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J. Perinat. Med. 2017; 45(7): 829–835

Ulrich Pecks*, Werner Rath, Dirk O. Bauerschlag, Nicolai Maass, Thorsten Orlikowsky, Markus G. Mohaupt and Geneviève Escher

Serum cholesterol acceptor capacity in intrauterine growth restricted fetuses

Abstract

Aim: Intrauterine growth restriction (IUGR) is an independent risk factor for the development of cardiovascular diseases later in life. The mechanisms whereby slowed intrauterine growth confers vascular risk are not clearly established. In general, a disturbed cholesterol efflux has been linked to atherosclerosis. The capacity of serum to accept cholesterol has been repeatedly evaluated in clinical studies by the use of macrophage-based cholesterol efflux assays and, if disturbed, precedes atherosclerotic diseases years before the clinical diagnosis. We now hypothesized that circulating cholesterol acceptors in IUGR sera specifically interfere with cholesterol transport mechanisms leading to diminished cholesterol efflux.

Methods: RAW264.7 cells were used to determine efflux of [^{3}H]-cholesterol in response to [umbilical cord serum (IUGR), n = 20; controls (CTRL), n = 20].

Results: Cholesterol efflux was lower in IUGR as compared to controls [controls: mean 7.7% fractional [^{3}H]-cholesterol efflux, standard deviation (SD) = 0.98; IUGR: mean 6.3%, SD = 0.79; P < 0.0001]. Values strongly correlated to HDL (ρ = 0.655, P < 0.0001) and apoE (ρ = 0.510, P = 0.0008), and mildly to apoA1 (ρ = 0.3926, P = 0.0122) concentrations.

*Corresponding author: Ulrich Pecks, Department of Obstetrics and Gynecology, University Hospital of Schleswig-Holstein Campus Kiel, Arnold-Heller-Straße 3, 24105 Kiel, Germany, Tel.: + 49 431 597 2100, E-mail: Ulrich.Pecks@uksh.de; and Department of Obstetrics and Gynecology, University Hospital of the RWTH, Aachen, Germany
Werner Rath, Dirk O. Bauerschlag and Nicolai Maass: Department of Obstetrics and Gynecology, University Hospital Schleswig-Holstein, Kiel, Germany
Thorsten Orlikowsky: Section Neonatology, Department of Pediatric and Adolescent Medicine, University Hospital of the RWTH, Aachen, Germany
Markus G. Mohaupt and Geneviève Escher: Department of Nephrology, Hypertension and Clinical Pharmacology and Department of Clinical Research, Inselspital University Hospital Bern, Bern, Switzerland

Conclusions: Reduced cholesterol efflux in IUGR could account for the enhanced risk of developing cardiovascular diseases later in life.

Keywords: Atherosclerosis; cholesterol; efflux; fetal programming; IUGR; lipids.

Introduction

Intrauterine growth restriction (IUGR) refers to a condition in which the fetus does not reach its genetically given growth potential. It is an interdisciplinary challenge for obstetricians and pediatricians affecting approximately 3%–8% of all pregnancies and contributing majorly to fetal and neonatal morbidity and mortality. Early (preterm) delivery is the only treatment option to prevent intrauterine death and fetal asphyxia [1]. Apart from its immediate clinical relevance, IUGR has an enormous socioeconomic impact [2] and is generally considered as an independent risk factor for the development of cardiovascular diseases later in life [3, 4].

