Prevalence of ANGPTL3 and APOB Gene Mutations in Subjects With Combined Hypolipidemia

Davide Noto, Angelo B. Cefalu', Vincenza Valenti, Francesca Fayer, Elisa Pinotti, Mariangela Ditta, Rossella Spina, Giovanni Vigna, Pin Yue, Sekar Kathiresan, Patrizia Tarugi, Maurizio R. Averna

Objective—Mutations of the ANGPTL3 gene have been associated with a novel form of primary hypobetalipoproteinemia, the combined hypolipidemia (cHLP), characterized by low total cholesterol and low HDL-cholesterol levels. The aim of this work is to define the role of ANGPTL3 gene as determinant of the combined hypolipidemia phenotype in 2 large cohorts of 913 among American and Italian subjects with primary hypobetalipoproteinemia (total cholesterol <5th percentile).

Methods and Results—The combined hypolipidemia cut-offs were chosen according to total cholesterol and HDL-cholesterol levels reported in the ANGPTL3 kindred described to date: total cholesterol levels, <2nd percentile and HDL-cholesterol, levels <2nd decile. Seventy-eight subjects with combined hypolipidemia were analyzed for ANGPTL3 and APOB genes. We identified nonsense and/or missense mutations in ANGPTL3 gene in 8 subjects; no mutations of the APOB gene were found. Mutated ANGPTL3 homozygous/compound heterozygous subjects showed a more severe biochemical phenotype compared to heterozygous or ANGPTL3 negative subjects, although ANGPTL3 heterozygotes did not differ from ANGPTL3 negative subjects.

Conclusion—these results demonstrated that in a cohort of subjects with severe primary hypobetalipoproteinemia the prevalence of ANGPTL3 gene mutations responsible for a combined hypolipidemia phenotype is about 10%, whereas mutations of APOB gene are absent. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: epidemiology lipoproteins genetics hypobetalipoproteinemia

Primary hypobetalipoproteinemia (pHBL) is a monogenic condition inherited as a dominant or recessive trait characterized by total cholesterol (TC) and/or LDL cholesterol (LDL-C) and/or apolipoprotein B (APOB) levels below the 5th percentile of the reference population. Heterozygous APOB gene mutations are responsible for the majority of the dominant pHBL,1,2 causing the familial hypobetalipoproteinemia (FHBL). The clinical phenotype of heterozygous FHBL is usually mild, being frequently characterized by fatty liver and levels of APOB and LDL-C reduced by 60%.1,2 The homozygous or compound heterozygous APOB mutations are in some cases responsible for a more severe biochemical and clinical phenotype, similar to the abetalipoproteinemia (ABL) due to homozygous mutations in the MTP gene, characterized1,4 by intestinal malabsorption, pigmented retinal degeneration, ataxic neuropathy, and almost undetectable levels of LDL-C and APOB.

Recently mutations in the PCSK9 gene have been identified as cause of FHBL in kindred of African American6 and Caucasian6 descent. These mutations are associated to a pHBL phenotype that is not complicated by fatty liver as shown in FHBL due to APOB gene mutations. 

Mutations in the ANGPTL3 gene have been recently identified in a kindred affected by “familial combined hypolipidemia.”7 The affected members of the kindred were found to be compound heterozygous for 2 ANGPTL3 nonsense mutations and their lipid profile was characterized by low levels of TC, triglycerides (TG), LDL-C, and high-density lipoprotein cholesterol (HDL-C). The ANGPTL3 gene emerges as a promising candidate for severe pHBL associated with low plasma HDL-C level.

The aim of this study was to evaluate the prevalence of ANGPTL3 and APOB mutations in a subset of pHBL subjects with a combined hypolipidemia (cHLP) phenotype (low TC,
LDL-C, and HDL-C belonging to 2 large cohorts of unrelated pHLB subjects.

