Effect of Early Enteral Feeding on Apolipoprotein AI Levels and High-Density Lipoprotein Heterogeneity in Preterm Infants

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Key Words
Neonates, parenteral nutrition - High-density lipoprotein heterogeneity, premature infants - Apo AI levels

Abstract

Background/Aim: We have previously shown that infants receiving total parenteral nutrition have low apolipoprotein AI levels which are associated with high-density lipoprotein (HDL) class distributions as in lecithin:cholesterol acyltransferase deficiency. This study investigates the influence of early enteral feedings on apolipoprotein AI and HDL subclasses. Methods: Apolipoprotein AI and HDL distributions were determined in 15 total parenterally fed preterm infants (TPN group) receiving early feedings, in 28 enterally fed preterm infants (ENT group), and in 26 term infants at birth and on day 5. The HDL subclasses were determined by gradient gel electrophoresis. Results: In the TPN group, the apolipoprotein AI levels increased significantly postnatally (from 73 ± 16 to 104 ± 23 mg/dl) to levels found in the term and ENT groups on day 5 (88 ± 16 and 96 ± 19 mg/dl). The HDL subclass distributions at birth and on day 5 were similar in both TPN and ENT groups with more large HDL2b and less small HDL3c than in term infants. whereas the HDL subclass distribution of term infants remained unchanged, in TPN and ENT infants, a shift from HDL2b to HDL3c was observed, with no difference between term and preterm infants on day 5. Conclusion: In contrast to exclusively parenterally fed infants, infants receiving early enteral feedings exhibited a significant rise of apolipoprotein AI and HDL subclass distributions as fully enterally fed preterm infants.

Introduction

The benefits of early enteral nutrition in parenterally fed preterm infants have received increased attention. The levels of gut hormones and regulatory peptides, such as motilin, neurotensin, enteroglucagon, gastrins, and gastric inhibitory peptide, increase, when preterm infants are started on enteral feedings soon after birth, as opposed to very low levels seen in infants that are not yet fed [1, 2]. Minimal enteral feedings also improve the maturation of the intestinal motility [3–5]. Nevertheless, parenteral nutrition with glucose, amino acids, and intravenous fat emulsion is essential to meet the substrate needs of sick preterm infants during the first weeks of life.

High-density lipoproteins (HDL) represent a heterogeneous population of particles based on both density and size. Two major ultracentrifugal density classes are recognized within the HDL spectrum: the less dense HDL2 and the more dense HDL3. The HDL heterogeneity has been redefined on the basis of particle size using non-denaturing
gradient gel electrophoresis [6, 7]. There are at least five
distinct HDL subpopulations within the normal adult
density region. In the HDL3 fraction (density 1.125–1.20
g/ml), three subpopulations are found: HDL3a (8.8–8.2
nm), HDL3b (8.2–7.8 nm), and HDL3c (7.8–7.2 nm). The
HDL2 fraction (density 1.063–1.125 g/ml) contains the
following two subpopulations: HDL2b, (12.9–9.7 nm) and
HDL2a (9.7–8.8 nm). In adults, HDL3a is the predomi-
nant HDL subspecies, while in cord blood this is a minor
subspecies [8, 9]. The increase in cholesterol as observed
in the cord blood samples of preterm infants consists
mainly of HDL cholesterol and is associated with an
increase of particles in the HDL2b region [8]. We have pre-
viously shown [10, 11] that within the first days of life
fasting preterm neonates have low HDL cholesterol and
apolipoprotein (apo) AI levels and low HDL subclass dis-
butions, not unlike lecithin:cholesterol acyltransferase
(LCAT) deficient patients. Intravenous parenteral nutri-
tion with lipid emulsions is associated with increases in
apo AI and LCAT concentrations and changes in HDL
subclass distribution. The apo AI level is low prior to par-
ental nutrition, in particular in a subgroup of infants
with enteral problems like necrotizing enterocolitis, and
increases with parenteral nutrition, but only reaches nor-
mal levels after the introduction of enteral nutrition. Two
adult patients maintained on total parenteral nutrition
after total small-bowel resection had apo AI and HDL
cholesterol levels 70–50% of normal. Alterations of the
amounts of infused phospholipid influenced cholesterol,
phospholipid, and apo B plasma levels, but not apo AI
and HDL cholesterol levels. The small intestine is impor-
tant as source of apo AI and for HDL cholesterol metabo-
lism [12], since significant amounts of apo AI are secreted
by the intestine [13].

