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Original Article

Molecular pathogenesis of spontaneous abortions – Whole genome copy number analysis and expression of angiogenic factors

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ABSTRACT

Objective: To study two major molecular alterations in spontaneous abortions (SA) with unexplained etiology – fetal genomic anomalies and the endometrial expression of main angiogenic factors VEGFA/VEGFR2 and chemokines SDF-1/CXCR4.

Materials and methods: Whole genome copy number analysis by arrayCGH or Next Generation Sequencing (NGS) was applied for detection of fetal genomic imbalances. The abortive decidua of SA without fetal aneuploidies was further investigated for expression levels of the abovementioned factors using real time PCR analysis. A total of 30 abortive materials were collected from spontaneous abortions after exclusion of known predisposing factors.

Results: In 21 of 30 spontaneous abortions (70%), genomic anomalies were discovered by whole genome copy number analysis. Numerical anomalies were detected in 90% of aberrant cases, and in 10% - structural aberrations were revealed. An increased expression for essential factors of angiogenesis was identified in spontaneous abortions' tissues - 3.44 times for *VEGFA* and 10.29 times for *VEGFR2*. We found an average of 14 times increase in the expression levels of *SDF-1* and 3.21 times for its receptor *CXCR4*.

Conclusion: We could suggest the occurrence of increased angiogenesis in SA without fetal aneuploidies, compared to the control tissues, which could lead to increased oxidative stress and fetal loss.

Introduction

Spontaneous abortions are among the most common reproductive problems in clinical practice - their incidence in clinically recognized pregnancies is 15–20%. Most often they happen in the first 7–8 weeks of pregnancy. More than half of them (50–60%) is due to unbalanced chromosome aberrations. Other known and clinically identifiable causes are: anatomical anomalies of uterus, immunological problems (eg antiphospholipid antibodies), thyroid dysfunction, other endocrine problems (polycystic ovary, diabetes), bacterial infections (eg Mycoplasma Hominis and Ureaplasma urealiticum), toxic substances. In many cases, however, the reason for spontaneous abortions can not be established and clinical behavior is unclear.

The continuous blood flow to the maternal-fetal área is essential for a normal pregnancy. Shortly after the implantation of the embryo, the decidual neoangiogenesis begins. The capillary growth around the syncytiotrophoblast in humans is reported at the 7th day of pregnancy [1]. In mice, the primary decidualization and angiogénesis around the embryonic crypt begin at gestation day 5, about 12 h after the implantation [2,3]. The formation of these vessels during the early decidualization of the endometrium proceeds quickly by vascular growth and branching. These events appear well before the maturation of the placenta with the onset of the uteroplacental circulation that occurs at about week 12 in humans and around GD9.5–10.5 in mice [4,5].

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The physiological modification of the terminal branches of the uterine artery, also known as spiral arteries (SpA), is closely linked to the process of normal development of the human and mouse placenta. The remodeling of SpA is considered essential for increasing mother's supply of blood and nutrients to the placenta thus assisting in the development and quick growth of the embryo. The impaired vessel development of the decidua throughout early angiogenesis together with the remodeling of the myometrial SpA leads to pregnancy complications (recurrent miscarriages [6], pre-eclampsia [7], fetal growth restriction [8]). Although these are common problems, the treatment approaches are still limited [9]. A detailed understanding of the mechanisms causing these impairments could inspire the development of innovative treatment methods. For this reason, it is important to understand the regulation of the early, normal, decidual neoangiogenesis and vessel remodeling.

It is now well known that earlier vessel development is mediated by immune cells, especially uNK cells. Research of multiple mice strains with NK cell insufficiency, identify the uNK cells as particularly important agents for the remodeling of SpA [10]. Although many factors secreted from the uNK cells during the remodeling were included as possible triggers of these changes in the vascular structure, we focused on the role of the chemokine receptor CXCR4, produced by the uNK cells, and his ligand SDF-1 as well. Their level of expression could be modulated during the activation of the NK cells.

