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Technical note

Trophoblast inclusions in the human placenta: Identification, characterization, quantification, and interrelations of subtypes

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ABSTRACT

We sought to examine placentas enriched for trophoblast inclusions (TIs) in order to characterize, quantify, and examine the interrelations between subtypes of TIs to better understand their underlying biology. We examined a cohort of 600 placentas from deliveries between 20^0 and 43^0 weeks of gestation. Forty-five percent of the placentas had at least one TI in the two slides examined. Four percent of the placentas had 10 or more TIs and two placentas had more than 70 TIs. Four distinct TI subtypes were observed: inclusionoids (early forming inclusions), inclusions, calcified inclusions, and calcified bodies. We suggest this reflects a developmental trajectory of TI maturation, the timing of which might be useful when comparing TI expression to clinical outcomes.

1. Introduction

In the last 10–15 years, researchers have shown that trophoblast inclusions (TIs) are found with increased frequency in placentas from children with autism spectrum disorder (ASD) [1] and from children with increased familial risk for ASD [2]. TIs are also more prevalent in cases of placenta accreta, increta, and percreta [3] as well as preterm delivery [4]. This study aimed to identify, characterize, quantitate, and study the interrelations between TI subtypes to better understand their biology. To achieve this, we performed histologic examination on a subset of 600 placentas from the Safe Passage Study (SPS) of the Prenatal Alcohol and SIDS and Stillbirth (PASS) Network [5,6]. This sample is highly enriched for risk of pregnancy loss and prematurity, outcomes which are associated with TIs [4,7–20].

2. Materials and methods

2.1. Subjects and study design

Placentas were obtained from 591 pregnancies that were part of the NICHD/NIAAA/NIDCD-funded SPS, a prospective, multicenter cohort study designed to evaluate the hypothesis that prenatal exposure to alcohol is associated with increased risk of SIDS or stillbirth [5]. This secondary analysis included only data obtained from the South African site [6]. Paraffin-embedded tissue sections were cut and two hematoxylin and eosin stained slides were produced for histological examination of each placenta. Of the 591 pregnancies included in this analysis, there were 582 singleton pregnancies and 9 twin pregnancies, resulting in a total of 600 placentas. Gestational age (GA) at delivery ranged from 20.9 to 43.0 weeks with a mean of 35.9 weeks (SD = 5.01).

The primary outcome of interest was the frequency of TIs per slide. TIs are defined as cross sections of invaginations of the trophoblast bilayer, resulting in the appearance of trophoblasts within the villous core characterized by central syncytiotrophoblast nuclei surrounded by one or more cytotrophoblasts, always away from the villus edge [1,2,4,

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Abbreviations

TIs	Trophoblast inclusions
ASD	Autism spectrum disorder
SIDS	Sudden infant death syndrome
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NICHD	National Institute of Child Health and Human Development
NIDCD	National Institute on Deafness and Other Communication Disorders
SPS	Safe Passage Study
PASS	Prenatal Alcohol and SIDS and Stillbirth
GA	Gestational age
CalcTIs	Calcified TIs

2.2. Statistical analysis

We examined the frequencies of all TI subtypes in the 600 placentas and the associations between the TI subtypes. Correlations were determined using the Spearman rank-order method to avoid undue influence from outliers. To get approximate confidence intervals for the correlation, as well as approximate p-values, we used statistical bootstrapping [23].

Intra-rater and inter-rater test-retest reliability was established by re-reading 10% of both slides from a randomly-selected subset of the placentas (60 out of 600 placentas, yielding 120 out of the original 1200 slides) and quantitating the four TI subtypes described above in each slide. The percent agreement between reads for both the expert (HJK) and novice (KMH) reader were calculated. Using the stringent criteria of an exact numeric intra-rater agreement, HJK had an exact agreement ranging between 81.5 and 94.1% for the four TI subtypes, while KMH's exact agreement ranged between 69.2 and 91.7%. We also evaluated the

