

## OBSTETRICS

# The placenta in fetal death: molecular evidence of dysregulation of inflammatory, proliferative, and fetal protective pathways

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**BACKGROUND:** It is estimated that over 2 million cases of fetal death occur worldwide every year, but, despite the high incidence, several basic and clinical characteristics of this disorder are still unclear. Placenta is suggested to play a central role in fetal death. Placenta produces hormones, cytokines and growth factors that modulate functions of the placental-maternal unit. Fetal death has been correlated with impaired secretion of some of these regulatory factors.

**OBJECTIVE:** The aim of the present study was to evaluate, in placentas collected from fetal death, the gene expression of inflammatory, proliferative and protective factors.

**STUDY DESIGN:** Cases of fetal death in singleton pregnancy were retrospectively selected, excluding pregnancies complicated by fetal anomalies, gestational diabetes, intrauterine growth restriction and moderate to severe maternal diseases. A group of placentas collected from healthy singleton term pregnancies were used as controls. Groups were compared regarding maternal and gestational age, fetal sex and birth-weight. Placental messenger RNA expression of inflammatory (interleukin 6), proliferative (activin A, transforming growth factor  $\beta$ 1) and regulatory (vascular endothelial growth factor, vascular endothelial growth factor receptor 2, ATP-binding cassette transporters (ABC) *ABCB1* and *ABCG2*, sphingosine 1-phosphate signaling pathway) markers was conducted using real-time polymerase chain reaction. Statistical analysis and graphical representation of the data were performed using the GraphPad

Prism 5 software. For the statistical analysis, Student's *t* test was used, and *P* values  $<.05$  were considered significant.

**RESULTS:** Placental mRNA expression of interleukin 6 and vascular endothelial growth factor receptor 2 resulted significantly higher in the fetal death group compared to controls ( $P<.01$ ), while activin A, *ABCB1*, and *ABCG2* expression resulted significantly lower ( $P<.01$ ). A significant alteration in the sphingosine 1-phosphate signaling pathway was found in the fetal death group, with an increased expression of the specific receptor isoforms sphingosine 1-phosphate receptor 1, 3, and 4 (sphingosine 1-phosphate<sub>1</sub>, sphingosine 1-phosphate<sub>3</sub>, sphingosine 1-phosphate<sub>4</sub>) and of sphingosine kinase 2, 1 of the enzyme isoforms responsible for sphingosine 1-phosphate synthesis ( $P<.01$ ).

**CONCLUSION:** The present study confirmed a significantly increased expression of placental interleukin 6 and vascular endothelial growth factor receptor 2 mRNA, and for the first time showed an increased expression of sphingosine 1-phosphate receptors and sphingosine kinase 2 as well as a decreased expression of activin A and of selected ATP-binding cassette transporters, suggesting that multiple inflammatory and protective factors are deranged in placenta of fetal death.

**Key words:** activin A, ATP-binding cassette transporters, BCRP, cytokines, fetal death, fibrosis, growth factors, inflammation, IL-6, P-gp, placenta, S1P, sphingosine-1-phosphate pathway, stillbirth, VEGFR2

## Introduction

The World Health Organization defines stillbirth as fetal death occurring at 28 weeks of gestation or more, before or during birth.<sup>1</sup> Over 2 million fetal deaths occur each year worldwide and 80% of these occur at the end of pregnancy.<sup>1</sup> Many cases are thought to result from preventable causes mostly correlated to

known maternal, placental, or fetal risk factors.<sup>2</sup> Despite that, many late fetal losses are associated with a failure to identify risk factors, leading to approximately 60% of unexplained fetal deaths, with a larger proportion of these cases occurring during the third trimester of pregnancy.<sup>3</sup> While infections and congenital anomalies are mainly responsible for early fetal deaths,<sup>4</sup> placental causes are implicated in losses that occur after 26 weeks of gestation,<sup>5</sup> by mechanisms correlated with placental vascular malperfusion<sup>6–11</sup> and placental senescence.<sup>12–14</sup>

A systematic review examining placental pathological findings of fetal death reported that 11% to 65% of cases of fetal death likely originated from placental dysfunction correlated with unknown mechanisms that originated in

the very early stages of pregnancy.<sup>15,16</sup> However, placental lesions associated with fetal death are extremely varied, and poorly defined, and their relation to pathologic processes leading to fetal death are difficult to evaluate.<sup>17</sup>

