AN APPLICATION OF LOGISTIC REGRESSION AND MULTIFACTOR DIMENSIONALITY REDUCTION ANALYSES FOR DETECTING GENOTYPE-PHENOTYPE INTERACTIONS ASSOCIATED WITH DEVELOPING OF ATHEROSCLEROSIS IN BULGARIAN COHORT

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ABSTRACT

Inflammation and genetic predisposition play a critical role in the initiation and progression of atherosclerosis, especially in the process of plaque destabilization. The apolipoproteinA-I component of High-density lipoproteins, Plasminogen activator inhibitor-1 and C-reactive protein (CRP) have been implicated in participation of vascular inflammation regulation. Our aim was to examine interactions among a panel of selected gene polymorphisms with serum CRP level on risk of developing of atherosclerotic in carotid and femoral artery using data from 42 patients with stable angina pectoris, 42 with acute coronary syndrome and 122 age-matched healthy subjects. Data examination using multifactor dimensionality reduction methods showed significant role of C-1562T MMP9, C373G PECAM-1, IL-1RA intron 2 polymorphism, 4G/5G PAI-1, C677T MTHFR, C-2123G SELP, T495G LPL polymorphisms in the epistatic interactions connected with increased intima-media thickness in the patients with stable angina pectoris and acute coronary syndrome. These analyses also showed the association of the selected polymorphisms mentioned above (gene-gene interaction) with serum CRP level (genotype-phenotype interaction) with developing of atherosclerosis.

Keywords: atherosclerosis, gene polymorphisms, multifactor dimensional reduction

Abbreviations: SAP: stable angina pectoris; ACS: acute coronary syndrome; MI: myocardial infarction; HDL: high-density lipoproteins; LDL: low-density lipoproteins; CVD: cardiovascular disease; CAD: coronary artery disease; SNP: single nucleotide polymorphisms; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; MDR: multifactor dimensionality reduction; APOA-I: apolipoprotein A-I; PAI-1: plasminogen activator inhibitor-1; CRP: C-reactive protein; IL1Ra: interleukin-1 receptor antagonist; PON1: Paraoxonase 1; PECAM-1: platelet endothelial cell adhesion molecule; SELP: Selectin P; GP IIIa: Glycoprotein IIIa; MTHFR: methylenetetrahydrofolate reductase; PPARα: Peroxisome proliferator-activated receptor alpha; LPL: lipoprotein lipase; MMP9: matrix metalloproteinase 9; TG: triglycerides; VLDL: very low density lipoproteins; IMT: intima-media thickness; CCA: common carotid artery; CFA: common femoral artery

Introduction

Atherosclerosis is the major underlying cause of cerebrovascular and cardiovascular diseases (39). It is considered that this disease is linked to hypercholesterolemia and the accumulation of inflammatory cells in the artery wall. Inflammation is involved in the atherosclerotic process by recruiting leukocytes, promoting plaque growth and inducing plaque destabilization. Formation of the plaque usually continues over decades, starting with early lesions formation which may occur in early adolescence. The velocity of progression depends on many factors, such as gender, genetics and some well recognized risk factors- obesity, diabetes, hypertension, smoking, ageing and etc. (8). The development of advanced atherosclerosis is a slow progressive process that starts in childhood and remains asymptomatic for many decades, with complications such as myocardial infarction, stroke or peripheral ischemia usually occurring in later life (9). However, different epidemiological studies suggest that a positive family history together with major genetic determinants of traditional cardiovascular risk factors could be associated with the incidence of cardiovascular events - unstable angina pectoris and/or myocardial infarction (8). It is likely that a large number of additional genetic factors could interact with environmental factors to determine overall cardiovascular risk progression.

