelial damage—specifically, EC apoptosis and endothelial regeneration.

Human parvovirus B19 (B19V), a erythrovirus and the infectious agent of fifth disease in children, is often detected in the tissues of asymptomatic individuals, and there is ongoing debate about the functional relevance of this finding [1–3]. On the other hand, B19V infection is diagnosed [4] in the majority of patients with the clinical symptoms of atypical angina [5] and impaired exercise capacity [6] who undergo endomyocardial biopsies. Furthermore, we and other have shown that this vasculotropic virus targets endothelial cells (ECs) with consequent endothelial dysfunction [7–9], which indeed suggests a pathogenetic role for B19V. Little is known about the mechanism of endothelial damage in humans with persistent B19V infection. The underlying mechanisms by which B19V might cause endothelial damage involves the cytotoxicity of nonstructural protein 1 [10], transactivation of interleukin 6 [11] and tumor necrosis factor α [12], and induction of apoptosis (which has been demonstrated in vitro) [13–15].

Endothelial vasodilator dysfunction predicts long-term disease progression in chronic heart failure [16] and atherosclerosis [17]. The close relationship between coronary artery endothelium–dependent vasomotor responses to acetylcholine and the noninvasive flow-me-
HMEC-1 cells were infected with B19V virions as described elsewhere [13, 33]. All experiments were performed concurrently with the infection. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel), in accordance with the manufacturer’s instructions. Complementary DNA of B19V RNA was obtained using Superscript II reverse transcriptase (Invitrogen) and a B19-VP2–specific primer (VP5; 3′-ATTCTTTAGATAATCCCTAGA-5′), in accordance with quantitative real-time TaqMan polymerase chain reaction (PCR) protocol described elsewhere [7].

**Cell viability and proliferation assay.** HMEC-1 cells were infected with B19V virions, and interferon beta (100–200 U/mL) was added to the cell culture medium 24 h after infection, as described above. Cell proliferation was analyzed using the ViaLight Cell Proliferation kit (Cambrex Bio Science Rockland), in accordance with the manufacturer’s instructions.

**Patients and control subjects.** Inclusion criteria were age 18–75 years, documented presence of B19V genome and increased endothelial activation in at least 1 endomyocardial biopsy sample, onset of clinical symptoms (atypical angina, fatigue, and impaired exercise capacity), and stable clinical condition and receipt of medication for chronic heart failure for at least 6 months. Nine consecutive patients with a prolonged history of cardiac symptoms (>0.5 years) despite receiving symptomatic medications and with high numbers of B19V copies (median [range], 300 [189–896] copies/μg of DNA) in their endomyocardial biopsy samples, as measured by real-time PCR, were allocated for interferon beta therapy (Beneferon; Schering) and prospectively studied between September 2005 and November 2006 in a phase 1 study. To assess the natural course of disease with regard to endothelial dysfunction in B19V persistence, 5 consecutive patients (median [range] B19V copy number, 316 [214–360] copies/μg of DNA) were recruited between April 2006 and November 2007 after completion of the initial pilot study. In these patients, only FMD was performed.

Exclusion criteria were diabetes; acute myocarditis; other causes of left ventricular dysfunction; coronary heart disease; pregnancy; antiviral, immunomodulatory, or immunosuppressive therapy within the last 6 months; severe heart failure; clinical or biochemical evidence of the presence of concomitant chronic inflammatory disease; chronic renal insufficiency (creatinine level, ≥1.4 mmol/L); inability to understand the consent form; participation in or consent to participate in another study; or malignant disease.

Nine patients without signs or symptoms of chronic heart failure or coronary artery disease, diabetes, severely impaired left ventricular function, and no more than 1 arteriosclerotic risk factor were recruited as control subjects. Cardiovascular...
risk factors were defined as follows: active smoking, hypercholesterinemia, positive family history (cardiovascular events in <55-year-old male or <65-year-old female next of kin). There were no changes in medication during the last 6 months (ie, anti-hypertensive drugs).

All study participants provided written informed consent, and the study was approved by the ethics committee of the Charité, Berlin.

Physical examination, clinical assessments (including echocardiography and electrocardiography), and laboratory controls were conducted every second month to monitor for interferon beta–associated side effects and adverse cardiac events. Assessment of clinical complaints (patient diary) and heart failure symptoms (according to the New York Heart Association classification system) and the completion of a questionnaire inquiring about both interferon beta–associated adverse effects (flu-like symptoms, headache, and signs of inflammation) and specific adverse cardiac symptoms (eg, angina and dyspnea) were done at every visit.

