Serum cholesterol acceptor capacity in intrauterine growth restricted fetuses

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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 [3H]-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 [3H]-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.

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 \textit{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 life.

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%.
were overweight to obese per definition of the World Health Organization with a prepregnancy BMI $>25$ kg/m$^2$. 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 were overweight to obese per definition of the World Health Organization with a prepregnancy BMI $>25$ kg/m$^2$. Five (25%) of the 20 mothers of the IUGR group were hypertensive.

### Table 2: Lipid, lipoprotein, and apolipoprotein concentrations.

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>IUGR</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>1.86 ± 0.45</td>
<td>1.29 ± 0.29</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>0.70 ± 0.33</td>
<td>0.53 ± 0.27</td>
<td>0.0622</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>0.92 ± 0.19</td>
<td>0.37 ± 0.11</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>0.78 ± 0.39</td>
<td>1.44 ± 0.67</td>
<td>0.0002</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.19 ± 0.12</td>
<td>0.55 ± 0.42</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>ApoA1 (µmol/L)</td>
<td>28.39 ± 7.40</td>
<td>23.62 ± 6.65</td>
<td>0.0157</td>
</tr>
<tr>
<td>ApoE (µmol/L)</td>
<td>2.96 ± 2.41</td>
<td>1.57 ± 1.34</td>
<td>0.0002</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>47.90 ± 8.57</td>
<td>48.25 ± 8.52</td>
<td>0.8670</td>
</tr>
</tbody>
</table>

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

### 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/m$^2$. Five (25%) of the 20 mothers of the IUGR group were hypertensive.
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 and LDL, respectively (TC: ρ = 0.638, P < 0.0001) and mildly to moderately correlated to 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.

**Table 3:** Confounder analysis.

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

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 persistent 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 intraperinatal development despite of a low-cholesterol availability.

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Author’s statement
Conflict of interest: Authors state no conflict of interest.

Material and methods: Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations and institutional policies, is in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee. All authors have done final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Author contributions: Ulrich Pecks: initial scientific idea and concept of the work, patient acquisition, interpretation of data, and drafting of the manuscript. Werner Rath: concept of the work and study design, interpretation of data, and revision of the manuscript. Dirk O. Bauerschlag: patient acquisition, interpretation of data, and revision of the manuscript. Nicolai Maass: interpretation of data and revision of the manuscript. Thorsten Orlikowsky: interpretation of data and revision of the manuscript critically for important intellectual content. Markus Mohaupt: concept of the work and study design, interpretation of data, and revision of the manuscript critically for important intellectual content. Geneviève Escher: concept of the work, CE Assay analysis, and interpretation of data, and drafting of the manuscript.

References


