CIRCULATION/AHA/2004/449421

A Prominent Large High-Density Lipoprotein At Birth Enriched In Apolipoprotein C-I Identifies A New Group of Infants of Lower Birth Weight and Younger Gestational Age

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Short Title. ApoC-I- Enriched HDL at Birth

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Word Count 4491 Abstract 242. Condensed Abstract 80. Figures 7.

Abstracts and Key Words.

Background. Low birth weight is associated with increased cardiovascular disease death in adulthood. We therefore studied lipoprotein heterogeneity at birth in 163 white and black infants.

Methods and Results. A prominent lipoprotein peak between density 1.062 to 1.072 g/ml was identified using sucrose density gradient ultracentrifugation (DGU) and corresponded to the largest subclass of HDL by nuclear magnetic resonance spectroscopy and gradient gel electrophoresis. This HDL subclass was enriched in apolipoprotein C-I (apoC-I), as judged by capillary electrophoresis, isoelectric focusing, mass spectrometry, and immunochemical measurement. Based on the amount of this apoC-I-enriched HDL peak, 156 infants were assigned to one of four groups: 0 (none detected), 17%; 1 (possibly present), N = 41%; 2 (probably present), N = 22%; 3 (prominent), 19%. Group 3 infants had significantly lower birth weights and younger gestational ages than Group 0,1, or 2 infants, a difference not explained by small-for-gestational age infants. After correction for age, Group 3 infants had significantly higher levels of total and large HDL cholesterol, and of total and large low-density lipoprotein (LDL) cholesterol and total LDL particle number, but lower levels of small HDL, very low-density lipoproteins and triglycerides, than class 0,1 and 2 infants.

Conclusions. A prominent, large apoC-I-enriched HDL identifies a new group of low birth weight infants. The physiological significance of apoC-I-enriched HDL, and apoC-I alone, in vitro and in vivo, is under investigation.

Key Words: lipoprotein subclasses, apolipoproteins, triglycerides, gestational age, birth weight

Condensed Abstract.

Because low birth weight is associated with adverse cardiovascular risk and death in adults, lipoprotein heterogeneity at birth was studied. A prominent, large high-density lipoprotein (HDL) subclass enriched in apolipoprotein C-I (apoC-I) was found in 19% of infants, who had significantly lower birth weights and younger gestational ages and distinctly different lipoprotein profiles than infants with undetectable, possible or probable amounts of apoC-I-enriched HDL. An elevated amount of an apoC-I-enriched HDL identifies a new group of low birth weight infants.

Low birth weight is associated with cardiovascular risk and death in adults (1). Differences in size, molecular weight, chemical composition, and quantity of lipoprotein subclasses are associated with coronary artery disease (2). We examined lipoprotein heterogeneity in cord blood. We found that small-for-gestational age (SGA) infants had higher triglyceride-rich lipoproteins than appropriate-for-gestational age (AGA) infants (3), extending the observations of others in SGA infants (4-7).

We report here the novel observation that the large HDL subclass found in cord blood (8-15) is enriched in apolipoprotein C-I (apoC-I). Infants with prominent apoC-I-enriched HDL (group 3) were further unique in that they had impressively lower birth weights and younger gestational ages and significantly different plasma levels of lipids, lipoproteins, apolipoproteins, and lipoprotein subclasses and lipoprotein size than infants with undetectable (group 0), possible (group 1) or probable (group 2) apoC-I-enriched HDL.

ApoC-I is a 6.6 kDa apolipoprotein associated with both apolipoprotein B- and apolipoprotein A-I-containing lipoproteins (16). ApoC-I decreases the receptor-mediated removal of the apolipoprotein B-containing lipoproteins, inhibits cholesterol ester transfer protein (CETP), and stimulates lecithin cholesterol acyl transferase (LCAT) (17). The function of apoC-I on a cellular level, and its possible atherogenicity, is not established. We found recently that both apoC-I-enriched HDL, and purified apoC-I, promotes apoptosis of cultured human arterial smooth muscle cells, through the induction of neutral sphingomyelinase (N-SMase), and the subsequent steps involved in apoptosis (16).

Methods.

Patient Population. The group of 163 infants (31 white males, 39 white females; 47 black males and 46 black females) studied was previously characterized (3). There were 23 small-for-gestational age (SGA) infants, defined as a birth weight for gestational age < or = 10% (3). The Joint Committee on Clinical Investigation at Johns Hopkins approved the study.

Lipid, Lipoprotein and Apolipoprotein Measurement. Plasma from cord blood was analyzed for levels of cholesterol, triglycerides, LDL and HDL cholesterol, Lp (a) lipoprotein, and apolipoproteins A-I, A-II, B, C-I, C-III, and apoE as described (3,16). Fifteen lipoprotein subclasses, the total number of LDL particles, and the average sizes (nm) of VLDL, LDL and HDL were determined by nuclear magnetic resonance (NMR) spectroscopy (3,18). Lipoprotein density profiles for VLDL, LDL and HDL were obtained after sucrose density gradient ultracentrifugation (DGU) as described (19,20).

