Developmental neuropathology of brainstem respiratory centers in unexplained stillbirth: What’s the meaning?

Anna M. Lavezzi\textsuperscript{a,\ast}, Stefano Ferrero\textsuperscript{a,\ast}, Luigi Matturri\textsuperscript{a}, Luca Roncati\textsuperscript{c,d}, Teresa Pusiol\textsuperscript{c}

\textsuperscript{a} "Lino Rossi" Research Center for the Study and Prevention of Unexpected Perinatal Death and SIDS; Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy
\textsuperscript{b} Division of Pathology, Fondazione IRCCS Ca’ Granda, Ospedale Maggiore Policlinico, Milan, Italy; Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy
\textsuperscript{c} Institute of Pathology, Hospital of Rovereto (Trento), Italy
\textsuperscript{d} Department of Diagnostic and Clinical Medicine and of Public Health, Section of Pathology, University of Modena and Reggio Emilia, Policlinico Hospital, Modena, Italy

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\textbf{A B S T R A C T}

Stillbirth is one of the most stressful life events affecting over 3 million pregnancies per year throughout the world. An accurate autopsy of the stillborn fetus, including the placenta and umbilical cord examination, should be performed promptly after delivery. A thorough maternal history also should be taken, including exposures to risk factors. In many cases a death cause, attributable to fetal, maternal, or placental pathology, is clearly identified. However, in 50% or more of cases the cause remains unknown.

The purpose of this study is to highlight possible developmental alterations of the autonomic nervous system in unexplained stillbirths to provide an explanation of the pathogenetic mechanism of their death.

We conducted a careful neuropathological study of the brainstem, where the main vital centers are located, in 85 unexplained stillbirths and 52 age-matched controls died of known cause. Information on the maternal lifestyle, including the smoking habit, was collected in all cases. Hypodevelopment of neuronal centers involved in breathing control, all connected together in a "respiratory network", precisely hypoplasia of the facial/parafacial complex, Kölliker-Fuse nucleus, pre-Bötzinger nucleus and intermedio-lateral nucleus, were frequently observed in unexplained deaths, significantly related to maternal cigarette smoking.

We support the hypothesis of a strong action of maternal smoking during pregnancy on the development of brainstem respiratory nuclei and suggest an explanation of the high incidence of the respiratory network alterations in unexplained fetal death, when breathing not represents a vital function.

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\textsuperscript{\ast} Corresponding author at: "Lino Rossi" Research Center for the Study and Prevention of Unexpected Perinatal Death, SIDS—Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via della Commenda 19, 20122 Milano, Italy.
E-mail address: anna.lavezzi@unimi.it (A.M. Lavezzi).

1. Introduction

Stillbirth is generally considered as the death of a baby before or during delivery, or in any case before their first birthday (Fretts, 2009; Goldenberg et al., 2004). Each year about 24,000 babies are stillborn in the United States and over 3 million throughout the world (Macedo and Gregory, 2015). Because of its apparent randomness, and the lack of any warning, stillbirth cuts across socio-economic classes, ethnicities, religions, and maternal age groups. However, even if no woman is immune from stillbirth, there are some maternal factors associated to the condition that can increase the risk for stillbirth, including black race, age under 20 or over 35 years, obesity, nulliparity or a previous miscarriage (McClure et al., 2009; Flenady et al., 2011; Yudkin et al., 1987; Liu et al., 2014). A well-documented risk factor for unexplained stillbirth is maternal cigarette smoking during pregnancy. A huge number of works in the literature have, in fact, reported the harmful effects exerted by the crossing of nicotine into the bloodstream of the fetus (Lambers and Clark, 1996; Marufu et al., 2015; Raymond et al., 1994; Wickström, 2007).

It is universally recognized that the most important test in the search for a possible death cause in stillbirth is gross and microscopic fetal autopsy, complete with placental, umbilical cord and membrane examination, genetic analyses, and a detailed medical...
history evaluation (Faye-Petersen et al., 1999; Saller et al., 1995; McPherson and Valdes-Dapena, 1998; Bove, 1997).