Placental lesions associated with fetal death may originate from infectious diseases, genetic disorders, metabolic alterations, placental inflammation, acute chorioamnionitis, fetal chorionic plate vascular degeneration, as well as, thrombi and fibrin peri or intervillous depositions.<sup>17,18</sup> In addition, vascular disorders, such as fetal and maternal vascular malperfusion are common findings in fetal and neonatal deaths.<sup>19–21</sup> In addition, defective maturation of the placental villous tree in fetal deaths, results in reduction of vascular-syncytial membranes and fetal

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## AJOG at a Glance

**Why was this study conducted?**

The study aims to investigate placental molecular changes in inflammatory and protective pathways in cases of fetal death, to further increase the knowledge of placental alterations in fetal death and possibly define new potential pathogenetic factors.

**Key findings**

The present study confirmed that placentas of pregnancy that ended in fetal death show an upregulated expression of the proinflammatory cytokine interleukin 6 and the vascular endothelial growth factor receptor 2, and showed for the first time an increased expression of receptors and kinases involved in the sphingosine 1-phosphate (S1P) signaling pathway, as well as a downregulation of activin A and the 2 ATP-binding cassette transporters *ABCB1* and *ABCG2*.

**What does this add to what is known?**

The upregulation of the S1P signaling pathway and the downregulation of *ABCB1* and *ABCG2* transporters in placentas from fetal death represent new factors associated with placental inflammation/fibrosis and transport mechanisms in this complex condition.

and inflammation and fibrosis processes.<sup>38,39</sup> S1P is produced upon phosphorylation of sphingosine by sphingosine kinases (sphingosine kinase 1 [SK1], sphingosine kinase 2 [SK2]) and it can act as an intracellular messenger or be exported and interact with 5 specific G-protein coupled receptors (S1P<sub>1-5</sub>).<sup>35</sup>

The present study aimed to investigate placental gene expression levels of genes involved in S1P metabolism and signaling, as well as genes conferring fetal protection against drugs and toxins, together with the expression of pathogenic factors known to be involved in placental dysfunction, such as IL-6, activin A and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), vascular endothelial growth factor (VEGF) and its receptor (vascular endothelial growth factor receptor 2 [VEGFR2]).

## Material and methods

### Study design and selection of samples

This is a retrospective case-control study of placental paraffin-embedded samples from singleton pregnancies ended in fetal death (n=10) compared with a control group of placental samples of physiological pregnancies (n=10).

We selected 10 cases of patients diagnosed with antepartum fetal death after 30+0 weeks of pregnancy. In all cases, the altered perception started less than 24 hours before the diagnosis. Antepartum fetal death was defined as “fetal death occurring during pregnancy and prior to delivery, before the onset of labor.” Fetal death was defined by Apgar scores of 0 at 1 and 5 minutes, and no signs of life by direct observation.<sup>40</sup> In all cases, labor was immediately induced after diagnosis of fetal death; otherwise, a cesarean section was immediately performed if indicated. All patients delivered between 4 and 24 hours after the diagnosis. We excluded pregnancies complicated by fetal anomalies, gestational diabetes, preexisting IUGR, and moderate to severe maternal diseases that might affect placentation—such as obesity, autoimmune diseases, hypercholesterolemia,

hypoxia, suggesting that hypoxia secondary to placental dysfunction, is a central cause in most deaths that occur in structurally normal fetuses.<sup>22–24</sup> In this connection, maternal vascular malperfusion was confirmed in almost 70% of cases of fetal death.<sup>25–27</sup>

Vascular lesions of malperfusion may be predicted in the maternal plasma by an imbalance between the concentration of angiogenic (placental growth factor, PIGF) and antiangiogenic (soluble vascular endothelial growth factor receptor, also known as soluble fms-like tyrosine kinase 1, sFlt1) factors,<sup>26</sup> which have been proposed to be the leading markers of fetal death in the late second and third trimester.<sup>26,27</sup>