Preparation of Lipoprotein Fractions from Sucrose DGU for Capillary Electrophoresis. Fractions from the lipoprotein density profile were thawed and a portion subjected to delipidation (19,20). The samples were then analyzed in duplicate by capillary electrophoresis using the Beckman P/ACE 5510 instrument at 17kV for 30 minutes.

Preparation of Lipoprotein Fractions for MALDI- TOF mass spectrometry (MS) and immobilized pH gradient (IPG) isoelectric focusing (IEF). The lipoprotein fractions were thawed, centrifuged to pellet particulate matter, and subjected to solid phase extraction delipidation (19,20). The apolipoproteins were eluted, concentrated, and an aliquot taken for MALDI-TOF MS analysis, using a Voyager Elite XL DE mass spectrometer. The remaining samples were evaporated to dryness, reconstituted in 250

μL 8.0 M urea containing 2% CHAPS, sonicated, degassed, and electrophoresis performed using the IPG phor unit (Amersham Pharmacia, Sweden) as described (19,20).

Activity of Cholesterol Ester Transfer Protein. CETP activity was determined by using the CETP Activity Kit by Roar Biomedical, Inc., according to the manufacturer's specifications.

Statistical Methods. The relationships between lipids, lipoprotein cholesterols, apolipoproteins, lipoprotein subclasses and lipoprotein sizes in the four groups of infants were evaluated, first using ANOVA on data that were not adjusted for age, and then linear regression to correct for influence of gestational age. P values were also estimated using the Kruskal-Wallis test, due to the small size of two of the groups (n = 5 each in group 0 and 3). To evaluate differences in these lipid- related variables between white and black and male and female infants, a CHI squared test was performed. All p values found to be significant at the $p < 0.05$ levels.

Results.

Lipoprotein density profiling after sucrose DGU. . A prominent feature of the lipoprotein density profiles was the presence or absence of a distinct peak of d 1.062 and 1.072 g/ml between the major peaks for LDL and HDL (Figure 1a, 1b). The peak density of Lp (a) in adult plasma is close to 1.055 gm/ml, and thus potentially occurring within the density range between 1.062 and 1.072 gm/ml. Lp (a) levels in cord blood, however, are very low (3), and the mean Lp (a) levels (mg/dL +/- 1 SD) in group 3 (1.2 +/-1.3)) and group 0 (0.6 +/- 0.9) were not significantly different.

Characterization of lipoprotein peak of d 1.062-1.072 g/ml. Two infants from group 3 and group 0, respectively, were selected for detailed analyses of this lipoprotein peak. The Lp (a) levels in these two infants were low (3 mg/dL in 013 and undetectable

in 021). Three lipoproteins, namely, LDL, the lipoprotein with a peak of d 1.062-1.072 g/ml, and HDL, were isolated by sucrose DGU, delipidated and prepared for the following analyses (see Methods).

Capillary electrophoresis and isoelectric focusing (IEF). After capillary electrophoresis, apoA-I (47.7 %) was the major apolipoprotein in the lipoprotein of d 1.062-1.072 g/ml from the group 3 infant, and apoC-I, ordinarily a minor component of HDL, was the second most prevalent apolipoprotein (37.6 %). Negligible amounts of apolipoproteins were detected in the same lipoprotein density segment from the group 0 infant. These results were confirmed by IEF, showing clearly apoA-I (pI 5.43) and apoC-I (pI 6.70) bands in the prominent lipoprotein peak from the group 3 infant, but not the group 0 infant.

MALDI-TOF MS analyses. In the prominent lipoprotein peak of d 1.062 – 1.072 g/ml (Figure 2a) from the group 3 infant, the intensity of apoC-I, relative to the intensity of apoA-I, was notably greater than in HDL (Figure 2c). The apolipoproteins in the lipoprotein peak of d 1.062 –1.072 from the group 0 infant were barely detectable (Figure 2b). There was little difference in the spectra for HDL of usual density between the group 3 (Figure 2c) and the group 0 infants (Figure 2d). The above observations were confirmed in another group 3 infant compared to a normal control. There was no difference detected in LDL spectra (data not shown).

Gradient Gel Electrophoresis of HDL. Plasma from four infants in group 3, one infant in group 2, and three infants in group 0 were ultracentrifuged at d < 1.21 g/ml and GGE performed as described (13,15). As shown in representative densitometric scans of the gels (Figure 3), group 3 infants differed from group 0 infants. The largest HDL

subclass in group 3 infants had a mean diameter of 11.6 nm (range 11.5 to 11.8), compared to 9.4 (range 8.8 to 10.8) in group 0 infants, and 10.8 in one group 2 infant.

ApoC-I was found in each of different HDL subclasses (Figure 3), as judged by immunoblots of the GGE gels using an anti-apoC-I antibody. These results are consistent with those from MALDI-TOS MS (see above), and indicate that all HDL subclasses contained apoC-I.

Immunochemical Characterization of the Apolipoproteins in Infants with Prominent Versus Undetectable Amounts of the Lipoprotein of d 1.062 – 1.072 g/ml.

