DE GRUYTER

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Mechanisms of death in structurally normal stillbirths

Abstract

Objectives: To investigate mechanisms of *in utero* death in normally formed fetuses by measuring amniotic fluid (AF) biomarkers for hypoxia (erythropoietin [EPO]), myocardial damage (cardiac troponin I [cTnI]) and brain injury (glial fibrillary acidic protein [GFAP]), correlated with risk factors for fetal death and placental histopathology.

Methods: This retrospective, observational cohort study included intrauterine deaths with transabdominal amniocentesis prior to induction of labor. Women with a normal pregnancy and an indicated amniocentesis at term were randomly selected as controls. AF was assayed for EPO, cTnI and GFAP using commercial immunoassays. Placental histopathology was reviewed, and CD15-immunohistochemistry was used. Analyte concentrations $>90^{th}$ centile for controls were considered "raised". Raised AF EPO, AF cTnI and AF GFAP concentrations were considered evidence of hypoxia, myocardial and brain injury, respectively.

Results: There were 60 cases and 60 controls. Hypoxia was present in 88% (53/60), myocardial damage in 70% (42/60) and brain injury in 45% (27/60) of fetal deaths. Hypoxic fetuses had evidence of myocardial injury, brain injury or both in 77% (41/53), 49% (26/53) and 13% (7/53) of cases, respectively. Histopathological evidence for placental dysfunction was found in 74% (43/58) of these cases.

Conclusion: Hypoxia, secondary to placental dysfunction, was found to be the mechanism of death in the majority of fetal deaths among structurally normal fetuses. Ninety-one percent of hypoxic fetal deaths sustained brain, myocardial or both brain and myocardial injuries *in utero*. Hypoxic myocardial injury was an attributable mechanism of death in 70% of the cases. Non-hypoxic cases may be caused by cardiac arrhythmia secondary to a cardiac conduction defect.

Keywords: amniotic fluid; brain injury; cardiac troponin I; CD15; delayed villous maturation; erythropoietin; fetal hypoxia; glial fibrillary acidic protein; myocardial damage; placental dysfunction.

Introduction

The rate of fetal death in the United States is 5.96 per 1000 live births [1], and it is twice as common in African-American than in Caucasian women [1, 2]. Several maternal and fetal disorders, such as maternal hypertension [3–9], diabetes mellitus [3, 10, 11], intrauterine infection [12–14] or inflammation [15–17], placental abruption [3,

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7, 18–22], placental disorders associated with maternal vascular lesions of malperfusion [23–25], massive perivillous fibrin deposition [26, 27] and small-for-gestational-age (SGA) fetuses [3, 28–31], have been associated with fetal death. However, 25%–62% of all fetal deaths are not attributed to known maternal, placental or fetal risk factors [32–38]. These "unexplained" fetal deaths comprise a progressively larger proportion of all fetal deaths as pregnancy advances [39], given that fetal deaths associated with congenital anomalies occur more frequently before the third trimester [32, 34, 35].

The term "unexplained fetal death" is an unfortunate designation applied to cases of fetal death that occur in the absence of known risk factors: it implies that the mechanism of death in cases associated with maternal, placental and fetal risk factors is known. Although our understanding as to why fetuses may die *in utero* has improved over time, this knowledge has allowed only educated guesses about possible causes [40–43].

Hypoxia, secondary to placental dysfunction, is believed to play an important role in most fetal deaths, yet evidence is indirect [25, 44–59]. The more proximal factors that lead to fetal cardiac arrest are largely conjectural. Understanding of the causes of hypoxia, and the intermediary steps between hypoxia and fetal death, may allow identification of biomarkers that could be used to predict and prevent fetal death in women at risk for this complication [23, 60–62].

There is evidence that erythropoietin (EPO), cardiac troponin I (cTnI) and glial fibrillary acidic protein (GFAP) detected in maternal serum or plasma are specific biomarkers for hypoxia [44, 63–65], myocardial damage [66–68] and brain injury [69–71], respectively, and that prenatal myocardial and brain damage occur in 20%–66% of stillborn fetuses [72–81]. Furthermore, studies have shown that EPO is of both fetal [82–85] and placental [86, 87] origin and remains elevated in amniotic fluid (AF) after the fetus dies [63]; that cTnI is specific to myocardial damage [66, 88, 89], and its concentration is elevated in hypoxic neonates [90–92]; and that serum concentrations of GFAP are elevated in newborns with hypoxic-ischemic encephalopathy (HIE), which correlate with the severity of HIE [70, 71, 81]. Although intrauterine brain injury is not generally regarded as a mechanism of death, abnormalities of the cardiovascular center in the brainstem, and of the cardiac conduction system, have been reported in 92% of a series of unexplained fetal or infant deaths [93].

With these considerations in mind, we measured the concentrations of EPO, cTnI and GFAP in samples of AF collected from 60 structurally normal fetuses that died *in utero* to determine if, and to what extent, hypoxic myocardial and brain injury were implicated in these fetal deaths. We also correlated the findings with the histopathology of the placenta and known risk factors for intrauterine death.

Materials and methods

This was a retrospective observational study of fetal deaths that occurred among pregnant women recruited into cohort studies conducted between January 2004 and January 2016 at Hutzel Women's Hospital, Detroit, MI, USA. All study participants provided written informed consent and were followed until delivery. The use of clinical data and biological specimens obtained from these women for research purposes was approved by the Institutional Review Boards of Wayne State University and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services (NICHD/NIH/DHHS).

The study group of cases comprised all fetal deaths for which an amniocentesis had been performed prior to induction of labor, and for those patients who had sufficient AF available for analysis. Women who had a normal pregnancy and an amniocentesis at term to assess fetal lung maturity prior to a scheduled cesarean section were randomly selected as controls, provided they also had sufficient AF available for analysis. We selected elective cesarean deliveries because labor is known to increase EPO concentrations in umbilical cord blood among live births [94]. Some of the women had been subjects in prior studies.

Clinical definitions

The following definitions were used:
1. **Gestational age** was determined by the last menstrual period and confirmed by ultrasound examination or by ultrasound examination alone if the sonographic determination of gestational age was inconsistent with menstrual dating by more than 1 week in the first trimester [95];
2. **Fetal death or stillbirth** death of the fetus diagnosed after 20 weeks of gestation confirmed by ultrasound examination prior to delivery [96];
3. **Hypertensive disorders of pregnancy** included four categories: preeclampsia, chronic hypertension, chronic hypertension with superimposed preeclampsia and gestational hypertension, defined as follows:
   – Preeclampsia: presence of new-onset systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg on at least two occasions, 4 h to 1 week apart, after 20 weeks of gestation, and of proteinuria ≥300 mg in a 24-h urine collection or one dipstick with ≥1+ for protein [97];
   – Chronic hypertension: maternal hypertension that predates pregnancy [98];
   – Chronic hypertension with superimposed preeclampsia: presence of chronic hypertension in association with preeclampsia [98];
   – Gestational hypertension: new-onset systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg on at least two occasions, 4 h to 1 week apart, after 20 weeks of gestation in the absence of systemic maternal organ damage (proteinuria, thrombocytopenia,

elevated liver enzymes, elevated creatinine, pulmonary edema and/or cerebral or visual disturbances) [98];