The influence of gut priming with small amounts of
enteral feedings from the 1st day of life on apo AI levels
and HDL subclass distribution has not been previously
investigated in preterm infants receiving parenteral nutri-
tion including lipid emulsions.

In the present report we evaluated HDL subpopula-
tions and apo AI levels in parenterally fed preterm infants
with early enteral feedings in comparison to fully enterally
fed preterm and term infants.

Patients and Methods

Patients

Preterm infants with a gestational age ≤ 32 weeks (as defined by
ultrasound examinations early in gestation) were enrolled after par-
ental consent (total parenteral nutrition; TPN group). The infants
received parenteral and early enteral nutrition by the standard proto-
col of the intensive care nursery; TPN with glucose, amino acids,
and calcium gluconate was started immediately after admission and sup-
plemented with parenteral fat emulsion (20%, Intralipid®; Pharma-
cia) and parenteral multivitamin solution on days 2–3 of life. Parent-
eral fat emulsion (20% soybean oil, 1.2% egg yolk phospholipid, and
2.2% glycerol) was started at 0.5 g/kg/day and advanced by 0.5 g/
kg/day to a maximum of 3 g/kg/day if tolerated. Fat was adminis-
tered intravenously continuously over 24 h. The infants received 1–
3 ml of diluted preterm formula every 3 h, starting on the 1st day of
life, as gavage feeding, and feedings were advanced daily as tolerated.
Parenteral and enteral nutrition was advanced by clinical status and
by discretion of the attending neonatologist. All infants received
pumped breast milk as soon as available. Cord blood was obtained as
baseline sample. Further blood samples were drawn on days 2 and 5
of life at times of routine blood sampling from indwelling catheters or
by venipuncture. Preterm infants with gestational ages >32 weeks
who tolerated enteral feedings and did not require parenteral nutri-
tion were also recruited (enteral nutrition; ENT group), and cord
blood and further blood samples were obtained on days 2 and 5 at
times of routine blood sampling just prior to their next feeding.
The preterm infants were gavage- or bottle-fed every 3 h, but were started
on breast-feeding as soon as possible. To prevent hypoglycemia, pre-
term infants with a birth weight <2,000 g routinely received glucose
solutions intravenously on days 1 and 2. If parenteral lipid emulsion
was given, the infant was excluded. Healthy term infants represented
the high risk group (TERM group). In addition to cord blood, blood sam-
ple was obtained on day 5 at the time of the mandatory newborn
screening for inborn metabolic and endocrine diseases and on day 2
if clinically indicated. The majority of the TERM group infants were
breast-fed on demand. The study underwent review by the Ethics
Committee of the Medical Faculty of the Ludwig Maximilians Uni-
versity, Munich.

Lipid Concentrations

Plasma was stored at −80 °C before analysis. Plasma triglyceride
(TG) and total cholesterol (TC) levels were determined on a Kodak
autoanalyzer by the glycerophosphate-oxidase/cholesterol-oxidase
peroxidase method (Kodak Vitros 250; Johnson & Johnson,
Braunschweig, Germany). HDL/IDL cholesterol was determined by
the HDL cholesterol polyethylene glycol precipitation method
with subsequent cholesterol analysis (Immuno, Heidelberg, Ger-
many). Apo AI and apo AII concentrations were measured by single
radial immundiffusion (Immuno). The phospholipid concentration
was determined by using a glycerophosphate-oxidase/cholesterol-oxi-
dase-peroxidase/phospholipase D-choline-oxidase phospholipid kit
(Wako, Neuss, Germany).

