Lipoprotein Profile and Cholesteryl Ester Transfer Protein in Neonates

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Undernourishment in utero appears to be associated with persisting changes in the metabolic, endocrine, and immune functions. In this study, we determined the influence of birth weight on the lipoprotein profile and cholesteryl ester transfer protein (CETP), which promotes a proatherogenic lipoprotein profile in plasma by determining the chemical, physical, and biologic properties of the respective lipoprotein particles. Triglyceride (TG) concentrations were highest and high-density lipoprotein $(HDL)_2$-cholesterol levels were lowest in small for gestational age (SGA) neonates. CETP-mass was determined by enzyme-linked immunosorbent assay (ELISA) and CETP-activity by using exogenous lipoproteins. Cholesteryl ester transfer was determined as transfer of radiolabeled cholesteryl esters (CE) from HDL to apolipoprotein B-containing lipoproteins. CETP mass was lowest and cholesteryl ester transfer was highest in SGA neonates. CETP-activity did not differ among the neonates. Our results suggest that increased and decreased nourishment in utero affects the lipoprotein profile and CETP in neonates. High TG and low HDL_2 levels in SGA neonates might result from increased cholesteryl ester transfer and, may in part, explain the increased risk of coronary heart disease (CHD) of small for gestational age neonates in later life.

IN HUMANS, LOW birth weight is a marker of lack of nutrients at particular stages of gestation.[1] Undernourishment in utero seems to be associated with persisting changes in cholesterol metabolism, insulin response to glucose, and a range of other metabolic, endocrine, and immune functions in later life.[1]

Cholesteryl ester transfer protein (CETP) plays an important role in reverse cholesterol transport[2] by mediating the transfer of cholesteryl esters (CE) from CE-rich lipoproteins (high-density lipoprotein [HDL], low-density lipoprotein [LDL]) to triglyceride (TG)-rich lipoproteins (chylomicrons, very–low-density lipoprotein [VLDL]) in exchange for triglycerides.[3,4] Data about the role of CETP in atherogenesis are controversial. CETP deficiency[5-7] has been described as a possible genetic reason for high HDL levels considered protective against coronary heart disease (CHD). Other studies suggested that higher HDL levels due to mutations in CETP are associated with an increased risk from CHD.[8,9]

In several studies, a positive correlation between mass and activity of CETP and body mass index (BMI) in obese subjects was reported.[10-12] Besides CETP concentration in plasma, TG concentration is a major determinant of CETP-mediated exchange of neutral lipids.[13,14]

The lipoprotein profiles of neonates and adults differ profoundly. In cord blood cholesterol, TG, LDL-C, and HDL-C concentrations are low when compared with adults.[15] In neonatal plasma, only a minor part of total cholesterol is transported in LDL particles.[16] Furthermore, apolipoprotein distribution differs between neonates and adults. Overall apolipoprotein E (apoE) concentrations in plasma are similar in neonates but, in contrast to adults, more than 80% of apoE is associated with HDL.[15] Apolipoprotein B (apoB) and apolipoprotein C-III (apoC-III) levels are low at birth and increase during the first week of life.[17] At the same time, TG levels increase, resulting in VLDL enriched with TG, apoE and apoC-III, respectively.

To investigate whether nourishment in utero affects the lipoprotein profile and whether these changes might be mediated by CETP, we determined plasma lipoprotein concentrations, CETP-mass, CETP-activity, and CE-transfer in neonatal plasma.

MATERIALS AND METHODS

Subjects

Cord blood was taken from V. umbilicalis immediately after birth, and venous peripheral blood was drawn from respective mothers within 1 hour before birth of the neonate. Blood was collected into EDTA tubes (1.6 $mg.mL^{-1}$). The inclusion criterium was birth between 37th and 43rd gestational week. Gestational diabetes, edema/proteinuria/hypertension (EPH) gestosis, insufficiency of placenta, hydramnion, and birth by caesarean section were exclusion cirteria. Informed consent was obtained from the mothers. Procedures were performed in accordance with institutional guidelines.

