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A proper placental sampling for syncytin-1 analysis

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ABSTRACT

Syncytin-1 (gene *ERVW-1*) has been proposed as a marker of pre-eclampsia and malfunctions in placental development. Placenta is heterogeneous tissue, hence the method of biopsy can significantly affect the outcome of analyses. A total of 44 placentae were analyzed by taking 3–30 samples from each. Relative levels of *ERVW-1* expression in the placental biopsies were characterized by RT-qPCR. Evaluation of ten biopsies from one placenta individually (not pooling them) is recommended due to the high variability of expression. No significant correlation was found between biopsy localization and level of *ERVW-1* expression; therefore, random sampling is recommended. A long cut from the umbilical cord to the edge of the placenta is a convenient approach to placental sampling.

METHOD SUMMARY

A modified protocol for placental sampling for gene expression studies was designed. Taking into account the heterogeneity of placental tissue, at least 30 samples should be taken along the axis from the umbilical cord to the edges of the placenta for pilot experiments and statistical analyses. Analyses of samples from one placenta have to be made separately, without pooling them, and at least ten samples have to be taken for *ERVW-1* expression assessment.

KEYWORDS:

ERVW-1 ● gene expression analysis ● placenta ● placental sampling ● pre-eclampsia ● syncytin-1

Hypertensive disorders in pregnancy can be categorized into: chronic hypertension, transient gestational hypertension, gestational hypertension and pre-eclampsia (either *de novo* or superimposed on chronic hypertension) [1]. Pre-eclampsia is a multifactorial disorder and still one of the main causes of maternal and perinatal morbidity and mortality worldwide [2,3]. Multiple processes lead to the development of pre-eclampsia, such as impaired implantation, systemic inflammation, endothelial dysfunction and tissue damage caused by repeated ischemia-reperfusion [4]. It is possible that genetic predisposition also plays a role in the occurrence of the disease [5]. Diagnosis of pre-eclampsia has limitations; high false negativity or positivity is typical, as the clinical presentation is highly variable. Even those with severe disease can remain asymptomatic [4]. The root cause of pre-eclampsia is probably the placenta, as symptoms disappear shortly after delivery [6,7]. High elevated liver and low platelets (HELLP) syndrome is a syndrome in pregnant and postpartum women, characterized by hemolysis with a microangiopathic blood smear, elevated liver enzymes and a low platelet count. It probably represents a severe form of pre-eclampsia, but the relationship between the two disorders remains controversial [7].

The placenta at term is a complex organ that includes numerous cellular components of fetal origin surrounded by maternal cells [8–10]. The localization of the biopsy reflects the cellular composition and activity of the placenta [11]. Studies have concluded that when many biopsies are used (n = 12), the distribution of all variables measured was homogeneous throughout the placenta. However, all the variables examined showed a large intraindividual variation, so the particular locations of biopsies can be a crucial factor in results evaluation [10,12]. Placenta-wide levels of expression of the genes studied and the way in which biopsy localization correlates with expression of the gene of interest, are commonly not known. Therefore it is hard to evaluate if there is significant change between normal and pathological samples as they are compared in research articles.

Aberrations during this cell fusion process in placenta are associated with intrauterine growth restriction, pre-eclampsia and HELLP syndrome. *ERWV-1* is one of the most important genes involved in cell fusion and shows decreased gene expression during these pathological pregnancies [13]; thus the gene and its product, syncytin-1, can be used as a potential marker in diagnostics. It has been observed that expression of *ERVW-1* is downregulated in pre-eclamptic cases and this downregulation correlates with severity of symptoms [14–

17]. It is not clear how (or whether) syncytin-1 has a role in pre-eclampsia development, but it is known that a physiologically normal amount of syncytin-1 is crucial for proper development of trophoblast in placentation [18]. On other hand, syncytin-1 expression is activated and upregulated in a variety of malignancies including breast cancer, endometrial carcinomas, ovarian cancer, colorectal cancer, leukemia and lymphoma [19,20]. Several studies have suggested that measurement of syncytin-1 expression levels in cancer tissues may carry some prognostic value for certain tumor types and stages.

Despite information about the extremely high variability of gene expression in the placenta and recommended standardized methods of placental sample collection [21–23], most studies have used a small biopsy size; usually one or three samples are taken from one placenta. Details about the exact site of sampling and the method of biopsies are rarely given. The majority of pre-eclampsia studies have analyzed only three samples (taken near the cord, near the edge and at a point somewhere between these areas) from one placenta [16,24,25]. These three samples are typically pooled together and analyzed.