Methods

Study Sample

pHLB subjects have been selected in 3 outpatients clinics: the Inherited Dyslipidemias Clinic at the University of Palermo, Italy; the Department of Biomedical Sciences at the University of Modena and Reggio Emilia, Italy; and the Lipid Clinic at the Washington University School of Medicine, St. Louis, MO. The 3 clinics collaborated over the last 2 decades collecting a large number of pHLB samples. In the present work we searched a CHLP phenotype in a sample of 390 Italian and 523 American asymptomatic subjects with TC <5th percentile of the relative population distributions. To define the criteria for the selection of CHLP subjects in our cohorts we took into account the plasma lipid levels of the only ANGPTL3 defective kindred described so far. 

Because one of the probands of the published kindred with familial combined hyperlipidemia7 belong to the American pHLB cohort of the present study, we could calculate the kindred plasma lipids percentiles relative to the American cohort distributions. In particular, we chose the TC and HDL-C percentile thresholds according to the highest TC and HDL-C values of the affected members of the kindred. The cut-offs for TC and HDL-C were below the 2nd percentile and the 2nd decile respectively, corresponding to: TC = 2.95 mmol/L (115 mg/dL) and HDL-C = 0.72 mmol/L (28 mg/dL) for the Italian cohort; TC = 3.34 mmol/L (130 mg/dL) and HDL-C = 0.87 mmol/L (34 mg/dL) for the American cohort. Because of the variability of the lipid profile in childhood and youth, subjects under 18 years of age were excluded in order to minimize the effect of nongenetic influences on HDL-C levels. At the end of the selection process, 51 Italian and 27 American subjects with the CHLP phenotype were studied. These subjects, selected by the low TC and HDL-C levels, also had low levels of LDL-C (1.41 mmol/L, range 0.27–1.65) and TG (1.02 mmol/L, range 0.19–4.27). Written informed consent was obtained from all subjects investigated. The study was approved by the institutional human investigation committees of each participating institution.

Plasma Lipid Determination and Genetic Analysis

TC and TG were analyzed by standard commercial enzymatic-colorimetric kits (Roche Diagnostic). LDL-C was measured by an automated analyzer. HDL-C was measured by a direct enzymatic colorimetric kit (Roche Diagnostic). LDL-C values were calculated by the Friedewald formula.

ANGPTL3 and PCSK9 genes were analyzed by direct DNA sequencing of genomic DNA; in subjects with TC below the 1st percentile values. Differences between groups were assessed by the Kruskal Wallis nonparametric test. Statistical analyses were performed using the SYSTAT 10 software (SYSTAT, CA).

Results

No APOB, PCSK9, and MTP gene mutations were found in the study sample.

The sequence of ANGPTL3 gene in 78 subjects (51 Italian and 27 American) with the CHLP phenotype led to the identification of 8 subjects carrying 9 different mutations with a raw prevalence of 10.25% (Table 1). Three novel frameshift mutations (E95del, N147fsX1, and E119fsX8) and 3 novel missense mutations (F295L, G56V, and R332Q) were identified in the Italian sample. In the American sample, a novel ANGPTL3 frameshift mutation (N121fsX9) was identified in addition to the 2 ANGPTL3 nonsense mutations (S17X and E129X) already described in the proband belonging to the published kindred.7

As shown in Table 1, 2 subjects were homozygotes for multiple bases deletions (c.283_285delGAA and c.439_442delAAACT), 2 were compound heterozygotes (subject 3 was the compound heterozygote previously described7 carrying the 2 nonsense mutations S17X and E129X), whereas subject 4 was carrier of a mutation introducing a premature stop codon plus a nonconservative missense mutation (N147fsX1 and F295L, respectively). Four subjects were simple heterozygotes, 2 for frameshift mutations (E119fsX8 and N121fsX9) and 2 for missense mutations (G56V and R332Q). The 3 novel missense mutations (F295L, G56V, and R332Q) were not reported as common polymorphisms in the “Exome Variant Server” repository of the NHLBI Exome Sequencing Project. More, they were not found in a control group of 200 normolipemic individuals.