The distribution of apolipoproteins A-I, A-II, B, C-I, and C-III, between apoB-containing lipoproteins (VLDL, IDL, LDL and Lp (a)), and non-apoB-containing lipoproteins (HDL) was determined in infants from group 3 and group 0 without prior ultracentrifugation. The apoB-containing lipoproteins in one ml plasma from five group 3 infants and five group 0 infants were precipitated with heparin-manganese chloride (16). The apolipoprotein levels were then measured in plasma, heparin-manganese supernatants (non-apoB containing lipoproteins) and resolubilized precipitates (apoB-containing lipoproteins), using rocket immunoelectrophoresis as described (16).

Apolipoprotein B. The plasma levels of total apoB were higher in group 3 than in group 0 infants, but did not reach statistical significance (Figure 4). All of the apoB was in the heparin manganese precipitates and none was detected in the supernatants.

Apolipoprotein C-I. The mean levels of apoC-I in both whole plasma and the heparin-manganese supernatants were about twofold higher in group 3 than in group 0 infants (Figure 1). In a larger subset of infants, the mean (SD) plasma level of apoC-I (umol/L) of 11.6 (4.8) in 17 group 3 infants was significantly higher ($p = 0.036$) than that of 7.7 (4.7) in 13 infants from group 0. All of the apoC-I was in the supernatants and

absolutely none was detected in the precipitates, distinctly different than later in life when a significant portion of apoC-I is associated with the apoB-containing lipoproteins (21). These immunochemical results further indicate that the apoC-I-enriched lipoprotein peak is an HDL subclass rather than a LDL subclass.

Apolipoprotein C-III. The distribution of apoC-III was quite different between the groups. Infants in group 3 had significantly more of their apoC-III associated with the non-apoB-containing lipoproteins, while infants in group 0 had significantly more of their apoC-III associated with the apoB-containing lipoproteins (Figure 4).

Apolipoprotein A-I and A-II. The apoA-I levels were significantly higher in both the supernatants and precipitates in the infants in group 3 than in group 0 (Figure 4). The mean apoA-II levels between groups 3 and 0 were very similar for whole plasma, supernatants and precipitates (Figure 4).

Apolipoprotein E. In a separate experiment, the concentration (umol/L) of apoE in group 3 infants was higher than in group 0 infants in pooled whole plasma (3.8 v 1.7) and heparin-manganese supernates (2.3 v 1.4).

Lipid, Lipoprotein, Apolipoprotein and Lipoprotein Subclasses and Lipoprotein Sizes in Group 0, 1, 2, and 3 Infants.

Sucrose DGU and lipoprotein density profiling were performed in 156 of the 163 infants (95.7%) previously reported (3), to determine the frequency of appearance and degree of enrichment of the d 1.062 – 1.072 g/ml peak. The frequency (%) of the four groups was: 0, 17.3 %; 1, 41.0 %; 2, 22.4 %; and 3, 19.2 % (Figure 5).

The levels of the lipid-related variables were determined in the four groups of infants (Figure 5). Because of the known influence of gestational age on the apoB- and apoA-I lipoproteins in this population (3), the p values were determined using data non-

adjusted and adjusted for gestational age. Before age adjustment, all the variables except apoB and small VLDL were significantly different (data not shown). After age adjustment, the only LDL variables that remained significantly higher in group 3 were L3, L1 and LDL size (Figure 5). In contrast, all the HDL and VLDL related variables remained significantly different after correction for gestational age (Figure 5). Large HDL levels were higher while small HDL and the VLDL related variables were lower in group 3.

We also examined age-corrected means (data not shown), which were very similar to the measured mean levels shown in Figure 5 for the HDL- and VLDL- related variables. Despite the fact that the gestational ages were very similar in groups 0, 1 and 2, there were also impressive dose-response relationships for the levels of these lipid-related variables as one progressed from class 0 through class 1, 2 and 3 infants (Figure 5). These analyses further support the conclusion that the differences shown in Figure 5 were independent of age.

Distribution of Gestational Age in Infants in Groups 0, 1, 2 and 3. The gestational ages (mean (1SD), in weeks) in groups, 0, 1, 2 and 3 were: 39.7 (1.75); 39.3 (1.28); 38.8 (1.68); and 36.2 (4.16), respectively, and differed significantly ($p < 0.0001$). The mean gestational age in group 3 infants was not only younger, but had a distribution that was clearly broader than those in groups 0, 1, and 2 (Figure 6).

The birth weights (grams) in group 0,1, 2 and 3 infants were (mean (1SD)): 3268.6 (631.9); 3412.2 (548.3); 3240.6 (609.2); and 2683.7 (783.3), respectively, and differed significantly ($p < 0.0001$), being particularly low in group 3. After correcting for gestational age, the birth weights were no longer significant ($p = 0.15$). There were no

significant differences in the numbers of male and female (p = 0.38), white and black (p = 0.88), or SGA and AGA (p = 0.34) infants between the four groups.

To determine further if the elevated apoC-I-enriched HDL might be a normal concomitant of gestational age, we plotted the levels of large LDL (L3) and largest HDL (H5) against gestational age for the group 3 and group 0 infants (Figure 7). Group 3 infants had higher values of L3 than group 0 infants, but these L3 levels decreased dramatically with increasing gestational age. In distinct contrast, the higher H5 levels in group 3 did not fall with gestational age, indicating strongly that the elevated amount of apoC-I-enriched HDL in group 3 persisted and was not simply a consequence of younger gestational age (Figure 7).

