Apo B Insertion/Deletion Polymorphisms Are Associated With Atherosclerosis in Young Black but Not Young White Males

James E. Hixson, C. Alex McMahan, Henry C. McGill Jr., Jack P. Strong, and the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group*

Investigators in eight communities collected aortas, right coronary arteries, blood and liver samples, and associated information from 872 young males, aged 15–34 years, who died of external causes. Pathologists graded the arteries for atherosclerotic lesions, and a central laboratory measured lipoprotein cholesterol concentrations. Apolipoprotein (apo) B sequences were amplified in hepatic DNA samples to determine genotypes for length polymorphisms in the signal peptide of apo B. In addition to the insertion (ins) allele (27-amino acid signal peptide) and the deletion (del) allele (24 amino acids), we detected a rare allele (ins*) in whites with an in-frame insertion of two Leu codons in a region that normally contains six Leu codons. The frequency for the apo B del allele was lower in blacks than in whites (p<0.0001). In blacks, homozygotes for the ins allele had the lowest levels of serum cholesterol and very low plus low density lipoprotein cholesterol (VLDL+LDL-C), homozygotes for the del allele had the highest levels, and heterozygotes had intermediate levels (p=0.0509 for cholesterol, p=0.0530 for VLDL+LDL-C), but no differences were found in whites. In blacks, homozygotes for the ins allele had the least involvement of the thoracic and the abdominal aorta with lesions, homozygotes for the del allele had the greatest involvement, and heterozygotes had intermediate involvement (p=0.0328 for thoracic aorta, p=0.0104 for abdominal aorta), but no differences were found in whites. In blacks, apo B ins/del genotype accounted for 1.2% of the observed variation in lesions of the thoracic aorta and 1.7% of the variation in those of the abdominal aorta. The association of apo B ins/del genotypes with lesions in young blacks but not in young whites may contribute to explaining the excess of fatty streaks previously observed in young blacks. (Arteriosclerosis and Thrombosis 1992;12:1023-1029)

KEY WORDS • apolipoprotein B • apolipoprotein E • polymorphisms • serum cholesterol • lipoprotein cholesterol • atherosclerosis • abdominal aorta • thoracic aorta • right coronary artery

Atherosclerosis is a lifelong process that begins early in life and results in clinically manifest coronary heart disease in middle age and later. Risk factors for coronary heart disease in adults (age, smoking, and serum lipoprotein cholesterol levels) are associated with the extent and severity of atherosclerosis in young males 15–34 years of age.1 Common genetic variants of human apolipoprotein (apo) E also are associated with differences in lipid risk factors and atherosclerosis in young males.2 In this study, we report the association of common length polymorphisms in the signal peptide of human apo B with atherosclerotic lesions in young people. The common alleles are the insertion (ins) allele, which contains a 27-amino acid signal peptide, and the deletion (del) allele, with a 24-amino acid signal peptide due to the deletion of Ala-Leu-Ala.3–5

Methods

Study Organization

In the multicenter cooperative project, "Pathobiological Determinants of Atherosclerosis in Youth" (PDAY), 14 cooperating centers in eight communities adopted standardized procedures to collect tissues, blood, and information from autopsied cases and to submit them to central laboratories for analysis as previously described.1

Subjects

The study subjects were males 15–34 years of age who died of external causes (accidents, homicides, or suicides) within 72 hours after injury and were autopsied in one of the cooperating medical examiners’ laboratories within 48 hours after death. The age and race of each subject were obtained from the death certificate. Persons excluded from the study were those who died of heart disease (Down's syndrome, acquired immunodeficiency syndrome, or hepatitis. We analyzed data from 872 cases collected from 1987 to 1990 for which liver DNA was extracted and for which pathological evaluation of at least one artery was available.
Typing and Sequencing of Apo B ins/del Polymorphisms in Hepatic DNA

