A Common Mutation of the Insulin Receptor Substrate-1 Gene Is A Risk Factor for Coronary Artery Disease

Marco Giorgio Baroni, Maria Pina D’Andrea, Anna Montali, Gaetano Pannitteri, Francesco Barillà, Filomena Campagna, Ercole Mazzei, Sarah Lovari, Fulvia Seccareccia, Pietro Paolo Campa, Giorgio Ricci, Paolo Pozzilli, Giancarlo Urbinati, Marcello Arca

**Abstract**—Insulin resistance is associated with increased risk of atherosclerosis. Insulin receptor substrate-1 (IRS-1) plays a key role in tissue insulin sensitivity. A common mutation (G972R) of the IRS-1 gene has been shown to impair IRS-1 function, and it has been associated with reduced insulin sensitivity and lipid abnormalities. This led us to investigate the role of the G972R mutation in predisposing individuals to coronary artery disease (CAD). The DNA of 318 subjects with angiographically documented coronary atherosclerosis (>50% stenosis) and 208 population control subjects was analyzed for the presence of the G972R mutation. This mutation was detected by nested polymerase chain reaction and BstNI restriction enzyme digestion. The frequency of the G972R mutation was significantly higher among patients with CAD than controls (18.9% versus 6.8%, respectively; P<0.001). After controlling for other coronary risk factors, the relative risk of CAD associated with the G972R mutation was 2.93 (95% CI 1.30 to 6.60; P<0.02) in the entire cohort. This risk was found to be even higher in the subgroups of obese subjects (odds ratio [OR] 6.97, 95% CI 2.24 to 21.4; P<0.001) and subjects with clinical features of insulin resistance syndrome (OR 27.3, 95% CI 7.19 to 104.0; P<0.001). The IRS-1 gene variant was associated with a higher frequency of diabetes mellitus (14.9% among carriers versus 6.5% among noncarriers; P<0.01) and with a 60% increase of plasma total triglycerides (P<0.001). Also, plasma concentrations of total cholesterol and the ratio of total cholesterol to HDL cholesterol were significantly higher among carriers than noncarriers, although to a lesser extent. These effects were independent of CAD status. The G972R mutation in the IRS-1 gene was found to be a significant independent predictor of CAD. Moreover, this mutation greatly increased the risk of CAD in obese subjects and in patients with the cluster of abnormalities of insulin resistance syndrome. Besides the increased frequency of diabetes, carriers showed a more atherogenic lipid profile, suggesting a potential role of the IRS-1 gene in the pathogenesis of lipid abnormalities associated with CAD. (Arterioscler Thromb Vasc Biol. 1999;19:2975-2980.)

**Key Words:** insulin resistance ■ IRS-1 gene ■ coronary artery disease ■ obesity ■ hyperlipidemia ■ association studies

There is increasing evidence that genetic factors contribute significantly to the risk of coronary artery disease (CAD). However, except for genetically determined lipid abnormalities, little is known about genetic factors leading to coronary atherosclerosis. Insulin resistance and hyperinsulinemia are conditions known to be associated with an increased risk of CAD. Therefore, genetic variants affecting tissue sensitivity to insulin may represent candidate genes for CAD.

The insulin receptor substrate-1 (IRS-1) gene has been proposed as having a role in the insulin-resistant disorders. Its gene product, the IRS-1 protein, is a cytoplasm molecule expressed in most insulin-sensitive tissues and has been demonstrated to play a pivotal role in modulating the cellular effects of insulin. After the binding of insulin to its receptor, the intrinsic tyrosine kinase activity of the receptor β-subunit is activated, thus catalyzing the phosphorylation of specific tyrosine residues on the IRS-1 protein. Thereby, phosphorylated IRS-1 binds with high affinity to several cellular signal proteins, thus functioning as a multisite “docking” protein linking the receptor kinase to the variety of cell functions regulated by insulin. The genetic analysis of the IRS-1 gene has revealed several base-pair changes that result in amino acid substitutions. The most common amino acid change is a glycine to arginine substitution at codon 972 (G972R), which has an overall frequency of ~6% in the general population. This mutation has been reported to significantly impair IRS-1 function in experimental models, and clinical studies have shown that this genetic variant is associated with reduced insulin sensi-
tivity. Moreover, earlier observations have indicated that the presence of a mutated IRS-1 gene is associated with dyslipidemia, further suggesting that this gene variant may have a significant effect on several risk factors for CAD.