Based on the important role of angiogenesis for decidualisation, placentation and embryo development, we aimed to investigate the expression of major angiogenic factors VEGFA/VEGFR2 and chemokines SDF-1/CXCR4 in decidua of spontaneous abortions with unexplained etiology and compare it with their expression in endometrial tissues of the same period of pregnancy as received by elective abortions. Before doing this expression analysis, we screened the fetal tissues for unbalanced genomic aberrations, thus we determined the type and frequency of fetal chromosomal aberrations as well.

Materials and methods

Materials

A total of 30 abortive materials were collected from spontaneous abortions (after exclusion of known predisposing factors) and 15 from elective abortions. The age and gestation parameters were as follows: the average age of women was 34.0 ± 6.8 in the cases' group and 31.4 ± 4.0 in the control group; the gestation week was 8.8 ± 1.4 in the cases' group and 9.0 ± 1.5 in the control group. All known factors predisposing to spontaneous abortions were excluded in both groups: anatomical anomalies of uterus, immunological problems (eg antiphospholipid antibodies), thyroid dysfunction, other endocrine problems (polycystic ovary, diabetes), bacterial infections (eg Mycoplasma Hominis and Ureaplasma urealiticum), toxic substances. The women in the control group had at least one previous successful pregnancy and no family or personal history of reproduction failure. For all materials, separation of the fetal from mother's tissues (decidua) was carried out and the materials were stored for further analysis. From the fetal tissues of spontaneous abortions, DNA was isolated to search for genetic defects. A commercial kit-Rneasy Mini Kit, Qiagen, Cat No.74104, was used to isolate RNA from the decidual tissues.

The study was approved by Ethical Committee of Medical University Sofia.

Whole genome copy number analysis

24 sure, Illumina microarray assay

The purified DNA samples were evaluated by aCGH (array-based comparative genomic hybridization) 24 sure V3 microarray assay (Illumina, Inc.). The gDNAs and reference DNAs (male and female) were labeled with Cy3 and Cy5 fluorophores using random primers for 2–4 h. After that Cy3 to Cy5 mixes were prepared. Then the labeling mixes were combined and ethanol precipitated with COT Human DNA in preparation for hybridization. Labeled DNA mixes were re-suspended in a dexsulphate hybridization buffer and hybridized onto the 24sure chip for up to 12 h. Thereafter, the chips were washed and dried. To read the resulting images and to analyze the scan data a laser scanner and BlueFuse Multi Software (Illumina, Inc.) were used. Autosomal chromosomes were analyzed for gain or loss whole chromosomal ratios, using a 3 × SD assessment, greater than ±0.3log2 ratio call, or both.

NGS VeriSeq assay by Illumina

The gDNA of each sample was processed to prepare DNA libraries by following the manufacturer's guidelines for VeriSeq assay by Illumina, Inc. The gDNAs were diluted (0.2 ng/μl, 1 ng total) and “tagmented”- tagged and fragmented using the Nextera XT transposome (Amplicon Tagmentation Mixture and Tagmentation DNA Buffer). Limited-cycle PCR reaction was used. Then the index sequences to the samples to enable dual-indexed sequencing (2 × 36 bp) were added. The tagmented DNAs with added indexes were amplified using the Nextera PCR Master Mix (NPM) through a PCR program: 1 cycle of 72 °C for 3 min, and 12 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s, 1 cycle at 72 °C for 5 min, and holding at 4 °C.

Using the AMPure XP beads (A63881, Beckam Coulter, USA) the PCR products were cleaned. After processing, the purified libraries were washed with 80% ethanol solution, then they were eluted by Nextera XT Resuspension Buffer. The purified DNA libraries were then normalized to equalize the quantity of each sample in the final pooling using the Library Normalization Additive and beads. Then, the normalized samples with equal volumes were pooled, denatured, and then sequenced. On a Miseq System (Illumina, Inc) the Miseq Reagent Kit v.3 (Illumina, Inc) was used. The generated bioinformatics data was also analyzed by BlueFuse Multi Software (Illumina, Inc). Every of the samples was identified if displayed a median chromosomal copy number deviated from the default copy number, and a possible trisomy or monosomy of autosomal chromosomes was seen as a copy number >2 or <2, respectively.

