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Characterization of lipoproteins in human placenta and fetal circulation as well as gestational changes in lipoprotein assembly and secretion in human and mouse placentas

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Abstract

In the maternal circulation, apoB-containing low-density lipoproteins (LDL) and apoA1-containing high-density lipoproteins (HDL) transport lipids. The production of lipoproteins in the placenta has been suggested, but the directionality of release has not been resolved. We compared apolipoprotein concentrations and size-exclusion chromatography elution profiles of lipoproteins in maternal/fetal circulations, and in umbilical arteries/veins; identified placental lipoprotein-producing cells; and studied temporal induction of lipoprotein-synthesizing machinery during pregnancy. We observed that maternal and fetal lipoproteins are different with respect to concentrations and elution profiles. Surprisingly, concentrations and elution profiles of lipoproteins in umbilical arteries and veins were similar indicating their homeostatic control. Human placental cultures synthesized apoB100-containing LDL-sized and apoA1-containing HDL-sized particles. Immunolocalization techniques revealed that ApoA1 was present mainly in syncytiotrophoblasts. MTP, a critical protein for lipoprotein assembly, was in these trophoblasts.

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Author contributions

TN performed experiments, analyzed and interpreted data, generated graphs, and summarized results. XL performed immunofluorescence microscopy, participated in analysis of results, read the manuscript and provided comments. EG was responsible for timed pregnancy, collection of placentas at different times during pregnancy, and quantification of apoB and apoA1 mRNA in mouse placentas. LI performed the explant culture studies with EMG. EMG performed immunohistochemistry microscopy and provided comments. SS performed western blot analyses for proteins in human and mouse placentas. DK collected, maternal blood, cord blood and amniotic fluids at term. LQ secured funding, supervised mouse experiments, and critically read and edited the manuscript. NH discussed experiments, provided human placental samples and critically reviewed and edited the manuscript. MMH conceived the idea, supervised the project, secured funding, generated the study hypothesis and design, and extensively edited the manuscript.

Declaration of interest.

Authors have no conflicts to report.

ApoB was in the placental stroma indicating that trophoblasts secrete apoB-containing lipoproteins into the stroma. ApoB and MTP expressions increased in placentas from the 2nd trimester to term, whereas apoA1 expression was unchanged. Thus, our studies provide new information regarding the timing of lipoprotein gene induction during gestation, the cells involved in lipoprotein assembly and the gel filtration profiles of human placental lipoproteins.

Next, we observed that mouse placenta produces MTP, apoB100, apoB48 and apoA1. The expression of genes gradually increased and peaked in late gestation. This information may be useful in identifying transcription factors regulating the induction of these genes in gestation and the importance of placental lipoprotein assembly in fetal development.

Keywords

Microsomal triglyceride transfer protein; apolipoproteins; maternal-fetal barrier; apolipoprotein B; apolipoprotein A1; triglyceride; cholesterol

INTRODUCTION

The placenta is a physiological and functional interface that serves as a barrier between the mother and the fetus. Its main functions are to exchange nutrients, oxygen and waste products, as well as to protect the fetus from the maternal immune system [1–7]. The functional unit of the placenta consists of a stromal core surrounded by two layers of trophoblasts: the inner cytotrophoblast and outer multinucleated syncytiotrophoblast. The stromal cells include macrophages, mesenchymal stromal cells, fibroblasts and fetal endothelial cells. The cytotrophoblasts proliferate and undergo cell fusion, thereby forming multinucleated syncytiotrophoblast. The syncytiotrophoblast is in contact with the maternal circulation and form the maternal-fetal barrier. The human placenta contains a single syncytiotrophoblast layer, whereas the mouse placenta has two layers [3]. In addition, mouse placenta shows significant differences with regard to molecular mechanisms, hormonal regulation and microRNA expression levels with human placenta [8]. Nevertheless, studying mouse and human placentas may enhance our understanding about similarities and differences in lipid and lipoprotein synthesis and transport in these species.

Fetal development is critically dependent on the supply of nutrients, including lipids and fat-soluble vitamins, from the maternal circulation. Lipoproteins in the maternal circulation are hydrolyzed on syncytiotrophoblast cell membranes, and free fatty acids are taken up by placental cells *via* diffusion or protein-mediated facilitated transport [9–11]. Several fatty acid-binding proteins are present in the cytoplasm of syncytiotrophoblasts. Within these cells, fatty acids are associated with acyl-CoA and esterified into complex lipids, such as triglycerides, or oxidized as a source of energy. In addition, fatty acids are exported from the basal membranes of these cells through unknown mechanisms, then reach the endothelium of fetal blood vessels. Other mechanisms of fatty acid transport in the placenta involve assembly and secretion of apolipoprotein B (apoB)-containing lipoproteins. The human placenta expresses apoB and microsomal triglyceride transfer protein (MTP), and it secretes apoB-containing lipoproteins [12] and apolipoprotein A1 (apoA1) [13, 14]. Madsen *et al.* have demonstrated that human placental trophoblast cell cultures assemble apoB100-

containing lipoproteins [12]. Subsequently, Kamper *et al.* showed that differentiated BeWo, human placental choriocarcinoma, cells in culture secrete apoB-containing lipoproteins on the maternal and fetal sides [15]. These lipoproteins are possibly crucial in the transport of lipids from the mother to the fetus [16, 17].

Beyond apoB-containing lipoproteins, high-density lipoproteins (HDL) also play a role in cholesterol transport [18–20]. In the maternal circulation, HDL is recognized primarily as a vehicle that transports cholesterol from peripheral tissues to the liver for catabolism and excretion from the body. During fetal development, systemic HDL is believed to deliver maternal cholesterol to the placenta. In this process, the syncytiotrophoblasts take up maternal cholesterol from HDL. These cells then excrete free cholesterol to acceptors such as apoA1 and HDL on the fetal side [18–20]. However, this concept has been challenged. Melhem *et al.* have shown that apoA1 concentrations are high toward the maternal apical side of the syncytiotrophoblast, and have advanced the hypothesis that HDL may be involved in cholesterol efflux from the placenta [13]. HDL is also a major cholesterol-carrying lipoprotein in the fetus [21].

Separately from maternal circulation and the placenta, the fetal circulation also contains lipid-carrying lipoproteins. Dolphin *et al.* have detected apolipoproteins and lipids in umbilical cord and maternal blood [22]. Nagasaka *et al.* have characterized HDL and LDL in human cord venous serum by using agarose gel electrophoresis and gel filtration chromatography [23]. However, these studies focused on neither placental lipoproteins nor the origin of fetal lipoproteins. Hence, a need exists to compare lipoproteins present in the placenta, the fetus and the maternal circulation, to address their origins, similarities and differences. Therefore, we characterized lipoproteins secreted by human placental explants and those present in the maternal and fetal circulation. We observed that the lipoproteins in fetal circulation are similar to those secreted by placental explants and dissimilar to those in the maternal circulation. Furthermore, we studied changes in mRNA expression of placental apoA1, apoB and MTP during gestation in humans and mice, and analyzed their protein expression.

MATERIALS AND METHODS

Study participants

All study participants provided informed consent to participate, and the study was approved by Institutional Review Board of NYU Langone Long Island Hospital, Mineola, NY. Placental tissues, amniotic fluid, umbilical venous cord blood, umbilical arterial cord blood and maternal blood samples were obtained from 20 healthy pregnant women delivering by cesarean section at term pregnancy. In addition, early pregnancy placental samples from the second trimester were collected after elective termination of presumed normal pregnancies.

Human hepatoma Huh-7 cell cultures

Human hepatoma Huh-7 cells were cultured in humidified 5% CO_2 incubators using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% L-glutamine at 37°C [24, 25].

Placental explant culture

Placental tissues were collected from the maternal side from random locations, as previously described [26]. Small biopsies were collected from middle regions of human placentas collected at term and placed in phosphate buffered saline (PBS). Before use, fetal membranes and decidual linings were removed, rinsed with PBS and sliced into 10 × 10-micrometer pieces with a Mc-Ilwain tissue chopper (Stoelting Co. Wood Dale, IL, USA). Sliced pieces were weighed and cultured in 10% FBS containing DMEM. For apoB and apoA1 secretion analysis, culture plates were incubated in a 5% CO_2 incubator overnight at 37°C. Conditioned medium (placental explant supernatant) was collected and stored at −80°C until apoB and apoA1 ELISAs were performed. For comparison, human hepatoma Huh-7 cells were cultured as previously described [25].

ELISA

Human apoB (Mabtech, 3715-1H-6) and apoA1 (R&D Systems, DY3664-05) concentrations in maternal serum, cord vein and cord artery serum, amniotic fluid, placental tissue lysate and cell culture supernatants were measured by ELISA in 96-well plates (Thermo Fisher Scientific, 07-200-640) and detected with 3,3',5,5'-tetramethybenzidine substrate (Thermo Fisher Scientific, 34041) according to the respective manufacturer's protocols. Standard curves were prepared in media containing 10% FBS used for cell culture. Placental tissue lysates were homogenized in RIPA (Bio Basic, RB4475) buffer containing protease inhibitor cocktail (Sigma, P2714). Homogenization was performed on ice with a tissue grinder (Wheaton, Potter-ELV). Homogenates were centrifuged at 14,000 × g for 20 minutes at 4°C to remove cell debris. Supernatants were collected, and total protein concentrations were measured with a BCA kit (Thermo Scientific™, 23225); the values were then used to normalize apoB and apoA1 concentrations in cell culture experiments.

