Efficacy of a Simplified Primary Screening Procedure for Detection of Hyperlipoproteinemias in a Pediatric Population

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We examined the efficacy of a simple primary screening procedure for detecting β- and pre-β-lipoprotein abnormalities in 3183 children, ages 5–14, residing in Bogalusa, Louisiana. This procedure is based on the ability of β- and pre-β-lipoproteins to form insoluble complexes with heparin in the presence of Ca^{2+}; the turbidity produced by the reaction was considered as an index of the concentration of these two classes of lipoproteins. Our results indicate a close relationship (r = 0.88) between the β- + pre-β-lipoprotein index (turbidity) and the concentrations of these lipoproteins. Comparison of serum lipid and β- + pre-β-lipoprotein values of 5% of the children whose results fell outside the normal limits (upper and lower 5%) indicated that serum total cholesterol was not reflecting the β- + pre-β-lipoprotein concentration of a given child. The variability of α-lipoprotein concentration in these children accounted for this discrepancy. Measuring the serum β- + pre-β-lipoprotein index may be more useful for large-scale screening and for detecting subtle abnormalities than are determinations of either cholesterol or triglycerides.

Additional Keyphrases: serum cholesterol and triacylglycerols (triglycerides) • lipoproteins • β- + pre-β-lipoprotein index • screening • pediatric chemistry

Usually, total cholesterol and triglycerides (triglycerols) in serum are measured in large-scale screening of persons to detect lipid abnormalities. These measurements provide some indication of hyperlipidemias, but in many instances—for example, in neonates and children—lipid abnormalities may not be detected if only serum lipids are determined (1–4). Because susceptibility to coronary artery disease is related to increased concentrations of the lower-density lipoproteins in serum, and protection is related to those of higher density, methods that are simple, inexpensive, and practical are in demand for evaluating lipoprotein abnormalities in the general population. Several simple methods are currently in use to assay serum lipoproteins for clinical purposes (5). The most commonly used is the electrophoretic-based phenotyping system for differential diagnosis of hyperlipoproteinemia (6). But, for detecting subtle lipoprotein differences or abnormalities in a group of individuals, quantitative rather than qualitative methods are required. These methods include selective precipitation by polyions in the presence of divalent cations, quantitative agarose gel electrophoresis, and membrane ultrafiltration (7–10).

Recent studies from this laboratory indicate that the characteristics and concentrations of polyanions (glycosaminoglycans) and the divalent metal ions (e.g., Ca^{2+}, Mg^{2+}, or Mn^{2+}) are all critical for quantitative differentiation of β- and pre-β-lipoproteins from α-lipoproteins (11, 12). In the presence of appropriate concentrations of heparin and Ca^{2+}, both β- and pre-β-lipoproteins are involved in forming an insoluble complex, producing a turbidity that is linearly related to the concentrations of these two lipoprotein classes (11). From this finding, we developed a simple and reproducible method for quantitating serum lipoproteins individually (13, 14). Based upon our clinical application of this technique (15, 16), we have adapted the initial turbidity part of this method as an adjunct for “primary” screening purposes to detect hyperlipoproteinemias (17). This study examines the efficacy of this primary screening procedure in a large pediatric population (The Bogalusa Heart Study), whose serum lipid and lipoprotein concentrations have been reported (2, 18).

Materials and Methods

Serum

Three thousand five hundred and twenty-four children, representing 93% of children ages 5–14 residing in Bogalusa, Louisiana, participated in the screening program for coronary artery disease risk factors, the Bogalusa Heart Study. Each child was asked to fast for 12–14 h. Fasting compliance, determined by interview on the morning of the examination, indicated 7.5% (263) of the children were nonfasting, and they were not included in the present study. Venous blood was collected [blood samples could not be obtained in 2.2% (78) of the children] and was allowed to clot. After centrifugation, sera were collected in tubes containing thimerosal, an antibacterial agent (Aldrich Chemical Co., Inc., Milwaukee, WI 53233), placed in a shipping box containing frozen packs, and sent by bus to New Orleans, where they were refrigerated overnight at 4 °C.

