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Genetics, not the uterine environment, drive the formation of trophoblast inclusions: Insights from a twin study

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ABSTRACT

Introduction: Trophoblast inclusions (TIs) are associated with aneuploidy and pregnancy loss and have thus been considered to be a marker of genetic abnormality. However, to date, no study has specifically explored whether TIs are a manifestation of fetal genetics or, rather, the result of the intrauterine environment. The goal of this study was to compare the frequency of TIs in the placentas of monozygotic (MZ) and dizygotic (DZ) twin pairs in order to determine whether the formation of TIs is genetically driven or not.

Methods: We performed a retrospective case series of placentas from 48 twin pairs. The placentas were grouped based on zygosity: MZ, DZ, or unknown (UZ). The average number of total TIs per slide was calculated for each twin individual and the mean absolute difference in the total TIs per slide between the twin pairs was calculated for each zygosity group and compared.

Results: The mean difference in the total TIs per slide for DZ twins was significantly greater than the mean difference in the total TIs per slide for MZ twins ($p = 0.003$). The mean difference in the total TIs per slide for the UZ group was also significantly greater than the mean difference in total TIs per slide between MZ twin pairs ($p = 0.028$).

Discussion: Our finding that MZ twins were significantly more concordant than DZ twins for the average number of TIs per slide supports the conclusion that TIs are intrinsic to the genetics of the fetus, not the uterine environment.

1. Introduction

Trophoblast inclusions (TIs) are microscopic morphological abnormalities of the placenta due to abnormal infolding of the trophoblast bilayer into the villous core [1–3]. By convention, TIs are characterized by a core of syncytiotrophoblasts surrounded by a layer of cytotrophoblasts [4–8]. Recently, researchers have identified a total of 4 TI subtypes: inclusionoids, inclusions, calcified inclusions, and calcified bodies [3]. It has been suggested that these 4 subtypes are temporally related, reflecting the dynamic nature of the formation and aging process of TIs.

Over the years, TIs have been associated with a number of genetic abnormalities, such as triploidies, trisomies, and other genetic conditions [9–14]. It is important to note that a normal karyotype does not inherently rule out the presence of a genetic anomaly [15–17]. Therefore, it is not surprising that TIs have also been seen in placentas of gestations associated with more subtle genetic abnormalities, such as spontaneous pregnancy losses [16–25], individuals with, and at risk for, autism spectrum disorder [6,7], placenta accreta [26], and preterm birth [8].

Since many genes regulate the processes of cytotrophoblast proliferation and fusion, it has been hypothesized that intrinsic genetic abnormalities are responsible for the formation of TIs [2,3]. Considering the fact that the placenta shares the same genetic composition as the fetus in over 98% of all gestations [27], it has been suggested that the presence of TIs may serve as a marker for fetal genetic abnormalities [1,3,26]. However, up until now, no study has confirmed that fetal

Abbreviations: TIs, Trophoblast inclusions; MZ, Monozygotic; DZ, Dizygotic; di-di, Dichorionic-diamniotic; GA, Gestational age.

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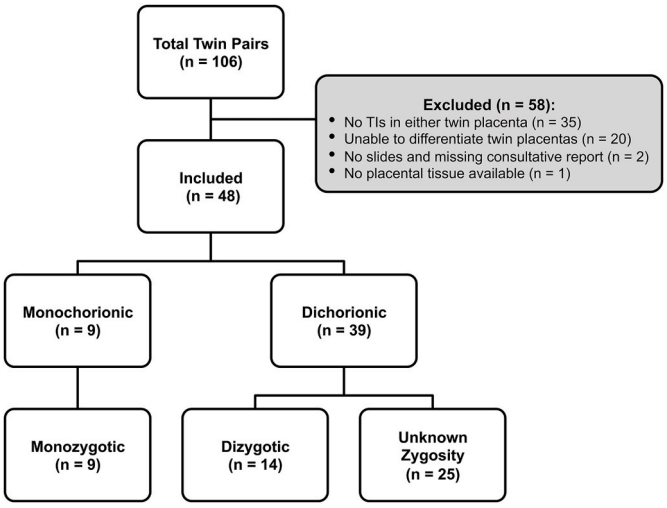

Fig. 1. Study population inclusion, exclusion, and zygosity classification.

genetics, rather than exogenous exposures of the uterine environment, drive the formation of TIs.