A central goal of our study was to examine whether and how several single nucleotide polymorphisms (SNPs) in a selected panel of genes are associated with susceptibility to develop atherosclerosis in carotid and femoral arteries. We hypothesized that the success of this effort will
critically depend of the degree of nonlinearity in the relationship between genotype and phenotype. Nonlinearities could arise from gene-environment interactions, epistasis, locus heterogeneity and/or gene-gene interaction. Several SNPs in different genes have been implicated to be somehow connected with cardiovascular disease (CVD). Among them to test our hypothesis we selected the follows: Apolipoprotein-A-I (APOA1, rs2893157), Lipoprotein lipase (LPL, rs320), Paraoxonase 1 (PON1, rs854560), C-reactive protein (CRP, rs3091244), Peroxisome proliferator-activated receptor alpha (PPARα, rs1800206), Methylene-tetrahydrofolate reductase (MTHFR, rs1801133), Matrix metalloproteinase - 9 (MMP-9, rs3918242), Matrix metalloproteinase - 3 (MMP-3, rs35068180), Platelet endothelial cell adhesion molecule-1 (PECAM-1, rs6668), Selectin-P (SelP, rs1800807), Selectin-E (SelE, rs5361), Glicoprotein IIIa (GPIIIa, rs5918), Plasminogen activator inhibitor (PAI-1, rs7997628), Interleukin-1 receptor antagonist (IL1Ra, rs315952).

APOA1 is the major apoprotein constituent of high-density lipoprotein (HDL). Multiple genetic variations in APOA1 have been shown to influence lipoprotein metabolism and the plasma concentration of total cholesterol (36). APOA1 (-75 G/A, rs2893157) polymorphism is relatively common and is positively associated with higher serum concentrations of lipoprotein-A and therefore may confer as a potential risk for CVD (1).

LPL catalyses the hydrolysis of the triacylglycerol component of circulating chylomicrons and very low density lipoproteins (VLDL), thereby providing non-esterified fatty acids and 2-monoacylglycerol for tissue utilization (31). Given the evidence above, we also hypothesized that the selected SNPs may be the strong candidate genes for CVD and might influence the risk of its developing. Several SNPs in different genes have been associated with various human diseases, including coronary heart disease (35), but other investigation did not indicate effects of PON1 55Leu/Met polymorphism (rs854560), either alone or in combination with the Q192R polymorphism, on CVD risk (5).

Materials and Methods

Study population

The study population was composed of 42 patients with stable angina pectoris (SAP), 42 with acute coronary syndrome (ACS) and 122 age-matched healthy subjects. This study was approved by the

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Ethical Committee of “St. Anna” Hospital in Sofia, Bulgaria, No 54/28.02.2005 and confirmed with the principles outlined in the Declaration of Helsinki (4).

SAP was diagnosed in the presence of typical chest pain during exercise, stable for at least 3 months before study entry and ≥0.1 mV ST-segment depression during the electrocardiogram exercise test. Acute coronary syndrome patients had ischemic chest pain at rest within the preceding 48 hours that had developed in the absence of an extracardiac precipitating cause with either ST segment depression of >0.1 mV or T wave inversion in two or more contiguous leads on the presenting 12 lead electrocardiography. Patients with myocardial infarction had similar diagnostic criteria along with elevation of cardiac troponin I, without the evolution of pathological q-waves. The control group comprised of 122 healthy volunteers in the same age of distribution, without angina symptoms and with normal physical examination and exercise stress test.

Ultrasonographic examinations
Ultrasonographic examinations were performed with a Fukuda Denshi UF-750 XT, equipped with 7 or 10 MHz transducers, with subjects in the supine position. An ECG signal synchronized the image analysis to the end of diastole. The left and right carotid and femoral arteries were examined. The carotid artery was scanned at the level of the carotid bulb, and 10 mm each of the internal and external carotid arteries. The femoral artery was examined distally to the femoral bifurcation. The regions were scanned approximately 30 mm proximal and 10 mm distal to the femoral bifurcation. The area scanned was perpendicular to the far vessel wall. The ultrasonographic images were analyzed with a Fukuda Denshi UF-750 XT, equipped with 7 or 10 MHz transducers, with subjects in the supine position. An ECG signal synchronized the image analysis to the end of diastole. The left and right carotid and femoral arteries were examined. The carotid artery was scanned at the level of the carotid bulb, and 10 mm each of the internal and external carotid arteries. The femoral artery was examined distally to the inguinal ligament at the bifurcation into the superficial and profound femoral arteries. The area scanned was approximately 30 mm proximal and 10 mm distal to the femoral bifurcation. The regions were scanned with both longitudinal and transverse projections, in order to assess the occurrence of plaques. Three B-mode images from the longitudinal view, as well as a short sequence of real-time images, were recorded. Doppler ultrasound was used for information on blood flow velocity, and for vessel identification. Three ‘frozen’ images were recorded for assessments of intima-media thickness and lumen diameter. Optimal image projection was considered to be achieved when ultrasound beams were perpendicular to the far vessel wall.

