



Fetal and Pediatric Pathology

ISSN: 1551-3815 (Print) 1551-3823 (Online) Journal homepage: http://www.tandfonline.com/loi/ipdp20

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**To cite this article:** Luca Roncati, Teresa Pusiol, Francesco Piscioli, Giuseppe Barbolini, Antonio Maiorana & Anna Lavezzi (2016): The First 5-Year-Long Survey on Intrauterine Unexplained Sudden Deaths from the Northeast Italy, Fetal and Pediatric Pathology, DOI: <u>10.1080/15513815.2016.1185751</u>

To link to this article: <u>http://dx.doi.org/10.1080/15513815.2016.1185751</u>



Published online: 16 Jun 2016.

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# The First 5-Year-Long Survey on Intrauterine Unexplained Sudden Deaths from the Northeast Italy

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#### ABSTRACT

Purpose: Sudden intrauterine unexplained death syndrome (SIUDS) represents one of the main open issues in the scientific and social setting of the modern medicine, and our efforts have aimed to understand its possible causes and risk factors. Methods: A 43-case series of consecutive unexplained fetal deaths coming from Northeast Italy, collected in a 5-year period (2011–2015), has been submitted to an in-depth investigation, based on neuropathological and cardiopathological examinations, immunohistochemistry for neuronal nuclear antigen (NeuN), genetic characterization for the serotonin transporter (5-HTT) gene polymorphisms, and toxicological environmental analyses. Results: The overall survey from the neuropathological findings highlights one or more congenital morphological abnormalities of the autonomic nervous system in 77% of cases of sudden fetal deaths. Conclusions: From our results emerges the need to perform a complete autopsy of all SIUDS victims with an in-depth examination of the neuronal centers of the brainstem, which modulate the vital functions.

#### **ARTICLE HISTORY**

Received 21 February 2016 Revised 31 March 2016 Accepted 5 April 2016

#### **KEYWORDS**

sudden intrauterine unexplained death syndrome (SIUDS); neuropathology; neuronal nuclear antigen (NeuN); serotonin transporter (5-HTT); cardiopathology; autopsy; pesticides

# Introduction

Sudden intrauterine unexplained death syndrome (SIUDS) and sudden infant death syndrome (SIDS) represent main open issues in the scientific and social setting of the modern medicine. The definition of SIDS is "the sudden unexpected death of an infant < 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and clinical history" [1]. SIDS is therefore a diagnosis of exclusion, striking one infant every 750–1000 live births.

In developed countries, one in 100–200 pregnancies, particularly at or near term, ends in stillbirth. The death remains unexplained in high percentages of cases (40–80%) even after the performance of a routine autopsy, including placental examination [2]. The acronym SIUDS, likewise to SIDS, has been proposed to define unexplained stillbirths [3]. SIUDS has an eightfold greater prevalence than SIDS. The frequency of these pathologies (both SIUDS)

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and SIDS) has not significantly decreased in the last 20 years, despite the modern advances in maternal-infant care [4]. Their causes are still unknown, although a large number of studies have reported morphological abnormalities of the brainstem and biochemical defects of the neurotransmission, above all in SIDS.

First of all, to fully understand the pathogenesis of these deaths, it is essential that the stillbirths and infants, unexpectedly dying in the first months of life, are submitted to autoptic examination, and that the diagnostic criteria used by investigators are appropriate and standardized, including an in-depth examination of the autonomic nervous system and cardiac conductions.

In 2006, the Italian special law "*Regulations for diagnostic postmortem investigation in victims of SIDS and unexpected fetal death*" [5] was enacted in order to meet this need (Law no. 31/2006).

In addition, the Law requires that the autoptic findings, the informations about pregnancy, fetal development, and delivery, and in SIDS, about the environmental and familial situation in which the death occurred, besides information related to the potential risk factors (such as maternal smoking, maternal obesity, feeding, position the baby was last left in) must be collected by the obstetrician-gynecologist, neonatologist, pediatrician, and pathologist involved in the case and recorded in a specific data bank. The data bank includes two subsections: one for fetal loss and another for infant deaths.

The Northeast Italy, with the Autonomous Province of Trento (APT) in the lead, was the first to enact a program in response to Law 31 in its territory, through the stipulation, in 2012, of a convention with the *Lino Rossi* Research Center from the Milan University, recognized by the Italian Ministry of Health as reference center for the application of the Law 31, thus formalizing a collaboration already ongoing.