The main focus of our study is the characterization of *ERVW-1* expression as a potential diagnostic marker for pre-eclampsia in 41 placentae (12 pre-eclamptic and 29 physiologically normal). Three samples were taken from each placenta and samples were either analyzed separately or pooled together. One of the goals of our study was to determine whether the pooling of samples and the separate evaluation of each biopsy from one placenta give the same results.

Informative genome-scale studies using few or pooled human placenta samples have been done with a focus on pathological conditions rather than variation in normal placentae. Gene expression studies usually focus on differences between physiologically normal and pathological placentae, even when the variations in expression of studied genes in the population are not known. Hence three full-term placentae without pathological condition were also analyzed (up to 30 samples from each placenta) to determine normal variation in *ERVW-1* gene expression. The goal was to establish what number of biopsies from one placenta is essential for representation of placental expression and whether the three samples usually taken are enough for gene expression studies.

Materials & methods

Study design & ethical approval

Placentae were transported on ice from the Obstetrics and Gynecology department within 30 min after cesarean section and biopsy samples were collected in Department of Medical Biology, Genetics and Clinical Genetics of Faculty of Medicine (Comenius University in Bratislava, Slovakia). The study has been approved by the local ethical committee of University Hospital Bratislava, Slovakia. Participants have signed a written informed consent.

Placental sampling & *ERVW-1* expression analyses

Full-thickness tissue samples were taken from 41 placentae from locations near the cord, near the edge of the placenta and in the space between them. The chorionic plate, including overlying membranes, was removed. Biopsy samples (small cubes of 75 mm^3) were collected from the fetal site at a depth of 1.5 cm to avoid contamination by decidua. A total of 12 samples came from patients with gestational hypertensive disorders (HD) – gestational hypertension, pre-eclampsia or HELLP syndrome – classified according to guidelines from the International Society for the Study of Hypertension in Pregnancy [1] and 29 were from mothers with uncomplicated pregnancies (physiologically normal, PN). Patients with chronic hypertension, diabetes mellitus, chronic kidney disease, lupus erythematosus, antiphospholipid syndrome, cancer, multiple pregnancy, gravidity with a fetus with chromosomal and structural abnormalities or a history of smoking were excluded from the PN group. All samples from the placenta were evaluated at the same time, either separately or pooled together. Additionally, samples were taken from three placentae from mothers with uncomplicated pregnancies (control placentae), all at the same gestation (week 39). Up to 30 samples from each of the control placentae were taken from different parts of the placenta, along the long axis of the placenta and axis perpendicular to it (Figure 1A). Biopsies were immediately placed in RNAlater (Sigma-Aldrich, MO, USA) and stored at -20°C according to the manufacturer's instructions. Human placental tissues were provided by pregnant women after informed consents.

Total RNA was extracted by using the GeneJET RNA Purification Kit (Thermo Scientific, USA) following the standard protocol. Total RNA was eluted in 50 μl of sterile RNase-free water. Total RNA concentration was calculated after the absorbance measurement at 260 nm (NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific). Protein contamination was monitored by A260/A280 ratio. All samples ranged in concentration from 70 to 350 μg/μl and had A260/A280 ratio of >1.8. They were aliquoted and stored at -80°C.

Isolated RNA was treated by DNase I, Amplification Grade (Sigma-Aldrich, MO, USA) following the standard protocol. First strand cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) according to the manufacturer's instructions.

Real-time PCR was performed in triplicate of 25 μl mixtures, 12.5 μl of Maxima Probe/ROX qPCR Master Mix (2×) (Thermo Scientific) and 1.25 μl of each Taqman Gene Expression Assay (Thermo Scientific) for *ERVW-1* and *YWHAZ* (reference gene). The gene expression was relatively quantified as ΔCt, the difference between the Ct of the gene of interest (*ERVW-1*) and the reference gene (*YWHAZ*). ΔΔCt is the difference between ΔCt of the HD and PN groups.