4. **A small-for-gestational-age (SGA)** neonate was defined as having a birthweight <10th percentile for gestational age at delivery according to a US reference population [99];

5. **An appropriate-for-gestational-age (AGA)** neonate was defined as having a birthweight between the 10th and 90th percentiles for gestational age at delivery according to a US reference population [99];

6. **Diabetes mellitus** in pregnancy included two categories: pregestational and gestational, defined as follows [100]:
   – Pre-gestational diabetes mellitus: diabetes mellitus diagnosed prior to pregnancy based on a fasting blood glucose >126 mg/dL or A1C >6.5%;
   – Gestational diabetes mellitus diagnosed in a pregnant woman without a prior history of diabetes and with at least two values greater than or equal to the following on a 3-h, 100 g oral glucose tolerance test performed between 24 and 28 weeks of gestation: fasting ≥95 mg/dL, 1-h ≥180 mg/dL, 2-h ≥155 mg/dL and 3-h ≥140 mg/dL;

7. **Placental abruption** was diagnosed clinically by the presence of vaginal bleeding, abdominal pain and a retroplacental blood clot not associated with vasa previa, placenta previa or uterine rupture [101];

8. **Placenta previa** was defined as a placenta that overlies or is proximate to the internal cervical os based on transvaginal ultrasound examination [102];

9. **Intra-amniotic inflammation** was diagnosed when the interleukin (IL)-6 concentration in the AF was ≥2.6 ng/mL [103–112];

10. **Intra-uterine infection** was defined by the presence of intra-amniotic infection or cytomegalovirus (CMV) placentitis. Intra-amniotic infection was defined as a positive AF culture for microorganisms using cultivation techniques [113–116]. CMV placentitis was defined by the presence of focal segmental chronic lymphoplasmacytic villitis, hemosiderin deposition and an occasional presence of owl-like CMV inclusions [117]. The latter was subsequently confirmed by immunohistochemistry for CMV [118];

11. **Control group** comprised women who had no medical, obstetrical or surgical complications, who were not in labor and who had an elective cesarean delivery of a structurally normal singleton fetus at 37–42 weeks of gestation, whose birthweight was between the 10th and 90th percentiles for gestational age [99].

Amniotic fluid samples

AF was collected by transabdominal amniocentesis for medical indications, such as karyotype studies or fetal lung maturity, or to rule out intra-amniotic infection, using standard cultivation techniques for aerobic and anaerobic microorganisms, genital mycoplasmas and fungi. Samples of AF not required for clinical purposes were centrifuged to remove cellular and particulate matter. Aliquots of AF were stored at −70 °C until analysis.

Analyte assays

Assay for erythropoietin: Concentrations of EPO in AF were determined with a commercially available specific immunoassay (American Laboratory Products Company [ALPCO], Salem, NH, USA). The sensitivity of this assay was 1.8 mIU/mL, and the coefficients of variation for intra- and inter-assays were 6.1% and 9.2%, respectively. All samples were assayed in duplicate.

Assay for cardiac troponin I: Concentrations of cTnI in AF were determined with a commercially available specific immunoassay (LifeSpan BioSciences, Seattle, WA, USA). The sensitivity of this assay was 26.89 pg/mL, and the coefficients of variation for intra- and inter-assays were 6.1% and 8.1%, respectively. All samples were assayed in duplicate.

Assay for glial fibrillary acid protein: Concentrations of GFAP in AF were determined using an enzyme-linked immunosorbent assay (Cloud Clone, Katy, TX, USA). The sensitivity of this assay was 0.19 ng/mL, and the coefficients of variation for intra- and inter-assays were 8.2% and 15.1%, respectively. All samples were assayed in duplicate.

Placental histopathological analysis

The placental disc of each patient was randomly sampled using a random sequence generator and software designed for this purpose as previously described [119]. Slides stained with hematoxylin and eosin as well as cytokeratin-7 from cases and controls were reviewed by a placental pathologist (SJ) blinded to the pregnancy outcome and any previous histopathological diagnoses. The nomenclature adopted by the Amsterdam Placental Workshop Group was used to describe the vaso-occlusive lesions and villous maturational defects in these cases [120]. Slides were also stained for the marker CD15; "immature" CD15-positive endothelium is a diagnostic marker of persisting villous immaturity and chronic placental dysfunction [121]. The level of CD15 expression was considered as pathological placental villous immaturity in both macrovasculature (chorionic plate and stem vessels) and microvasculature (terminal villi) with ≥50% villi exhibiting positive expression.

Accelerated villous maturation was defined as premature maturation of terminal chorionic villi (resembling term villi at more than 38 weeks of gestation) with conspicuous syncytial knotting in preterm placentas [122]. Delayed villous maturation is a developmental placental abnormality defined by a monotonous villous population (at least 10 such villi) with centrally placed capillaries and decreased vasculosyncytial membranes, recapitulating the histology in early pregnancy [120, 123]. The presence of stromal villous-vascular karyorrhexis, avascular villi and stem villous vessel obliteration was noted but considered as post-mortem changes secondary to cessation of the fetal circulation, and used only to estimate the time of death prior to delivery, as previously described [124].

In cases for which light microscopy and hematoxylin and eosin-stained sections displayed focal segmental chronic lymphoplasmacytic villitis, hemosiderin deposition and the occasional presence of owl-like inclusions in the stromal cells suggestive of CMV placentitis [117], the diagnosis of CMV placentitis was subsequently confirmed by immunohistochemistry [118]. Cases of chronic villitis that did not exhibit lymphoplasmacytes in the villi, nor had any evident inclusions or hemosiderin deposition, were not considered as CMV villitis. Immunohistochemistry was performed in cases with lymphoplasmacytic villitis by the streptavidin-biotin complex method using a monoclonal anti-CMV antibody (Leica BOND, Buffalo Grove, IL, USA).

Statistical analysis

We analyzed the results as multivariate discrete data. The analyte concentrations below the limit of detection were replaced by 99% of the minimum observed value for the analyte. The analytes were then categorized at the 90th centile for the controls, and values above the 90th centile were considered "raised". Fetal deaths were dichotomized based on the presence of known risk factors (i.e. placental abruption, chronic hypertension, preeclampsia, gestational hypertension, gestational diabetes mellitus, pre-gestational diabetes mellitus, SGA neonate, intra-amniotic infection and placenta previa) to determine whether analyte concentrations differed between what have traditionally been labeled "explained" and "unexplained" deaths. Because the proportion of "unexplained" fetal deaths has been found to increase with gestational age [32, 34, 35, 125, 126], we also examined the effect of gestational age on analyte concentrations and dichotomized gestational age arbitrarily at 28 weeks of gestation so that this analysis could be as close as possible to equal numbers in the two gestational age categories.

We used the statistical package R to fit hierarchically nested log linear models to multi-way contingency tables of the data to analyze associations, partial and conditional, between the three analytes, presence of risk factors and gestational age. The relationship between the variables was first examined by fitting a three-way contingency table to the three analytes dichotomized at the 90th centile for the controls. We next examined the effects of risk factors and gestational age individually in a model containing informative analytes and retained significant terms to arrive at a final model.

