Maternal vs Fetal Origin of Placental Intervillous Thrombi

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ABSTRACT

Objectives: To determine maternal vs fetal origin for blood in placental intervillous thrombi (IVTs).

Methods: We used comparative analysis of microsatellites (short tandem repeats [STRs]), sex chromosome fluorescence in situ hybridization (FISH), and immunohistochemistry (IHC) for fetal (α-fetoprotein [AFP]) and maternal (immunoglobulin M [IgM]) serum proteins to distinguish the origin of IVTs. Using an informatics approach, we tested the association between IVTs and fetomaternal hemorrhage (FMH).

Results: In 9 of 10 cases, the preponderance of evidence showed that the thrombus was mostly or entirely maternal in origin. In 1 case, the thrombus was of mixed origins. STR testing was prone to contamination by entrapped fetal villi. FISH was useful but limited only to cases with male fetuses. IgM showed stronger staining than AFP in 9 cases, supporting maternal origin. By informatics, we found no association between IVTs and FMH.

Conclusions: Evidence supports a maternal origin for blood in IVTs. IHC for IgM and AFP may be clinically useful in determining maternal vs fetal contribution to IVTs.

KEY POINTS

- This study addresses the issue of whether placental intervillous thrombi are maternal or fetal in origin.
- Immunohistochemistry for α-fetoprotein and immunoglobulin M is useful for testing maternal vs fetal origin of thrombi. Microsatellite testing is prone to contamination by fetal villi.
- We found that most thrombi are maternal in origin, but some have mixed maternal and fetal contributions.

KEY WORDS

Placenta; Perinatal; Thrombi; STR; FISH; Immunohistochemistry; COVID-19

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INTRODUCTION

The placental intervillous space is occupied by maternal blood arising from the remodeled spiral arterioles of the uterus. Adequate perfusion of this intervillous space is essential to the maintenance of healthy fetal gas exchange across the vasculosyncytial interface. Perturbations of this blood flow result in placental parenchymal lesions associated with maternal vascular malperfusion (MVM), including increased perivillous fibrin and villous infarcts.

Another common intervillous lesion that is not thought to be associated with MVM is the intervillous thrombus (IVT). This lesion is characterized by large deposits of fibrin that contains lines of Zahn, suggestive of true thrombi.[1] The origin of IVT is unclear, although there is a current, broad understanding that they represent a consequence of fetal hemorrhage into the intervillous space. Previous studies have investigated whether these lesions are maternal or fetal in origin. Kaplan et al[2] evaluated erythrocytes within IVT and found expression of hemoglobin F in 85% of examined thrombi. Frequently, fetal erythrocytes outnumbered maternal erythrocytes in thrombi. Fetal erythrocytes were also noted in IVT by Basnet et al,[3] although with relatively lower numbers of F cells—12 per 10 high-power field, on average. Further evidence for fetal origin of IVTs has been adduced from their reported association

with fetomaternal hemorrhage (FMH).[4] In the German literature, Gille et al[5] compared blood group antigens in IVTs between mother and fetus, concluding that approximately 88% of intervillous material was maternal in origin, while 12% was fetal. They hypothesized an initial fetal insult leading to a maternal coagulative response.

We employed 3 modern techniques to distinguish whether thrombi originate from the maternal or fetal circulation: microsatellite comparison between the thrombus and villous tissue of known fetal origin, fluorescence in situ hybridization (FISH) analysis for the XX and XY chromosomal complement in cases of discordant maternal-fetal sex (male fetuses), and immunohistochemistry (IHC) for 2 serum proteins—α-fetoprotein (AFP) and immunoglobulin M (IgM). DNA microsatellite comparison is an established technique for identifying whether formalin-fixed, paraffin embedded (FFPE) tissue contains DNA of maternal origin, as in the diagnosis of complete and partial moles.[6] XY FISH has previously been used in the investigation of maternal vs fetal origin of inflammatory cells within the placenta.[7] AFP is the main fetal serum protein, analogous to albumin in the postnatal state. At delivery, median umbilical arterial AFP has been reported as 72,062 ng/mL, nearly 100-fold greater than the reported maternal median of 95 ng/mL.[8] IgM is a pentameric immunoglobulin and the first isotype produced in the adaptive immune response. Unlike IgG, IgM is not transported across the placenta.[9] Accordingly, fetal serum levels are significantly lower. At vaginal delivery, maternal serum IgM levels are reported as 131.5 ± 41 mg/dL, while fetal levels are almost 10-fold lower, at 15.6 ± 7.4 mg/dL.[10] Thus, we hypothesized that differential staining of AFP and IgM would signify fetal vs maternal origin of IVTs.

MATERIALS AND METHODS

Cases

We identified placentas from 10 patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in pregnancy and a placental pathologic examination showing IVT. H&E-stained slides were retrieved and the diagnosis confirmed. This study was approved by the institutional review board (STU00212232) and clinical specimen release committee (#1507).