To realize the objectives of this cooperation, the APT drew up an operational protocol for the epidemiological overseeing (surveillance) of stillbirths and SIDS, with the aim to ensure to all healthcare professionals involved, operating procedures for the correct management of each case, thus allowing the study and design of preventive strategies of these deaths, whose inherent emotional consequences among families are very devastating. Here, we present the overall analysis of the information and pathological results obtained from 43 SIUDS victims, coming from the Northeast Italy in a 5-year period and collected according to the aforementioned agreement.

# **Materials and methods**

#### Study subjects

The study included 43 cases of SIUDS, aged from 19 to 40 gestational weeks, occurring in the Northeast Italy during a 5-year period (2011–2015), and in whom a complete autopsy and analysis of the clinical history did not establish any cause of death. The brain and heart of all the victims were sent to the *Lino Rossi* Research Center for the specific study, by serial histological sections, of both the autonomic nervous system and cardiac conduction system.

For each case, family interviews provided information about pregnancy, fetal development, and potential risk factors (such as maternal smoking or maternal obesity). This was collected and categorized during postmortem family interviews. The risk factors have been classified as preventable and unpreventable, according to Guntheroth [6]. The unpreventable factors are sex, race, and gestational age. The preventable risk factors include maternal cigarette smoking, alcohol, and drug abuse. The information sheets were recorded in a dedicated data bank,

Table 1. The 43 fetuses unexplainably died in utero are subdivided for gender and gestational weeks at the time of intrauterine death and compared to 40 control cases. The death diagnoses in controls were: severe chorioamnionitis (five cases), pulmonary dysplasia (five cases), umbilical cord accidents (five cases), congenital heart disease (four cases), placenta abruption (four cases), congenital toxoplasmosis (three cases), karyotype alterations (three cases), gestational diabetes (three cases), preeclampsia (two cases), trauma (two cases), Potter's syndrome (one case), systemic lupus erythematosus (one case), septicemia (one case), and severe uterine infection (one case).

	Gestational weeks										
Fetuses	19–23	M/F	24–28	M/F	29–33	M/F	34–38	M/F	39–40	M/F	
SIUDS (43 cases) Controls (40 cases)	2 cases 3 cases	1/1 0/3	3 cases 2 cases	1/2 1/1	9 cases 10 cases	3/6 4/6	18 cases 13 cases	11/7 8/5	11 cases 12 cases	5/6 6/6	

M/F: males/females (ratio).

provided by the APT. As controls, we used a group of 40 fetuses which died with known causes, selected from a wide set of cases, previously collected at the *Lino Rossi* Research Center. These controls were referred to the medical institutions involved in the application of the law, with a specific purpose of contributing to research studies.

The 40 control cases have been matched with the SIUDS cases for gestational age, sex, and area of origin (Northeast Italy). Table 1 summarizes the SIUDS and control cases enrolled in the present study.

# Methodology

#### Neuropathological protocol

The neuropathological study particularly includes the histological examination in the brainstem of the main nuclei and structures involved in the control of the vital functions. Figure 1 shows a scheme of the applied methodology for the brainstem analysis. At the right, the sampling of four specimens is shown. The first specimen, mesencephalon, includes the upper third of the pons and the adjacent portion of the caudal mesencephalon. The second extends from the upper portion of the medulla oblongata to the adjacent caudal portion of the pons. The



**Figure 1.** Schematic representation of the neuropathological protocol applied for the study of all SIUDS cases from our series: midbrain, pons, medulla oblongata, spinal cord, and the related main neuronal nuclei are reported.

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third specimen extends 2–3 mm above and below the obex. A fourth sample is taken from the rostral tract of the spinal cord. At the left, a scheme of the more representative histological sections obtained from the earlier-described specimens is shown, indicating the great part of the main nuclei and structures to be examined. Overall they are the hypoglossus, dorsal motor vagus, tractus solitary, ambiguus, inferior olivary, pre-Bötzinger, arcuate, dorsal and ventral cochlear, medial inferior vestibular, obscurus, and pallidus raphé nuclei in the medulla oblongata; locus coeruleus, facial/parafacial complex, retrotrapezoid nucleus, superior olivary complex, superior and lateral vestibular nuclei, Kölliker-Fuse, median, and magnus raphe nuclei in the caudal mesencephalon. In the spinal cord, the intermediate lateral nucleus is of great interest.