A two-tailed Fisher's exact test was used to test 2×2 marginal tables, and the Mann-Whitney U-test was used to compare median analyte concentrations between the different causes of death and placental perfusion categories. Values for statistics below the 5% significance level were considered statistically significant.

Results

Of 279 fetal deaths in the database, 45 had congenital anomalies. The proportion of fetal deaths associated with congenital anomalies was significantly higher before, rather than after, 28 weeks of gestation (20% [27/134] vs. 11.6% [18/155], P = 0.03). Cases with congenital anomalies were excluded from further analysis because AF was available for only two fetal deaths associated with congenital malformations.

Of the 234 cases of structurally normal singleton fetuses that died *in utero*, 119 had undergone amniocentesis, and AF was available for analysis in 60 cases. Among these, clinical abruption occurred in 10 (16.7%) cases, diabetes mellitus in 11 (18.3%; pre-gestational diabetes in five, gestational diabetes in six cases), hypertensive disorders of pregnancy in 16 (26.7%; gestational hypertension in six, chronic hypertension in four, preeclampsia in four, chronic hypertension with preeclampsia in two cases), SGA fetuses in 18 (31%, 18/58), intra-uterine infection in six (10%; intra-amniotic infection in four and CMV-chronic villitis in two cases) and one case with placenta previa (1.7%). Fifteen (25%) patients had more than one of these risk factors, and 15 (25%) had no risk factors (Figure 1). Birthweight was registered in 58 cases and autopsy reports were available for 37 cases. None of the fetal deaths was hydropic.

Sixty controls were randomly selected from 535 patients who had an amniocentesis at term when not in labor. There were no significant differences in median maternal age, body mass index, frequency of smoking status or self-reported ethnicity (African American) among the 60 cases and 60 controls. Cases had significant lower median gestational ages at amniocentesis, at delivery, as well as lower birthweights, and a higher frequency of nulliparity than controls (Table 1). The estimated time interval between death and delivery in cases estimated from placental histopathology is shown in Table 2. The percentile distribution of birthweight between cases and
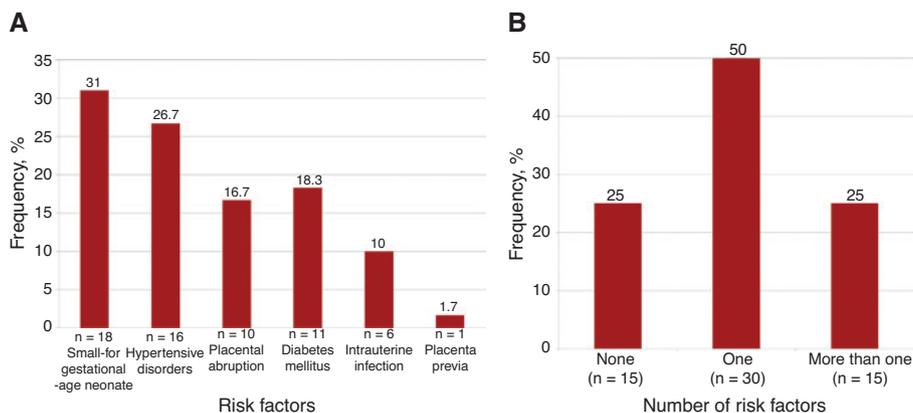

Figure 1: Frequency of risk factors in cases of fetal death.
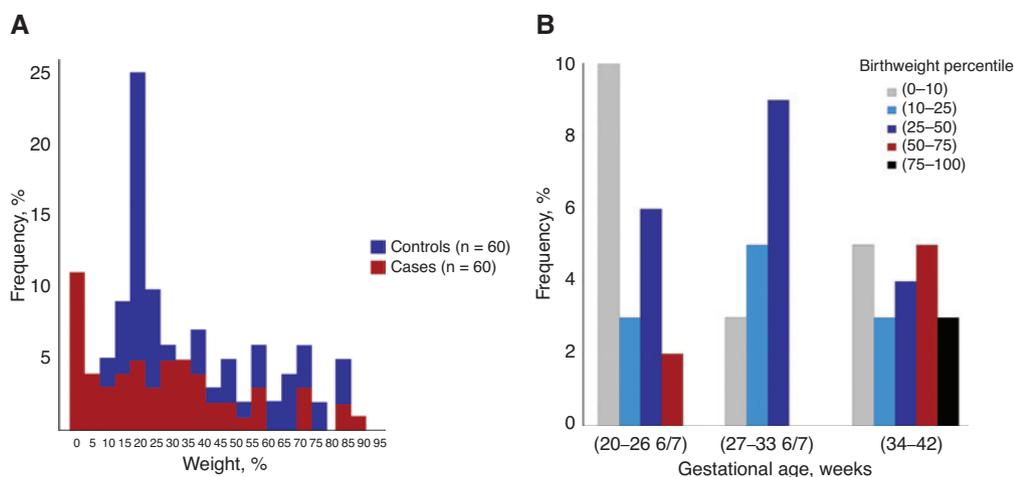(A) Frequency of different risk factors. (B) Frequency of none, one or more than one risk factor.

Table 1: Demographic and clinical characteristics of the study group.

Characteristics	Cases of fetal death (n = 60)	Controls (n = 60)	P-value[a]
Maternal age, years	25.0 [16.0–42.0]	26.0 [17.0–42.0]	0.77
Body mass index, kg/m[2,b]	28.3 [15.5–47.9]	30.1 [15.5–55.8]	0.38
Smoking status	19 (31.7)	13 (21.7)	0.30
Nulliparity	22 (35.5)	0	<0.001
African-American ethnicity	50 (83.3)	50 (83.3)	1.0
Gestational age at amniocentesis, weeks	29.9 [20.1–40.9]	39.1 [37.0–41.4]	<0.001
Gestational age at delivery, weeks	30.0 [20.3–41.3]	39.1 [37.0–41.4]	<0.001
Birthweight, g	1265.0 [91.0–3885.0]	3017.5 [2670.0–3970.0]	<0.001
Birthweight-to-placental weight ratio	3.94 [0.6–9.66]	5.72 [3.4–7.6]	<0.001

Continuous variables are expressed as median [range], and categorical variables are expressed as number (percentage). [a]Kruskal-Wallis test for continuous variables and a Fisher's exact test for categorical variables. [b]Body mass index (BMI) was calculated as follows: BMI, (maternal pre-pregnancy weight [kg]/maternal height [m][2]).

Table 2: Pattern of raised amniotic fluid concentrations of cardiac troponin I (cTnI) and glial fibrillary acidic protein (GFAP) according to the estimated death-to-delivery time interval in cases of fetal death.