In order to gain insight into whether the formation of TIs is driven by, or relatively independent of, genetic influences, we conducted a twin study to compare the average number of TIs among dizygotic and monozygotic twin pairs. Dizygotic (DZ) twins are derived from two zygotes and share about 50% of their genes; monozygotic twins (MZ) are derived from a single zygote and are, therefore, genetically identical [28]. Regardless of zygosity, all twin pairs share the same intrauterine environment. This creates the perfect "natural experiment:" if a trait is influenced by genetics, then the within-pair resemblance for this trait is expected to be higher in MZ twins than in DZ twins [29,30].

2. Materials and methods

2.1. Subjects and study design

Cases were selected from a subset of consultation reviews conducted at the Yale University Reproductive and Placental Research Unit. As part of these clinical consultations for either pregnancy losses or complications, hematoxylin and eosin (H&E) recut slides of archived paraffin-embedded placental tissue specimens, pathology reports, and clinical information included with the consultation request were available for review. Complete medical records were not available. The analysis of this retrospective case series was approved by the Yale University Human Research Protection Program Institutional Review Board (protocol ID 2000029781).

During the histopathologic examination of each placenta, each slide was analyzed in a row-by-row raster pattern. Features recorded included placenta type (multiple versus singleton), chorionicity and amnionicity (if applicable), and quantification of TIs present per slides. TIs were identified as previously described [3]. The average number of total TIs per slide were calculated and qualitatively recorded as None (no TIs identified in all slides examined), Mild (an average of >0 to 5 TIs estimated per slide), Moderate (an average of >5 to 10 TIs estimated per slides), or Marked (an average of more than 10 TIs estimated per slide) on the consultative report created at the time of evaluation.

For our current retrospective case series, 106 twin pairs were identified as a subset of the consult cases sent to the Yale University Reproductive and Placental Research Unit for review. Cases were included based on the following criteria: 1) twin gestation; 2) must be able to differentiate twin placentas upon microscopic evaluation; 3) copy of the original pathology report; 4) at least one twin must have had a non-zero average of total TIs per slide; 5) copy of the consultative report created at the time of initial histopathologic evaluation at Yale.

Out of the 106 twin pairs, 58 pairs were excluded from the study (Fig. 1). Thirty-five twin pairs (60%) were excluded because neither twin placenta had TIs. Twenty twin pairs (35%) were excluded because it was impossible to differentiate the placenta of Twin A from the placenta of Twin B upon histopathological examination. This was likely due to loss at an early gestational age (GA range of these excluded cases: 6.9–19 weeks; average GA: 12.4 ± 4.0 weeks). Two twin pairs (3%) were excluded because the consultative reports were missing and the placenta slides had already been returned to their originating hospitals. One twin pair (2%) was excluded because there was no placental tissue available for analysis (the slides only contained umbilical cord tissue).

We next classified the 48 twin pairs that were included in our study based on zygosity. Nine (19%) twin pairs were monochorionic, and therefore monozygotic [31]. Thirty-nine (81%) twin pairs were dichorionic (two distinct placental disks). Of these, 14 (36%) were definitively diagnosed as being dizygotic twins based on the discordant sexes of the associated children or stillbirths. The zygosity for the remaining 25 (64%) dichorionic twin pairs was indeterminate, as they were same-sex dichorionic-diamniotic twin pairs (di-di). Therefore, for the purpose of this study, we divided the 48 twin cases into the following three groups based on zygosity classification: MZ (n = 9), DZ (n = 14), unknown zygosity (UZ) (n = 25). Relevant demographic information, including placentation, fetal sex, gestational age at birth, maternal age, gravidity, and parity was recorded for each zygosity group. Birth and

Table 1
Demographics stratified by twin pairs.

