Increased placental expression and maternal serum levels of apoptosis-inducing TRAIL in recurrent miscarriage

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ABSTRACT

Introduction: Recurrent miscarriage (RM; ≥3 consecutive pregnancy losses) occurs in 1–3% of fertile couples. No biomarkers with high predictive value of threatening miscarriage have been identified. We aimed to profile whole-genome differential gene expression in RM placental tissue, and to determine the protein levels of identified loci in maternal sera in early pregnancy.

Methods: GeneChips (Affymetrix®) were used for discovery and Taqman RT-qPCR assays for replication of mRNA expression in placentas from RM cases (n = 13) compared to uncomplicated pregnancies matched for gestational age (n = 23). Concentrations of soluble TRAIL (sTRAIL) and calprotectin in maternal serum in normal first trimester (n = 35) and failed pregnancies (early miscarriage, n = 18, late miscarriage, n = 4; tubal pregnancy, n = 11) were determined using ELISA.

Results: In RM placentas 30 differentially expressed (with nominal P-value < 0.05) transcripts were identified. Significantly increased placental mRNA expression of TNF-related apoptosis-inducing ligand (TRAIL; P = 1.4 × 10−3; fold-change 1.68) and S100A8 (P = 7.9 × 10−4; fold-change 2.56) encoding for inflammatory marker calprotectin (51008A/A9) was confirmed by RT-qPCR. When compared to normal first trimester pregnancy (sTRAIL 16.1 ± 1.6 pg/ml), significantly higher maternal serum concentration of sTRAIL was detected at the RM event (33.6 ± 4.3 pg/ml, P = 0.00027), and in pregnant women, who developed an unpredicted miscarriage 2–50 days after prospective serum sampling (28.5 ± 4.4 pg/ml, P = 0.039). Women with tubal pregnancy also exhibited elevated sTRAIL (30.5 ± 3.9 pg/ml, P = 0.035).

Maternal serum levels of calprotectin were neither diagnostic nor prognostic to early pregnancy failures (P > 0.05).

Conclusions: The study indicated of sTRAIL as a potential predictive biomarker in maternal serum for early pregnancy complications.

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1. Introduction

Recurrent miscarriage (RM), defined as three or more consecutive pregnancy losses before 20–22 weeks pregnancy, affects 1–3% of couples aimed at childbirth [1–3]. While fetal chromosomal abnormalities represent the major factor behind sporadic miscarriages [4,5], they account for a smaller fraction of miscarriage events in RM couples [6,7]. Currently available diagnostic procedures allow to identify clinical conditions increasing their risk to pregnancy failure and to offer appropriate management options only in 50% of RM couples [1]. The known risk factors for developing of RM are maternal thrombophilic disorders or anti-phospholipid syndrome (5–15% in RM vs 2–5% in control women), uterine malformations (10–25% in RM vs ~5% in controls), maternal immunological and endocrine disturbances, parental balanced chromosomal rearrangements (3–6% of couples) [1]. Nevertheless, half of RM couples are classified as idiopathic with no identifiable cause [8]. Early recognition of a potential risk to pregnancy loss and systematic monitoring has beneficial effect in increasing live birth rates in RM couples [8,9]. The preferred
medical supportive care includes determination of hCG rising serum concentrations in early pregnancy and frequent ultrasound examinations [9]. However, only a limited number of potential predictive biomarkers of threatening miscarriage have been proposed [10–13].

Successful implantation, trophoblast invasion and placental development are key processes in early pregnancy, and placental insufficiency increases a risk of miscarriage and other pregnancy complications [14,15]. Due to the difficulties in obtaining first trimester placental tissue material, only a limited number of investigations on targeted gene expression in placentas from RM cases have been conducted [16–18]. To our knowledge, no whole-genome gene expression profile has been reported for RM placental tissue. The current study aimed (i) to address the whole-genome differential gene expression in the first trimester placental tissue in cases of RM compared to gestational-age matched uncomplicated pregnancies, and (ii) to determine the protein levels of identified RM related genes in maternal serum in order to evaluate their potential as biomarkers for predicting early pregnancy complications. In RM placentas, the study detected increased expression of TNF-related apoptosis-inducing ligand (TNFSF10; TRAIL) and S100A8 encoding for inflammatory marker calprotectin (heterodimer S100A8/A9). The study indicated sTRAIL as a potential predictive biomarker in maternal serum for early pregnancy complications.