Death-to-delivery time interval	Not raised cTnI or GFAP (n = 10)	Raised cTnI only (n = 21)	Raised GFAP only (n = 8)	Raised cTnI and GFAP (n = 19)
≤6 h	0	0	0	1
6–24 h	3	1	1	2
≥48 h	7	9	4	12
2–7 days	0	6	3	3
7–14 days	0	5	0	1

Figure 2: Percentile distribution of birthweight in cases of fetal death and controls.
(A) There was no significant difference in the percentile distribution of birthweight between cases of fetal death and controls (P = 0.05). (B) Distribution of birthweight centiles by gestational age for cases of fetal deaths. There was a significant correlation between birthweight centile and gestational age (Spearman's ρ = 0.3, P = 0.03): earlier fetal deaths had lower birthweight centiles than later fetal deaths.

controls is shown in Figure 2A; the difference between the groups was not statistically significant (P = 0.05). The distribution of birthweight centiles by gestational age for cases is shown in Figure 2B; there was a significant correlation between birthweight centile and gestational age (Spearman's ρ = 0.3, P = 0.034): earlier fetal deaths had lower birthweight centiles than later fetal deaths. There was a significant difference between cases and controls in the birthweight-to-placenta weight ratio (3.94 vs. 5.72, respectively, P < 0.001; Table 1).
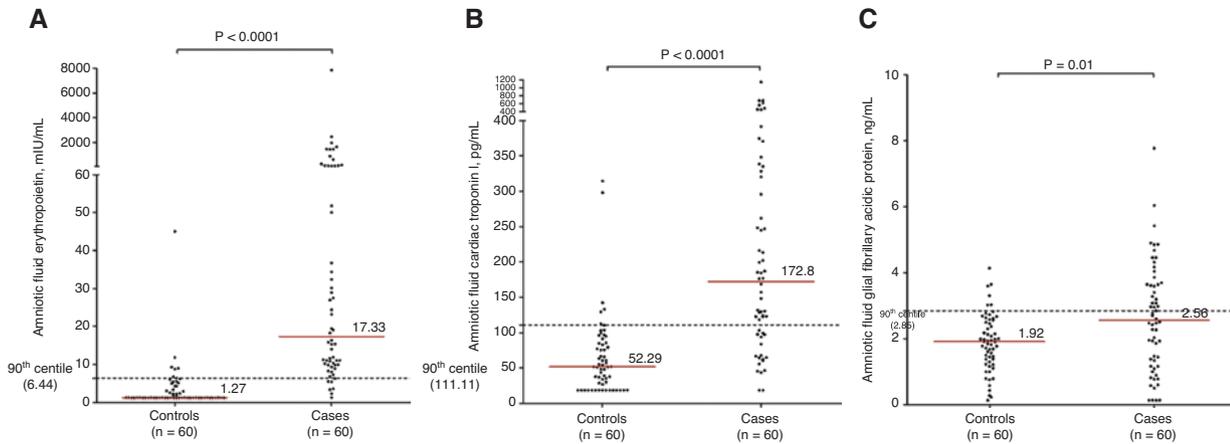
Figure 3: Amniotic fluid concentration of erythropoietin (EPO), cardiac troponin I (cTnI) and glial fibrillary acidic protein (GFAP) in the controls and cases of fetal death.
(A) The median amniotic fluid concentration of EPO in fetal death was significantly higher compared to controls (median [interquartile range]: 17.33 mIU/mL [9.89–77.08] vs. 1.27 mIU/mL [1.27–4.03], P < 0.0001). The concentration at the 90th centile in amniotic fluid EPO for controls was 6.44 mIU/mL (dashed line). (B) The median amniotic fluid concentration of cTnI in fetal death was significantly higher compared to controls (median [interquartile range]: 172.8 pg/mL [97.51–326.30] vs. 52.29 pg/mL [21.07–85.60], P < 0.0001). The concentration at the 90th centile in amniotic fluid of cTnI for controls was 111.11 pg/mL (dashed line). (C) The median amniotic fluid concentration of cTnI in fetal death was significantly higher compared to controls (median [interquartile range]: 2.56 ng/mL [1.21–3.65] vs. 1.92 ng/mL [1.32–2.56], P = 0.01). The concentration at the 90th centile in amniotic fluid of GFAP for controls was 2.84 ng/mL (dashed line).

Analyte concentrations

Distribution of the analyte concentrations in the cases and controls is shown for each analyte in Figure 3A–C. AF EPO was below the level of detection in 60% (36/60) of controls and in one (1.7%) case; AF cTnI was below the level of detection in 25% (15/60) of controls and two (3.3%) cases; and AF GFAP was below the level of detection in one (1.7%) control and four (6.7%) cases.

Median AF concentrations of EPO, cTnI and GFAP were significantly higher for cases than controls (EPO: 17.33 mIU/mL vs. 1.27 mIU/mL, P < 0.001; cTnI: 172.8 pg/mL vs. 52.29 pg/mL, P < 0.001; GFAP: 2.56 ng/mL vs. 1.92 ng/mL, P = 0.01) (Figure 3A–C). Median AF concentrations of EPO were significantly higher among cases with risk factors than cases without risk factors (cases with risk factors: 23.19 mIU/mL vs. cases without risk factors 10.89 mIU/mL, P = 0.03), but median AF cTnI and GFAP concentrations were not significantly different between cases that had risk factors and those that did not (AF cTnI: cases with risk factors: 177.08 pg/mL vs. cases without risk factors 124.48 pg/mL, P = 0.29; AF GFAP: cases with risk factors: 2.71 ng/mL vs. cases without risk factors: 2.48 ng/mL, P = 0.53). There was no significant association between the presence of risk factors and gestational age (dichotomized ≥28 weeks of gestation at amniocentesis).

The percentile distribution of the analytes in AF is shown in Table 3. The 90th centile concentration of AF EPO,

Table 3: Percentile distribution of amniotic fluid concentrations of erythropoietin, cardiac troponin I and glial fibrillary acidic protein in cases of fetal death.

Percentile	Erythropoietin (n = 60)	Cardiac troponin I (n = 60)	Glial fibrillary acidic protein (n = 60)
>95th	48	33	19
90th–95th	5	9	8
80th–90th	3	4	2
70th–80th	2	2	5
60th–70th	1	5	3
50th–60th	0	2	3
≤50th	1	5	20

cTnI and GFAP in controls was 6.44 mIU/mL, 111.11 pg/mL and 2.85 ng/mL, respectively. AF EPO, cTnI and GFAP concentrations were raised, i.e. above the 90th centile for the controls, in 88% (53/60), 70% (42/60) and 45% (27/60) of fetal deaths, respectively (Table 3). The proportion of cases in which AF EPO, cTnI or GFAP was raised was not significantly different between cases that had or did not have risk factors for fetal death.