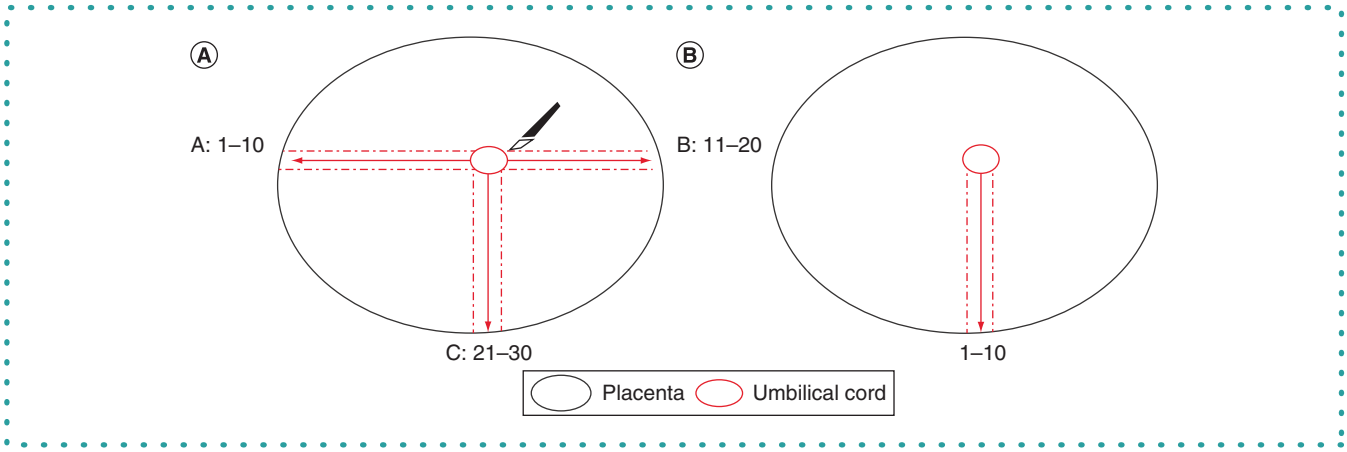

Figure 1. Localization of placental biopsies. (A) Localization of biopsies from three control placentae. The line A-B represents the long axis of the placenta and the line C is perpendicular to it. **(B)** Recommended localizations based on *ERVW-1* expression variability.

Table 1. Characteristics of mothers and pregnancies.

Characteristic	PN	HD
Maternal age (years, mean ± SD)	33.2 ± 4.7	32.9 ± 4.8
Gestational week (mean ± SD)	38.3 ± 2.6	39.4 ± 0.5
BMI (mean ± SD)	29.2 ± 4.7	31.6 ± 4.6
Gravidity (median)	2	2
Parity (median)	1	1
Birth weight (g, mean ± SD)	3115 ± 704	3057 ± 633
Placental weight (g, mean ± SD)	577 ± 158	550 ± 162
Baby's sex	M: 51.6% F: 48.4%	M: 50% F: 50%

HD: Gestational hypertensive disorder; PN: Physiologically normal; SD: Standard deviation.

Statistical analyses

Results from the QuantStudio 3 & 5 qPCR Data Analysis Software using the relative Quantitation method with auto thresholds and baselines were exported into Excel (Microsoft Corporation, WA, USA) for data analysis. If the Ct values of the triplicates differed by a >0.5 standard deviation, the sample was retested. Ct value triplicates differing by ≤ 0.5 standard deviations were averaged.

An analysis of variance (ANOVA) test, *t*-test, F-test, Mann–Whitney test, Friedman test or Bartlett test were performed to identify significant differences in gene expression of *ERVW-1* in placental samples. All calculations were performed using the SPSS software package (SPSS v10.0, SPSS Inc, IL, USA).

Results & discussion

Expression of *ERVW-1* in placentae from pooled placental biopsies from single RNA isolation

A total of 41 placentae were collected: 29 from uncomplicated (low-risk pregnancies) and 12 from high-risk pregnancies (gestational hypertension, pre-eclampsia and HELLP). Characteristics of the mothers and pregnancies are given in Table 1. First, the method based on protocols from published articles [23,26,27] was used, whereby three samples (C – near the chord, L – near the edge of placenta, S – space approximately midway between C and L) taken from analyzed placentae were pooled together for RNA isolation. No significant difference was detected between gestational hypertensive, pre-eclamptic and HELLP placentae and they were therefore grouped together in analyses. The mean level of *ERVW-1* expression in HD placentae was lower by 33% compared with PN (Figure 2), which correlates with already published data. However, the change of *ERVW-1* expression was not statistically significant in our samples (F-test, p = 0.2625; *t*-test, p = 0.2271; ANOVA, p = 0.2271).