ATP-binding cassette (ABC) efflux transporters are involved in fetal protection from a wide range of structurally unrelated drugs (antibiotics, antidepressants, antiretrovirals, synthetic glucocorticoids, sulfonyleureas, nonsteroidal antiinflammatory drugs [NSAIDs] and proton pump inhibitors [PPIs]), toxins (organochlorines, organophosphate, herbicides, mercuric species, estrogenic mycotoxins, and carcinogens phototoxic compounds), hormones (endogenous glucocorticoids, estrogens, progestogens, and androgens), cytokines (interleukin 1 beta, interleukin 6 [IL-6], interferon-gamma,

tumor necrosis factor), chemokine (C-C motif ligand 2), and endogenous metabolites (bilirubin, porphyrins, uric acid, and prostaglandins), across the placenta; limiting fetal over-exposure to these toxic compounds.<sup>28–30</sup> P-glycoprotein (P-gp) (encoded by the gene *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*) are the best-described protective ABC transporters within this superfamily and are highly enriched in the placenta.<sup>29,31</sup> They function as placental gatekeepers localized at the apical membrane of the syncytiotrophoblast, facing the maternal blood, where they efflux back to the maternal circulation potentially maternally-derived toxic substrates that may have reached the fetal circulation.<sup>32</sup> Expression and function of P-gp/*ABCB1* and BCRP/*ABCG2* are potentially inhibited by infection and inflammation<sup>28,33,34</sup> and may therefore be potentially altered in fetal death placentas, playing a role in the pathomechanisms of fetal death.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that modulates various cellular processes (cell survival, proliferation, migration, fibrosis, and inflammation),<sup>35</sup> involved in pregnancy complications such as intrauterine growth restriction (IUGR) and preeclampsia,<sup>36</sup> as well as regulating trophoblast migration<sup>37</sup>

and thrombophilia, as well as maceration at postmortem evaluation. Information regarding maternal demographics, medical and obstetrical history was extracted, using a Microsoft Excel software (Microsoft Corporation, Redmond, WA) database. Birthweight percentiles by gestational age and sex were calculated with World Health Organization Fetal Growth Calculator. The study was approved by the Ethic Committee (Protocol 13744).

### Placental pathology

Ten placental samples from pregnancies ending in idiopathic fetal death and 10 placental samples of physiologic pregnancy were retrospectively analyzed considering macroscopic and microscopic aspects. All the placentas were sampled using systematic sampling techniques.<sup>41</sup> For each placenta a minimum of 4 blocks was submitted, including a roll of the extraplacental membranes with a cross section of the umbilical cord and 3 or more blocks containing a full-thickness section of normal-appearing placenta parenchyma. In addition, if gross lesions were identified, they were described and an estimation of the percentage of the total placental volume was made. The lesions were sampled too for histological evaluation. Then, slides from cases and controls were stained with hematoxylin and eosin and were reviewed by a placental pathologist blinded to the previous diagnosis. The terms recommended

by the Amsterdam Placental Workshop Group were used to describe the lesions.<sup>41</sup>

### RNA extraction and quantification

Samples of formalin-fixed and paraffin-embedded placental tissue were made according to standardized protocol and conventional histopathological examination and total RNA was extracted from the selected samples using MagCore Total RNA FFPE One-Step Kit (RBC Bioscience Corp, New Taipei City, Taiwan). The RNA was reverse transcribed to complementary DNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, by Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. The quantification of mRNA expression levels was performed using real-time polymerase chain reaction (PCR). The analysis was performed in duplicate using TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA). VEGFR2: Hs00911700\_m1, VEGF: Hs00900055\_m1, INHBA: Hs01081598\_m1, TGF $\beta$ 1: Hs00998133\_m1, IL-6: Hs00174131\_m1, S1P<sub>1</sub>: Hs01922614\_s1, S1P<sub>2</sub>: Hs01003373\_M1, S1P<sub>3</sub>: Hs00245464\_s1, S1P<sub>4</sub>: Hs02330084\_s1, S1P<sub>5</sub>: Hs00928195\_s1, SK1: Hs00184211\_m1, SK2: Hs01016543\_g1, ACTB: Hs01060665\_g1 and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, Hercules, CA). Relative quantification was performed with the  $2^{-\Delta C_t}$  method,<sup>42</sup> using the housekeeping gene  $\beta$ -actin for normalization.

### Statistical analysis

Statistical analysis and graphical representation of the data were performed using the GraphPad Prism 5 software (GraphPad Software Inc, Boston, MA). For the statistical analysis of mRNA expression, groups were tested for normal distribution using the Shapiro-Wilk normality test. Multiple Student's *t* test analysis was then conducted, using the false discovery rate approach with the Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli, and *P* values < .05 were considered significant. For the comparison of clinical characteristics, continuous variables were compared using Student's *t* test or Mann-Whitney *U* test based on their distribution, and categorical variables were compared using Fisher's exact test.

