A novel approach for the *in vivo* isolation of fetal cells directly from the maternal circulation for prenatal diagnostics using a functionalized structured medical wire (FSMW)

Klaus Luecke[1], Mateusz Madejczyk[2], Judith Schniedermann[1], Maren Keller[1], Steffi Mewes[1], Andreas Bollmann[1], Ekkehard Weber[1], Pornpimol Ruangvutilert[3], Angkana Lurngnateetape[3], Supornchai Kongpatanakul[3], Grzegorz H. Bręborowicz[2]

Abstract

The prenatal diagnosis of chromosomal defects gains in importance due to the fact, that the average maternal age increases and therefore the risk of chromosomal aberrations. In the presented approach, a functionalized and structured medical wire (FSMW) was developed for the *in vivo* isolation of fetal trophoblasts directly from the maternal circulation. The FSMW was coated with human or murine antibodies directed against the HLA-G-antigen, which is highly expressed on the surface of fetal trophoblasts. The safety of the FSMW was tested with 18 (6+12) healthy non-pregnant female volunteers in two studies with different antibodies. Subsequently, 36 (24+12) healthy pregnant subjects participated in the study with the FSMW. The medical wire was inserted through a standard venous cannula into the cubital vein for 30 to 45 minutes. After removal of the FSMW trophoblasts were identified by PCR or immunocytochemical staining and subsequent counting under a fluorescence microscope. The FSMW showed very good biocompatibility and no side effects in all healthy volunteers and all pregnant subjects. After the application of the detector functionalized with the murine antibody no human anti-mouse antibodies (HAMAs) were detected in all 24 participating non-pregnant and pregnant women. The detection of male fetal cells on the FSMW was accomplished by PCR.

Key words: prenatal diagnostics, circulating fetal cells, minimal invasive detection device

Introduction

In the last two decades a growing tendency of advanced age in pregnant women is observed. This tendency is one of the reasons for increasing numbers of fetal chromosomal abnormalities.

At present there are a number of invasive methods used for prenatal diagnostics like amniocentesis and chorionic villous sampling (CVS), which are used depending on the time of gestation. The obtained fetal cells can be analyzed for chromosomal aberrations.

Transabdominal amniocentesis is the most commonly used procedure to take a sample of amniotic fluid to obtain fetal cells for cytogenetic analyses. The procedure is mostly performed at week 15 to 18 (second trimester), but was also applied between 11 and 13 weeks of gestation [1, 2]. An adequate sample for the analysis is provided in more than 99% of the cases. Results are usually available within one to two weeks. Risks of this method include fetal loss, fetal injury and maternal Rh-sensitization; however each one is very uncommon. The highest risk is fetal loss at less than 0.5%. The rate of miscarriage after the procedure at 15 weeks of gestation varies and is most commonly quoted to be 1% [3]. This risk increases in twin pregnancies up to 2.73%. Early amniocentesis, even by an experienced examiner, before week 13 of gestation increases the risk of fetal loss up to 1 to 2%. This also increases the risk of congenital foot deformities [3-6].

Chorionic villous sampling (CVS) has some disadvantages compared with amniocentesis. First, the incidence of limb deficiencies is greater in cases of CVS performed before the completed ninth week of pregnancy. Second, there is a 2% false-mosaicism rate during the laboratory evaluation of the chorionic tissue [3]. The risks for CVS greatly depend on the time in pregnancy when the procedure is carried out. However Evans and Wapner conclude, that even in the hands of an experienced examiner first trimester CVS and mid-trimester amniocentesis are comparably safe for mother and child.

[1] GILUPI GmbH, Potsdam, Germany

[2] Department of Perinatology and Gynaecology, Poznan University of Medical Sciences, Poznań, Poland

[3] Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Therefore, the safest method before week 13 of gestation is CVS [5].

Further methods for prenatal diagnosis include the sampling of fetal blood and preimplantation genetic diagnosis (PGD). Fetal blood sampling is applied only under special circumstances and carries a risk of spontaneous abortion of between 1 and 2%, i.e. higher than amniocentesis or CVS [2]. PGD is performed in cases where the family carries a known genetic disorder. In this procedure a single cell is taken from the early embryo and analyzed using PCR.

Non-invasive prenatal diagnostic techniques include those using visualization techniques (like ultrasound) and maternal serum screening. With sonography a screening for fetal trisomy 21 by measuring the thickness of a fluid-filled space behind the fetal neck can be applied, otherwise known as the nuchal translucency (NT) measurement. In a study of 96127 singleton pregnancies, 5% of the participants combined the risk of an elevated maternal age with a striking result in the NT measurement and enabled a detection rate of 77% of fetuses with trisomy 21. Furthermore in approximately 73% of the examined fetuses with trisomy 21 and in 0.5% of fetuses with a normal karyotype an absence of the nasal bone was detected by ultrasound [7]. Combining the nasal bone identification with first-trimester NT and serum screening scores resulted in the detection of 90% of fetuses with Down syndrome with a false-positive rate of 2,5% [8]. However, while these approaches are useful in screening for the Down syndrome, they are rarely used in diagnosing fetal aneuploidy.

When the presence of fetal DNA was discovered in maternal plasma it was used to determine the fetal gender and the fetal RhD blood type in Rh-negative pregnant women. Lo et al. examined the presence of cell-free fetal DNA in maternal plasma using SNP genotyping and sequencing of plasma DNA [9]. Although a non-invasive method with promising results, there are still a lot of methodological and financial disadvantages for the detection of fetal genetic disorders using fetal DNA in maternal plasma. First, only 10% of cell-free DNA is of fetal origin, therefore a sequencing of the fragmented fetal DNA in maternal plasma is required for the detection of e.g. trisomy 21 and second for a reliable result DNA samples of both parents should be analyzed too [10].