Assessments of intima-media thickness (IMT)
The ultrasonographic images were analyzed with a computerized system, and blind evaluation was made in terms of treatment. Intima-media thickness was defined as the distance from the leading edge of the lumen -intima interface to the leading edge of the media -adventitia interface of the far wall. Intima-media thickness was measured in a 10-mm long segment just proximal to the carotid bulb in the common carotid artery (CCA) and in a 15-mm long segment just proximal to the bifurcation in the femoral artery (CFA). The computer program calculated the minimum, maximum and mean values of intima-media thickness from three separate images.

Laboratory methods
Cholesterol and triglycerides levels were determined according to routine laboratory techniques. Serum CRP levels were measured by immunoturbidimetric method and immunonephelometry, and confirmed with high-sensitive ELISA (IBL Int., USA).

DNA preparation
Genomic DNA was extracted from whole peripheral blood cells using sodium extraction protocol (32). The purity of the isolated DNA was confirmed with an A260/280 ratio of 1.8-2.00 for all samples tested measured on NanoDrop1000. The DNA quality was also checked on agarose gel, followed by ethidium bromide staining (43).

Polymorphism analysis
The polymorphisms were screened using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). The primers and the conditions for the PCR reactions and restriction enzymes are previously described (2, 7, 11, 13, 14, 16, 23, 26, 30, 41, 45, 46).

The detailed sequence information for SNPs studied is available at: http://www.ncbi.nlm.nih.gov/SNP/.

Statistical analysis
Chi-square test was used to detect differences in genotype and allele distribution from Hardy-Weinberg equilibrium.

The between-group data were compared with Student’s unpaired t-test for continuous data and with the χ² test for categorical data. Allele frequencies were calculated from the genotypes of the subjects. Differences in allele and genotype frequencies between the cases and controls were compared with the χ² test or Fisher’s exact test. For the entire study population, we examined the genotype-phenotype correlation with additive, dominant model, and recessive models, and used logistic regression to control for the various traditional risk factors of CAD (age, gender (male), hypertension, diabetes mellitus, smoking status, dyslipidemia, and family history). Odds ratios and their 95% confidence intervals were calculated accordingly.

The statistical analysis was carried out using the PASW Statistics 18.0 software package (IBM, USA) for Windows.
The MDR method was used for the evaluation of gene-gene and genotype-phenotype interactions (20, 33). This method includes a combined cross-validation/permutation-testing procedure that minimizes false-positive results which might otherwise result from multiple examinations of the data (12). Models that are true-positives are likely to generalize to independent datasets and will have estimated testing accuracies greater than 0.5. Here, the case-control labels are randomized 1000 times and the entire MDR model fitting procedure were repeated on each randomized dataset to determine the expected distribution of testing accuracies under the null hypothesis. This process yields an empirical distribution of testing accuracies under the null hypothesis that is in turn used to calculate a p-value (3, 6). Permutation testing was performed to assess the probability of obtained testing accuracy as large as or larger than observed in the original data given the null hypothesis of no association is true. An interaction graph using entropy (measurement of randomness) estimates as described by Jakulin and Bratko (24) was created to confirm, visualize and interpret the results obtained by logistic regression analysis and MDR. The MDR analysis was carried out using version 2.0 beta7 of the open-source MDR software package that is freely available.