Lipoprotein Isolation and Nondenaturing Gradient Gel
Electrophoresis

To accommodate to the very small blood samples, the methods
were modified so that only 100 μl of plasma was necessary. The plas-
ma was adjusted to a density of 1.21 g/ml with a NaBr solution and
preservation agents, as described by Schumaker and Puppione [14].
For analysis of the HDL subgroup distribution, the total lipoprotein
fraction of the plasma was separated by ultracentrifugation. The den-
sity ≤ 1.21 g/ml fraction was isolated in a single ultracentrifugation
step utilizing only 100 μl of plasma to which 130 μl of a solution
containing plasma conservation agents [14] and sodium bromide
was added. Samples with added salt solution and preservatives were
stored in the ultracentrifugation test tubes at −20 °C until ultracentrifugation. Ultracentrifugation was carried out in an LP 42.2 Ti rotor and an L8-55 M ultracentrifuge (all from Beckman, Munich, Germany) at 42,000 rpm and 20 °C for 6 h, and subsequently the 50-µl top fraction containing the total lipoprotein fraction was harvested by tube cutting (CentriTube Slicer, Beckman). Frozen and stored samples were compared with refrigerated (4 °C) samples stored for 24 h, and no difference with regard to HDL subclass distribution was found. Gradient gel electrophoresis was carried out on precast polyacrylamide gradient gels (4–30%; David Rainwater, San Antonio, Calif., USA) according to the procedure of Nichols et al. [6]. The gels were stained with Coomassie brilliant blue to identify protein peaks, and densitometric scans were obtained with a TLC Scanner II (Camag, Berlin, Germany). The reference proteins used to determine the particle diameter were thyroglobulin, apoferritin, catalase, lactate dehydrogenase, and bovine serum albumin. The area under the curve was calculated for the five HDL subclasses. Blanche et al. [7] found a good correlation between percent area of HDL subgroups and plasma concentrations obtained by analytical ultracentrifugation [7].

Statistics
Mean values for cord blood and for blood sampled on days 2 and 5 were compared using ANOVA for the three groups. Changes of the values from cord blood to day 5 were assessed using paired t tests of mean values of each group. For the analysis of the values for infants with three samples, ANOVA was employed. Adjustment for multiple comparisons was done by the appropriate post tests (Dunnett and Bonferroni). For all calculations, Graphpad Prism 2.01 for Windows 3.1 (GraphPad Software, San Diego, Calif., USA) was used. Statistical significance was assumed with p < 0.05.

Results
Fifteen preterm TPN infants, 28 healthy preterm ENT infants, and 26 healthy TERM infants (birth weights and gestational ages: TPN 1,521 ± 304 g and 30 ± 2 weeks; ENT 2,253 ± 296 g and 34 ± 1 weeks; TERM 3,242 ± 489 g and 39 ± 1 weeks, respectively) were included in the study after obtaining parental consent.

In table 1, parenteral and enteral caloric intakes for the TPN group are shown. Of the 28 infants of the ENT group, 13 were breast-fed very early on, and as in the healthy TERM group, the caloric intake could not be recorded reliably. The remaining 15 infants of the ENT group were either bottle- or gavage-fed, and the intake could be recorded. In comparison to the TPN group, their intravenous caloric intake was minimal on day 2, and they were on full enteral feedings on day 5.

### Lipid Levels
The lipid and apo levels in cord blood and on day 5 of the three groups are shown in Table 2. Since the lipid levels on day 2 were not available in all infants (TERM group n = 6; ENT group n = 15; TPN group n = 8), they are not included in the table.