Expression of *ERVW-1* in randomly selected placentae from pooled placental biopsies from single RNA isolation

We wanted to test whether pooling of samples from one placenta provides reproducible results; hence seven PN samples and eight samples with gestational HD were randomly selected from 41 placentae. This number of samples was chosen based on sample sizes in published studies [13,16,28,29]. Mean (median and quartiles) of gene expression of selected samples are shown in Figure 3A. Mean values of expression in placentae with gestational hypertensive disorder were higher by 86% compared with PN (Figure 3D). Mean,

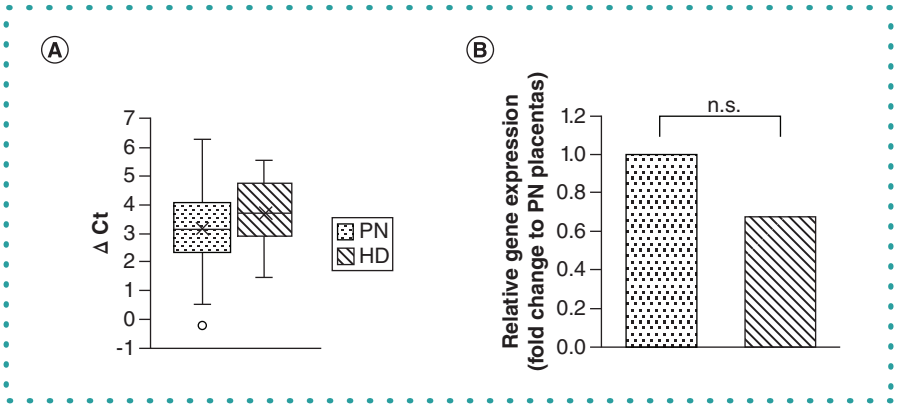

Figure 2. Relative *ERVW-1* expression. (A) ΔCt and **(B)** $2^{-\Delta\Delta Ct}$. ΔCt is the difference between Ct of *ERVW-1* and reference gene (*YWHAZ*); ΔΔCt is the difference between ΔCt of HD and PN. n.s.: $p > 0.05$.
HD: Hypertensive disorders; PN: Physiologically normal; n.s.: Not significant.