The neuropathological study includes also the examination of the cerebellum, in particular its cortex layers (external granular layer, molecular layer, Purkinje cell layer, and internal granular layer) and the medullary deep nuclei (dentate nucleus, fastigial nucleus, globose nucleus, and emboliform nucleus). All the nuclei and/or structures, earlier listed, have a tridimensional structure, with different extent into the brainstem, and they have been deeply examined in specific histological sections, selected on the basis of over 20 years of research activity in this field. These sections are easily achievable, following specific landmarks, such as the superior cerebellar peduncle decussation in the rostral pons, the medial nucleus of the superior olivary complex in the caudal pons and the dorsal accessory of the inferior olivary nucleus in the medulla oblongata. A diagnosis of hypoplasia of a given nucleus has been formulated when it showed a significant decrease in the number of neurons and/or a decreased area [7], compared to the mean values obtained from a group of age-matched controls, previously collected and stored at the *Lino Rossi* Research Center [8–12]. The morphometric evaluations were quantitatively performed using an Image-Pro Plus Image Analyzer (Media Cybernetics, Silver Spring, MD, USA). All the histological examinations of the brain samples have been carried out by two independent and blinded observers and the comparison of results performed employing K statistics (Kappa Index), to evaluate the interobserver reproducibility. The Landis and Koch system [13] for the K interpretation has been used, where 0–0.2 indicates slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 strong or substantial agreement, and 0.81-1.00 very strong or almost perfect agreement (a value of 1.0 being perfect agreement). Specific immunohistochemical techniques, according to the needs, have been used in order to evaluate the expression of the neuronal nuclear antigen (NeuN).

Genetic studies have been performed on fresh specimens of cerebral cortex treated with RNA-later using polymerase chain reaction method in order to analyze, in detail, the sero-tonin transporter (5-HTT) [14]. Direct evidence of exposure to environmental contaminants, such as pesticides (organochlorine and organophosphate pesticides, belonging to the endocrine disruptors) specifically used in agricultural areas of the Northeast Italy, have been evaluated in cerebral cortex samples through a gas chromatography-mass spectrometry (GC-MS) method [15]. The applied guidelines included also the removal of a lock of the victims' hair for the search of traces of cotinine, the main metabolite of nicotine, with the aim to demonstrate the maternal cigarette smoking habit.

#### Cardiopathological protocol

The protocol for the study of the cardiac conduction system essentially consists in the removal of two samples, including the sino-atrial node and the atrio-ventricular system, respectively. The first sample, or sino-atrial block, contains the junction of the superior vena cava and right atrium encompassing the entire area of the sino-atrial node. The second sample, or

atrio-ventricular junctional block, contains the atrio-ventricular node, the His bundle, its bifurcation and the bundle branches located in the superior two thirds of the interventricular septum. The sino-atrial block has been serially sectioned in a plane parallel to the *crista terminalis*. The atrio-ventricular junctional block has been serially sectioned in a plane parallel to the two atrio-ventricular valve rings. All sections have been cut at intervals of 40  $\mu$ m (levels). For each level, 5 sections have been retained, mounted, and stained alternatively with hematoxylin/eosin and Heidenhain's Azan trichrome.

#### Ethics

Ethics approval for this study was granted by the Italian Health's Ministry in accordance with the Italian Law no. 31. The parents of all the SIUDS victims provided written informed consent to autopsy and related researches under protocols approved by the Milan University's committee.

#### Results

All the results obtained in the present study have been compared with those of a wide series of control cases with known causes of death, previously collected at the *Lino Rossi* Research Center of the Milan University (Table 1). All the pathological results, obtained from the indepth anatomo-pathological examination of the brainstem, cerebellum, and cardiac conduction system, and the results of the immunohistochemical, genetic, and toxicological analyses, are summarized in Table 2. All the diagnoses, performed by experienced pathologists, according to the Landis and Koch system [13], revealed a very satisfactory Kappa Index (KI = 0.86).