Serum Total Cholesterol, Triglycerides, and Lipoprotein Cholesterol

Cholesterol and triglycerides were determined simultaneously in an AutoAnalyzer II (Technicon Instrument Corp., Tarrytown, NY 10591) in the Core Laboratory of SCOR-A according to the protocol developed by Lipid Research Clinics in collaboration with the Center for Disease Control (CDC), Atlanta, GA (19). An isopropanol extract of 0.2 mL of whole serum or of the β- + pre-β-lipoprotein fraction (see below) was used for the determination. We used a calibrator provided by the CDC to convert the cholesterol value obtained with the AutoAnalyzer II to values consistent with the method of Abell–Kendall (20). (This laboratory participates in the Cooperative Lipid Standardization Program of the CDC.) Serum β- + pre-β-lipoprotein cholesterol values were determined after selective precipitation of serum β- and pre-

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β-lipoproteins with heparin and Ca\textsuperscript{2+} (11, 13). Briefly, this method consists of mixing serum (0.2 mL), distilled water (3.2 mL), beef lung heparin (0.1 mL of 2.5 g/L solution, \( \approx 140 \) USP units/mg; Upjohn Co., Kalamazoo, MI), and CaCl\(_2\) (0.5 mol/L, 0.5 mL), in the order given. After the mixture was allowed to stand for 15 min, the precipitate was obtained after centrifugation (1500 \( \times \) g, 30 min) and analyzed for the corresponding β- and pre-β-lipoprotein cholesterol content by dissolving it in 0.2 mL of 0.15 mol/L NaCl.

The value for α-lipoprotein cholesterol was obtained by subtracting β- + pre-β-lipoprotein cholesterol from the serum total cholesterol (2, 14). A detailed description of this method's application on 3183 school-age children is reported elsewhere (2).

**Serum β- + Pre-β-lipoprotein Index**

This primary screening procedure consists of mixing serum, distilled water, heparin, and Ca\textsuperscript{2+} in the proportion described above and measuring the turbidity after 15 min at 600 nm vs. a blank containing a similar mixture but with heparin omitted. Because the turbidity obtained by adding Ca\textsuperscript{2+} and heparin was quantitatively related to the serum β- and pre-β-lipoprotein concentrations (determined by analytical ultracentrifuge), it was considered an index of serum β- and pre-β-lipoprotein concentrations (17).

**Electrophoretic Ratio of β- and Pre-β-lipoprotein**

Serum (10–20 μL) was electrophoresed on agar–agarose gel plates (8.3 × 10 cm), with use of a barbital buffer (pH 8.6, 0.05 mol/L) and 22 mA per plate (13, 21). The lipoprotein bands, stained with Oil Red O, were scanned in a densitometer to assess the relative proportion of β- and pre-β-lipoprotein.

**Estimation of β- and Pre-β-lipoprotein Cholesterol**

The estimation of serum β- and pre-β-lipoprotein concentrations was based on the densitometric ratio of β- to pre-β-lipoprotein, β- + pre-β-lipoprotein cholesterol concentration, and the reported mean values for the cholesterol content of β-lipoprotein (46.9%) and pre-β-lipoprotein (22.2%) molecules (13–15). Any changes in lipoprotein estimations owing to variations in cholesterol content of these molecules in normal individuals, including children, were considered to be negligibly small (16). The β- and pre-β-lipoprotein cholesterol concentrations were estimated indirectly as follows: β-lipoprotein cholesterol = mg β-lipoprotein \( \times \) 0.469; pre-β-lipoprotein cholesterol = mg pre-β-lipoprotein \( \times \) 0.222. The factors 0.469 and 0.222 represent mean cholesterol content per milligram of β- and pre-β-lipoprotein, respectively. We separately evaluated split specimens of serum for lipoprotein cholesterol in 38 children, ages 5 to 16, by the present method and by the ultracentrifugation/dextran sulfate precipitation method and we found excellent agreement between the two methods (22).