DNA Extraction

Serial unstained, 5-µm sections of FFPE tissue for each study case were examined microscopically. Identified clot sections within fetal tissues were selected together with corresponding areas with fetal-only tissue for genotype comparison. DNA was extracted using the Covaris truXTRAC FFPE total NA Plus Kit. All available DNA material was used for genotyping study.

Genotyping

To establish the genotype of cells in the clot, DNA extracted from the clots and DNA extracted from fetal material were tested concurrently within the same analysis to allow for a direct comparison of results. Thrombi as well as maternal and fetal tissue were identified on H&E-stained sections by an experienced pathologist and marked accordingly. Unstained slides were aligned to the H&E section for marking and macrodissection. Thrombus appeared red/brown on unstained slides because of native pigment, further simplifying the task. The regions were manually scraped with a scalpel blade into separate microcentrifuge tubes for DNA extraction. Three slides were used for scraping. The marking area can be seen in Supplemental Figure 1 (all supplemental materials can be found at *American Journal of Clinical Pathology* online).

Genotyping of 15 microsatellite short tandem repeat (STR) loci and an X/Y-specific marker (amelogenin) was performed using the AmpFLSTR Identifiler PCR Amplification Kit (ThermoFisher Scientific), following the manufacturer's protocol. This method allows distinction between fetal DNA and DNA from the clot. By examining these samples in parallel and comparing the relative ratios of alleles at each marker, we were able to determine the relative proportion of maternal and fetal DNA in the clot **FIGURE 1**.

FISH Analysis

FISH analyses were performed on the FFPE tissue sections for 5 cases in which the fetus was known to have a male sex chromosome complement. We used commercially available DXZ1 probes for the X chromosome (SpectrumGreen) and DYZ3 probe for the Y chromosome (SpectrumOrange) (Vysis/Abbott Molecular) according to the manufacturer's instructions.

Immunohistochemistry

Staining was performed at the Pathology Core Facility using a Leica BOND-MAX autostainer. IgM was Dako A042501-2, rabbit polyclonal antihuman IgM diluted 1:2,000; AFP was Dako A0008, polyclonal rabbit antihuman α-1-fetoprotein diluted 1:200. The bulk of the thrombus, excluding entrapped villi, was scored semiquantitatively as 0-3+ with half-scores by 2 perinatal pathologists and averaged.

Informatics

Patients undergoing flow cytometry testing of maternal blood for fetal hemoglobin were identified and the results retrieved from the institutional electronic data warehouse. Placental pathology reports from July 2011 to December 2020 were retrieved from the laboratory information system, and diagnoses were parsed.[11,12] Flow cytometry results were extracted. FMH was considered present when measured fetal hemoglobin was greater than the limit of detection of 0.1% (ie, 5 cm^3). Association between the presence of FMH and IVT was tested using Fisher exact test. Association between FMH and IVT size and IVT and FMH volume was tested with the Mann-Whitney *U* test. Association of FMH volume and IVT size was performed using linear regression. Statistical testing was performed using SciPy, version 1.6.1, software.

RESULTS

We studied 10 cases of IVT from placentas of mothers with SARS-CoV-2 in pregnancy **TABLE 1**. The average gestational age at delivery was 38.1 weeks (range, 35-40 weeks). The average latency from positive SARS-CoV-2 test result to delivery was 25 days (range, 0-113 days). Five of 10 (50%) patients were symptomatic. None had a clinical history of abruption or risk factors for FMH (eg, abdominal

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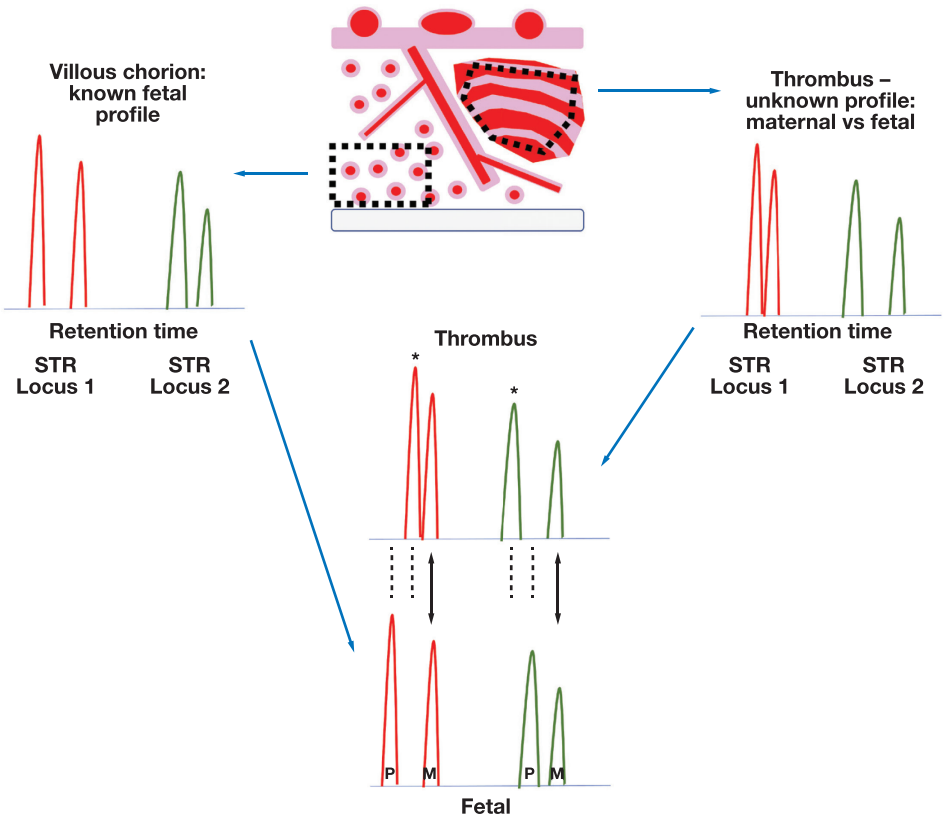