### Results and Discussion

The baseline characteristics of the study population are shown in Table 1. No differences were found in triglyceride level between the patients with SAP and ACS compared to control subjects (P1 and P2 >0.05). The level of cholesterol was significantly higher in ACS group compared with the controls (P2=0.002) while the cholesterol level between SAP and controls was no significantly different. The factors of hypertension, obesity and family history were significantly higher in patients with SAP and ACS compared to controls (P1 and P2 <0.05). No significant differences were found in most characteristics between the patients with SAP compared to ACS (P1 >0.05) except the cholesterol level which is significantly higher in patients with ACS (P3=0.030).

The common carotid artery intima-media thickness and IMT of CFA were used as markers for atherosclerosis. IMT is considered a useful marker for generalized atherosclerosis and has been shown to predict future cerebrovascular and coronary events (38). Our results showed that there are significant differences between IMT of CCA and IMT of CFA in patients with SAP and ACS compared to the control subjects (Table 1). We also observed a significant difference (p=0.005) when compare the IMT of CCA between patients with SAP and ACS, indicating that these patients could be at risk for stroke.

### Table 1

Demographic and biochemical characteristics of the groups studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=122)</th>
<th>SAP (n=42)</th>
<th>ACS (n=42)</th>
<th>P1-value*</th>
<th>P2-value*</th>
<th>P3-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male) (%)</td>
<td>43.7</td>
<td>75.0</td>
<td>62.2</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.091</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>53.4</td>
<td>86.0</td>
<td>75.6</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.192</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>14.3</td>
<td>59.2</td>
<td>60.0</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.533</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>44.3</td>
<td>69</td>
<td>66.3</td>
<td>0.006</td>
<td>0.002</td>
<td>0.458</td>
</tr>
<tr>
<td>Family history of CVD (%)</td>
<td>7.4</td>
<td>27.5</td>
<td>33.7</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.460</td>
</tr>
<tr>
<td>Plasma concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.3±1.2</td>
<td>5.3±1.0</td>
<td>6.0±1.7</td>
<td>0.985</td>
<td>0.002</td>
<td>0.030</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>2.0±1.4</td>
<td>1.9±1.1</td>
<td>2.0±1.3</td>
<td>0.960</td>
<td>0.987</td>
<td>0.960</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.23±0.24</td>
<td>2.12±0.39</td>
<td>21.0±3.95</td>
<td>0.0996</td>
<td>0.166</td>
<td>0.023</td>
</tr>
<tr>
<td>Markers of atherosclerosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima-media thickness (mm) CCA</td>
<td>0.069±0.005</td>
<td>1.170±0.017</td>
<td>1.250±0.014</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Intima-media thickness (mm) CFA</td>
<td>0.091±0.005</td>
<td>1.280±0.012</td>
<td>1.320±0.010</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.267</td>
</tr>
</tbody>
</table>

*P1, P2 and P3 were calculated with chi-square tests and ANOVA

Data are reported as mean (± S.D.) for continues variables and as percent for categorical variables. Because of a skewed distribution, CRP (C-reactive protein) values are reported as median with an interquartile range.