The TC and TG values increased significantly from cord blood values to day 5 in all three groups. On day 5, the TG values of the TERM infants were significantly higher than those of both groups of preterm infants. Since the TERM infants were breast-fed on demand, not all samples were obtained 3 h after the last meal. Both groups of preterm infants had significantly higher TC values not only at birth, but also on day 5. The HDL cholesterol levels were also significantly higher at birth in both groups of preterm infants (ENT and TPN groups) in comparison to the TERM group, but decreased thereafter, so that on day 5, there was no significant difference between the three groups. For all preterm infants there was no significant correlation between gestational age and any of the lipid levels or the apo AI and AII levels. The TERM infants did not show any significant changes in their HDL cholesterol concentration from cord blood to day 5. The apo AI and apo AII levels increased significantly in all three groups and did not differ at any time between the three groups. At birth, the phospholipid levels were higher in both groups of preterm infants as compared with the TERM group. The phospholipid levels increased postnataally in all three groups. For the preterm

<table>
<thead>
<tr>
<th></th>
<th>Intravenous</th>
<th>Enteral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>calories</td>
<td>protein</td>
</tr>
<tr>
<td></td>
<td>kcal/kg/day</td>
<td>g/kg/day</td>
</tr>
<tr>
<td>Day 2</td>
<td>24±6.9</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Day 5</td>
<td>29±7.8</td>
<td>0.8±0.3</td>
</tr>
</tbody>
</table>

### Table 1. Parenteral and enteral calories, protein, and fat of the 15 preterm infants (TPN group) receiving parenteral nutrition with lipid emulsion and minimal enteral feeding as gut priming from day 1 of life (mean ± SD)
Table 2. Lipid (mmol/l) and apo AI and apo AII (mg/dl) values (mean ± SD) in cord blood and on day 5 for the three groups TERM (n = 26), ENT (n = 28), and TPN (n = 15)

<table>
<thead>
<tr>
<th></th>
<th>Cord blood</th>
<th>Day 5</th>
<th>Cord blood</th>
<th>Day 5</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TG</td>
<td></td>
<td>TC</td>
<td></td>
</tr>
<tr>
<td>TERM</td>
<td>0.46 ± 0.22</td>
<td>1.66 ± 0.8*</td>
<td>1.40 ± 0.4</td>
<td>2.75 ± 0.12*</td>
</tr>
<tr>
<td>ENT</td>
<td>0.38 ± 0.21</td>
<td>1.14 ± 0.4*a</td>
<td>2.22 ± 0.6a</td>
<td>3.00 ± 0.9*a</td>
</tr>
<tr>
<td>TPN</td>
<td>0.37 ± 0.14</td>
<td>1.25 ± 0.74*a</td>
<td>2.60 ± 0.8a</td>
<td>3.60 ± 1.08*a</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol</td>
<td></td>
<td>HDL A III cholesterol</td>
<td></td>
</tr>
<tr>
<td>TERM</td>
<td>0.62 ± 0.2</td>
<td>0.70 ± 0.2</td>
<td>0.40 ± 0.13</td>
<td>0.45 ± 0.13</td>
</tr>
<tr>
<td>ENT</td>
<td>1.08 ± 0.3*a</td>
<td>0.80 ± 0.25*a</td>
<td>0.65 ± 0.15a</td>
<td>0.52 ± 0.15*a</td>
</tr>
<tr>
<td>TPN</td>
<td>1.08 ± 0.3*a</td>
<td>0.78 ± 0.2*a</td>
<td>0.72 ± 0.2a</td>
<td>0.55 ± 0.15*a</td>
</tr>
<tr>
<td></td>
<td>apo AI</td>
<td></td>
<td>apo AII</td>
<td></td>
</tr>
<tr>
<td>TERM</td>
<td>70 ± 16</td>
<td>88 ± 16*</td>
<td>23 ± 6</td>
<td>28 ± 6*</td>
</tr>
<tr>
<td>ENT</td>
<td>78 ± 14</td>
<td>96 ± 19*</td>
<td>26 ± 5</td>
<td>31 ± 6*</td>
</tr>
<tr>
<td>TPN</td>
<td>73 ± 16</td>
<td>104 ± 23*</td>
<td>23 ± 8</td>
<td>29 ± 7*</td>
</tr>
</tbody>
</table>