Gene expression analysis

A whole-genome copy number analysis identified chromosome aneuploidies in 21 of the abortive materials tested and the remaining 9 were used in further analysis. The RNA isolated from decidual tissues was tested for the required quality via spectrophotometer-Nanodrop. All RNA samples were analyzed for expression levels of the genes *VEGFA*, *VEGFR2*, *SDF-1* and *CXCR4* by real time PCR analysis. The normalization was carried out relative to the expression of the housekeeping gene *GAPDH*. The expression in the spontaneous abortions group was compared to the expression in the group of the abortive control samples (calibrators). The average relative expression in the group of spontaneous abortions was defined.

For relative expression measurement (RQ) we used Comparative Ct method - the most common method for expression analysis. The method gives information regarding the differences in

expression levels between two samples which are calculated with the following equations:

ΔCt sample = Ct target gene − Ct endogenous control

ΔCt control = Ct target gene − Ct endogenous control

ΔCt sample - ΔCt control = $\Delta\Delta Ct$

$RQ = 2^{-\Delta\Delta Ct}$

Results

The abortive foetuses were subjected to DNA analysis using whole genome copy number analysis. In 21 of the tested samples (70%) different aneuploidies were detected, which are listed in Table 1. Numerical anomalies were detected in 90% of aberrant cases, and in 10% - structural aberrations were revealed.

Fig. 1 graphically presents some of the identified numerical chromosome aberrations in the fetuses investigated and Fig. 2 – structural chromosome aberrations. Since most of the unbalanced structural chromosome aberrations are due to balanced translocations in the parents, we tested by cytogenetic analysis the parents in these cases. We detected balanced translocation in the mother of the fetus with 7p duplication (Fig. 2). After performing preimplantation genetic diagnostics in this case, an ongoing pregnancy was achieved.

Table 1
Chromosome aneuploidies in the foetuses investigated.

Chromosome aneuploidy	Number of cases	%
Monosomy X	4	13.2%
Trisomy 22	3	9.9%
Trisomy 16	2	6.6%
Trisomy 21	2	6.6%
Trisomy 19	2	6.6%
Trisomy 20	2	6.6%
Trisomy 18	1	3.3%
Trisomy 15	1	3.3%
Trisomy 8	1	3.3%
Double trisomy 18 и 19	1	3.3%
duplication 7p	1	3.3%
tetrasomy 9p	1	3.3%
TOTAL	**21**	**70%**

RNA was isolated from the decidual tissue of the remaining abortive materials. Qualitative evaluation of the RNA samples was carried out prior to the following expression analysis. Only the samples with the required quality (ratio A260/A280 between 1.8 and 2) were subjected to expression analysis.

After the real-time PCR, normalization according to the Ct value of the universally expressed *GAPDH* gene was done. The ΔCt values for each of the analyzed genes were calculated in test samples and controls. After that, the relative levels of expression of each of the genes were identified according to the $\Delta\Delta$Ct values of the test samples and the controls. For each of the genes was calculated the average value of the relative expression and the standard deviation (SD) along the all samples tested.

The average levels of expression of *VEGFA* in spontaneous abortions' tissues were 3.44 times higher than in the tissues from elective abortions ($p = 0.056$), and those of *VEGFR2* – 10.29 times higher ($p < 0.001$) - Fig. 3. Regarding the expression levels of SDF-1, we found an average of 14 times increase compared to the controls ($p = 0.004$), and for its receptor – 3.21 an average increase ($p = 0.026$) - Fig. 4.