An alternative research method of non-invasive prenatal diagnosis is the analysis of intact fetal cells circulating in maternal blood. In 1893 Schmorl observed fetal-maternal traffic of trophoblasts into the maternal circulation [11]. Different subtypes of trophoblasts are known, like syncytiotrophoblasts, extravillous cytotrophoblasts and anucleated trophoblasts [12].

Whereas the number of fetal cells in maternal blood increases in pregnancies with aneuploid fetuses only a limited number of fetal cells circulate in maternal blood with healthy fetuses [13]. Therefore, procedures to enrich the cells and enable single cell analysis with high sensitivity are required. Based on the isolation of intact fetal cells, the GILUPI GmbH developed a minimal invasive medical device for the application in prenatal diagnosis in order to permit the analysis of fetal chromosomal aberrations. The device, also called Nanodetektor (ND) or functionalized structured medical wire (FSMW), inserted into a maternal vein is supposed to enrich circulating fetal extravillous cytotrophoblasts on the surface of the detector via specific antibodies against the target cells. The captured fetal cells serve as a source for fetal DNA material for further diagnostic methods.

Due to the low number of target cells in the volumetrically limited blood samples used in the conventional cell isolation methods this novel *in vivo* cell-enrichment technique has a decisive advantage over these methods.

Methods

Development and production of the functionalized structured medical wire (FSMW)

Wire, gold and hydrogel

The structure of the FSMW is based on a smooth stainless steel medical wire of 0.5 mm diameter and 160 mm length (EPflex, Germany). The first 20 mm are covered with a 0.5-1 μm thick gold layer deposited by galvanization (OTEK, Brieselang, Germany).

A 1 μm thick hydrogel consisting of a linear, synthetic polycarboxylate is subsequently attached onto the gold layer (Xantec bioanalytics, Duesseldorf, Germany). This coating has to be both protein repelling and able to bind high amounts of antibody.

Antibodies

For the development of a fetal cell accumulating FSMW highly specific antibodies are necessary. Until 2006 no specific human antibody against a fetal cell surface antigen was commercially available. Therefore, human Fab-antibodies specific against the human leukocyte antigen G (HLA-G) were developed because HLA-G shows a high expression rate on the surface of extravillous trophoblasts [14].

The functionality of the antibodies was determined with a HLA-G-positive cell line by flow cytometry and

compared with the commercially available mouse anti-HLA-G antibody MEM-G/9. Quality and quantity of the antibody binding to the surface were measured by ELISA and surface plasmon resonance (SPR) experiments.

The cell binding ability of the FSMW functionalized with these antibodies was tested by incubating cells in different solutions with the FSMW. To simulate the venous blood flow an artificial dynamic flow system was used.

Flow cytometry with the anti-human HLA-G-Fab antibody

For the clinical trial (safety and functionality) the human anti-HLA-G Fab-antibody AbD10521.3 was used. This antibody was selected out of a panel of various antibodies, and it was used in *in vitro* tests. The selected antibody AbD10521.3 demonstrated a 23 fold higher affinity to the HLA-G-positive target cell line K562 (ACC 488, DSMZ, Braunschweig, Germany) than Fab AbD7 109.25 (Fig. 1). Both antibodies were produced by MorphoSys AG (Munich) on the basis of their HUCAL technology.

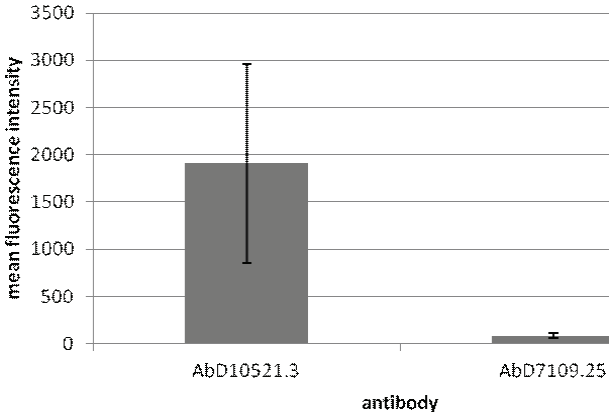

Fig. 1. Flow cytometric analysis of human anti-human HLA-G Fab antibodies with HLA-G-positive cells. HLA-G-positive K562 cells were incubated with two recombinant human anti human HLA-G Fab antibodies (AbD10521.3 and AbD7109.25) followed by the incubation with a secondary fluorescent anti-human Fab antibody. Data show the mean fluorescence intensity of 2 independent experiments with standard deviation. HLA-G-negative K562 cells were used as references (not shown)

Activation of the hydrogel, antibody binding and application of the FSMW

The carboxyl groups of the hydrogel are activated with EDC/NHS allowing the functionalization by covalent coupling of anti-HLA/G Fab antibody AbD10521 or anti-HLA/G clone MEM-G/9 (Exbio) at pH 4.5 in sodium acetate buffer.

The amount of antibody present on the FSMW was analysed by SPR and found to be 10.2 ng/mm^2 on the activated surface. The complete amount of bound antibody on the FSMW was 321 ng. The highest possible amount of antibodies bound to the FSMW was estimated to be 3 μg.

A schematic presentation of the functionalized tip of FSMW is shown in Figure 2 while Figure 3 represents an overall view of the FSMW. The device fits into a standard 18GA or 20GA intravenous cannula (FSMW Fab, 1 x BD Venflon™ 18GA 45 mm (BD 391453) or 20GA 32 mm (BD 391452)).