Known causes of stillbirth can generally be subdivided into three broad categories, namely: 1) congenital fetal defects (including genetic abnormalities), 2) placenta and/or umbilical cord pathologies and 3) maternal disorders (such as obesity, hypertension, diabetes, etc.) (Bendon, 2001; Bukowski et al., 2011; Fatima et al., 2014).

However, despite the progress in medical diagnostics and antenatal care over the last 30 years, after a careful investigation one-half to two-thirds of stillbirths are still listed as deaths for undeterminable reasons. Because these deaths cannot be attributed to a specific identifiable cause, they are called “unexplained stillbirths” (Gardosi et al., 2005; Yudkin et al., 1987; Warland and Mitchell, 2014). We have proposed that a sudden death during pregnancy, that remains unexplained after a thorough study, should also be considered as a syndrome and referred to with the acronym “SIUDS”, i.e., “Sudden Intrauterine Unexplained Death Syndrome”, like “SIDS” for “Sudden Infant Death Syndrome” (Matturri et al., 2014). This suggested definition is based on the realization that several conditions simultaneously occurring may contribute to a given stillbirth and on the presence in these unexplained deaths of common developmental abnormalities of the autonomic nervous system, associated to the same risk factors related to SIDS.

It is necessary to point out that the lack of uniform post-mortem protocols applied worldwide for evaluating and classifying stillbirths has hindered significant studies in this field.

The “Lino Rossi” research Center of Milan University has developed an investigative postmortem protocol that includes, in particular, an in-depth examination of the autonomic nervous system (available at the webpage http://users.unimi.it/centrolinorossi/en/guidelines.html), according to the Italian Law 31/2006 “Regulations for Diagnostic Post Mortem Investigation in Victims of Sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death.” This law states in particular that all stillbirths that died suddenly without any apparent cause after 25 weeks of gestation must be submitted to a depth diagnostic postmortem investigation.

Herein, we report the autonomic nervous system findings obtained according to the above-mentioned protocol in a wide group of stillbirths previously diagnosed as “unexplained” after a routine fetal autopsy. Surprisingly, we frequently have observed in the brainstem of these victims hypoplasia of neuronal centers known to be involved in the autonomic control of breathing. So, our main purpose was to provide an explanation of the high incidence of these developmental alterations, also suggesting how they can lead to death in the womb, before breathing is yet required for life.

2. Material and methods

We studied 85 stillbirths, aged from 25 to 40 gestational weeks, sent to the “Lino Rossi” research Center of the Milan University from hospitals and health Institutions of Italian regions, in accordance with the abovementioned national Law 31/2006.

The fetuses had previously been submitted to a complete autopsy, including examination of the placental disk, umbilical cord and membranes, but without identifying a possible death cause. So, they were classified as “unexplained stillbirths” or, more appropriately, “SIUDS”.

For each case, a complete clinical history, with particular reference to the maternal lifestyle, was given. None of the mothers of the 85 victims had any significant pathology. The mothers were also asked for information about a smoking habit before and during pregnancy. Forty-eight mothers (56%) claimed to be active smokers before and during pregnancy, while 37 (44%) declared no history of cigarette smoking. Since, as is well known (Shipton et al., 2009), retrospective assessment of a mother’s smoking habit, mainly after the death of a child, is sometimes unavoidable, the negative self-reports were verified by testing for cotinine, the main metabolite of nicotine, that has a long half-life, in the hair of victims. In 4 stillbirths among the 37 mothers who denied a smoking habit the cotinine-test was positive, thereby leading to a total of 52 (61%) of the cases with nicotine absorption in pregnancy and reducing the actual number of non-smoking mothers to 33 (39%).

For every case, the material to be sent to the “Lino Rossi” Center consists of the brain, heart (for the specific study of the cardiac conduction system), a lock of the victim’s hair and, when possible, lung samples to evaluate the pulmonary maturation stage. A group of 52 fetuses who died of known causes, selected from a wide set of cases previously collected at the “Lino Rossi” Research Center, were designated as “Controls”. The same material and information, including the smoking habit, required for unexplained stillbirths was sent for the control cases from the medical Institutions involved in application of Law 31, for the specific purpose of carrying out comparative analyses.