2. Methods

2.1. Study subjects

Study participants were recruited at Women’s Clinic of Tartu University Hospital, Estonia during 2003–2011. The inclusion and analysis of collected human samples (placenta, blood serum) in each study stage are detailed on Fig. 1. The study was approved by the Ethics Review Committee on Human Research of the University of Tartu, Estonia (protocols no 17/9, 16.06.2003 and 180/M-15, 23.02.2009) and was conducted according to Declaration of Helsinki principles. A written informed consent to participate in the study was obtained from each individual.

2.1.1. Study subjects for placental gene expression experiments

Subjects included into the analysis of differential gene expression in trophoblastic tissue comprised of women (i) with recurrent miscarriage with no identifiable cause (RM patients; age 31.8 ± 1.5 years, gestational age at the miscarriage 67.7 ± 6.6 days) and (ii) electively terminated normal first trimester pregnancies with no maternal or fetal clinical complications until the termination of the pregnancy (ETP controls; age 26.7 ± 1.4 years, gestational age 63.0 ± 2.7 days) (Fig. 1A). The recruited RM patients (n = 13; parity 0–2, mean 0.7; gravidity 3–8, mean 4.3) had experienced at least three consecutive miscarriage events. The ETP control group (n = 23; parity 0–4, mean 1.5; gravidity 2–9, mean 3.8) had no clinically confirmed miscarriages in their clinical reproductive history. The following risk factors of pregnancy loss had been excluded in RM cases: abnormal menstrual cycle (>21 days and >35 days), genital infections, antiphospholipid syndrome (positive anticardiolipin and/or glycoprotein 1 antibodies), thrombophilic mutations (Factor V Leiden c.6191G>A; p.Arg506Gln; Coagulation factor II c.20210G>C), and gestational tract anomalies (ultrasound examination or hysterosonography) in RM women, and abnormal karyotype of either of the partners. Neither anticoagulant nor immunological treatment was applied during the pregnancy. Three patients had

Fig. 1. Study design. A. Patient groups analyzed in the study included patients diagnosed with recurrent miscarriage (RM), women with no miscarriages in their reproductive history as well as or patients with tubal pregnancy. RM was defined as three and more consecutive pregnancy losses. Index pregnancies at the recruitment were classified as ongoing or terminated. B. Biological material analyzed in the study included placental tissue (boxes in gray background, solid line) and maternal serum samples (unfilled boxes, dashed line). Trophoblastic tissue for genome-wide expression profiling was obtained during the surgical evacuation of uterus in patients with recurrent (>3rd case) miscarriage or elective termination of normal uncomplicated pregnancy (ETP) before 12th gestational weeks. Serum samples were taken during an office visit at the first trimester of pregnancy. The course of the index pregnancy was followed until successful delivery or pregnancy loss. Treatment with methotrexate or laparoscopic surgery due to tubal pregnancy was applied after blood sampling. C. Experimental design included mRNA expression analysis and functional assays. Differentially expressed genes identified from genome-wide expression profile in trophoblastic tissue were confirmed and replicated by real-time RT-qPCR using locus-specific Taqman assays. Concentration of proteins sTRAIL and S100A8/A9 (calprotectin) coded by the loci exhibiting significant overexpression in RM placentas, was measured in maternal blood serum samples.
2.1.2. Study subjects for maternal serum ELISA

Enzyme-linked immunosorbent assay (ELISA) was applied for the quantification of sTRAIL and S100A8/A9 (calprotectin) proteins in maternal serum obtained from: (i) same individuals studied for in the placental gene expression analysis (13 RM cases, 23 ETP controls); (ii) patients with ongoing pregnancies recruited at the 4–13th weeks of gestation (n = 32) (Fig. 1). All maternal serum samples have been stored at −80 °C.