### Results

Cases and controls were compared regarding maternal and gestational age, fetal sex, mode of delivery, smoking habit, birthweight and birthweight percentile, placental weight (in fresh conditions), and birthweight-to-placenta-weight ratio (Table 1). No significant difference was found, except for gestational age at delivery and birthweight (*P* < .05).

### Placental pathology

Table 2 shows the most relevant macroscopic and microscopic findings in placentas collected from fetal death.

**TABLE 1**  
**Comparison of characteristics between cases and controls**

Characteristics	Cases of fetal death (n=10)	Controls (n=10)	<i>P</i> value
Maternal age (y)	33±4.6	35±3.4	.36
Gestational age at delivery (wk)	35+6 (31+6–41+2)	39+3 (37+5–41+0)	<.05 (0.027)
Male fetal sex	5/10	5/10	1
Vaginal delivery	8/10	5/10	.35
Current smoking status	2/10	0/10	.47
Birthweight (g)	2383±800	3306±427	<.05 (0.019)
Birthweight (percentile): World Health Organization	24.3 (2–74.7)	40.6 (11.6–84.2)	.17
Birthweight-to-placenta-weight ratio	5.504 (3.340–6.964)	5.997 (4.762–7.838)	.33

**TABLE 2**  
**Macroscopic and microscopic placental examination of fetal death cases**

Case	Macroscopic examination	Placental weight	Birthweight-to-placenta-weight ratio	Microscopic relevant findings
1	Placental weight 410 g (underweight) Opaque-whitish membranes	420 g	6.8	Villous immaturity with predominance of mature villi and cluster of immature intermediate villi. Stem vessel sclerosis and terminal villous deficiency.
2	Placental weight 210 g (underweight)	280 g	7.0	Predominance of dysmorphic and hypovascular mature intermediate villi. Lack of mature intermediate villi.
3	Placental weight 500 g (overweight)	500 g	3.3	Villar immaturity with deficiency of terminal villi. Choriodecidualitis. Maternal dysmetabolic and dysglycemic state.
4	Opaque membranes Umbilical cord length 68 cm	600 g	4.9	Thrombosis of the umbilical vessels and intervillar hemorrhage.
5	Placental weight 280 g (underweight) Opaque membranes	340 g	4.4	Avascular villi with moderate intervillitis and edema of terminal villi.
6	Greenish membranes and opaque fetal side	390 g	6.6	Focal choriodecidualitis. Chronic histiocytic intervillitis with villitis and necrosis of villar branches.
7	Placental weight 200 g (underweight) Greenish membranes	270 g	6.2	Severe chorioamnionitis. Long-term hypoxic-ischemic suffering with ischemic infarctions and hemorrhages. Necrotic villi
8	Placental weight 320 g (underweight)	420 g	3.9	Acute villitis and intervillitis.
9	Placental weight 630 g (overweight) Greenish membranes	700 g	4.8	Choriodecidualitis. Tenney-Parker changes. Hypoxic-ischemic suffering with ischemic infarctions.
10	Opaque and greenish membranes	600 g	6.2	Spiral artery atherosclerosis. Thickening of the capillary basal membranes and trophoblasts.

The control placentas were also pathologically evaluated. They did not show any severe macroscopic or microscopic pathological features.

### Placental mRNA expression

Placental mRNA expression of IL-6 was significantly higher ( $P=.0495$ ) in fetal death compared to controls. Activin A ( $\beta$ A-subunit) expression was lower ( $P=.0098$ ) in fetal death compared to control, while TGF- $\beta$ 1 expression was not significantly different between the 2 groups (Figure 1). VEGF expression was also not significantly altered in fetal death placentas, but VEGFR2 expression levels resulted significantly higher ( $P=.0305$ ) in the fetal death group compared to controls (Figure 1).

The analysis of the placental expression of the 2 ABC transporters P-gp (*ABCB1*) and BCRP (*ABCG2*), which have a protective role for the fetus, preventing fetal exposure to several molecules through their expression in the placenta,<sup>28,29</sup> was also carried out. Both

the transporters were found to be less expressed in fetal death compared to the control placentas (*ABCB1*  $P=.0450$  and *ABCG2*  $P=.0232$ ) (Figure 2).