The relationship among the three analytes, dichotomized as above or equal or below the 90th centiles for the controls, is shown in Table 4. No AF analyte was raised in five (8.3%) cases. Only AF EPO was raised in five (8.3%) cases; only AF cTnI or only AF GFAP was raised in one case each (1.7%). It should be noted that in these two latter cases,

Table 4: Distribution of amniotic fluid concentrations of erythropoietin (EPO), cardiac troponin I (cTnI) and glial fibrillary acidic protein (GFAP) according to the presence or absence of EPO raised (>90th percentile for controls) in cases of fetal death.

cTnI	EPO >90th percentile			cTnI	EPO ≤90th percentile		
	GFAP		Total (n=53)		GFAP		Total (n=7)
	>90th percentile (n=26)	≤90th percentile (n=27)			>90th percentile (n=1)	≤90th percentile (n=6)	
>90th percentile	19	22	41	>90th percentile	0	1	1
≤90th percentile	7	5	12	≤90th percentile	1	5	6

Table 5: Birthweight below the 50th percentile, small-for-gestational-age neonate, birthweight-to-placenta weight and mean amniotic fluid concentrations of erythropoietin (EPO), cardiac troponin I (cTnI) and glial fibrillary acidic protein (GFAP) according to the pattern of raised amniotic fluid concentration of EPO in cases of fetal death.

	Raised EPO only (n=5)	Raised EPO and cTnI (n=23)	Raised EPO and GFAP (n=8)	Raised EPO, cTnI and GFAP (n=19)	P-value[a]
Birthweight <50th centile	4	18/21	6	15	0.87
Small-for-gestational-age neonate	1	10/21	2	5	0.47
Birthweight-to-placenta-weight ratio	4.95	3.75	4.35	3.68	0.40
Mean amniotic fluid concentration					
EPO, mIU/mL	27.24	226.93	261.62	724.30	0.41
cTnI, pg/mL	67.27	232.48	70.93	380.38	<0.001
GFAP, ng/mL	1.1	1.65	3.66	4.24	<0.001

[a]Kruskal-Wallis test for continuous variables and exact tests for categorical variables.

AF EPO was above the 80th and 70th centiles, respectively. Therefore, these two cases were considered as cases with hypoxic cardiac and brain injury occurring at slightly lower concentrations of EPO than the criteria used in this study.

Thus, when including these two cases (raised cTnI and EPO >80th and raised GFAP and EPO >70th centiles), the concentration of GFAP in hypoxic fetal deaths was raised in the same proportion of cases regardless of whether the concentration of cTnI was or was not raised (45.2% [19/42] vs. 44.4% [8/13], P=0.36). In addition, there was no significant difference in median GFAP concentration between these two groups of fetal deaths (2.65 ng/mL vs. 2.98 ng/mL, P=0.95).

There was no association between cases with raised AF cTnI and those with raised AF GFAP concentrations; AF GFAP was raised in 45.2% (19/42) of fetal deaths with raised AF cTnI and in 44.4% (8/18) of those that did not have raised AF cTnI (P=1). The difference between the proportion of SGA and AGA neonates with fetal death that had raised AF cTnI concentrations did not reach statistical significance (15/40 [37.5%] vs. 3/18 [16.7%], P=0.14; data not shown).

The mean AF concentrations of EPO, cTnI and GFAP, and the proportion of fetal deaths with birthweights below the 50th centile for gestational age, and of SGA neonates, as well as the birthweight-to-placenta-weight ratio for each raised AF EPO combination are shown in Table 5. The mean AF cTnI concentration was significantly higher among cases with raised concentration for all analytes (AF EPO, cTnI and GFAP) than among cases that had AF with either raised cTnI or GFAP or raised EPO alone (P<0.001). Furthermore, the mean AF cTnI concentration in cases with raised AF EPO, cTnI and GFAP was higher than in those for which AF EPO and cTnI were raised (380.38 pg/mL vs. 232.48 pg/mL, P=0.03).

Model selection

The three AF analytes were not mutually independent (Pearson's χ^2=18.124, df=4, P=0.001). EPO was conditionally dependent on cTnI (P=0.001) – i.e. the interaction among the biomarkers was statistically significant – but the interactions between GFAP and cTnI, and between GFAP and EPO, were not significant (P=0.96, and P=0.07, respectively). When entered into the model containing the main effects of the three analytes and the interaction between EPO and cTnI, the effects of gestational age dichotomized at 28 weeks of gestation and the presence of prognostic factors were not statistically significant.

Histopathology

Placental histology was available for 58 of 60 cases and all 60 controls. The frequency of placental lesions consistent with maternal and fetal vascular malperfusion and of placental inflammatory lesions was significantly higher in cases compared to controls (Table 6).

Two cases with missing pathology slides had raised AF EPO and cTnI concentrations: one patient had late-onset preeclampsia with an SGA neonate and delivered at 37.1 weeks; the other patient had placental abruption and delivered at 22.4 weeks.

Sections from normal term placentas may display immature villi, and this physiological immaturity occurs in less than 10% of cases [127]. Therefore, to establish the pathogenicity of these immature villi, immunohistochemistry with CD15 was performed in any placental section that showed monotonous villous population (defined as at least 10 such villi) with centrally placed capillaries and decreased vasculosyncytial membranes, recapitulating the histology in early pregnancy. CD15 immunohistochemistry was used in 53 samples, including 40 controls and 13 cases. In the control group, three of the 40 samples examined showed immunohistochemistry consistent with pathological delayed villous maturation, while 12 of the 13 cases examined displayed this pathological pattern.

Of 53 hypoxic fetal deaths (EPO >90th percentile), placental histology was available for 51 cases. Among these 51 hypoxic fetal deaths (EPO >90th percentile), vaso-occlusive lesions, accelerated or delayed maturational villous defects, retroplacental hemorrhage or fetal vascular lesions were present in 38 (74.5%) cases. There were 12 cases of fetal deaths with delayed villous maturation: 11 (92%) had raised EPO and one case (8%) was associated with placental abruption. Four of the five fetal deaths in which only the AF EPO concentration was raised had delayed or accelerated villous maturation in the placenta, and the fifth had hypervascular villi, a characteristic response to hypoxia. Three of the five fetal deaths not associated with any raised analytes in AF had histological evidence of abruption consisting of retroplacental and/or intravillous hemorrhage, one had a massive subchorial hematoma (a Breus' mole) and one displayed delayed villous maturation. Therefore, a histopathological explanation for placental dysfunction was found in 74% (43/58) of cases.

Eleven fetal deaths showed evidence of acute histologic chorioamnionitis: four had intermediate (stage 2) disease with intra-amniotic inflammation diagnosed in two, and seven cases were mild (stage 1), and six of them had intra-amniotic inflammation [128]. AF cultures were negative for nine cases of histological chorioamnionitis, except for one case with candida placentitis, which was culture positive for *Candida* spp. and had intra-amniotic inflammation, and another with coagulase-negative *Staphylococcus* species without intra-amniotic inflammation.

Two 22-week-old fetal deaths displayed chronic villitis due to CMV infection with raised AF EPO and cTnI concentrations, as well as intra-amniotic inflammation.

The proportion of cases that had no significant placental pathology was not significantly different between the various AF analyte groups, nor was there a difference between the groups in the type of pathological lesions present.

Table 6: Placental histopathological findings of the study group.