To investigate whether this common variant of the IRS-1 gene may be a genetic marker for risk of CAD, we studied the distribution of the G972R mutation in patients with angiographically defined coronary atherosclerosis and assessed its relation with clinical and metabolic abnormalities.

Methods

Subjects
A total of 526 subjects were included in the present study. The case population consisted of 318 consecutive patients (266 men and 52 women, aged 40 to 78 years) selected for having angiographic evidence of coronary atherosclerosis (one or more stenoses >50% in at least one major coronary artery). One hundred fourteen of these patients underwent coronary angiography for myocardial infarction, and 204 for stable or unstable angina. None of them was enrolled during the acute phase of the ischemic syndrome. All angiograms were independently evaluated by 2 cardiologists who did not know that the patients were to be included in the study. Patients with concurrent thyroid, liver, or renal disease and those on lipid-lowering medications were excluded. Control subjects were 208 unrelated individuals (108 men and 100 women, aged 40 to 80 years) randomly selected from a population of free-living individuals screened for coronary risk factors. In these subjects, CAD was excluded by the Rose questionnaire and ECG (Minnesota coding), the use of which to classify CAD patients for population-based screenings has been well established. A structured questionnaire was used to characterize both case patients and controls. History included questions about known CAD risk factors (smoking habits, alcohol consumption, and exercise habits), medical history (hypertension, diabetes, and cardiac history), and current medications. Diagnosis of non—insulin-dependent diabetes mellitus (NIDDM) was based on history of hypoglycemic treatment and/or fasting blood glucose (>110 mg/dL or >6.1 mmol/L) and/or the current use of antihyper- tensive drugs. Data collection included height and weight (to determine body mass index [BMI], assessed as weight in kilograms divided by height squared in meters) and systolic and diastolic blood pressures. Fasting blood samples for laboratory measurements and DNA isolation were obtained early in the morning after an overnight fast.

DNA Analysis
Blood was collected in 10 mL Na-EDTA and kept frozen at −20°C. DNA was extracted by the salting-out method and stored at 4°C in 10 mL Tris-HCl and 1 mL EDTA (pH 8.0) until analysis. To ensure privacy, DNA extraction and analysis were carried out anonymously with the use of code numbers. The presence of a glycine to arginine substitution at codon 972 of the IRS-1 gene (G972R) was determined according to the method of Almind et al with some modifications. A primary polymerase chain reaction (PCR) was performed in a final volume of 25 μL containing 100 to 200 ng of genomic DNA, 200 μmol/L dNTPs, 1.5 mmol/L MgCl2, 0.2 μmol/L of primers, and 0.05 U of Taq DNA polymerase (Applied Biotechnologies) in the standard buffer. Sequences of primers have been already reported. A standard thermocycling procedure was carried out at annealing temperatures of 64°C for 1 minute. In this primary PCR reaction, fragments of 479 bp were amplified. A secondary nested PCR reaction was performed with 1 μL of a 1:10 diluted primary PCR product as template. Primers for this second PCR were those described by Almind et al. A fragment of 263 bp was obtained during the secondary PCR. Restriction enzyme digestion was carried out at 60°C for 12 hours in 15 μL reaction buffers containing 10 μL of the secondary PCR product, 1.5 μL of 10× NEB buffer 2 (New England Biolabs Inc), and 10 U of the restriction enzyme BstNI (New England Biolabs Inc). The fragments were analyzed by 4.5% high-resolution agarose gel electrophoresis. Digestion patterns obtained in wild-type and heterozygous IRS-1 mutation carriers are shown in Figure 1. Genotypes were scored by 2 independent investigators who did not know whether the samples were from a case patient or from controls. Ambiguous samples were analyzed a second time.

Other Laboratory Measurements and Techniques
Cholesterol and triglyceride concentrations in whole plasma and lipoprotein fractions were measured with a Technicon RA-1000 Autoanalyzer. HDL cholesterol was determined in the whole plasma after precipitation of apoB-containing lipoproteins with phosphotungstic acid/MgCl2. Plasma glucose was measured with a glucose oxidase method adapted for the Technicon RA-1000 Autoanalyzer. Plasma insulin concentrations were measured in frozen samples by radioimmunoassay (Biodata Insulin Kit). The interassay coefficient of variation was 7.5%.