# Neuropathological findings

In only eight cases, the marked autolysis of the brain did not allow us to analyze the main neuronal centers; in the brainstem of the remaining 35 cases, the hypoplasia of one or more nuclei of the raphe system (above all the obscurus raphe nucleus) was the more frequent alteration (Figure 2), seen in 15 of 35 SIUDS (43%). In 11 SIUDS victims (31%), the raphe nuclei hypoplasia was associated to the hypoplasia of the parafacial nucleus in the caudal pons.

Hypodevelopment of other nuclei was less common, such as the hypoplasia of the Kölliker– Fuse complex, present in eight SIUDS, the hypoplasia of the arcuate nucleus seen in six cases (Figure 3), the hypoplasia of the retrotrapezoid nucleus (three cases), the hypoplasia of both the superior and inferior olivary nuclei (four cases), and the hypoplasia of the pre-Bötzinger nucleus (two cases). Noteworthy was also the agenesis of the intermediate lateral nucleus in the spinal cord, detected in two cases. The cerebellar cortex was often relatively immature for age [16,17]. In no control case, the examination of the autonomic nervous system has disclosed developmental alterations.

Immunohistochemistry has revealed that the NeuN antigen is almost always unexpressed in SIUDS cases, precisely in 28 of the 35 analyzable cases (80%).

#### **Genetic findings**

Investigation in the nervous system for the polymorphisms of the 5-HTT gene, the major determinant of serotoninergic function, has highlighted a heterozygote genotype S/L in eight cases. In these cases, there was correlation with severe hypoplasia of the serotonergic raphe nuclei. More in detail, in eight cases, the hypoplasia of the obscurus nucleus was associated with the genotypic presence of the L allele.

Pathological results	Cases
Brain autolysis	8
Neuropathological findings (35 analyzable cases)	
Pons mesencephalon Hypoplasia of the parafacial nucleus Hypoplasia of the Kölliker–Fuse nucleus Hypoplasia of the retrotrapezoid nucleus Hypoplasia of the superior olivary nucleus	11 8 3 2
Medulla oblongata	
Hypoplasia of the raphe obscurus nucleus Hypoplasia of the arcuate nucleus Hypoplasia of the pre-Bötzinger nucleus Hypoplasia of the inferior olivary nucleus Hypoplasia intermediolateral nucleus Ependyma alterations	15 6 2 2 2 7
Spinal cord	
Agenesis of the intermediate lateral nucleus	2
Cerebellum	
Cortex delayed maturation	10
Immunohistochemical findings (35 analyzable cases) NeuN poor expression	28
Genetic findings (35 analyzable cases)	
5-HTT polymorphism	8
Toxicological findings (35 analyzable cases)	
p,p'-DDE p,p-DDT Chlorpyrifos Chlorfenvinphos $\alpha$ -chlordane $\gamma$ -chlordane Heptachlor	1 1 2 4 4 1
Cardiopathological findings (43 analyzed cases)	
Mahaim fibers Cartilaginous metaplasia of the central fibrous body Sino-atrial node malformations	15 7 2

Table 2. Pathological results from our case series: individual victims frequently displayed a combination of these alterations.



**Figure 2.** The hypoplasia of the obscurus raphe nucleus in a SIUDS victim, aged 40 gestational weeks, is noticeable (A, Klüver–Barrera stain;  $100 \times$ ); a transversal section of the medulla oblongata, showing the localization of the obscurus raphe nucleus, is also provided (A, insert;  $5 \times$ ). The normal structure of the obscurus raphe nucleus in an age-matched control case is highlighted by a red circle (B, Klüver–Barrera stain;  $100 \times$ ).



**Figure 3.** The hypoplasia of the arcuate nucleus in a SIUDS victim, aged 36 gestational weeks, is observable (A, Klüver-Barrera stain;  $100 \times$ ); a transversal section of the medulla oblongata, showing the localization of the arcuate nucleus, is also provided (A, insert;  $5 \times$ ). The normal structure of the arcuate nucleus in an agematched control case is pointed out by black arrows (B, Klüver-Barrera stain;  $100 \times$ ).

# **Cardiopathological findings**

The more frequent observation was the presence of accessory atrio-ventricular pathways, mainly Mahaim fibers (Figure 4), frequently associated with cartilaginous metaplasia of the central fibrous body in 15 SIUDS (35%). Noteworthy was the presence of a dualism of the sino-atrial node, observed in two cases.