ApoC-I and CETP Activity. ApoC-I levels in adults are associated with decreased CETP activity and larger HDL particles (17, 22). We examined if higher apoC-I levels in cord blood inhibited CETP activity, accounting for the different amounts of the large HDL particles. In a subset of 40 infants (group 0 N = 17; group 3 N = 13; and groups 1 and 2, N = 10), there was no relationship between CETP activity (uM/ml) (range 0.0620 to 1.398) and apoC-I levels (umol/L) (range 2.3 to 20.7), r = 0.087, NS. In a larger group (N = 123) of infants, there was also no significant difference (p = 0.149) in CETP activity between the four groups.

Discussion.

A prominent lipoprotein peak in density range 1.062-1.072 g/ml, identified in about one in five infants in this population (3), was a large HDL particle enriched in apoC-I. At birth all the plasma apoC-I was found in HDL, and group 3 infants had higher apoC-I levels than group 0 infants. The distributions of the quantity of apoC-III and apoA-I in the apoB- and apoA-I-containing particles were also significantly different in

the group 3 and group 0 infants, suggesting other differences between their HDL particles. We also found, consistent with our prior work (16) and that of others (9,10), that apoE is a major component of HDL from group 3 infants.

ApoC-I is influences the activities of CETP, LCAT, and hepatic lipase HL (17). Adults deficient in CETP (23) have large HDL particles, but the large HDL in cord blood does not appear to be due to a deficiency of CETP (5). ApoC-I in HDL inhibits CETP (22). We found however that the elevated amount of apoC-I-enriched HDL was not due to inhibition of CETP by apoC-I. The association of D442G mutation of the CETP gene with increased HDL cholesterol in adults was not seen in cord blood (24). Although LCAT activity is low in cord blood (25,26), cholesterol esterification is normal and does not appear to account for the presence of the large HDL species in cord blood (5).

Conde-Knape et al (27) described an apoC-I-enriched HDL in a moderately expressing APOC-I transgenic on an APOE null background. ApoC-I-enriched HDL (but not VLDL) had a marked inhibitory effect on HL but not LPL (27). In regard to the APOC-I gene, we found no relationship between a common HpaI RFLP (28) in the promotor of this gene and groups 0, 1, 2 and 3 infants (data not shown). A –514 C to T RFLP in the promotor of the HL gene (LIPC) explains about 30% of HL activity (29), but we found that no relationship between this DNA variant and apoC-I- enriched HDL (data not shown).

Nichols et al (13), using GGE, in 13 umbilical cord bloods, found that 15.4 % of the infants had a prominent HDL 2b peak. In our larger population the prevalence of group 3 infants was 19.6 %. Prominent apoC-I-HDL was expressed in males and females, and white and black infants.

We examined in detail whether the prominent apoC-I-HDL might be a normal concomitant of the earlier gestational age observed in group 3 infants. Sixty percent of the group 3 infants were in the 36 + week age group, and their large HDL levels were as elevated as those in the younger age groups, both indicating that the expression of prominent apoC-I-enriched HDL was not simply a normal consequence of younger gestational age.

An unexpected finding was that group 3 infants had a birth weight that was 584.8 grams (1.29 lbs) lower than group 0 infants. Group 3 infants appear distinctly different than SGA infants. The lipoprotein profile of group 3 infants was characterized by higher total and large HDL cholesterol but lower total and VLDL triglycerides, in distinct contrast to that of our relatively hypertriglyceridemic SGA infants (3). Furthermore, of the 30 infants in group 3, only six were SGA infants, and their mean gestational age of 37.5 weeks was actually higher than that of 35.9 weeks in the 24 AGA infants. The low mean birth weight (grams) in group 3 infants was present in both the SGA (2402.1) and the AGA (2754.0) infants. Thus, prominent apoC-I-enriched HDL identifies a new subgroup of low birth weight infants, distinct from SGA.

The relationship between low birth weight and adult arteriosclerotic cardiovascular disease is attributed to intrauterine effects on fetal tissue development (1), but might also be explained by the actions of genes that influence birth weight and cardiovascular risk in later years. There is substantial evidence that genes influence birth weight (30). We postulate that the low birth weight and prominent apoC-I-enriched HDL in group 3 infants may be an important marker to predict atherosclerosis. The apoC-I-enriched HDL from group 3 infants, and purified apoC-I, promote apoptosis in cultured human aortic smooth muscle cells (16), an effect that might produce rupture of an unstable plaque.

ACKNOWLEDGMENTS. This work was supported by The Thomas Wilson Foundation, and by grants from the National Institutes of Health (M01-RR-00052; HL 18574; HL 54566).

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Legends for Figures.

Figure 1. Lipoprotein profiles from cord blood were obtained after sucrose density gradient ultracentrifugation (see Methods). The profile on the left is from a group 3 infant while that on the right is from a group 0 infant.

Figure 2. MALDI-TOF MS of apoC-I-enriched HDL and normal HDL. ApoC-I-enriched HDL (top panels) and normal HDL (bottom panels) were isolated from plasma of a group 3 infant (left panels) and group 0 infant (right panels) and prepared for MALDI-TOF MS (see Methods).