	Monozygotic N = 9 (%)	Dizygotic N = 14 (%)	Unknown Zygosity N = 25 (%)
Placentation:			
Dichorionic-diamniotic, fused	0 (0%)	6 (43%)	18 (72%)
Dichorionic-diamniotic, not fused	0 (0%)	8 (57%)	7 (28%)
Monochorionic-diamniotic	8 (89%)	0 (0%)	0 (0%)
Monochorionic-monoamniotic	1 (11%)	0 (0%)	0 (0%)
Sex:			
Male-Male	6 (67%)	0 (0%)	15 (60%)
Female-Female	3 (33%)	0 (0%)	8 (32%)
Male-Female	0 (0%)	14 (100%)	0 (0%)
Unknown	0 (0%)	0 (0%)	2 (8%)
Adverse outcomes/fetal disorders (AO/FD) in one or both of the twins	8 of 9 (89%)	13 of 14 (93%)	22 of 25 (88%)
Concordant for AO/FD	6 of 8 (75%)	8 of 13 (62%)	13 of 22 (59%)
GA at birth, weeks (mean ± SD)	26.4 ± 7.3	27.2 ± 6.7	28.5 ± 8.0
Maternal Age, years (mean ± SD)	30.9 ± 7.5	33.1 ± 5.0	34.5 ± 5.5
Gravidity (mean ± SD)	2.9 ± 1.9*	2.5 ± 1.7 **	2.5 ± 1.7***
Parity (mean ± SD)	1.4 ± 1.8*	0.8 ± 1.1**	1.3 ± 1.3***

Abbreviations: SD, standard deviation; GA, gestational age; AO, adverse outcomes; FD, fetal disorders. *Data NA for 1 set of twins. ** Data NA for 2 sets of twins. ***Data NA for 3 sets of twins.

developmental outcomes were documented as well; stillbirths, neonatal losses, developmental delays and genetic abnormalities were categorized as adverse outcomes or fetal disorders. These outcomes were only based on parental reporting, as the medical records were not available.

A placental pathologist (HJK) systematically reanalyzed the available twin placenta slides. The outcome of interest was the number of total TIs per slide in each twin pair. Out of the 48 twin pairs included in our study, placenta slides for 34 (71%) twin pairs were available for reanalysis. HJK was blinded to all identifying information, including demographic information, maternal clinical history, twin type, and birth and developmental outcome data. Inclusionoids, inclusions, calcified inclusions, and calcified bodies [3] were counted and summed across the slides and the average total TIs per slide were calculated.

Slides for the remaining 14 (29%) twin pairs were unavailable, as they had already been returned to their originating institutions. Although we were unable to obtain an exact average number of TIs per slide for these placentas, we were able to extrapolate information about the average number of total TIs based on the previously recorded TI classification data found in the consultative report. In regards to the average number of total TIs per slide, 7 (25%) twin individuals had a classification of None and 21 (75%) twin individuals had a classification of Mild. We decided, a priori, to assign a numerical value for each classification category. Since the Mild classification indicated that the average number of total TIs per slide was between >0 and 5 TIs, we estimated that these cases had an average of 2 TIs per slide, and used this designated value for the purpose of statistical analysis.

2.2. Statistical analysis

Microsoft Excel 2011 software was used to calculate the total TIs per slide for the twin individuals, the absolute difference of the total TIs per slide between each twin pair, the mean absolute difference of the total TIs per slide between twin pairs in each zygosity group, as well as standard deviation (SD) values. Further statistical analysis was performed using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

After calculating the absolute differences of the total TIs per slide between the twin pairs for each zygosity group, we examined the data distributions. We found that the data for each zygosity group displayed exponential distributions. Normally, when comparing differences between groups, one would use a Student *t*-test; however, this test is only applicable if the data display normal distributions. Since the data for each zygosity group displayed exponential distributions, we performed Generalized Likelihood Ratio Tests for Exponential Distributions [32] to compare the mean absolute difference in the total TIs per slide between MZ and DZ twin pairs, MZ and UZ twin pairs, and DZ and UZ twin pairs. Details of this statistical test are provided in supplementary material. We used a one-way ANOVA to compare the mean maternal age, mean gravidity, mean parity, and mean gestational age (GA) at birth between the zygosity groups. A two-sample *t*-test was used to compare the average TIs between the DZ females and the DZ males.

