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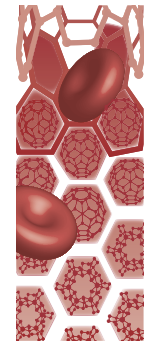

Novel chemical-physical autopsy investigation in sudden infant death and sudden intrauterine unexplained death syndromes

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Aim: Verify the presence of inorganic nanoparticle entities in brain tissue samples from sudden infant death syndrome (SIDS)/sudden intrauterine unexplained death syndrome (SIUDS) cases. The presence of inorganic debris could be a cofactor that compromises proper brain tissue functionality. **Materials & methods:** A novel autopsy approach that consists of neuropathological analysis procedures combined with energy dispersive spectroscopy/field emission gun environmental scanning electron microscopy investigations was implemented on 10 SIDS/SIUDS cases, whereas control samples were obtained from 10 cases of fetal/infant death from known cause. **Results:** Developmental abnormalities of the brain were associated with the presence of foreign bodies. Although nanoparticles were present as well in control samples, they were not associated with histological brain anomalies, as was the case in SIDS/SIUDS. **Conclusion:** Inorganic particles present in brain tissues demonstrate their ability to cross the hemato–encephalic barrier and to interact with tissues and cells in an unknown yet pathological fashion. This gives a rationale to consider them as cofactors of lethality.

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Keywords: inorganic particulate matter • nanoparticle electron microscopy • neuropathology • SIDS/SIUDS

Sudden intrauterine unexplained death syndrome (SIUDS) and sudden infant death syndrome (SIDS) are unresolved social and health problems even today [1–3]. There are no specific pathognomonic or diagnostic findings identified so far at the routine autopsy of SIUDS and SIDS victims. Cause of death remains largely unexplained; however, in-depth brain examination has increasingly disclosed a variety of developmental abnormalities of brain centers that are crucial for the control of autonomic and cardiorespiratory functions. Such structures include the Kölliker-Fuse nucleus, the facial/parafacial complex in the pons and the pre-Bötzinger nucleus in the medulla oblongata [4–6]. Defective synthesis and deregulated expression of various neurochemicals (catecholamines, serotonin, somatostatin, orexin, nicotinic acetylcholine receptors, growth factors etc.) are frequently found in both SIUDS and SIDS [7–11]. However, morphological and functional alterations highlighted in SIUDS and SIDS are frequently not sufficient to explain their etiopathogenesis. It is increasingly clear that sudden fetus or infant deaths are due to multiple factors. Before the modern age of metabolic diseases, death was caused mostly by environmental events, infection, pollution or disasters. Today, in the Pandora's box of environmental pollutants influencing human well-being, one would need to add nano-sized environmental pollutants (NEPs), whose abundance and significance increased dramatically in the past few years. NEPs derived from nanomaterials used in biomedicine, biotechnology and environmental industry [12,13] are known to cross the blood–brain barrier [14]. As metal nanoparticles have already been found in fetal kidney and liver tissues [15], it is easy to suspect that if taken up by a pregnant woman (through inhalation, ingestion, injection, skin contact etc.), NEPs can enter the mother's blood, easily cross the

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Table 1. List of sudden infant death syndrome, sudden intrauterine unexplained death syndrome and control cases with their brief history.

Case no.	Sex/age	Death cause	Mother's history			
			Pathologies	Smoking during pregnancy	Drug/alcohol during pregnancy	Cigarette use
SIDS 1 (snud)	F / 1 pn h	Unknown	Hashimoto thyroiditis, Addison disease	No	No	No
SIDS 2	F / 1 pn month	Unknown	No	Yes	Paracetamol, alcohol	Yes
SIDS 3	M / 1 pn month	Unknown	Obesity	No	No	No
SIDS 4	M / 2 pn months	Unknown	No	No	?	No
SIDS 5	F / 6 pn months	Unknown	No	No	No	Yes
SIUDS 1	M / 36 gws	Unknown	2 previous miscarriages	No	No	No
SIUDS 2	M / 37 gws	Unknown	No	No	Alcohol	Yes
SIUDS 3	M / 38 gws	Unknown	No	Yes	Sleeping pills	Yes
SIUDS 4	F / 38 gws	Unknown	No	Yes	No	No
SIUDS 5	M / 40 gws	Unknown	Respiratory infection, 1 previous miscarriage	No	Antibacterial drugs	Yes
Ctrl SIDS 1	M / 1 pn day	Prematurity, low birth weight	No	?	No	No
Ctrl SIDS 2	M / 2 pn days	Cardiomyopathy	No	No	No	No
Ctrl SIDS 3	F / 1 pn month	Cardiomyopathy	Diabetes	Yes	Alcohol	Yes
Ctrl SIDS 4	M / 1 pn month	Severe pneumonia	No	?	Drugs	No
Ctrl SIDS 5	F / 11 pn months	Severe pneumonia	Hypertension	?	?	No
Ctrl SIUDS 1	M / 34 gws	Placental abruption	No	No	No	No
Ctrl SIUDS 2	F / 37 gws	Placental abruption	Obesity, hypertension	Yes	No	No
Ctrl SIUDS 3	F / 37 gws	Umbilical cord knotted	No	No	No	No
Ctrl SIUDS 4	M / 39 gws	Congenital malformation	Preeclampsia, autoimmune diseases	No	Antidepressant drugs	Yes
Ctrl SIUDS 5	M / 41 gws	Infection	No	No	No	No