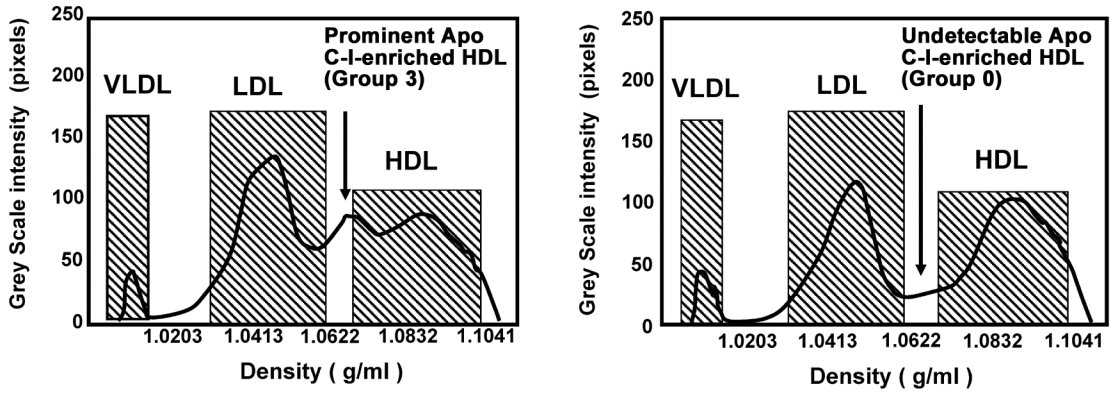
Figure 3. Gradient gel electrophoresis of HDL. Plasma lipoproteins were isolated by ultracentrifugation at d > 1.21 g/ml and prepared for GGE (see Methods). Following GGE, gels were stained for protein and densitometric scans performed. Scans of HDL from a group 3 infant are depicted by the solid line and from a group 0 infant by the broken line. Sizes of the HDL subclasses are shown in nm.

Figure 4. The levels of apolipoproteins A-I, A-II, B, apoC-I and apoC-III were determined by rocket immunoelectrophoresis (16) of plasma, heparin manganese supernatants and precipitates in five group 3 infants and five group 0 infants.

Figure 5. Measured mean (1SD) plasma levels of lipids, lipoprotein cholesterols, apolipoproteins, lipoprotein subclasses, and lipoprotein sizes were determined by nuclear magnetic resonance (NMR) spectroscopy (18) in cord blood from group 0,1,2 and 3 infants. The p value given for each variable was corrected for the influence of gestational age by linear regression.

Figure 6. Gestational age in group 0, 1, 2, and 3 infants. The median and 25^{th} and 75^{th} percentiles (box) and 5^{th} and 95^{th} percentiles (whiskers) for gestational age are shown.

Figure 7. Plots of gestational age versus large (L3) LDL cholesterol (left) and largest (H5) HDL cholesterol (right) in group 3 (solid circles) and group 0 (open circles) infants. Regression lines are depicted for each group.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VLDL
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Prominent Apo
C-I-enriched HDL
(Group 3)
1.0203
1.0413
1.0622
1.0832
1.1041
Density (g/ml)
Grey Scale intensity (pixels)
250
200
150
100
50
0
VLDL
LDL
HDL
Undetectable Apo
C-I-enriched HDL
(Group 0)
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1.0413
1.0622
1.0832
1.1041
Density (g/ml)

Figure 1.