The Controls were matched with the unexplained stillbirths for gestational age, sex and area of origin. Table 1 summarizes the SIUDS/Control case study, indicating the age-ranges, sex distribution and the death diagnoses.

Consent- Parents of all the infants included in the study provided written informed consent to autopsy and related researches; study approval was granted by the institutional review board of the Milan University (Lino Rossi Research Center).

2.1. Protocol for the anatomopathological study of the brain

After fixation in 10% phosphate-buffered formalin, the brains were processed and embedded in paraffin. Target of this study was the in-depth microscopic study of the brainstem. Transverse serial sections of the midbrain,pons, medulla oblongata and spinal cord (rostral cervico-thoracic tract), where the main structures controlling the vital functions are located, were made at intervals of 60 µm. For each level, six-seven 5 µm sections were obtained, two of which were stained using hematoxylin-eosin and Klüver-Barrera for histological examination, while two sections were treated for immunohistochemical detection of the neuronal nuclear antigen (NeuN), a marker of neuronal functionality. The remaining sections were saved for further investigations and stained as deemed necessary.

The routine histological evaluation of the brainstem was performed on the locus coeruleus, Kölßiker-Fuse nucleus, and median raphé nucleus in the rostral pons/mesencephalon; on the parafacial/facial complex, superior olivey complex, ambiguous, pre-Bötzingert, inferior olivary, arcuate, obscurus, pallidus raphé nuclei and solitary tract complex in the medulla oblongata; the intermediolateral nucleus in the spinal cord junction with the brainstem.

Many of these nuclei (and precisely the Kölßiker–Fuse nucleus, the facial/parafacial complex, the pre-Bötzingert nucleus and the intermediolateral nucleus) are anatomically and functionally interconnected via interneuronal synapses, modulating one another, within a network extending from the rostral spinal cord through the medulla oblongata, pons, to the caudal pars of the midbrain. This nervous complex is defined as “respiratory network” (RN), given its primary involvement in the control of breathing (Cohen, 1979; Bianchi et al., 1995; Viemari et al., 2003).

Fig. 1 presents a scheme of the human breathing control mechanism in perinatal life, proposed by one of the Authors in a recent paper (Lavezzi, 2015), indicating the more representative
In the human fetus, episodic respiratory activity aimed at promoting lung development is generated by the intermediolateral nucleus (ILN) in the upper spinal cord. At the same time, during intrauterine life, the Kölliker–Fuse nucleus (KFN), located in the rostral pons, plays an important function by inhibiting the response of central and peripheral chemoreceptors and therefore any respiratory reflex, while allowing the occasional breathing activity headed by the ILN. The facial/parafacial complex (F/PFC), in the caudal pons, starts working at birth, under the stimulation of the KFN, which drastically changes its function, giving rise to the first inspiratory act. The activity of the F/PFC is called “pre-inspiratory” because it is limited to activating, in its turn, the proper inspiratory nucleus in the medulla oblongata: the pre-Bötzinger nucleus (pBN) (4), responsible for starting postnatal breathing. (B) Brainstem schematic representation showing the localization of the RN components. (from: Lavezzi, Front. Neuro. 2015, 6, 220).

2.2. Neuropathology diagnostic criteria

All the above-listed nuclei and/or structures are examined in serial histological sections throughout their extension. A diagnosis of “hypoplasia” of a given nucleus is formulated when it shows a significantly decreased number of neurons and/or decreased area in transverse histological sections, compared to the mean values obtained in age-matched controls. The morphometric evaluations are quantitatively performed using an Image-Pro Plus Image Analyzer (Media Cybernetics, Silver Spring, MD).

All the histological examinations of the brain are carried out by two independent and blinded observers and comparison of the results performed employing K statistics (Kappa Index–KI) to evaluate the inter-observer reproducibility. The Landis and Koch system (Landis and Koch, 1977) for the K interpretation is used, where 0–0.2 is slight agreement, 0.21–0.40 indicates fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 strong or substantial agreement, and 0.81–1.00 indicates very strong or almost perfect agreement (a value of 1.0 being perfect agreement). The application of this method in the present study revealed a very satisfactory KI (0.86).