Immunohistochemistry

For immunohistochemistry by peroxidase labeling, formalin-fixed paraffin embedded sections were deparaffinized. Antigen retrieval was performed with sodium citrate (10 mM, pH 6.0), and endogenous peroxidase was blocked with 3% hydrogen peroxide. After being blocked with 10% goat serum, sections were incubated with primary antibodies to human apoA1 (Abcam, ab52945, 1:100 dilution); apoB (R & D Systems, MAB4124, 1:400 dilution); and human MTP (Abcam, ab63467, 1:500 dilution). Normal rabbit and mouse IgG were used as negative controls for apoA1/MTP and apoB, respectively. After incubation with biotinylated goat anti-rabbit IgG (Vector, BA-1000, for apoA1 and MTP) and biotinylated goat anti-mouse IgG (Vector, BA-9200, for apoB), the primary antibody staining was visualized with a Vectastain Elite ABC kit (Vector, PK-6100) with ImmPact DAB as the substrate (Vector, SK-4105). The sections were counterstained with hematoxylin.

For immunofluorescence detection of apoA1, apoB and MTP, cryosections (10-μm thickness) of human placentas collected at term through cesarean section were fixed with buffered formalin. Antigen retrieval was performed by heating sections in 10 mM citrate and 0.05% Tween 20, pH 6.0, for 10 minutes in a microwave, and 30 minutes of cooling. After being washed in PBS, sections were incubated in 0.3 M glycine at room temperature for

20 min and blocked in blocking solution (5% normal goat serum and 2% BSA in PBS) for 1 hour. The primary antibodies mouse anti-apoA1 (Mabteck, 4H1, 1:200 dilution), mouse anti-apoB (R & D Systems, 4124, 1:100 dilution), rabbit anti-MTP (Abcam, ab86759, 1:100 dilution), mouse anti-cytokeratin (Thermo Fisher, MA5-11986, 1:100 dilution), rabbit anti-β-tubulin (Cell Signaling Technology, 2146, 1:200 dilution) and rabbit anti-HAI (Abcam, ab189511, 1:100 dilution), diluted in blocking solution were applied on the sections and incubated at 4°C overnight. Normal mouse IgG and rabbit serum were used as negative controls for apoA1/apoB and MTP, respectively. After being washed with 0.1% Triton X-100 in PBS, the sections were incubated with goat antimouse IgG-Dylight 488 (Abcam, ab96879, 1:1000 dilution) and goat anti-rabbit IgG-Dylight 550, (Abcam, ab96879, 1:1000 dilution) diluted in blocking solution at room temperature for 1 hour. The sections were then washed in 0.1% Triton X-100 in PBS and mounted in Prolong Gold Antifade reagent with DAPI (Life Technologies, P36935). The slides were analyzed with a Nikon Eclipse Ti confocal microscope (Nikon, Melville, NY USA).

Fast performance liquid chromatography

Lipoproteins were separated by fast performance liquid chromatography (FPLC) with a Superose 6 column. PBS was applied at a 0.2 mL/min flow rate, and 200 μL fractions were collected to measure apoB and apoA1 concentrations.

Western blot analysis

Biopsies from human placentas collected at term were homogenized in RIPA buffer (Fisher Scientific, j63306ap). Proteins (15–50 μg) were separated on gels to detect human apoB (Academy Biomedical Company, 205-G2, 1:1000 dilution), human apoA1 (Novus Biologicals, 2083D, 1:1000 dilution) and human β-actin (Cell Signaling Technology, 4967S, 1:1000 dilution). For human MTP detection (Abcam, ab63467, 1:1000 dilution), 250 μg of protein was immunoprecipitated with 1 μg of antibody and then subjected to western blotting.

For detection of these proteins in the mouse placenta, placentas were collected from wild type (WT) dams at 17.5 days post-coitum (dpc). ApoB (My Biosource, MBS2006107, 1:1000 dilution), apoA1 (Novus Biologicals, 2083D, 1:1000 dilution), MTP (Abcam, ab63467, 1:1000 dilution) and β-actin (Cell Signaling Technology, 4967S, 1:1000 dilution) were detected with commercial specific primary antibodies. The following corresponding secondary antibodies tagged with peroxidase enzymes were used at a dilution of 1:5000: goat anti-mouse IgG HRP (Invitrogen, 626520), anti-rabbit IgG HRP-linked antibody (Cell Signaling technology, 7074S) and rabbit anti-goat IgG HRP (Invitrogen, 81-1620). The bands were visualized with chemiluminescent substrates (Fisher Scientific, PI34578), and images were recorded with a ChemiDoc Touch Imaging System (Bio-Rad).

Real-time quantitative PCR

Total RNA was isolated from placental biopsies with TRIzol reagent (Thermo Scientific™, USA, 15596018), and concentrations were measured with a NanoDrop ND-2000 instrument (Thermo Scientific™, USA, ND2000). cDNA was synthesized with an Applied Biosystems™, High-capacity cDNA Reverse Transcription kit (Thermo Scientific™, USA,

4368813) from 1 μg RNA, and gene expression was determined by quantitative real time PCR (QuantStudio3, Applied Biosystems) with SYBR Green, as described previously [27]. Relative gene expression was quantified with the ΔΔCt method after normalization to Cyc1 mRNA for human samples or β-actin mRNA for mouse samples (Supplementary Table 1).

Timing of pregnancy and collection of placental samples in mice

These studies were approved by the Institutional Animal Care Committee at Rutgers University. WT mice (mixed background C57Bl/6 × 129Sv) were maintained on a 12:12 h light:dark cycle with lights on at 7:00 AM and fed a chow diet (PicoLab Rodent Diet 20, LabDiet Catalog number 5053) *ad libitum*. At 3 months of age, WT females were mated with WT males. Vaginal plug detection was timed as 0.5 days *post coitum* (dpc), the beginning of gestation.

Statistical analysis

Statistical analysis was performed in GraphPad Prism software (version 8.0.2). The data are shown as mean ± standard deviation. Numerical variables are compared with Student's *t*-test. P-values less than 0.05 are considered statistically significant.

RESULTS

Detection and localization of apoA1, apoB and MTP in human placental cells

We detected apoB100 (Fig. 1A), MTP (Fig. 1B) and apoA1 (Fig. 1C) by western blotting in human placental samples; these proteins had similar molecular weights to those in Huh-7 human hepatoma cells, which served as a positive control (Fig. 1A–C). Next, we investigated the presence of apoB, apoA1 and MTP in human placental samples with immunohistochemistry to identify their cellular localization. ApoB staining was observed in the trophoblast layer and the stromal area of the villi (Fig. 2A). ApoA1 protein was located primarily on the trophoblast layer in the placental villi (Fig. 2B). MTP was found predominantly in the trophoblast layer (Fig. 2C) but was also weakly expressed in the stromal region and the villous vessels (Fig. 2C). Furthermore, we investigated the cellular localization of these key proteins in fixed sections through immunofluorescence microscopy and assessed their co-localization with specific cellular markers (Fig. 3). Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a marker of cytotrophoblasts but not the syncytiotrophoblast layer [28–30]. Control IgG did not show any staining (Fig. 3A). Most apoB staining was immediately below the HAI-1 staining cells, thus indicating that apoB localized primarily in the stromal area of the villi and to a lesser extent in the trophoblasts (Fig. 3A, red arrowhead). We next investigated the cellular localization of apoA1 in placental sections (Fig. 3B). Tubulin staining revealed all cells present in the stroma [31]. ApoA1 staining was localized only to the outer layer of the syncytiotrophoblasts. MTP colocalized with cytokeratin-7, thus indicating the presence of this protein in the trophoblast layer (Fig. 3C), but MTP was also seen in mesenchymal cells (Fig. 3C).

Characterization of lipoproteins secreted by human placental explant cultures

We quantified apolipoproteins secreted by human term placental explant cultures by FPLC and compared them with those in the medium of human hepatoma Huh-7 cells, again

used as a control to determine elution profile of apolipoproteins (Fig. 4). The apoB in Huh-7 cell medium showed a homogeneous peak corresponding to plasma LDL (Fig. 4A). Most apoB from human term placentas was observed in a peak corresponding to LDL, but apoB also appeared in earlier fractions, thus indicating its presence in larger lipoproteins (Fig. 4A). These data indicate that human placental cultures may secrete heterogeneous apoB-containing lipoproteins differing in size.

Analysis of apoA1 revealed that Huh-7 cell medium contained apoA1 in fractions corresponding to plasma HDL (Fig. 4B). Similarly, apoA1 in the medium of human term placental explants was found in the same fractions. Thus, the conditioned medium of human placental explant cultures contains apoA1 as HDL, very similar to those found in Huh-7 medium.