3. Results

In this retrospective case series, we examined the placentas of 48 twin pairs. We classified the twin pairs based on zygosity: MZ (9 twin pairs), DZ (14 twin pairs), and UZ (25 twin pairs) (Fig. 1). Demographic data for each zygosity group are shown in Table 1. In regards to the placentation of these twins, 8 (89%) of the MZ twins were monochorionic-diamniotic and 1 (11%) set of MZ twins was monochorionic-monoamniotic. Six (43%) of the DZ twins were di-di, fused, and 8 (57%) were di-di, not fused. Eighteen (72%) placentas associated with UZ twins were di-di, fused, and 7 (28%) were di-di, not fused. We also recorded the biological sexes of the twin pairs. Six (67%) of the MZ twins were male-male and 3 (33%) were female-female. Fourteen (100%) DZ twins were male-female. Fifteen (60%) UZ twins were male-male, 8 (32%) were female-female; the sex of 2 (8%) of the twin pairs was unknown. Eight (89%) of the MZ twins had adverse outcomes or fetal disorders, 6 of these 8 (75%) were concordant for these outcomes. Thirteen (93%) of the DZ twins had adverse outcomes or fetal disorders, 8 of these 13 (62%) were concordant for these outcomes. Twenty-two (88%) of the UZ twins had adverse outcomes or fetal disorders, 13 of these 22 (59%) were concordant for these outcomes. There were no statistically significant differences between the mean maternal age, mean gravidity, mean parity, and mean gestational age of

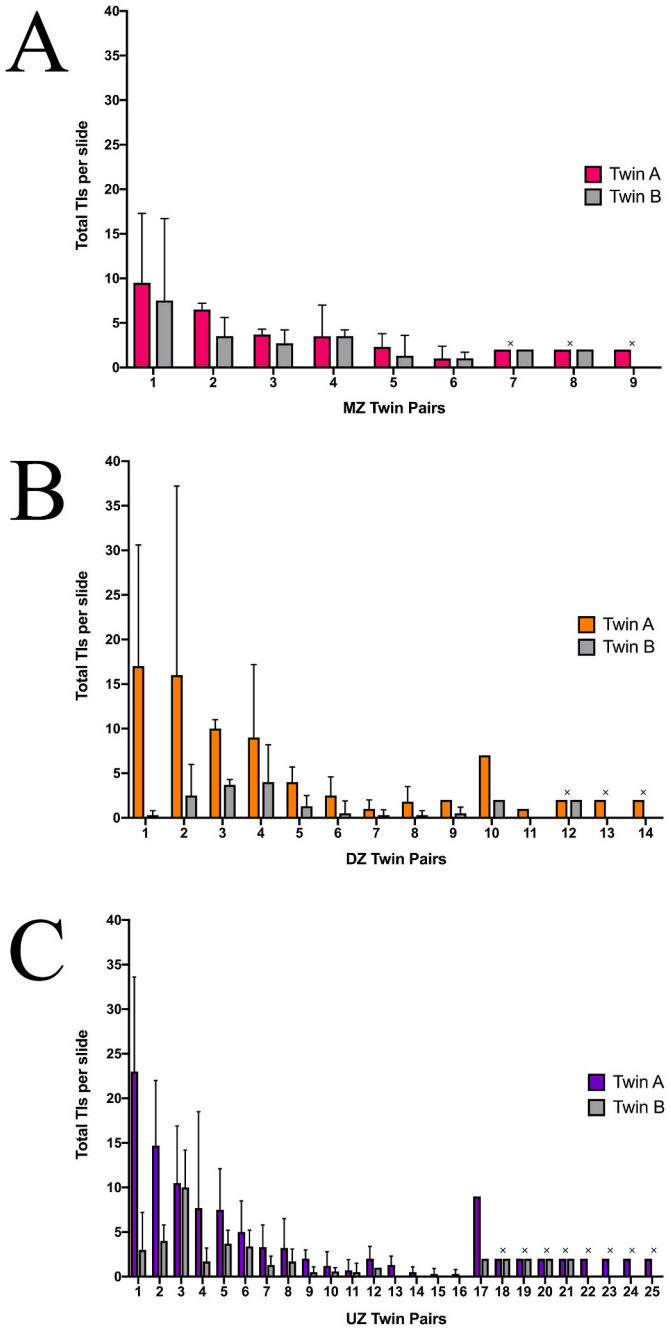

Fig. 2. Total TIs per slide for A) MZ twin pairs, B) DZ twin pairs, and C) UZ twin pairs. Note: the designations of "Twin A″ and "Twin B″ do not correspond to birth order; rather we designated "Twin A″ to be the twin with the higher number of total TIs per slide and "Twin B″ to be the twin with the lower number of total TIs per slide. SD bars are depicted when exact total TI averages were calculated. Missing SD bars indicated by × represent estimated value based on TI classification. The remaining missing SD bars are cases in which the SD was zero.