2.3. NeuN immunohistochemistry

Representative sections from paraffin-embedded tissue blocks were stained using commercially supplied mouse monoclonal antibodies against the neuronal nuclear antigen NeuN (Chemicon International, MAB377). A standard avidin-biotin complex (ABC) technique was used with peroxidase-diaminobenzidine to visualize and develop the antigen-antibody reaction. The antibody dilution was 1:1500. Incubating solutions were boiled in 10 mM citric acid at pH 6.5, in a microwave oven, for 5 min at high power, then 5 min at 50% power, and finally cooled for 20 min. Sections were lightly counterstained with Mayer’s hematoxylin. Negative controls were prepared by replacing the primary antibody with phosphate-buffered saline (PBS) in the incubation. In these procedures staining always failed to occur.

1. These selected histological sections, identified on the basis of over twenty-years of research at the Lino Rossi Center in this field, are easily recognizable on the basis of precise landmarks, that are the superior cerebellar peduncle decussation in the rostral pons (for the Kölliker–Fuse nucleus analysis), medial nucleus of the superior olivary complex in the caudal pons (for the facial/parafacial complex), dorsal accessory of the inferior olivary nucleus in the medulla oblongata (for the pre-Bötzinger nucleus) and dorsal and ventral horns, adjacent to the central canal in the rostral spinal cord (for the intermediolateral nucleus).
2.3.1. Evaluation of the NeuN immunohistochemical results

Even if in health status the NeuN immunopositivity is diffused in almost the entire brain, we specifically examined at the light microscope the areas including the nuclei of interest.

For every structure, only cells with intense brown immunostaining were considered to be really positive. Moreover, also a weak brown intensity was taken into account.

We quantified the scoring using a x 40 lens, as follow:

- = no positive cell (negativity)
-/+ = a number of cells with only weak positivity ≤30% per unit area (weak positivity)
+ = a number of positive cells ≤30% per unit area (moderate positivity)
++ = a number of positive cells >30% per unit area (strong positivity)

The unit area was represented by square millimeter (mm²).

2.4. Protocol for the histological examination of the lungs

The stage of lung development in each case was evaluated according to microscopic criteria, i.e. the presence of cartilaginous bronchi up to the distal peripheral level and the radial alveolar count (RAC). This last is a reliable index of lung maturation in intrauterine life, closely related to the gestational age in weeks (Emery and Mithal, 1960).

The RAC was evaluated in terminal lung units (distal portions of parenchyma to the last respiratory bronchioles, identifiable by an incomplete epithelial lining), examining at least 10 random histological fields for each case in order to estimate the number of airspaces cut by a straight line drawn from the center of the most peripheral bronchiole to the nearest connective tissue septum or the pleura. Emery and Mithal had also provided the reference RAC values at different ages, as reported in Table 2.

2.5. Statistical analysis

Quantitative data were expressed as means ± SEM. The significance of differences between different parameters in the groups (SIUDS and Controls) were evaluated by Student-t test, chi-square test or Fisher’s test. In cases of a skewed distribution, a nonparametric Whitney rank sum test was applied. One-way ANOVA is used for quantifying and partitioning variance between groups. Statistics was performed using SigmaPlot® statistical software (version 13, Systat Software Inc, Chicago, IL). The selected level of significance was p < 0.05, two-tailed.

3. Results

The histological examination performed on the brainstem of 85 SIUDS children showed a marked hypodevelopment of one or more structures making up the RN in 75% of cases. Hypoplasia of the facial/parafacial complex in all its extension into the caudal pons was observed in 51 SIUDS (60%), frequently associated to hypoplasia of the pre-Bötzing nucleus in the medulla oblongata. Hypoplasia of the Kölliker–Fuse nucleus was present in 40 SIUDS (47%), sometimes as the only alteration, and of the intermediolateral nucleus in 36 cases (42%). Figs. 2 and 3 illustrate the cytoarchitecture of the two developmental alterations more frequently observed (i.e. hypoplasia of the facial/parafacial complex and hypoplasia of the Kölliker–Fuse nucleus) in the specific transversal histological sections.