ApoB and apoA1 containing lipoproteins in human maternal and fetal circulation

We quantified lipoproteins present in maternal and fetal blood at term in normal pregnancies. ApoB and apoA1 concentrations were measured in paired blood samples from the maternal vein, umbilical cord vein, umbilical cord artery and amniotic fluid collected at term (Fig. 5). We observed no differences in the apolipoprotein concentrations in the serum in umbilical veins and arteries (Fig. 5A, 5B). However, the amounts of apoB and apoA1 in cord veins and arteries were 10-fold lower than those in the maternal circulation. The lowest concentrations of apoB and apoA1 were found in the amniotic fluid.

Next, we plotted apoB and apoA1 levels in cord veins and arteries in paired samples and observed no specific directionality (Fig. 5C–D). In addition, we plotted differences between cord veins and cord arteries against cord vein apolipoprotein concentrations (Fig. 5E–F). These studies showed a significant linear correlation for apoB, but not for apoA1. We interpret these studies to suggest that apoB concentrations are under tight homeostatic control in fetal circulation. It is likely that when there is a decrease in cord artery apoB-containing lipoproteins, the CV will compensate by carrying more lipoproteins to the fetus from the placenta.

We then characterized lipoproteins present in the maternal venous blood, cord venous blood and cord arterial blood (Fig. 6). FPLC analysis of maternal serum revealed the presence of apoB in several fractions with two distinct peaks corresponding to VLDL and LDL (Fig. 6A), thereby indicating a heterogeneous population of apoB-containing lipoproteins. In cord venous and arterial serum, apoB was present in one homogeneous peak corresponding to LDL (Fig. 6A). ApoA1 in the maternal circulation was associated with several lipoproteins (Fig. 6B). Small amounts were found in the VLDL fraction, but substantial amounts were observed in intermediate-density lipoprotein fractions. In addition, a major broad peak of apoA1 was observed in the LDL and HDL fractions (Fig. 6B). In contrast, in cord venous and arterial serum, apoA1 was found mainly in the HDL fraction. Thus, lipoproteins in cord venous and arterial serum showed elution profiles distinct from those of maternal lipoproteins. The apolipoprotein concentrations were too low to determine lipoprotein profiles in amniotic fluid. In summary, apoB- and apoA1-containing lipoproteins in the fetal circulation, compared with those present in maternal circulation, were more homogeneous and eluted as one peak. Moreover, lipoproteins in the fetal circulation were similar to those

in the medium obtained from placental explants (Fig. 4), thus indicating that the placenta might be a source of apoB- and apoA1-containing lipoproteins in the fetal circulation.

Gestational age difference in apolipoprotein synthesis in human placentas

Next, we studied changes in gene expression in the human placenta during gestation. For this purpose, we compared human placental samples from 2nd trimester and term pregnancies, and measured different mRNA levels with quantitative real time PCR. The values of 18S, β-actin and Gapdh significantly differed between groups, as previously reported [32]. However, the Ct values of cytochrome C1 (Cyc1), TATA box-binding protein (Tbp) and DNA topoisomerase 1 (Top1) were not different between 2nd trimester and term placentas (Supplementary Table 2), in agreement with findings from other reports [32]. Therefore, the expression levels of apoB, apoA1 and MTP were normalized to those of Cyc1. ApoB (Fig. 7A) and MTP (Fig. 7B) mRNA levels increased significantly from 2nd trimester to term pregnancies. In contrast, apoA1 mRNA levels did not differ between 2nd trimester and term pregnancies (Fig. 7C). We also measured apoB and apoA1 protein levels in the placental explant tissue culture medium. In agreement with the changes in mRNA levels, apoB concentrations were significantly higher in term than 2nd trimester placental explant cultures (Fig. 7D). Moreover, similarly to mRNA levels, apoA1 protein concentrations did not significantly differ between the 2nd trimester and term placental culture supernatants (Fig. 7E). These findings suggest that the assembly and secretion of apoB-containing lipoproteins, but not apoA1-containing lipoproteins, increase in the placenta with gestational age. The highest expression of apoA1 might already be achieved by the 2nd trimester in the human placenta.

Developmental changes in apoB, MTP and apoA1 expression in mouse placentas

A limitation of the above human studies was that we could only obtain samples at two different time points during pregnancy. We hypothesized that a detail knowledge about the expression of these proteins can be obtained in experimental models used to study physiology and pathology. Therefore, to obtain a better indication of developmental changes, we measured apoB, MTP and apoA1 mRNA levels in mouse, the most used experimental animal model, placentas at different dpc, beginning from the time when the placenta is fully formed. We observed three phases in the induction of apoB expression during gestation (Fig. 8A). ApoB expression was low but still detectable at 9.5 and 10.5 dpc. Significantly higher levels were seen at 12.5 to 14.5 dpc than at 9.5 dpc. A sudden and significant (2- to 4-fold) increase in apoB expression was seen at 17.5 dpc, and expression peaked at 18.5 dpc (Fig. 8A). Overall, a >1,000-fold increase in apoB mRNA was observed from 9.5 dpc to 18.5 dpc. These data indicate that apoB is highly induced late in pregnancy close to term in mice.

Similar changes were observed for MTP (Fig. 8B). Mouse MTP mRNA levels were low until 10.5 dpc, then started to increase at 12.5 dpc. The highest induction of MTP expression was observed at 14.5 dpc. Subsequently, modest increases continued until 18.5 dpc, and maximal levels were seen at 18.5 dpc. The increases in MTP expression were approximately 20-fold from 9.5 dpc to 18.5 dpc.

ApoA1 mRNA expression levels were detectable during the early stages of gestation at 9.5 and 10.5 dpc in mice. A significant elevation was seen at 12.5 dpc, which was followed by gradual increases until 17.5 dpc. A sudden increase in apoA1 expression was seen at 18.5 dpc. Overall, a 3,000-fold increase in apoA1 mRNA was observed from 9.5 dpc to 18.5 dpc (Fig 8C).

Expression of apoB, MTP and apoA1 protein in mouse placentas

Subsequently, we confirmed the expression of these proteins in mouse term placentas (Fig 9). For comparison, we used mouse liver and plasma. Mouse placentas expressed ~100 kDa MTP (Fig. 9A) and ~25 kDa apoA1 (Fig. 9B) proteins similar to those in the liver and plasma, respectively. Analyses of apoB in mouse placentas revealed the presence of both apoB100 and apoB48 (Fig. 9C). The presence of apoB48 was unexpected. Therefore, we measured the expression of Apobec1, an enzyme that post-transcriptionally edits apoB100 mRNA [33]. We were able to measure Apobec1 mRNA levels in mouse placentas (Fig. 9E). These levels were approximately 5–10-fold lower than those in the liver and intestine. Attempts to detect Apobec1 protein levels with commercial antibodies were unsuccessful. Similarly, Ct values for Apobec1 mRNA levels were not detectable in Huh-7 cells and human placentas using two different primer sets, thereby indicating that the human liver and placenta do not express Apobec1 (not shown). In short, Apobec1 is expressed in mouse placenta, and the mouse placenta is likely to synthesize both apoB100 and apoB48, whereas the human placenta does not express Apobec1 and therefore synthesizes only apoB100.

DISCUSSION

In maternal circulation, fatty acids are transported via two distinct mechanisms. Adipose tissue hydrolyzes stored triglycerides and mobilizes free fatty acids for use by other tissues. In contrast, dietary and endogenous fatty acids are esterified, packaged and secreted as lipoproteins by the intestine and liver. This process is dependent on apoB and MTP. Significant efforts in the past have addressed the transport of free fatty acids in the placenta. Here, we concentrated on understanding the transport of esterified fatty acids, mainly triglycerides. Our studies showed that human apoB was present mainly in the stroma of the human placenta and to a lesser extent in the trophoblastic layer, whereas MTP was detected primarily in the trophoblastic layer and to a lesser extent in the stroma. The presence of MTP in the trophoblasts indicated that these cells may assemble and secrete apoB-containing lipoproteins, whereas apoB in stromal cells may represent secreted lipoproteins. Therefore, the placental system of triglyceride transport may be akin to the maternal system, in which lipoproteins are synthesized by the intestinal and liver cells, then deliver exogenous and endogenous lipids to peripheral cells. Placental trophoblasts synthesize and secrete apoB-containing lipoproteins, perhaps to deliver triglycerides and cholesterol to other placental and fetal cells. This system may also be responsible for the delivery of β-carotene from maternal circulation to the fetus that we have described in the past [16, 17].

Our studies may indicate directionality of apoB-lipoprotein transport in the placenta with important caveats. First, we show that apoB and MTP are in syncytiotrophoblasts.

Second, we performed immune-localization studies and observed that MTP is mainly in the syncytiotrophoblasts indicating that these cells can assemble and secrete apoB-containing lipoproteins. Third, we observed apoB in the stroma of the placenta. ApoB-containing lipoproteins may be a source of energy for stromal cells such as fibroblasts. In addition, these may represent lipoproteins that are in transit to fetal circulation. Fourth, we found apoB-containing lipoproteins in the cord blood. These studies suggest that apoB-containing lipoproteins are assembled and secreted by trophoblasts into the stroma. However, it is unclear how these lipoproteins travel through the stroma and reach cord blood. Previously, we have shown that β-carotene injected into pregnant dams reaches fetal circulation and have proposed that lipoproteins produced by placenta may transport β-carotene to embryo [16, 17]. More extensive studies involving development of novel technologies are needed to refine understanding about the trafficking of lipoproteins through stroma and entry of these lipoproteins into the fetal circulation.