Lipoprotein Analysis, Plasma Insulin

Plasma was separated from erythrocytes by centrifugation at 3,000 rpm for 10 minutes at 4°C immediately after collection. Plasma samples were stored frozen at -80°C until assayed. Plasma TG and cholesterol concentrations were quantified using a commercially available enzymatic kit (Roche Diagnostic Systems, Basel, Switzerland) on a Cobas Mira analyzer (Roche Diagnostic Systems). HDL-C concentrations were measured after precipitation of apoB-containing lipoproteins with dextrane sulphate (Pharmacia Biotech, Brussels, Belgium)/$MgCl_2$.[18] HDL_3-C concentrations were determined after an additional precipitation step[18,19] by increasing the $MgCl_2$ concentration in the HDL precipitation reagens. LDL-C was calculated according to the formula of Friedewald et al.[20] Plasma insulin concentration was determined by a microparticle enzyme immunoassay on an IMx analyser (IMx system No.2A10-20; Abbott Diagnostics, Abbott Park, IL).

Gradient Gel Electrophoresis

Size of plasma lipoproteins was determined by 0.75% to 16% gradient polyacrylamide gel electrophoresis (LaboMed, Waldkirch, Germany). Gels were fixed and stained with Sudanblack (LaboMed) before analysis on a Molecular analysis densitometer (Bio-Rad, Hercules, CA).

CETP-Mass

CETP-mass concentrations were measured by capture enzyme-linked immunosorbent assay (ELISA) as described elsewhere.[21] Wells

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of a microtiter plate were coated with monoclonal anti-CETP antibody 2F8. CETP was detected using a polyclonal anti-CETP antibody conjugated to alkaline phosphatase.

CETP-Activity

CETP-activity was measured as the transfer of radiolabeled CE from exogenous LDL to exogenous HDL in lipoprotein-depleted plasma. LDL were labeled as described.[22] [4-^{14}C]cholesteryl-oleate (specific activity, 55.00 mCi/mmol, Amersham, Buckinghamshire, England) was transferred from synthetic micelles to LDL via CETP in lipoprotein-depleted plasma. For isolation of ^{14}C-LDL, the labeling mixture was adjusted to a density of 1.019 with NaBr and ultracentrifuged at 50,000 rpm for 12 hours in a Type 50.4 rotor (L8-M Ultracentrifuge; Polycarbonate Thick Wall Centrifuge Tubes, Beckman, Palo Alto, CA). The infranatant was adjusted to a density of 1.063 g/mL with NaBr (Sigma, St Louis, MO) and ultracentrifuged as described above. The supernatant was recovered and used for the CETP activity assay.[22]

Lipoproteins were precipitated,[22] and the resulting lipoprotein-depleted plasma was incubated with 200 nmol/L HDL_2 and 500 nmol/L LDL (radiolabeled and not labeled LDL) with 5,5′-dithio-bis-(2-nitrobencoic acid) in a phosphate buffer. The mixture was incubated for 16 hours at 37°C. ApoB-containing lipoproteins were precipitated after incubation with $MgCl_2$, the radioactivity of the supernatant was measured in a β-counter (Beckman, LS 6500). CETP-activity was expressed as nmol.mL^{-1}.h^{-1} CE transfer from LDL to HDL_2.[22]

CE-Transfer

CE-transfer in total plasma was measured as the capacity of the sample to promote the transfer of radiolabeled CE from exogenous HDL_3 (less than 7% of the plasma HDL concentration) to apoB-containing lipoproteins. HDL were labeled as previously described.[23] A total of 21 mL of human EDTA plasma was adjusted to a density of 1.13 g/mL with NaBr and ultracentrifuged for 10 hours at 50,000 rpm in a Type 50.4 rotor by using an L8-M ultracentrifuge (Beckman). The plasma fraction greater than 1.13 g/mL was dialyzed against phosphate-buffered saline (PBS) overnight. A total of 40 nmol of 7(n)-^{3}H cholesterol (specific activity, 2 Ci/mmol, Amersham) was evaporated under gaseous nitrogen and resuspended in 200 μL ethanol. The dialyzed plasma fraction was added and incubated for 24 hours at 37°C to allow lecithin:cholesterol acyltransferase (LCAT) esterification of ^{3}H-cholesterol. After incubation, the mixture was adjusted to a density of 1.13 g/mL with NaBr and ultracentrifuged at 50,000 rpm for 14 hours in a Type 50.4 rotor, and the infranatant was subsequently adjusted to a density of 1.21 g/mL with NaBr and further ultracentrifuged for 14 hours as described above. The fraction less than 1.21 g/mL was finally recovered. More than 96% of total radioactivity was esterified cholesterol as determined by thin-layer chromatography (TLC) (data not shown).