The expression levels of the 2 sphingosine kinases responsible for S1P production (SK1 and SK2) and of the 5 receptors involved in S1P signaling (S1P<sub>1-5</sub>) were also investigated, and a significant alteration in the S1P signaling pathway was found. Indeed, an increased expression of the S1P receptors 1, 3, and 4 (S1P<sub>1</sub>  $P=.0421$ ; S1P<sub>3</sub>  $P=.0399$ ; S1P<sub>4</sub>  $P=.0494$ ) and of the enzyme SK2 ( $P=.0038$ ) in fetal death (Figure 3) was shown. The expression of the other 2 S1P receptors (S1P<sub>2</sub> and S1P<sub>5</sub>) and of SK1 was not significantly different between the 2 groups (data not shown).

## Discussion

### Principal findings

The present study showed significant changes in placental mRNA expression of new inflammatory and protective mediators in cases of fetal death. In

particular, the mRNA expression of the 2 best studied ABC transporters, *ABCB1* and *ABCG2*, was lower in fetal death, whereas S1P<sub>1</sub>, S1P<sub>3</sub>, and S1P<sub>4</sub>, as well as SK2, mRNA expression was higher, suggesting a dysregulation of the placental mechanism of fetal protection and S1P signaling pathway. Moreover, higher expression of IL-6 and VEGFR2 and a lower expression of Activin A was confirmed. Altogether, these findings show several molecular alterations in placentas of fetal death.

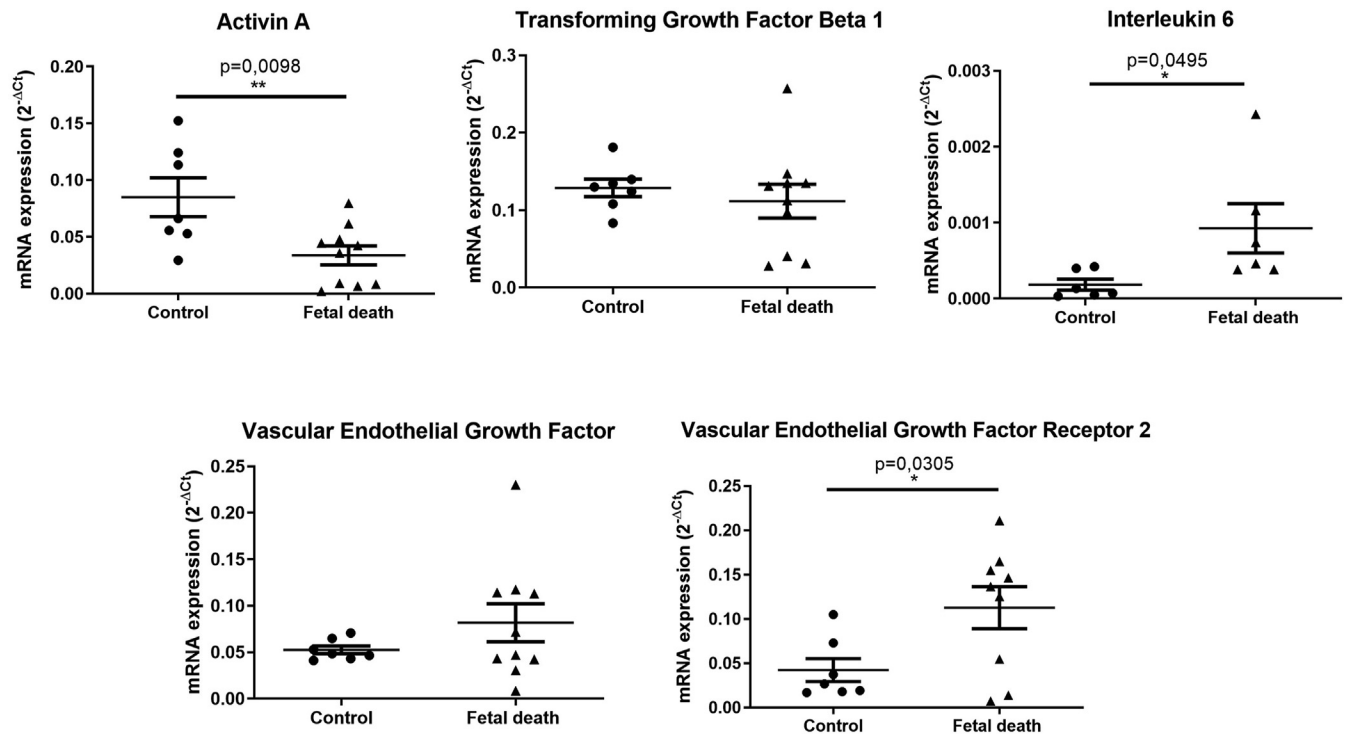
### Results in the context of what is known