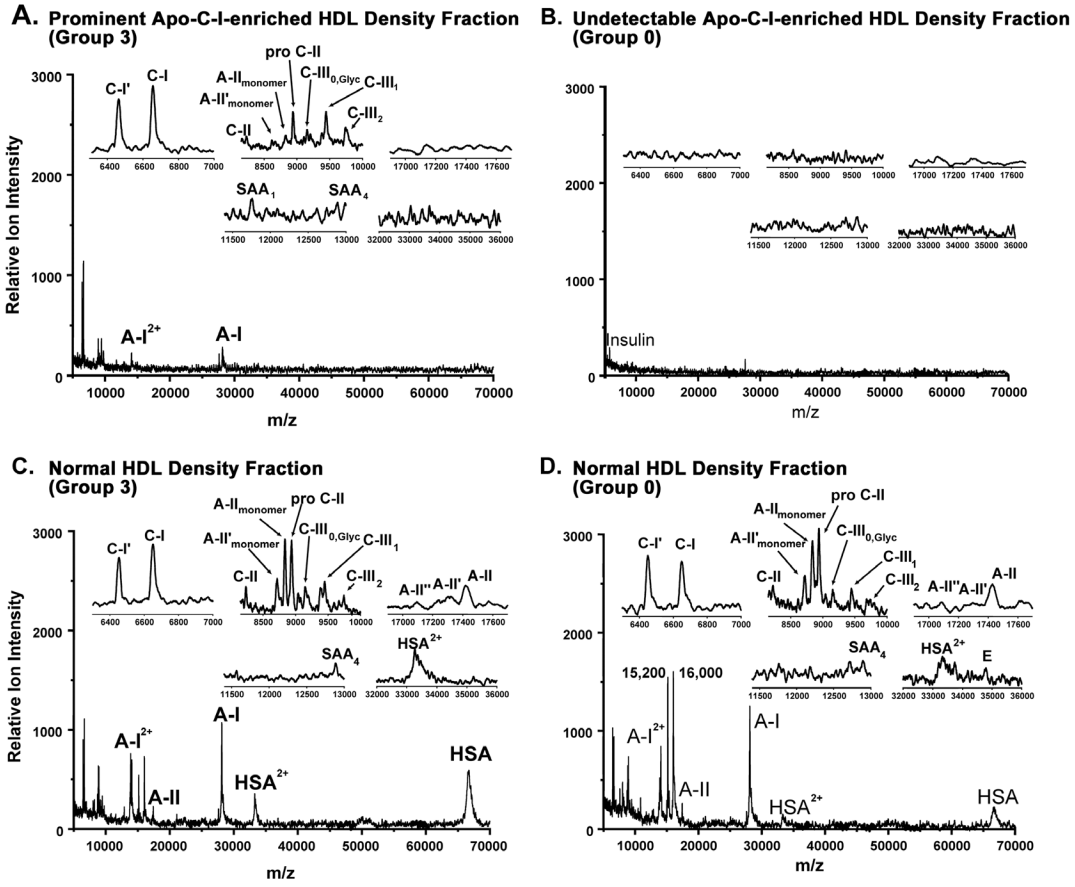
A. Prominent Apo-C-I-enriched HDL Density Fraction (Group 3)
B. Undetectable Apo-C-I-enriched HDL Density Fraction (Group 0)
C. Normal HDL Density Fraction (Group 3)
D. Normal HDL Density Fraction (Group 0)
Relative Ion Intensity
m/z
C-I'
C-I
pro C-II
A-II monomer
A-II' monomer
C-III 0,Glyc
C-III 1
C-III 2
C-II
SAA 1
SAA 4
A-I 2+
A-I
Insulin
A-II'' A-II'
A-II
HSA 2+
HSA
15,200
16,000
E
A-II

Figure 2.

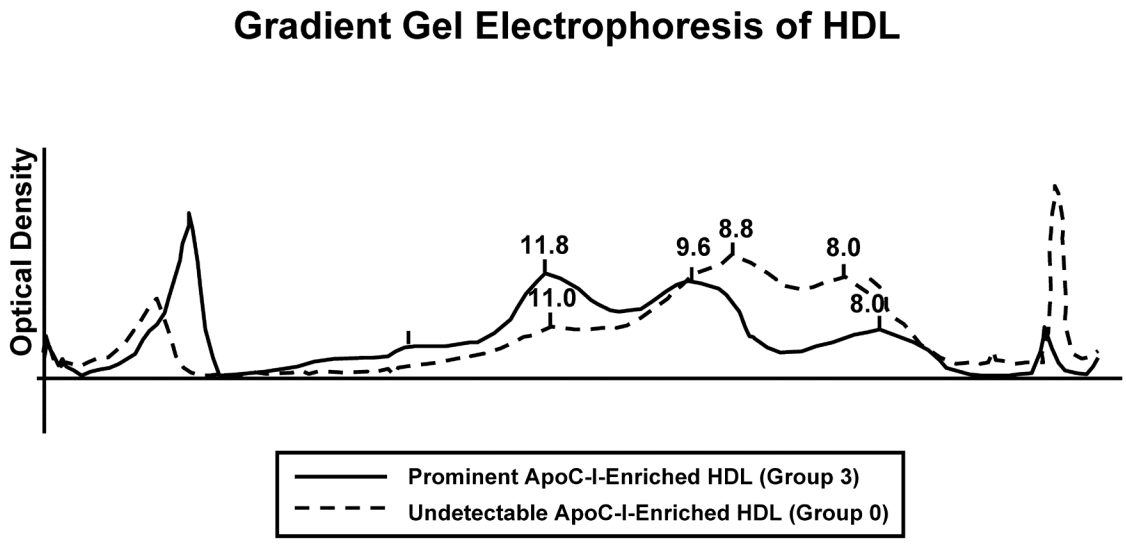
Gradient Gel Electrophoresis of HDL
Optical Density
11.8
11.0
9.6
8.8
8.0
8.0
Prominent ApoC-I-Enriched HDL (Group 3)
Undetectable ApoC-I-Enriched HDL (Group 0)

Figure 3.