FIGURE 1 Identity testing via short tandem repeat (STR) analysis using 2 illustrative color-coded loci. Although no maternal DNA was available for comparative analysis, the genotype of thrombus cells can be determined by sizing the peaks on thrombus vs fetal electropherograms. The fetus profile matches the thrombus at 1 allele for each locus (double-headed arrows) corresponding to the allele inherited from the mother (M) with the other allele being paternally inherited (P). One of thrombus alleles (labeled with *) is not present in the fetus profile; thus, it is not of fetal origin and must have been of maternal origin.

TABLE 1 Clinicopathologic Features of Cases

Study No.	GA	Method of Delivery	SARS-CoV-2 Diagnosis to Delivery, d	Symptomatic	Fetal Sex	MVM Features	FVM Features
1	39	SVD	3	No	F	—	HCUC
2	39	SVD	0	No	F	Infarct	AV
3	39	CS	0	No	M	MHTY	—
4	40	SVD	25	Yes	M	Infarct, Agg	Patchy DVM
5	35	SVD	31	Yes	F	—	—
6	39	CS	4	Yes	M	MHTY, PMBPA	Patchy DVM
7	38	SVD	113	No	M	—	—
8	38	SVD	0	No	M	Infarct	—
9	37	Failed VAVD, CS	67	Yes	M	—	AV
10	37	SVD	7	Yes	M	—	—

Agg, villous agglutination; AV, avascular villi; CS, cesarean section; DVM, delayed villous maturation; F, female; FVM, fetal vascular malperfusion; GA, gestational age; HCUC, hypercoiled umbilical cord; M, male; MHTY, mural hypertrophy of membrane arterioles; MVM, maternal vascular malperfusion; PMBPA, persistent muscularization of basal plate arterioles; SVD, spontaneous vaginal delivery; VAVD, vacuum-assisted vaginal delivery.

trauma). None were diagnosed with a maternal deep vein thrombosis or pulmonary embolism. Five of 10 patients had features of MVM, and 6 of 10 had features of fetal vascular malperfusion, although none had a topline diagnosis. There were no cases of vertical transmission of SARS-CoV-2.

Cases had 1 to 4 thrombi ranging in size between 0.7 cm and 3.2 cm TABLE 2. Thrombi were central in 6 cases; peripheral in 2 cases; multiple, involving both central and peripheral placenta, in 2 cases; and in 1 case the central vs peripheral location was unstated. Thrombi were midparenchymal in 6 cases, parabasal in 2 cases, and

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TABLE 2 Thrombus Characteristics and Findings.

Case No.	Thrombus Size, cm	Central—Peripheral Position	Chorionic—Basal Position	Molecular Genotyping	FISH Analysis[a]	AFP	IgM	Summary
1	1.2, 1.5	Peripheral	Midparenchymal	Mismatch	—	0.25	1.75	Maternal
2	0.8	Unstated	Parabasal	Mismatch	—	0	2.5	Maternal
3	1.4, 1.6, 1.7, 1.8	Central and peripheral	Subchorionic	Mismatch	—	0.25	2.75	Maternal
4	0.7, 1.2	Central	Parabasal	Mismatch	—	0.75	2.75	Maternal
5	1.2	Central	Subchorionic, cyst adjacent	Weak match	—	0.25	2.5	Likely maternal
6	1.7	Central	Midparenchymal	—	50:50 XX:XY	0	2.5	Likely maternal
7	1.5-3.2	Central and peripheral	Midparenchymal	—	XX	1	2.75	Maternal
8	1	Peripheral	Midparenchymal	—	XX	1.25	2.75	Maternal
9	1.7	Central	Midparenchymal	Match	75:25 XX:XY	1.25	2	Mixed
10	1.4	Central	Subchorionic	Mix	XX	0.5	2.5	Likely maternal

AFP, α-fetoprotein; FISH, fluorescence in situ hybridization; IgM, immunoglobulin M.
[a]Result of XY FISH testing.