Other developmental defects were the hypoplasia of the arcuate nucleus in the medulla oblongata, observed in 15 cases (13%), almost always associated to hypoplasia of one or more raphé nuclei, and hypoplasia of the pontine retrotropezoid nucleus, detected in 8 cases (9%). Individual victims frequently displayed a combination of all the above mentioned alterations.

A high correlation was evident between hypoplasia of the RN components and the lack of NeuN expression. In fact, in 62 SIUDS with various developmental morphological defects of respiratory nuclei, only immunonegativity or, more rarely, weak positivity of the NeuN protein was found.

In Controls, the examination of the brainstem did not highlight developmental alterations of the RN centers, except for two cases (both died of severe choorioamnionitis) in which there was partial hypoplasia of the Kölliker–Fuse nucleus. Arcuate nucleus hypoplasia was observed in 7 control cases (13%) and hypoplasia of the obscurus raphé nucleus in 3 cases (6%).

The degree of maturation of the pulmonary parenchyma was evaluated in order to highlight a possible correlation between anomalies of the RN and lung hypodevelopment. In 28 SIUDS with hypoplasia of the intermediolateral nucleus and/or hypoplasia of the Kölliker–Fuse nucleus, an interesting finding was the lung hypoplasia, characterized by a markedly decreased RAC index (almost always below 2.2) and frequent presence of cartilaginous bronchi at peripheral level. The lung developmental stage was, on the contrary, above the reference values in nearly all control cases. Only in two fetuses, who died at the 36th and 38th gestational week, respectively, a RAC value corresponding to 3.0 was obtained (normal reference value for age = 3.6).

Finally, we related the neurodevelopmental defects observed in the brainstem to the mother’s smoking habit. A significant correlation was evident between maternal smoking and delayed maturation of respiratory nuclei in SIUDS, compared to controls (p < 0.01). In fact, in all SIUDS with a smoker mother, hypoplasia of one or more components of the RN was highlighted.

Table 3 summarizes all the results.

4. Discussion

Stillbirth, occurring in particular in the last trimester of an uncomplicated pregnancy, when the developing baby could survive outside the womb, is a very distressing event. Parents want to know why their baby has died, so clinicians should be ready to call for an autopsy in cases of stillbirth, also considering that the results may be valuable in planning future pregnancies.

However, despite the recognized importance of a thorough autopsy in cases of stillbirth so as to individuate the morphological substrate of death, the rate of autopsies is very poor worldwide. In addition, there is no uniform stillbirth post-mortem protocol in use anywhere today, and every autopsy is done according to local practice (Chichester, 2007; McPherson and Valdes-Dapena, 1998).

This state has been improved in Italy, thanks to Law 31/2006 that imposes, in cases of death of a fetus without any apparent cause after the 25th week of gestation, an autopsy performed in the referral centers of each Region following unified guidelines and, when the diagnosis is not clarified (i.e. in cases of “unexplained stillbirth”), the autopsy samples, mandatorily including the brainstem, must be sent to the “Lino Rossi” Research Center of Milan University for further in-depth studies (Roncati et al., 2016a).

Here we illustrate how the thorough histological examination of the brainstem may reveal specific developmental defects in these deaths. In fact, in a wide range of SIUDS we very frequently observed hypoplasia of neuronal centers that preside over respiratory function, namely of the Kölliker–Fuse nucleus and facial/parafacial complex in the pons, the pre-Bötzing nucleus in the medulla oblongata and the intermediolateral nucleus in the upper spinal cord, all components of the so-called “respiratory network” (RN). These centers can coordinate each other through excitatory and/or inhibitory connections, in relation to the need to control breath-
Table 2
Histological diagnostic criteria of lung hypoplasia in fetuses.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Gestational weeks</th>
<th>RAC normal reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Radial alveolar count (RAC)(^a) minor than reference value for gestational week</td>
<td>24–27</td>
<td>2.2±</td>
</tr>
<tr>
<td></td>
<td>28–31</td>
<td>2.6±</td>
</tr>
<tr>
<td></td>
<td>32–35</td>
<td>3.2± 0.9</td>
</tr>
<tr>
<td></td>
<td>36–39</td>
<td>3.6± 0.9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4.4±</td>
</tr>
<tr>
<td>2) presence of cartilaginous bronchi up to the distal level</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) This parameter represents the mean number of alveoli transected by a perpendicular line drawn from the center of the most peripheral bronchiole (recognizable by not being completely covered by epithelium) to the pleura or the nearest interlobular septum. Below, an histological picture showing the RAC method.