Liver samples were collected at autopsy, frozen at −70°C, and shipped on dry ice for long-term storage at −80°C. DNA was extracted from 0.5 g homogenized liver samples as previously described. DNA samples were subjected to amplification by the polymerase chain reaction (PCR) in a DNA thermal cycler. PCR used oligonucleotide primers (Genosys Inc., Houston, Tex.) to amplify the signal peptide region of the apo B gene (forward primer, 5'-CCCTGCAGCTGGCGATGGACCCCGCGA-3'; reverse primer, 5'-ACCGGCCCTGGGCAGCA-3') as described by Boerwinkle and Chan. In addition to the buffer and nucleotide components described by the supplier of Thermus aquaticus (Taq) polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), each amplification reaction contained 1 μg hepatocyte DNA, 1 pmol/μl of each primer, 10% dimethyl sulfoxide, and 0.025 unit Taq polymerase/μl in a final volume of 20 μl. Each reaction mixture was heated at 95°C for 5 minutes for denaturation and subjected to 30 cycles of amplification by primer annealing and extension (60°C for 1.5 minutes) and denaturation (95°C for 1 minute). Each reaction mixture was loaded onto 8% polyacrylamide non-denaturing gels and electrophoresed for 3 hours under constant current (35 mA). After electrophoresis, gels were treated with ethidium bromide (0.2 mg/l) for 10 minutes, and DNA fragments were viewed by UV illumination.

For sequencing of apo B alleles, synthetic cleavage sites for Pst I and BamHI (underlined in primer sequences) were added to the amplification primers (forward primer, 5'-CCTTGCGAAGGCGAGTGAGCCCGCGA-3'; reverse primer, 5'-CCTGGATCCGGCCTGGGCGCCCGAGA-3'). Hepatic DNA samples from individuals bearing each allele were subjected to PCR, and the amplification products were digested with Pst I and BamHI (3 hours at 37°C). The digested PCR products were then ligated into Bluescript SK+ phagemid vectors (Stratagene, San Diego, Calif.). Single-stranded templates from recombinant clones were sequenced by the chain-termination method with modified T7 polymerase and 35S-labeled deoxyadenosine triphosphate.

Grading of Arterial Specimens

The aorta and right coronary artery were removed at autopsy and prepared as previously described. A central laboratory stained the arteries with Sudan IV. Three pathologists independently evaluated left aortic halves and right coronary arteries for percentage of intimal surface involved with fatty streaks, fibrous plaques, complicated lesions, and calcified lesions. The sum of all types of lesions was designated as the total percent involvement of intimal surface for each artery. The sum of fibrous plaques, complicated lesions, and calcified lesions was designated as percent involvement with raised lesions. Three independent gradings were averaged to obtain consensus gradings of lesions. For total percentage of intimal surface area involved, the intraclass correlation coefficients between pairs of pathologists ranged from 0.734 to 0.945. For the percentage of intimal surface area involved with raised lesions in the abdominal aorta and the right coronary artery, the intraclass correlation coefficients between pairs of pathologists ranged from 0.672 to 0.917. For involvement with raised lesions in the thoracic aorta, the intraclass correlation coefficients ranged from 0.313 to 0.668.

Serum Measurements

Serum derived from approximately 20 ml blood collected at autopsy was shipped to a central laboratory, which measured total serum cholesterol concentrations by the cholesterol oxidase enzymatic method. The laboratory precipitated very low density lipoprotein (VLDL) and low density lipoprotein (LDL) by heparin...
Mn$^{12}$ and measured high density lipoprotein cholesterol (HDL-C) in the supernate by the same enzymatic method. The cholesterol assay was standardized with reference material obtained from the College of American Pathologists. The coefficient of variation between blind duplicates for the serum cholesterol measurement was 1.1%, and that for the HDL-C was 5.5%. Concentration of VLDL-C and LDL-C was calculated as the difference between serum cholesterol and HDL-C. To assess smoking status, serum levels of thiocyanate were measured by a modification of the Bowler method.$^9$ A smoker was defined as one who had a serum thiocyanate concentration $\geq$90 $\mu$M/l. The coefficient of variation between blind duplicates for serum thiocyanate was 5.6%. Because emergency medical teams administer large quantities of fluids to some individuals immediately before death, serum data from cases with serum cholesterol concentration $<$100 mg/dl were not included in the statistical analyses. Sera met the foregoing acceptability criterion in 503 cases.