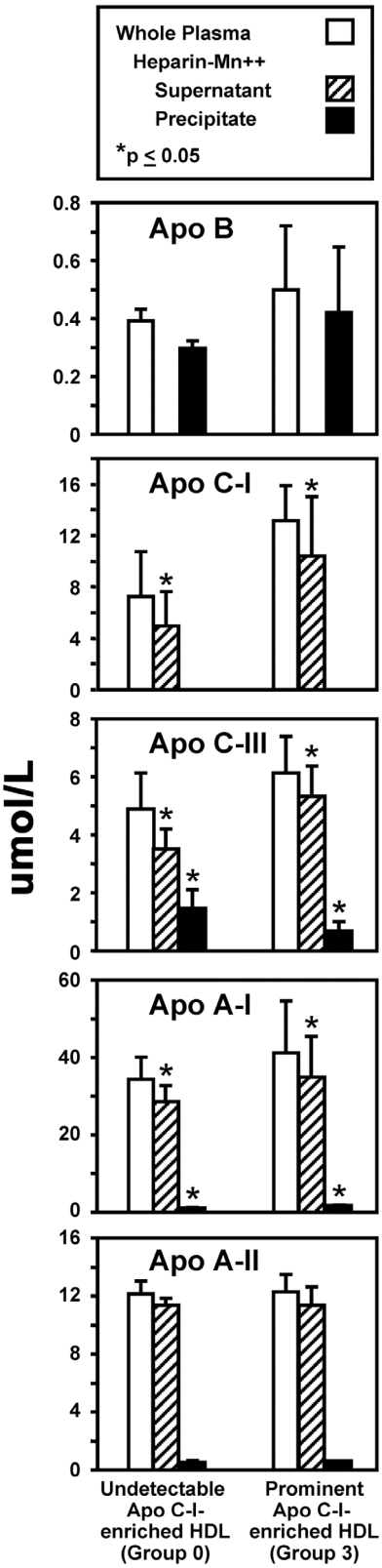
Whole Plasma
Heparin-Mn++
Supernatant
Precipitate
*p ≤ 0.05
Apo B
Apo C-I
Apo C-III
Apo A-I
Apo A-II
umol/L
Undetectable Apo C-I-enriched HDL (Group 0)
Prominent Apo C-I-enriched HDL (Group 3)

Figure 4.

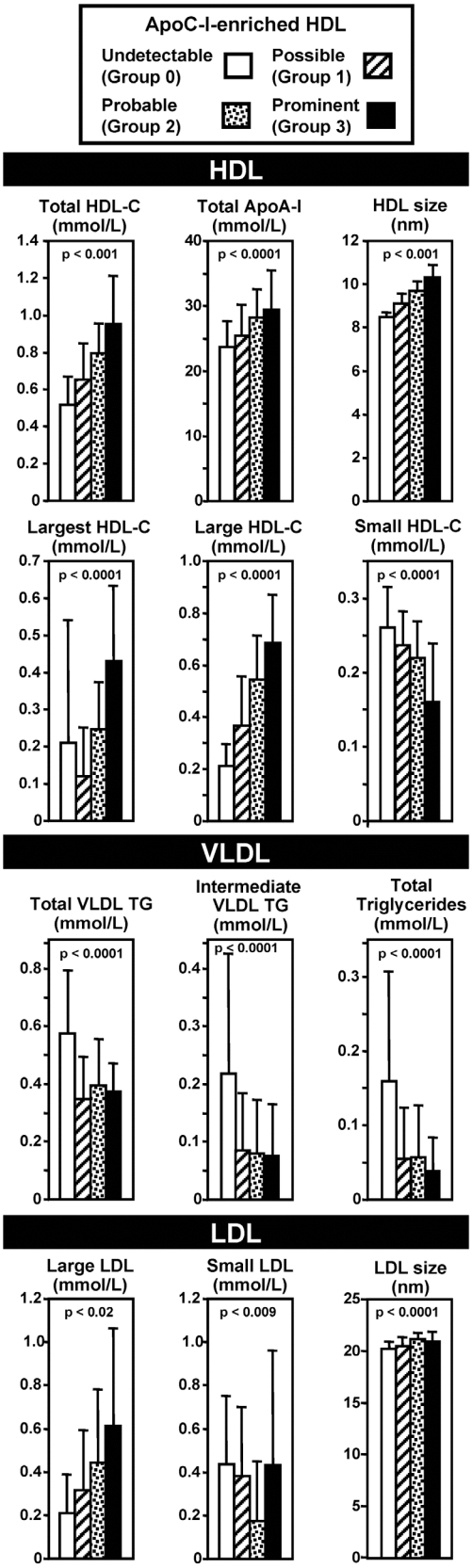
ApoC-I-enriched HDL
Undetectable (Group 0)
Possible (Group 1)
Probable (Group 2)
Prominent (Group 3)
HDL
Total HDL-C (mmol/L)
p < 0.001
Total ApoA-I (mmol/L)
p < 0.0001
HDL size (nm)
p < 0.001
Largest HDL-C (mmol/L)
p < 0.0001
Large HDL-C (mmol/L)
p < 0.0001
Small HDL-C (mmol/L)
p < 0.0001
VLDL
Total VLDL TG (mmol/L)
p < 0.0001
Intermediate VLDL TG (mmol/L)
p < 0.0001
Total Triglycerides (mmol/L)
p < 0.0001
LDL
Large LDL (mmol/L)
p < 0.02
Small LDL (mmol/L)
p < 0.009
LDL size (nm)
p < 0.0001

Figure 5.