Fig. 2. (A) Representative histological section of caudal pons showing the localization of the facial/parafacial complex; (B) normal cytoarchitecture of this nucleus; (C) Hypoplasia of the Facial/Parafacial complex in a SIUDS case (36 gestational weeks). Klüver-Barrera staining. Magnification: (A): 0.5x; (B) and (C): 20x.

ing before and after birth. Based on experimental studies, we have hypothesized a chronological functional sequence of action of these nuclei (as depicted in Fig. 1). The intermediolateral nucleus has the task of promoting the initial episodic breathing patterns in early fetal developmental stages and the spread of these spontaneous bursts into the spinomedullary neuronal axis to the other respiratory centers, even before the establishment of a synaptic drive, through the release of neurotrophic signals, transmitted by an extracellular ionic flux (Hanson and Landmesser, 2003; Ren and Greer, 2003).

The intermediolateral nucleus then plays a leading role by inducing the early rhythmic respiratory movements aimed, above all, to promote lung development in utero and then to monitor the respiratory output pattern in postnatal life.

During intrauterine life, at the same time, the Kölliker–Fuse nucleus plays an important function by inhibiting any respiratory reflex, allowing only, at intervals, the occasional breathing activity triggered by the intermediolateral nucleus (Damasceno et al., 2014). At birth, the Kölliker–Fuse nucleus function changes drastically, giving rise to the first inspiratory act through stimulation of the facial/parafacial complex which, in turn, triggers the pre-Bötzinger nucleus, the effective activator of breathing (Cohen, 1979; Bianchi et al., 1995; Viemari et al., 2003).

Other alterations highlighted in this study in addition to hypoplasia of the RN components, although with a lesser frequency,
Fig. 3. (A) Representative histological section of rostral pons showing the localization of the Kölliker–Fuse nucleus; (B) normal cytoarchitecture of this nucleus; (C) Hypoplasia of the Kölliker–Fuse nucleus in a SIUDS case (38 gestational weeks). Klüver-Barrera staining. Magnification: (A): 0,5x; (B) and (C): 20x.

Table 3
Pathological results from case series: individual victims frequently displayed a combination of these alterations. In all, 75% of SIUDS (n=64 cases) and 15% (n=8 cases) of Controls showed developmental alterations of one or more respiratory nuclei.

<table>
<thead>
<tr>
<th>Pathological Results</th>
<th>SIUDS (85 cases)</th>
<th>Controls (52 cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoplasia of brainstem nuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory network</td>
<td></td>
<td></td>
</tr>
<tr>
<td>facial/parafacial complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kölliker-Fuse nucleus</td>
<td>51 (60%)</td>
<td>–</td>
</tr>
<tr>
<td>pre-Bötzing nucleus</td>
<td>40 (47%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>intermediolateral nucleus</td>
<td>22 (26%)</td>
<td>–</td>
</tr>
<tr>
<td>raphé nuclei</td>
<td>36 (42%)</td>
<td>–</td>
</tr>
<tr>
<td>Other nuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>arcuate nucleus</td>
<td>15 (18%)</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>raphé nuclei</td>
<td>11 (13%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>retrotrapezoid nucleus</td>
<td>8 (9%)</td>
<td>–</td>
</tr>
<tr>
<td>NeuN poor/negative immunoexpression</td>
<td>62 (73%)</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>Lung hypoplasia (RAC)</td>
<td>28 (33%)</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Maternal smoking in pregnancy</td>
<td>52* (61%)</td>
<td>10* (19%)</td>
</tr>
</tbody>
</table>

* In all 52 SIUDS cases, and in 3 of the 10 Controls with a smoking mother, developmental alterations of one or more respiratory nuclei were present.

were developmental defects of the arcuate, the retrotrapezoid and the raphé nuclei, all involved to varying degrees in chemoreception, a fundamental mechanism for an efficient uptake and delivery of oxygen and removal of carbon dioxide (Wong-Riley and Liu, 2005; Nattie, 1998; Feldman et al., 2003). Then chemoreception takes part in the respiratory control system, preserving normoxic ventilator parameters through a variety of responses.