The pathomechanisms by which the intrauterine condition increases the atherosclerotic burden is still a matter of debate [5, 6]. We and others recently showed that cord blood lipid profile of IUGR fetuses favors a high-density lipoprotein (HDL) depleted phenotype leading to an increase in low-density lipoprotein (LDL)/HDL ratio. This clearly distinguishes IUGR from constitutional small for gestational age and adequate for age-weighted preterm and term born fetuses [7, 8]. In general, a low level of HDL-cholesterol is a major independent risk factor for atherosclerotic cardiovascular disease in adults [9]. The antiatherogenic properties of HDL are mainly related to its role in reverse cholesterol transport from the periphery to the liver and its rate-limiting step, the cholesterol efflux from the cells into the circulation, which protects the cells from cholesterol overload. On the cellular level, cholesterol efflux from macrophages or endothelial cells involves a stepwise cascade with cholesterol being effluxed to its circulating acceptors in the blood, namely, apolipoprotein A1 (apoA1) and HDL, by the ATP binding cassette transporters ABCA1 and ABCG1, and the scavenger receptor B1 (SR-B1) pathways [10, 11]. The capacity of blood to accept

cholesterol from the cells depends on the concentration and functionality of the circulating cholesterol acceptors and can be measured *ex vivo* using cell-based cholesterol efflux assays. Clinical studies revealed inverse correlations between cholesterol acceptor capacity and prevalent coronary artery disease [12–14]. Moreover, the HDL-mediated acceptor capacity has been demonstrated to be a more powerful determinant of future incident cardiovascular outcome over a 9-year observation period than classic risk factors or HDL concentration alone [15].

The aim of the present investigation was to characterize cholesterol acceptor capacity of cord blood from IUGR and a control (CTRL) group by the use of a well-established mouse-macrophage-based cholesterol efflux assay [10, 16, 17]. We hypothesized that cholesterol homeostasis in IUGR fetuses is disturbed by reduced acceptor capacity, taking into account the unique acceptor composition with apoE beside HDL and apoA1 playing a functional role in fetal cholesterol transport [11, 17]. We speculate that a lower fetal cholesterol acceptor capacity during a critical fetal developmental phase is a determinant of atherosclerotic diseases later in live.

Materials and methods

Study cohort – umbilical vein serum samples

Venous cord blood was sampled at a single university hospital between March 2008 and March 2012. The study was approved by the Institutional Ethics Committee (EK 119/08 and 154/11). The authors complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. Written informed consent was obtained from all participating parents.

IUGR was diagnosed antenatally as recently described [8] and defined in accordance with the guidelines of the American College of Obstetricians and Gynecologists [1] as an estimated fetal weight < 10th percentile in addition to at least one of the following criteria: (1) deceleration of fetal growth rate > 40th percentiles, (2) elevated resistance index in umbilical artery Doppler sonography above the 95th percentile, (3) head-to-abdominal circumference ratio > 95th percentile, or (4) amniotic fluid index < 6 cm. Five of the IUGR cases additionally were complicated by preeclampsia as defined by the ISSHP criteria [18].

A total of 299 patients giving birth at the university hospital were enrolled during the study period. Of those, we identified 20 IUGR cases with sufficient serum volume to be further used for the planned analyses. Sixteen of the IUGR fetuses needed mandatory preterm delivery before 37 weeks of gestation (WOG). The IUGR cases were matched with CTRL as closely as possible for maternal age, gestational age, fetal gender, betamethasone administration, maternal smoking habits, and maternal body mass index (BMI). Neonatal

weight in the CTRL group was within the 10th and 90th percentiles. Fourteen of the 20 CTRL fetuses were born preterm before 37 WOG for various reasons (premature rupture of the membrane, spontaneous onset of labor, and vaginal bleeding). None of the CTRL mothers suffered from hypertension or preeclampsia.

Exclusion criteria were defined as multiple gestation, fetal anomalies, abnormal fetal karyotype, patients with clinical or biochemical signs of infection, positive toxoplasmosis, rubella, cytomegalovirus, herpes simplex, and HIV (TORCH) screening results, maternal diabetes mellitus/gestational diabetes or other severe maternal metabolic disorders, and the patient's withdrawal from the study. All neonates were delivered by caesarean section. Sample storage times and conditions were equal for all groups.

Blood sampling, serum generation, and storage of aliquots

Blood samples (up to 4.9 mL each) were taken postnatally from a double-clamped umbilical cord vein using Monovette syringes (Serum 4.9 mL Monovette; Sarstedt, Nümbrecht, Germany). After incubation at room temperature for 15–30 min, samples were centrifuged at 2000 g for 15 min. Serum was aliquoted and stored at − 80°C.