Discussion

In this study, an expression analysis for the factors involved in angiogenesis of decidual tissues from spontaneous abortions was carried out in comparison to controls, after screening for chromosomal fetal aberrations. This is a preliminary study and the number of investigated samples is still small. It's estimated that more than 20 percent of pregnancies may have an aneuploidy, but many of these pregnancies are not viable and therefore will not result in a baby or will result in miscarriage before the tenth week of

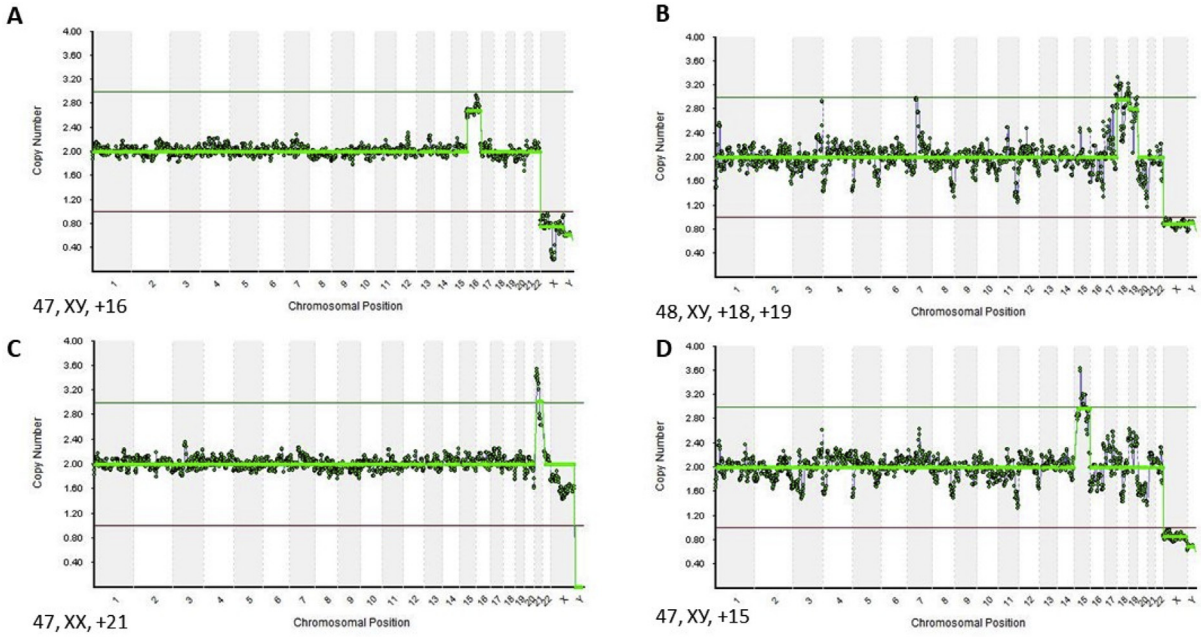
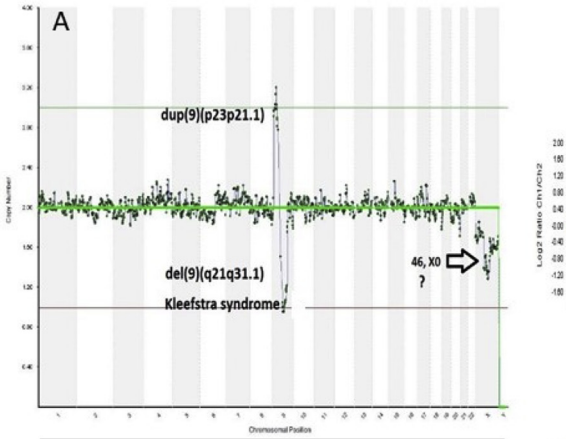
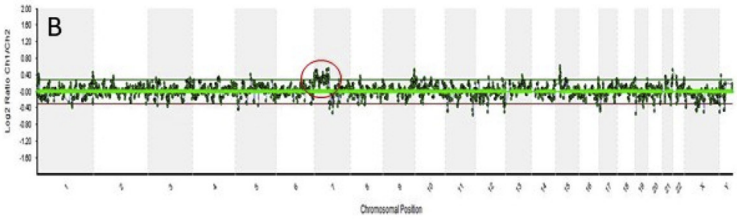
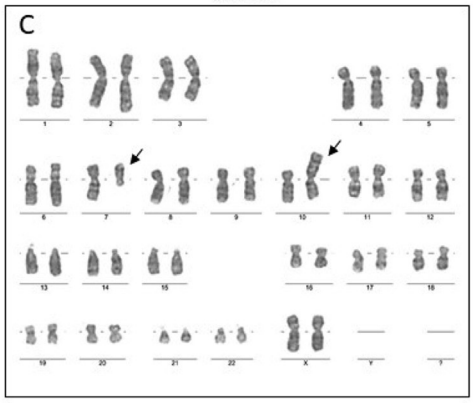