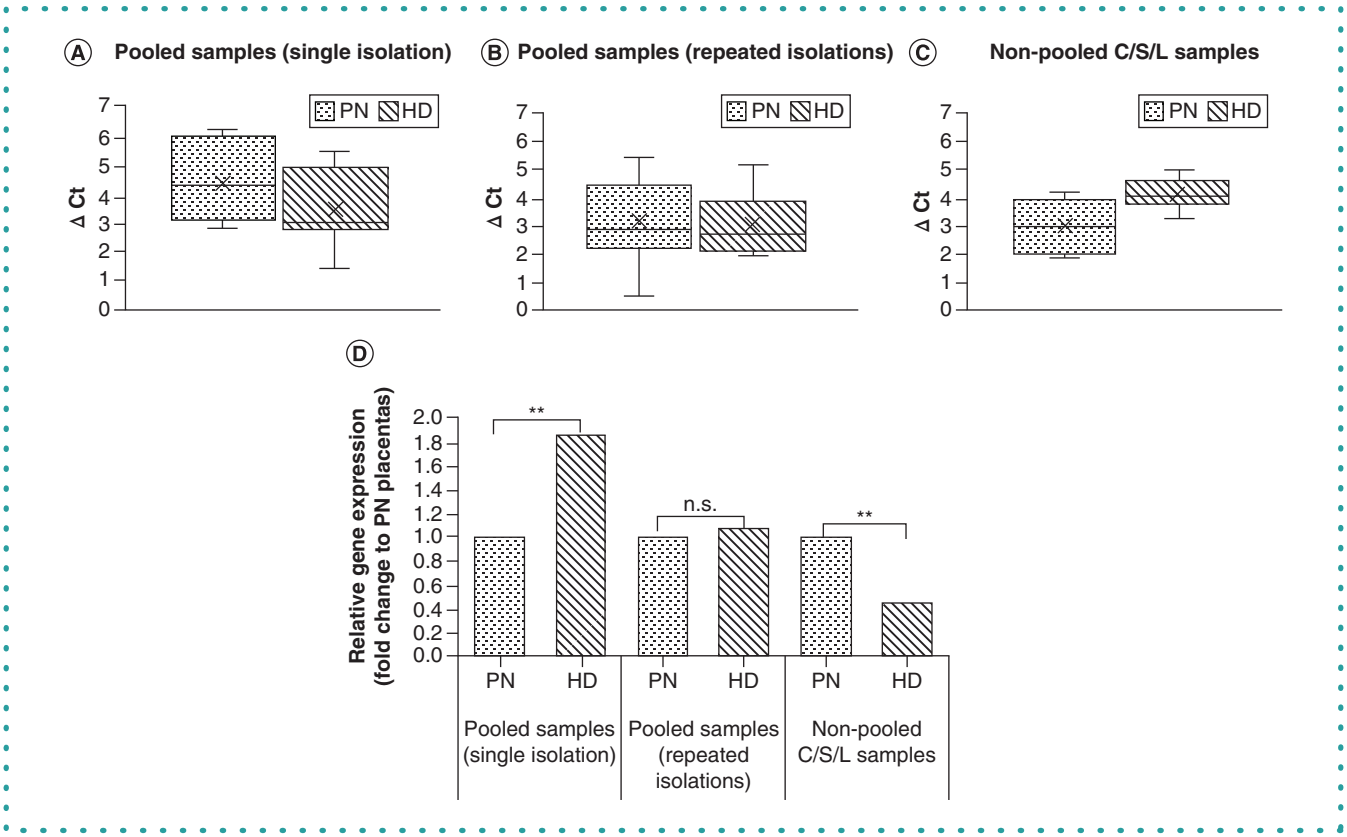

Figure 3. Relative *ERVW-1* expression of selected samples. (A) Pooled samples from single isolated RNAs expressed as ΔCt. **(B)** Pooled samples from repeatedly isolated RNAs expressed as ΔCt. **(C)** Nonpooled C/S/L samples (analyzed separately) expressed as ΔCt. **(D)** Samples expressed as fold change compared with PN placentae.
C: Samples taken near the cord; L: Samples taken near the edge of the placenta; S: Samples from space approximately between C and L. ΔCt is the difference between Ct of *ERVW-1* and reference gene (*YWHAZ*); ΔΔCt is the difference between ΔCt of HD and PN.
HD: Hypertensive disorder; PN: Physiologically normal; n.s.: Not significant.

variance and median of ΔCt for PN and HD were not significantly different (*t*-test, p = 0.22; F-test, p = 0.9992; Mann–Whitney test, p = 0.20). *ERVW-1* expression in pooled samples from one placenta was higher in HD samples, but not significantly so. If another set of samples had been chosen, the results of level of *ERVW-1* expression and differences would not be the same, highlighting that sample size has a direct impact on the result. Although previously published articles have shown unambiguous conclusions with small sample

sizes [13,15,16,25], our results prove that it is extremely difficult to say if changes in *ERVW-1* expression are significant or not significant in pre-eclamptic cases when a small number of samples is used. On average, 33.2, 58.9 and 7.8% of placental transcriptome variation is caused by variation within individuals, among individuals and among human groups, respectively [30]. If the between-group differences at a gene of interest are close to normal biological variability within a placenta, the sample sizes for every group have to be big enough to reliably prove significant differences between normal and pathological placentae.

Mean expression of *ERVW-1* in randomly selected placentae from pooled placental biopsies from repeated RNA isolations

To verify reproducibility of the method when biopsies (C/S/L) from one placenta are pooled together and RNA is isolated from the mixture, RNAs from seven PN and eight HD samples were repeatedly isolated (two- to four-times). The means (median and quartiles) of gene expression of selected samples, which were repeatedly analyzed, are shown in Figure 3B. Mean expression in HD placentae was higher by 10% compared with PN (Figure 3D). The mean, variance and median of ΔCt for PN and HD were not significantly different (*t*-test, p = 0.86; F-test, p = 0.31; Mann–Whitney test, p = 0.69; Figure 3). An interesting finding was that the variance of obtained ΔCt was extremely high for repeatedly isolated samples from the same placenta. These results suggest that any method where samples are pooled from one placenta has low reproducibility. Biopsy of the same amount of every part of tissue (C, S or L) is extremely hard to achieve, even with analytical balances and even small changes of input materials can change the outcome of gene expression. The main reason is presumably high variability of *ERVW-1* expression in placenta. Determination of the level of *ERVW-1* expression was highly affected by repetition of RNA isolation steps from pooled samples. The conclusion is that pooling of samples is not an optimal and reproducible method for *ERVW-1* gene expression analysis.

