Video Article

The 4-vessel Sampling Approach to Integrative Studies of Human Placental Physiology *In Vivo*

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Abstract

The human placenta is highly inaccessible for research while still *in utero*. The current understanding of human placental physiology *in vivo* is therefore largely based on animal studies, despite the high diversity among species in placental anatomy, hemodynamics and duration of the pregnancy. The vast majority of human placenta studies are *ex vivo* perfusion studies or *in vitro* trophoblast studies. Although *in vitro* studies and animal models are essential, extrapolation of the results from such studies to the human placenta *in vivo* is uncertain. We aimed to study human placenta physiology *in vivo* at term, and present a detailed protocol of the method. Exploiting the intraabdominal access to the uterine vein just before the uterine incision during planned cesarean section, we collect blood samples from the incoming and outgoing vessels on the maternal and fetal sides of the placenta. When combining concentration measurements from blood samples with volume blood flow measurements, we are able to quantify placental and fetal uptake and release of any compound. Furthermore, placental tissue samples from the same mother-fetus pairs can provide measurements of transporter density and activity and other aspects of placental functions *in vivo*. Through this integrative use of the 4-vessel sampling method we are able to test some of the current concepts of placental nutrient transfer and metabolism *in vivo*, both in normal and pathological pregnancies. Furthermore, this method enables the identification of substances secreted by the placenta to the maternal circulation, which could be an important contribution to the search for biomarkers of placenta dysfunction.

Video Link

The video component of this article can be found at https://www.jove.com/video/55847/

Introduction

According to the National Institutes of Health, USA, the placenta is the least understood organ in the human body [1,2,3]. It is difficult to access and study the human placenta *in vivo* without imposing unethical risks on the ongoing pregnancy. Studies of placental function in the human are therefore largely based on *in vitro* and *ex vivo* models. The majority of previous *in vivo* studies of placental transport and metabolism have been performed in animals [4,5,6]. However, as placental structure and functions vary considerably between species, extrapolation of results from animals to humans must be done with caution. Only a few smaller human *in vivo* studies have investigated placental and fetal uptake and transport under normal physiological conditions, and none have explored the integrated transfer of several compounds [7,8,9,10,11,12,13]. These fundamental studies illustrate that *in vivo* studies of the human placenta are feasible, and that they may serve several purposes. First, current concepts of placental functions mainly derived from *in vitro*, *ex vivo* and animal studies may be tested in a human setting and thus provide novel and more specific insight into the human placenta. Second, properties of the dysfunctional placenta associated with aberrant fetal growth, preeclampsia, maternal diabetes, metabolic syndrome and other maternal metabolic disturbances may be better characterized. Third, human *in vivo* studies provide an opportunity to develop diagnostic and predictive tools of placental function.

On this background we aimed to establish a comprehensive collection of physiological data to investigate human placental function *in vivo*. During a planned cesarean section, we exploit the intraabdominal access to the uterine vein to collect blood samples from the incoming and outgoing vessels on the maternal and fetal sides of the placenta (the 4-vessel sampling method). These samples are used to calculate the paired arteriovenous concentration differences of nutrients and other substances [14]. In addition, we measure volume blood flow on both sides of the placenta by ultrasound. Consequently, placental and fetal uptake of any compound may be quantified. Further, it is possible to determine substances released by the placenta to the maternal and fetal circulations [15,16,17]. When combined with clinical parameters of mother and child, and analyses of placental and other relevant tissues, this method has the exciting potential to integrate many aspects of placental functions *in vivo* in the same mother-fetus pairs.

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Protocol

The study was approved by the data protection officials at Oslo University Hospital and the Regional Committee for Medical and Health Research Ethics, Southern Norway 2419/2011. All participants signed a written informed consent at inclusion.

1. Preparations

NOTE: A timeline for the procedures is outlined in **Figure 1**.