Computational analyses to predict the effects of these amino acid changes on protein function by the PolyPhen and SIFT algorithms (see Methods) gave comparable results, indicating that the F295L (PSIC score: 2.403, SIFT score: 0) and G56V (PSIC score: 2.338, SIFT score: 0) amino acid substitutions had a “damaging effect”, whereas the R332Q variant was predicted to be benign (PSIC score: 1.478, SIFT score: 0.27). Mutationtaster software predicted all the 3 missense mutations to be possibly damaging (G56V, Score: 2.97; F295L, Score: 0.60; R332Q, Score: 1.17).

Figure shows that these amino acid substitutions fall in highly conserved regions of the ANGPTL3 protein, with the
exception of the F295L mutation in which the conserved F (triptophan) residue is located in a nonconserved region.

Table 2 shows the characteristics of the ANGPTL3 mutation (m) carriers in comparison with the ANGPTL3 negative (w) subjects. The table shows that the homozygous and compound heterozygous carriers had lower TC, TG, HDL-C, and LDL-C levels in comparison with the heterozygous ANGPTL3 mutations carriers and ANGPTL3 negative subjects. With respect to ANGPTL3 mutation negative pHBL subjects, ANGPTL3 mutations heterozygotes had lower TG but comparable levels of TC, LDL-C, and HDL-C.

The analysis of ANGPTL3 gene also revealed the presence in the Italian cohort of 3 subjects heterozygotes for the intronic polymorphism rs72649577 with a frequency of 3.8%.

Discussion

Loss of function mutations of ANGPTL3 gene have been recently associated with a phenotype defined as “familial combined hypolipidemia” in a kindred originally identified in view of low plasma TC and LDL-C.7 In this family the compound heterozygotes for ANGPTL3 mutations (S17X and E129X) had plasma LDL-C, TG, and HDL-C levels lower than heterozygous carriers of both mutations; moreover the comparison of the not affected family members with the heterozygotes and compound heterozygotes suggested that the complex phenotype is not inherited as a homogeneous trait. In fact, the low LDL-C and TG phenotype seems to be inherited as a codominant trait, whereas the low HDL-C phenotype seems to be inherited as a recessive trait.7

In this study we investigated the role of ANGPTL3 gene on the chLP phenotype. The cut-offs to define the chLP were the following: 2nd percentile for TC and 2nd decile for HDL-C (see Methods). By using these criteria the prevalence of ANGPTL3 mutation carriers was 10.25% (as raw prevalence rate). The strength of association between our definition of the chLP phenotype and the probability to find ANGPTL3 mutations is reinforced by the finding that a relative (brother) of subject 5 (table 1) carrying the same heterozygous E119fsX8 mutation also shares the chLP phenotype as defined by our criteria (data not shown). However, the high prevalence of ANGPTL3 mutations found in subjects with the chLP phenotype does not exclude that ANGPTL3 mutations are likely to be found in pHBL subjects with a different phenotype. The finding of low TG but not low HDL-C values in carriers of ANGPTL3 “loss of function” mutations shown by others8 supports this possibility. No APOB gene mutations were found in the subjects with HDL-C/2nd decile of the present study. We have previously searched for APOB mutations, both in the Italian and the American cohort,5–9 in subjects with TC/2nd percentile, and APOB mutations were found with a prevalence of 6.8% and 7.9%, respectively, in the subgroup of subjects with HDL-C was above the 2nd decile (unpublished data). This finding suggests that APOB mutations are likely to be found in pHBL subjects with HDL-C >2nd decile, whereas ANGPTL3 mutations are highly prevalent in subjects with chLP in whom the very low TC levels (<2nd percentile) are associated with low levels of HDL-C (<2nd decile).

Figure. Interspecies ANGPTL3 protein alignment near the three novel missense ANGPTL3 gene mutations. Arrows show the amino acid substitutions encoded by the identified mutations.