Statistical Analysis
The means were compared by ANOVA or the 2-tailed Student t test. For comparing discrete variables, χ2 analysis or the Fisher exact test was used. Logarithmic transformation was used to normalize distribution of plasma triglycerides and insulin values. All comparisons were made after age and sex standardization. The frequencies of the alleles and genotypes in case patients and controls were determined by direct count and compared by the χ2 test with the values predicted assuming Hardy-Weinberg equilibrium. To estimate the relative risk of CAD associated with IRS-1 genotypes, odds ratios (ORs) were calculated by multivariate analysis. These analyses were performed in the whole group as well as in the subgroups of obese subjects and of those showing features of insulin-resistance syndrome (IRS). Obese subjects were defined on the basis of a BMI value >25 kg/m2. Subjects with IRS were defined as those showing at least 2 of the following clinical characteristics: hypertension, obesity, high triglycerides (2.26 mmol/L [200 mg/dL]), and high fasting insulin (>9.2 μU/mL, corresponding to the 75th percentile of fasting insulin distribution in the controls). Age, sex, smoking, obesity, total cholesterol, hypertension, diabetes, and fasting insulin were included in the logistic model as confounding variables. Obesity, hypertension, and fasting insulin were not considered for adjustment in the obese and IRS subgroups. For each OR, a 2-tailed probability value and 95% confidence intervals were estimated. The association of concomitant variables with genotypes was tested by 1-way ANOVA. Data for continuous variables were expressed as mean±SD; a 2-tailed value of P<0.05 was considered statistically significant. Bonferroni correction was used for repeated measurements. All computations were carried out with the BMDP Statistical package.

Results

Study Subjects
The distribution of selected risk factors in CAD patients and controls are reported in Table 1. The 2 groups were compa-
TABLE 1. Prevalence of Selected Coronary Risk Factors in Subjects With CAD and Controls

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Subjects with CAD (N=318)</th>
<th>Controls (N=208)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59.8±5.1</td>
<td>59.3±6.7</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.7±3.4</td>
<td>26.2±3.3</td>
<td>NS</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>266 (83.6)</td>
<td>108 (51.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Menopause, n (%)†</td>
<td>47 (90.6)</td>
<td>166 (79.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>156 (49.2)</td>
<td>33 (15.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>141 (44.3)</td>
<td>60 (29.0)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>38 (11.9)</td>
<td>4 (1.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>87 (27.3)</td>
<td>68 (32.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertriglyceridemia, n (%)</td>
<td>101 (31.8)</td>
<td>44 (21.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.11±0.29</td>
<td>1.37±0.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>4.90±1.4</td>
<td>4.34±1.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting plasma insulin, U/mL</td>
<td>13.5±7.7</td>
<td>8.1±5.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Age, BMI, blood glucose, and fasting insulin values are given as mean±SD. Other values represent number of individuals (n) with percentage (n/N) in parentheses. Hypercholesterolemia has been defined as calculated LDL cholesterol ≥160 mg/dL.† Hypertriglyceridemia has been defined as total triglycerides ≥200 mg/dL. To convert cholesterol values to mg/dL units, multiply by 0.02586; to convert triglyceride values to mg/dL units, multiply by 0.0113; to convert fasting blood glucose values to mg/dL units, multiply by 18; and to convert fasting insulin values to pmol/L units, multiply by 7.175.

†Percentage has been calculated over the female sample.

TABLE 2. Genotype and Allele Frequencies for G972R Mutation in IRS-1 Gene in Patients With CAD and Controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients With CAD (N=318)</th>
<th>Controls (N=208)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>257/317</td>
<td>81.1</td>
<td>191/205</td>
</tr>
<tr>
<td>GR</td>
<td>60/317</td>
<td>18.9</td>
<td>14/205</td>
</tr>
<tr>
<td>RR</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>574/634</td>
<td>90.5</td>
<td>396/410</td>
</tr>
<tr>
<td>R</td>
<td>60/634</td>
<td>9.5</td>
<td>14/205</td>
</tr>
</tbody>
</table>

n indicates number of subjects (for genotype frequencies) or alleles (for allele frequencies). Data on G972R were missing for 1 subject with CAD and 3 controls.

In each case, the P value is for the overall difference between groups; χ²=14.98 for comparison of genotypes and χ²=13.83 for comparison of allele frequencies.