Cholesterol is also transported by apoB-containing lipoproteins. However, its transport via HDL is considered more important for cholesterol homeostasis. Although cholesterol can be secreted from the liver and intestine as part of HDL, the main function of HDL is reverse cholesterol wherein HDL picks up cholesterol from peripheral tissues (cholesterol efflux) and delivers (cholesterol influx) it to the liver for excretion out of the body. The presence of apoA1 in the syncytiotrophoblast suggests its potential involvement in either cholesterol efflux or cholesterol influx. On the basis of the anticipated needs for lipids in growing fetuses, we propose that trophoblast control lipid trafficking by synthesizing and secreting apoB-containing lipoproteins into the stroma and in importing cholesterol from the maternal side. However, it is also possible that apoA1 may be involved in cholesterol efflux to avoid high cholesterol accumulation in the fetus.

Western blot analyses revealed that human placentas express apoB100, similarly to the human liver. In contrast, we observed the presence of both apoB100 and apoB48 in the mouse placenta, similarly to the mouse liver. Thus, the gene expression patterns in the placenta and maternal liver in both mice and humans are similar, and differ from those in the intestines, which synthesize exclusively apoB48.

The umbilical vein carries fresh oxygenated and nutrient-rich fetal blood to the fetal systemic circulation, whereas the umbilical arteries carry deoxygenated fetal blood and waste products toward the placenta for replenishment. Umbilical vein concentrations of retinol are higher than those in the umbilical artery [34]. The lower levels in the umbilical artery have been interpreted to be a consequence of consumption/uptake of retinol by the fetus. We measured apoB and apoA1 protein levels in umbilical arteries and veins, and did not find significant differences. No significant changes could be due to low contribution of placental lipoproteins to fetal circulation. Yet another possibility is that fetal circulatory lipoproteins are under tight homeostatic control to maintain their concentrations. We compared lipoprotein levels in the umbilical arteries and veins in individual babies and did not find any directionality (Fig. 5C–D). However, when we plotted differences in CV and CA against CV lipoprotein concentrations (Fig. 5E–F), we found a significant linear relationship for apoB-containing lipoproteins, but not for apoA1. Therefore, we suggest that homeostatic control mechanisms exist to maintain similar apoB-containing lipoprotein

concentrations in the fetal circulation. It is likely that when there is a decrease in CA apoB-containing lipoproteins, the CV will compensate by carrying more lipoproteins to the fetus from the placenta.

Our observation that apoB production is increased throughout gestation might suggest that the fetus is more dependent on maternal supply in early gestation till placental production is established as pregnancy advances. In short, there could be three sources of apoB-containing lipoproteins in the fetal circulation; mother, placenta and fetus. In early gestation, fetus may be dependent on fatty acid supply from the lipoproteins in the maternal circulation, but later in pregnancy it may shift to lipoproteins derived from placenta and/or the fetus.

We showed that the induction of apoB, MTP and apoA1 mRNA in gestation varies in humans and mice. ApoB and MTP mRNA levels increase over time during gestation, in both humans and mice. In contrast, we observed differences in the expression of apoA1 in human and mouse placentas. No significant changes in the expression of apoA1 occurred from the 2nd trimester (equivalent to 12.5 dpc in mouse) to term in humans; however, significant increases in apoA1 expression were observed beyond 13.5 dpc in the mouse placenta. Maximum expression of apoA1 might already be reached by the 2nd trimester in humans. If supported by additional studies, this finding may indicate that apoA1's role is more critical in early fetal development in humans than in mice.

Madsen *et al.* have studied secretion of apoB-containing lipoproteins after metabolic labeling with ^{35}S-methionine. Through sucrose density ultracentrifugation, they have shown that ^{35}S-labeled apoB100 lipoproteins have a density of 1.02–1.04 g/ml, corresponding to the buoyant density of human plasma LDL. They have ruled out the possibility that these lipoproteins are smaller because of lipolysis in the extracellular milieu. Beyond immunohistochemistry and mRNA quantification, we demonstrated that placental explant cultures secrete apoB and apoA1 as two discrete LDL and HDL sized particles. Similar lipoproteins were detected in the fetal circulation. On the basis of gel exclusion chromatography, these placental and fetal lipoproteins are distinct from those present in the maternal circulation, thereby indicating local placental production rather than systemic delivery of intact maternal plasma lipoproteins. Furthermore, the amounts of apoB and apoA1 in the fetal circulation were significantly lower than those in the maternal circulation. Thus, the amounts of apolipoproteins and the sizes of lipoproteins found in the fetal circulation differ from those in the maternal circulation and more closely resemble those secreted by placental trophoblasts. Lipoproteins found in the fetal circulation are likely to be of placental origin.

We compared the concentrations of apoA1 in maternal and fetal serum, and found that apoA1 in the maternal circulation is approximately 10-fold higher than that in the fetal circulation. Melhem *et al.* have compared the concentrations of apoA1 in maternal and fetal serum samples, and have found that apoA1 is ~6-fold higher in maternal serum than in the fetal circulation [13]. In contrast, apoE levels have been found to be similar in the maternal and fetal circulation [23]. We extended these comparative studies and examined the distribution of apoA1 in different lipoprotein fractions. ApoA1 was found exclusively in HDL-like particles in the fetal circulation.

ApoA1 was observed mainly on the maternal side of the trophoblasts. This finding agrees with studies demonstrating that apoA1-ABCA1 mediated cholesterol efflux occurs mainly toward the maternal side of the syncytiotrophoblast [19, 35]. Immunohistochemistry and mRNA quantification indicated that the placenta synthesizes apoA1. However, from our studies, whether apoA1 on the trophoblast surface is of placental or maternal origin, and whether this apoA1 is involved in cholesterol efflux are unclear. ApoA1 might potentially also be involved in cholesterol influx via its interaction with SR-B1. More studies are needed to establish the physiologic importance of the presence of apoA1 on the trophoblast surface and its role in the exchange and transfer of maternal-fetal lipids across the maternal-fetal barrier.

We show that placental explant cultures secrete apoA1, which is associated mainly with HDL-size particles in the medium. Recently, two groups have shown that the placenta synthesizes and secretes apoA1 [13, 14]. Using placental perfusion and primary trophoblast cells, Melhem *et al.* [13] have demonstrated that apoA1 is secreted toward both the apical and basal sides of primary trophoblast cells; however, most apoA1 was secreted toward the apical side. Secretion toward the apical side decreased during culture, whereas the secretion toward the basal side remained stable. However, they did not determine the form of apoA1 present in the conditioned medium. ApoA1 is known to be secreted as a lipid-free protein [36]. Our placental culture studies indicated that the placenta might possibly assemble and secrete HDL-like particles.

In the mouse placenta, the mRNA expression of MTP, apoB and apoA1 increased from 9.5 to 17.5 days during gestation, thus confirming the importance of placental lipoprotein assembly and secretion in trafficking lipids and other lipid-soluble molecules, such as vitamins—for instance, from the mother to the fetus—not only in humans but also in mammals in general. Of note, we identified mouse placental MTP and apoB as the downstream targets of a feed forward mechanism induced by β-carotene to enhance β-carotene transfer from mother to fetus to ensure proper embryogenesis [16, 17].

A notable limitation of our studies is that we examined the localization of different proteins through immunohistochemical techniques relying on commercial antibodies. *In situ* hybridization studies involving the detection of corresponding mRNA levels will be required to positively identify the cells that express and synthesize these proteins. Furthermore, gene ablation studies are needed to confirm and extend the knowledge and importance of these genes in placental and fetal lipid transport as well as in fetal development.

In this study, we used both human and mouse placenta to learn about placental lipid metabolism with a focus on acquiring information about lipid transport and lipoprotein biology in two different species. The aim was neither to compare and contrast species differences nor to suggest that mouse is a model to study human placental biology. We are aware of significant differences in mouse and human placental with respect to placentation, anatomy, endocrinology, microRNA expression etc. [8]. These studies are aimed to acquire knowledge and appreciate similarities and differences in two different species.

In summary, our studies identified cells that express apoB, MTP and apoA1 in the placenta and provided information about their temporal induction in the placenta during gestation. Our studies suggest that syncytiotrophoblasts assemble and secrete apoB-containing lipoproteins. These lipoproteins are also in the stroma and may represent their transit to the fetal circulation or may be a source of energy for stromal cells. We observed that apoB-containing lipoproteins are tightly controlled in fetal circulation. Studies in mice revealed significant induction in the expression of genes involved in lipoproteins production. This information should aid in identifying transcription factors that up-regulate gene expression and in generating placenta-specific knockout mice to address the roles of these genes in fetus development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations used:

apoA1	apolipoprotein A1
apoB	apolipoprotein B
BSA	bovine serum albumin
cyc1	cytochrome C1
dpc	days post-coitum
ELISA	enzyme linked immunoassay
HDL	high-density lipoproteins
IDL	intermediate density lipoproteins
LDL	low-density lipoproteins
MTP	microsomal triglyceride transfer protein
VLDL	very low-density lipoproteins

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Highlights

1. Human syncytiotrophoblasts synthesize apoB and apoA1 containing lipoproteins.
2. ApoB-lipoprotein concentrations are tightly controlled in fetal circulation.
3. Placental apolipoprotein expression is induced in late gestation in mice.

