ASSOCIATED OBSTETRIC AND PERINATAL DETERMINANTS OF VANISHING TWIN PREGNANCIES
Ph.D. Thesis

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LIST OF PUBLICATIONS

I. **Márton V**, Zádori J, Kozinszky Z, Keresztúri A. Prevalences and pregnancy outcome of vanishing twin pregnancies achieved by in vitro fertilization versus natural conception. Fertil Steril. 2016 Nov.;106(6):1399-1406. **IF_{2016}:4,447**

II. **Márton V**, Németh G., Keresztúri A., Iker vagy sem? A vanishing twin fenomén. Magyar Nőorvosok Lapja. 2018. 81 (6). pp. 330-337

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1. LIST OF ABBREVIATIONS

AOR	adjusted odds ratio
ART	assisted reproduction technique
BMI	body mass index (kg/m2)
BW	birth weight
CI	Cronfield's 95% confidence interval
DZ	dizygotic
GDM	gestational diabetes mellitus
IUGR	intrauterine growth restriction
IUI	intrauterine insemination
IVF	in-vitro fertilization
IVF/ICSI	in-vitro fertilization/intracytoplasmic sperm injection
LBW	low birth weight
MZ	monozygotic
NICU	neonatal intensive care unit
OR	odds ratio
VT	vanishing twin
VTS	vanishing twin syndrome

2. INTRODUCTION

Twins and twinnings as a unique result of human reproduction have been followed with interest through the centuries. From the aspect of development, there are two types of twins, dizygotic (DZ) and monozygotic (MZ) twins, each with a very different etiology. Growing infertility, delayed childbearing and the strong demand for assisted reproductive techniques have led to an increased rate of DZ twin pregnancies and have been cited as the main iatrogenic cause in the past two decades. The twin ratio refers to twin live births and does not correlate with the twin conception rate. Indeed, twin conception does not necessarily result in twin birth; spontaneous loss could affect one or both of the fetuses.

The rate of spontaneous loss in twin pregnancies varies widely, between 33 and 62%, and is influenced by maternal age, mode of conception and gestational age. The true incidence of spontaneous abortion is unknown because many abortions occur before pregnancy was clinically recognized and it makes more difficult especially by naturally conceived pregnancies.

Spontaneous loss in twin gestation with the survival of the "remaining" embryo was suggested more than 70 years ago and has been recognized since the advent of sonography, but the data on the true reduction rate are limited. The definition of the vanishing twin phenomenon as an early gestational loss of one twin and survival of the co-twin originated from Levi and has not changed since the first description in 1976. The etiology of the VT phenomenon remains vague; however, placental degeneration and chromosomal abnormality in the vanishing embryo have been confirmed pathologically. Other possible causes, including inappropriate implantation, placental "crowding," intrauterine bleeding and chronic maternal diseases, have previously been suggested. Vanishing twin syndrome (VTS) is not unusual; however, despite its importance to anxious parents and to the obstetrician, the exact prevalence of VT after spontaneous conception has remained unidentified. The incidence of VT pregnancies varied between 10 and 39% in pregnancies resulting from IVF/ICSI. It has been noted that spontaneous pregnancy loss mainly occurs between 8 and 9 weeks of gestation. In VT pregnancies achieved with the IVF/ICSI technique, the disappearing embryo has a detrimental effect on the surviving co-twin, leading to intrauterine growth disturbances. The VT phenomenon is also associated with an array of pregnancy complications (GDM, pregestational diabetes and hypertensive disorders) and neonatal sequelae (cerebral palsy).

3. HYPOTHESIS

We hypothesized that there is a difference in perinatal outcomes between VT pregnancies following artificial conception and those following natural conception, possibly reflecting the potential differences between underlying pathomechanisms.