Expression of *ERVW-1* in randomly selected placentae from nonpooled biopsies

C, S and L parts of placental biopsies from one placenta were analyzed separately, without pooling and values were averaged. The mean (median and quartiles) of *ERVW-1* expressions are shown in Figure 3C. Mean values of expression in HD placentae were lower by 54% compared with PN (Figure 3D). Differences in the mean and median of ΔCt between HD and PN were significant (*t*-test, p = 0.01; Mann–Whitney test, p = 0.03), contrary to variance (F-test, p = 0.12). Lower variance of ΔCt values was observed compared with ΔCt from pooled biopsies, confirming better suitability of the method for expression studies. Variance among the C, S and L samples from one placenta was relatively high, with standard deviations for samples from 0.03 to 2.57 (mean and median 0.87). A total of nine out of 15 samples had standard deviations over 0.5 which can be considered as significant difference.

Comparison of *ERVW-1* gene expression by three methods of placental biopsy

The levels of *ERVW-1* expression of selected samples that were achieved by three approaches (pooled samples from single or repeated isolations and C/S/L samples) are shown in Figure 4. Selected samples are sorted from the highest to lowest level of *ERVW-1* expression in the single isolation experiments. The means of *ERVW-1* gene expression for PN or HD samples obtained by the different methodical approaches were not significantly different (PN: ANOVA, p = 0.1196; Friedman test, p = 0.3679; HD: ANOVA, p = 0.01158; Friedman test, p = 0.1969). Lack of correlation or trend between the methods and level of *ERVW-1* expression can explain statistical nonsignificance. When levels of *ERVW-1* expression for individual samples achieved by different methods were compared, the majority differed by more than one cycle (Figure 4), indicating that expression was two-times higher or lower depending on the sampling methods used; this would be considered as significant difference in gene expression studies.

Wide variability of *ERVW-1* expression in our samples was repeatedly observed; evaluation of biopsies separately from one placenta is recommended because pooling of samples can lead to distortion of results. Slightly lower variability of HD samples compared with PN samples was observed (F-test, p = 0.008) in our experiment. This is an interesting finding because the HD group consisted of pathological samples with different severities of disorder and it has previously been stated that *ERVW-1* expression is altered depending on the severity [16]. Our results do not correlate with this suggestion. On the other hand, we observed higher variability in the PN group. This only emphasizes the need for higher sample numbers, primarily to evaluate natural variability of *ERVW-1* expression in population. After this, differences between PN and pathological samples can be tested appropriately. It seems that higher sample numbers than are used in the majority of the research studies have to be collected, for the elucidation of the possible role of *ERVW-1* in pre-eclampsia.

Intraplacental & interplacental variability of *ERVW-1* expression in healthy population

Physiologically normal *ERWV-1* expression is not precisely characterized, not only in the population, but also within one placenta. Without evaluation of the gene expression variability in higher numbers of samples from different locations within one PN placenta, it is not possible to decide whether three samples, frequently used in *ERVW-1* expression studies, are enough for representation of *ERVW-1* expression. There are several methodical standards for placental sampling. Mayhew has recommended systematic uniform random sampling, in which the first item is chosen randomly but then a predetermined pattern decides the sites of other samples. This approach can eliminate bias because it gives all parts an equal chance of selection, assuming the sampling interval does not correspond with an inherent pattern of gene expression within the placenta [22]. Roberts *et al.* describe a standardized method for the collection of placental tissue samples along the long axis of the placenta orientated around the umbilical cord insertion site [31]. The overall variation within a study depends on biological variation (e.g., between placentae in a group, between sample sites within a placenta) and introduces errors