Table 1. ANGPTL3 Gene Mutations Identified in the Study Sample

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gene Mutation</th>
<th>Effect on Protein</th>
<th>TC (Percentile %)</th>
<th>HDL-C (Percentile %)</th>
<th>TG</th>
<th>LDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ho. c.283_285delGAA</td>
<td>E95del</td>
<td>1.43 (&lt;0.01)</td>
<td>0.25 (1.9)</td>
<td>0.32</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>ho. c.439_442delAACT</td>
<td>N147fsX1</td>
<td>1.38 (&lt;0.01)</td>
<td>0.43 (5.8)</td>
<td>0.85</td>
<td>0.56</td>
</tr>
<tr>
<td>3</td>
<td>het. c.50–51CC/GA</td>
<td>S17X</td>
<td>1.38 (0.013)</td>
<td>0.56 (6.0)</td>
<td>0.19</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>het. c.85G/T</td>
<td>E129X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>het. c.439_442delAACT</td>
<td>N147fsX1F295L</td>
<td>2.13 (&lt;0.01)</td>
<td>0.59 (7.6)</td>
<td>0.44</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>het. c.883T/C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>het. c.355_360delAACT</td>
<td>E119fsX8</td>
<td>1.49 (0.14)</td>
<td>0.48 (12.5)</td>
<td>0.30</td>
<td>0.84</td>
</tr>
<tr>
<td>6</td>
<td>het. c.362insCTCAT</td>
<td>N121fsX9</td>
<td>2.33 (0.6)</td>
<td>0.74 (15.2)</td>
<td>0.29</td>
<td>1.43</td>
</tr>
<tr>
<td>7</td>
<td>het. c.167G/T</td>
<td>G56V</td>
<td>2.72 (0.9)</td>
<td>0.61 (14.1)</td>
<td>1.01</td>
<td>1.64</td>
</tr>
<tr>
<td>8</td>
<td>het. c.995G/A</td>
<td>R332G</td>
<td>1.54 (0.019)</td>
<td>0.61 (14.1)</td>
<td>0.82</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Values are expressed in mmol/L, percentiles in brackets.

*This subject belongs to the kindred already described in Ref. 7.

TC indicates total cholesterol; HDL-C, HDL-cholesterol; TG, triglycerides; LDL-C, LDL-cholesterol; ho., homozygous state; het., heterozygous state.

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In the familial combined hypolipidemia kindred described by Musunuru et al., a gene dose effect on LDL-C and TG was shown, the ANGPTL3 heterozygotes having intermediate plasma levels between homozygotes and not affected members. Differently, our results suggest that gene dosage affects only the TG plasma levels (Table 2).

It is to be noticed that the ANGPTL3 negative subjects of the present study are represented by pHBL subjects. Then, a clear difference of lipid profile between ANGPTL3 negative and heterozygous subjects is not expected. In fact, the ANGPTL3 negative subjects of the published kindred7 had mean LDL-C plasma levels of 109 mg/dL (2.79 mmol/L), whereas common and rare (mostly loss of function) ANGPTL3 gene variants have been mainly associated to TG in heterozygous carriers. Studies in mice from ANGPTL4, which promotes the conversion of active lipases inhibition. Further studies are required to address this issue.

We have demonstrated in a setting of primary hypocholesterolemia that ANGPTL3 gene mutations exert a strong and significant gene dose effect on TG levels (Table 2). These results might suggest that the loss of inhibition of LPL and HL activities represents the unifying mechanism able to explain the LDL-C, TG, and HDL-C reduction seen in the familial combined hypolipidemia.7

Table 2. Clinical and Biochemical Data of the Study Sample According to the No. of ANGPTL3 Mutated Alleles