Among the 21 women with ongoing intrauterine pregnancy, 12 patients had no history of miscarriage and 9 patients had experienced RM before the index pregnancy; additionally 11 women were recruited at their diagnosis of a tubal pregnancy.

Among the 12 women with no history of RM, in nine cases the index pregnancy resulted in the birth of a single live baby and in three cases the women experienced early or delayed miscarriage one to two weeks after serum sampling. Among the nine prospectively monitored patients with the clinical history of RM, the index pregnancy ended with a birth of single live baby in one-third of the cases (n = 3). The rest of them experienced recurrent miscarriages in the index pregnancy and were included in this study. Two cases had an early miscarriage one week after recruitment. Four patients experienced late pregnancy loss between 16 and 27th gestational weeks due to placental infarct, choioamnionitis or severe Parvovirus infection. Blood serum samples of the 11 women with ongoing tubal pregnancy was performed a day before treatment of the patients with methotrexate or laparoscopic surgery.

2.2. Tissue collection and RNA extraction

Placental tissue samples were obtained during elective surgical abortion of uncomplicated pregnancy (ETP individuals) or uterine curettage due to recurrent or delayed miscarriage (RM individuals), performed in Women’s Clinic of Tartu University Hospital in 2003–2006. Karyotype of the collected placental samples was unavailable. Placental samples were immediately washed with 0.9% saline solution to remove maternal blood, snap-frozen in liquid nitrogen and stored at −80 °C or placed into RNAlater solution (Ambion, Austin, TX, USA) and kept at −20 °C for RNA analysis. RNA was extracted from 200 mg of homogenized placental tissue (containing trophoblastic and decidual material) of each subject using TRIzol® Reagent (Invitrogen, Paisley, UK) according to manufacturer’s recommendations. To remove the residual phenol from TRIzol®-based extraction, 100 μl of extracted total RNA was further cleaned with NucleoSpin RNA II mini spin columns (MACHEREY-NAGEL, Germany) following the manufacturer’s protocol. Purity level and concentration was measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of the RNA used for microarray analysis were evaluated by the Agilent RNA 6000 Nano LabChip kit on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

2.3. Microarray hybridization

Differential gene expression in placental tissues in RM (n = 4) compared to normal first trimester uncomplicated pregnancies (n = 6) was addressed using Affymetrix Human Genome U133 plus 2.0 GeneChip according to manufacturer’s recommendations (Affymetrix, Santa Clara, CA, USA). In brief, 1 μg of purified total RNA was used to generate the first strand cDNAs using SuperScript II Reverse Transcriptase (Invitrogen) and T7-oligo (dT) primer. Next, in vitro transcription reaction for cRNA amplification and biotin labeling was performed in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix. Biotin-labeled cRNA targets were cleaned up, fragmented, and hybridized to GeneChip expression arrays.

2.3.1. Microarray data analysis

The microarray data generated in the study is Minimum Information About a Microarray Experiment (MIAME) compliant and the raw data has been deposited to a MIAME compliant database the Gene Expression Omnibus (GEO) data repository (accession no GSE22490). Quality control (QC) was performed by Affy package of Bioconductor, an open-source software project based on R [21]. QC criteria included correlation with average expression, correlation with median intensity of other GeneChips, GAPDH 3′−5′ and β-actin 3′−5′; scaling factor, percentage of presence calls, average background, intensities of positive and negative border elements (Fig. S1). GeneChip cel-files were imported to dChip [22] (http://biosou1.harvard.edu/complab/dchip/) and after QC analyzed using perfect match (PM)/mismatch (MM) modeling and invariant set normalization as previously described [23,24]. We filtered the genes with high signal intensity (dChip signal threshold > 100). Sample comparison (ETP against RM) was done with t-test, and fold-changes were calculated to evaluate differential expression of genes. Estimation of the empirical False Discovery Rate (FDR) [25] was described in Supplemental Data. Test 3. Hierarchical clustering of probe sets was performed by centroid linkage and the distance between two genes was defined as 1 − r. Allocation of identified genes to biological processes was performed using Gene Ontology Annotation Database (http://www.ebi.ac.uk/GOA/).