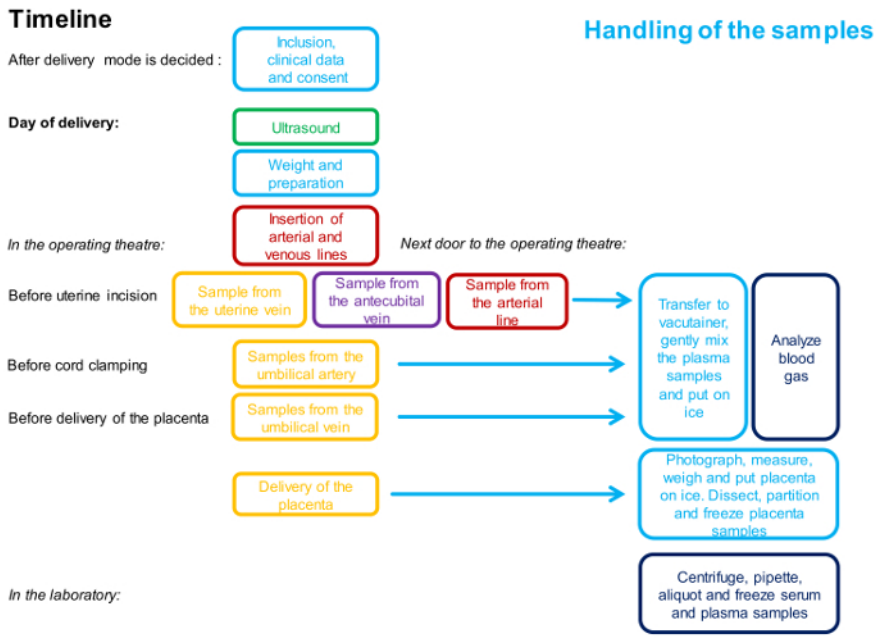

Figure 1: **Flowchart Describing the Timing and the Personnel Involved in the 4-vessel Sampling Procedure.**
One color represents one person. Detailed description of the method is given in the protocol. Please click here to view a larger version of this figure.

1. **Staff**
    1. Make sure all required personnel are available: a highly skilled Fetal Medicine specialist conducting the ultrasound measurements, two obstetricians conducting the surgery, one of the obstetricians and two nurses collecting the samples, one assistant handling the blood gas analyses and one assistant handling the other samples consecutively and immediately following the collection.
    NOTE: In cases of more advanced collection of placental tissue, an additional person is required.

2. **Equipment**
    1. Prepare the equipment, 50 mL of ice cold 1 M phosphate buffered saline (PBS), 25 mL of cold RNA stabilizing solution and 5 x 0.5 mL of optimal cutting temperature compound (OCT). Label the vacutainers and tubes. See tentative list of equipment.

2. Maternal Characteristics

1. Record the maternal clinical and non-clinical characteristics at inclusion and repeat relevant questions and measurements, including weight, at the time of delivery. Record the duration of the fasting period prior to the cesarean section, and any hypotensive episodes occurring during the surgery.
Note: Include the minimal maternal clinical dataset reported in a recent publication from Global Pregnancy CoLaboratory (COLAB). This article also includes some very important aspects in choosing study populations and should be addressed while planning the study [18].
2. Consider recording paternal characteristics, including ethnicity, age and body mass index (BMI).

3. Ultrasound

1. Perform the Doppler ultrasound examination on the day of the delivery, with the women in a fasting state. Perform the examination during a period of fetal quiescence, with the woman in semi-supine position, tilted slightly laterally opposite to the region of interest in order to avoid compression of the aorta and vena cava. Monitor the output intensity by the mechanical and thermal indices on the display.
2. **Umbilical vein**

1. Visualize the umbilical vein in a sagittal or oblique transection of the fetal abdomen. Measure the internal vessel diameter in the straight portion of the intra-abdominal umbilical vein, before any visible branches. Use regular B-mode and visualize the vessel in a perpendicular insonation angle for diameter measurements and keep several optimal frames for later measurements to minimize the effect of pulsatile diameter changes.
    1. Repeat the measurements five to ten times [19].

2. At the same site, use Doppler ultrasound and adjust the probe to get an insonation angle as low as possible (always <30°) in order to measure the time-averaged maximum velocity (TAMX). Obtain the velocity over a period of 3 - 5 s (non-pulsating flow).