Fig. 1. Graphical representation of the numerical chromosomal aberrations - on the X-axis are represented all chromosomes, and on the Y-axis-the change in their copy number. A. Trisomy 16 in a male fetus; B. Double trisomy 18 and 19 in a male fetus; C. Trisomy 21 in a female fetus; D. Trisomy 15 in a male fetus.







D

Segregation	Possible chromosomal combinations in the oocyte	Expected karyotype
2:2	N7/N10	46, XY
	A7/A10	46, XY, t(7;10)
	N7/A10	46, XY, dup(7)(q11;qter), del(10)(p12;pter)
	A7/N10	46, XY, del(7)(q11;qter), dup(10)(p12;pter)
	N7/A7	46, XY, dup(7)(pter;q11), del(10)(p12;qter)
	N10/A10	46, XY, del(7)(pter;q11), dup(10)(p12;qter)
3:1	N7	45, XY, -10
	A7	45, XY, -10, der(7)
	N10	45, XY, -7
	A10	45, XY, -7, der(10)
	N7/A7/N10	47, XY, +der(7)
	N7/A10/N10	47, XY, +der(10)
	A7/N10/A10	47, XY, t(7;10), +10
	A7/N7/A10	47, XY, t(7;10), +7

Fig. 2. Structural unbalanced chromosome aberrations, detected by aCGH: A. Tetrasomy 9p-we detected increased copy number for the short arm 9p and decreased copy number for the long arm 9q; B. Duplication of 7p (increased copy number for the short arm 7p) - 46,XY,arr (7p)×3; C. Karyotype of the mother of the fetus from B, showing balanced translocation between 7p11 and 10p12 – 46, XX, t (7; 10) (p11; p12) and D. The possible segregation of chromosomes during gametogenesis. ArrayCGH result (B) correspond to the segregation 3:1.

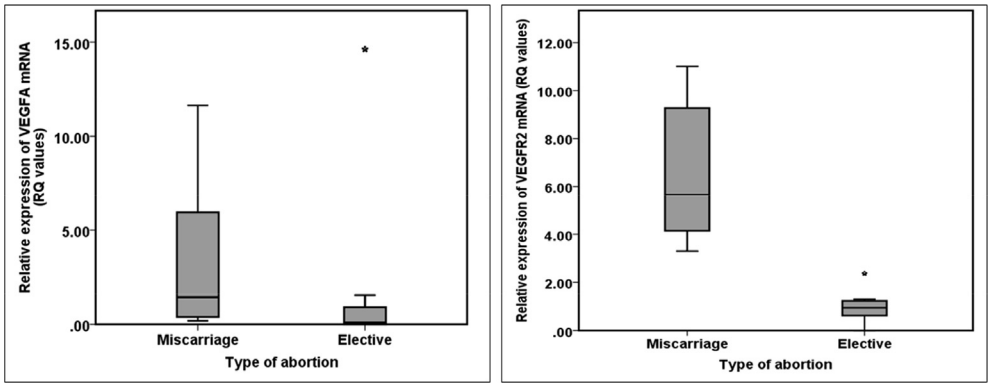


Fig. 3. Expression levels of *VEGFA* and its receptor *VEGFR2* in decidual tissue from spontaneous abortions vs. controls from elective abortions.

pregnancy. The frequency of the chromosomal aneuploidies at birth is 0.3%, i.e. 1 in about 350. We assumed that in our control group the risk for having an aneuploidy is extremely low.