2.4. Reverse transcription quantitative PCR (RT-qPCR) and gene expression data analysis

One μg of total RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis SuperMix for RT-PCR kit (Invitrogen) in accordance with manufacturer’s instructions. Quantitative gene expression analyses were performed with commercially available pre-made TaqMan Gene Expression Assays (Table S1; Applied Biosystems, Carlsbad, CA, USA) using a duplex qPCR of target sequence and endogenous control (HPRT, Applied Biosystems). The qPCR reactions (95 °C for 15 min; 40 cycles of 15 s at 95 °C and 1 min at 60 °C) were performed in triplicate with ABI 7900HT Real-time PCR system (Applied Biosystems) using ABIsolution® qPCR ROX Mix (Thermo Fisher Scientific) or HOT FIREPol® Probe qPCR Mix (Solis BioDyne, Tartu, Estonia). Negative controls contained either non-reverse transcribed RNA or lacked template inputs.

Relative quantification of RT-qPCR data was determined by comparative Ct method, mean value of normalized expression was calculated by averaging three independently calculated normalized expression values of the triplicate (Equation No 2 in [26]). The calculations and efficiency corrections were carried out with qGENE software [27]. The median expression level of the control group was selected as a calibrator. All statistical analyses were performed by R 2.8.1, a free software environment for statistical computing and graphics (http://www.r-project.org/). Logistic regression model adjusted by maternal and gestational age was applied to assess differential gene expression between RM cases and controls quantified by RT-qPCR. The stringent Bonferroni significance threshold for multiple testing correction was calculated as s = 0.05/N, where N = 10 represents the number of independent Taqman RT-qPCR gene-probes.

2.5. ELISA analysis of sTRAIL and S100A8/S100A9 heterocomplex in maternal serum

Enzyme-linked immunosorbent assay (ELISA) was used to assess the concentration of sTRAIL (Duoset ELISA development kit #DY375, lot #1240906; R&D Systems Europe, Ltd., Abingdon, UK) and S100A8/A9 (MRPS14/1 ELISA kit #S-1011, lot #14E-1101; BMA Biomedicals, Augst, Switzerland) proteins in maternal serum. ELISA assays were conducted following manufacturer’s instructions, details are provided in Supplemental Data, Text S2. The raw data were analyzed by curve fitting ReaderFit software with 5-parameter logistic model (www.readefit.com). All statistical analyses were performed by R 2.13.1. Logistic regression models were applied to assess the differences between the study groups in the concentration of sTRAIL and S100A8/A9 in maternal serum. The analyses were adjusted for gestational and maternal age. Natural log-transformation was applied to all quantitative data to improve the approximation of normal distribution. Correlation of the relative mRNA expression (RT-qPCR) in placenta and protein level in maternal serum was assessed with Pearson correlation test (coefficient r). P < 0.05 was considered as statistically significant.

3. Results

3.1. Genome-wide differential gene expression in RM placentas

Whole-genome differential gene expression patterns in placental tissue obtained from four recurrent miscarriage (RM) and six uncomplicated first trimester pregnancies were addressed using GeneChip (Affymetrix) expression arrays (Fig. 1). In total, 27 differentially expressed genes (representing 30 transcripts with nominal P-values < 0.05) in RM compared to control placentas were identified, 12 genes with increased and 15 loci with reduced transcript levels (Table 1, Fig. S3). However, none of the P-values remained significant after correction for multiple testing with FDR and thus, GeneChip profiling was considered as exploratory analysis for hypothesis generation. For the further confirmatory experiments of the discovery observations, genes were selected based on their fold-changes. In GeneChip dataset, gene alterations in general expression in RM placentas were averaged from 1.2-2.9 fold decrease in ASMTL mRNA level (P-value 0.048) to more than 4.1 fold increased expression of S100A8 transcripts (P-value 0.040). The expression level of S100A9, the dimerization partner of S100A8 in
the assembly of heterocomplex S100A8/A9 protein (calprotectin) was also up-regulated (fold-change 1.81; P-value 0.040). It is noteworthy, that three probes targeting the TRAIL (TNFSF10) gene were among the six transcripts with the highest fold-change (fold-changes 2.16–7.25; P-value ≤0.032). The ‘Volcano’ plot positioned TRAIL (TNFSF10) among the genes exhibiting the highest differential expression (top fold-change) in RM combined with the best statistical support (lowest P-values) (Fig. S2). Among the detected 27 differentially expressed genes in RM placenta, an enrichment of genes involved in cell communication and signaling (CALR, CCR1, LYN, NENF, PTEF, S100A9, TRAIL), inflammatory and immune response (CD163, CCR1, SECTM1, S100A8, S100A9, TRAIL) was observed (Table S2).