Basic serum lipid profiling

Analysis of serum triglycerides (TG), TC, LDL- and HDL-cholesterol, and total protein (TP) was performed by colorimetric enzymatic methods using an automated photometric measuring unit (Roche/Hitachi Modular P800; Roche Diagnostics, Basel, Switzerland) as described [8].

Enzyme-linked immunosorbent assay (ELISA)

ApoA1 and apoE concentrations were determined by ELISA (Mabtech, Nacka Strand, Sweden) in duplicates according to the manufacturer's protocol [17].

Cholesterol efflux assays

Cholesterol efflux analysis was determined as described [16, 17] with RAW264.7 (ATCC, USA) using the various sera as acceptor. Cells were labeled for 48 h with 0.2 µCi/mL [³H]-cholesterol (Anawa, Switzerland), washed six times with phosphate-buffered saline (PBS, pH 7.4), and further equilibrated for 18 h in OptiMem. Cells were washed with PBS and further incubated in the presence or absence of 5% serum 120 min. Radioactivity was measured in medium and cells. Fractional [³H]-cholesterol efflux was calculated as the percentage of labeled cholesterol released to the medium divided by the amount of total labeled cholesterol in the medium and cells. A standard serum pool was prepared from three healthy (nonpregnant) volunteers, aliquoted and frozen at − 80°C, and used with each assay as a reference. Intra- and interassay variance was less than 10%.

Table 1: Clinical characteristics of the study cohort.

		CTRL		IUGR		P-value
		Mean or n	SD or %	Mean or n	SD or %	
Total number of neonates	n	20		20		
Maternal age (years)	mean (SD)	32.6	6.0	29.5	7.2	0.2036
Maternal prepregnancy BMI (kg/m²)	mean (SD)	22.6	3.5	26.1	6.6	0.1591
Maternal parity (primiparity)	n (%)	6	30	12	60	0.1110
Maternal hypertension > 140/90 mm Hg	n (%)	0	0	5	25	**0.0471**
Maternal prepregnancy smoking status (smoker)	n (%)	10	50	7	35	0.5230
Maternal smoking status during pregnancy (smoker)	n (%)	4	20	5	25	1.0000
Gestational age at birth (weeks)	mean (SD)	34.2	3.4	34.2	3.2	0.9947
Neonatal gender (female)	n (%)	11	55		55	1.0000
Neonatal weight at birth (g)	mean (SD)	2349	788	1540	533	**0.0011**
Neonatal birth weight percentile	mean (SD)	45	15	4	3	**<0.0001**
Neonatal umbilical artery pH	mean (SD)	7.343	0.035	7.301	0.038	**0.0006**
Neonatal Apgar score	mean (SD)	9.5	0.7	9.3	0.9	0.6063
Betamethasone application during pregnancy	n (%)	8	40	10	50	0.7512
Betamethasone exposure (days prior to delivery)	mean (SD)	14.5	14.7	12.3	10.6	1.0000
Storage time at − 80°C (years)	mean (SD)	2.39	0.77	2.03	1.04	0.3044

P-value < 0.05 (bold) are considered as significant.

Statistics

nQuery Advisor 7.0 [Janet D. Elashoff (2007), CA, USA] was used for sample size calculation. The minimal required sample size was estimated on the basis of cord blood HDL-cholesterol concentrations published previously [8]. Choosing a power of 80% and a level of significance less than 0.05, at least five patients had to be included in each group.

Data analysis was conducted using the statistical packages Prism Version 6.0e Software (GraphPad Software Inc., CA, USA). Clinical data are presented as means ± standard deviations (SD) if metric. Categorical data are presented as frequencies and percentages. Laboratory variables are expressed as means ± SD. A two-tailed Mann-Whitney test was conducted for comparison of metric variables. Fisher's exact test was used for categorical data. Correlations were analyzed by Spearman's correlation coefficient. Values of P < 0.05 were regarded as significant.