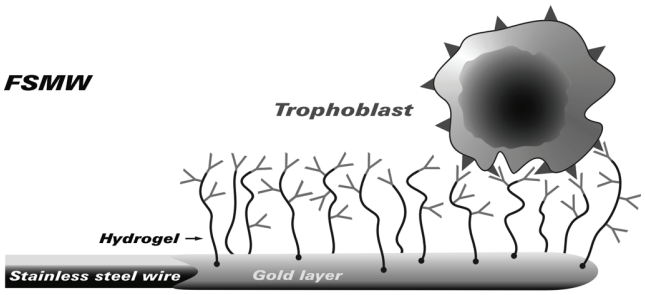

Fig. 2. Schematic presentation of the functionalized tip of the 160 mm long FSMW. 20 mm of one end of the stainless steel wire are coated with a 0.5-1 μm thick gold layer to which a linear synthetic polycarboxylate gel (black threads) is covalently attached. To this gel antibodies (light gray) are bound which recognize trophoblast specific surface antigens

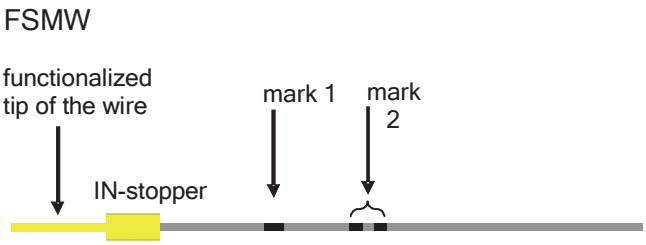

Fig. 3. Schematic representation of the FSMW with its functionalized tip, the IN-stopper for fixing the FSMW to a Venflon cannula and the two insertion marks

Sterilization process

Sterilization of the hydrogel coated medical wire is done through the application of 121°C heat for 20 min (Systec D65, ARA Service Mohnke, Seriennummer D 0801, Berlin, Germany). The functionalization process of the complete FSMW is then carried out in a germ poor environment. Sterility and Limulus-Amebocyte-Lysate assays (LAL-test for endotoxins) were performed for each production lot by culturing all parts of the medical device (SLM-Speziallabor für angewandte Mikrobiologie, Berlin, Germany).

Preclinical testing

Artificial dynamic flow system

For the preclinical examination of the functionality and the sensitivity of the established medical wire a dy-

namic (hemodynamic) fluid model system was used. In this *in vitro* artificial flow system blood or PBS were enriched with fluorescence stained (Tracker for living cells CFSE [C1157] or CMTMR [C2927], Invitrogen) Jeg-3 cells (ACC 463, DSMZ, Braunschweig, Germany). With the flow system it was possible to determine the enrichment of cells on the FSMW under defined conditions. Immobilized anti-HLA-G antibodies bind cells floating in the suspension medium. With this model system cell adhesion can be investigated as it is found under flow conditions in a vein. A flow rate of 0-38 ml/min can be simulated which is corresponding to the flow rate of blood in a vein which was estimated by ultrasound tests. With a diameter of the vein of 0.4 cm and a velocity of approximately 5 cm/s we calculated a volumetric flow rate of 37.7 ml/min.

Cell binding experiments in the peripheral blood of pregnant women (*in vitro*)

To test the ability of the GILUPI FSMW of catching cells in the human blood we used blood of pregnant women in the artificial flow system. In these experiments we were able to isolate fetal cells. After exposing the wires to the blood they were stained by the fluorescence labelled antibody MEM-G/9-PE (1P-292-C100, Exbio, Prague, Czech Republic) against HLA-G and anti-CD14-FITC (1F-212-T100, Exbio, Prague, Czech Republic) against CD14 expressed on macrophages which could also express HLA-G (double stained cells).

Preclinical *in vitro* and *in vivo* safety and biocompatibility tests (according to European safety guidelines for medical products – EN ISO 10993)

Relevant inspections

For the intended use of the medical product FSMW the period of contact with the circulating blood is less than 24 h. According to EN ISO 10993-1, Table 1, the following tests were considered necessary to assess the biocompatibility of the FSMW.

Cytotoxicity test (EN ISO 10993-5)

To assess possible effects of the FSMW on cells the cytotoxicity test was adapted to the conditions of the application of the product in humans to improve its expressiveness.

Normal human dermal fibroblasts (NHDF, C-12302, Promocell, Heidelberg, Germany) were directly treated with the eluates of the FSMW for 48 hours. Eluates of reference materials known to be toxic (positive controls) and non-toxic (negative controls) were tested in parallel to the eluates of the test detectors.

Undiluted eluates of the detector showed no reactivity (reactivity of grade 0), like the negative control. The cells did not show morphological alterations. They remained adherent and typically spindle shaped like the controls. By contrast cytotoxic effects were observed in the positive controls, which were evaluated with an average score of 4 (severe reactivity). A sample meets the guideline requirement of being non-toxic if the response to the sample preparation is not greater than grade 2 (mildly reactive), while negative controls are of grade 0 and positive controls at least of grade 3.

Hemocompatibility test (EN ISO 10993-4)

Hemocompatibility was determined and evaluated by a certified test laboratory. Included in the analyses were free hemoglobin, a small blood count (erythrocytes, leukocytes and thrombocytes) and the coagulation parameter fibrin. These tests revealed no serious disturbances. The FSMW caused, with the exception of a very low level of hemolysis, no effects on hemostasis and the overall conclusion is that the FSMW is not raising the risk of side effects such as thrombus formation.

Tests for systemic toxicity (EN ISO 10993-11, *in vivo* animal trials)

The acute systemic toxicity is a potential general adverse effect of *in vivo* applied medical devices. Its assessment provides general information on health hazards likely to arise from an acute exposure by the intended clinical route. Generalized effects, as well as organ and organ system effects can result from absorption, distribution and metabolism of leachates from the device or its materials in parts of the body with which they are not in direct contact.

With the FSMW no compound related mortalities or other signs of toxicity were recorded within 72 hours post-dose for any of the animals. Therefore, according to ISO 10993-11 (and cross referencing ASTM F 750-87 and USP), a sufficient estimate of the acute toxicity of the test item is provided. For the conditions of the present study it can be stated that the test items FSMW-human Fab antibody AbD10521.3 or FSMW-murine antibody MEM-G/9 showed no acute toxic characteristics.