Each incubation mixture for measuring the CE-transfer contained 50 μL plasma, 1.2 nmol ^{3}H-CE-HDL, 150 nmol Na-iodoacetat (Sigma), and was filled up with 5% bovine serum albumin (BSA) (Sigma)-PBS to a final volume of 100 μL. Mixtures were incubated at 37°C in a shaking waterbath. Nonincubated controls were stored at 4°C. The reaction was stopped by chilling the tubes on ice. Transfer reaction was linear up to 3 hours (initial rate of CE-transfer) and reached its maximum after 16 hours (total transfer of CE). ApoB-containing lipoproteins were precipitated with dextrane sulphate/$MgCl_2$.[18] CE-transfer was measured as the rate of total radiolabeled CE transferred to apoB-containing lipoproteins compared with controls stored at 4°C. Results are expressed as the percentage of decrease of ^{3}H-CE in the supernatant of total radiolabeled CE.

Statistical Analysis

Descriptive data are expressed as mean values ± SD. Statistical difference between means was estimated by unpaired *t* test and 1-way ANOVA. Correlation coefficients were calculated with Pearson's method. Statistical significance was inferred at a 2-tailed *P* value of less than .05. Statistical analyses were calculated using SPSS for Windows software (version 8.0; SPSS, Chicago, IL).

RESULTS

Subjects

Clinical characteristics of neonates and respective mothers are shown in Table 1. Neonates were subdivided into 3 groups with respect to their birth weight: (1) small for gestational age (SGA) less than 10th percentile of birth weight; (2) appropriate for gestational age (AGA), between 10th and 90th percentile of birth weight; (3) large for gestational age (LGA), greater than 90th percentile of birth weight. Age, weight, height, and BMI in mothers, respectively, did not differ between respective mothers of SGA, AGA, and LGA neonates, respectively.

Lipoprotein Profile in Mothers and Respective Neonates

Plasma concentrations of lipoproteins were significantly lower in neonatal plasma (Table 2). Thirty percent of total cholesterol was transported in LDL in neonatal plasma, whereas in maternal plasma, half of total cholesterol was transported in LDL (Table 2). LDL/HDL ratio was significantly higher in mothers (1.47) than in neonates (0.50; $P < .01$). With respect to gender, LDL-C and total cholesterol concentrations were significantly lower in male than in female neonates (Table 3).

With respect to size of LDL particles mothers exhibited, as expected, either a preponderance of smaller LDL particles (pattern B) or large particles (pattern A). In contrast, LDL particles of neonates were uniformly small, even smaller than the small LDL particles dominating in pattern B of maternal plasma (Fig 1).

Lipoprotein Profile in SGA, AGA, and LGA Neonates

As shown in Table 4, SGA neonates had significantly higher TG levels than both AGA) and LGA neonates. HDL_2 levels

Table 1. Clinical Characteristics of Neonates and Respective Mothers (mean ± SD)

	SGA Neonates (n = 14)	AGA Neonates (n = 38)	LGA Neonates (n = 12)
Birth weight (g)	2,518 ± 410	3,263 ± 382	3,962 ± 470
Gestational week	40 ± 1	41 ± 1	40 ± 1
	Mothers of SGA Neonates	Mothers of AGA Neonates	Mothers of LGA Neonates
Height (m)	168 ± 8	166 ± 7	169 ± 5
Weight (kg)	76 ± 21	75 ± 10	81 ± 10
BMI (kg/m^2)	27 ± 6	27 ± 3	29 ± 4
Age (yr)	33 ± 4	30 ± 3	31 ± 4
Smoke behavior (smoker/nonsmoker)	3/11	7/31	2/10

Abbreviations: SGA, small for gestational age (birth weight < 10th percentile); AGA, appropriate for gestational age (birth weight between 10th and 90th percentile); LGA, large for gestational age (birth weight > 90th percentile).

Table 2. Plasma Concentrations of Total Cholesterol, TG, LDL-C, HDL-C, HDL_2-C, HDL_3-C, Insulin and CETP-Mass, CETP-Activity, and CE-Transfer in Neonates and Their Mothers (mean ± SD)