Association Between G972R Mutation and CAD

Genotype and allele frequencies of G972R mutation in CAD patients and controls are reported in Table 2. This mutation was found to be almost 3 times more frequent in CAD patients than in controls (18.9% versus 6.8%, respectively), and the difference was highly significant (χ²=14.9, P<0.0001). Also, allele frequencies were significantly different between the 2 groups (P<0.0002), with the mutated R allele showing a frequency of 9.5% and of 3.5% in CAD patients and controls, respectively. ORs were calculated as measure of risk of CAD attributable to the presence of the G972R mutation by logistic regression analysis (Table 3). After controlling for other risk factors, heterozygosity for the R allele was associated with a significantly higher risk of coronary atherosclerosis (OR 2.93, 95% confidence interval 1.30 to 6.60; P<0.01). Sex (P<0.001), smoking (P<0.001), history of diabetes (P<0.005), and fasting insulin (P<0.001) were strongly and independently associated with CAD. Also, obesity and hypertension were associated with increased risk of CAD.

TABLE 3. Logistic Regression Analysis of Determinants of CAD in Group of Studied Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR</th>
<th>95% CI*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>1.02</td>
<td>0.99–1.05</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>5.99</td>
<td>3.31–10.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Obesity, Y/N</td>
<td>1.33</td>
<td>0.77–2.27</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, Y/N</td>
<td>6.61</td>
<td>3.62–12.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus, Y/N</td>
<td>32.9</td>
<td>3.88–279</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Hypertension, Y/N</td>
<td>1.68</td>
<td>0.98–2.90</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>1.00</td>
<td>1.00–1.00</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>1.20</td>
<td>1.14–1.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IRS-1 G972R mutation†</td>
<td>2.93</td>
<td>1.30–6.60</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Y indicates yes; N, no. Obesity has been defined as BMI ≥25 kg/m².

*The analysis included 495 subjects (295 with CAD and 200 controls). For age, total cholesterol, and fasting insulin, ORs indicate the increase in the relative risk of CAD for each unitary increase in the variable.

†The relative risk associated with mutant GR genotype has been estimated vs wild-type GG genotype.

Frequency of G972R Mutation in the IRS-1 Gene

In the total cohort, 74 (14.2%) of 522 subjects were found to be heterozygous for the G972R mutation in the IRS-1 gene; no homozygous carrier was found (Table 2). In the CAD group, the control group, and the total cohort, the observed frequencies were in Hardy-Weinberg equilibrium. The frequency of G972R mutation carriers in our control population was 6.8%, which is similar to that previously reported in normal subjects from different ethnic groups.12 No sex-related differences were found in the frequency of the G972R mutation in both case patients and controls.
Although the association did not reach statistical significance, total cholesterol was not shown to be significantly associated with CAD, and this is in agreement with the lack of difference in the prevalence of hypercholesterolemia between case patients and controls.

To test the potential interaction between the IRS-1 gene variant and the conditions known to be associated with hyperinsulinemia and insulin resistance, we repeated the analyses by subdividing the sample according to BMI (above or below 25 kg/m²) and to the presence or absence of IRS. The results, presented in Figure 2, indicate that obesity (BMI > 25 kg/m²), per se, did not significantly affect CAD risk. However, when obesity was associated with the presence of the G972R mutation, the risk of CAD increased up to 6 times (OR 6.92, 95% CI 2.24 to 21.4; P < 0.001) for these individuals compared with nonobese/noncarrier individuals. After stratification for IRS, the affected noncarriers already showed a significantly increased risk of CAD (OR 5.10, 95% CI 3.04 to 8.58; P < 0.001), and this effect was further enhanced by the presence of the G972R mutation in the IRS-1 gene (OR 27.3, 95% CI 7.19 to 104.0; P < 0.001).

**Association Between G972R Mutation and Clinical and Metabolic Characteristics**

Table 4 compares clinical and biochemical data according to the presence of the G972R mutation. Carrier individuals showed significantly higher values of plasma total cholesterol (P < 0.001) and triglycerides (P < 0.001), total triglycerides (2.02 ± 0.95 versus 3.38 ± 1.34 mmol/L, respectively; P < 0.001), and ratio of total cholesterol to HDL cholesterol (5.5 ± 1.5 versus 8.2 ± 0.9, respectively; P < 0.05). Also, the frequency of diabetes was higher among CAD subjects with the IRS-1 mutation compared with those with wild-type IRS-1 (17% versus 7.1%, respectively; P < 0.01).

**Discussion**

The rationale for including the IRS-1 gene in the list of candidate genes for CAD was based on the demonstration that defects in tissue sensitivity to insulin may cause several metabolic alterations leading to the development of coronary atherosclerosis. Because the IRS-1 protein appears to have a pivotal role in modulating tissue response to insulin, a structural defect in this protein may well influence the risk of CAD.