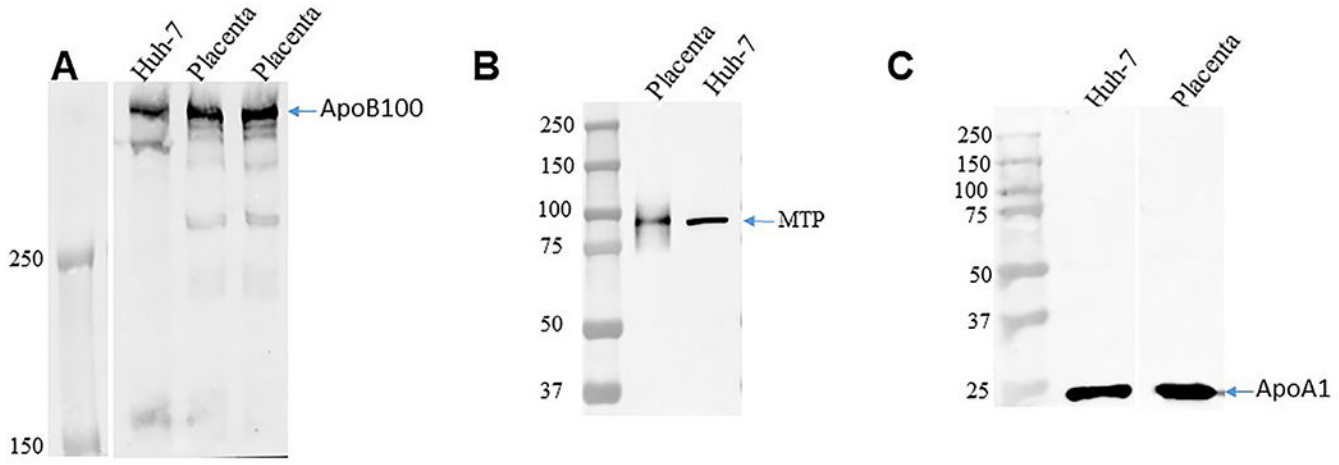


Figure 1: Detection of MTP, apoB and apoA1 proteins in human placentas.

Protein extracts were prepared from Huh-7 hepatoma cells (for control) and from placentas collected from normal term pregnancies, as described in the Materials and Methods. ApoB (6% gel) (**A**), MTP (10% gel) (**B**), and apoA1 (12% gel) (**C**) were detected with specific antibodies, as detailed in the Materials and Methods section. Huh-7 cells and placental lysates were run in different lanes on the same gels. When samples were not run side by side, lanes were separated by spaces. (A) For apoB, Huh-7 cell (25 μg, control for comparing sizes of different proteins and not concentrations) and placental (30 and 50 μg, experimental to determine whether placenta expresses same size proteins as in liver cells) proteins were used. (B) Placental proteins (250 μg) were immunoprecipitated with anti-MTP and then applied to gels. For comparison, Huh-7 cell proteins (25 μg) were applied. Blots were first treated with anti-MTP antibodies and then with VeriBlot IgG HRP-linked antibody (Abcam, 131366). (C) For apoA1, 30 μg of placental and Huh-7 cell proteins were used. Both samples were run on the same gel. However, in between these samples, there were samples that were unrelated to this study. Therefore, intervening lanes have been removed.

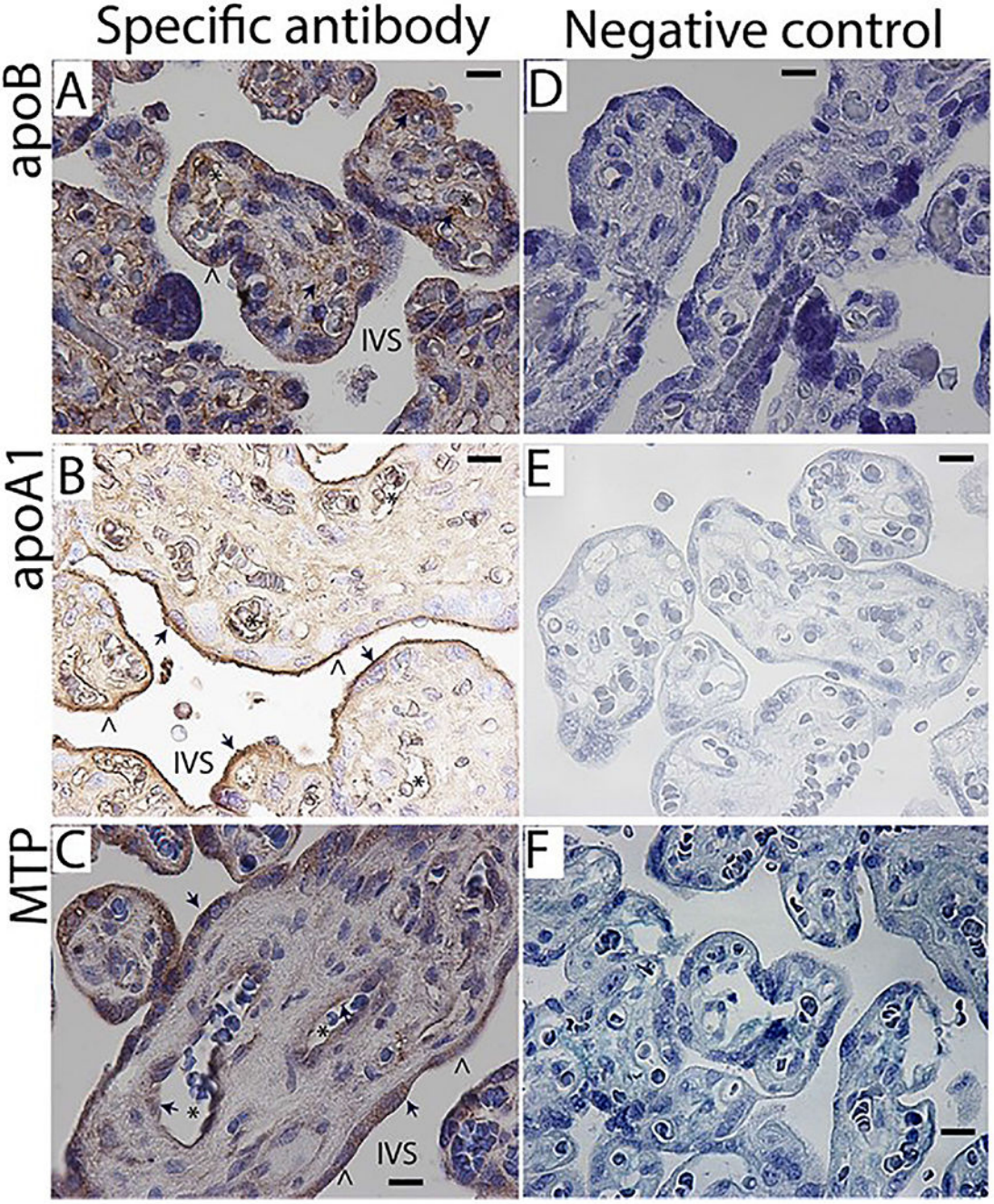


Figure 2: Immunohistochemical detection of apoB, apoA1 and MTP in term placentas.

Term placentas were fixed in paraformaldehyde and stained with primary antibodies against the specified proteins and counterstained with hematoxylin, as detailed in the Materials and Methods. Expression of apoB (**A**), apoA1 (**B**), and MTP (**C**) proteins in term placentas. Control IgGs do not show specific labeling (right panels). IVS, intervillous space; ^, syncytiotrophoblast; *, villous capillary. Scale bar, 10 μm. Black arrows indicate specific staining.