Methods for hemodynamic, histological, and immunohistochemical evaluations and endothelial function measurements are given in the Appendix, which appears only in the online version of the Journal.

**Interferon beta treatment.** Interferon beta therapy followed a stepped regimen to reduce the flulike side effects typical of the initial phase of interferon beta therapy. Subcutaneous administration was initiated at a dose of 4 × 10⁶ U of interferon beta 3 times a week on alternate days and was increased to 8 × 10⁶ U during the second week and to 16 × 10⁶ U after the fourth week. By the end of week 24, interferon beta therapy was discontinued. Follow-up visits after completion of treatment were prespecified to occur after 7 months (±30 days; visit 1) and after 12 months (±15 days; visit 2).

**Isolation of mononuclear cells and flow cytometry.** Mononuclear cells (MNCs) were isolated from peripheral blood by means of a ficoll gradient (Histopaque 1077; Sigma). Circulating mature apoptotic ECs (CMAECs) were analyzed by a 4-channel flow cytometry performed in accordance with the methods of Mancuso et al [34] and Monstiroli et al [35] (Figure 1). After exclusion of debris and platelets, only CD45⁻ cells (CD45–peridinin chlorophyll protein [PerCP]; Becton Dickinson) were further analyzed by flow cytometry via double staining with antibodies against the EC-specific epitopes CD146 (fluorescein isothiocyanate [FITC] conjugated; Chemicon) and against von Willebrand factor (vWF) (goat; Calbiochem), followed by a phycoerythrin (PE)–conjugated secondary antibody. These CD45⁻ CD146⁺ vWF⁺ cells were then assessed for apoptosis with primary annexin V–allophycocyanin (APC) conjugates (BD Pharmingen) [36]. As an individual negative control for the CD146⁺ vWF⁺ double-positive cells, a gate was set on lymphocytes. Annexin binding was controlled by incubation with streptavidin-coupled APC. At least 100,000 events were counted. Measurements were done in duplicate.

CPCs were detected by flow cytometry, as described elsewhere [26]. In brief, MNCs were double stained with PE-conjugated monoclonal antibodies against human KDR (R&D Systems) and against PerCP-conjugated CD34 (Becton Dickinson) to depict CPCs, in accordance with the methods of Rafii [37]. Additionally, the more immature CD133⁺ KDR⁺ CPCs were stained with PE-conjugated anti–human KDR (R&D Systems) and APC-conjugated anti–human CD133 (Becton Dickinson). Isotype-identical antibodies served as controls (immunoglobulin G [IgG] 2a–FITC and IgG1-PE; Becton Dickinson). Each analysis included 200,000 events.

**Cytokines.** Levels of cytokines (stromal-derived factor 1 [SDF-1] and vascular endothelial growth factor [VEGF]) were measured using commercially available enzyme-linked immunosorbent assay kits (R&D Systems), in accordance with the manufacturer’s instructions.

**Statistical analysis.** Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test. Comparisons between the 2 groups were analyzed by the t test (2-sided) for normally distributed variables. Nonnormally distributed continuous variables (FMD, CMAECs, and CD34⁺ KDR⁺ CPCs) were compared by the Mann-Whitney U test. Comparison of categorical variables was generated by the Pearson χ² test. Statistical significance was assumed if a null hypothesis could be rejected at P < .05. All statistical analysis was performed with SPSS software (version 14; SPSS).

**RESULTS**

**Infection of cultured ECs.** As shown in Figure 2A, there was a statistically significant increase in replication intermediates of B19V in HMEC-1 cells that was significantly (P = .008) suppressed by interferon beta. B19V infection resulted in significantly reduced cell viability and proliferation (P = .007) of HMEC-1 cells. However, after treatment with interferon beta the viability of infected HMEC-1 cells was comparable to that of uninfected cells, as shown in Figure 2B.