Hypertension: blood pressure >140/90 mm Hg; SAP: Patients with stable angina pectoris; ACS: Patients with acute coronary syndrome; CCA: Common carotid artery; CFA: Common femoral artery.
It has been shown that PAI-1 is elevated in the presence of atherosclerosis, and that the elevated levels could be connected with an appearance of 4G/5G SNP. The allele frequencies and the genotype distributions in the cases (patients with SAP and ACS) and controls are shown in Table 2. The genotype distribution fulfilled the criteria of a Hardy-Weinberg distribution (p>0.05). The overall allele frequencies for PAI-1 4G/5G were 0.38 and 0.62 for the 4G and 5G alleles in control subjects, which significantly differ from the distribution among patients with SAP (0.54:0.46, p=0.033), but not in those with ACS (0.40:0.60, p=0.885). A significant different distribution of allele frequencies were also observed between patients with SAP and ACS (Table 2, p =0.033), assuming the association between 4G allele and SAP. A linear regression showed that PAI-1 can be predicted from a linear combination of markers for CAD adjusting for cholesterol level, sex, age, and IMT of CCA (F=3.663, p=0.010). The 4G-allele was not associated with a higher prevalence of atherosclerosis, neither connected with IMT of CCA nor IMT of CFA. Although not significantly, there was a trend towards a higher probability of a high IMT of CCA (>0.9 mm) for both the 4G/5G + 4G/4G-genotypes compared to the 5G/5G-group (4G/5G: OR=1.778 (95%-CI: 0.3; 11.8), p=0.053). Adjustments for possible classical risk factors did not materially alter the results. It is know that PAI-1 is an inhibitor of fibrinolysis in the vessel wall. It was suggested that 4G-deletion polymorphism of the promoter region increases the risk of CVD (22) by removing the extra guanosine base in the binding site, which attenuate the response to the transcription repressor factor and enhance the PAI-1 transcription (22), and hence reduce fibrinolytic activity (38). Several other factors have been implicated in the regulation of the fibrinolytic and thrombotic processes in patient with CVD, among them is the elevated level of homocysteine (15).

![Table 2](image)

SAP: Patients with stable angina pectoris; ACS: Patients with acute coronary syndrome; PAI-1: Plasminogen activator; MTHFR: Methylene-tetrahydrofolate reductase; ILRa: Interleukin-1 receptor antagonist; SELP: Selectin P

It has also been shown that MTHFR 677 polymorphism (C677T, rs1801133) can result in elevated homocysteine levels, which may lead to an increased risk of thrombotic events. In our study the mutant homozygote for MTHFR C677T is 9.5%, 9.5%, 16.9%, and the T allele frequency is 26%, 29% and 40%, respectively for control subjects, patients with SAP and ACS. There is only a
A significant difference between the patient with ACS and the control group in the genotype distribution of this SNP (Table 2, \( p = 0.035 \)), but the allele frequency significantly differ between all groups studied (Table 2: Controls:SAP, \( p = 0.001; \) Controls:ACS, \( p = 0.001; \) SAP:ACS, \( p = 0.001 \)).

A binary logistic regression was carried out to evaluate the role of MTFHR C677T polymorphism as potential predictor of CAD. When 11 independent risk factors (obesity, hypertension, family history, male sex, smoking, total cholesterol, triglycerides, diabetes, age over 50 years, IMT of CCA, and dominant genotype for MTFHR) were considered together they predicted significantly and correctly 78% of the cases with increased IMT of CFA (\( \chi^2 = 4.4818, df = 7, p = 0.043 \)) only.

Some inflammatory factors like ILRa play role in CVD development (27, 37), ILRa genotypes BB, BC, BD and DD were significantly more frequent among patients with SAP and ACS compared to control subjects (Table 2, \( P_1 = 0.002 \) and \( P_2 = 0.009 \)). ILRa alleles B, C and D were significantly more frequent among SAP and ACS patients than control subjects (\( P_1 = 0.004 \) and \( P_2 = 0.034 \)). The most frequent allele observed among the three studied groups (SAP, ACS and controls) was A (controls-0.75; SAP- 0.55; ACS- 0.61) followed by B (controls- 0.23; SAP- 0.34; ACS- 0.32), C and D alleles were rare (for C: controls-0.02; SAP- 0.03; ACS- 0.03 and for D: controls- 0.00; SAP- 0.08; ACS- 0.04). There was no significant difference in the genotype and allele frequencies between the groups of patients with SAP and ACS. Allele-E was not observed in our population. Following adjustment for the risk factors mentioned above, the binary logistic regression analysis showed the association of allele- B of the ILRa and the development of atherosclerosis in the femoral artery (adjusted OR=12.67, (95% -CI: 0.998- 160.979), \( p = 0.049 \)).