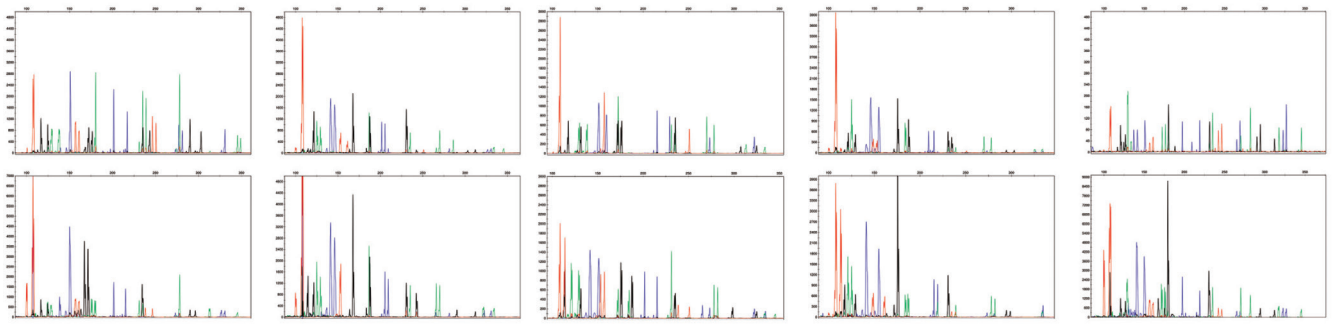

FIGURE 2 Comparative molecular genotyping of fetal tissues vs clot areas. Electropherograms of all tested short tandem repeat (STR) loci and the amelogenin sex typing are from the AmpFLSTR Identifiler PCR amplification test (ThermoFisher Scientific). The bottom panels show a set of peaks from fetal DNA. The top panels show a set of peaks from DNA extracted from clots. Molecular genotyping reveals STR loci mismatch in cases 1 through 4 and match in case 5.

subchorionic in 2 cases. One subchorionic thrombus was adjacent to a small subchorionic cyst.

Molecular Genotyping

Cases 1 through 5, 9, and 10 were tested by molecular genotyping of STR markers **FIGURE 2**. Cases 1 through 4 showed a mismatch between the thrombus and villous tissue, consistent with maternal origin of cells within clots. Cases 5 and 9 initially showed fetal origin for the thrombus, but review of the H&E slides from both cases showed fetal villi in the area marked for scraping (Supplemental Figure 2). Given the hypocellularity of thrombus material, even a small area of contamination could cause a thrombus to be read as fetal in origin, highlighting the challenges of bulk genotyping methods. Case 10 showed a mixture of maternal and fetal material.

FISH Testing

Cases 6 through 10 were tested by FISH for XX and XY complement **FIGURE 3**. In all these cases, the fetus was male. In cases 7, 8, and 10, all thrombus cells had XX complement, consistent with maternal origin. In case 6, nuclei in the thrombus were a mixture (~50% XX), and in case 9, nuclei were mostly XY (~75%).

IHC Testing

IHC for AFP and IgM was performed on all cases. Serum staining in fetal vessels was calibrated as 3+ for AFP, while serum or material clinging to erythrocytes in the nonthrombotic intervillous space was calibrated as 3+ for IgM **FIGURE 4** (Supplemental Figure 1). IgM staining was more intense than AFP in all cases, consistent with maternal blood.

Integration of Methodologies

We summarized the findings for each thrombus using the different techniques. In 7 of 10 cases, molecular genotyping of STR markers, FISH analysis, and IHC testing were concordant that the thrombus was of maternal origin. In case 5, STR analysis showed low signal of fetal origin, shown on review to consist of scant fetal villi. IHC strongly indicated maternal origin, summarized as "likely maternal." Likewise, case 6 showed a mixture by FISH testing, while IHC