Abrasion tests with the FSMW

High abrasion resistance is very important for a medical device like the FSMW. Therefore, it had to be

tested whether the hydrogel or the gold can be scratched off during the application of the product.

In our abrasion experiments wires coated with gold or with gold and hydrogel were tested by single- and double-pass sliding through 18GA and 20GA cannulas. If pulled through the cannulas carefully none or virtually none abrasion could be observed microscopically. On the contrary if the wire was tilted against the metal sleeve of the catheter the gold along with the hydrogel got scraped off. A comparison test between different gold coatings confirmed galvanized wires as the best.

Diagnostics after the *in vivo* application of the FSMW

Macroscopic examination

After the application of the FSMW, the device was examined macroscopically by a physician. Intensive unspecific cell deposits on the wire and abrasions from the wire were documented in the CRFs.

Microscopic examination (Haema-Kit)

After its *in vivo* application the FSMW was washed in PBS before preparing the device for the Haema test (Haema-Schnellfärbung, Labor und Technik, Eberhard Lehmann GmbH, Berlin, Germany). The detector was removed from the transport container and 5 × dipped first into the fixation solution, second into staining solution I and third into staining solution II (all mentioned solutions are components of the kit). After each staining step, the solutions had to drip off the wire. Then the device was dipped twice for 1 sec in water and was dried afterwards. The microscopic examination of the cell number was done with a 40 × magnification.

Fibrin detection

After the Haema cell staining the FSMW was washed in PBS. The first antibody (anti-fibrin antibody UC45, SCBT, sc-53284) was diluted 1:100 in PBS. The FSMW was incubated for 30 min at room temperature in the anti-fibrin antibody solution. Afterwards the device was washed in PBS and incubated for 30 min at RT in the dark with the second antibody (goat anti-mouse IgM-FITC, SCBT, sc-2082) at a dilution of 1:250 in PBS. Before the microscopic analysis the FSMW was washed again in PBS. The detector was examined for netlike fibrin fibers in the FITC/GFP-channel of the Zeiss Axio Imager Z1 AX10 microscope (Zeiss, Jena, Germany), equipped with an AxioCam digital camera system and AxioVision 4.6 software (Zeiss).

Immunocytochemical analysis

After removal of the FSMW from the circulation it was briefly washed in PBS, followed by incubation in 2% (w/v) BSA for 30 min at RT. The analysis of the bound cells captured on the device was done by immunocytochemistry.

Cells on the FSMW were labeled with mouse monoclonal antibodies to HLA-G (MEM-G/9-PE) and to CD14 (FITC) for 45 min at RT. After washing in PBS for 5 min cells were counterstained for 3 min with the nuclear dye 4, 6-diamidino-2-phenylindole (DAPI, D1306, Invitrogen, Carlsbad, California, USA), diluted 1:10.000 in PBS, and washed again in PBS for 5 min. The presence of trophoblasts was confirmed by MEM-G/9-PE-staining (red) and the blue color of DAPI.

Determination of the gender – PCR (polymerase chain reaction)

For the molecular diagnosis of the fetal gender amelogenin gene regions were selected. The amelogenin protein is present in developing tooth enamel, which is an extracellular matrix protein [15].

The amelogenin gene is available on the X and Y-chromosomes. However, on the Y-chromosomes a deletion of 184 bp exists. Therefore, in a sensitivity increased nested PCR one band (412bp) for female and two for the presence of male (412 and 232bp) DNA will be visible after the electrophoresis of the PCR products.

After the *in vivo* application for the determination of the functionality in the clinical trial, the wire was washed in PBS and cut into small pieces for the PCR. Bound fetal cells were destroyed by the temperature changes during the PCR and fetal DNA was set free for amplification. The gender of the fetus was unknown. In every PCR-experiment one no-template-control (NTC, negative control) as well as positive control (known concentrations of male/female DNA) is included. In Figure 4 an example for a gel electrophoresis of PCR products is given.

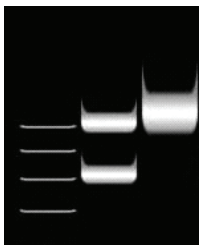

Fig. 4. Results of the amelogenin gene amplification of female and male DNA. Electrophoresis of the amplification products (female fetus: one fragment for X-chromosomes (412 bp); male fetus: two fragments (412 bp for X-chromosome and 232 bp for Y-chromosome))

QF-PCR

The ChromoQuant® QF-PCR (Cybergene, Stockholm, Sweden) is a quantitative technique which identifies changes in the number of chromosomes (e.g. chromosome 21). The method is based on the PCR amplification of repetitive DNA segments called microsatellites or STR (short tandem repeats), which differ from person to person. The technique was developed for the analysis of DNA of fetal cells out of the amniotic fluid. The amplified DNA fragments are marked with different fluorescent dyes. Capillary electrophoresis allows the simultaneous analysis of multiple STRs by the appropriate selection of fluorescent molecules labeled to the different DNA fragments. In the clinical studies only the X/Y chromosomes and chromosome 21 were analyzed. The analysis of the PCR products of healthy volunteers is represented in Figure 5, a+b.

Fig. 5. Examples of peak patterns of different genetic characteristics; a) normal heterozygote 1:1 (informative), b) homozygote (non-informative), c) trisomy 1:1:1 (tri-allelic pattern) and d) trisomy 2:1 (di-allelic pattern); (http://www.cybergene. se/res/Downloads/q07121d04ifu311.pdf, modified from ChromoQuant®)

To diagnose a trisomy at least 2 markers must have a corresponding pattern for a trisomy (see Figure 5, c+d). 4 different genetic markers for the allosomal chromosomes X and Y and 6 different markers on chromosome 21 were analyzed. The different fluorescent primers are starting points for different lengths of PCR amplicons. This allows their recognition in the subsequent capillary electrophoresis of the PCR products.