Histologic lesions	Cases of fetal death (n=58)	Controls (n=60)	q-Value[a]
Acute inflammatory lesions	11 (19%)	2 (3.3%)	0.01
Chronic inflammatory lesions	36 (62.1%)	10 (16.7%)	<0.001
Maternal vascular malperfusion	41 (70.7%)	21 (35%)	<0.001
Vascular	35 (60.3%)	17 (28.3%)	<0.01
Villous lesions	19 (32.8%)	7 (11.7%)	0.01
Retroplacental hemorrhage	13 (22.4%)	0 (0%)	<0.001
Infarction	10 (17.2%)	1 (1.7%)	<0.01
Accelerated villous maturation	9 (15.5%)	0 (0%)	<0.01
Distal villous hypoplasia	2 (3.4%)	0 (0%)	0.24
Decidual arteriopathy	23 (39.7%)	13 (21.7%)	<0.05
Fetal vascular malperfusion	48 (82.8%)	20 (33.3%)	<0.001
Vascular	40 (69%)	9 (15%)	<0.001
Villous	36 (62.1%)	12 (20%)	<0.001
Delayed villous maturation	12 (20.7%)	3 (5%)	<0.02

[a]q-Values are P-values adjusted for multiple comparisons using Benjamini-Hochberg procedure.

Discussion

Principal findings of the study

The study indicated that 88% (53/60) of fetal deaths were hypoxic; that 91% (48/53) of hypoxic fetal deaths had sustained brain, myocardial or both brain and myocardial injuries *in utero*; and that circulatory failure and cardiac arrest, secondary to hypoxic myocardial damage, were the mechanisms of death in 70% (42/60) of cases.

Given that we considered the two isolated cases, in which only the concentration of cTnI or GFAP was raised, as instances of hypoxic cardiac and brain injury occurring at slightly lower concentrations of EPO than the criteria used to conduct this study (>80th centile and >70th centile, respectively), we may conclude that: (1) 92% (55/60) of fetal deaths were hypoxic; (2) 91% (50/55) of hypoxic fetal deaths sustained myocardial, brain or both myocardial and brain injuries *in utero*; (3) myocardial injuries alone occurred significantly more frequently than brain injury alone (23/50 vs. 8/50, P < 0.01), but myocardial and brain injuries occurred independent of each other; and (4) vaso-occlusive lesions, villous maturational defects, histologic evidence of abruption or fetal vascular lesions were present in 74.5% (38/51) of hypoxic fetal deaths.

Difficulty in determining the cause of fetal death

The use of objective data to assess the cause of fetal death, including clinical information, placental examination and fetal autopsy, allows for the identification of potential etiologies of fetal death in 40% of cases [42]. Given that the risk factors for stillbirth are also present in live born neonates, one cannot attribute the presence of these risk factors as direct causes of fetal demise [40]. Instead, potential etiologies of fetal death should be classified as a probable cause, a possible cause or a present condition based on the best available scientific evidence [41]. The Stillbirth Collaborative Research Network found that 31% (161/512) of fetal deaths in 59 tertiary care and community hospitals from 2006 to 2008 had more than one probable or possible cause of death [59]. In the current study, we found that 25% (15/60) of cases with fetal death had more than one risk factor. The result of these two studies supports the notion that the cause of death is multifactorial in at least one-quarter of cases of fetal death. Because multiple conditions may contribute to a stillbirth, the practice of recording the chain of events that led to death, rather than the single most probable cause of death, is recommended [40, 129, 130].

In the current study, 75% (45/60) of fetal deaths had a known risk factor, and these fetuses had significantly increased median concentrations of EPO than fetuses without a risk factor. The clinical implication of this finding is that pregnancies with risk factors for fetal death are more likely to present placental dysfunction due to fetal hypoxia in the index pregnancy. Previous studies reported that pregnancies with risk factors for fetal death, such as diabetes mellitus [131], hypertensive disorder of pregnancies [64] and fetal growth restriction [132] have increased AF/umbilical cord concentration of EPO as compared to normal pregnancies without these risk factors. Furthermore, Lamont et al. [133] reported that, compared to women who had a live birth in their first pregnancy, those who experienced a stillbirth were almost five times more likely to experience a fetal death in their second pregnancy. In addition, a recent systematic review and meta-analysis has demonstrated that neonates of mothers who had a previous preterm birth or an SGA neonate are more likely to be stillborn; the risk of stillbirth in the following pregnancy is doubled if the previous neonate was preterm and SGA, and neonates of mothers who had a previous fetal death were more likely to be preterm or SGA [134]. Collectively, these findings are consistent with the concept that the outcome of previous pregnancies influences the risk of a poor outcome in the subsequent pregnancy [133, 134].

Birth occurring before 28 weeks of gestation appear to be strongly associated with an intra-amniotic infection, whereas late preterm births are less likely to have an associated intrauterine infection [135, 136]. Indeed, our study showed that 66.7% (4/6) of cases with intrauterine infection occurred before 28 weeks of gestation. In addition, we found that CMV infection accounted for 33.3% of intrauterine infection in cases with fetal death and was present in 3% of cases with fetal death. An Australian study showed that 9% of blood samples taken from fetal deaths by cardiac puncture were polymerase chain reaction (PCR) positive for CMV [137]. A study from Greece, using PCR, showed significantly increased levels of CMV (16%) in the placentas of fetal deaths compared to controls (3%) [138]. As CMV is the most common cause of congenital infection associated with fetal growth restriction and central nervous system damage, and as a recent meta-analysis has demonstrated that maternal CMV infection increases the risk of spontaneous abortion and fetal death [139], we suggest that every placenta and fetus submitted for autopsy should be examined for CMV infection.

The usefulness of placental histopathology in evaluating fetal death

Normal placental maturation involves transition of immature intermediate villi in the first and second trimesters of pregnancy to mature intermediate and terminal villi, defined by small-caliber (40–100 μm), minimal stroma and abundant syncytio-capillary membrane formation in the third trimester [140]. This transition results from an increasing number of peripherally placed, thin-walled vascular sinusoids and formation of syncytio-capillary membranes in villi, thereby shortening the distance between the maternal and fetal circulations and increasing the efficiency of gas exchange across the placenta [123, 141]. In contrast, delayed villous maturation involves a higher percentage of centrally placed villous vessels, a reduced number of syncytio-capillary membranes and an increase in the distance between the maternal and fetal circulations [121, 142, 143]. A reduced number of syncytio-capillary membranes prevents maternal supply from meeting fetal demand for oxygen and nutrients, increasing the risk of antenatal fetal hypoxia [121, 142], unexplained fetal death [121, 142] and increased neonatal mortality [121, 142].

Although an assessment of the presence of delayed placental villous maturation is subjective, and the concordance for this lesion is poor among pathologists and subject to much inter-observer variability [144], the subjectivity and variability can be enhanced by the use of CD15 immunohistochemistry, a diagnostic marker of persisting villous immaturity and chronic placental dysfunction. The level of CD15 expression in the macro- and microvasculature reflects the degree of pathological placental villous immaturity [121]. The usefulness of placental pathology for identification of potential causes of fetal death in the current study was enhanced by the use of an immunohistochemistry marker of CD15 to diagnose delayed villous maturation as reported by Seidmann et al. [121, 145–147]. Indeed, the contribution of placental pathology for the identification of potential causes of fetal death in the current study was higher (74%) compared to that reported by Page et al. [148] who also reported that the most useful test in the diagnostic evaluation of fetal death is placental pathology (65% of the cases). Therefore, we propose that the evaluation of late fetal death should include placental pathology with the use of a CD15 immunohistochemistry marker to identify delayed villous immaturity as a marker of fetal hypoxia. Indeed, all 12 cases of fetal deaths with CD15-confirmed delayed villous maturation were hypoxic: 11 (92%) had raised AF EPO concentration, and one case

(8%), associated with placental abruption, did not have raised AF EPO concentration as fetal death from an acute event prevents the elevation of AF EPO concentration [63]. Raised AF EPO concentration indicates chronic fetal hypoxia [65].