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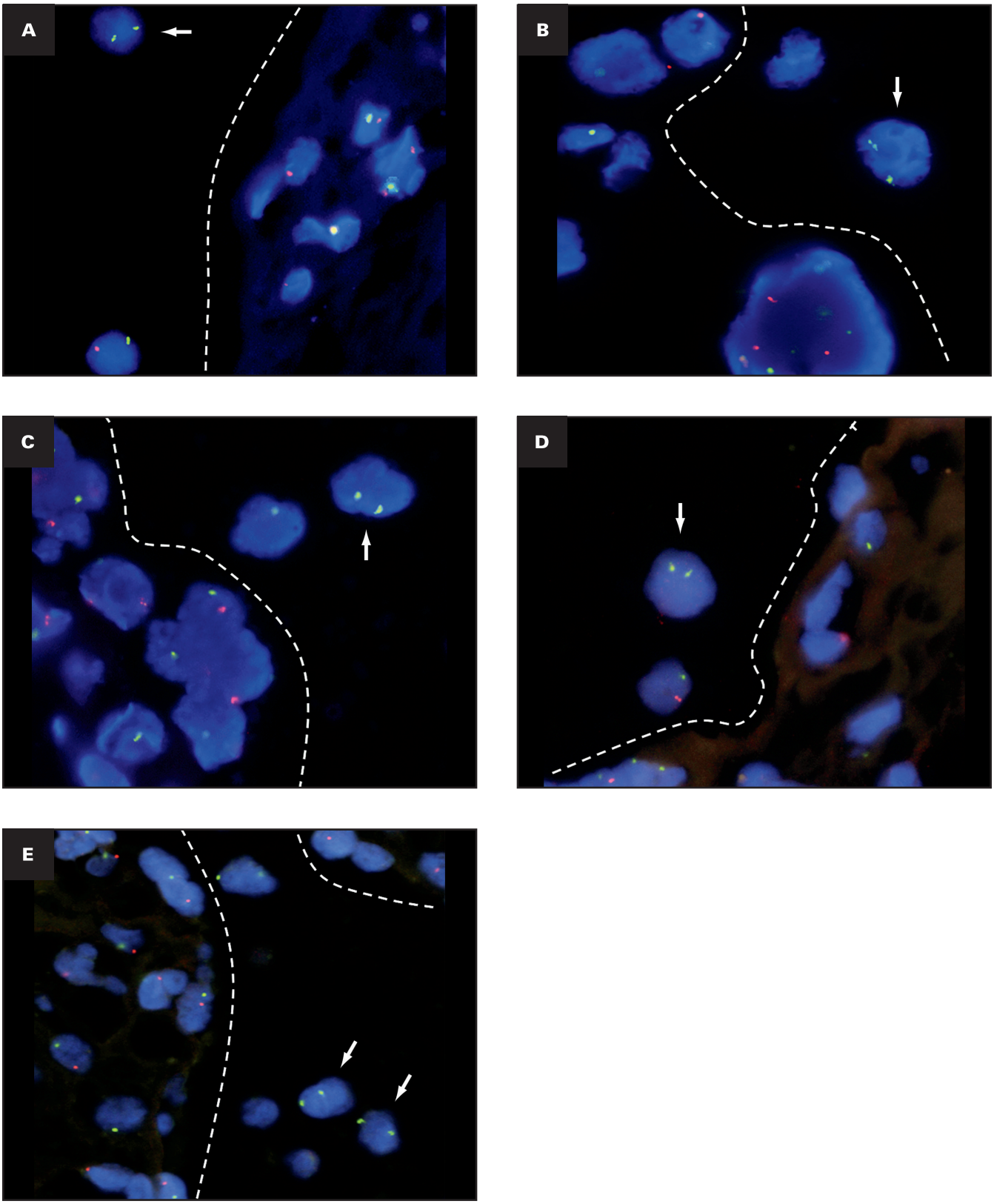

FIGURE 3 XX/XY fluorescence in situ hybridization (FISH) of fetal tissues vs clot areas. FISH analysis of interphase nuclei in formalin-fixed, paraffin-embedded sections using X (green) and Y (red) probes. FISH reveals the predomination of XX cells (arrows). Dotted lines separate fetal tissues from clot.

testing was strongly maternal, summarized as "likely maternal." In case 9, STR showed fetal origin, while FISH testing showed a mix of maternal and fetal. Review of case 9 on high power showed fibrotic fetal villi within the thrombus, suggesting fetal contamination in STR testing. Although AFP was less intense than IgM, case 9 showed the highest AFP intensity of the thrombi examined. Thus, case 9 was