An appropriate placental inflammatory response is of fundamental importance in maintaining pregnancy and reacting to any infective or exogenous stressors.<sup>43-45</sup>

The present study showed that the S1P pathway, involved in trophoblast migration<sup>37</sup> cell fusion,<sup>38</sup> inflammatory responses,<sup>38</sup> and fibrosis,<sup>46</sup> is significantly dysregulated in fetal death

FIGURE 1

The RNA extracted from paraffinized placenta samples (control N = 8, fetal death N = 10) was analyzed using real-time polymerase chain reaction



The mRNA expression of IL-6, activin A (Inhibin Subunit Beta A, INHBA), TGF- $\beta$ 1 (TGFB1), VEGF, and VEGFR2 was evaluated. Results were analyzed with the use of the 2<sup>-ΔCt</sup> method, using the housekeeping gene  $\beta$ -actin for normalization. Normal distribution of the values within each group was verified using the Shapiro-Wilk normality test. Differences are statistically significant according to multiple Student's *t* test analysis, using the false discovery rate approach with the 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (\**P* < .05, \*\**P* < .01).

IL-6, interleukin 6; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

placentas. In particular, increased expression of S1P<sub>1</sub>, S1P<sub>3</sub>, and S1P<sub>4</sub> was found. Moreover, the augmented expression of the S1P-generating enzyme, SK2, may be related to reduced proliferation and induction of apoptosis of trophoblastic cells.<sup>47,48</sup> In fact, S1P has been identified as a negative regulator of trophoblast differentiation,<sup>38</sup> as well as regulating cell viability and apoptosis in primary cytotrophoblasts.<sup>49</sup> These findings are consistent with the pathological signs of villous immaturity, and terminal villous deficiency found in our cohort. Furthermore, these data suggest an involvement of the placental S1P signaling pathway in fetal death, stimulating local inflammation and fibrosis. This placental inflammatory state is confirmed by the increased expression of IL-6 and by the

diverse pathological signs of inflammation in fetal death placentas, such as choriodecidualitis, intervillitis, villitis, and chorioamniosis.

There is increasing evidence that prenatal exposure to different environmental factors affects pregnancy outcomes. Prenatal alcohol exposure can increase the risk of fetal death,<sup>50</sup> as well as air pollutants, such as particulate matter and ozone, heat exposure,<sup>51</sup> smoking, and drugs of abuse.<sup>52</sup>

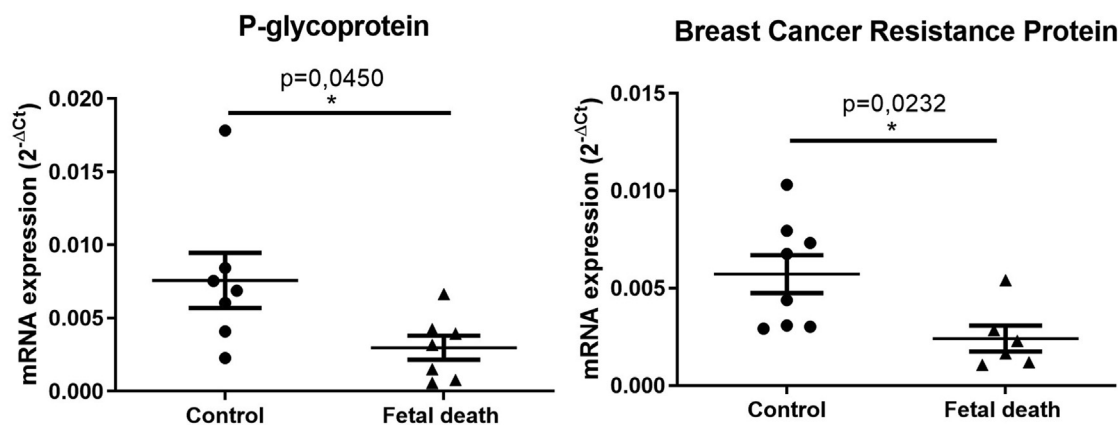
The present study also showed decreased mRNA expression of the 2 best-described ABC transporters, P-gp (*ABCB1*) and BCRP (*ABCG2*) in placentas of fetal death. Infection and inflammation are potent inhibitors of P-gp and BCRP,<sup>28,33,34,53</sup> therefore, it is likely that the local inflammation detected in fetal death placentas is