In the present study, subjects with angiographic evidence of coronary atherosclerosis were compared with population controls for whom coronary disease was excluded on the basis of clinical history and noninvasive tests. Even though the possible enrollment of asymptomatic CAD subjects in the control group cannot be definitely ruled out, its effect on

![Figure 2. Relative risk of CAD attributable to G972R mutation in different subgroups. IRS indicates subjects with clinical features of insulin-resistance syndrome. Reference groups are nonobese/noncarrier subjects and non-IRS/non-carrier subjects, respectively; ORs for reference groups are set to 1. Definition of obesity and IRS are reported in Methods. Obese group included 332 subjects (214 with CAD and 118 controls); IRS group included 309 subjects (228 with CAD and 81 controls). OR was adjusted for age, sex, smoking, total cholesterol, hypertension, and diabetes. Hypertension was not considered for adjustment in IRS subjects.](image-url)
comparisons appears to be negligible. In fact, compared with CAD patients, controls showed the expected lower prevalence of risk factors for atherosclerotic disease, similar to that observed in the same age group of the general Italian population. Therefore, our control group is representative of the general population of healthy individuals. This is further supported by the observation that the 6.8% frequency of the G972R mutation in controls is very similar to that previously reported in normal subjects.

A large number of consecutive subjects with well-defined coronary status and the expected higher frequency of classical coronary risk factors were selected as case patients. Sex, diabetes, fasting insulin levels, and smoking were the strongest predictors of coronary stenosis in our cohort. It is noteworthy that hypertriglyceridemia was the most prevalent lipid disorder in our CAD group, a finding already reported in other large angiographically based case-control studies.

We found that the G972R mutation in the IRS-1 gene was significantly associated with increased risk of CAD and that this effect was independent of the other coronary risk factors. Overall, subjects heterozygous for the mutated R allele showed a CAD risk almost 3 times as high as that for wild-type carriers. More notably, the most substantial increase in the risk of CAD was observed within the subgroups of individuals with obesity or IRS, suggesting that the G972R mutation in the IRS-1 gene may worsen or induce these abnormalities.

The mechanisms responsible for these associations remain speculative. We observed that the G972R substitution in the IRS-1 gene was significantly related to increased whole-body insulin resistance. 

One possible explanation is that the G972R mutation may influence insulin binding to IRS-1. The IRS-1 gene may represent a useful genetic marker of CAD risk factors. Furthermore, it may, per se, reduce tissue insulin sensitivity. Although further studies are needed to better define the role of the IRS-1 gene in modulating triglycerides and in fatty acid metabolism, our findings clearly indicate that this IRS-1 gene variant may predispose to proatherogenic alterations in plasma lipids.

An additional interesting finding of the present study was that the G972R mutation significantly contributes to increasing the risk of CAD in subjects with obesity or IRS. This phenomenon was particularly evident in obese subjects, in whom only those carrying the G972R mutation showed a significantly increased risk of CAD. Obesity is associated with CAD risk only in the presence of a visceral distribution of adipose tissue. In the present study, we did not assess the regional distribution of body fat, and so far, no data indicating that the presence of IRS-1 variant is associated with the accumulation of visceral adipose tissue are available. Another possibility might be that the G972R mutation represents a genetic marker in obese individuals, who are more predisposed to develop the abnormalities associated with insulin resistance. Additional work is warranted to clarify these important issues. Nevertheless, our results suggest that the identification of the G972R IRS-1 gene mutation might be particularly useful in the presence of obesity or IRS for the detection of individuals at higher risk of CAD.

In conclusion, we demonstrated that a common mutation in the IRS-1 gene may represent a useful genetic marker of increased risk of CAD. Even though the mechanisms underlying this effect cannot be inferred from the present study, our findings suggest that mutations in the IRS-1 gene may increase CAD risk by predisposing individuals to the development of metabolic risk factors such as diabetes and dyslipidemia. Further studies are needed to investigate the clinical and therapeutic implication of our results.

Acknowledgments

This study was supported by grant E252 from Telethon Italy and from the Ente Cassa di Risparmio di Roma (to Dr Baroni) and by grants 95.0000982.PF40, 95.00910.PF41, 96.05269.74, and 95.00924.41 from the Italian National Research Council (CNR) (to Drs Arca and Urbinati). We are indebted to L. Porcu and R. Cantini for their excellent technical support, to Drs A. Barbato and N. Di Lecce for their invaluable contribution to the population screening, and to Dr A. Candeloro for assistance in the recruitment of patients.

References


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doi: 10.1161/01.ATV.19.12.2975

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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