The aims of the present study were as follows:

- To determine the incidence of VT pregnancies following spontaneous conception and after assisted reproductive techniques.
- To find the average gestational length at the time of vanishing.
- To determine whether we should consider VTS as a risk for various pregnancy complications or adverse perinatal outcome.
- To evaluate whether VT pregnancies after assisted reproductive techniques have a more adverse perinatal outcome than those after natural conception.
- To examine whether the impact of the VT phenomenon is the sole contributor to the adverse perinatal outcome in IVF/ICSI VT pregnancies.
- To demonstrate the effect of the IVF/ICSI technique on the VT phenomenon.
- To collect potential risk factors for VT pregnancies by mode of conception.

4. MATERIALS AND METHODS

This thesis involves two retrospective studies for VT pregnancies. In an attempt to prove the hypothesis, we initiated the first study to compare obstetric and neonatal outcomes between the survivors of VT pregnancies and matched originally singleton control pregnancies and the second study to evaluate whether vanishing twin pregnancies after spontaneous conception have a more adverse perinatal outcome than those conceived after assisted reproductive techniques.

The first retrospective case-control study included data on VT pregnancies after IVF/ICSI and after spontaneous conception and their matched singleton control pregnancies detected between 1 January 1994 and 30 November 2014 at the Department of Obstetrics and Gynecology, University of Szeged. The first study included 65 237 singleton deliveries, 1563 twins and 306 VT pregnancies. As regards the type of conceptions, 78 VT cases resulting from

IVF/IVF-ICSI and 228 VT cases were identified from spontaneously conceived twin pregnancies.

The second retrospective cohort study processed data on VT pregnancies, and perinatal and neonatal outcomes for these deliveries from an extended period with two more years after IVF/ICSI and after spontaneous conception (between January 1994 and January 2016) in the same single center. The vast majority of the assisted reproductive treatments were performed at the Center for Assisted Reproduction, Kaáli Institute, Szeged. The second study population consisted of 67 827 singleton deliveries, 1941 twins and 316 VT pregnancies. 81 VT cases resulting from IVF/IVF-ICSI, and 235 VT cases were identified from spontaneously conceived twin pregnancies. Based on Protocol Nos. 14/2013 and 5/2015, the research project was approved by the Ethics Committee at the University of Szeged in accordance with the Code of Ethics of the Declaration of Helsinki for scientific research involving humans.

VT cases were identified by a manual systematic search of the entire sonography database, which was managed between the 5^{th} and 14^{th} gestational weeks during the study periods at our outpatient clinic at the Department of Obstetrics and Gynecology, University of Szeged. In the first study for each VT case, three controls were matched according to the following criteria: singleton pregnancies started as singleton gestations achieved by natural conception or IVF/ICSI, which were as similar as possible in maternal age, previous gravidity and parity and pre-pregnancy body mass index (BMI), and delivered after VT pregnancies in chronological order.

4.1 Inclusion criteria

In the study design, we used the classification for vanishing twin based on Landy's diagnosis. Vanishing twin was diagnosed in cases where two viable embryos with spontaneous reduction of one embryo or a single viable embryo and an additional gestational sac with or without a nonviable embryo were demonstrated before 14 weeks of gestation.

4.2. Exclusion criteria

All cases of single fetal loss identified after 14 gestational weeks or pregnancies after ovulation induction or intrauterine insemination (IUI) were excluded from the analyses. MC twins or singleton pregnancies after artificial fetal reduction were also excluded from both of the studies.

4.3. Statistics

All statistics were calculated using SPSS 22 (SPSS Inc., Chicago, IL, USA). The non-parametric design of the continuous variables was verified with the Shapiro–Wilk test. Univariate comparisons between VT pregnancies and controls (Paper 1) and between VT pregnancies (Paper 2) both for IVF/ICSI and spontaneously conceived pregnancies were assessed with the Mann–Whitney U-probe for continuous variables. Categorical variables were compared between the subgroups using χ^2 tests, while odds ratios (ORs) and Cornfield's 95% confidence intervals (CIs) were also calculated. The resultant ORs for IVF and spontaneous pregnancies were compared with Mantel–Haenszel tests. Multivariable logistic regression was performed to evaluate the factors determining VT pregnancies in both the IVF/ICSI and the spontaneous groups separately. The adjusted odds ratios (AORs) were also calculated with 95% CI. All tests were two-tailed, and significance was accepted at $p<0.05$. P-values were adjusted using the Holm–Bonferroni correction for multiple comparisons (Mann–Whitney U tests and logistic regression analyses).