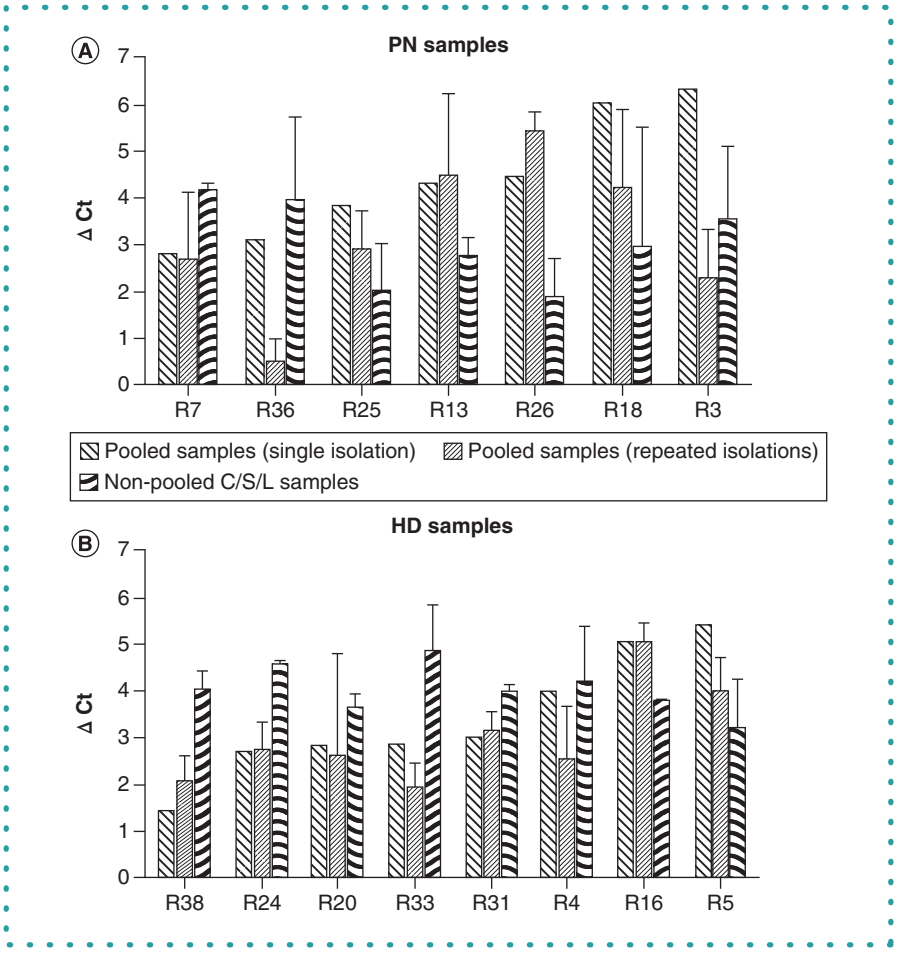

Figure 4. Relative *ERVW-1* expression of pooled samples and samples from selected placentae. (A) Physiologically normal placentae. **(B)** Placentae from patients with hypertensive disorders (gestational hypertension, pre-eclampsia, HELLP syndrome).
R numbers represent repeated isolations of pooled samples.
C/S/L: Nonpooled samples (C: samples near the cord; L: samples near the edge of the placenta; S: Samples from a space approximately between C and L).
HD: Hypertensive disorders; HELLP: High elevated liver and low platelets; PN: Physiologically normal.

including those due to sampling [21]. So another important question, apart from biopsy localization, is how many samples are needed for accurate characterization of *ERVW-1* expression. Burton *et al.* proposed that taking multiple samples from at least four placental sites/quadrants, pooling samples from each of the four sites and generating a global estimate for the analyte concerned is the most cost-effective method [21]. Pidoux *et al.* concluded that when 12 biopsies were used, the distribution of measured specific transcripts was homogeneous throughout the placenta. However, it is not known what number is enough for characterization of *ERVW-1* expression [10].

In our study, 30 biopsies were taken from each three PN placentae (C1–C3) to evaluate variability of *ERVW-1* expression in healthy placental tissue (Figure 5). Samples were taken along the long axis of placenta (A + B) and axis perpendicular to it (C) (Figure 1). Variability of *ERVW-1* expression was relatively wide, but differences in variability and level of gene expression among placentae were statistically insignificant (ANOVA, $p = 0.5214$; Bartlett's test, $p = 0.2154$) (Figure 5A). There were no differences in gene expression between parts A, B or C (Figure 1) when compared among the placentae (ANOVA, $p = 0.7571$; Bartlett's test, $p = 0.1442$) (Figure 5B). No relationship was found between the level of *ERVW-1* expression and localization of the placental biopsy (testing of correlation coefficient, $p = 0.1319$; Spearman's rank correlation coefficient, $p = 0.0641$).

We set the 95% CIs for different sample sizes from one placenta based on the level of *ERVW-1* expression in healthy placentae (Table 2). We tested the hypothesis that three samples are enough for characterization of *ERVW-1* expression. Based on variability in control placentae, if three samples are taken from one placenta, the 95% CI is from -0.73 to 3.54 for ΔCt (Table 2). Considering the expression variability in placenta, it can be concluded that low numbers of samples (i.e., three to nine) give wide 95% CIs and are inapplicable in gene expression studies. If financial and methodical expenses are taken into account, ten biopsies from one placenta can be considered as a compromise, because 95% CIs are not significantly changed for more than ten samples (Table 2).