3.2. Significantly increased levels of S100A8 and TRAIL transcripts in RM placentas

Although GeneChip profiling had failed to identify any transcripts exhibiting statistically significant (after FDR) evidence for differential expression in RM placentas, it provided a list of genes with the highest fold-changes, and thus with potential importance. Top genes in this list were subjected to further experimental confirmation by an independent method for mRNA quantification, and by analyzing an independent sample set. First, ten genes (ASMTL, BRD1, CALR, CCR1, CD163, NENF, PTEF, S100A8, SNAI2, TRAIL) showing a fold-change greater than two (up- and down-regulated) in RM placentals were subjected to experimental confirmation by RT-qPCR using identical samples as analyzed at the expression microarray (Table 2). One gene, SECTM1, was excluded from RT-qPCR due to incompatibility with the pre-made Taqman assay. mRNA quantification by Taqman confirmed significantly altered expression in RM placental tissue for five genes (logistic regression: S100A8, P = 2.4 × 10⁻⁴; fold-change 4.98; TRAIL, P = 2.7 × 10⁻³, fold-change 2.46; CD163, P = 2.5 × 10⁻²; fold-change

| Gene (abbreviation) | Fold-change | 90%CI       | Nominal P-value |
|---------------------|-------------|-------------|-----------------|
| S100 calcium binding protein A8 (S100A8) | 4.11*       | 2.13; 10.05 | 0.03937         |
| CD163 molecule (CD163) | 2.94*       | 1.56; 7.19  | 0.04662         |
| Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10 or TRAIL) | 2.75*       | 1.93; 3.82  | 0.02344         |
| Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10 or TRAIL) | 2.57*       | 1.79; 3.69  | 0.02016         |
| Secreted and transmembrane 1 (SECTM1) | 2.38       | 1.60; 3.61  | 0.02776         |
| Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10 or TRAIL) | 2.16*       | 1.49; 3.14  | 0.03204         |
| Bromodomain containing 1 (BRD1) | 2.12*       | 1.25; 5.25  | 0.03722         |
| Chemokine (C–C motif) receptor 1 (CCR1) | 2.03*       | 1.50; 2.69  | 0.02940         |
| Potassium voltage-gated channel, Isk-related family, member 3 ( KCNHI2) | 1.97       | 1.35; 2.94  | 0.03691         |
| Bromodomain containing 1 (BRD1) | 1.92*       | 1.34; 3.11  | 0.01233         |
| S100 calcium binding protein A9 (S100A9) | 1.81       | 1.24; 3.25  | 0.02793         |
| Branched chain aminotransferase 1 (BCAT1) | 1.63       | 1.32; 2.10  | 0.00362         |
| RAB11 family interacting protein 1 (RAB11FIP1) | 1.52       | 1.26; 1.87  | 0.00502         |
| CCAAT/enhancer binding protein (C/EBP), delta (C/EBPD) | 1.5       | 1.21; 1.84  | 0.02857         |
| 3.2. Significantly increased levels of S100A8 and TRAIL transcripts in RM placentas