3. **Uterine artery**
    1. Use Doppler ultrasound to visualize the uterine artery as it crosses the external iliac artery, immediately after it branches from the internal iliac artery. Adjust the probe at this site to get a low insonation angle (always <30°) and measure TAMX. Obtain the velocity as the mean velocity of three heart cycles.
    2. As it is unlikely to get a perpendicular angle at the same site as TAMX is measured, follow the vessel distally to get a correct angle for diameter measurements as close to the sites of diameter measurements as achievable. Exclude the diameter measurements if any visible vessels branch off before this site as evaluated by color Doppler ultrasound.
        1. Use regular B-mode and visualize the vessel in a perpendicular insonation angle for diameter measurements and keep several optimal frames for later measurements to minimize the effect of pulsatile diameter changes.
        2. Repeat the measurements five to ten times [19].

4. Note the position of the placenta.

4. 4-vessel Blood Sampling

NOTE: The timeline for the procedures is outlined in **Figure 1** and an overview of the samples is illustrated in **Figure 2**.

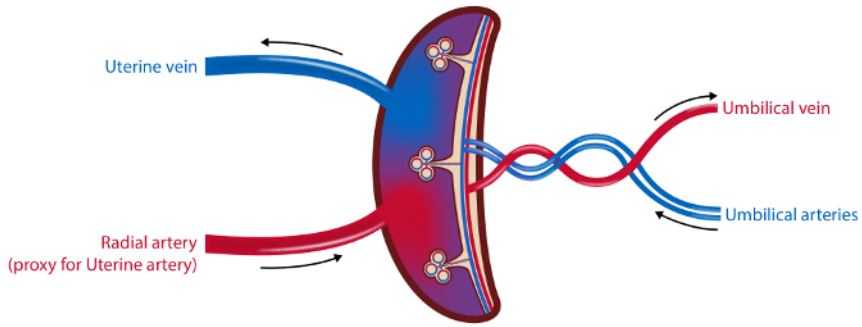

Figure 2: **Schematic Illustration of the Placental Vasculature and the Sampling Sites.**
In the 4-vessel sampling method blood samples are drawn from the uterine vein, the radial artery (as a proxy for the uterine artery) and the umbilical arteries and vein. Blood flow in the uterine artery and the umbilical vein is measured by ultrasound. Tissue samples from the placenta are collected. Illustration: Øystein H. Horgmo, University of Oslo. Please click here to view a larger version of this figure.

1. **Safety procedures**
    1. Provide all personnel in the operation theater with gloves, surgical scrub suits, masks and headwear.
    2. Provide the surgeons and research personnel in contact with the operation field with surgical scrub suits, masks, headwear, gowns and double gloves. Glasses are optional.
    3. Provide personnel handling the blood samples with gloves.
    4. Provide personnel handling the placenta samples with gloves and surgical mask. Homogenization requires the use of hoods.

2. **Preparation in the operation theater**
    1. Give a briefing and hand the equipment to all personnel that will assist the sampling before onset of surgery.
    2. Address the anesthesiologist and anesthesiology nurse who will assist with the necessary peripheral arterial and venous access, and ensure that no liquids are given intravenously before sampling.
    3. Give three syringes (10 mL) without needles to the person assisting with the antecubital vein sample and two syringes (one 20 mL and one 10 mL) and one blood gas syringe (with heparin) to the person assisting with the radial artery.
    4. Prepare two sterile syringes (20 mL), five sterile syringes (10 mL), three "butterfly needles" and two blood gas syringes for the operation field.

3. **Access to blood vessels.**
    1. Follow standard procedure before the cesarean section to assure peripheral intravenous (iv) access.
    NOTE: The antecubital vein is preferable because it is easier to draw samples from this site.
    2. Localize the radial artery at the wrist by ultrasound or by palpation. Following 0.5 mL of subcutaneous lidocaine analgesia, place an arterial line into the radial artery. Abandon the sampling from this site in case of three failed insertions, or if the woman experiences pain during the insertion.

NOTE: Perform the surgical procedure of cesarean section according to standard procedure. Only the adjustments needed for the sampling procedure are underlined below.