**Results**

To ascertain that the β- + pre-β-lipoprotein index (the turbidity value) is a measure of the concentration of these two classes of lipoproteins, approximately 10% of the serum samples were randomly assigned on every screening day for β- + pre-β-lipoprotein cholesterol determination. Figure 1 shows the close relationship (\( r = 0.83 \)) between serum β- + pre-β-lipoprotein index and the concentration of cholesterol contained in these two classes of lipoproteins. It should be mentioned that sera from different individuals with different relative proportions of β- and pre-β-lipoproteins could give a similar β- + pre-β-lipoprotein index. These sera with a similar index could have different β- + pre-β-lipoprotein cholesterol concentrations because of variations in their fractional cholesterol contents. Therefore, when the β- + pre-β-lipoprotein index was plotted vs. β- + pre-β-lipoprotein concentrations (Figure 2), an even stronger relationship (\( r = 0.88 \)) was seen. The serum β- + pre-β-lipoprotein index correlated less with serum total cholesterol (\( r = 0.65 \)) and triglycerides (\( r = 0.57 \)).

To evaluate the usefulness of this method in detecting increases in serum β- and (or) pre-β-lipoprotein concentrations in comparison to conventional serum cholesterol and triglyceride measurements, we arbitrarily selected 5% of the children who fell outside the statistical normal limits (i.e., the upper and lower 5%) with respect to each of the above variables (Table 1) and studied the degree of overlap in their

| Table 1. Upper and Lower 5% Values\(^a\) of Serum Total Cholesterol (n = 159), Triglycerides (n = 159), and β- + Pre-β-Lipoprotein Index (n = 160) in 3188 Children, Ages 5–14 |
|---------------------------------|----------------|----------------|
|                                | mg/L           | mg/L           |
| Total cholesterol              | 2150           | 1260           |
| Triglycerides                  |                | 340            |
| β- + pre-β-lipoprotein index\(^b\) | 0.29           | 0.12           |

\(^a\) Obtained from the frequency distribution.

\(^b\) Turbidity (A at 600 nm) produced by heparin–Ca\textsuperscript{2+}.
distribution. Figure 3 shows the distribution of serum total cholesterol of all the children superimposed on the distributions of serum total cholesterol of children in the upper and lower 5% of the serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index. More than half the children with the upper and lower 5% of serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index values fell, respectively, below or above the 5% level of serum total cholesterol distribution. Likewise, when the distribution of serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index of all the children was superimposed on the distributions of serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index of children in the upper and lower 5% of the serum total cholesterol values (Figure 4), more than half of the children did not stay in their respective 5% limits of serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index. These results suggest that serum total cholesterol is not reflecting the \( \beta^- \) + pre-\( \beta^- \)-lipoprotein concentration of a given child.

Figure 5 shows a Venn diagram depicting the overlap of serum total cholesterol, triglycerides, and \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index in children belonging to the upper or lower 5% levels of the respective variables. In the upper 5% limit, while 28.8% of the children had both serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index and total cholesterol values above the 5% upper limit, only 1.3% of them had both serum total cholesterol and triglycerides above this limit. In 10% of the children, all of these three variables were above the upper 5% limit. It can also be seen that for a considerable number of children, only one of the serum lipid variables exceeded the upper limit (total cholesterol: 59.4%; triglycerides: 58.8%; \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index: 31.9%). Similar observations can be made regarding children belonging to the low lipid category (lower 5% of the distribution). In this category, although the degrees of overlap between \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index and total cholesterol as well as between total cholesterol and triglycerides were similar to those in the high lipid category, the overlap between \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index and triglycerides was considerably less. This resulted in large percentages of children having either a low \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index (50.1%) or low triglycerides (75.6%) with no overlap with other variables.