	Neonates (n = 64) (23 F/41 M)	Mothers (n = 64)	*P* Values
Total cholesterol (mg/dL)	58 ± 15	238 ± 46	<.01
TG (mg/dL)	46 ± 16	217 ± 74	<.01
LDL-C (mg/dL)	17 ± 8	113 ± 44	<.01
HDL-C (mg/dL)	35 ± 17	77 ± 21	<.01
HDL_2-C (mg/dL)	12 ± 7	23 ± 11	<.01
HDL_3-C (mg/dL)	24 ± 12	53 ± 17	<.01
Insulin (μU/mL)	7.3 ± 5.2	21.6 ± 28.0	<.01
CETP-mass (μg/mL)	1.04 ± 0.32	1.24 ± 0.36	<.01
CETP-activity ($nmol \cdot h^{-1} \cdot mL^{-1}$)	134.41 ± 24.18	162.23 ± 24.80	<.01
Initial rate of CE-transfer (%)	20.71 ± 8.52	27.01 ± 10.00	<.01
Total transfer of CE (%)	41.87 ± 12.17	61.63 ± 10.60	<.01

Abbreviations: F, female; M, male.

were lower in SGA neonates when compared with both AGA and LGA neonates. The ratio of HDL_2/HDL_3 was lowest in SGA without reaching statistical significance.

CETP in Mothers and Respective Neonates

CETP-mass, CETP-activity, and both initial rate of CE-transfer and total transfer of CE were all significantly lower in neonatal plasma when compared with maternal plasma, respectively (Table 2). In contrast to CETP-activity and CE-transfer, respectively, CETP-mass in mothers correlated significantly with CETP mass in respective neonates ($r = .34$; $P = .01$). In female neonates, CETP-mass was higher than in male neonates (1.10 ± 0.31 μ/mL *v* 1.01 ± 0.32 μg/mL) (Table 3).

Table 3. Metabolic Parameters in Male and Female Neonates

	Female Neonates (n = 23)	Male Neonates (n = 41)	*P* Values
Total cholesterol (mg/dL)	64 ± 14	54 ± 13	*P* = .03
TG (mg/dL)	47 ± 20	43 ± 13	NS
LDL-C (mg/dL)	21 ± 9	14 ± 5	*P* = .01
HDL-C (mg/dL)	37 ± 16	36 ± 20	NS
HDL_2-C (mg/dL)	13 ± 7	12 ± 9	NS
HDL_3-C (mg/dL)	25 ± 12	24 ± 14	NS
Insulin (μU/mL)	8.2 ± 4.5	6.8 ± 4.1	*P* = .04
CETP-mass (μg/mL)	1.10 ± 0.31	1.01 ± 0.32	NS
CETP-activity ($nmol \cdot h^{-1} \cdot mL^{-1}$)	135.70 ± 22.90	132.79 ± 22.65	NS
Initial rate of CE-transfer (%)	20.35 ± 8.17	20.89 ± 7.75	NS
Total transfer of CE (%)	43.32 ± 12.98	39.7 ± 12.24	NS

NOTE. Data are expressed as means and ±SD. Differences in total cholesterol, LDL-C, and insulin concentrations were statistically significant.

Abbreviation: NS, not significant.

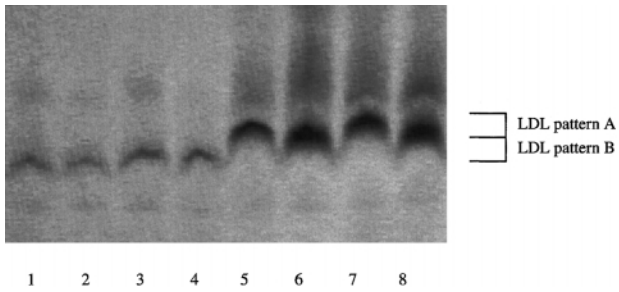


Fig 1. LDL particle size distribution in neonates and mothers. Lanes 1 to 4, LDL particle size in plasma samples of neonates; lanes 4 to 8 in plasma samples of mothers, respectively. LDL pattern A is shown in lanes 5 and 7. Lanes 6 and 8 show pattern B with dominating small, dense LDL particles. The figure is representative of all tested neonates independent of their birth weight.

CETP in SGA, AGA, and LGA Neonates

Within neonates, CETP-mass was lowest in SGA neonates when compared with AGA neonates (0.70 ± 0.28 μg/mL *v* 1.11 ± 0.29 μg/mL, $P < .01$) (Fig 2). CETP-activity did not differ between LGA, AGA, and SGA neonates.

Both initial rate and total transfer of CE were highest in SGA neonatal plasma (initial rate of CE-transfer, 23.47% ± 6.57%; total transfer of CE, 49.81% ± 10.51%) and lowest in LGA neonatal plasma (initial rate of CE-transfer, 15.54% ± 10.16%; total transfer of CE, 39.32% ± 12.38%) reaching statistical significance in initial rate between SGA and AGA neonates, AGA and LGA neonates, and between SGA and AGA neonates and SGA and LGA neonates in total transfer of CE, respectively (Fig 3).