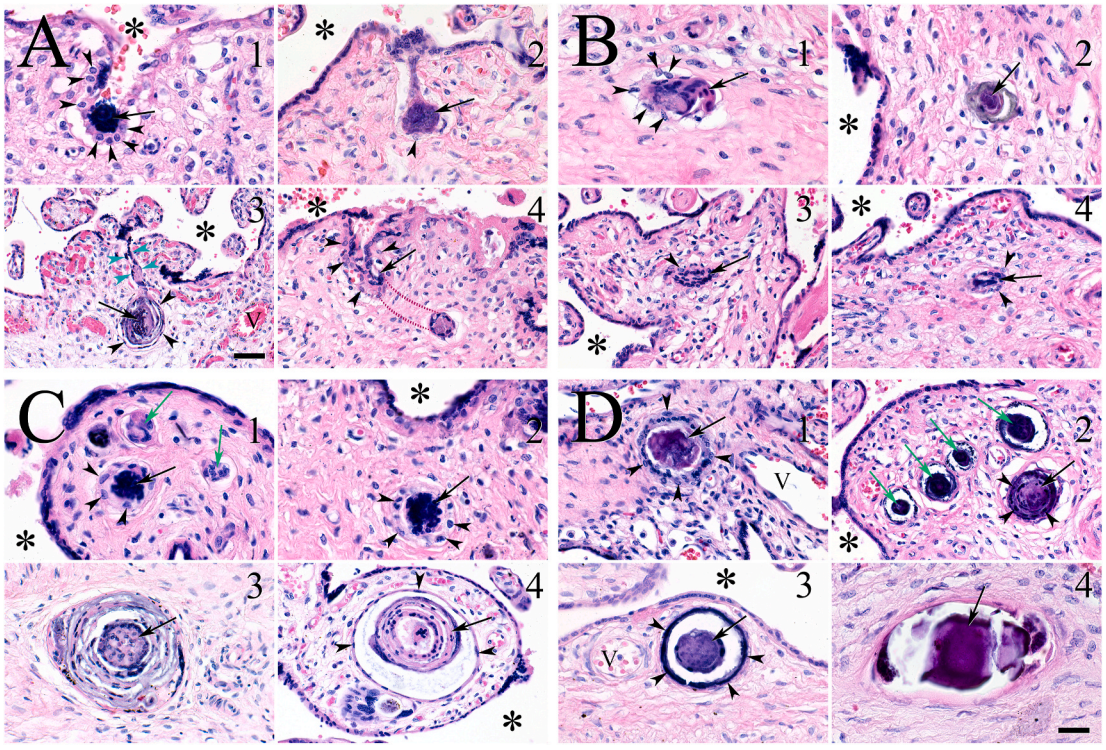

Fig. 1. Trophoblast invaginations and inclusion subtypes. (A) Panel showing four examples of invaginations of the trophoblast bilayer. (A1) A concentrated mass of syncytiotrophoblast nuclei (arrow) at the base of an invagination formed by an excess of cytotrophoblasts (arrowheads). Intervillous space (*). (A2) Dumbbell shaped invagination with syncytiotrophoblast core (arrow) and only a rare surrounding cytotrophoblast (arrowhead). (A3) Multilayered TI core (arrow), surrounded by thinned out cytotrophoblasts (arrowheads). Note the tenuous invagination channel (green arrowheads) demonstrating the connection between the surface trophoblast layers and a forming inclusion. (A4) Portion of an invagination with a central syncytiotrophoblast core (arrow), surrounded by cytotrophoblasts (arrowheads) that leaves the plane of the section (red dashed lines), to eventually make contact with an inclusion. (B1-4) Panel showing four examples of inclusionoids. Irregular inclusions with syncytiotrophoblast cores (arrows), surrounded in some cases by cytotrophoblasts (arrowheads). (C1-4) Panel showing fully developed inclusions, with syncytiotrophoblast cores (arrows), surrounded by cytotrophoblasts of varying thicknesses (arrowheads). Inclusionoids also seen in (C1; green arrows). (D) Panel showing calcified inclusions and an inclusion body. (D1) What appeared to be an inclusion, had, on closer inspection, a calcified core (arrow) with surrounding cytotrophoblasts (arrowheads). Vessel (V). (D2) Calcified inclusion with calcified core with persistent syncytiotrophoblast nuclei (black arrow), surrounded by a dense ring of cytotrophoblasts (arrowheads). The remaining calcified inclusions (green arrows) had such dense calcified cores that syncytiotrophoblast nuclei could not be identified. (D3) Single calcified inclusion with only faint syncytiotrophoblast nuclei seen in the core (arrow), surrounded by a very dense layer of calcified cytotrophoblasts (arrowheads). (D4) Calcified body with a completely calcified core with no evidence of nuclei present (arrow). Magnification bar = 50 μ for all images except image (A3), for which it = 100 μ.

21,22]. In our previous studies, two types of TIs were assessed: inclusions and calcified inclusions [2,4]. For this study we further subdivided the TIs into four groups: inclusionoids (incipient inclusions), inclusions, calcified inclusions, calcified bodies (Fig. 1).

percent agreement between reads using a Poisson derived standard deviation. The two slides were considered to be in agreement if both were no further from their average than the square root of their average. Using this approach we found that HJK had an adjusted percent agreement ranging between 99.2 and 100%, while KMH's agreement