5. SUMMARY OF RESULTS

During the total study period between 1994 and 2016, 67 827 singleton and 1615 dichorionic twin live births were registered at the Department of Obstetrics and Gynecology. Of these, 316 were VT live births, exhibiting a rate of 4.53 out of 1,000 total pregnancies. ART was registered from 1992, from the opening of the IVF center in Szeged, and a total of 640 twin pregnancies and 81 VT pregnancies were conceived by ART until the end of the study period. 5.2% of all singleton deliveries originated from a VT pregnancy after assisted conception, and 0.37% did so after spontaneous single gestation. The VT live birth rate varied between 36.8 and 28.8/1000 live births in VT pregnancies after IVF/ICSI and was stable at 3.6–3.55/1000 live births in spontaneously conceived VT pregnancies ($p<0.001$). The proportion of VT pregnancies among twins was significantly higher after spontaneous conception.

In the first study, 78 VT cases resulting from 617 IVF/ICSI dichorionic twins (12.6%) and 228 VT cases were naturally conceived for 1252 spontaneous dichorionic twin pregnancies (18.2%) ($p=0.002$).

In the second study, 81 VT cases originated from 640 IVF/ ICSI dichorionic twins (12.7%), and 235 VT cases were conceived without medical assistance for 1301 dichorionic twin pregnancies (18.1%) (p=0.002).

5.1. Maternal characteristics

Maternal age was higher for the IVF/ICSI pregnancies (mean maternal age: 34.8±3.71) compared to spontaneously conceived pregnancies (mean maternal age: 33.20±4.38). All the women in the IVF/ICSI subgroups were non-smokers, and negligibly small proportions (2.6%) smoked in the spontaneously conceived subgroups. BMI at delivery was significantly lower among spontaneous VT pregnant women (p=0.049) than their matched controls. All of the chronic maternal diseases examined were significantly more prevalent among VT pregnancies than in the matched control groups. The number of previous miscarriages was not different in VT and non-VT pregnancies, but termination of pregnancy and second trimester fetal loss previously came into prominence as risk factors for single fetal loss. IUGR (intrauterine growth restriction) in a previous pregnancy was associated with a significantly higher risk of VT in the spontaneous group, but there was no recorded IUGR in the previous IVF/ICSI pregnancies. However, previous maternal diabetes was an independent contributing factor to VT pregnancy in IVF/ICSI pregnancies, whereas it was not a significant determinant in the spontaneous group.

Maternal obstetric history and characteristics of previous pregnancies were also investigated. A comparison with control groups, and differences between the two VT groups were found. Generally, maternal and neonatal morbidity in previous pregnancies was less frequent among IVF/ICSI mothers due to a low percentage of previous pregnancies. The number of previous miscarriages was not different in VT and non-VT pregnancies, but previous termination of pregnancy and second trimester fetal loss came into prominence as risk factors for single fetal loss. IUGR in a previous pregnancy was associated with a significantly higher risk of VT in the spontaneous group, but there was no recorded IUGR in the previous IVF/ICSI pregnancies. However, previous maternal diabetes was an independent contributing factor to VT pregnancy in IVF/ICSI pregnancies, whereas it was not a significant determinant in the spontaneous group. Congenital anomalies of the uterus or fibroids were not observed in the VT groups.