Exploratory confounder analyses were done by linear regression. In case of association, the respective variable was further tested by subgroup analysis.

Results

Clinical characteristics of study cohort

Maternal and neonatal characteristics are summarized in Table 1. Maternal age, smoking habits, gestational age at delivery, days after betamethasone administration, and fetal gender were kept well adjusted. Seven patients in the IUGR group and five patients in the CTRL group were overweight to obese per definition of the World Health Organization with a prepregnancy BMI > 25 kg/m². Five (25%) of the 20 mothers of the IUGR group were hypertensive.

Serum biochemistry

Lipid and apolipoprotein profile

Lipid and apolipoprotein profile is displayed in Table 2. Mean TC concentration was 30.8% lower in IUGR as compared with CTRL (P < 0.0001). Mean HDL concentration was 59.4% lower (P < 0.0001), and mean LDL concentration

Table 2: Lipid, lipoprotein, and apolipoprotein concentrations.

	CTRL		IUGR		P-value
	Mean	SD	Mean	SD	
TC (mmol/L)	1.86	0.45	1.29	0.29	**<0.0001**
LDL-C (mmol/L)	0.70	0.33	0.53	0.27	0.0622
HDL-C (mmol/L)	0.92	0.19	0.37	0.11	**<0.0001**
LDL-C/HDL-C	0.78	0.39	1.44	0.67	**0.0002**
TG (mmol/L)	0.19	0.12	0.55	0.42	**<0.0001**
ApoA1 (µmol/L)	28.39	7.40	23.62	6.65	**0.0157**
ApoE (µmol/L)	2.96	2.41	1.57	1.34	**0.0002**
TP (g/L)	47.90	8.57	48.25	8.52	0.8670

P-value < 0.05 (bold) are considered as significant.

was lower by 24.0% (P = 0.0622) in the IUGR group as compared with CTRL. Consequently, the mean LDL/HDL ratio was 83.3% higher in the IUGR group as compared with CTRL (P = 0.0002).

Although mean apoA1 concentration was slightly (16.8%) lower in the IUGR than in the CTRL group (P = 0.0157), apoE levels were reduced by 46.8% in IUGR as compared with CTRL fetuses (P = 0.0002). Correlation analyses revealed a moderate correlation of apoA1 concentrations to TC (ρ = 0.480, P = 0.0017) and HDL (ρ = 0.344, P = 0.03) values. ApoE concentrations were highly correlated to HDL (ρ = 0.638, P < 0.0001) and mildly to moderately correlated to TC and LDL, respectively (TC: ρ = 0.466, P < 0.0025; LDL: ρ = 0.358, P < 0.0235).

Cholesterol efflux assays

Fractional cholesterol efflux was 18.4% lower when using serum of the IUGR group (mean = 6.25%, SD = 0.79; units: % fractional [³H]-cholesterol efflux) in comparison with the CTRL group (mean = 7.66%, SD = 0.98, P < 0.0001) (Figure 1). Overall fractional cholesterol efflux from RAW264.7 cells correlated moderately to highly to concentrations of the cholesterol acceptors HDL (ρ = 0.655, P < 0.0001) and apoE (ρ = 0.510, P = 0.0008), and mildly to apoA1 (ρ = 0.3926, P = 0.0122).
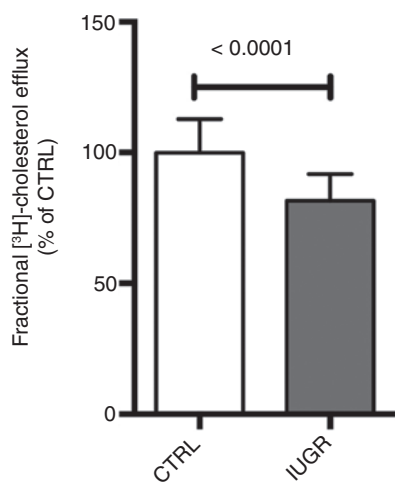

Figure 1: Estimating cholesterol acceptor capacity by cell-based [³H]-cholesterol efflux assay.
Cells were incubated with 5% serum for 2 h, and the percentage of cholesterol removal from the cells to the medium (fractional [³H]-cholesterol efflux) was calculated. IUGR (gray, n = 20) vs. CTRL (white, n = 20). Displayed are mean and SD values in % of CTRL. Statistical analysis was done by Mann-Whitney test. Significance is indicated by P-value.