**Patient characteristics.** The baseline characteristics of the 23 subjects are summarized in Table 1. The 14 patients and 9 control subjects were matched for age and sex. As expected, patients with B19V persistence had a higher incidence of angina (n = 7 for B19V with treatment; n = 1 for B19V without treatment), arrhythmias (n = 4 for B19V with treatment; n = 1 for B19V without treatment), and fatigue (n = 7 for B19V with treatment; n = 3 for B19V without treatment). The control subjects either were healthy individuals recruited from the outpatient clinic after exclusion of signs or symptoms of cardiac disease (n = 6) or had intermittent atrio-ventricular block 2 (n = 1) or intermittent atrial fibrillation (n = 2; last episode, ≥1 month ago; duration of episode, <4 h). Control subjects...
had a mean ± standard deviation (SD) number of cardiovascular risk factors of 0.7 ± 1.1, patients with B19V persistence who received treatment had 1.8 ± 1.1, and patients with B19V persistence who did not receive treatment had 1.8 ± 1.3 (Table 1). There were no differences with respect to concomitant medication. In endomyocardial biopsy samples, 70% of patients had elevated levels of the endothelial activation marker intercellular adhesion molecule 1 (ICAM-1) and HLA. Interferon
beta therapy was mostly well tolerated by all patients. All of the patients experienced at least 1 symptom classified as moderate associated with interferon; in 2 patients, symptoms were classified as severe (leukopenia and thrombocytopenia). Interferon therapy had to be interrupted in 4 patients for between 5 days and 2 weeks because of adverse events—namely, leukopenia (n = 2), anemia (n = 1), and local skin reaction (n = 1). Adverse effects in the 9 patients were flulike symptoms (n = 8), leukopenia (n = 4), local skin reaction (n = 6), headache (n = 3), fever (n = 2), dyspnea (n = 2), palpitations (n = 2), fatigue (n = 2), and intermittent increase in atypical angina symptoms (n = 2). Athralgia, nausea, diarrhea, conjunctivitis, increase in liver enzyme levels, and sore throat were present in 1 patient each. All of the patients completed the full course of interferon. Anti–heart failure treatment was kept constant throughout the follow-up period.

The mean ± SD duration of follow-up in patients with B19V persistence who did not receive treatment was 8 ± 3 months.

**Clinical end points and echocardiographic measurements.**

There was a significant reduction in the number of patients with B19V persistence reporting angina pectoris after 6 months of therapy (from 7 to 2; P = .046), which persisted over the actual treatment period, whereas the frequency of the symptoms of fatigue, dyspnea, and arrhythmias remained unchanged. In patients with B19V persistence who were not treated, symptoms remained unchanged. There were 3 patients with B19V persistence in the therapy arm and 2 patients with B19V persistence in the no-therapy arm who experienced suppressed (<45%) left ventricular ejection fraction (LVEF), whereas none of the control subjects had a LVEF of <45% (Table 1).

Cardiac measures were similar between groups (mean ± SD angiographic left ventricular end diastolic pressure, not available for control subjects, 7.25 ± 3.2 mm Hg for B19V with treatment, and 17 ± 9 mm Hg for B19V without treatment; mean ± SD left ventricular end diastolic diameter, 48% ± 2% for control subjects, 55% ± 9% for B19V with treatment, and 51% ± 13% for B19V without treatment; and mean ± SD intraventricular septal thickness, 10 ± 1 mm for control subjects, 10 ± 1 mm for B19V with treatment, and 11 ± 2 mm for B19V without treatment). There was a strong trend between the 2 subgroups of patients with B19V persistence in E/A value (mean [range], 1.1 [1.1–2.2] for control subjects, 1.33 [0.69–1.4] for B19V with treatment, and 1.7 [1.4–1.9] for B19V without treatment; P = .05). After interferon beta therapy, there was no significant improvement in echocardiographic left ventricular systolic or diastolic function.