5.2 Pregnancy characteristics and intrapartum complications

GDM developed in a substantially higher percentage of women with VT pregnancies (odds ratio (OR): 3.0; 95% CI: 1.6–5.6) than in the non-VT pregnancies after IVF/ICSI

(p=0.01), while it was inversely less common among their naturally conceived counterparts (OR: 0.46; 95% CI: 0.2–1.1). Even after adjusting for confounders, IVF/ICSI was significantly more associated with GDM; this was more pronounced for VT cases (p<0.001) due to its very low occurrence in the spontaneous group. Pregestational diabetes mellitus represented an increased risk for VT after IVF/ICSI (Adjusted (A)OR: 1.07; 95% CI: 1.04–1.2), while it was not a risk for the spontaneously conceived subgroups (AOR: 1.00; 95% CI: 0.94–1.00), thus exhibiting a significant difference between the IVF/ICSI and non-IVF VT pregnancies (p<0.001). Preeclamptic pregnancies in VT subgroups following IVF/ICSI (AOR: 1.6; 95% CI: 0.7–6.1) and spontaneous conception (AOR: 1.00; 95% CI: 0.8–1.8) showed similar proportions compared to matched controls.

Placentation abnormalities were more frequent in VT pregnancies after IVF/ICSI than their counterparts who conceived spontaneously. Placenta previa was significantly more frequent in VT pregnancies after IVF/ICSI than after natural conception (p<0.001), and almost significantly more prevalent in VT pregnancies in the IVF/ICSI group (p=0.08, AOR: 3.8; 95% CI: 1.0-9.3), compared to the non-VT IVF/ICSI subgroup. The occurrence of retained placenta was significantly more frequent in VT pregnancies after IVF/ICSI (AOR: 7.2; 95% CI: 3.1-19) compared to matched controls or spontaneous pregnancies (AOR: 0.67; 95% CI: 0.3-1.2). There was a negligible low occurrence of placental abruption among VT pregnancies. Although the spontaneous VT pregnancies were significantly more likely to be complicated by placental abruption than the matched controls (AOR: 10.6; 95% CI: 2.5-39), this was less marked after IVF/ICSI (AOR: 3.6; 95% CI: 1.5-9). The incidences of operative deliveries were non-significantly lower among the IVF/ICSI pregnancies compared to their spontaneously conceived counterparts.

The average gestational length at the time of vanishing in the IVF/ICSI group was 9.86±2.06 weeks, whereas VT was confirmed at 8.86±2.70 weeks in the spontaneous group (p=0.057).

5.3. Neonatal outcomes

Similar gestational age at time of delivery was noted in all of the study groups. In the first study the incidences of preterm birth were equally low among VT cases and controls in both subgroups. Furthermore, there were no very preterm deliveries in the study groups. In the second study unexpectedly, the rate of preterm birth was higher among spontaneously conceived VT cases than after IVF/ICSI procedure.

The rate of IUGR was statistically higher in the VT pregnancies in the spontaneous group (AOR: 3.0; 95% CI: 1.8-5.2) and even more so among the IVF/ICSI cases compare to the control groups (AOR: 9.2; 95% CI: 5-22). Comparing the prevalence of IUGR between the two VT groups, it is significantly higher in the IVF/ICSI group than in their spontaneously conceived counterparts.

The incidences of low birth weight (LBW) were threefold higher in the IVF/ICSI VT pregnancies and two times higher in the naturally conceived VT pregnancies compared to the control groups. Comparing the incidence of LBW between the two VT subgroups, the rate was significantly higher in the IVF/ICSI VT group than in their spontaneously conceived counterparts. There were no IVF/ICSI VT pregnancies registered as very low birth weight, and the rate was extremely scant in the spontaneous VT group.

The proportion of macrosomia was slightly lower in the VT pregnancies than in the controls in the IVF/ICSI group, but VT was associated with a significantly lower rate of macrosomia after spontaneous conception.

The VT phenomenon in the first study resulted in a significantly higher male sex rate in spontaneous pregnancies (AOR: 1.4; 95% CI: 1.2-1.9) but not in the IVF/ICSI group (AOR: 1.1; 95% CI: 0.8-1.9). Unlike in the second study, the study groups presented a similar neonatal gender rate.

In the first study, the frequencies of NICU (neonatal intensive care unit) admission were not more common in the VT cases; however, in the second study, a significantly higher rate of NICU admission was found in the spontaneously conceived VT group than in the IVF/ICSI VT group.