DISCUSSION

Recent findings suggest that undernourishment in utero may be associated with disorders such as CHD, stroke, or diabetes mellitus in later life.[1] In 16,000 persons born during 1911 and 1930, a 2-fold decrease of death rates from CHD between those with low and those with high birth weight has been reported.[24]

In this study, we set out to determine whether lipoprotein metabolism in neonates is affected by nourishment in utero. Alterations in lipoprotein profile could partly explain the proposed higher risk of SGA neonates for CHD, stroke, or diabetes mellitus in later life.

TG concentration was highest in SGA neonates when compared with AGA and LGA neonates. Several investigators, as well our laboratory, proved TG to act as independent predictors of coronary artery disease (CAD).[23-26] Higher TG levels in SGA neonates could partly explain the proposed increased risk of CHD in those subjects in later life.

Higher concentrations of TG in plasma of SGA neonates might be due to increased secretion of TG-rich lipoproteins by the liver or decreased lipolysis or reuptake of TG-rich lipoproteins. In a state of undernourishment, it is conceivable that impaired clearance of TG-rich lipoproteins rather than increased secretion of TG-rich lipoproteins contributes to the higher TG levels observed in SGA neonates. Lipoprotein concentrations were similar in LGA and AGA neonates suggesting

Table 4. Lipoprotein Profile in SGA, AGA, and LGA Neonates (mean ± SD)

	SGA (n = 14) (3 F/11 M)	AGA (n = 38) (16 F/22 M)	LGA (n = 12) (4 F/8 M)	*P* Values SGA *v* AGA	*P* Values SGA *v* LGA
Total cholesterol (mg/dL)	56 ± 17	59 ± 15	62 ± 13	.54	.28
TG (mg/dL)	60 ± 23	43 ± 11	43 ± 14	<.01	.04
LDL-C (mg/dL)	16 ± 9	18 ± 8	18 ± 8	.59	.58
HDL-C (mg/dL)	27 ± 12	37 ± 19	36 ± 9	.07	.06
HDL_2-C (mg/dL)	7 ± 4	13 ± 8	13 ± 5	.02	<.01
HDL_3-C (mg/dL)	20 ± 8	25 ± 13	25 ± 8	.21	.14
HDL_2/HDL_3 ratio	0.37 ± 0.14	0.58 ± 0.57	0.52 ± 0.17	.17	.11
Insulin (μU/mL)	4.7 ± 1.5	7.3 ± 3.6	10.3 ± 9.4	.01	.04

NOTE. Differences in lipoprotein profile as well as in insulin concentrations between AGA and LGA neonates did not reach statistical significance.

Abbreviations: F, female; M, male.

that birth weight is not the sole determinant of lipoprotein profile in neonates.

CETP is a key enzyme that affects reverse cholesterol transport from peripheral tissues to the liver. Exchange of neutral lipids by CETP leads to enrichment of apoB-containing lipoproteins with CE resulting in small dense LDL and TG-enriched smaller HDL.

Subjects homozygous for CETP-deficiency had markedly increased levels of HDL cholesterol.[26] A possible role of CETP in atherogenesis was investigated in CETP transgenic mice. Marotti et al[27] reported increased severity of atherosclerotic lesions in transgenic mice expressing simian CETP. Hayek et al[28] found a decrease of early atherosclerotic lesions in hypertriglyceridemic mice expressing CETP compared with control mice. Although these data appear confusing and in part controversial, they indicate an important role of CETP in atherogenesis. Besides CETP mass, TG concentration is a major determinant of CETP-activity in plasma. In SGA neonates, CETP-mass was lowest and CE-transfer was highest, suggesting that the environment of CETP rather than the quantity of CETP predicts CE-transfer.