Confounder analysis

Possible confounding factors were considered based on previous reports on cord blood lipid concentrations. These were gestational age at delivery and birth weight, neonatal weight percentile, gender of the neonate, fetal distress during labor as assessed by postpartum pH and a 5-min Apgar score, maternal smoking status, and betamethasone application to the mother [8, 17, 19–23]. We also analyzed the influence of blood pressure, maternal age, BMI, and parity. R^2 values of the linear regression analysis are presented in Table 3. Associations were found for BMI, parity, umbilical artery pH, and betamethasone application, and for neonatal birth weight and birth weight percentiles. As part of the definition of IUGR, the latter is not considered as confounders. The differences between IUGR and CTRL persisted when subgrouping lean (BMI < 25 kg/m²) and overweight (BMI ≥ 25 kg/m²) patients [lean: CTRL 7.88% (SD = 0.90) vs. IUGR 6.46% (SD = 0.85), P = 0.0004; overweight: CTRL 6.98% (SD = 1.00) vs. IUGR 5.85% (SD = 0.48), P = 0.048] and primiparous and multiparous mothers [primiparity: CTRL 7.34% (SD = 0.97) vs. IUGR 6.12% (SD = 0.87), P = 0.0320; multipara: CTRL 7.80% (SD = 0.99) vs. IUGR 6.46% (SD = 0.63), P = 0.0029]. Likewise, subgrouping patients by median of pH [higher than median pH: CTRL 7.84% (SD = 1.04) vs. IUGR 6.38% (0.94), P = 0.0052; lower pH: CTRL 7.44% (SD = 0.91) vs. IUGR 6.09% (SD = 0.57), P = 0.004] or by betamethasone application [betamethasone: CTRL 7.24% (SD = 0.79) vs. IUGR 5.96% (SD = 0.40), P = 0.0021; no betamethasone: CTRL 7.94% (SD = 1.03) vs.

Table 3: Confounder analysis.

	R²	P-value
Maternal age	0.059	0.131
Maternal prepregnancy BMI	**0.140**	**0.018**
Maternal primiparity	**0.125**	**0.026**
Maternal systolic blood pressure	0.077	0.084
Maternal diastolic blood pressure	0.041	0.211
Maternal prepregnancy smoking status	0.013	0.487
Maternal smoking status during pregnancy	0.010	0.531
Gestational age (weeks) at birth	0.032	0.267
Fetal gender	0.006	0.644
Fetal weight at birth	0.194	0.005
Fetal birth weight percentile	0.297	0.000
Fetal umbilical artery pH	**0.199**	**0.004**
Fetal Apgar score	0.043	0.200
Betamethasone application	**0.120**	**0.029**
Storage time at − 80°C	0.063	0.118

Higher R² values with a P-value < 0.05 (bold) are considered as a potential confounder.

IUGR 6.54% (SD = 0.98), P = 0.0071] persistently showed significant differences between the IUGR and CTRL group.

Discussion

In the present investigation, we have characterized the functional relevance of a low HDL concentration in the IUGR fetus with respect to the role of HDL and associated apolipoproteins in triggering cholesterol efflux. Uniquely, sera from IUGR fetuses were less likely to accept cholesterol. Both HDL and apoE concentrations are associated with cholesterol efflux values, but we observed only mild associations with apoA1. In a recent prospective study, the serum cholesterol acceptor capacity has been demonstrated to be a more powerful determinant of atherosclerosis than HDL concentration alone and an independent predictor of cardiovascular events over a 9-year observation period [15]. Yet the difference between IUGR and CTRL in the present study was twice as high as the effect recently reported by Khera et al. [12] who evaluated serum cholesterol acceptor capacity in adult patients with incident coronary artery disease.