The umbilical cord blood analysis had shown a higher prevalence of acidemia (umbilical artery pH less than 7.20) in the IVF/ICSI VT group, but the 5-min Apgar scores, as another

independent marker for neonatal outcome, had not shown the same unfavorable results. The frequencies of congenital malformation reported were not common in the VT cases; the rate was higher among those who conceived after assisted reproductive techniques but did not reached statistical significance.

5.4 Independent risk factors associated with vanishing twin phenomenon

The results of the multiple logistic regression analyses in the first study pointed out the different risk factor structure for VT pregnancies achieved by ART and natural conception. Previous and present GDM influenced the occurrence of VT in the IVF/ICSI group with AORs of 5.41 and 2.33, respectively, while chronic maternal disease was also a predictor, with an AOR of 3.48. The women with IVF/ICSI VT pregnancies had an overall 4.35-fold higher risk of placental abruption. Within the IVF/ICSI VT group, there was 8.00-fold higher risk of retained placenta and 28.2-fold higher risk of an IUGR neonate. The risk of VT rose to 2.10-fold when a chronic maternal disease was present, and the surviving fetus had 3.65-fold higher odds for IUGR in spontaneous VT pregnancies.

In the second study, multivariable regression analysis was also performed, with factors associated with the infertility treatment among those with VT pregnancies were summarized. Women were significantly older (AOR: 1.30) and primiparity was also a predictor with an OR of 3.8 in the IVF/ICSI group. Like the results in the first study, GDM and hypertensive disorders developed at a substantially higher percentage of pregnant women with VT pregnancies following IVF/ICSI (AOR: 2.10 and 3.54, respectively). VT pregnancies after ART had an increased risk of placenta anomalies (placental abruption with an AOR of 4.9 and retained placenta with an AOR of 5.69). Mode of conception was a distinguishable determinant in birth weight (BW), and neonates after IVF/ICSI had a lower BW even after controlling for relevant cofactors (AOR: 0.98). The neonates after IVF/ICSI VT pregnancies had an overall 1.10-fold higher risk of unfavorable arterial cord blood pH and an AOR of 1.13 for prolonged labor.

6. DISCUSSION OF SPECIFIC RESULTS

The principal finding of the present study is that VT carries a higher risk of various pregnancy complications, particularly following the IVF/ICSI procedure.

Embryonic loss has a significant effect on more twin pregnancies after natural implantation (18.2%) than after the iatrogenic transfer of two embryos (12.6%). Early pregnancy loss and

the vanishing twin phenomenon both share a chromosomal defect in the conceptus, and this explains the fact that the artificial selection procedure for morphologically normal embryos decreases the rate of VT after IVF/ICSI.

The incidence of VT pregnancies increases with maternal age due to a higher risk of chromosomal abnormalities in the oocytes, which is the strongest predictor of embryo potential. After natural conception without any embryo selection, age-related chromosomal abnormalities and the derived embryo pose a higher risk of VTS in advanced maternal age. In line with the concept that twinning frequency increases with maternal age, we found that the VT phenomenon is also associated with advanced age in both groups in IVF/ICSI pregnancies. Although the vanishing twin phenomenon occurs more often after natural conception, the prevalence of VTS is significantly higher among twins after ART. In our study 5.2% of all singleton deliveries originated from a VT pregnancy after assisted conception, and only 0.37% did so after spontaneous single gestation. The observed difference in the incidences of VT supports the idea that an artificial selection procedure favors embryo with high developmental potential. This raises the possible higher risk of the implantation of a genetically impaired vanishing embryo in naturally conceived pregnancies, as suggested earlier. An adverse obstetric history comprising induced abortion and second trimester fetal loss as classical miscarriage-related factors is also strongly associated with single loss in twins in spontaneous pregnancies.

Another major finding of the thesis is that chronic maternal diseases and a history of certain high-risk pregnancies might contribute to the absorption of a single embryo in twin pregnancy, particularly in IVF/ICSI pregnancies. An important observation is that a significantly higher recurrence rate for GDM and an increased incidence of pre-gestational and gestational diabetes mellitus was found among IVF/ICSI VT pregnancies, which are in line with the fact that IVF/ICSI presents a higher risk for diabetes mellitus than spontaneous conception.