4. **Maternal blood samples**
NOTE: Obtain all three maternal blood samples (uterine vein, radial artery and antecubital vein) simultaneously before the uterine incision.
    1. For the uterine vein, after opening the abdominal cavity, use a retractor to lift the abdominal wall and expose the main branches of the uterine veins on the anterolateral sides of the uterus. Obtain blood from uterine vein branches at the same side as the placenta whenever possible or use the most prominent vein plexus if the placenta is located in the uterine midline.
        1. Insert a butterfly needle on a blood gas syringe in the uterine vein at an angle of approximately 30 degrees and collect blood through gentle aspiration to avoid hemolysis. While carefully securing the iv position of the butterfly needle, replace the filled blood gas syringe by a 20 mL and a 10 mL syringe consecutively.
        NOTE: Optimal access is best ensured when standing on the contralateral side of the chosen uterine vein.
    2. For the radial artery, aspirate from the intra-arterial line. Discard the first 5 mL, and then aspirate 3 mL in heparin syringe for blood gas analyses, followed by 3 mL in two syringes (20 + 10 mL).
    3. For the antecubital vein, aspirate gently from the intravenous catheter. Discard the first 5 mL, and then aspirate 30 mL in three syringes (10 mL).
    4. Perform a final inspection of the sampling site on the uterine vein before starting to close the abdomen.

5. **Fetal blood samples**
    1. When the child is born, immediately aspirate blood from the umbilical artery, without clamping the umbilical cord or delivering the placenta. Start with the syringe for blood gas analysis, and follow with three 10 mL syringes if possible.
    2. When the arterial samples are secured, clamp the cord and hand the child to the midwife before sampling from the umbilical vein (blood gas and 20 + 10 mL syringes).
    NOTE: Obtain all umbilical samples within seconds of delivery and with the placenta *in situ* unless it has detached spontaneously.
    3. Follow the Norwegian recommendations on late cord clamping. In case of a distressed child, clamp and cut the cord immediately and the hand the child to the midwife and neonatologist.

6. **Handling of blood samples**
    1. Put the blood gas syringes on ice while preparing the rest of the blood samples, and analyze them in a blood gas analyzer within 5 min.
    2. Transfer the blood samples immediately to vacutainers and place the plasma tubes on a rocker for 1 - 2 min before putting them on ice. Leave the serum tubes on the laboratory bench to settle for 30 minutes.
    NOTE: This is a critical step in the procedure that needs extra attention because samples from all five sites have to be prepared simultaneously to ensure good quality.
    3. Centrifuge the plasma samples as soon as possible, and within 30 min, at 6 °C, 2,500 x g for 20 min.
    4. After 30 min, centrifuge the serum samples at room temperature for 10 min at 2,500 x g.
    5. Aliquot the supernatants carefully to 2 mL cryo tubes, leaving 0.5 mL of the supernatant above the pellet to ensure platelet free plasma.
    6. Store the samples at -80 °C.

5. Collection of Placental Tissue

1. Place the placenta flat down on an ice chilled dissection tray as soon as possible after it has been delivered. Photograph*and measure the longest diameter and the diameter at 90 degrees.*
2. Weigh the placenta.
3. Record the weight, the two diameters, any gross pathology, number of vessels in the cord and the time interval from delivery to when the placenta was placed on ice.
NOTE: Send the placenta to pathological examination if clinically indicated.
4. Place the placenta with the maternal surface facing up and identify 4 - 5 sampling sites randomly located in each quadrant of the placenta, avoiding areas of frank pathology. Remove the decidua using scissors to cut away 3 - 5 mm from the maternal surface. Collect a 1 - 2 cm^3 piece of villous tissue from each site.
5. Wash the collected tissue gently in 50 mL of cold 1M PBS. Divide into several pieces from each sampling site and aliquot.
Note: The size of the placenta pieces will depend on the planned analyzes.
6. Add aliquots of 0.1 - 0.5 cm^3 tissue samples to 5 cryo tubes and snap freeze in liquid nitrogen.
7. Add small pieces of 0.1 - 0.2 cm^3 to the tube with 25 mL of RNA stabilization solution. Store at 4 °C for 24 h, discard the RNA stabilization solution and replace it. Freeze.
8. Add pieces of 0.5 cm^3 to the 5 cryo tubes with 0.5 mL of OCT, top up with OCT, mix and freeze.
9. Store the samples at -80 °C until analysis.
NOTE: Burton et al. provides an excellent overview of practical aspects of placental sampling depending on the analyses planned. [20]
Consider to prepare the remaining tissue for isolation of the microvillous and basal membranes, and to collect decidual tissue by vacuum suction technique. [21,22]