**Statistical Analyses**

Apo B $\text{ins/del}$ allele frequencies were estimated by the gene counting method. Arterial lesions and serum lipid and lipoprotein data were analyzed by analysis of variance and analysis of covariance.$^{10}$ To better satisfy the assumptions of the statistical analysis, serum lipid and lipoprotein concentrations were transformed with a logarithmic transformation. For the lesion data, we added 0.1 to each percent surface area involvement before applying the logarithmic transformation. Results for whites and blacks were obtained in separate analyses. The genetic variance was estimated as described by Boerwinkle and Sing.$^{11}$

**Results**

**Apo B $\text{ins/del}$ Genotypes in PDAY Cases**

Figure 1 shows amplification of the apo B signal peptide region in hepatic DNA from PDAY cases representing the common apo B $\text{ins/del}$ genotypes and from heterozygotes for the rare $\text{ins}^*$ allele. Examples of each apo B allele were cloned and sequenced to identify the nucleotide sequences responsible for allelic size differences. As previously reported,$^{14}$ the $\text{ins}$ allele contained a 27-amino acid signal peptide, and the $\text{del}$ allele contained a 24-amino acid signal peptide due to an in-frame deletion of 9 bp encoding Leu-Ala-Leu (Figure 1). $\text{del}$ alleles were sequenced from whites and blacks. The larger $\text{ins}^*$ allele contained a 29-amino acid signal peptide due to the addition of 6 bp. The $\text{ins}^*$ allele adds two Leu codons in a region that normally contains six identical codons for Leu (Figure 1).

Table 1 shows the numbers and relative frequencies of genotypes for the apo B signal peptide length polymorphisms for blacks and whites. There were significant differences in genotypic frequencies between blacks and whites ($p<0.0001$). Table 2 shows the estimated allele frequencies for each apo B length polymorphism. Allele frequencies were different between blacks and whites ($p<0.0001$). The frequency of the $\text{ins}$ allele was higher and the frequency of the $\text{del}$ allele lower in blacks than in whites. Four of the seven PDAY cases carrying the $\text{ins}^*$ allele had Spanish surnames.

**Effects of Apo B $\text{ins/del}$ Genotypes on Serum Lipid and Lipoprotein Concentrations**

Postmortem serum concentrations of cholesterol, HDL-C, and VLDL+LDL-C were measured in 503 cases. Table 3 shows the mean levels (in milligrams per deciliter) of these lipoproteins for each genotype in blacks and whites after adjustment for age. In addition, Table 3 shows significance levels for effects of age (adjusted for genotype) and genotype (adjusted for age). There were no significant differences in age distributions between whites and blacks. The effects of age were similar for whites and blacks for VLDL+LDL-C levels, although the differences were not statistically significant in blacks. There was no association of serum cholesterol and VLDL+LDL-C concentrations with apo B $\text{ins/del}$ genotypes in whites. In blacks, apo B $\text{ins/del}$ genotypes were associated with differences in serum cholesterol concentration ($p=0.0509$) and VLDL+LDL-C concentration ($p=0.0530$). In blacks, homozygotes for the $\text{del}$ allele had the highest levels of serum cholesterol and VLDL+LDL-C, homozygotes for the $\text{ins}$ allele had the lowest levels, and heterozy-
gotes had intermediate levels. HDL-C levels were not significantly affected by apo B ins/del genotypes. We were not able to assess the effects of the ins* allele because of the small number of cases that carried this allele.