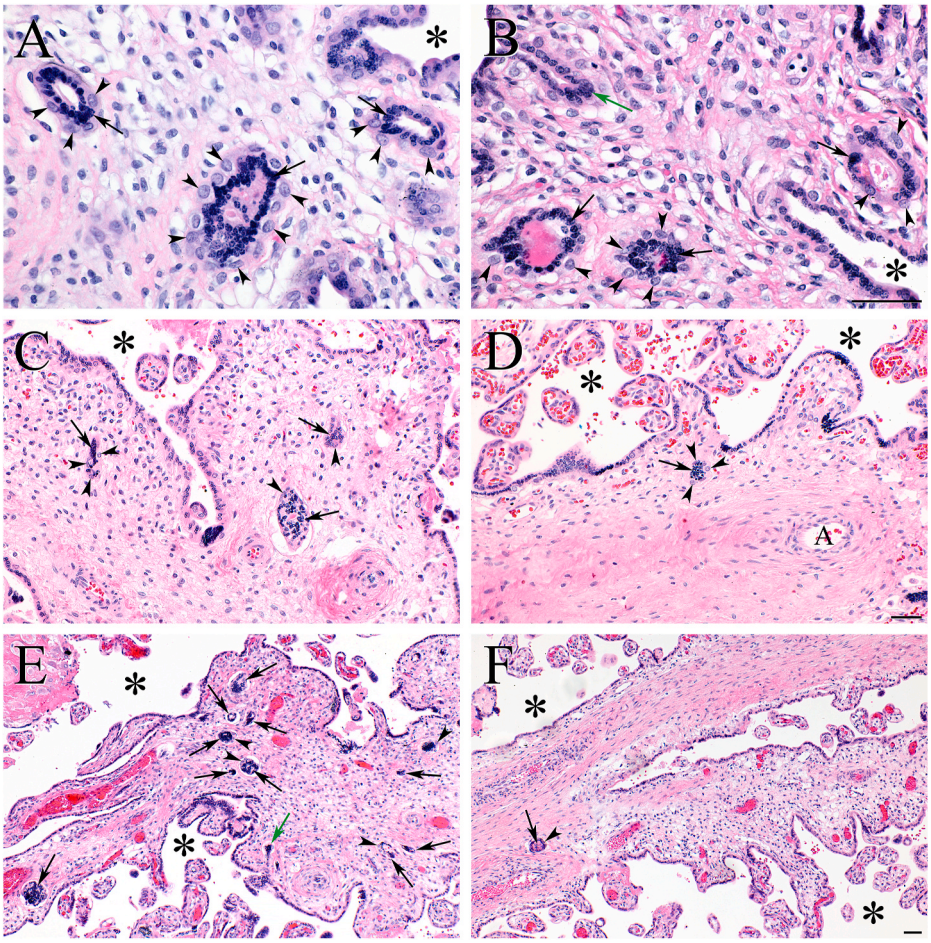

Fig. 3. Trophoblast inclusions (TIs) in representative twin pairs from each zygosity group. Monozygotic twin pair (A, B). (A) Representative field with 3 TIs, each with the defining syncytiotrophoblast centers (arrows), surrounded by multiple cytotrophoblasts (arrowheads). (B) Similar field as the co-twin, again with 3 TIs (same labels as in (A)). Note invagination (green arrow). Dizygotic twin pair (C, D). (C) Representative field with 3 TIs again with syncytiotrophoblast core (arrow), surrounded by cytotrophoblasts (arrowheads). (D) Representative field with 1 TI (same labels as in (C)). Fetal arteriole (A). Unknown zygosity twin pair (E, F). (E) Representative field with 11 TIs, again with syncytiotrophoblast core (arrow), surrounded by cytotrophoblasts (arrowhead). Note invagination with forming TI at its base (green arrow). (F) Representative field with 1 TI (same labels as in (E)). Intervillous space (*). Magnifications are the same for each twin pair. Bars represent 20 μM.

the zygosity groups.