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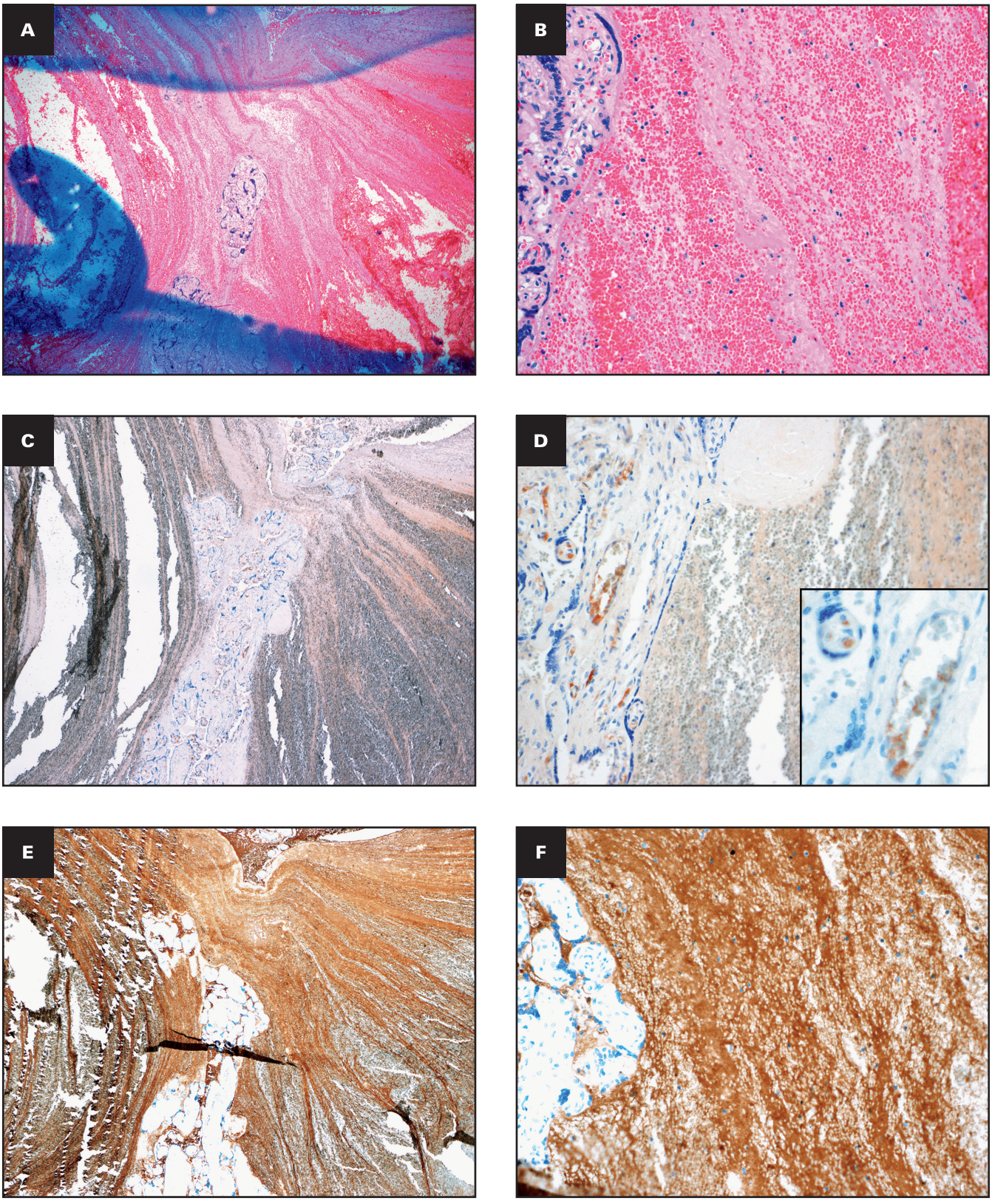

FIGURE 4 Histology and immunostains in thrombus (case 4). Thrombus showing lines of Zahn (**A**, H&E, blue ink marks boundary for short tandem repeat extraction; ×4). Entrapped villi may be a source of contaminating fetal DNA in molecular testing (**B**, H&E; ×4). The thrombus shows minimal staining for α-fetoprotein (**C**, ×4; **D**, ×20), but serum in fetal capillaries is a positive internal control (**D**, inset). Conversely, immunoglobulin M shows strong staining in the fibrinous portions of the thrombus and negative staining in villi (**E**, ×4; **F**, ×20).

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summarized as a mixture of maternal and fetal. Case 10 showed a mixture of material on STR, but FISH and IHC testing were more consistent with maternal origin.

Informatics Approach

If thrombi are of fetal origin, they represent hemorrhage of fetal blood into maternal space, with subsequent clotting. If so, they should be associated with nonclotted circulating fetal blood, detectable as FMH.[13] We first hypothesized that if fetal blood causes or comprises IVT, then cases with known fetal bleeding into the maternal system will have increased rates or sizes of thrombi. To test this hypothesis, we identified patients delivering at our institution with flow cytometry testing for FMH and placental examination. We identified 592 patients with FMH flow cytometry and placental examination. Of them, 97 patients had clinically detectable FMH, of whom 7 had IVT (7.2%). In total, 45 of 450 patients without FMH had IVT (10%; Fisher exact test, odds ratio, 0.78; $P = .70$). These findings do not support an association of detectable FMH and IVTs.

Our lower limit of flow cytometry detection for FMH is 5 cm^3, while a typical 1-cm diameter (0.5-cm radius) thrombus, if assumed spherical, has a volume of ~0.5 cm^3 ($\frac{4}{3}\pi 0.5^3$). Therefore, this technique does not rule out that small FMH causes IVT. If small FMH causes IVT, however, a reasonable hypothesis is that larger FMH is more likely to cause IVT. We examined the amount of FMH