Placental dysfunction

Decreased placental function, due to either abnormal placental development, placental damage or both, leads to decreased blood flood, oxygen and nutrient transfer to the fetus [149]. The results of the current study establish that placental dysfunction was the underlying cause of death in more than 90% of cases of fetal deaths. Consistent with this conclusion, birthweight was below the 50th centile in 81% (47/58) of the cases for which it was recorded, and 31% (18/58) of fetal deaths were SGA neonates. Although the apparent birthweight centile may have been affected by the death-to-delivery time interval [150], the fact that the birthweight-to-placenta-weight ratio for the cases was significantly lower than that for the controls (3.94 vs. 5.72, $P < 0.001$) makes it unlikely that this was a significant factor affecting the birthweight centile. Placental pathology "explained" 74% (43/58) of these fetal deaths, and the proportion may have been higher, but for the inability to differentiate post-mortem from ante-mortem histologic changes of fetal vascular malperfusion in the chorionic plate, stem villous and terminal villous vessels which, therefore, were excluded.

Fetal response to acute and chronic hypoxia

The likely mechanisms of death can be inferred from the fetal physiological responses to acute and chronic hypoxia, which have been investigated extensively *in vivo* in chronically instrumented, unanesthetized fetal lambs. Because fetal arterial partial pressure of oxygen (PO_2) is only about 25% of maternal arterial PO_2, comparable to the PO_2 on Mount Everest [151], and fetal oxygen supply is limited by uterine and umbilical blood flow, the fetus is vulnerable to sudden interruptions of its oxygen supply, and has thus evolved cardiovascular and neuro-endocrinal mechanisms to maintain oxygen supply to its vital organs during episodes of acute hypoxic episodes and to survive in a chronically hypoxic intrauterine environment. The net effects of these compensatory mechanisms are to redistribute the fetal cardiac output away from peripheral organs and the carcass to the brain, myocardium and

adrenal glands ("centralization of the circulation"), and to reduce oxygen consumption [152, 153].

Mechanisms for fetal blood redistribution

Redistribution of blood to vital organs is mediated partly by vasoconstriction and increased peripheral vascular resistance, and partly by increased perfusion of vital organs, which is at first passive and pressure dependent, but is maintained by active vasodilation and increased conductance in the cerebral, myocardial, adrenal and umbilical circulations [154, 155]. These and other adaptations allow the fetus to withstand moderate reductions in PO_2 and to maintain cerebral and myocardial oxygen consumption at normal values and centralization of its circulation more or less indefinitely. However, this comes at the cost of reducing fetal growth [156–163], and reprogramming tissue development [164], which predisposes the fetus to develop a variety of diseases in adult life [164–166].

The increase in peripheral vascular resistance in response to acute hypoxia involves cardiovascular, neuroendocrine and local vascular mechanisms. The cardiovascular response consists of transient fetal bradycardia, systemic hypertension and peripheral vasoconstriction triggered by carotid sinus chemoreceptors that transmit afferent impulses to the cardiovascular center in the medulla, which then sends parasympathetic and α- and β-adrenergic efferent discharges to the heart, peripheral vasculature and adrenal glands [154]. Blood flow to the brain, heart and adrenal glands increases, as do plasma concentrations of catecholamines, adrenocorticotropic hormone (ACTH) and cortisol, and the cortisol response to ACTH [154, 167, 168], but cardiac output and umbilical blood flow are unaltered unless there is acidosis, in which case both umbilical blood flow and cardiac output are reduced [152, 169].

If hypoxia continues, redistribution of blood flow to vital organs is maintained by continued peripheral vasoconstriction mediated by the circulating vasoconstrictors norepinephrine, arginine vasopressin, neuropeptide Y and angiotensin II [155], and also by the local balance between vasoconstriction and vasodilation in peripheral vascular beds [170]. Hypoxia increases both nitric oxide synthesis by vascular endothelium by a mechanism involving calcitonin gene-related peptide (cGRP) [170, 171] and the production of oxygen free radicals that quench nitrogen oxide [172, 173]. The ratio of oxygen free radicals to nitric oxide determines the balance between vasoconstriction and vasodilation. In the peripheral vascular beds, this balance usually favors vasoconstriction, but this balance

can change in fetuses compromised by chronic hypoxia [170–173].

Nitric oxide

Chronic hypoxia increases nitric oxide production and attenuates the cardiovascular response to acute hypoxia by diminishing the vasoconstrictor response [174]. Baseline cerebral and femoral blood flow are higher, but cerebral blood flow does not increase, and femoral blood flow increases less during acute hypoxia [175]. These findings have been interpreted to indicate a shift in the compensatory mechanism to chronic hypoxia from increased peripheral vasoconstriction to enhanced central vasodilation [175]. This effect is seen even if the "chronic" hypoxia is transient, as when induced by reduction of umbilical flow by 30% for 3 days, and the effect lasts up to 1 week [176].

The carotid chemoreceptor

The carotid chemoreceptor response that initiates the response to acute hypoxia and the humoral response that maintains it are actually enhanced by chronic intrauterine conditions, including chronic hypoxia [177, 178]. However, the vasoconstrictor effect of these responses is offset by increased nitric oxide production; the net vascular response, i.e. whether there is vasodilation or vasoconstriction, will be determined by the balance between the catecholaminergic and nitrergic activities, which may be different in different vascular beds [174]. In the femoral circulation, the increased nitric oxide activity in chronically hypoxic fetuses overcomes the vasoconstrictor influences, and these fetuses have a markedly diminished capacity to increase peripheral vascular resistance in response to an episode of acute hypoxia [174–176].

Centralization of the fetal circulation

Centralization of the circulation occurs if uterine blood flow is reduced experimentally to 50% of the baseline for 15 min, but fails if uterine blood flow is reduced to 25% of the baseline. Respiratory and metabolic acidosis then develop, and there is generalized vasoconstriction, reduction of blood flow in all vascular beds, followed by hypotension, bradycardia and decreased cardiac output prior to fetal death [179]. Centralization of the circulation is maintained for about 5 min following complete occlusion

of the umbilical cord, after which loss of vasoconstriction, increase in femoral blood flow, further decrease in fetal heart rate and progressive hypotension develop as peripheral vasoconstriction is lost, cardiac glycogen stores are depleted and acidosis impairs cardiac contractility [180].

Failure of centralization of fetal blood as a mechanism of fetal death

In 91% (50/55) of hypoxic fetal deaths in this study, centralization of the circulation failed or could not be maintained. In 35% (19/55) of cases, there was global failure of centralization resulting in both myocardial and brain injuries; in 56% (31/55) of cases, blood flow was insufficient to meet the metabolic requirements of the heart or brain. Myocardial damage occurred in 70% (42/60) of cases, and brain damage occurred in 45% (27/60) of cases.