responsible for lowering the mRNA expression of these transporters. Placental P-gp and BCRP protect the fetus from exposure to drugs and environmental toxins of obstetric relevance<sup>29</sup> and the decreased expression of these transporters may expose the fetus to an abnormal quantity of toxicants making it more vulnerable and leading to fetal death. The loss of these 2 protective mechanisms in fetal death placenta may be a critical factor which exposes the fetus to other placental dysfunction. Interestingly, we found an increased VEGFR2 mRNA expression. VEGFR2 stimulates and mediates a variety of biological responses and pathological processes in angiogenesis.<sup>54</sup> sVEGFR2, the soluble form of the receptor, is decreased in plasma but increased in the amniotic fluid of patients with fetal



FIGURE 2

The RNA extracted from paraffinized placenta samples (control N = 8, fetal death N = 10) was analyzed using real-time polymerase chain reaction



The mRNA expression of the 2 ABC transporters P-gp (ABCB1) and BCRP (ABCG2) was evaluated. Results were analyzed with the use of the  $2^{-\Delta Ct}$  method, using the housekeeping gene  $\beta$ -actin for normalization. Normal distribution of the values within each group was verified using the Shapiro-Wilk normality test. Differences are statistically significant according to multiple Student's *t* test analysis, using the false discovery rate approach with the 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli ( $*P < .05$ ).

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein.

death.<sup>55</sup> Surprisingly, the present data do not show significant changes in VEGF expression, while decreased VEGF levels in the amniotic fluid of women who subsequently had fetal death before 20 weeks was previously observed.<sup>56</sup> The mRNA expression levels of this growth factor in the placenta, however, do not necessarily reflect the levels of secreted protein; moreover, VEGF expression changes might have happened in earlier pregnancy stages. In fetal death placentas, we also confirmed decreased mRNA expression of activin A, which supports a poor trophoblast invasion and placental angiogenesis<sup>57</sup> and is in agreement with low amniotic fluid levels of activin A in women who later had fetal death.<sup>58</sup>

### Clinical implications

The present study shows and confirms how different inflammatory, proliferative and protective pathways are dysregulated in placentas of pregnancy ended in fetal death, suggesting the possible use of circulating levels of the 2 ABC transporters P-gp and BCRP and of S1P as potential future fetal death biomarkers. Of importance, it is challenging to tease apart 1 specific P-gp/

BCRP clinical substrate that leads to fetal death. P-gp and BCRP prevent the fetal accumulation of a myriad of exogenous and endogenous compounds of obstetric relevance, including antibiotics, antidepressants, antiretrovirals, endogenous and synthetic glucocorticoids, estrogens, progestogens, androgens, sulfonyleureas, nonsteroidal NSAIDs, PPIs, organochlorines, organophosphate, herbicides, mercuric species, estrogenic mycotoxins, carcinogens phototoxic compounds, cytokines, chemokines, bilirubin, porphyrins; uric acid prostaglandins, among others.<sup>28–30,59</sup> It is likely that greater fetal exposure to combined P-gp and BCRP substrates, associated with a hypoxic-ischemic state, placental high-grade inflammation, deranged trophoblastic proliferation, and diverse placental pathologic lesions, altogether, lead to fetal death. One important aspect of our research is that we show, consistently with previous research,<sup>28,33,34,53,60,61</sup> that pregnancies threatened by infection and inflammation (ie, with different grades of chorioamnionitis; or exposed to bacterial and viral pathogen-associated molecular patterns) are altogether at

greater risk of fetal drug/toxin accumulation, which associated with a hypoxic-ischemic state may lead to fetal death.