<table>
<thead>
<tr>
<th>ANGPTL3 Mutations Carriers Status</th>
<th>(w/w)</th>
<th>(w/m)</th>
<th>(m/m)</th>
<th>KW Test P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>70</td>
<td>4</td>
<td>4</td>
<td>.075</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51.7 (18–93)</td>
<td>45 (40–45)</td>
<td>36 (19–45)§</td>
<td>.48*</td>
</tr>
<tr>
<td>Gender (Male/Female)</td>
<td>57/13</td>
<td>3/1</td>
<td>3/1</td>
<td>.05</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>2.57 (1.13–3.36)</td>
<td>2.23 (1.54–2.72)</td>
<td>1.41 (1.38–1.49)§</td>
<td>.002</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>1.03 (0.32–4.27)</td>
<td>0.63 (0.39–1.00)‡§</td>
<td>0.31 (0.19–0.84)‡§</td>
<td>.007</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>0.64 (0.18–0.85)</td>
<td>0.61 (0.59–0.74)</td>
<td>0.46 (0.25–0.56)‡</td>
<td>.07</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>1.42 (0.27–2.50)</td>
<td>1.40 (0.65–1.65)</td>
<td>0.80 (0.56–1.03)§</td>
<td>.02</td>
</tr>
<tr>
<td>Median TC percentile (%)</td>
<td>0.6 (0.004–2.1)</td>
<td>0.4 (0.02–0.9)</td>
<td>0.01 (0.001–0.01)§</td>
<td>.002</td>
</tr>
<tr>
<td>Median HDL-C percentile (%)</td>
<td>11 (0.4–20)</td>
<td>14 (12–15)</td>
<td>6 (2–7)‡</td>
<td>.05</td>
</tr>
</tbody>
</table>

Data are shown as median values (minimum–maximum values in brackets). Missense and nonsense mutations are pooled together.

w indicates wild type; m, mutated; KW Test P, Kruskall Wallis test P value, except *chi-square test. (w/m) vs (m/m), Mann Whitney test (**P<0.05, †P<0.01). (w/m) or (m/m) vs (w/w), Mann Whitney test (**P<0.05, §P<0.005).

In familial combined hypolipidemia kindred described by Musunuru et al., a gene dose effect on LDL-C and TG was shown, the ANGPTL3 heterozygotes having intermediate plasma levels between homozygotes and not affected members. Differently, our results suggest that gene dosage affects only the TG plasma levels (Table 2).

In familial combined hypolipidemia kindred described so far, FL was not detected, so that ultrasonography emerged as a tool to discriminate ANGPTL3 gene variants have been mainly associated to TG in heterozygous carriers. Studies in mice from ANGPTL4, which promotes the conversion of active lipases inhibition. Further studies are required to address this issue.

We have demonstrated in a setting of primary hypocholesterolemia that ANGPTL3 gene mutations exert a strong and significant gene dose effect on TG levels (Table 2). These results might suggest that the loss of inhibition of LPL and HL activities represents the unifying mechanism able to explain the LDL-C, TG, and HDL-C reduction seen in the familial combined hypolipidemia.7

In FHBL due to APOB gene mutations severe fatty liver (FL) was detected with a prevalence rate around 20%.18 FL is caused by an impaired export of APOB-containing lipoprotein causing TG accumulation in the liver. In the only ANGPTL3 mutated kindred described so far, FL was not detected, so that ultrasonography emerged as a tool to discriminate ANGPTL3 from APOB genetic form of pHBL, but not from pHBL due to PCSK9 mutations, where severe FL is usually not detected. We were not able to screen FL in every pHBL subject of our sample, but we detected severe FL in at least 1 ANGPTL3 mutations carrier out of 3 investigated by ultrasonography. The few clinical data available in our study sample, though not conclusive, suggest that the presence of FL should not be considered a tool able to discriminate FHBL due to ANGPTL3 mutations from other genetic causes of pHBL.

In pHBL subjects with very low TC values (below the 2nd percentile), the detection of HDL-C below the 2nd decile remains the only parameter suggesting that ANGPTL3 analysis should be privileged, whereas higher HDL-C levels should lead to the search for APOB gene mutations first.

In conclusion our results demonstrated that in a cohort of subjects with severe pHBL the prevalence of ANGPTL3 gene mutations responsible of a combined hypolipidemia pheno-
type is about 10%, although mutations of APOB gene are absent. This finding indicated for the first time that the HDL-C plasma levels can help to set a phenotype-oriented candidate genes cascade screening of pHBL.