In terms of atherosclerosis in human adults, the protective effect of HDL by promoting reverse cholesterol transport and its first and rate-limiting step, the efflux of cholesterol from cells of the periphery into an acceptor in the circulation, is evident [12, 15]. Studies in mice have shown that macrophage-specific cholesterol efflux and reverse cholesterol transport are inversely correlated with atherosclerotic lesion size [24]. Of note, HDL-mediated cholesterol efflux not only reduces atherosclerosis by preventing cells from cholesterol overload. In endothelial cells, HDL-promoted cholesterol efflux also activates endothelial nitric oxide synthase and interferes with the vascular endothelial growth factor receptor 2, thus promoting endothelial repair and angiogenesis [25, 26]. Defective cholesterol efflux has also been linked to increased platelet reactivity and proliferation *in vitro* [27]. Thus, HDL-mediated cholesterol efflux may have multiple atheroprotective functions. Regrettably, therapies targeting HDL and HDL composition to prevent cardiovascular events have been disappointing to date [28]. Therefore, pharmacologic interventions in adults mainly aim at lowering total and LDL cholesterol concentrations and at restoring LDL/HDL balance using statins and/or PCSK9 inhibitors [29].

Several studies suggest that atherogenesis in the fetus and in childhood is similar to those observed in adults involving LDL oxidation and the formation of fatty streaks [30]. In post mortem analyses of children aged 1–13 years, the number of aortic atherosclerotic lesions is inversely correlated to birth weight [31]. Clinically, in children born with IUGR, atherosclerosis is evident by ultrasound-based measurement of aortic intima-media thickening as early as postnatally and intrauterine [32–34]. Zanardo et al. [33] followed 25 IUGR children and 25 CTRL up to the age of 18 months. They found that persisting early childhood aortic thickening was associated with higher blood pressure values, suggesting a causal alteration of the vascular structure and function in IUGR-children.

Paradoxically to its future risk of atherosclerosis, and by contrast to the atherogenic profile in adults, beside lower HDL levels, lower cord blood total cholesterol concentrations in IUGR fetuses compared with CTRL fetuses were observed [7, 8]. This may be explained by several profound differences between the fetal and the adult lipid composition and metabolism. HDL is the most dominant lipoprotein in the fetus, and this results into a much lower LDL/HDL ratio than in adults [8]. Moreover, compared with other lipid and lipoprotein fractions, fetal HDL concentration and function is a remarkable constant throughout the second half of gestation, supporting a fundamental and highly conserved role for HDL in the maintenance of fetal cholesterol homeostasis [17]. Notably, apoE compared with other apolipoproteins is highly concentrated in the fetal circulation as levels are as high as in adults [35]. Moreover, fetal HDL-particle has been demonstrated to contain remarkably high amounts of apoE. Enrichment of HDL3 with apoE enhances cholesterol efflux as compared with apoE-free HDL3 particles [11]. These findings are supported by the present study because apoE levels in addition to HDL highly determined cholesterol efflux values. The source of cord blood apoE is still a matter of debate. Term placental trophoblasts are principally enabled to synthesize and secrete apoE. However, placental perfusion experiments revealed that more than 80% of the apoE synthesized in the placenta is secreted toward the maternal circulation [36]. A more likely source of fetal apoE may therefore be the fetal liver or the mother, possibly via placental uptake of maternal apoE-rich very low-density lipoprotein particles [37]. This interesting hypothesis merits further investigations and current studies are underway to address this question.

Taken together, our data are in concert with the present literature on subclinical atherosclerosis in IUGR neonates. We provide evidence for a well-established mechanistic link promoting premature atherosclerosis being applicable in the light of intrauterine development despite of a low-cholesterol availability.

Aachen and the University Hospital Bern have funded the project. The authors thank Brigitte Dix-Kuessner for her English language corrections.

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