* Cord blood vs. day 5, a in comparison to TERM infants, b in comparison to the ENT group (p < 0.001).

infants of the TPN group the phospholipid concentration became even higher than for the infants of the ENT group. The phospholipid levels increased significantly already on day 2 in all three groups (TERM 1.2 ± 0.2 vs. 1.89 ± 0.28 mmol/l; ENT 1.71 ± 0.49 vs. 2.36 ± 0.36 mmol/l; TPN 1.74 ± 0.44 vs. 2.3 ± 0.49 mmol/l). In the ENT group, the TC levels increased significantly (1.1 ± 0.04 vs. 1.3 ± 0.04 mmol/l), and the HDL cholesterol level decreased significantly (1.1 ± 0.28 vs. 0.8 ± 0.26 mmol/l) on day 2, whereas the other lipid concentrations and apo AI and AII levels were not significantly different from cord blood levels (data not shown).

Gradient Gel Electrophoresis

The values for the areas under the curve of the gradient gel electrophoresis scans are listed in table 3. The HDL subclass distribution as seen in table 3 did not change significantly in the TERM group. Cord blood samples of both groups of preterm infants (ENT and TPN) showed an increase in mass in the HDL2b region and less mass in the HDL3c region. On day 5, the scans of both groups (ENT and TPN) showed a statistically significant shift of particles from the large less dense HDL2b region to the smaller more dense particles in the HDL3c region. Therefore, on day 5, there was no longer a difference in HDL
subclass distribution between TERM group and both groups of preterm infants. Only in a minority of the TPN infants persistently low HDL cholesterol levels were observed, similar to the pattern observed in our previous study [10]. As the lipids levels, the areas under the curve did not correlate with the gestational age.

**Discussion**

The postnatal development of the HDL subclass distribution in parenterally nourished infants who are started on early enteral nutrition has previously not been studied. We were able to show that preterm infants with low-level enteral feedings during the first days of life in addition to their parenteral nutrition do not exhibit the abnormal HDL pattern previously reported in solely parenterally nourished infants, but show the same transformation of large HDL to small HDL3c particles as completely enterally fed preterm infants [11]. The HDL distribution previously reported in infants not fed enterally resembles most closely the HDL distribution of LCAT-deficient patients [10]. Exclusively parenterally fed rats exhibit mucosal hypoplasia and hypofunction of the intestine within days, with changes in the protein metabolism. In particular, the urea cycle amino acid concentrations were altered in plasma and mucosa of solely parenterally fed pigs, suggesting that the arginine synthesis by an atrophied gut may have been limited. Gut atrophy resulted in significantly lower concentrations of all indispensable amino acids as compared with enterally fed pigs [15–18]. Feeding small amounts of enteral nutrition to parenterally nourished preterm infants may not only facilitate better enteral feeding tolerance, but also influence the metabolism of a parenteral lipid emulsion. This might be of particular interest in extremely preterm infants who frequently are nourished parenterally for weeks and in whom enteral feeding is often delayed for the fear of necrotizing enterocolitis. Starting enteral nutrition very early allows a more rapid increase in enteral feedings. Our group of TPN infants received a fairly large part of their nutrition enterally on day 5. Since feeding very-low-birth-weight infants enterally is a standard procedure in our unit, it was not feasible to randomize infants to a later start or to a restricted increase in enteral feedings.