In 21 of 30 spontaneous abortions (70%), genomic anomalies were discovered by whole genome copy number analysis. About 60% of them could be detected by the rapid DNA analysis (offered in our country as a rapid QF-PCR analysis for aneuploidies 13, 15, 16, 18, 21, 22, X and Y) – that means, using this analysis, aneuploidies will be discovered in 42% of the analyzed abortions. Numerical anomalies were detected in 90% of aberrant cases, and in 10% - structural aberrations were revealed. For couples with recurrent pregnancy loss and evidence of a structural genetic abnormality in one of the parents, preimplantation genetic diagnosis with transfer of unaffected embryos or the use of donor gametes might be considered for therapy.

An increased expression for essential factors of angiogenesis was identified, which suggests a role of the excessive angiogenesis in the pathogenesis of spontaneous abortions with unclear reason. The average levels of expression of *VEGFA* in spontaneous abortions' tissues were 3.44 times higher than in the tissues from elective abortions, and those of *VEGFR2* – 10.29 times higher. A similar increase in the levels of *VEGFA* expression compared to controls was

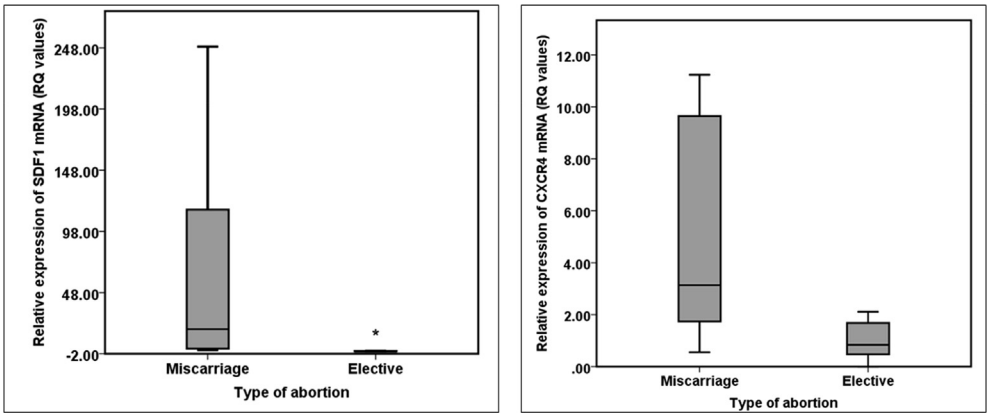


Fig. 4. Expression levels of *SDF-1 (CXCL12)* and its receptor *CXCR4* in decidual tissue from spontaneous abortions vs. controls from elective abortions.

identified in endometrial biopsies from women with recurrent spontaneous abortions and it is believed that this takes part in the pathogenesis of spontaneous abortions [11]. The expression of *VEGFA* is strongly represented in the uteroplacental area, especially in early pregnancy and is the main mediator of the decidual angiogenesis [12]. VEGFA carries out the signalization via VEGFR1 and VEGFR2. The independent knockout of each of the VEGF receptors is lethal during the development of mice [13]. At the same time, strong angiogenic balance is needed for the proper embryo development. The impaired foeto-placental blood circulation in increased angiogenesis is most likely related to increased oxidative stress and loss of pregnancy. Recent researches prove the role of genetic polymorphisms in vascular factors for the oxidative stress such as rs2779249 (−1290G > T) in the gene *NOS2* for predisposition to recurrent spontaneous abortions [14]. The research of angiogenesis in spontaneous abortions opens the path towards the diagnostic introduction of the angiogenesis-related polymorphisms in this reproductive pathology.