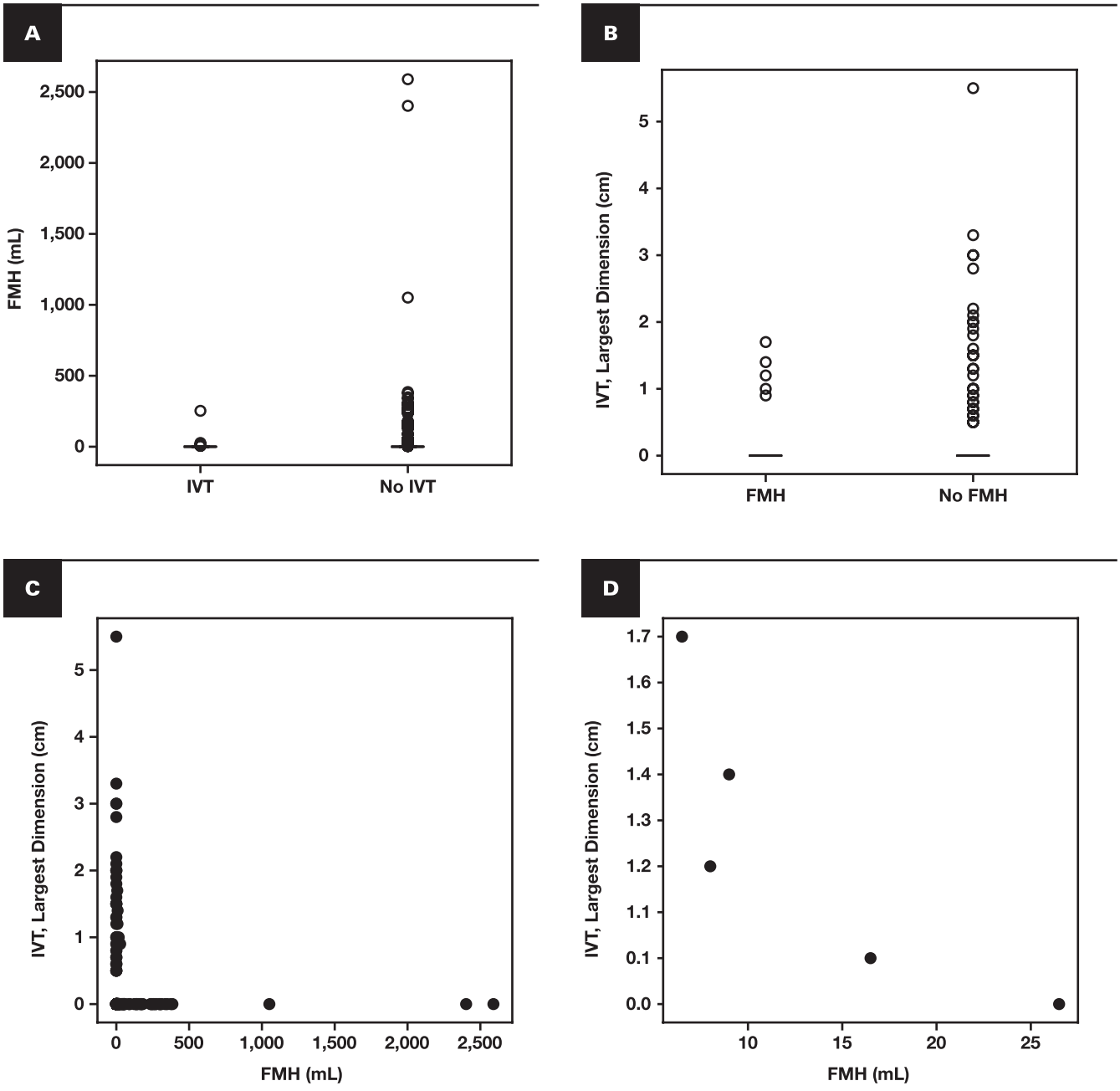

FIGURE 5 Association of clinically detectable fetal maternal hemorrhage (FMH) with intervillous thrombus (IVT). **A**, FMH volumes are not significantly different between patients with and without IVT. **B**, Conversely, IVT size is not significantly different between patients with and without FMH. **C**, Few patients have both IVT size >0 cm and FMH volume >0. **D**, In those who do, IVT size is nonsignificantly negatively correlated with FMH volume.

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measured in patients with and without IVT. In patients with IVT, the mean (standard deviation [SD]) FMH was 6 (35) cm^3, while the median was 0 cm^3. In patients without IVT, the mean (SD) FMH was 24 (166) cm^3, with a median of 0 cm^3. Mann-Whitney *U* test showed a *P* value of .24 **FIGURE 5A** (Supplemental Figure 3). These findings do not support the hypothesis that larger-volume FMH is associated with IVT.

A complementary hypothesis is that larger IVTs are seen more frequently in the context of FMH. We examined the greatest dimension of IVTs (where reported) in patients with and without clinically detectable FMH. In patients with FMH, the mean (SD) IVT diameter was 0.06 (0.29) cm, with a median of 0 cm. In patients without FMH, the mean (SD) IVT diameter was 0.11 (0.49) cm, with a median of 0 cm. Mann-Whitney *U* test showed $P = .21$ **FIGURE 5B**.