The lack of postnatal changes in the HDL subclass distribution of healthy term infants despite increased TG levels is not readily explained, since an increase in TG is usually accompanied by an increase in small, dense HDL3. In particular the persistent lack of material in the HDL3c region is of interest. This region contains the main peak in adults and the least amount of material in cord blood [8, 9]. Cord blood lipoproteins consist predominantly of HDL, small amounts of low-density lipoprotein, and nearly no very low density lipoproteins and chylomicrons. Since there is an inverse relation between HDL2 and very low density lipoproteins, the postnatal increase of TG and the concomitant increase in very low density lipoproteins should lead to a decrease of HDL2 particles. Even though enteral nutrition led to increased TG, low-density and very low density lipoprotein, and chylomicron levels in all three groups, the HDL subclass distribution changed only in the preterm infants but not in term infants. Davis et al. [8] and Silliman et al. [19, 20] reported a shift in the HDL subclass distribution during late pregnancy with an increase of particles in the HDL2b region. Estrogen levels were associated strongly with the HDL2b mass. Estrogen has a half-life of a few days, and possibly the observed postnatal changes in our preterm infants were caused by decreasing estrogen levels.

Previous studies [9, 21–24] demonstrated that the HDL cholesterol levels are elevated in cord blood in preterm infants, but declined within the first 5 days of life to levels as seen in term infants. The HDL cholesterol values found in our study are comparable to those reported by Decsi et al. [25] in preterm infants during the first 10 days of life.

The apo AI levels increased postnatally in all three groups. The influence of the gestational age on the apo AI concentration is under debate. Parker et al. [23] described an influence of gestational age on apo AI, but found a positive correlation with increasing levels only between 21 and 32 weeks of gestation. The overall cord blood apo AI levels are approximately 60–70% of those of adults [26, 27]. The apo AI levels increase in plasma after a fatty meal. A postnatal increase of apo AI has been observed previously in healthy enterally fed term and preterm infants [8, 10, 26–29]. Apo AI as a cofactor of LCAT is essential for metabolizing intravenous lipid emulsion constituents, but the level of this enzyme is very low in parenterally fed preterm infants [10, 29, 30]. It is of importance that in contrast to our previous study on entirely parenterally fed preterm infants [10], the preterm infants in the present study who received parenteral nutrition along with a small amount of enteral nutrition for gut priming had postnatal apo AI levels comparable to those of term infants and displayed the same rise in apo AI as exclusively enterally fed preterm infants. The postnatal increase of apo AI happens predominantly in the HDL3b+3c region [28]. Since significant amounts of apo AI

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are secreted by the intestine [13], the early enteral nutrition in the TPN group facilitated the previously not reported postnatal rise in apo AI in this group.

Parallel to the increase of apo AI, we also observed a rise of mass in the HDL3b+3c region which is likely to be caused by an increase in protein content and not in lipid particle levels, since the HDL3 cholesterol levels did not increase. Postnatally unchanged HDL3 cholesterol levels are in accordance with the findings of Decsi et al. [31].

Data on apo AI levels in infancy are sparse [27, 31, 32], but are similar to our values. There is also little information on plasma phospholipid concentrations in newborns. Dolphin et al. [33] reported slightly lower levels, but his method did not exclude lysolecithin. Jain and Díaz [34] did not find a correlation between gestational age and phospholipid levels in cord blood which is in contrast to our data, showing that preterm infants had significantly higher levels than the TERM group infants. Our cord blood data and the increased levels during parenteral lipid emulsion are in accordance with the findings reported by Haumont et al. [35, 36], as are the levels of phospholipids in the enterally fed preterm infants. The increased phospholipid levels in parenterally fed infants are mostly likely caused by the phospholipids of the lipid emulsion.

To conclude, early enteral feeding in parenterally nourished preterm infants may prevent intestinal atrophy and, therefore, lead to an increase in apo AI levels. Early enteral feeding may facilitate the same postnatal changes in HDL subclass distribution, including a shift from large to small HDL particles as in fully enterally fed preterm infants. These findings are opposite to previously reported changes observed in exclusively parenterally nourished infants. Therefore, early feeding not only facilitates better enteral nutrition, it also enhances parenteral nutrition tolerance. Whether improved nutrition during the 1st week of life has implications on the long-term outcome still needs to be proven.

Acknowledgement

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