Table 2 lists serum total, \( \alpha^- \), \( \beta^- \), and pre-\( \beta^- \)-lipoprotein cholesterol values (mean ± SD) for those children who had only one serum lipid variable in either the high or low lipid category (i.e., having no overlap with any other lipid variable in the corresponding category). We determined these in order to explain the reason for lack of overlap in these groups. Although 51 children had only the \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index in the high lipid category, they had serum total cholesterol values less than the upper 5% limit (2150 mg/L) and high \( \beta^- \) and pre-\( \beta^- \)-lipoprotein cholesterol values. Low values for \( \alpha^- \)-lipoprotein cholesterol accounted for the low serum total cholesterol values in these children. Similarly, 81 children with a low \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index had serum total cholesterol values exceeding the lower 5% limit (1220 mg/L). In this case, high \( \alpha^- \)-lipoprotein cholesterol accounted for the discrepancy. Similar reasoning holds for children having high or low serum total cholesterol values with no corresponding overlap of the \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index. Because serum total cholesterol represents all three classes of lipoproteins, it is less discriminatory than the \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index. The 94 children for whom only triglycerides were high had rather high pre-\( \beta^- \)-lipoprotein cholesterol but low values for total, \( \beta^- \), and \( \alpha^- \)-lipoprotein cholesterol. It should be mentioned that the data represent "reported" fasting only, and such may not reflect 100% true fasting. To our surprise, 34% of the children in this group were chylomicron-positive (by agar–agarose gel electrophoresis criteria). Therefore, measuring serum triglycerides alone may give false-positive results with respect to triglyceride abnormalities. In the low serum triglycerides category, these children (n = 121), rather, had higher total \( \beta^- \) and \( \alpha^- \)-lipoprotein cholesterol, thus accounting for the lack of overlap with either serum \( \beta^- \) + pre-\( \beta^- \)-lipoprotein index or total cholesterol.

**Discussion**

There currently is no general agreement as to the most useful and effective means to screen serum lipids to assess risk of coronary artery disease, but it is recognized that measurement of serum total cholesterol or triglycerides alone is inadequate for such an evaluation. Much earlier, Gofman and co-workers (23) emphasized differences in serum lipoproteins
that could be characterized by ultracentrifugation and the use of an atherogenic index as a measure of risk. While above-normal values for β- and pre-β-lipoproteins are positively related to coronary artery disease, an increased concentration of α-lipoprotein has recently been considered as a negative risk factor (3, 4). Therefore, any simple method for differentiating serum α-lipoprotein from other lipoproteins should be very useful for primary screening purposes. Our results show that measuring serum β- pre-β-lipoproteins in terms of a β- pre-β-lipoprotein index may be more useful and discriminatory in screening for subtle abnormalities than is the mere determination of cholesterol or triglycerides alone. The total cholesterol fractions in serum at birth as well as during childhood are distributed predominantly in the β- and α-lipoproteins (2, 24, 25), while pre-β-lipoproteins remain low during childhood. In neonates or children, it is not uncommon to find serum total cholesterol within acceptable “normal” limits (by any criteria) but increased β-lipoproteins, or high serum total cholesterol but normal β-lipoprotein concentration. The latter is due to the presence of high α-lipoprotein in children (1, 2, 24–26).

The β- pre-β-lipoprotein measurement as described is easier to perform than serum total cholesterol or triglyceride determinations and is more reproducible (2, 15). It could aid as a rapid, inexpensive initial screening procedure in large-scale population studies. Also, this procedure can be easily automated for mass screening (27, 28). The analysis will reduce the need to run a battery of lipid studies on all subjects. Only individuals who are “abnormal” at selected cutoff values by any criteria need be studied in depth for definitive characterization and therapeutic purposes.

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References

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