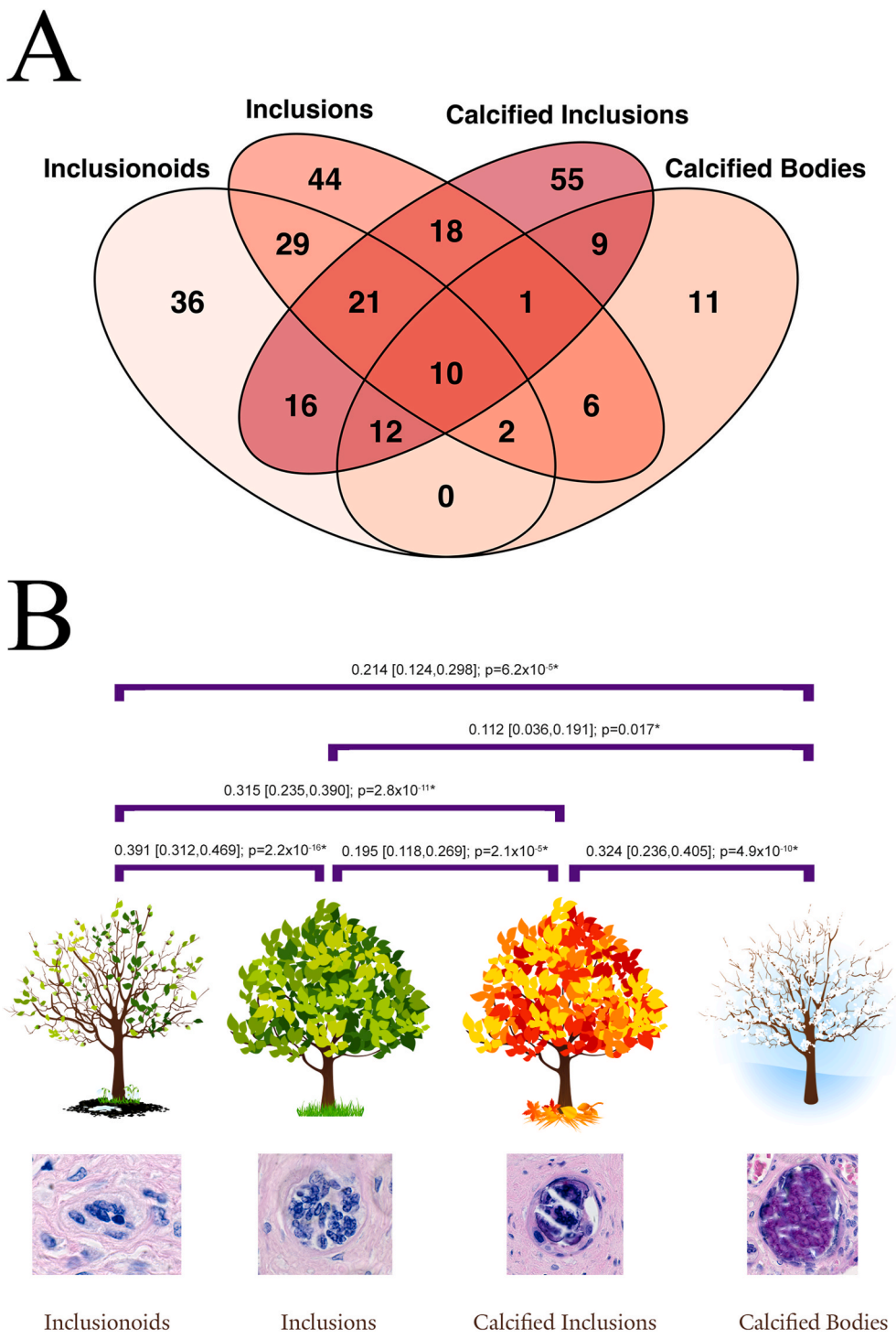

Fig. 2. Interrelations between inclusion subtypes. (A) Venn diagram showing the number of placentas with each inclusion subtype and their overlaps with the other inclusion subtypes. The total adds up to 270, the number of placentas with at least one TI in two slides. (B) Spearman correlations between inclusion subtypes (with 95% confidence limits and p values), all of which were significant (*). The inclusion subtypes have been placed in their temporal formation order with inclusionoids first (analogous to the buds forming on a tree), inclusions next (as in the leaves on a tree), calcified inclusions (now aging like the colored leaves of autumn), and finally calcified bodies (as in a tree in winter).

increased to between 95.8 and 100%. Analyses were carried out using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

Of the 600 placentas examined, 270 (45%) had at least 1 TI of any subtype in the two slides examined, while 330 (55%) had no TIs in the two slides. 438 placentas (73%) had fewer than 2 TIs across the two slides (our previous *a priori* definition of a negative placenta [1]). Twenty-four placentas (4%) had 10 or more TIs of any subtype and two placentas had more than 70 TIs in the two slides examined.