The leukocytes represent a great part of the cells in the basal decidua, where the uterine natural killer cells (uNK) are 70% of the early decidual leukocytes [15]. The histological researches prove that uNK cells in mice are spatially and temporarily found in the area of the active microvascular development close to the supplying uterine artery [16,17]. The immunohistological studies show that the uNK cells express multiple angiogenic factors. Human uNK cells express vascular endothelial growth factor A (VEGFA), VEGFC, PGF, angiopoietin 1 (ANGPT1), ANGPT2, Matrix metallopeptidase 2 (MMP2), transforming growth factor beta 1 (TGFβ1) and NKG5 (currently known as granulysin) [17,18]. In humans, in vitro analyses using isolated uNK cells in the first trimester substitute the researches for early implantation. These analyses clarify the angiogenetic properties of the early human decidual cells. An important study reported increased endothelial cell migration from the umbilical vein (HUVEC) and tubular structure generation in response to uNK cell supernatants [19]. Mechanisms for a direct relationship between the impaired angiogenesis, triggered by the uNK cells, and the human reproductive health have been suggested. The high number of uNK cells (>5%) in the secretory phase of the endometrial biopsies is related to the increased density of the decidual vessels in women, suffering from recurrent spontaneous abortions [6]. It is believed that excessive decidual angiogenesis in early pregnancy leads to increased oxidative stress in the embryogenesis-a mechanism resulting in spontaneous abortions. Apart from changes in the number of vessels, this can be also caused by a mismatch in the maturation and differentiation of the vessels, which are important for the blood flow. Both in normal women and those with spontaneous abortions, the number of uNK cells is proportional to the number of mature vessels (containing mature myosin-expressing vascular smooth muscle cells). These vessels lead to indications for high vascular resistance in ultrasound examination [6]. Clinical trials with Prednisolone were performed as an intervention for decreasing the number of uNK cells and therefore spontaneous abortions [20]. The Prednisolone decreases the number of uNK cells in some women and in the treated women, which later had a successful pregnancy, the density of the secretory endometrial vessels was decreased [21]. The uNK cells are prompted as particularly important agents for the remodeling of SpA, which are independent of the trophoblasts [10]. Although many factors secreted from the uNK cells during the remodeling were included as possible triggers of these changes in the vascular structure, we focused on the role of the chemokine receptor CXCR4, produced by the uNK cells. The receptors of chemokines, including CXCR4, are differentially expressed from NK cellular subgroups and their level of expression could be modulated during the activation of the NK cells.

Recently, a new subgroup CD56lowCD16low uNK cells was identified, which shows strong capacity for generation of IFNy and has high levels of expression of CXCR4 and CXCR3 in comparison with the other subgroups [22]. A main ligand of the CXCR4 receptor is CXCL12 (SDF-1), which shows a constitutive, but limited model of expression in trophoblast cells and endothelial cells [23–28]. In our study, we found an average of 14 times increase in the expression levels of *SDF-1* compared to the controls, and for its receptor *CXCR4* – 3.21 an average increase. Taking into account that VEGFR2 and SDF-1 are molecules, which are expressed in the endothelial cells, we could suggest the occurrence of increased angiogenesis, i.e. increased density of the vascular network in decidua of spontaneous abortions compared to the control tissues. This could be due to the higher expression of *VEGFA* from the extracellular tissues or due to an increased number of a specific subpopulation of uNK cells, which express *CXCR4*. Conversely, the latter could be accumulated as a result of the chemotaxic action of *SDF-1,* which is obviously overexpressed in the endothelium.

A key question is the therapeutically implications of the impaired angiogenesis, which could have an effect on the clinical management of recurrent spontaneous abortions. A recent study discovered a relationship between the levels of Vitamin D in the serum and the angiogenic factors, whose high expression threatens the development of the fetus. It was demonstrated that levels ≥100 nmol/L of Vitamin D in the mother's circulation decreases the expression of VEGFA and its receptor thus decreasing the risk of pregnancy complications [29]. This shows that every research on

the angiogenesis in early pregnancy contributes to the discovery and application of new treatments for pregnancy loss.

Declaration of Competing Interest

None declared.

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