Effects of Apo B ins/del Genotypes on Arterial Lesions

Table 4 shows the mean percent surface area involvement with all lesions and raised lesions in the aorta and right coronary artery for each apo B ins/del genotype. Table 4 also shows significance levels for effects of age (adjusted for genotype) and genotype (adjusted for age). There was no association of apo B ins/del genotype with atherosclerotic lesions in whites. After adjustment for age, statistically significant differences among apo B ins/del genotypes were observed for total percent involvement of the thoracic (p=0.0104) and abdominal (p=0.0001) aorta in blacks. In blacks, homozygotes for the ins del allele had the highest level of total involvement, homozgyotes for the ins allele had the lowest involvement, and the heterozygotes were intermediate. Although not statistically significant, the same pattern of differences was observed for total involvement of the right coronary artery. There was no consistent pattern among genotypes for raised lesions.

Table 5 shows the estimated percent variance for lesions explained by apo B ins/del genotype after adjustment for age. For total lesions in abdominal and thoracic aortas, the variance due to apo B ins/del genotype was approximately 1-2% in blacks.

Effects of Apo B ins/del Genotypes on Arterial Lesions After Adjusting for Apo E Genotype, Smoking Status, and Serum Cholesterol Levels

Table 6 shows the percent surface area involved with lesions after adjusting for age and apo E genotype. There were no significant differences among apo B ins/del genotypes in whites, whereas significant genotypic differences in blacks were observed for total involvement in the thoracic aorta (p=0.0222) and the abdominal aorta (p=0.0067). Apo B genotypic effects were also detected in the abdominal aorta after adjustment for age, apo E genotype, serum cholesterol levels, and smoking status (p=0.0067) (results not shown).

After adjusting for age and apo B ins/del genotype, there were significant apo E genotype effects on total involvement in the thoracic and abdominal aortas in both races (p=0.0001) as previously reported.

Discussion

Apo B, a key apolipoprotein in lipid metabolism, is secreted on chylomicrons from the small intestine or on VLDL particles from the liver and is a ligand for the LDL receptor. Many variants have been identified in the apo B gene, including polymorphisms that alter the length of the apo B signal peptide. In 872 PADDY cases, we detected the two common length polymorphisms (ins/del) and a rare allele (ins*) that adds two Leu codons. Nucleotide sequencing showed that the ins and del alleles were identical to those from other studies. The del allele from a black PADDY case was the same as that in whites. The ins* allele was found in Mexican Americans in previous studies and was suggested as a diagnostic marker of Amerindian origins. Our study provides further support for this hypothesis, as four of the seven heterozygotes carrying the ins* allele had Spanish surnames.

The ins del alleles alter the signal sequence required for biosynthesis of apo B and may affect production of apo B. In our study, we detected an association between apo B ins/del alleles and serum levels of apo B-containing lipoproteins (VLDL+LDL-C) and total cholesterol in blacks but not in whites. In a study of 106 Finnish individuals, Xu et al found that the del allele was associated with lower levels of serum triglycerides, but no differences were found for cholesterol or LDL-C. In a random sample of Mexican Americans, Boerwinkle et al found that the del allele was associated with elevated levels of glucose. The same study found that apo B ins/del genotypes in French whites were associated with differences in apo A-I and glucose levels, but the rank order of glucose levels among genotypes was not the same as that among the Mexican American population. In French whites but not Mexican Americans, apo B ins/del genotypes were weakly associated with
triglyceride levels.13 However, the del allele was associated with higher triglyceride levels rather than lower levels as reported for the Finnish population.14

This is the first study to test for associations of apo B ins/del genotype with atherosclerosis. We detected significant associations of apo B ins/del genotype with percent surface area involved with atherosclerotic lesions in both the thoracic and the abdominal aorta in blacks (Table 4). This association remained after adjustments for smoking status, serum cholesterol levels, and apo E genotype. In addition, del homozygotes among black cases had more lesions in right coronary arteries compared with ins homozygotes, and heterozygotes had intermediate levels (Table 4). However, these differences were not statistically significant, perhaps because right coronary arteries develop fewer lesions than the aorta in this age group.