**Endothelial dysfunction.** As illustrated in Figure 3A, highly impaired FMD in patients with B19V persistence compared with that in control subjects (P = .002 for B19V with treatment and P = .001 for B19V without treatment; data not shown) was observed in this study. Flow-mediated dilatation normalized after 6 months of interferon beta therapy (mean [range], 4.7% [2.3%–5.8%] vs 12.3% [11.3%–14.6%; P = .04). In contrast, endothelium-independent dilatation was similar in control subjects and both patient groups, as depicted in Figure 3B, and remained unchanged throughout the course of treatment. In contrast to patients with B19V persistence who received interferon beta therapy (P = .004), patients with B19V persistence who did not receive therapy showed persistently impaired
Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control subjects (n = 9)</th>
<th>Patients with B19V persistence Before therapy (n = 9)</th>
<th>No therapy (n = 5)</th>
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<tr>
<td>Age, mean ± SD, years</td>
<td>48 ± 15</td>
<td>50 ± 14</td>
<td>48 ± 9</td>
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<tr>
<td>I</td>
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<td>4 (44)</td>
<td>3 (60)</td>
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<tr>
<td>II</td>
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<td>III</td>
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<td>IV</td>
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<td>0 (0)</td>
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<td>Atypical angina</td>
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<td>7 (78)</td>
<td>1 (20)</td>
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<td>Arrhythmias</td>
<td>3 (33)</td>
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<td>1 (20)</td>
</tr>
<tr>
<td>Fatigue</td>
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<td>Cardiovascular risk factors</td>
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<tr>
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<td>3 (60)</td>
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<td>ACE inhibitors/AT1 blockers</td>
<td>2 (22)</td>
<td>8 (89)</td>
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<td>b-Blockers</td>
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<td>5 (100)</td>
</tr>
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<td>Hemodynamic and echocardiographic</td>
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<td>measurements, mean ± SD</td>
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<td>LVEF echocardiography, %</td>
<td>60 ± 7</td>
<td>54 ± 14</td>
<td>46 ± 25</td>
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<td>Laboratory parameters, mean ± SD</td>
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<tr>
<td>VEGF level, pg/mL</td>
<td>81 ± 51</td>
<td>292 ± 644</td>
<td>644 ± 81</td>
</tr>
<tr>
<td>SDF-1 level, pg/mL</td>
<td>105 ± 35</td>
<td>125 ± 75</td>
<td>75 ± 20</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of participants, unless otherwise indicated. There were no significant differences between the 3 groups. ACE, angiotensin-converting enzyme; AT1, angiotensin II receptor 1; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; SD, standard deviation; SDF-1, stromal cell derived factor 1; VEGF, vascular endothelial growth factor.

There was no correlation between systolic or diastolic dysfunction and FMD.

**CMAECs and CPCs.** Figure 4A illustrates that, at baseline, patients with B19V persistence before therapy had significantly higher levels of CMAECs than did control subjects (P = .004). EC apoptosis was significantly reduced after 6 months of interferon beta therapy in patients with B19V persistence (P < .008) and reached values comparable to those in control subjects. As shown in Figure 4B, there were significantly higher numbers of CPCs in patients with B19V persistence before therapy (P = .02), compared with those in control subjects. Similarly, the more premature subset of CPCs, characterized by the coexpression of CD133 and KDR, was significantly increased in patients with B19V persistence (mean ± SD, 0.0049% ± 0.003% vs 0.0013% ± 0.0009% of peripheral MNCs; P = .007). Further analysis showed that only patients with a history of angina (n = 7) showed a strong trend toward elevated levels of CD34+KDR+ CPCs (mean ± SD, 0.52% ± 0.48% vs 0.01% ± 0.009% of peripheral MNCs; P = .059). Indeed, after 6 months of treatment with interferon beta the number of CD34+KDR+ CPCs returned to normal (P = .03), which persisted over the actual treatment period. None of the cardiac medications at baseline were seen to have any effect on EC apoptosis.

**Levels of growth factors.** To assess potential mobilization of CPCs, VEGF and SDF-1 were analyzed. Plasma VEGF levels were nonsignificantly higher in patients with B19V persistence (mean ± SD, 292 ± 644 vs 81 ± 51 pg/mL; P = .2) and were lowered by interferon beta therapy (mean ± SD, 292 ± 644 vs 30 ± 21 pg/mL; P = .038). SDF-1 levels were similar in both groups and were not altered by treatment.

**Correlations among cytokines, circulating ECs, and endothelial damage.**
Figure 3.  A, Flow-mediated dilatation in 9 control subjects, in 9 patients with human parvovirus B19 (B19V) persistence before and after a 6-month course of interferon beta therapy, and in 5 patients with B19V persistence who did not receive interferon beta therapy. Significance levels are given for group comparisons and for changes in patients during interferon beta therapy.  B, Endothelial-independent dilatation in control subjects and in patients with B19V persistence at baseline. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers above and below the box mark the range. IFN, interferon beta.
Interferon Beta in B19V-Induced Endothelial Damage

There was an inverse correlation between circulating mature ECs and FMD ($r = -0.53; P = .02$). The CPC-mobilizing factors SDF-1 and VEGF ($r = .55; P = .02$) were correlated with each other, but no correlation was seen between the 2 cytokines and the 2 subsets of CPCs or FMD.