It was studied that cell adhesion molecules like Selectin P are important for the attachment of leukocytes to endothelial cells and platelets (11). Several polymorphisms in SELP gene were investigated for association with CVD (18). There was a significant difference between the genotype frequencies of the SELP -2123C/G polymorphism between controls and patients with SAP and ACS (Table 2, \( P_1 = 0.049 \) and \( P_2 = 0.033 \)). There was no significant difference in the genotype and allele frequencies between the groups of patients with SAP and ACS as well as in the allele frequencies between controls and patients with SAP and ACS. Binary logistic regression analysis did not reveal significant associations of SELP -2123C/G polymorphism with development of atherosclerosis in CCA and CFA.

Numerous epidemiological studies have suggested that several genetic variants increase the risk for atherosclerotic plaque progression (21, 40).
SNP and CRP-serum level had the weakest synergetic effect (Fig. 1B).

TABLE 3

<table>
<thead>
<tr>
<th>Best combination in each direction from SNPs and biochemical markers</th>
<th>Intima-media thickness of common carotid artery</th>
<th>Intima-media thickness of common femoral artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum CRP concentration</td>
<td>10/10</td>
<td>9/10</td>
</tr>
<tr>
<td>Serum CRP concentration /C677T MTHFR</td>
<td>8/10</td>
<td>9/10</td>
</tr>
<tr>
<td>Serum CRP concentration / C677T MTHFR/4G/5G PAI-1</td>
<td>10/10</td>
<td>9/10</td>
</tr>
<tr>
<td>Serum CRP concentration / C495G LPL/ C677T MTHFR/4G/5G PAI-1</td>
<td>10/10</td>
<td>9/10</td>
</tr>
</tbody>
</table>

CRP: C-reactive protein; MTHFR: Methylenetetrahydrofolate reductase; MMP9: Matrix metalloproteinase 9; PAI-1: Plasminogen activator inhibitor-1; PECAM-1: Platelet endothelial cell adhesion molecule; SELP: Selectin P

Fig. 1. Interaction circle graph for patients with atherosclerosis
(A) Model for intima-media thickness of the carotid artery; (B) Intima-media thickness of the femoral artery;
These interaction models describe the percent of the entropy in case–control status that is explained by each factor or two-way interaction. Two-way interactions between factors are depicted as a line accompanied by a percent of entropy explained by that interaction. Redundancy is depicted as a line between factors accompanied by a negative percent of entropy. All positive values in the nodes indicate independent main effect of the marker. CRP_serum: C-reactive protein concentration in the serum; MTHFR: Methylenetetrahydrofolate reductase; MMP9: Matrix metalloproteinase 9; PAI-1: Plasminogen activator inhibitor-1; PECAM-1: Platelet endothelial cell adhesion molecule; SELP: Selectin P
This study show that interacting genes involved in the development of atherosclerosis in CCA and CFA are closely associated with lipid metabolism, cell matrix adhesion and inflammation phenotypes. The results from MDR also showed several genetic interactions that were significant after permutation testing. Our findings appear to be of importance because they implicated involvement of genes, which have been shown previously to be connected with development of atherosclerosis (10, 11, 14, 15, 27, 30, 46). The use of MDR closely reassembles the complex nature and multifactorial base of the disease by evaluating multiple SNPs in an unbiased way. To confirm the present results for the Bulgarian population additional studies are needed with more patients at different stages of CAD and inflammation, respectively, and especially focusing on gene-gene interactions and including common polymorphisms of genes related with the inflammatory response.

Conclusions
In current study, we used candidate gene approach to explore the genetic contribution of set of polymorphisms and biochemical markers to essential atherosclerotic plaque formation in CFA and CCA in a Bulgarian cohort. We demonstrated a comprehensive analytical strategy for investigating the base of the disease by evaluating multiple SNPs in an unbiased way. To confirm the present results for the Bulgarian population additional studies are needed with more patients at different stages of CAD and inflammation, respectively, and especially focusing on gene-gene interactions and including common polymorphisms of genes related with the inflammatory response.

Acknowledgments
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