6. Neonatal Characteristics

1. Record the neonatal characteristics, including Apgar-score (1, 5 and 10 min), sex, weight, length, gestational age and admission to Newborn Intensive Care Unit (length and outcome of stay).
2. Consider measuring neonatal body composition by anthropometric measurements, air-displacement plethysmograph or dual X-ray absorptiometry.[23,24]

7. Calculations

1. Assume similar blood composition in the radial and uterine artery and calculate the uteroplacental arteriovenous concentration difference.
   Uteroplacental arteriovenous concentration difference = C_A - C_V
   Umbilical venous - arterial concentration difference = C_v - C_a
   Where C is concentration with subscripts: A, the radial artery; V, the uterine vein; v the umbilical vein and a, the umbilical artery.
2. Calculate the volume blood flow, mL/min (Q):

$$Q = h \times \left[\frac{D}{2}\right]^2 \times \pi \times TAMX$$

   Where D is the vessel diameter (cm), TAMX is time averaged maximum velocity and h is the coefficient for the spatial blood velocity profile. Use 0.5 as the coefficient for the umbilical vein and 0.6 for the uterine artery[25,26].
3. Calculate the placental uptake and release according to Fick's principle:
   Uteroplacental uptake = (C_A - C_V) x Qm
   Fetal uptake = (C_v - C_a) x Q_f
   Subscripts: m, maternal and f, fetal.

Representative Results

The 4-vessel sampling method is applicable in clinical practice and we have successfully obtained blood samples from 209 mother/infant-pairs. In 128 of these we also achieved to measure volume blood flow. Complete 4-vessel sampling and good quality flow measurements of both maternal and fetal vessels were obtained in 70 mother-fetus pairs (**Figure 3**). In addition, we have so far collected blood and placenta samples from 30 preeclamptic patients. We have previously published articles on human placental transfer of nutrients, as well as placental release of vasoactive factors, demonstrating two applications of the method [14,15,16].

Example of how the 4-vessel method is used to study placental transfer
There are significant arteriovenous glucose differences on both sides of the placenta demonstrating an *in vivo* uteroplacental and fetal uptake of glucose (**Table 1**). The placental transfer of glucose is dependent on the maternal-fetal glucose gradient, and thereby on the maternal glucose levels. However, we have previously demonstrated that this gradient, and thus the glucose transfer, is significantly influenced also by fetal insulin levels and glucose consumption. This is an example of how this method illustrates important maternal-fetal interplay [14].

Vessel	Glucose mmol/L	p-value*
Radial artery	4.49 [4.22, 4.84]	
Uterine vein	4.23 [3.94, 4.53]	
Umbilical vein	3.78 [3.52, 4.06]	
Umbilical artery	3.24 [2.95, 3.56]	
Paired differences		
Radial artery - uterine vein	0.29 [0.13, 0.41]	<0.001
Umbilical artery – umbilical vein	0.54 [0.29, 0.76]	<0.001
Radial artery – umbilical artery	1.25 [1.03, 1.51]	<0.001

Table 1: Median [Q1, Q3] Concentrations and Arteriovenous Differences of Glucose
* Wilcoxon Signed-Rank test

The fetal glucose uptake from (placental release to) the umbilical circulation is believed to be dependent not only on the maternal-fetal gradient, but on placental blood flow [5]. Likewise, it may be relevant to study the fetal glucose uptake as a function of placenta weight or birth weight. In n= 128 we found a median [Q1, Q3] total umbilical venous flow of 196.2 [158.3, 232.2] mL/min, and calculated a median [Q1, Q3] fetal glucose uptake from (placental release to) the umbilical circulation of 0.10 [0.05, 0.15] mmol/min. Normalized for birthweight this equals 0.03 [0.02, 0.04] (mmol/min)/kg. The placenta releases 0.16 [0.10, 0.26] (mmol/min) per kg placenta.