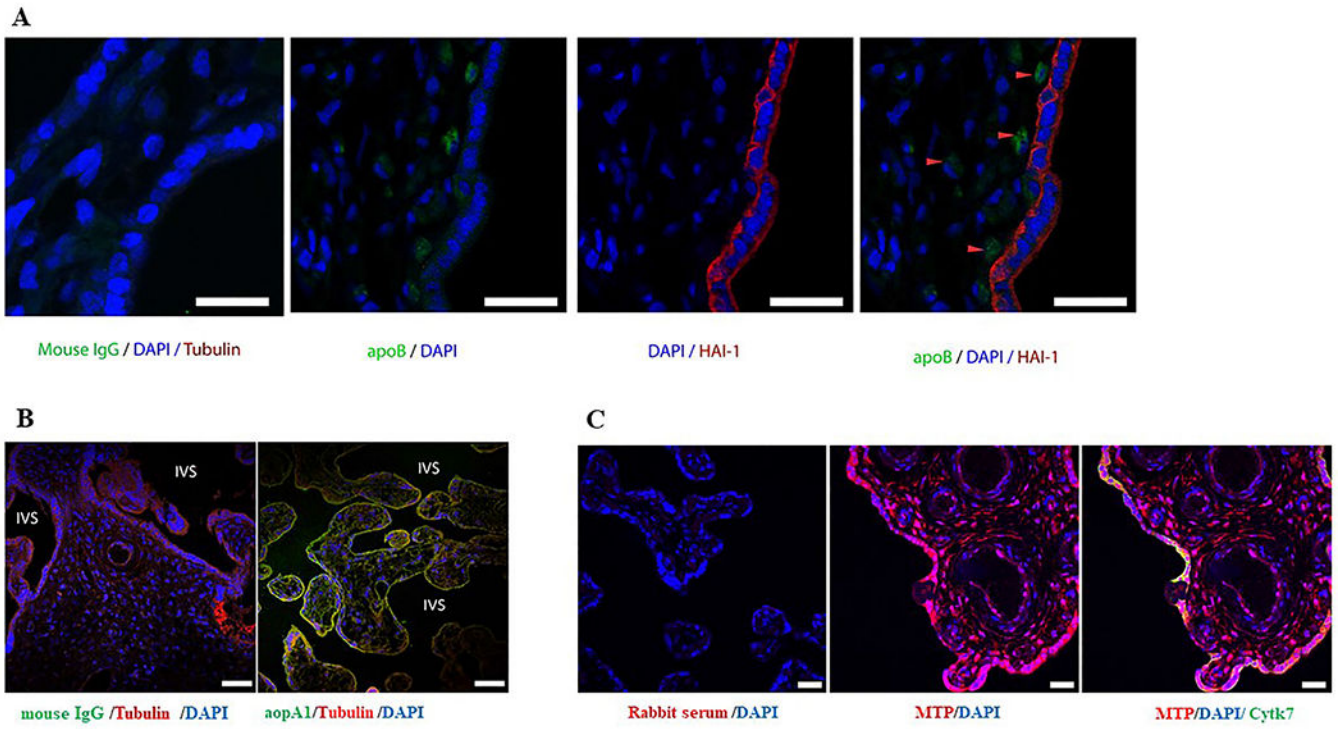


Figure 3: Cellular localization of apoB, apoA1 and MTP in term placentas, on the basis of immunofluorescence staining.

Human placental cryosections were stained with anti apoA1, apoB and MTP antibodies, and co-stained with tubulin, HAI-1 and cytokeratin 7. DAPI (blue) was used for nuclear staining. (**A**) Staining of human apoB: Anti-apoB staining (green) was observed in the placental stroma under the cytotrophoblasts stained with anti-HAI-1 antibodies (red). Scale bar, 20 μm. (**B**) Staining of apoA1: apoA1 stained green, and the placental tissue was delineated by anti-tubulin antibody, stained in red. ApoA1 localized to the maternal side of the trophoblast layer, as seen in (Fig. 2B). Scale bar, 50 μm. (**C**) Staining of MTP: anti-MTP antibodies recognize a protein in trophoblasts and in stromal cells (red). MTP co-localized with cytokeratin-7 (green). Scale bar, 20 μm.

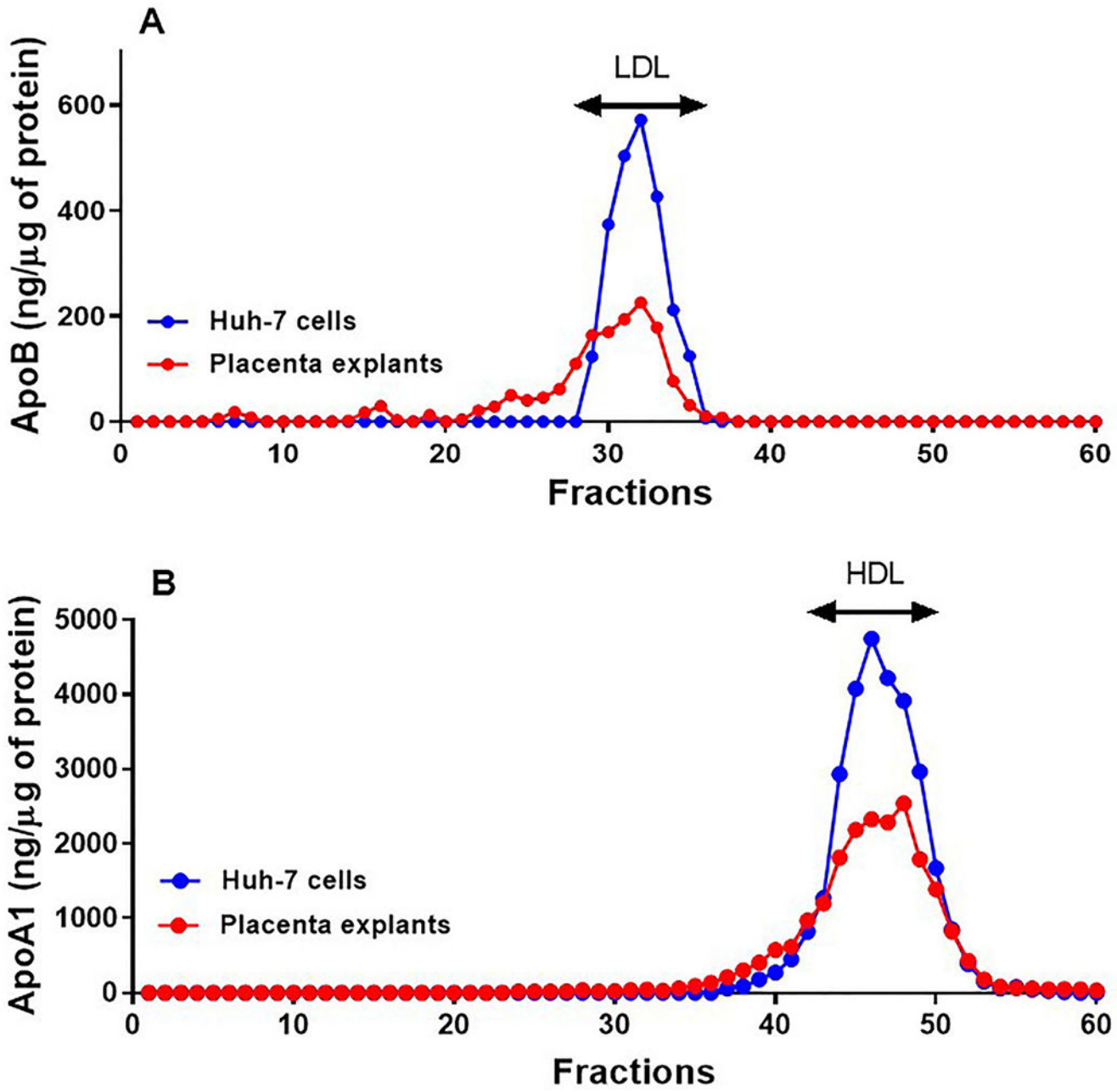
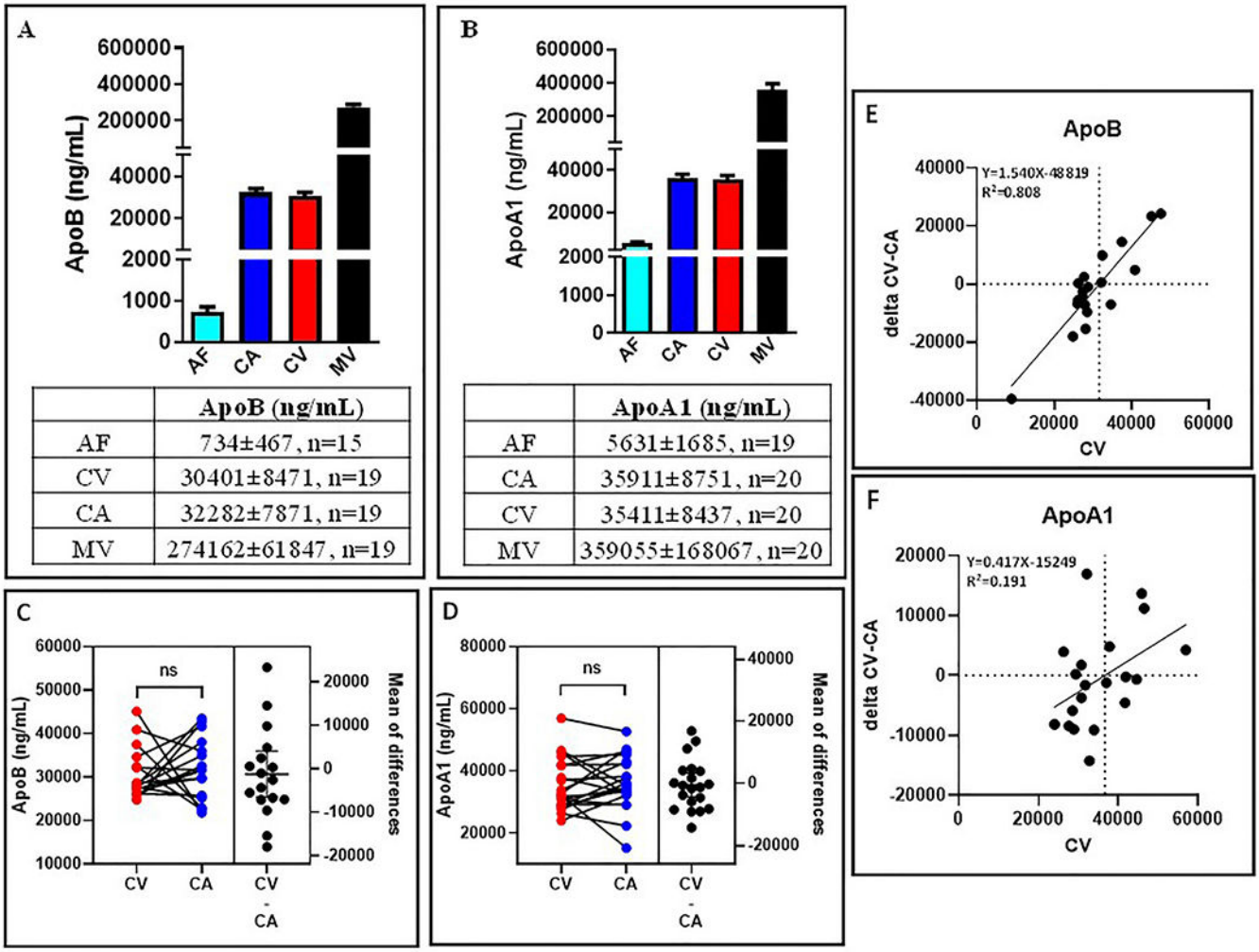