Finally, we hypothesized that greater FMH is associated with larger IVT. The great majority of patients had neither measurable FMH nor measurable IVT. Large fractions had 1 or the other, and a small number of patients had both measurable FMH and measurable IVT **FIGURE 5C**. Interpretation of a regression in that context is challenging. We postulated a modified hypothesis that when both FMH and IVT are present, more FMH should be associated with larger IVTs. In patients with FMH and IVT, larger-volume FMH was nonsignificantly associated with decreased IVT dimension, with a slope (SD) of −0.032 (0.012) cm of IVT diameter per 1 additional cubic centimeter of FMH ($r^2 = 0.687$; $P = .08$) **FIGURE 5D**.

DISCUSSION

All IVTs examined showed evidence of maternal contribution, with 9 of 10 definitely or likely entirely maternal in origin. Testing on 1 thrombus was consistent with a mix of maternal and fetal material.

We used 3 techniques to distinguish maternal from fetal origin for thrombi. Of these, IHC for AFP and IgM seems most useful, being easily correlated with H&E and technically feasible for most clinical and research labs. This panel may be useful for clinical applications, such as workup of suspected FMH in conjunction with flow cytometry or when flow cytometry is unavailable or not performed. Of the DNA-based tests, STR seems most definitive but is less widely available. More significantly, identifying and excluding potential fetal contaminants in hypocellular thrombi is challenging. Our experience shows that most thrombi contain entrapped fetal villi. Exfoliated syncytiotrophoblasts or ingrown extravillous trophoblasts are also likely to be present. The low cellularity of IVTs and the possibility of intermixed fetal material can result in null or incorrect results. Thus, we discourage the use of STR in future studies, despite its seeming definitiveness. FISH testing may be useful in male fetuses, but it requires the FISH scorer to exclude entrapped villi or trophoblasts with minimal counterstain clues.

Our study was conducted in patients with SARS-CoV-2 infection in pregnancy. Our design was motivated by our initial finding that IVTs were more common in placentas from patients with SARS-CoV-2 in pregnancy,[11] although this finding has not been replicated in the broader literature.[14] Nonetheless, we feel that our study contributes to the understanding of this common placental lesion. Future studies will examine generalization to the broader non–SARS-CoV-2 population.

We sought to examine the generalizability of our results by testing for an association between IVTs and FMH. If fetal blood is the source of IVT, we would expect associated leaking of unclotted fetal blood into maternal circulation, detectable as FMH. We performed a series of quantitative analyses linking the volume of FMH and size of IVT to one another, which resulted in no statistically significant link between the presence and volume of FMH with IVT, the presence and size of IVT with FMH, and the volume of FMH with the presence of IVT. One potential limitation of this approach is selection bias driven by the nonrandom assignment of patients for FMH testing and placental examination. Therefore, patients suspected of FMH may not be representative of the larger population. Comparison with broader populations may offer some insight. Our historical rate of IVT is 1,601 of 17,479 (9.2%), while the IVT rate in placentas from uncomplicated pregnancies with uncomplicated deliveries not selected for clinical examination was reported as 10.3%.[11,15] These rates are similar to the flow cytometry–tested population and greater than the FMH population.

IVT could be caused by fetal hemorrhage that is too small or slow to be detected by flow cytometry—for example, from fetal capillaries or veins—while clinically significant FMH would be from large arterioles. Our findings are compatible with a version of this mechanism in which a small fetal bleed triggers clotting of maternal blood.

Alternatively, IVT could be unrelated to fetal blood. IVTs have been associated with subchorionic cysts, which contain prothrombotic proteins.[16] One of our thrombi (case 5) was associated with a small subchorionic cyst. Subchorionic cysts are low in AFP, but IgM levels have not been reported.

The final possible mechanism is direct thrombosis of maternal blood. Although contrary to published reports, this mechanism is intuitive, given that IVTs arise in a maternal compartment. The mechanism would be driven by Virchow triad: stasis, hypercoagulability, and endothelial activation. Stasis is a strong explanation for subchorionic fibrin plaques and foci of perivillous fibrin deposition, which are invariably peripheral. Conversely, IVTs seem evenly distributed between the central and peripheral placenta. Hypercoagulable states, such as antiphospholipid syndrome or SARS-CoV-2 infection, are not associated with IVT, which suggests that endothelial activation or, in the placental context, trophoblast activation is the cause. The nature and cause of that activation, if it does exist, are obscure.

In comparison with published studies on fetal hemoglobin or blood type antigens in the entrapped erythrocyte portion of the thrombus, our study focused on the composition of the proteinaceous portion.[2,3,5] The diagnosis of IVT has not been significantly revised between 1982 and 2020, but thrombi as presently diagnosed may be biologically or epistemically different.[1]

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Placental IVTs are primarily maternal in origin in the COVID-19 population, with rare cases of intermixed fetal blood. This investigation as well as future investigation into the origin of placental thrombi outside the context of COVID-19 may help elucidate the underlying pathophysiology of this process.

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