Example of how the 4-vessel method is used to study placental uptake
Animal studies suggest that glutamic acid is important both in interconversion of amino acids in the placenta and the fetal liver, and as an oxidative fuel in other metabolic pathways [27]. Using the placenta 4-vessel sampling method we studied the uteroplacental and umbilical arteriovenous differences of glutamic acid in humans (**Table 2**). We found a placental uptake (thus a fetal release) of glutamic acid from the umbilical circulation. Further we found a placental uptake of glutamic acid from the maternal circulation. This placental uptake from both circulations is an example of how the 4-vessel method can be used to demonstrate *in vivo* in the human that placental metabolism of nutrients is a part of the regulation of the transplacental transfer.

Vessel	Glutamic acid µmol/L	p-value*
Radial artery	61.5 [51.0, 77.7]	
Uterine vein	51.0 [36.3, 65.0]	
Umbilical vein	39.3 [24.7, 52.8]	
Umbilical artery	44.7 [33.1, 59.3]	
Paired differences		
Radial artery- uterine vein	10.4 [1.6, 21.2]	<0.001
Umbilical artery –umbilical vein	-8.7 [-16.0, 0.2]	<0.001

Table 2: Median [Q1, Q3] Concentrations and Arteriovenous Differences of Glutamic Acid
* Wilcoxon Signed-Rank test

Example of how the 4-vessel method is used to study placental release
It is established that the placenta secretes progesterone and in order to validate our 4-vessel method on the maternal side of the placenta, we measured the *in vivo* release of progesterone at term [28]. We found a significant placental release of progesterone to the maternal circulation (**Table 3**). The observed arteriovenous difference demonstrates how the placental 4-vessel sampling method can be used to detect substances released by the placenta, and is highly relevant when studying pathological pregnancies.

Vessel	Progesterone nmol/L	p-value*
Radial artery	678 [514, 971]	
Uterine vein	1852 [1059, 2786]	
Paired differences		
Radial artery- uterine vein	-1187 [-1855, -404]	p<0.001

Table 3: Median [Q1, Q3] concentrations and uteroplacental arteriovenous difference of progesterone
* Wilcoxon Signed-Rank test

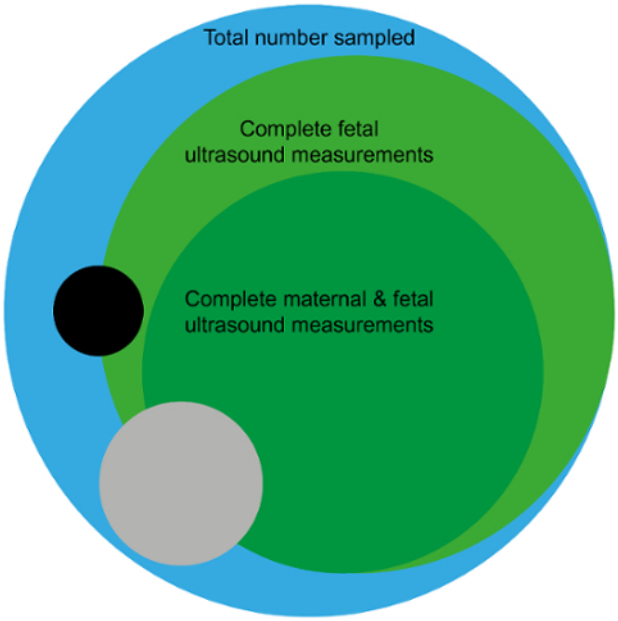

Figure 3: **Flowcharts and illustration of included and lost participants.**
A. Shows the inclusion of the participants, demonstrating that participants were lost mainly due to start of labor prior to caesarean section or lack of sufficient personnel to conduct the study. **B.** Of the 179 women with normal pregnancies (blue) complete 4-vessel blood samples were obtained in 162 (91%) (incomplete fetal blood samples: black, incomplete maternal blood samples: grey). Fifty-one (28%) participants were not included for ultrasound measurements due to logistical limitations. Of the 128 participants (72%) subjected to ultrasound, blood flow measurements at the fetal side of the placenta were obtained in all participants (light green), whereas complete blood flow measurements at both the maternal and fetal side were obtained in 77 (60%) (dark green). Illustration: Øystein H. Horgmo, University of Oslo. Please click here to view a larger version of this figure.