**DISCUSSION**

The results of the present phase 1 study confirm and extend previous findings of B19V-associated vascular damage. B19V persistence is associated with endothelial dysfunction [7], atypical angina pectoris [5], and impaired exercise capacity. However, the underlying complex pathogenetic mechanisms of B19V-induced endothelial dysfunction are unclear. To evaluate whether immunomodulation could improve HMEC-1 cell fate in B19V persistence, the effect of interferon beta was assessed. The viability of HMEC-1 cells was markedly reduced after infection with B19V. In vitro, interferon beta significantly reduced viral replication in HMEC-1 cells and significantly increased the viability of ECs, providing strong evidence that B19V-induced damage of HMEC-1 cells is at least partially mediated by direct virus-cell interaction. Clearly, the results of this study do not provide detailed insight into the pathogenetic mechanisms, which will be the aim of future studies.

To assess whether these preliminary in vitro data translate into better endothelial function in patients with B19V infection after interferon beta therapy, we designed a human phase 1 study to analyze different component of endothelial damage in vivo. The inverse correlation between a significantly increased level of circulating ECs and impaired FMD suggest that similar pathophysiological mechanisms led to EC apoptosis and endothelial dysfunction in this (albeit small) group of patients. Given that previous studies have shown that a procoagulant state is associated with the circulation of apoptotic microparticles [21, 22], we assume that, apart from vessel wall erosion, circulation of increased numbers of apoptotic mature ECs might be another component in the multifactorial pathogenesis of endothelial dysfunction and activation. Simultaneous measurements of different stages of maturation of CPCs and mature ECs allowed us to differentiate between vessel wall damage and regeneration capacity. Increased levels of CD34+KDR+ CPCs—as well as the more immature subset (CD133+KDR+)—in patients with B19V infection and angiina denote a potentially ischemia-triggered mobilization of these cells. Notably, patients with virally induced endothelial damage and angina mobilize CPCs, in contrast to the vascular damage evoked by classical risk factors [38] or typical coronary heart disease [26]. Future studies will be needed to address the functional capacity of CPCs in B19V infection. There may be secondary mechanisms responsible for myocardial damage in these patients.

Indeed, the results of our pilot study demonstrated that a 6-month course of immunomodulation significantly reduced the numbers of CMAECs and led to normalization of endothelial dysfunction in patients with B19V infection over the actual treatment period, pointing to a disease-modifying property of interferon beta therapy. In contrast, we documented persistent endothelial dysfunction in patients with B19V persistence who did not receive treatment, hence excluding the possibility of spontaneous improvement of endothelial damage. The underlying protective mechanisms of interferon beta remain to be
further elucidated. Simultaneously, after interferon beta therapy the clinical symptom of angina subsided in the majority of patients with B19V persistence, and the increased numbers of CPCs normalized. Further supporting that mobilization indeed influenced the increased numbers of CPCs in patients with B19V persistence was the reduction in the elevated levels of the ischemia-induced mobilizing growth factor VEGF [28] after interferon beta therapy. Clearly, the nature of our clinical study does not permit us to dissect the individual components leading to the presumably increased mobilization of CPCs in patients with angina associated with B19V persistence.

Thus, our data provide clinical evidence for the hypothesis that B19V contributes to ongoing vascular injury and that chronic endothelial damage evoked by an extrinsic viral stimulus can be improved by immunomodulation. However, the present study is a phase 1 study, and even if it is unlikely that the natural process of healing accounts for our findings after the long period of symptoms in these patients, a randomized, placebo-controlled phase 2 trial is warranted to confirm our results. For ethical reasons a second biopsy was not performed after the treatment course, so no information on the clearance of B19V or a reduction in viral load in endomyocardial biopsy samples after treatment can be provided. Given the majority of our patients had a normal or only slightly reduced ejection fraction and that there was a low incidence of concomitant cardiovascular risk factors as inducers of endothelial dysfunction, we presume, but cannot prove, that B19V was the cause of endothelial dysfunction.

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References