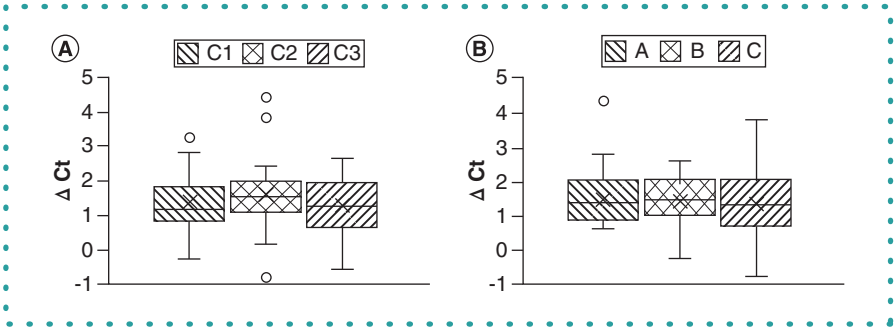

Figure 5. Relative *ERVW-1* expression in three physiologically normal placentae. (A) Comparison between placentae. **(B)** Comparison between parts of one placenta.
A/B/C: Parts of placenta (see Figure 1); C1–C3: Control (physiologically normal) placentae.

Table 2. 95% CI for the estimation of mean ΔCt based on level of *ERVW-1* expression in healthy placentae.

Samples from one placenta (n)	Lower limit of CI	Upper limit of CI
3	-0.73	3.54
5	0.34	2.47
7	0.61	2.20
10	0.79	2.02
15	0.93	1.88
20	1.00	1.81

Based on our experiments (30 biopsies from three physiologically normal placentae), four of seven C/S/L PN samples (57.14%) were out of the 95% interval for average value of ΔCt in healthy placentae, proving that three samples are not enough for determination of *ERVW-1* expression level.

No correlations between the localizations of biopsy and *ERVW-1* expression was found. There were no statistically significant differences between parts A, B or C within or between placentae. Based on that, taking ten biopsies from placenta randomly is recommended for *ERWV-1* expression studies. We propose a method of sampling by taking a long cut from the umbilical cord to the edge of the placenta, cutting the placental strip into ten equal parts and analyzing them separately.

Pre-eclampsia is a major medical problem worldwide and searching for new diagnostic markers or risk factors is a current topic of interest. Syncytin-1, the product of *ERVW-1*, is one of the investigated markers. Several studies have analyzed gene expression of *ERVW-1* in pre-eclamptic placentae and mainly detected decrease in *ERVW-1* expression. Three samples from one placenta have usually been taken and then pooled together for analyses of *ERVW-1*. We concluded that variability of *ERVW-1* expression is high across the placenta and this methodical approach has not been suitable. We encourage taking up to ten samples from one placenta and analyzing them separately for statistically significant results in the case of *ERVW-1*. We also recommend random sampling from every part of the placenta; taking a long cut from the umbilical cord to the edge of the placenta proved useful in our study. This approach can also be used for other gene expression studies, beginning with analyses of three or four normal physiological placentae with approximately 30 samples from each placenta to evaluate intraplacental variability of expression of the gene of interest. The number of biopsies necessary from one placenta should be determined by statistical testing of detected expression variability in PN samples. Given our findings, we suggest that exact description of the sampling method from placenta (i.e., number of samples and localization of biopsies) should be mandatory in research articles. If the between-group differences for a particular gene of interest do not exceed normal biological variability within a placenta, then the result is less likely to be clinically meaningful [11]. However, further research is necessary to decide whether *ERVW-1* and syncytin-1 represent this case and to determine their diagnostic potential.

Future perspective

Processing of huge amounts of data and information is one of the biggest challenges in the wide spectrum of analyses. Sophisticated methods based on genomics, transcriptomics, proteomics and so on are trying to find new possible markers for different diseases, including pre-eclampsia. However, there is no possibility of identifying such markers if we do not know precisely what is the normal state for the studied organisms and organs – in this case, placenta. Therefore unified methods for sampling, with regard to the intraorgan variability of studied organs, are essential for evaluation of data from rapidly evolving methods from various omics.

Author contributions

D Böhmer and V Repiská conceived the project. M Korbeľ and Z Nižňanská managed the patients and arranged the placental sample collection alongside K Letkovská. P Priščáková designed and performed experiments. P Priščáková and H Gbelcová interpreted the results of the study. K Sušienková statistically tested the data. P Priščáková wrote the original version of the manuscript. All authors critically revised the manuscript and approved the final version.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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