Discussion

The placenta 4-vessel sampling method is relevant for three main purposes. First, it can be used to study how specific substances are taken up by the placenta on the maternal side and possibly transferred to the umbilical circulation and the fetus, as demonstrated by our glucose and amino acid studies. Second, the method is highly relevant to study substances produced by the placenta and released to the maternal or the fetal circulations, as demonstrated by the progesterone results. Third, it may be useful to study how the fetus *in vivo* eliminates waste products during the rapid growth and tissue remodeling.

Critical steps and logistical issues of the 4-vessel method
Placenta 4-vessel sampling has previously been used to determine the placental transfer of macronutrients *in vivo* in normal physiologic pregnancies [8,10,12], but with a limited number of study subjects. The limited use of the 4-vessel sampling is probably due to the demanding logistics of the procedure. A successful coordination between the patient, the researchers and the personnel at the departments of fetal-medicine, obstetrics and anesthesiology is essential in order to employ this method. We believe it has been a great advantage that several of the main investigators are obstetricians, knowing clinical routines and key personnel. Thereby the study procedures have been implemented alongside the daily clinical practice. By coordinating and securing every step of the procedure, we have obtained samples from over 200 mother/newborn-pairs. Furthermore, our strategy has been to keep the sampling procedure on few hands because there are technical challenges in the procedure that are decisive for the successful outcome of the sampling, and too many samplers can introduce unnecessary sources of variation of the data. Consequently, we recommend that all sonographic examinations should be performed by the same examiner using the same ultrasound machine. The equipment should be chosen with care, as the resolution of the vessel wall is crucial. The uterine arteries in third trimester are especially technically challenging to measure because the uterine size and content makes it difficult to obtain both the perpendicular insonation angle used for the diameter measurements, and the low insonation angle for flow velocity measurements at the exact same site. Moreover, successful blood sampling demands identification of appropriate sampling site of the uterine vein and gentle aspiration. On the umbilical side the fragility of the fetal erythrocytes requires particular attention to the aspiration force. It is our experience that delayed delivery or minor stress to the infant was associated with early constriction of the umbilical artery resulting in reduced arterial sample volume.

Ethical issues of the 4-vessel method
The "placenta 4-vessel sampling" method is an invasive and demanding procedure. Inclusion and exclusion criteria should therefore be well defined according to the research question to avoid unnecessary sampling procedures. Patients should only be approached for inclusion after the decision about delivery mode has been made, to ensure that indications for cesarean delivery are not influenced by participation in the research project. Although the procedure requires little extra time at the operation theater, it requires the presence of more personnel making it mandatory to limit the inconvenience and disturbance caused by the sampling. We have used the radial artery as a proxy for the uterine artery as the insertion of an arterial line is less invasive and ensures simultaneous sampling from the artery and vein. Some groups use arterialized blood which is an even less invasive procedure [13]. However, apart from one incidence of a local hematoma in relation to the arterial line resulting in temporary paresthesia of the hand, we have not experienced adverse effects during sampling from any of the 4 sites. In particular, we have not observed any bleeding from the punctured uterine vein.

Methodological/ Analytical issues of the 4-vessel method
It is important to address several methodological issues in the interpretation of the results from a placenta 4-vessel study. First, if the aim is to calculate the mass of a substance taken up, or released, by the placenta it is important to consider the volume of blood passing. It should be kept in mind that the uterine vein not only drains the placenta, but also drains the uterine muscle, and that the uterine vasculature in various degrees anastomoses with the ovarian and vaginal vasculature. Next, it is important to consider that the exchange of water across the placenta may influence the concentrations measured, and thereby affect the arteriovenous concentration differences calculated. Ideally this is best addressed by adjusting the concentration differences for the water lost or gained in each mother-fetus pair. This may be achieved by measuring a substance which is not taken up or released by the placenta or the uterus. The hemoglobin concentrations or calculated percentages of erythrocytes (hematocrit) may serve as correction factors for water exchange. Further, when interpreting uptake or release of compounds on the maternal side of the placenta it may be of interest to obtain arteriovenous differences in other tissues for comparison. We have therefore included a blood sample from the antecubital vein to characterize specific features of the placenta by comparing the arteriovenous differences over the placenta with those of the capillary bed of the forearm. We found this comparison particularly interesting when we tested placental release of sFlt-1 and placental growth factor because systemic endothelial cells could be a potential source of these compounds [14]. Depending on the research question it might be of importance to relate the arteriovenous differences to the weight of the placenta to calculate the placental efficiency, *i.e.* in terms of (mmol/L)/kg or (mmol/min)/kg placenta.