Regarding the aspect that death rates from CHD in later life are higher in subjects with low birth weight, higher CE-transfer in SGA seems to be very interesting. High rate of CE-transfer results in low HDL-C, a negative risk factor of CHD,[29-32] increased TG concentrations, an independent risk factor of CHD,[25,33,34] and small, dense LDL particles, which are also associated with an increased risk of CHD.[35] This proatherogenic lipoprotein constellation might also partly explain the

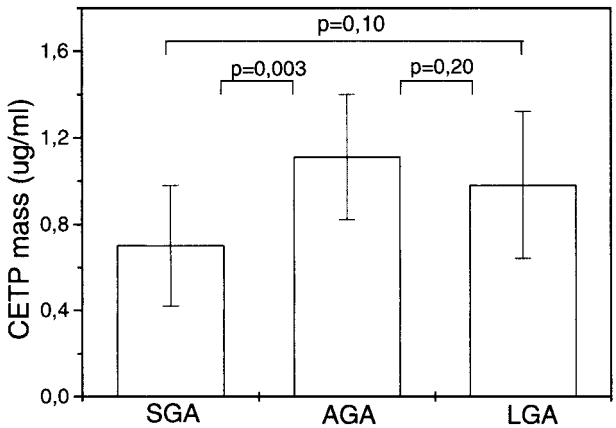
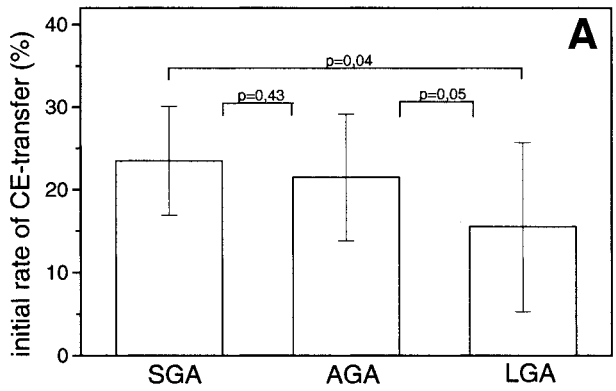


Fig 2. CETP-mass (μg/mL) with different birth weights in neonates. SGA (birth weight less than 10th percentile); AGA (birth weight between 10th and 90th percentile); LGA (birth weight greater than 90th percentile).



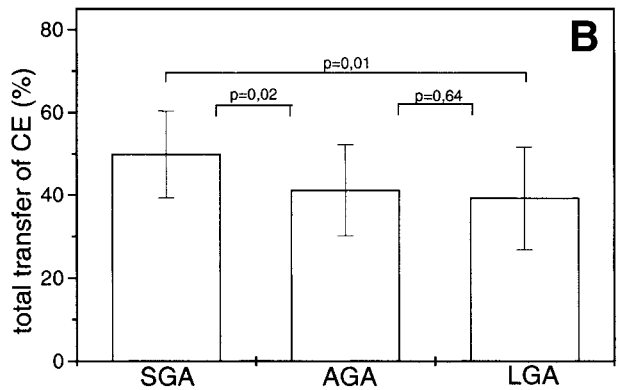


Fig 3. CE-transfer in SGA, AGA, and LGA neonates. Initial rate of CE-transfer (%) (A); (B), total transfer of CE (%). SGA (birth weight less than 10th percentile), AGA (birth weight between 10th and 90th percentile), LGA (birth weight greater than 90th percentile).

higher risk of CHD in SGA neonates in later life. Therefore, we hypothesize that high TG-concentrations in SGA neonates do not only reflect, but also determine the rate of CE-transfer.

Considering the relationship of lipoprotein concentrations in neonatal and maternal plasma, the rate of CE-transfer was relatively high in neonatal plasma when compared with maternal plasma. Differences in lipoprotein composition may explain the high rate of CE-transfer in neonates. Differences in distribution of apolipoproteins in lipoproteins of neonates have already been described.[15,17] In this study, we showed that LDL particles in neonates are smaller compared with LDL particles of adults, suggesting decreased CE and increased TG content of LDL particles in neonatal plasma.

In contrast to CETP-mass, no correlation was observed between CETP-activity and CE-transfer in plasma of mothers and those in plasma of respective neonates. Therefore, CETP-mass partly appears to be determined genetically, while CETP-activity and CE-transfer appear to be mainly influenced by exogenous factors, such as nourishment.

In contrast to the finding of Loughrey et al,[36] TG levels in neonates were independent of gender. We found significantly higher levels of total cholesterol in female neonates, which were due to increased LDL-C concentrations and not due to differences in HDL concentrations.

In conclusion, our study suggest that in neonates lipoprotein profile, CETP-mass, and CE-transfer, respectively, are influenced by undernourishment in utero. High TG levels in SGA neonates might partly contribute to high CE-transfer in SGA neonates and, as a consequence, to an atherogenic lipoprotein profile in neonates that might partly explain the proposed higher prevalence of deaths from CHD in later decades.

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