Figure 4: Gel filtration of apoB- and apoA1-containing lipoproteins secreted by human term placental cultures.

Human placentas were sliced (10 × 10-micrometers) and cultured overnight in 10% FBS containing DMEM. The medium was subjected to FPLC. ApoB (**A**) and apoA1 (**B**) were quantified in each fraction via specific ELISA, as detailed in the Materials and Methods. Medium obtained from Huh-7 cells was also subjected to FPLC and the elution profiles of apoB and apoA1 were plotted for comparison. The results are representative of four experiments.



	ApoB (ng/mL)
AF	734±467, n=15
CV	30401±8471, n=19
CA	32282±7871, n=19
MV	274162±61847, n=19

	ApoA1 (ng/mL)
AF	5631±1685, n=19
CA	35911±8751, n=20
CV	35411±8437, n=20
MV	359055±168067, n=20

Figure 5: ApoB and apoA1 concentrations in maternal serum, cord vein, cord artery and amniotic fluid.

Blood samples from the maternal vein (MV), cord vein (CV), cord artery (CA) and amniotic fluid (AF) were collected during normal term deliveries. Serum was isolated, and (**A**) apoB and (**B**) apoA1 concentrations were measured by specific ELISA, as detailed in the Materials and Methods. (**C-D**) paired apoB and apoA1 concentrations in CV and CA (left), and mean differences are plotted (right). (**E-F**) differences between CV and CA are plotted against concentrations in CV.

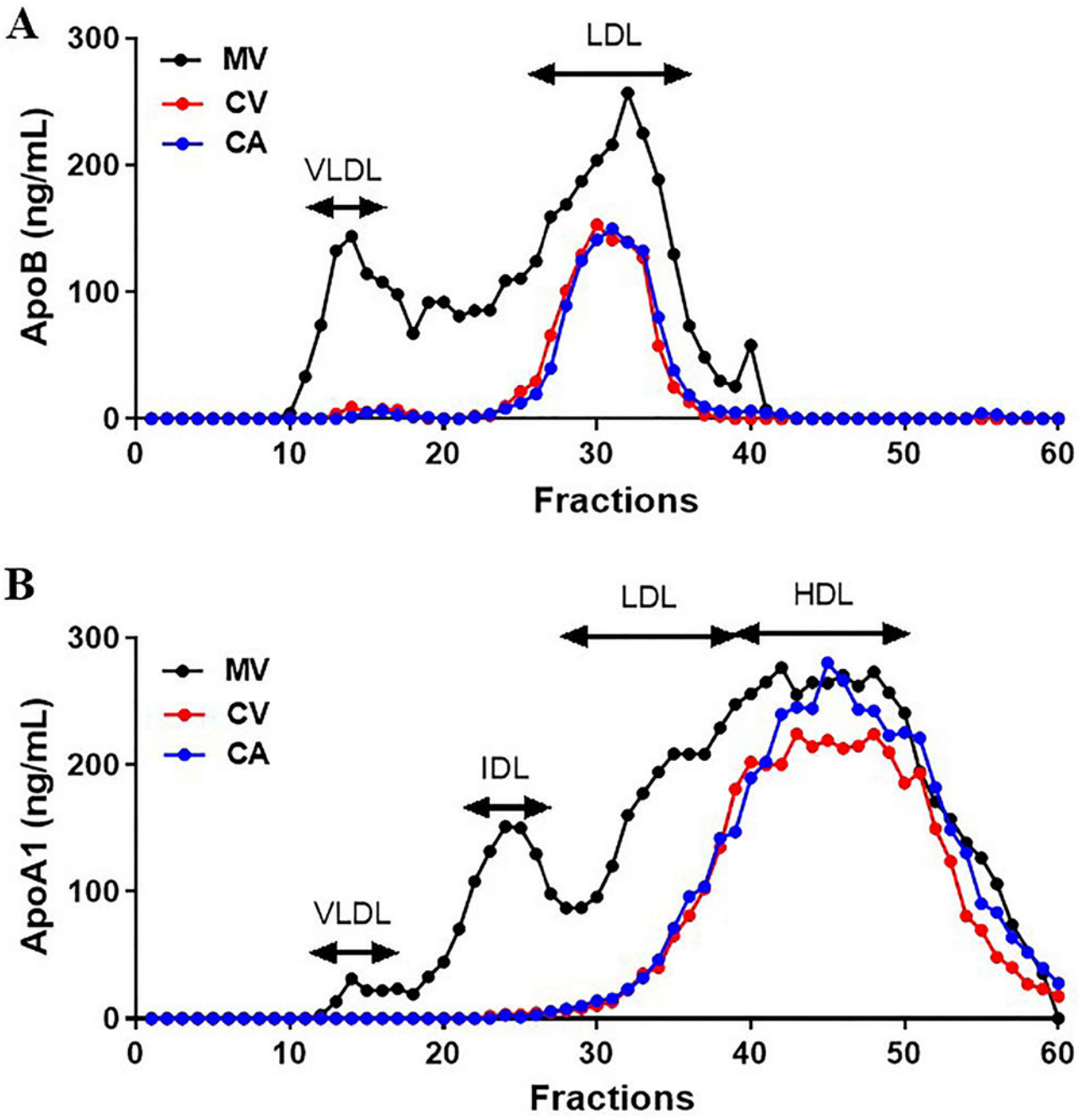


Figure 6: Characterization of lipoproteins in the maternal , cord vein and cord artery serum.

Serum was pooled from six mother-fetus pair-derived samples, and diluted 1:50. Subsequently, 250 μL serum was applied to the FPLC column. (**A**) ApoB and (**B**) apoA1 distribution in different fractions was measured by ELISA, as detailed in the Materials and Methods. CA, cord artery; CV, cord vein; MV, maternal vein.