Apo B ins/del genotypes were not significantly associated with raised lesions in the aorta or right coronary artery in blacks or whites. Therefore, apo B ins/del genotypes are associated only with the total extent of lesions in blacks. On the average, fatty streaks comprise >90% of the lesion area in the aorta and >80% of the lesion area in the right coronary artery. An excess of percent arterial surface involved by fatty streaks in young blacks compared with young whites has been observed in several previous studies,15,16 including the PDAY study.1 This excess in blacks was not explained by serum lipoprotein levels in the Bogalusa Heart Study16 nor by serum lipoprotein levels, smoking, or apo E genotype in the PDAY Study.1,2 Nevertheless, older blacks develop less extensive raised lesions than do older whites.17 Either fatty streaks in blacks are augmented by factors that do not promote progression to raised lesions or raised lesions originate independent of fatty streaks. The association of apo B ins/del genotypes with fatty streaks in blacks but not in whites and its lack of association with raised lesions in blacks and whites offer a genetic explanation for the incongruity between the two races in the relation of fatty streaks to raised lesions.

We do not know the mechanisms by which the apo B del allele is associated with increased atherosclerosis in blacks. Perhaps size differences in signal peptides alter production of apo B or apo B–containing lipoproteins and thus exert a positive effect on the extent of atherosclerosis. This idea is supported by our finding that effects of the ins/del alleles on levels of apo B–containing lipoprotein cholesterol are parallel to the effects on arterial lesions. The del allele, which is associated with increased lesions, is also associated with higher levels of the atherogenic apo B–containing lipoproteins (VLDL+LDL-C). However, apo B ins/del genotypes are not associated with atherosclerosis in whites. Perhaps the del allele is not directly responsible for the effects in blacks but is in linkage disequilibrium with a different polymorphism affecting apo B expression or function that is present in higher frequency in blacks. Alternatively, the effects of the del allele may result from higher-order interactions with other genes that affect susceptibility to atherogenesis.

Acknowledgment

We thank Patricia K. Powers for her excellent technical assistance.

Appendix: The PDAY Research Group

The investigators cooperating in the multicenter study, "The Pathobiological Determinants of Atherosclerosis in Youth" (PDAY), are listed below.

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TABLE 5. Estimated Percent Variance in Arterial Lesions Explained by Apolipoprotein B ins/del Genotype, Adjusted for Age

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<td>Thoracic aorta</td>
<td>Total</td>
<td>437</td>
<td>0.0</td>
<td>411</td>
<td>1.2</td>
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<td></td>
<td>Raised</td>
<td>437</td>
<td>0.0</td>
<td>411</td>
<td>0.0</td>
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<tr>
<td>Abdominal aorta</td>
<td>Total</td>
<td>437</td>
<td>0.1</td>
<td>403</td>
<td>1.7</td>
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<tr>
<td></td>
<td>Raised</td>
<td>437</td>
<td>0.0</td>
<td>403</td>
<td>0.0</td>
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<tr>
<td>Right coronary</td>
<td>Total</td>
<td>421</td>
<td>0.0</td>
<td>388</td>
<td>0.0</td>
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<tr>
<td></td>
<td>Raised</td>
<td>421</td>
<td>0.0</td>
<td>388</td>
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Apo, apolipoprotein.
TABLE 6. Percent Intimal Surface Area Involvement With Lesions by Apolipoprotein B ins/del Genotype and Race, Adjusted for Age and Apolipoprotein E Genotype