Acknowledgments

Maurizio R. Averna, Patrizia Tarugi, and Sekar Kathiresan conceived and designed the research. Vincenza Valentì, Rosella Spina, Mariangela Ditta, Elisa Pinotti, and Francesca Fayer acquired the data. Davide Noto, Angelo B. Cefalu, Pin Yue, and Giovannì Vigna analyzed and interpreted the data. Davide Noto performed statistical analysis. Maurizio R. Averna and Patrizia Tarugi handled funding and supervision. Davide Noto and Angelo B. Cefalu drafted the manuscript. Pin Yue, Sekar Kathiresan, and Giovannì Vigna made critical revision of the manuscript for important intellectual content.

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Acknowledgments

Maurizio R. Averna, Patrizia Tarugi, and Sekar Kathiresan conceived and designed the research. Vincenza Valentì, Rosella Spina, Mariangela Ditta, Elisa Pinotti, and Francesca Fayer acquired the data. Davide Noto, Angelo B. Cefalu, Pin Yue, and Giovannì Vigna analyzed and interpreted the data. Davide Noto performed statistical analysis. Maurizio R. Averna and Patrizia Tarugi handled funding and supervision. Davide Noto and Angelo B. Cefalu drafted the manuscript. Pin Yue, Sekar Kathiresan, and Giovannì Vigna made critical revision of the manuscript for important intellectual content.

Disclosures

None.

References


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### Supplemental table I: Primer sequences for ANGPTL3 gene sequence

<table>
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<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing Temp. (°C)</th>
<th>Size (bp)</th>
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<tr>
<td>Ex. 1-1</td>
<td>TGTTCACCTACCAACCTCCTT</td>
<td>CACTGTTTGCAAGCGATAGAT</td>
<td>60 °C</td>
<td>392 bp</td>
</tr>
<tr>
<td>Ex. 1-2</td>
<td>CGAAGGGCCAATAATAGAC</td>
<td>TTTAAAAGATAACCAGTGAAGAAACA</td>
<td>60 °C</td>
<td>360 bp</td>
</tr>
<tr>
<td>Ex. 2</td>
<td>GAAAAGAAAGAGGAAAGCAACTTA</td>
<td>GCTTCAGAGCTGCAATTTT</td>
<td>60 °C</td>
<td>303 bp</td>
</tr>
<tr>
<td>Ex. 3</td>
<td>CATCCTTACCTCAGATTCCCCCT</td>
<td>GCATCCAGTTAGGTTGATACATT</td>
<td>60 °C</td>
<td>318 bp</td>
</tr>
<tr>
<td>Ex. 4</td>
<td>TCAAGTGAATCTCAAGCTCCA</td>
<td>CAATGATAACCCAGTAAATCTTCC</td>
<td>60 °C</td>
<td>271 bp</td>
</tr>
<tr>
<td>Ex. 5</td>
<td>CTACCTTACAAACCACCAATTTAA</td>
<td>CTGGTCCATAAGAGGTTCTCAGTA</td>
<td>60 °C</td>
<td>275 bp</td>
</tr>
<tr>
<td>Ex. 6</td>
<td>ACTGTCAGTGCAAACCTGTACCT</td>
<td>AAACGCTTTATTTGGGATTCG</td>
<td>60 °C</td>
<td>440 bp</td>
</tr>
<tr>
<td>Ex. 7</td>
<td>GGAAGATAACCTACGGGGAAA</td>
<td>TAGACCACATTAACCTTGGAATGA</td>
<td>60 °C</td>
<td>332 bp</td>
</tr>
</tbody>
</table>
Supplemental Figure I a: TC distribution in the sample of Italian origin. (2nd percentile)

filled circled and dashed line: TC distribution frequency polygon.

solid line: fitted gaussian distribution.

fitted gaussian curve parameters: mean 197 mg/dL, standard deviation 40.9 mg/dL.

estimated percentile of the value used as true 2nd percentile (study cut-off value): 2.3 percentile
Supplemental Figure I b: TC distribution in the sample of American origin. (2nd percentile)

filled circled and dashed line: TC distribution frequency polygon.
solid line: fitted gaussian distribution.
fitted gaussian curve parameters: mean 252 mg/dL, standard deviation 64 mg/dL.
estimated percentile of the value used as true 2nd percentile (study cut-off) value: 2.9 percentile