Having parsed the TIs at the time of data collection, we decided to examine the relationships among these TI subtypes. A Venn diagram (see Fig. 2A) revealed that although many of the 270 placentas with TIs only had one subtype of TI, there was significant overlap with placentas simultaneously having two or more TI subtypes. This suggested that there might be significant relationships between these four subtypes. Therefore, we next calculated the correlation coefficients of each TI subtype compared to the other three subtypes, resulting in six correlation coefficients (see Fig. 2B). There were statistically significant correlations between all six pair-wise comparisons, the most significant three being between the inclusionoids and inclusions (Spearman's rank

correlation coefficient [ρ] = 0.391, 95% confidence interval [CI] 0.320, 0.467; p = 0), followed by calcified inclusions and calcified bodies (ρ = 0.324, 95% CI 0.237, 0.406; $p = 4.9 \times 10^{-10}$), and finally, inclusionoids and calcified inclusions (ρ = 0.315, 95% CI 0.239, 0.390; $p = 1.1 \times 10^{-11}$). These correlations, and their ordering, suggests the possibility of a temporal relationship between these inclusion subtypes, namely that inclusionoids form first, followed by inclusions, then calcified inclusions, and finally, calcified bodies (see Fig. 2B).

4. Discussion

The cohort examined in this study was highly enriched in TIs, allowing us to assess the interrelations between the four TI subtypes we identified (see Fig. 2). The pregnancies included in this secondary analysis were derived from the SPS Study, which was itself enriched to study maternal prenatal alcohol use, SIDS, and stillbirth [5]. With this enrichment, we were able to detect what appears to be the developmental relationships between the four TI subtypes.

Inclusionoids are imperfect inclusions due to several factors. They are frequently smaller than what a classic inclusion would look like (compare Fig. 1, panel B to panel C), more often irregular in shape, and may not have had an obvious cytotrophoblast outer layer, one of the features of a fully developed inclusion. Although our current approach is not sufficient to prove this hypothesis, we speculate that inclusionoids are the earliest form of TIs, possibly representing cross sections of very early invaginations. As such, we have placed them as occurring prior to the development of a full-fledged TI (Fig. 2B). Inclusionoids are highly correlated with both inclusions and calcified inclusions (see Fig. 2), suggesting that these TI subtypes are closely related. Although it is speculative to suggest that inclusionoids precede the other TI subtypes, the temporal relationship between calcified inclusions and calcified bodies is much more certain. The calcification process itself is irreversible. Therefore, once the syncytiotrophoblast core of an inclusion starts to become calcified, it cannot revert to either an inclusion, nor an inclusionoid, but will remain as a calcified inclusion or progress to a calcified body.

The slides in the present cohort were examined previously by other pathologists as part of the SPS study, however, TIs were not initially identified in these slides under the examination protocol based on the Amsterdam Placental Workshop Group Consensus Statement [24]. TIs are not part of routine pathologic examination, and therefore, they are often not noted by pathologists. To establish the feasibility and reliability of including TIs as a standard pathologic feature of placental examination, we performed an analysis of intra-rater and inter-rater test-retest reliability. Our results suggest that TIs can be easily and reliably identified.

Trophoblast inclusions are formed by abnormal folding of the trophoblast bilayer and represent dysmorphic development in the placenta, which is most likely associated with a genetic abnormality. As such, TIs may serve as a proxy/indicator for matched genetic abnormalities in the associated conceptus and newborn. Our study validates the reliability of identifying TIs in placentas and suggests that it may be useful to prospectively include TIs in the examination of all placentas to identify those pregnancies that may be associated with an occult genetic abnormality. More generally, TIs may be an important tool for both clinical and research applications. It is also clear from our study that when TIs are present—and specifically looked for—they will be seen.

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Ethical approval

Patient recruitment into the Safe Passage Study (SPS) and PASS Network was approved by Stellenbosch University Health Research Ethics Committee (project #9317) and the New York State Psychiatric Institute Institutional Review Board (protocol #5338). Microscopic examination of the deidentified slides sent for evaluation to the Yale Reproductive and Placental Research Unit was approved by the Stellenbosch University Ethics Board and the Yale University Human Investigation Committee (protocol #1003006495).

Author contributions

Study concept and design: Kliman, Firestein, Brink, Odendaal, Fifer.
Creation and management of SPS PASS cohort: Brink, Odendaal, Fifer.
Randomization, labeling and data management: Firestein, Milano.
Placenta sign out form: Kliman.
Slide review: Kliman, Hofmann.
Data analysis and statistics: Holzer, Kliman, Firestein.
Drafting of the manuscript: Kliman, Firestein.
Critical revision of the manuscript for important intellectual content: Kliman, Firestein, Hofmann, Brink, Odendaal, Fifer.
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Declaration of competing interest

The authors declare no competing interests.

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