### Research implications

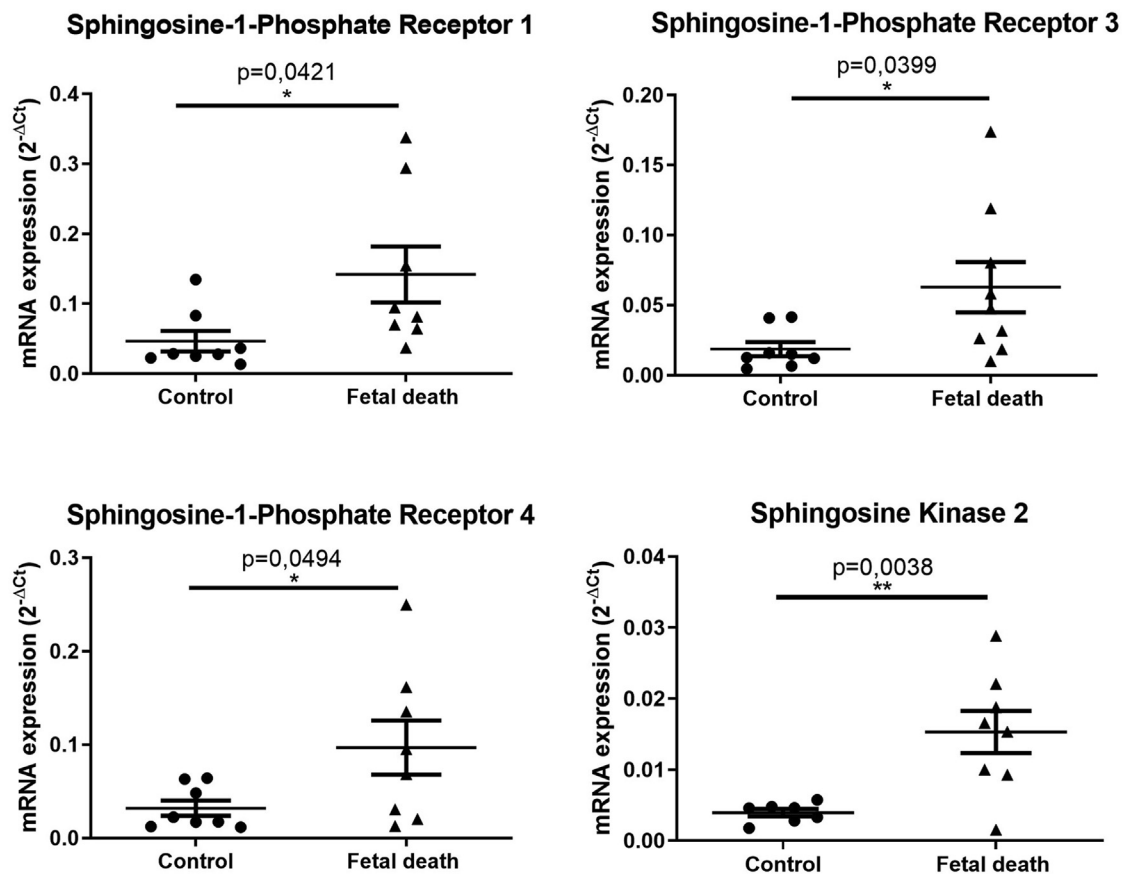
The present study highlights alterations in the expression of some molecules in placentas of fetal death. Dysregulation of a new inflammatory/fibrosis signaling pathway (S1P) and downregulation of ABC transporters were demonstrated. From a basic research point of view, further investigation of their molecular actions also using in vitro models might lead to a better understanding of the mechanisms underlying fetal death. Furthermore, evaluation of the protein levels of these pathogenic factors could lead to proposing S1P, P-gp, and BCRP as potential circulating markers in the blood of patients at risk.

### Strengths and limitations

The major strength of the study is the investigation of the altered expression of new potential placental pathogenic factors that may be involved in fetal death, while the main limitation is the limited sample number. Further studies with a larger sample number are

FIGURE 3

The RNA extracted from paraffinized placenta samples (control N = 8, fetal death N = 10) was analyzed using real-time polymerase chain reaction



The mRNA expression of enzymes and receptors involved in the S1P signaling pathway was evaluated. Results were analyzed with the use of the 2<sup>-ΔCt</sup> method, using the housekeeping gene  $\beta$ -actin for normalization. Normal distribution of the values within each group was verified using the Shapiro-Wilk normality test. Differences are statistically significant according to multiple Student's *t* test analysis, using the false discovery rate approach with the 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (\* *P* < .05, \*\* *P* < .01).

S1P, sphingosine 1-phosphate.

required to confirm the present results and further elucidate the molecular mechanisms involved in fetal death. Furthermore, a correlation between changes in mRNA expression and protein levels was not possible in paraffin-embedded samples, and requires further investigation.

## Conclusions

In conclusion, the present study shows a decreased expression of regulatory factors (P-gp, BCRP) implicated in fetal protection, as well as a dysregulation in the S1P signaling pathway, involved in inflammation and fibrosis, confirming an inflammatory state and impaired fetal protection mechanisms in

placentas of pregnancies ended in fetal death. ■

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