# **Toxicological findings**

The analysis of the endocrine disrupting compounds (EDCs) in brain specimens has highlighted in two cases of SIUDS the presence of high concentrations of dichlorodiphenyldichloroethylene (p,p'-DDE) and dichlorodiphenyltrichloroethane (p,p'-DDE) amounting to 3.5 and 63.5 ng/g, respectively. Among the target analytes,  $\alpha$ -chlordane



**Figure 4.** An atrioventricular accessory Mahaim fiber, bridging the atrioventricular node (AVN) with the ventricular septum (VS), throughout the central fibrous body (CF), is pointed by a black arrow in a SIUDS case, aged 39 gestational weeks (Azan stain;  $200 \times$ ).

and  $\gamma$ -chlordane, in a concentration of 1.2 and 1.7 mg/g, have been detected in brain samples of further eight SIUDS. Moreover, the presence of chlorpyrifos and chlorfenvinphos, the two most common pesticides used in apple cultivation, has been ascertained in four SIUDS victims.

#### Correlation of findings with smoke exposure

A 2010 dated report of the World Health Organization indicates that about 250 million women in the world are daily smokers [18]. About 22% of women in developed countries and 9% of women in developing countries smoke tobacco; 18.4% of Italian women were smokers in this study, but no most recent reliable data are available. The overall percentage of smoking mothers of subjects who died in the perinatal period for known causes (representative of the percentage in the general population) was 21%. Contrariwise, 17 of our 43 SIUDS mothers (40%) were active smokers by their own admission or after the cotinine test. In all cases, developmental alterations of the central nervous system and/or of the cardiac conduction system were present.

#### Discussion

This work represents the results obtained in 5 years of collaboration, in particular between the APT and the *Lino Rossi* Research Center of the Milan University in a large series of fetal deaths, on the basis of a specific agreement between the two institutions.

The overall analysis of the neuropathological findings, obtained from the 35 analyzable cases of SIUDS, highlights, in 77% of cases, one or more congenital morphological abnormalities of the autonomic nervous system.

The hypoplasia of the parafacial nucleus and Kölliker–Fuse nucleus in the pons, together with hypoplasia of the obscurus raphe nucleus and arcuate nucleus in the medulla oblongata, has been the most frequent abnormalities observed.

The parafacial nucleus consists of "preinspiratory" neurons, as they trigger the neurons of the pre-Bötzinger nucleus in the medulla oblongata that have the main function of initiating each inspiratory breath [19–22]. In other words, the inspiratory activity is always preceded by firing of preinspiratory neurons.

The Kölliker–Fuse nucleus has an important function during intrauterine life, inhibiting the response of central and peripheral chemoreceptors, which are already fully formed and potentially functional, and therefore any respiratory reflex. After birth, the Kölliker–Fuse abruptly reduces its inhibitory effects and becomes active as a respiratory center able to coordinate the pulmonary motor responses to hematic oscillations of pO<sub>2</sub>, pCO<sub>2</sub>, and pH [23,24]. Also the arcuate nucleus contributes to this chemoreceptive activity in the modulation of the eupneic breathing [25].

The hypodevelopment of these structures, all involved in the respiratory activity, in our fetal deaths, suggests a role in the SIUDS pathogenesis. It is reasonable to speculate whether alterations of the respiratory nuclei can lead to death during intrauterine life. It is known that occasional respiratory movements occur before birth, indispensable for the lung development. Therefore, the hypoplasia of the respiratory nuclei could determine defects of this occasional ventilatory activity in the prenatal life. This is supported by the frequent finding of lung hypoplasia in fetal deaths [26]. A normal breathing activity becomes vital only after birth, while the aforementioned breathing alterations in pregnancy can be related to lung

hypoplasia, but they appear not sufficient to justify a fetal death. One possibility is that the parafacial nucleus and other neuronal structures of the respiratory network participate not only in breathing control but, more extensively, in the modulation of all vital functions, the cardiocirculatory activity included. Another hypothesis could be that, in the last weeks of pregnancy, advancing toward the time of birth, a general check of all neuronal centers essential for extra-uterine life occurs. Intrauterine unexplained sudden deaths could be, therefore, ascribed to a selective process of natural suppression in the presence of developmental alterations of these vital centers.