We microscopically analyzed all available twin placenta slides for the total number of TIs. We subsequently calculated the average number of total TIs per slide. For the 34 twin pairs with available placenta slides, we calculated the average number of total TIs per slide. For the 14 twin pairs missing placenta slides, we assigned an estimated value for the total TIs per slide based on the TI classification data found in corresponding consultative reports. Fig. 2A–C depicts the average number of total TIs per slide for the MZ, DZ, and UZ pairs, respectively.

Representative images of the TIs identified in the chorionic villi slides of MZ, DZ, and UZ co-twins are displayed in Fig. 3. Fig. 3A and B are representative images of the TIs found in MZ twin pair #1 (see Fig. 2A for reference). The placenta from Twin A had an average of 9.5 TIs per slide, while the Twin B's placenta had an average of 7.5 TIs per slide. Fig. 3C and D are representative images of the TIs found in DZ twin pair #3 (see Fig. 2B for reference). The placenta from Twin A revealed an average of 10 TIs per slide, while the Twin B's placenta had an average of 3.7 TIs per slide. Fig. 3E and F are representative images of the TIs found in the UZ twin pair #1 (see Fig. 2C for reference). The placenta of Twin A revealed an average of 23 TIs per slide, while the placenta of Twin B revealed only 3 TIs per slides.

Next, we calculated the absolute difference between the total TIs per slide for each twin pair and then calculated the means of these differences for the MZ, DZ, and UZ groups. MZ twin pairs had a mean absolute difference of 1 (±1.1) TIs; DZ twin pairs had a mean difference of 4.3 (±5.0) TIs; UZ twin pairs had a mean difference of 2.7 (±4.4) TIs. We compared these means using the Generalized Likelihood Ratio Tests for Exponential Distributions. The mean difference of the average number of total TIs between DZ twin pairs was significantly greater than the mean difference of the average total TIs between MZ twin pairs ($p = 0.003$) (Fig. 4). The mean difference of the average number of total TIs between twin pairs in the UZ group was also significantly greater than the mean difference of the average TIs between MZ twin pairs ($p = 0.028$). There was no significant difference between the mean difference of the average number of total TIs between the DZ and UZ twin pairs.

We calculated the average number of total TIs of the DZ males (n = 7) to that of the DZ females (n = 7). DZ males had an average of 4.6 (±5.3) TIs; DZ females had an average of 2.2 (±3.2) TIs. This difference was not statistically significant.

4. Discussion

Trophoblast inclusions (TIs) are a dysmorphic feature of placentas that form as a result of improper infoldings of the trophoblast bilayer [3]. Increased cytotrophoblast proliferation, relative to the rate of cytotrophoblast fusion and subsequent syncytiotrophoblast formation, is likely the key etiology for TI formation [1]. Although this placental dysmorphology feature has most often been attributed to the endogenous genetics of the placenta and fetus [9–14,16,17,19–26], some have argued that prenatal environment impacts placental morphologic features [33]. To distinguish between these two potential pathogenic mechanisms, we examined nature's ideal experiment: a twin study. We investigated the concordance of TIs found in MZ and DZ twin pairs in order to gain better insight into the driving factors of TI formation.

In comparing the means of the absolute difference of the total TIs per slide found in the placentas of twin pairs, we found that the TI differences between DZ twin pairs were significantly higher compared to the TI differences between MZ twin pairs. This finding supports the