In the clinical trial with FSMW-Fab QF-PCR results were compared with the results from amniocentesis, also performed in more than 20 cases.

HAMA-detection

Due to possible immune reactions caused by the use of murine antibodies in humans a HAMA IgG-ELISA test (10018-A, Medac) was used for the detection of human anti-mouse antibodies (HAMA). This test system allows the quantitative detection of anti-mouse antibodies in human serum. The presence of HAMA can cause an anaphylactic reaction in the case of a second application of mouse-antibodies. Therefore, the subjects included in the clinical trial with the murine antibody on the FSMW were tested before and four weeks after the application of the FSMW.

Clinical trials

Study population

The medical device FSMW is designed to isolate and enrich fetal extravillous cytotrophoblast cells circulating only in small numbers in the maternal blood stream on the antibody covered surface of the device.

Two clinical trials were conducted. One with FSMW-Fab which was functionalized with a recombinant human Fab-antibody against HLA-G (Poznań) and the other with FSMW-MEM-G/9 carrying the mouse anti-HLA-G-antibody clone MEM-G/9 (Bangkok). An overview of both studies is given in Table 1.

Table 1. Overview. Study I (FSMW-Fab, Poznan, Poland) and study II (FSMW-MEM-G/9, Bangkok, Thailand)

	Study I human antibody	**Study I human antibody**	**Study II murine antibody**	**Study II murine antibody**
Subjects	Non-pregnant (safety)	Pregnant (functionality)	Non-pregnant (safety)	Pregnant (functionality)
Exposure time	30 min	30-40 (45) min	30 min	30 min
Haema- and Fibrin-staining			6	
Anti-HLA-G (MEM-G/9-PE), anti-CD14-FITC		3		
Amelogenin gene PCR		15 (4 contaminated with maternal blood)	6 (negative control)	12
QF-PCR		6 (2 contaminated with maternal blood)		
HAMA-antibodies			12	12

Healthy female non-pregnant and pregnant subjects were recruited at the Poznan University of Medical Sciences (Department of Perinatology and Gynecology) (13.08.09-04.08.10). The study was approved by the Institutional Ethics Committee and written informed consent was given by all participants. 6 non-pregnant and 24 pregnant subjects between 21 and 46 years of age (non-pregnant subjects till 51 years) were included in the study to determine safety and functionality of the FSMW-Fab (recombinant human anti-HLA-G antibody).

A second study was accomplished in the Department of Obstetrics and Gynaecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, including 12 non-pregnant and 12 pregnant subjects for the analysis of the safety and functionality of the FSMW-anti-HLA-G MEM-G/9 (commercially available murine monoclonal antibody, 11-292-M001, Exbio). The study was accomplished from 03/2010 to 01/2011.

In vivo application of the device

The FSMW was designed to fit into a standard 18GA or 20GA intravenous cannula, which is placed into a cubital vein of the subject. An IN-stopper allows the attachment to the intravenous cannula (Fig. 3). The wire is then slowly pushed forward into the cannula until the activated surface reaches out 2 cm into the lumen of the cubital vein. The correct length of insertion is indicated by marks on the distal part of the wire, which is not inserted into the cannula.

The FSMW remained in the cubital vein for 30 or 45 min. The patient remained in a flat or supine position during the procedure.

Results of the clinical trials

The first aim of the clinical trials was the confirmation of the safety of the medical device in healthy non-pregnant women. Therefore, 6 non-pregnant female subjects in Poznan (FSMW with human recombinant Fab-antibody) and 12 non-pregnant female subjects in Bangkok (FSMW with murine antibody MEM-G/9) were recruited to determine the tolerance of the product *in vivo*.

Results of the clinical trial with the FSMW functionalized with a human anti-HLA-G Fab-antibody (Poznan University of Medical Sciences, Department of Perinatology and Gynaecology Poznań, Poland)

The detector was applied in a forearm vein of the first six pregnant subjects for 30 min. Because of some difficulties during the insertion of the FSMW in the study an estimation of the blood flow in the elbow vein was accomplished by ultrasound. The examination showed that no homogenous blood flow was guaranteed if a 18GA (1.3 × 45 mm) cannula was used. When using a smaller 20GA (1.0 × 32 mm) cannula a continuous blood flow could be measured, and therefore, the application was adapted to the results of the ultrasound examination. From pregnant subject P013 till the end of the study the FSMW was inserted into the elbow vein for 40 min. In one case the detector could not be inserted entirely into the elbow vein (subject P021).

Safety assessment

The non-pregnant participants in the clinical trial in Poznan had one application of the FSMW in the forearm with an 18GA cannula. The application time was 30 min. Blood values of the subjects in the first trial were examined before and after the insertion and compared (Table 2). The hemoglobin value for the third subject (P003) decreased after the application of the medical wire. However, this decrease was not significant. A macroscopical and a microscopical examination of the FSMW were carried out after the procedure. In 3 out of 6 cases the FSMW showed macroscopically blood deposits and in 2 cases clinically irrelevant fibrin fibers on the surface. No adverse events (AE) were observed.