Although GeneChip profiling had failed to identify any transcripts exhibiting statistically significant (after FDR) evidence for differential expression in RM placentas, it provided a list of genes with the highest fold-changes, and thus with potential importance. Top genes in this list were subjected to further experimental confirmation by an independent method for mRNA quantification, and by analyzing an independent sample set. First, ten genes (ASMTL, BRD1, CALR, CCR1, CD163, NENF, PTEF, S100A8, SNAI2, TRAIL) showing a fold-change greater than two (up- and down-regulated) in RM placentals were subjected to experimental confirmation by RT-qPCR using identical samples as analyzed at the expression microarray (Table 2). One gene, SECTM1, was excluded from RT-qPCR due to incompatibility with the pre-made Taqman assay. mRNA quantification by Taqman confirmed significantly altered expression in RM placental tissue for five genes (logistic regression: S100A8, P = 2.4 × 10⁻⁴; fold-change 4.98; TRAIL, P = 2.7 × 10⁻³, fold-change 2.46; CD163, P = 2.5 × 10⁻²; fold-change
of sTRAIL exhibited its peak concentration at the 4th gestational week (mean 32.4 pg/ml, median 32.6 pg/ml, range 13.7–49.5 pg/ml; Fig. 3A) followed by a gradual decrease toward the end of the first trimester (mean level during the 8–13th gestational week 12.7 pg/ml, median 12.0 pg/ml, range 5.8–29.0 pg/ml). In contrast, no similar decrease in the maternal serum sTRAIL concentration was detected in pregnancies that ended with early miscarriage (Fig. 3B). In cases of pregnancy loss the circulating protein level during the 8–13th gestational week remained as high as at the beginning of the pregnancy (mean 32.5 pg/ml, median 29.8 pg/ml, range 17.3–50.1 pg/ml).

When compared to normal first trimester pregnancy (sTRAIL 16.1 ± 1.6 pg/ml, median 13.0 pg/ml), significantly higher maternal serum concentration of sTRAIL was detected both (i) at the recurrent miscarriage event (33.6 ± 4.3 pg/ml, median 29.7 pg/ml; P = 0.00027; Fig. 4), as well as (ii) in uncomplicated gestation at the time of serum sampling, which terminated with an unexpected first trimester pregnancy loss (28.5 ± 4.4 pg/ml, median 27.7 pg/ml; adjusted P = 0.039). In addition, women with diagnosed tubal pregnancy also exhibited significantly elevated concentration of sTRAIL in their circulation (30.5 ± 3.9 pg/ml, median 31.9 pg/ml; adjusted P = 0.035). In contrast to the cases with early pregnancy failure, sTRAIL concentrations measured in women with an uncomplicated pregnancy at the time of serum sampling, but developing a late miscarriage or extremely preterm delivery of a stillbirth baby (16–27 weeks of gestational age) did not differ statistically from the sTRAIL serum levels in normal pregnancy resulting in the birth of a healthy child (mean 7.6 ± 2.3 pg/ml, median 5.6 pg/ml; adjusted P > 0.05).

In the cases with data for both placental mRNA expression of TRAIL and maternal serum concentration of TRAIL, the two measurements were significantly correlated (Pearson’s correlation coefficient r = 0.60, P = 0.002).

No temporal dynamics during the first trimester of normal and failed pregnancies was detected in the maternal serum levels of heterodimeric complex of S100A8/A9 (calprotectin) by ELISA assays (Fig. 3C–D). Maternal serum levels of S100A8/A9 were neither diagnostic (sampled at the occurred miscarriage or tubal pregnancy, P > 0.05) nor prognostic (sampled prospectively in patients, who later experienced pregnancy loss, P > 0.05) to pregnancy failures, when compared to normal pregnancy at the same gestational age. In the cases with the available data for placental mRNA expression of S100A8 and maternal serum concentration of S100A8/A9, the two measurements were not correlated (r = 0.04, P = 0.8).