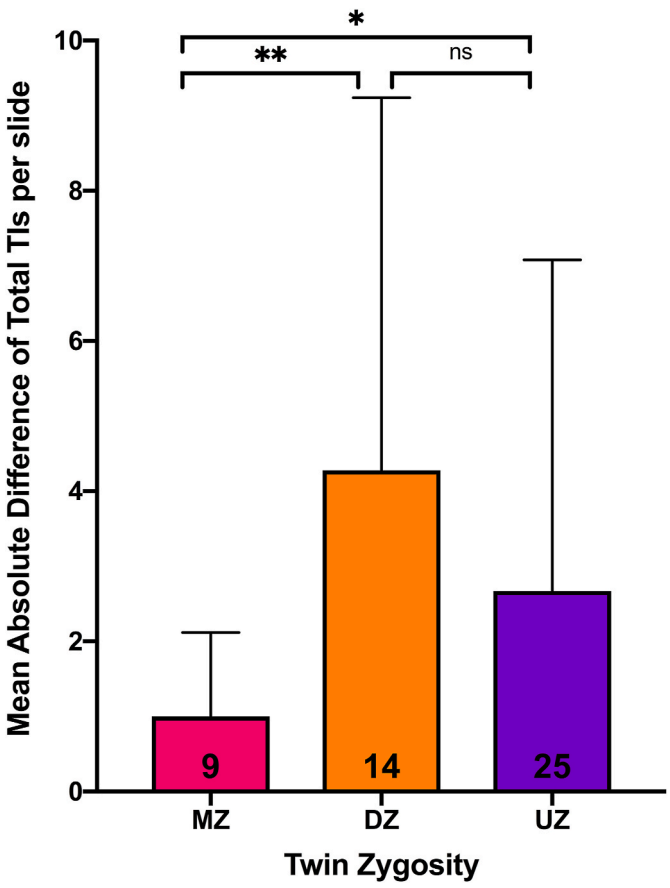

Fig. 4. Mean absolute difference of the total TIs per slide between zygosity groups. The number of twin pairs is identified at the base of each zygosity type column. **: $p = 0.003$; *: $p = 0.028$.

conclusion that the frequency of TIs is a manifestation of zygote genetics, rather than the prenatal maternal uterine environment.

Our study was limited by the fact that we were unable to definitively determine the zygosity of the 25 twin pairs, which were classified in the UZ group. The only information we had for these twins was that they were same-sex dichorionic-diamniotic (di-di), indicating that each fetus had its own placenta and amniotic sac. By definition, all DZ twins are di-di (some fused, some not fused). Given this, along with the fact that the mean difference of average total TIs between MZ twins and UZ was significantly different—whereas the mean difference of average total TIs between DZ twins and UZ twins was not—it is likely that our UZ group was composed mostly of DZ twins. Interestingly, the mean difference of average total TIs between the MZ and UZ twins was intermediate between the MZ and DZ difference, suggesting that some portion of the UZ twins were in fact MZ. This is consistent with the relative frequencies of monozygotic (28%) versus dizygotic (72%) twin pairs [34] and the fact that about 18% of spontaneously conceived MZ twins are di-di [31].

We do acknowledge that dichorionic twins may have slightly different prenatal environments due to the fact that they do not share the same chorion. However, due to the limitation discussed above, it was not possible to compare monozygotic dichorionic placentas to dizygotic dichorionic placentas. Future studies should be considered that are able to make these comparisons.

Although we did not definitively know the zygosity of the UZ group, comparing the frequency of TIs between the UZ twin pairs still revealed important information. If TI formation was the result of the intrauterine environment, then all twin pairs, regardless of zygosity, should have had a similar frequency of TIs. However, this was not the case in our study. MZ twins were significantly more concordant in regards to the average number of TIs per slide compared to both DZ twins and UZ twins.

To date, no study has examined the effect of fetal sex and hormonal milieu on TI formation. One may argue that the degree of TI concordance among the MZ twins could be attributed to the fact that MZ twins are concordant for sex as well. However, the fact that the same-sex di-di twins of the UZ group were significantly more discordant for TIs than the MZ twins suggests that the TI concordance of the MZ twins is not the mere result of being twins of the same sex.

While the presence of placental TIs is not diagnostic of a specific genetic abnormality [3], our finding that TIs are inherent to fetal genetics cannot be overlooked. The identification of TIs has the potential to serve as a proxy for early detection and diagnosis of individuals at risk for genetic abnormalities. It is therefore our hope that placental examination for the presence of TIs will become a standard in placental pathology.

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Author contributions

Study concept and design: Katz, Kliman.
Case management: Katz.
Slide review: Kliman.
Data analysis and statistics: Katz, Holzer.
Writing the manuscript: Katz, Kliman.
Obtained funding: Kliman.

Declaration of competing interest

All the authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2021.04.010.

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