<table>
<thead>
<tr>
<th>Race/genotype</th>
<th>Aorta (No.)</th>
<th>Total</th>
<th>Standard Error</th>
<th>Raised (No.)</th>
<th>Total</th>
<th>Standard Error</th>
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<tr>
<td>White del del</td>
<td>56</td>
<td>16.59 (13.20–20.85)</td>
<td>0.17 (0.13–0.23)</td>
<td>56</td>
<td>18.10 (14.17–23.11)</td>
<td>1.63 (0.91–2.92)</td>
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<tr>
<td>White ins del</td>
<td>183</td>
<td>16.25 (13.63–19.37)</td>
<td>0.19 (0.15–0.24)</td>
<td>183</td>
<td>20.76 (17.20–25.05)</td>
<td>1.71 (1.09–2.68)</td>
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<tr>
<td>White ins ins</td>
<td>198</td>
<td>17.16 (14.34–20.53)</td>
<td>0.18 (0.14–0.23)</td>
<td>198</td>
<td>20.84 (17.20–25.26)</td>
<td>1.77 (1.12–2.81)</td>
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<tr>
<td>Age</td>
<td>p=0.0058</td>
<td>p=0.0001</td>
<td>p=0.0001</td>
<td>p=0.0001</td>
<td>p=0.0001</td>
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<tr>
<td>Apo E genotype</td>
<td>p=0.0001</td>
<td>p=0.7131</td>
<td>p=0.0001</td>
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<td>p=0.0001</td>
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<td>Apo B ins/del genotype</td>
<td>p=0.7542</td>
<td>p=0.7634</td>
<td>p=0.4435</td>
<td>p=0.9489</td>
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<tr>
<td>Black del del</td>
<td>15</td>
<td>27.41 (19.69–38.15)</td>
<td>0.19 (0.11–0.30)</td>
<td>15</td>
<td>36.70 (24.55–54.88)</td>
<td>1.93 (0.77–4.84)</td>
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<td>Black ins del</td>
<td>135</td>
<td>21.77 (18.65–25.41)</td>
<td>0.19 (0.15–0.24)</td>
<td>130</td>
<td>26.70 (22.07–32.31)</td>
<td>1.72 (1.11–2.66)</td>
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<tr>
<td>Black ins ins</td>
<td>261</td>
<td>19.03 (16.65–21.76)</td>
<td>0.22 (0.18–0.26)</td>
<td>258</td>
<td>22.07 (18.75–25.98)</td>
<td>1.88 (1.30–2.74)</td>
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<tr>
<td>Age</td>
<td>p=0.4010</td>
<td>p=0.0001</td>
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<td>Apo E genotype</td>
<td>p=0.0001</td>
<td>p=0.1264</td>
<td>p=0.0001</td>
<td>p=0.1902</td>
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<td>Apo B ins/del genotype</td>
<td>p=0.0222</td>
<td>p=0.4794</td>
<td>p=0.0067</td>
<td>p=0.8837</td>
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Apo, apolipoprotein.

95% Confidence intervals in parentheses.

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References
1. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group: Relationship of atherosclerosis in...
### TABLE 6. (Continued)

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<th>No.</th>
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<td>54</td>
<td>6.63 (3.48–12.62)</td>
<td>1.25 (0.71–2.21)</td>
</tr>
<tr>
<td>177</td>
<td>5.31 (3.20–8.83)</td>
<td>1.02 (0.65–1.60)</td>
</tr>
<tr>
<td>190</td>
<td>5.67 (3.40–9.45)</td>
<td>1.03 (0.65–1.62)</td>
</tr>
<tr>
<td></td>
<td>p=0.0001</td>
<td>p=0.0001</td>
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<tr>
<td></td>
<td>p=0.4870</td>
<td>p=0.7401</td>
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<td></td>
<td>p=0.7661</td>
<td>p=0.7307</td>
</tr>
<tr>
<td>15</td>
<td>14.55 (4.80–44.09)</td>
<td>0.68 (0.28–1.66)</td>
</tr>
<tr>
<td>124</td>
<td>11.20 (6.28–19.97)</td>
<td>0.77 (0.48–1.22)</td>
</tr>
<tr>
<td>249</td>
<td>10.48 (6.43–17.11)</td>
<td>0.89 (0.60–1.32)</td>
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<td></td>
<td>p=0.0001</td>
<td>p=0.0001</td>
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<tr>
<td></td>
<td>p=0.2512</td>
<td>p=0.3982</td>
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<tr>
<td></td>
<td>p=0.8251</td>
<td>p=0.6398</td>
</tr>
</tbody>
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