The hypodevelopment of the respiratory nuclei in SIUDS was also combined with a low viability of neurons, testified by the immunonegativity of the NeuN antigen, a specific protein that is only expressed in post-mitotic healthy neurons (Sarnat et al., 1998). It has been reported that a decreased NeuN expression in postembryonic life can be indicative of neuronal degeneration, often resulting from a severe injury (Unal-Cevik et al., 2004).
From the analysis of the overall findings here obtained, at least two inevitable questions arise:

1) why the developmental abnormalities of the brainstem respiratory centers are significantly increased in unexplained fetal deaths, compared to controls, if breathing is not a vital function in utero? We suggest the following possible explanation on the basis of our experience: since, as well known, the fetal brain is particularly vulnerable to injuries in critical phases of its development, we detain that the respiratory centers are more susceptible to environmental factor exposure than other neuronal structures and so are more involved in the mechanism of intrauterine sudden death. Given that, in our study, all cases with a smoker mother showed these specific alterations, we are prone to believe that nicotine represents the major triggering factor.

In cases of maternal smoking in pregnancy, carbon monoxide, a gaseous combustion product of nicotine, may readily cross the placenta and bind to fetal hemoglobin (Lambers and Clark, 1996; Aubard and Magne, 2000; Prockop and Chichkova, 2007). The consequent carboxyhemoglobin is not able to release oxygen, causing an altered physiological development of fetal organs and tissues, especially those most susceptible to hypoxic damage, including the brain. In addition, nicotine is one of the few lipid-soluble substances that, by passing through the blood-brain barrier by passive diffusion, can act directly on the expression of genes checking the developing nervous system, leading to hypoplasia of the main vital centers (Lavezzi et al., 2003; Lichtensteiger et al., 1988; Gressens et al., 2003).

However, we cannot exclude the involvement of other environmental risk factors, too, such as air pollution in the defective brainstem development (Calderón-Garcidueñas et al., 2002; Genc et al., 2012; Roncati et al., 2016b).

2) The second question concerns the indisputable link that we observed between altered development and, obviously, function of the respiratory centers and stillbirth. While death related to improper breathing control is understandable in SIDS, it is reasonable to speculate whether breathing alterations can lead to death during intrauterine life, when breathing is not yet a vital condition.

This is our own theory: in the last weeks of pregnancy, advancing towards the time of birth, a general check of all the neuronal centers essential for extra-uterine life occurs. Sudden unexpected late fetal deaths could therefore be ascribed to a selective process of self-suppression in presence of developmental alterations particularly of the respiratory centers, preventing the even more serious and stressful event for parents of a neonatal death.

5. Conclusions

The developmental defects of the brainstem nuclei involved in the breathing control that we have identified in unexplained stillbirths can have serious consequences, ranging from pulmonary hypodevelopment that can, however, be compatible with fetal life, to deficits in essential autonomic functions, particularly respiratory activity, that can lead to sudden death.

Therefore, a systematic evaluation of the brainstem, possibly performed by experienced, reliable pathologists, is extremely important to highlight any possible pathogenic mechanism in SIUDS.

A last consideration in terms of preventing stillbirth: the good news is that even if many of the risk factors for unexplained stillbirth cannot be changed (such as race or a previous miscarriage), many others, and in particular cigarette smoking, are modifiable. Women planning a pregnancy should try to optimize their health, at least taking care to refrain from smoking, knowing that nicotine quickly passes in the fetal nervous system and first of all acts on the nervous centers that will be essential for the life of their child.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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