Noteworthy was also the frequent hypoplasia of the raphe system, in particular of the obscurus raphe nucleus in the medulla oblongata (observed in 15 cases). The raphe neurons, known as major producers of serotonin and responsible for the serotonergic transmission during intrauterine life, play a trophic role in the neuronal development of the fetal brain [27,28]. A dysfunction in serotonergic transmission during intrauterine life could affect all neuronal structures checking vital functions, thus causing the fetal death [29]. A notable datum was the observation that in eight cases the hypoplasia of the obscurus nucleus was associated with the genotypic presence of the L allele in the 5-HTT, quite rare in the general population, suggesting the involvement of a serotonin polymorphism in the development of the raphe system.

SIUDS may also occur in the setting of developmental disturbances of the cardiovascular system. These disturbances can interfere with the conduction of impulses, as in the cases of cartilaginous metaplasia of the fibrous body, observed in seven cases. Arrhythmias, caused by microscopic alterations of the cardiac conduction system, such as accessory atrio-ventricular communications (Mahaim fibers), are quite frequently associated with perinatal unexplained death. These lesions have been attributed to the variable outcome of a resorptive degenerative process that normally reshapes the functional pathways of the cardiac conduction system in the fetal and early neonatal period [30]. The Mahaim fibers, under particular conditions and/or neurovegetative stimuli, are liable to suddenly provoke electrical dyshomogeneity, instability and desynchronization, raising the risk of malignant functional arrhythmias [30]. However, a clinicopathologic assessment of their lethal arrhythmogenic potential is usually impossible. Clinically, these abnormalities can be present without hemodynamic impairment or detectable congestive heart failure. In our study, Mahaim fibers have been detected in 15 SIUDS (35%), and in all the cases the malignant arrhythmias leading to sudden death were not foreseeable.

From the analysis of the risk factors reported in the filled forms for the mothers, exogenous risk factors, affecting the intrauterine environment, are relevant, such as maternal smoking, maternal alcoholism, abuse of drugs, and likely atmospheric pollution [31-33]. In particular, exposure to tobacco smoke *in utero* is the most important preventable risk factor. The smoking, in fact, causes vasoconstriction and reduces the fetal oxygenation and blood flow [34–37]. In addition, air pollution, which features high rates of both gases (carbon monoxide, nitrogen dioxide, ozone, and sulfur dioxide) and particulate matter (above all PM<sub>10</sub>, with a median diameter of  $<10 \,\mu$ m), could have an important influence in determining SIUDS [32]. We have already postulated that pollutants, cigarette smoke included, can cross the placenta during pregnancy through the maternal blood, and lead to a hypoxic status responsible for structural and/or functional impairments of the central nervous system [38,39]. Moreover, the EDC, including a variety of chemicals such as pesticides, dioxins, and furans [39,40], have been recently defined environmental risk factors for SIUDS, given their recognized adverse effect on fetal growth and above all on brain development [38-40]. All these noxious agents in perinatal life cross the still immature fetal blood-brain barrier, the major interface between the blood and nervous system [39]. In this study, we have observed that 17 SIUDS victims 10 👄 L. RONCATI ET AL.

had a smoking mother, confirming the harmful role of the smoke in pregnancy [38]. In addition, traces of EDC, such as the pesticides used in the agricultural areas of the Northeast Italy, have been detected in brain specimens of 15 SIUDS cases [39,41]. In conclusion, we underline the need to perform a complete autopsy of all SIUDS victims with an in-depth examination of the neuronal centers of the brainstem which modulate the vital functions. The prevention of these deaths can be based only on a better recognition of the abnormalities found in the nervous system, indispensable to highlight the basic underlying mechanisms. Since this problem is highly complex, it is extremely important to obtain an exhaustive diagnosis of each case, together with full details of the familial and circumstantial background to the demise. The scientific advantages deriving from a better understanding of perinatal loss are certainly extremely significant. The data and arguments here illustrated provide an invitation to motivate all the professional involved in this field of interest (pathologist, gynecologist, obstetricians, neonatologist, and pediatrics). The systematic assessment of the risk factors will allow the best, most current information to the population through public education and prevention programs aimed at decreasing SIUDS.

# **Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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