4. Discussion

The current study represents the first genome-wide expression profiling of placental tissues from recurrent miscarriage (RM) compared to uncomplicated first trimester pregnancy. The identified 27 differentially expressed genes (nominal P < 0.05) act in the pathways involved in cell signaling and apoptosis, inflammation and environmental response (Table S2). The main limitation of the discovery study was small sample resulting in limited statistical power in whole-genome expression profiling. Additionally, possible inclusion of some aneuploid miscarriages may have weakened the analysis, as the karyotype of miscarried embryos was unavailable. The limitations of the GeneChip exploratory analysis were overcome by two rounds of confirmatory experiments using quantitative methods (RT-qPCR and serum measurements), which targeted individual loci. Significantly increased mRNA expression of
Apoptosis-inducing ligand TRAIL and inflammatory marker S100A8 was confirmed (Fig. 2; Table 2). Measurement of the soluble forms of coded proteins in maternal serum in the first trimester gestation revealed sTRAIL as a potential novel non-invasive predictive biomarker for pregnancy outcome (Figs. 3 and 4). Our pilot dataset suggests that the level of maternal serum sTRAIL is predictive of the potential risk of pregnancy failure from the 8th gestational week. It is noteworthy that a recent study has independently demonstrated significantly elevated levels of sTRAIL immediately after miscarriage in RM patients compared to that in first-trimester normal pregnant women [13].

Apoptosis plays an important role in the normal development of human placenta and an altered balance between proliferation and apoptosis of villous trophoblast is associated with abnormal pregnancies [28,29]. Increased levels of villous trophoblast apoptosis have been identified in several placental pathologies including early pregnancy loss, tubal pregnancy, preeclampsia, intrauterine growth restriction and gestational trophoblastic disease [17,28–32]. TRAIL encodes for TNF-related apoptosis-inducing ligand that is expressed on the surface of immune cells in many tissues. It is a type II transmembrane protein and can be cleaved by a cysteine protease to generate a soluble form sTRAIL [33]. In the villous placenta TRAIL is expressed constantly throughout gestation at the protein level [34]. The expression of TRAIL in trophoblast cell culture and release of sTRAIL into culture media was reported to decrease concordantly during differentiation and syncytialisation processes [35]. In the present study a gradual decrease of maternal sTRAIL during the first trimester of normal pregnancy was documented. Interestingly, TRAIL and its receptors appear to be differentially expressed in villous placenta. Expression of TRAIL and its two decoy receptors DcR1 and DcR2 is localized predominantly in the syncytiotrophoblast, whereas cytotrophoblast cells have been shown to express high levels of DR4 and DR5 [34,36] that trigger apoptotic signaling via activation of the classic caspase-dependent ‘death’ pathway [37]. In contrast, DcR1/DcR2 and a soluble TRAIL receptor osteoprotegerin antagonize apoptotic signaling and may protect resident cells of the fetal membranes against the pro-apoptotic effects of TRAIL during pregnancy [35,38]. Additionally, the trophoblast might use TRAIL as a non-apoptotic signal influencing the cytokine milieu of the maternal endometrium [39]. The presence of TRAIL has been also shown at the tubal implantation site, possibly contributing to trophoblast invasion in cases of extrauterine pregnancy [40]. Taken together, there is an important balance between cell death and survival signaling upon TRAIL binding [41]. The increased level of sTRAIL may reflect either already initiated process of fetal rejection or indicate to abnormal development and syncytialization of cytotrophoblast cells.

In addition to TRAIL, the current study detected significantly elevated placental mRNA expression of S100A8 in cases of RM. Heterodimeric S100A8/A9 protein (calprotectin) has antimicrobial, cytostatic, antiproliferative, apoptosis-inducing and chemotactic properties and is involved in many physiological and pathological
processes in female reproductive tract [42]. The significantly increased concentrations of S100A8/A9 in amniotic fluid or in maternal serum have been reported in intra-amniotic inflammation, preterm labor and preeclampsia [42–44]. However, in the present study the increased placental mRNA expression of S100A8 and S100A9 in RM placentas was not reflected in the level of calprotectin in maternal serum and may rather reflect a local inflammation process of the tissues at the maternal–fetal interface during the rejection process of the pregnancy.

In summary, we showed TRAIL and S100A8 as differentially expressed genes in placental tissue from recurrently miscarried compared to uncomplicated pregnancies. Significantly increased level of sTRAIL was measured in maternal serum during the first trimester of pregnancy with unfavorable endpoint, exhibiting potential as a predictive non-invasive biomarker for early pregnancy complications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2012.11.032.

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