Table 2. FSMW Fab study in Poznań.
Blood values (c = constant, d = decrease)

No.	1	2	3	4	5	6
Erythrocytes	c	c	c	c	c	c
Hemoglobin	c	c	**d**	c	c	c
Hematokrit	c	c	c	c	c	c
Leukocytes	c	c	c	c	c	c
Thrombocytes	c	c	c	c	c	c
INR	c	c	c	c	c	c
Fibrinogen	c	c	c	c	c	c
Partial thromboplastin time	c	c	c	c	c	c
CRP	–	c	–	–	–	–
Alanin-aminotransferase	c	c	c	c	c	c
Aspartat-aminotransferase	c	c	c	c	c	c
Creatinine	c	c	c	c	c	c

Functionality

Another major aim of the clinical trial was to estimate the functionality of the FSMW in catching HLA-G-positive trophoblast cells from the circulatory system of 24 pregnant women. The functionality of the detector was determined in three approaches to identify caught cells (Table 3) by:

- QF-PCR (detection of chromosomal abnormalities or confirmation of the male fetal gender)
- PCR of the amelogenin gene (confirmation of the male fetal gender)
- Immunocytochemistry (detection of fetal cells on the surface of the FSMW)

The results of these analyses were compared with analyses of the cultured cells obtained by amniocentesis (karyotyping).

The FSMW was applied to isolate fetal cells in 24 pregnant women and in 20 of them amniocentesis was performed. In 17 patients the cell cultures provided the karyotype and it was possible to determine the presence of chromosomal abnormalities and fetal gender. In patients without determined karyotype PCR analyses of the amniotic fluid were performed. In these cases the PCR results were used as the reference value.

Fetal gender and karyotype were available as reference values.

In the analysis data of 12 pregnant women were used. The data of the other 12 patients were not used because of the following reasons:

- the lack of amniotic fluid from amniocentesis for comparison
- the analysis of the FSMW revealed insufficient data

On 11 of 12 tested FSMW female fetal cells were detected by PCR (P007, P008, P012, P018-P020, P022-P025 and P028). After the analysis of the amniotic fluid the female gender of the fetal cells was confirmed 6 times. In 5 cases male fetal cells were detected by external amniotic fluid test.

Table 3. Summary of the analyses of the functionality (male fetal gender)

		Gender of the fetus (confirmed by the analysis of amniotic fluid cells or after ultrasound examination)	
Confirmation of the presence of cells of male origin on the surface of the FSMW	yes	Right positive outcome $n = 1$ (17%)	False positive outcome $n = 0$ (0%)
	no	False negative outcome $n = 5$ (83%)	Right negative outcome $n = 6$ (100%)

With our method it is so far difficult to reliably differentiate between female fetal and maternal cells. Only in one case we were able to detect the male origin of the fetal cells correctly.

In two cases the karyotype analysis of the fetal cells was used to confirm the fetal origin of the cells. It was concluded that cells with an abnormal number of chromosomes caught in the blood stream of healthy pregnant women were of fetal origin.

In patient number P007 we detected female fetal cells with a trisomy 21. This trisomy was detected after QF-PCR analysis of cells caught by the FSMW. The analysis of the karyotype of cultured cells confirmed this result.

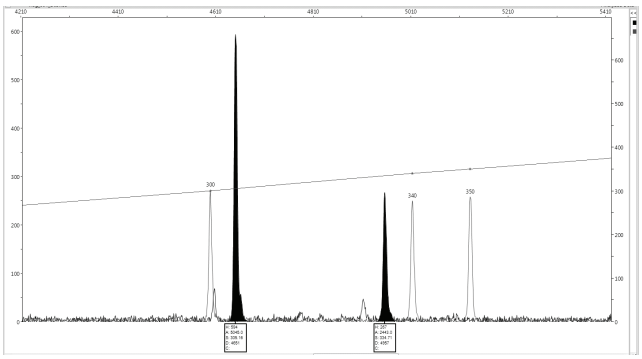

Fig. 6. Result of QF-PCR (genetic markers of chromosome 21). The ratio between the area of the peak of the long allele and that of the short allele is 2.06. The red peaks represent specific size markers

In the clinical trial with FSMW Fab QF-PCR results were compared with the results from amniocentesis, performed in more than 20 cases. In one of the pregnant subjects from the clinical study, two genetic markers of chromosome 21 could be found indicating a trisomy (Fig. 6). For both markers, either a ratio of 1:2 or 2:1 was found. As an example the peak pattern is shown in Figure 5.

In the PCR analysis of the amelogenin gene of patient P017 male cells were detected. This outcome was confirmed by ultrasound examination of the fetus.

Immunocytochemistry

In three cases (P026, P029, P030) immunocytochemistry was used to determine the presence of fetal cells on the FSMW.

Safety assessment

In the second part of the study with pregnant subjects no AEs were detected.

Results of clinical trial with the FSMW functionalized with mouse anti-HLA-G antibody clone MEM-G/9

(Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand)

This clinical trial with the FSMW functionalized with a murine antibody was based on the results of the clini-

cal trial conducted in Poznan. A thinner 20GA cannula was used for the insertion of the FSMW into the cubital vein instead of another forearm vein. The application period was 30 min. In comparison to the first study with the human Fab-antibody no blood values were determined but the presence of HAMAs was checked before and after the application of the FSMW. In all 12 non-pregnant and 12 pregnant subjects in the clinical trial with FSMW MEM-G/9 no HAMAs could be detected in the blood of the subjects four weeks after the application with the medical product.

***In vivo* safety**

In the safety study with 12 non-pregnant women the first six detectors were stained with an anti-fibrin antibody after the application. The other six detectors were used as negative controls for the amelogenin gene PCR. No signals for male DNA were detected. Only the band for the female DNA was visible in all six determined samples.

AEs (adverse events) were observed as deviations from the normal physical status (Table 4 and Table 5).