The mean EPO concentration in fetal deaths that had only myocardial injury was not significantly different from those that had only brain injury (Table 4), but significantly more fetal deaths had only myocardial injury than only brain injury (23/50 vs. 8/50, $P < 0.01$), which implies either that the fetal heart is more vulnerable to hypoxia than the fetal brain, or that the oxygen free radical-to-nitric oxide ratio in the cerebral and myocardial circulations was such as to cause greater blood flow and oxygen delivery to the brain than to the heart.

Poudel et al. [181] measured blood flow to the brain, heart and adrenal glands in fetal lambs rendered chronically hypoxic and growth-restricted by carunculectomy prior to pregnancy (a procedure that restricts placental size in a subsequent pregnancy), and found that blood flow, as well as oxygen and glucose delivery, increased only in the adrenal glands; blood flow and oxygen delivery to the whole brain did not increase; and oxygen and glucose delivery to the heart actually decreased compared to controls. A similar distribution of blood flow would explain why the frequency of myocardial injury alone was almost three times higher than brain injury alone among these fetal deaths (23/50 vs. 8/50, $P < 0.01$).

Myocardial injury is an insufficient cause of brain injury in fetal death

There was no association between the frequency of brain and myocardial injury, as the concentration of GFAP was raised in the same proportion of hypoxic fetal deaths in which the concentration of cTnI was raised as in those

in which cTnI was not raised; nor was there a significant difference in median GFAP concentration between these two groups of fetal deaths. Therefore, myocardial injury does not appear to have been the direct cause of the brain injury where both myocardial and brain injuries were present.

A role for fetal hypotension

The mean cTnI concentration in fetal deaths that had both myocardial and brain injuries was significantly higher than the mean concentration in fetal deaths that had only myocardial injury (380.38 pg/mL vs. 232.48 pg/mL, $P = 0.03$), implying that there was an indirect relationship between myocardial and brain injuries. Hypotension likely links these two types of injuries because the amount of brain damage during acute hypoxia correlates strongly with the depth and duration of hypotension [165], and hypotension is likely related to the extent of the myocardial injury, which in turn is reflected in the cTnI concentration.

When redistribution of the cardiac output fails to sustain myocardial oxygenation, there is cardiac glycogen depletion, anaerobic respiration, metabolic acidosis, myocardial depression, circulatory failure and cardiac arrest [176, 179]. Whether there is also brain injury will depend on the duration of hypotension prior to cardiac arrest [165]. When there is no evidence of intrauterine myocardial injury, the mechanism of brain injury and death is less clear.

If hypotension is a pre-requisite for brain injury, as appears to be the case [165], it suggests that the cause of hypotension in fetal deaths that have brain injury without myocardial injury is not cardiac failure, but rather inadequate peripheral vasoconstriction, although it is far from clear how hypotension might cause brain injury and death without cardiac damage. However, we speculate that increased nitric oxide production in response to chronic hypoxemia could simultaneously decrease systemic vascular resistance and cerebral blood flow while maintaining adequate myocardial perfusion long enough for brain injury to occur before cardiac glycogen stores are depleted, anaerobic respiration has run its course and the cycle of cardiac decompensation leading to circulatory failure and cardiac arrest commences.

Nitric oxide attenuates the sensitivity of the fetal baroreceptor reflex [182], which means that a drop in blood pressure caused by nitric oxide-mediated reduction in resistance in peripheral vascular beds would not elicit a sufficient increase in heart rate to restore blood pressure.

Cerebral blood flow can be expected to drop in parallel with the decrease in systemic arterial blood pressure as autoregulation of the cerebral vasculature operates within a narrow range in the fetus [165]. However, coronary blood flow could be maintained by nitric oxide-mediated coronary vasodilation sufficiently long enough for brain injury to occur before cardiac decompensation and death supervene, as the fetus has a significant coronary vasodilator reserve, 3.5-fold greater than in adults [183].

Fetal cardiac arrhythmia as a potential cause of fetal death in cases without evidence of myocardial and neuronal injuries

The mechanism of death in hypoxic fetal deaths that had no evidence of myocardial or brain injury cannot be determined from the data in this study. Each autopsy report showed some evidence of brain damage in all three of the five cases for which one was available: diffuse white matter and periventricular damage with calcifications in one case; bilateral ventriculomegaly with focal germinal matrix hemorrhage and scattered petechial hemorrhages most prominent in white matter in another; and evidence of brain stem gliosis in the third case. Brain injury, however, does not explain intrauterine death, and this was caused either by the previously described mechanisms without being reflected in an increase of cTnI and/or GFAP in AF, or, we suggest, more likely by a fetal cardiac arrhythmia. A fetal arrhythmia could have been caused by: (1) a cardiac conduction defect, which has been described in 39% of fetal deaths [93]; (2) autosomal recessive inheritance of a gene that predisposes to atrial fibrillation [184]; (3) mutation of the potassium voltage-gated channel subfamily H member 2 (*KCNH2*) gene that involves the pore region of the potassium channel [185]; or (4) mutation in the sodium channel alpha-subunit gene (*SCN5A* gene) that interferes with sodium transport in cardiac sodium channels that predisposes to long QT intervals and ventricular arrhythmias [186, 187].

Placental abruption

In the remaining five cases for which none of the analytes was raised, there was histological evidence of a placental abruption in three of them, delayed villous maturation in one case and a massive subchorial hematoma (Breus' mole) in the fifth. Autopsy of the brain and heart showed only autolytic changes. AF EPO concentrations can increase exponentially with severe hypoxia – by as much as

22–25 mIU/mL daily among high-risk pregnancies [64, 131]. Nevertheless, these fetal deaths were most likely hypoxic, given the histological findings, but death from cardiac arrest or a cardiac arrhythmia occurred too rapidly for the EPO or the markers for heart damage to rise in AF [63].

Limitations

The main limitation on the conclusions of this study is the lack of knowledge of the pharmacokinetics of cTnI and GFAP in AF given that the length of time it takes for cTnI and GFAP to become detectable in AF after brain or myocardial injury is not known; how frequently these analytes are detectable after brain or myocardial injury, i.e. their sensitivity in AF, is not known; and the rate at which the concentrations of these analytes can increase in AF, as well as their half-lives, is also unknown. The time of death prior to delivery was also not known in any of these cases, although it could be estimated based on the placental histological findings [124]. From these estimates, one can be reasonably certain that the analyte patterns observed in these fetal deaths were not materially affected by clearance of the analytes from AF.

Conclusion

Hypoxia, secondary to placental dysfunction, is the mechanism of death in the majority of structurally normal fetuses after 20 weeks of gestation. Ninety-one percent of hypoxic fetal deaths sustained brain, myocardial or both brain and myocardial injuries *in utero*. An attributable mechanism of death in 70% of the cases is circulatory failure and cardiac arrest secondary to hypoxic myocardial injury. A histopathological explanation for placental dysfunction was found in 74% of these cases.

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