Limitations and strengths of the 4-vessel method
Although placental physiology is less affected by cesarean section than by the stress of vaginal delivery, there are several limitations to this method. Vaginal delivery is recommended in most cases of common pregnancy complications (like preeclampsia, diabetes, obesity and moderate fetal macrosomia), which may limit and bias the recruitment. Even when optimizing every step of the method it is hard to obtain complete measurements and samples in each patient because of the technical difficulties in the blood sampling procedure and ultrasound volume flow measurements (**Figure 3**). In addition, although the ultrasound measurements are conducted as close in time to the surgery as possible, they are inherently conducted prior to the spinal anesthesia and the blood sampling. From this follows that maternal cardiac output (CO) may change and possibly affect uteroplacental (and even feto-placental) blood flow. The possible change in CO caused by spinal anesthesiacan be compensated by phenylephrine which was used in the current study. Preliminary data from a subset of our participants (n= 23) show no significant alteration in CO before spinal anesthesia and at the time of sampling (unpublished data). Using the 4-vessel sampling method in humans, as opposed to in animals, limits both the possibility to introduce a time variable and to manipulate the blood content [5,29,30]. From these considerations, it follows that the 4-vessel sampling method is cross-sectional and largely observational by nature, and the data obtained must be analyzed accordingly. On the other hand, the 4-vessel sampling method provides a unique possibility to study human placental physiology and pathophysiology *in vivo*, with all the interacting factors at play, a situation that can never be reproduced *in vitro*. It offers an excellent opportunity to test hypotheses that have emerged from animal or other experimental studies. Likewise, it can generate new hypotheses that need to be tested mechanistically *in vitro* and in animal studies.

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Potential applications of the 4-vessel method

In pathological pregnancies, the maternal and fetal arteriovenous concentration differences have, so far, been studied separately, and allowed for testing of some of the existing hypotheses *in vivo*[15,16,31]. The 4-vessel sampling method offers the appealing opportunity to study the maternal, placental and fetal unit together rather than as separate entities in pathologic pregnancies and may shed new light on both old and new questions within the scope of maternal-fetal interaction. The 4-vessel sampling method may be applied to a wide range of research questions in both normal and pathological pregnancies depending on the further analyses of the samples. The choice of vacutainers, the volume of blood, the range of placenta and other tissue samples should be decided according to the research question. Burton et al. have recently published an excellent paper describing procedures to ensure good quality samples of placental tissue, and to allow merging of different biobanks in order to address certain puzzles that need a large amount of samples to be solved [20]. The 4-vessel samples may be analyzed to study the specific release of exosomes to maternal circulation, the transfer of medication, metabolites and toxins. Big scale omics (metabolomics, proteomics and lipidomics) analyzes has the potential to identify substances and metabolites in maternal plasma that are secreted by the placenta. Thereby the establishment of the 4-vessel sampling method may identify placenta derived factors in the maternal circulation and possible tease out biomarkers of placental function. Combined with techniques to separate the maternal facing microvillous and fetal facing basal plasma membranes of the syncytiotrophoblast, transfer of a substance may be studied together with the activity and location of relevant transporter proteins [21]. Further, mechanisms regulating nutrient transfer *in vivo* may be elucidated by analyzing the levels of nutrients and micronutrients in the four vessels and relate the transfer of nutrients to measurements of the nutrient and energy sensing system in the placenta[32]. The combination of 4-vessel sampling with the use of stable isotopes may represent a powerful approach that would provide more dynamic and mechanistic information, as demonstrated for amino acid transfer [33]. Glucose infusion prior to delivery is another possible approach. Placental transfer could be related to maternal metabolic variables like BMI, glucose and plasma lipid profiles and to fetal outcomes like birthweight and body composition [18]. Together, these approaches will possibly illuminate the integrative role of the placenta, being situated in the center of the interplay between maternal and fetal conditions and needs.

Disclosures

The authors have nothing to disclose.

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