Table 4. AEs in non-pregnant subjects (clinical trial Bangkok)

Subject	Diagnosis (non-pregnant)	Begin of AE (Treatment)	End of AE
B005	White blood cells in urine	2h after the insertion, no treatment mentioned	3 days after the event
B006	"normal" headache	4 days after the application, no treatment mentioned	30 min later
B019	"normal" headache	25 days after the application, no treatment mentioned	6 hours after onset

Table 5. AEs in pregnant subjects (clinical trial, Bangkok)

Subject (pregnant)	Diagnosis (pregnant)	Begin of AE (Treatment)	End of AE
B025	Severe pre-eclampsia and preterm delivery	Not mentioned, no treatment mentioned	Preterm delivery, gestational week 34, 22 weeks after the application
B026	Ankle pain	25 days after application, treated with 120 mg bid. "Counterpain" topical	3 days later
B029	URI (URI is common in pregnancy. Occurrence of 1 in the cohort cannot be attributed to the detector application)	7 days after application, treated with 500 mg "Paracetamol" (per os)	4 days later
B030	Headache	31 days after the application, no treatment mentioned	7 hours later
B031	Mild URI	29 days after application, treated with 500 mg "Paracetamol" (per os)	4 days later
B032	Vaginal spotting	32 days after application, treated with "Proluton-Depot" 250 mg i.m.	2 hours later

Two subjects (B006 and B019) had "normal" headaches, treatable with pain killers and not accompanied by any other sign of physical impairments like vascular events that could be attributed to the application of the detector. Subject B005 had white blood cells (WBC) in her urine 2 hours after the insertion of the FSMW. Due to the fact that the initial laboratory screening (urinalysis) was conducted three days before the application of the wire the increase in WBCs is unlikely to be caused by the procedure.

Functionality

12 healthy pregnant women within the 10^{th} to 14^{th} week of pregnancy were included in the study.

Ten detectors were used for the amelogenin gene PCR, two for immunofluorescence staining. Although 7 of 10 participating subjects were carrying male fetuses, no signals for male DNA were detected in the PCR, only the band for the female DNA was visible in all ten determined samples.

FSMWs B031 and B034 were not used for PCR, but for immunofluorescence staining with anti-HLA-G antibody MEM-G/9-FITC. Due to interfering maternal blood cells the detection of MEM-G/9-FITC-positive cells was difficult.

In the trial with 12 pregnant women AEs were observed in six out of twelve cases, as deviations from the normal physical status.

Two subjects had upper respiratory infections (URI) after the application of the wire (B029, B031). Subject B030 complained of headaches and subject B032 observed vaginal spotting after the procedure. In one case severe pre-eclampsia with preterm delivery in gestational week 34 was noted for subject B025 and ankle pain was striking after the application for subject B026. The time period of AE-detection for pregnant women started with the application of the wire and finished with the birth of the child (up to 7 month after the application). However none of the detected AEs could be attributed to effects caused by the FSMW. The time period between the application of the detector and the events were several days up to weeks (Table 5). In the case of subject B025, the severe preeclampsia developed later in pregnancy. At the moment of study recruitment the inclusion criteria were fulfilled and no signs for a developing severe preeclampsia were given.

Discussion

Routine methods for the early detection of chromosomal aberrations in fetuses are based on the analysis of fetal cells. These cells are obtained by amniocentesis or chorionic villous sampling (CVS), two invasive procedures which carry a significant risk for both mother and fetus. Other methods for the acquisition of fetal tissue for the determination of the chromosomal status of the fetus are currently not available.

However, from the 10th week of gestation on, fetal trophoblasts are circulating in the maternal blood and the aim of this work was to isolate these cells and to use them for diagnostic analyses. In order to achieve this objective a functionalized structured medical wire was developed as a novel technology designed to isolate the trophoblasts *in vivo* out of the circulating maternal blood. This approach is different from all other cell sampling technologies which are enriching cells *ex vivo* out of a limited quantity of blood.

The structure of this FSMW is based on a common, FDA approved Seldinger medical wire as it is used for angiography. At its functional tip the FSMW is covered with covalently bound antibodies recognizing specific trophoblast surface antigens like HLA-G. In order to optimize the cell binding capacity of the FSMW different nanostructures were tested and human as well as murine antibodies were used. Several approaches were made to bind the antibodies tightly onto the FSMW. A polycarboxylate hydrogel proved to be the best matrix on the gold covered metal surface of the FSMW for binding a maximum amount of antibodies and for the reduction of the unspecific adherence of blood components. Specificity and affinity of the antibodies used on the FSMW were first determined by flow cytometry and SPR and the cell binding efficiency of the complete ND was characterized *in vitro* in a dynamic flow system simulating the *in vivo* venous blood flow. Before entering the clinical trials the biocompatibility (cytotoxicity, hemocompatibility, immunogenicity etc.) of the FSMW was intensively investigated according to mandatory rules. All these preclinical investigations revealed that the developed FSMW was able to bind cells out of a flowing medium and that it was fully biocompatible.

The FSMW is placed into the cubital vein potentially enabling the direct *in vivo* binding of trophoblasts from a woman's entire circulating blood volume.

In the two clinical trials the primary endpoint was to test feasibility and functionality of the medical device *in vivo.* The FSMW proved to be a non-hazardous, safe medical *in vivo* device causing no adverse effects. It had no negative impact on the blood coagulation system during its *in vivo* exposure, neither in healthy volunteers nor in pregnant women.

The FSMW was able to catch fetal cells out of the blood stream in three cases. The fetal origin of the trapped cells was proven by the determination of the gender of the fetal cells, the detection of a trisomy 21 or specific immunofluorescence staining. Despite this proof of concept it is obvious from the obtained results that there is still the necessity to improve the performance of the FSMW.

The design of the FSMW as it was applied in the studies offers the opportunity to adapt several of its characteristics in order to achieve this purpose. The functional area of the FSMW could be increased e.g. by introducing a braided wire composed of three individual wires and by additional microstructures which could also

contribute to slowing down the velocity of the bypassing blood. More affine antibodies are equally important and an array of several antibodies directed against different targets on the fetal cells could help to enhance the binding efficiency of the FSMW.