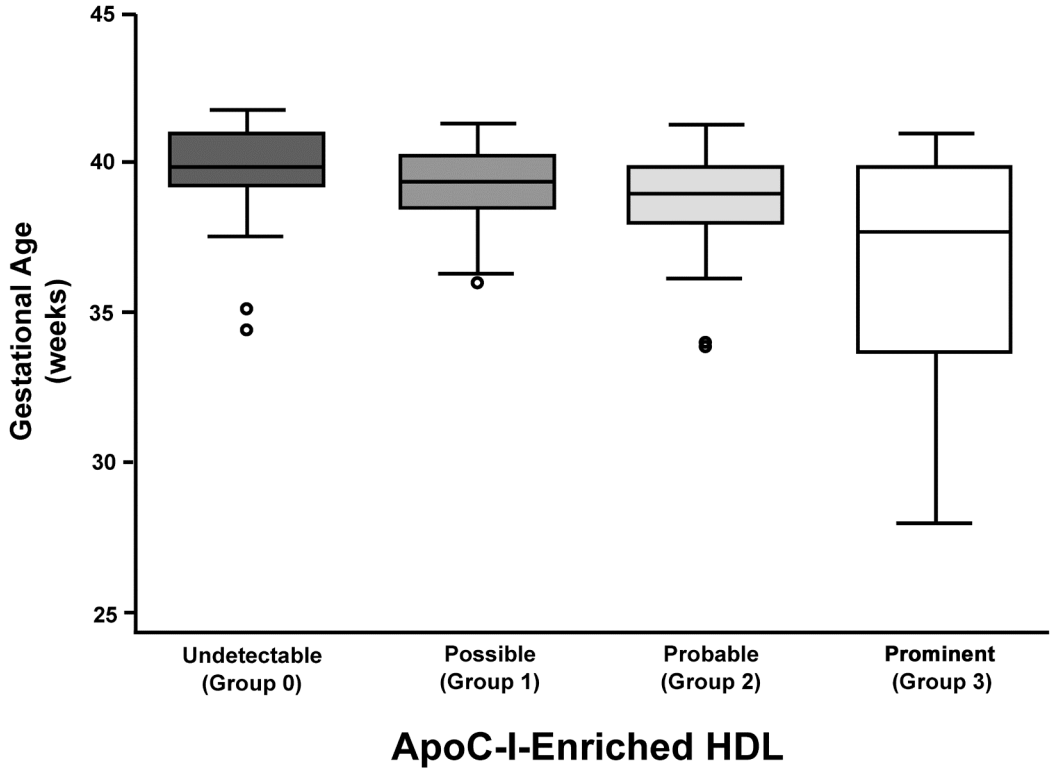
45
40
35
30
25
Gestational Age
(weeks)
Undetectable
(Group 0)
Possible
(Group 1)
Probable
(Group 2)
Prominent
(Group 3)
ApoC-I-Enriched HDL

FIGURE 6.

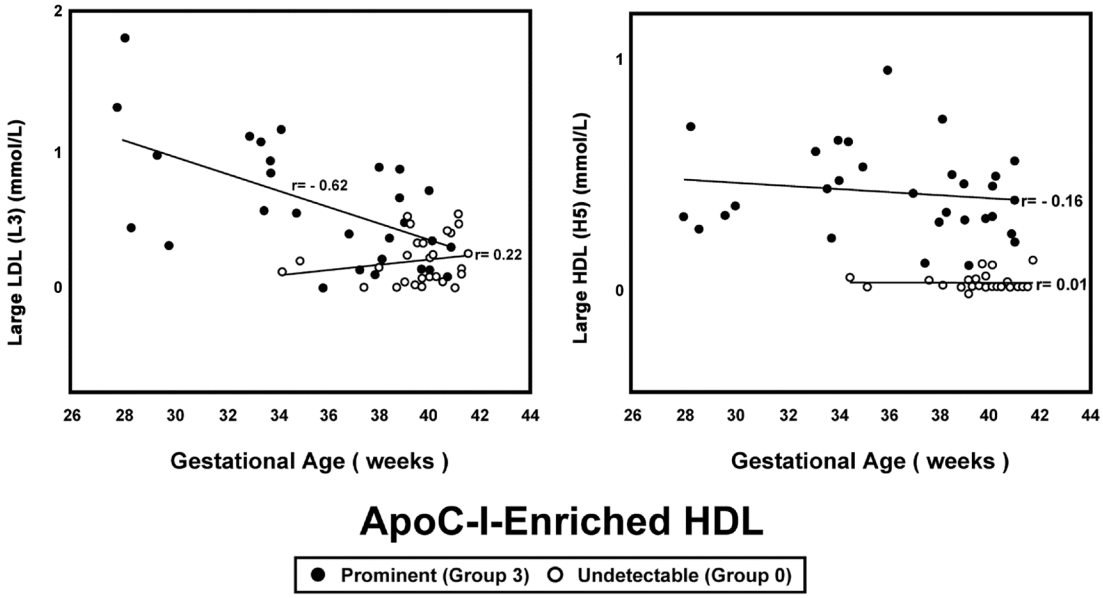
Large LDL (L3) (mmol/L)
2
1
0
r= - 0.62
r= 0.22
26 28 30 32 34 36 38 40 42 44
Gestational Age (weeks)
Large HDL (H5) (mmol/L)
1
0
r= - 0.16
r= 0.01
26 28 30 32 34 36 38 40 42 44
Gestational Age (weeks)
ApoC-I-Enriched HDL
Prominent (Group 3)
Undetectable (Group 0)

FIGURE 7.