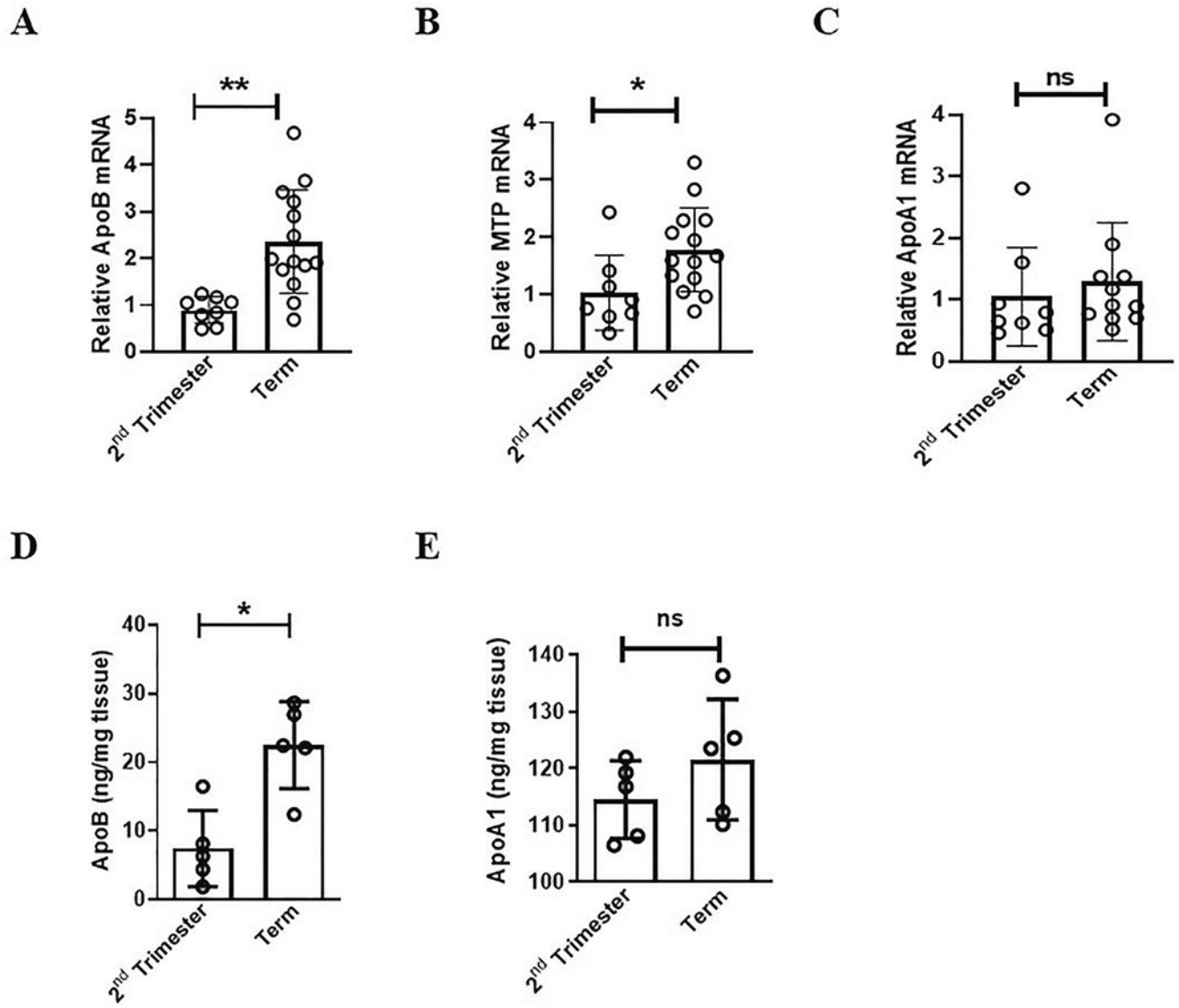


Figure 7: Expression levels, and synthesis and secretion, of apoB, MTP and apoA1 in placentas obtained from the 2nd trimester and at term.

(**A**-**C**) mRNA was isolated from placental biopsies from the 2nd trimester (n=8) and at term (n=14), and expression levels of apoB (**A**), MTP (**B**) and apoA1 (**C**) were measured. Human Cyc1 mRNA was used as a control. (**D**-**E**) Placental tissue explants (2nd trimester and term, n=5) were cultured overnight in 10% FBS containing DMEM, and supernatants were analyzed for apoB and apoA1 by ELISA, as detailed in the Materials and Methods. Statistical analysis was performed with Student's *t*-test, and data are represented as mean ± SD (error bars).

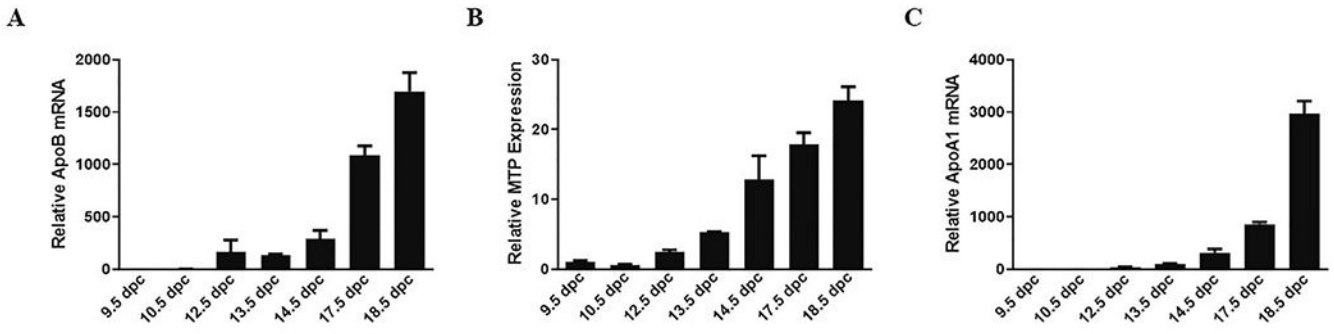


Figure 8: Changes in apoB, MTP and apoA1 mRNA levels in mouse placentas during gestation.

Pregnancy in female mice was timed, and placentas were collected at different gestation times from 9.5 to 17.5 dpc. Real-time qPCR analysis was performed to measure placental mRNA expression levels of apoB, MTP and apoA1. Data are mean ± SD, calculated with the $2^{-\Delta\Delta CT}$ method; n = 3/gestational time.

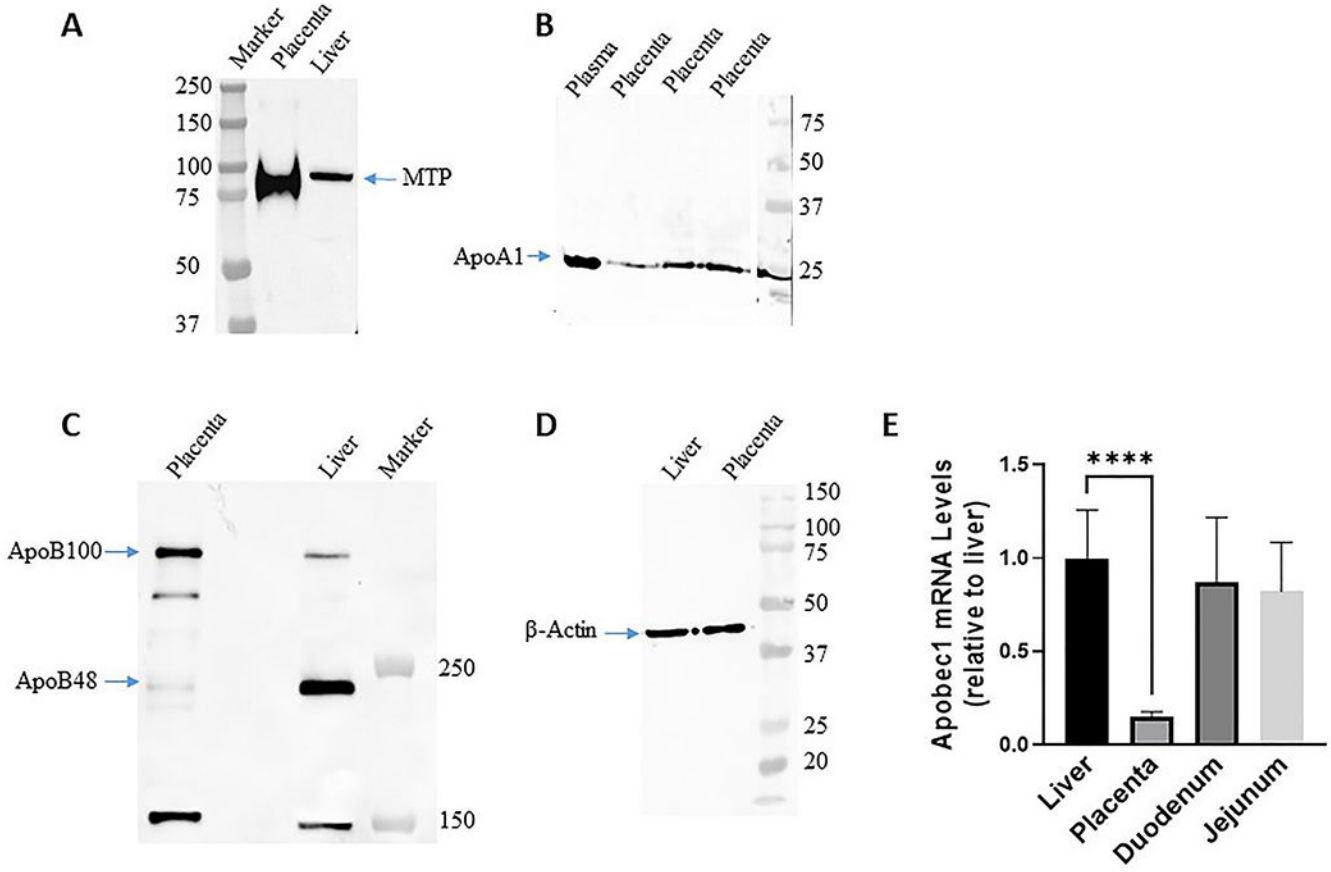


Figure 9: Mouse placental MTP, apoB, apoA1 and Apobec1 expression.

Placentas were collected from WT dams at 17.5 dpc. Representative western blots are shown. Mouse MTP (**A**), apoA1 (**B**), apoB (**C**) and β-actin (**D**) were detected with specific antibodies, as detailed in the Materials and Methods. (A) placental proteins (250 μg) were immunoprecipitated with anti-MTP antibodies (Abcam, ab63467) and applied to gels. Liver proteins (25 μg) were run for control. Membranes were probed with anti-MTP antibodies and VeriBlot (1:1000). (B) Placental proteins (30 μg) and mouse plasma was used for apoA1 detection. (C) For apoB, placental (50 μg) and liver (25 μg) proteins were used. (D) β-Actin was detected in 15 μg of placental and liver proteins. (E) Apobec1 and mRNA levels were measured in different mouse tissues by qRT-PCR.