Placentation anomalies such as placenta previa, placental abruption and retained placenta associated with VT via the reduced uteroplacental flow, represent a further factor that seems to contribute to the absorption of a conceptus in a twin pregnancy, and may also contributes to the adverse outcomes of VT pregnancies. Moreover, the IVF/ICSI technique promotes higher rates of placental anomalies.

Our most striking result is that the resorption of an embryo induces growth restriction in the remaining twin, particularly after IVF/ICSI, compared to age- and previous gestation-matched singletons. It is indeed a remarkable fact that IVF/ICSI has been associated independently with

an increased risk of IUGR, with the risk being more pronounced in IVF/ICSI VT pregnancies. One reason for this difference may be that the VT phenomenon in IVF/ICSI pregnancies was detected at a greater gestational length, suggesting that reduction occurs at a later stage.

One intriguing finding was that we did not find any very preterm births in our VT groups. Our study provides evidence that adverse outcomes of VT pregnancies are more likely to be associated with the greater odds of lower birth weight and an increased proportion of age-related morbidities, which are usually more common in IVF pregnancies.

The rate of congenital malformation was slightly higher in vanishing pregnancies after ART, but it does not cause an increase in the risk of adverse outcomes.

7. CONCLUSION

In conclusion, a more adverse pregnancy and neonatal outcome seems to be present in VT pregnancies following IVF/ICSI, thus confirming the concept of underlying maternal factors related to infertility or ART. Our results did not show whether VT is generated by the technique, the infertility due to underlying impairment or other IVF-related factors (i.e. fresh or frozen cycles or other stimulation protocol details).

Spontaneous reduction occurs more frequently in spontaneous twin pregnancies than in conceptions after assisted reproduction. The main findings of the high rate of IUGR neonates in connection with certain maternal illnesses are related to adverse perinatal outcome in VT pregnancies.

Conversely, VT is more likely to occur after spontaneous implantation of two embryos (2.9%) than after the iatrogenic transfer of two embryos (0.35%). Both pregestational and gestational diabetes and placentation/placental anomalies are associated with the VT phenomenon. To our knowledge, no study has yielded spontaneously conceived VT pregnancy outcomes with relatively high case numbers and compared with VT pregnancies after ART until now. Recognizing vanishing twin by mode of conception and treating VT pregnancies as a possible high risk for intrauterine growth restriction, placental abnormalities and gestational diabetes hold the key for more effective prenatal care for that significant subgroup of pregnancies.

In the future, we would like to continue our research, extend analysis of data from VT pregnancies after ART with special interest in culture medium, mode of ART, and improvement and effect of embryo cryopreservation or embryo transfer policy on VT pregnancy outcome. For further consistency, prospective epidemiological studies of neonatal outcome are necessary to investigate the role of less prevalent miscarriage-related factors.

9. SUMMARY OF NEW FINDINGS

- The incidence of VT pregnancies after natural implantation is 18.2%, which is higher than among VT pregnancies after assisted reproductive techniques (12.6%).
- The average gestational length at the time of vanishing in the IVF/ICSI group was 9.86±2.06 weeks and 8.86±2.70 weeks in the spontaneous group.
- A higher rate and increased incidence of pre-gestational and gestational diabetes mellitus, abnormal placentation and placental anomalies are associated with the VT phenomenon. VT was still noted as an independent risk factor for adverse perinatal outcome after controlling for all possible confounders.
- VTS poses a higher risk for an adverse perinatal outcome following IVF/ICSI as compared with spontaneously conceived counterparts.
- The IVF/ICSI technology and the underlying pathomechanisms which finally lead to infertility worsen the poorer outcome of vanishing twin pregnancies.
- VTS induces growth restriction in the remaining twin.
- Chronic maternal diseases and an anamnestic history of GDM or IUGR can contribute to the absorption of a single embryo in twin pregnancy, particularly in IVF/ICSI pregnancies.
- An adverse obstetric history comprising induced abortion and second- trimester fetal loss as risk factors are strongly associated with vanishing twins in spontaneous pregnancies.