In contrast to other cell trapping methods this type of *in vivo* device offers the decisive advantage of isolating cells from a larger volume of blood. Nucleic acid-based approaches to collect fetal DNA out of the maternal blood share the disadvantage of the other *in vitro* cell enriching methods because the assessment of cell morphology is not possible. Fetal DNA necessary for the detection of chromosomal abnormalities is only a minor fraction of the free DNA circulating in the mother's blood and is broken down to small fragments. While the analyses of single genes might be possible with this method an overall screening of the chromosomal status of the fetus for an all out analysis of abnormalities requires the complete DNA of the fetus which is only available with cells.

In addition to the advanced development of the FSMW itself, further clinical studies are needed to determine efficiency and reliability of this novel *in vivo* enrichment technology with the optimized product.

Conclusions

There is a worldwide tendency of an increase in the age of pregnant women. In association with this trend the number of fetal chromosomal aberrations is also increasing. Therefore, there is a great demand to improve methods of prenatal diagnosis and to find new ways of diagnosing fetal chromosomal abnormalities. In order to get genetic material of the fetus for analyses amniocentesis or chorionic villous sampling (CVS) are currently performed. These methods are associated with the danger of fetal loss. We have developed a completely new *in vivo* method for collecting fetal cells directly from the maternal circulation. A catheter functionalized with specific antibodies against fetal cell surface antigens is placed into the vein of a pregnant woman for 30 to 45 min coming into contact with a large volume of the maternal blood. The caught cells can then be identified by different methods and investigated for fetal chromosomal aberrations. This functionalized detector can be the basis for an effective *in vivo* device, as a tool for the detection and isolation of a rare cell type out of the peripheral blood. Covered with a hydrogel and specific antibodies (specific human F(ab) fragments or mAb MEM-G/9) against the HLA-G antigen the FSMW can be used for the *in vivo* isolation of trophoblast cells with high specificity. It was demonstrated that this device is safe (no side effects, low health risk) for mother and child, able to capture fetal cells for diagnostic analyses, easy to operate and it shows full hemocompatibility, is not cytotoxic and not immunogenic.The FSMW can be considered as an alternative method for obtaining fetal derived biological material for prenatal diagnostics avoiding the risks associated with amniocentesis and chorionic villous sampling for mother and child but its efficacy has to be improved.

References

[1] Assel B.G., Lewis S.M., Dickerman L.H., Park V.M., Jassani M.N. (1992) *Singleoperator comparison of early and mid-second-trimester amniocentesis.* Obstet. Gynecol. 79: 940-944.

[2] Cunniff C. (2004) *Prenatal Screening and Diagnosis for Pediatricians*. Pediatrics 114: 889-894.

[3] Papp C., Papp Z. (2003) *Chorionic villus sampling and amniocentesis: what are the risks in current practice?* Curr. Opin. Obstet. Gynecol. 15(2): 159-165.

[4] Yukobowich E., Anteby E.Y., Cohen S.M., Lavy Y., Granat M., Yagel S. (2001) *Risk of fetal loss in twin pregnancies undergoing second trimester amniocentesis.* Obstet. Gynecol. 98(2): 231-234.

[5] Evans M.I., Wapner R.J. (2005) *Invasive Prenatal Diagnostic Procedures 2005.* Semin. Perinatol. 29(4): 215-218.

[6] Snijders R.J., Noble P., Sebire N., Souka A., Nicolaides K.H. (1998) *UK multicentre project on assessment of risk of trisomy 21 by maternal age and fetal nuchal-translucency thickness at 10-14 weeks of gestation*. Fetal Medicine Foundation First Trimester Screening Group. Lancet 352(9125): 343-346.

[7] Cicero S., Curcio P., Papageorghiou A., Sonek J., Nicolaides K. (2001) *Absence of nasal bone in fetuses with trisomy 21 at 11-14 weeks of gestation: an observational study*. Lancet 358(9294): 1665-1667.

[8] Cicero S., Avgidou K., Rembouskos G., Kagan K.O., Nicolaides K.H. (2006) *Nasal bone in first-trimester screening for trisomy 21.* Am. J. Obstet. Gynecol. 195(1): 109-114.

[9] Lo Y.M.D., Corbetta N., Chamberlain P.F., Rai V., Sargent I.L., Redman C.W., Wainscoat J.S. (1997) *Presence of fetal DNA in maternal plasma and serum.* Lancet 350 (9076): 485-487.

[10] Lo Y.M., Chan K.C., Sun H., Chen E.Z., Jiang P., Lun F.M., Zheng Y.W., Leung T.Y., Lau T.K., Cantor C.R., Chiu R.W. (2010) *Maternal Plasma DNA Sequencing Reveals the Genome-Wide Genetic and Mutational Profile of the Fetus*. Sci. Transl. Med. 2, 61ra91.

[11] Schmorl G. (1893) *Pathologisch-anatomische Untersuchungen über Puerperal-Eklampsie*. Leipzig: Verlag FCW Vogel.

[12] Huppertz B., Kadyrov M., Kingdom J.C. (2006) *Apoptosis and its role in the trophoblast*. Am. J. Obstet. Gynecol. 195(1): 29-39.

[13] Bianchi D.W. (1998) *Current knowledge about fetal blood cells in the maternal circulation*. J. Perinat. Med. 26(3): 175-85.

[14] Hiby S.E., King A., Sharkey A., Loke Y.W. (1999) *Molecular studies of trophoblast HLA-G: polymorphism, isoforms, imprinting and expression in preimplantation embryo*. Tissue Antigens 53(1): 1-13
[15] Nakahori Y., Takenaka O., Nakagome Y. (1991) *A human X-Y homologous region encodes "amelogenin"*. Genomics 9(2): 264-9
[16] Firth H. (1997) *Chorion villus sampling and limb deficiency – cause or coincidence?* Prenat. Diagn. 17(13): 1313-1320.

✉ Klaus Luecke
GILUPI GmbH
Am Mühlenberg 11
14476 Potsdam, Germany