Ctrl: Control; F: Female; gws: Gestational week; M: Male; pn: Postnatal; SIDS: Sudden infant death syndrome; SIUDS: Sudden intrauterine unexplained death syndrome; snud: Sudden neonatal unexplained death.

placental barrier and finally enter the fetal bloodstream [16]. After crossing the blood–brain barrier, there is nothing between the NEPs and the fetal brain tissue. It can be assumed that NEPs may interfere with the normal development of vital centers and consequently participate in the pathogenic mechanism of death during pregnancy or shortly after birth.

In this study, the authors present a novel autopsy approach using a field emission gun environmental scanning electron microscope (FEGESEM) coupled with energy dispersive spectroscopy (EDS) performed on brain tissue samples from SIUDS, SIDS and respective control cases in search of inorganic lethality cofactors.

Materials & methods

Obtaining consent

Consent and institutional review board approval were not required for this study, as it complies with the requirements of the Italian law no. 31/2006 'Regulations for Diagnostic Postmortem Investigation in Victims of Sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death.' Parents of all infants included in the study provided written informed consent on performing the autopsy, research and publication of data.

Sample & data collection

Test samples were collected from 5 SIDS (2 males, 3 females; age 1 h–6 months) and 5 SIUDS cases (4 males, 1 female; age 36–40 gestational weeks) (Table 1). The diagnoses of SIUDS and SIDS were formulated after excluding any other cause (pathological, genetic, toxic, malformative, intentional etc.) and after a thorough death scene investigation in the case of infant death, as required by all international protocols. Control samples were collected from 10 cases (5 fetuses: 3 males, 2 females, age 34–41 gestational weeks and 5 infants: 2 males, 3 females, age 1 day–11 months) where the cause of death was not diagnosed as SIDS/SIUDS. In agreement with the Italian law

no.31/2006, article 3, the samples were analyzed at "Lino Rossi" Research Center of the University of Milan, that is the national law referral and data bank center regarding sudden fetal and infant deaths. The law decrees that all infant sudden deaths within the first year of age, or the deaths of fetuses post-25th week of gestation (with no apparent cause), undergo indepth anatomopathological examination.

For each case, a thorough maternal clinical history was collected, including information regarding potential risk factors such as maternal smoking and alcohol or drug abuse (before and during pregnancy) (Table 1). Residential air pollution data were collected when the area was considered at high risk, and it is included in the Report 'Sentieri' of the Superior Institute of Health (Italy).

Brainstem histological examination

The protocol for brain tissue preparation has been previously described [4–6]. All autopsies were performed between 1 and 2 days following death, according to Police Mortuary Regulations in Italy. In brief, the time elapsed between the subject's somatic death and the beginning of the immersion-fixation of tissues in 10% phosphate-buffered formalin was 24 to 48 h. Three specimens were taken from the brainstem: the first specimen included the upper third of the pons and the adjacent caudal portion of the midbrain; the second specimen included the caudal portion of the pons; the third specimen included the medulla oblongata around the obex. Routinely, transversal serial 4 μm sections were made at 30 μm intervals from each brainstem sample. Two of these sections were stained using hematoxylin & eosin and Kluver/Barrera staining, while other sections were used for immunohistochemical detection of neurotransmitters (catecholamines, orexin, serotonin) and specific receptors (nicotinic acetylcholine receptors). The microscopic examination of the histological sections was focused on the:

- Locus coeruleus, Kölliker Fuse and median raphe nuclei in the rostral pons
- Facial/parafacial complex, superior olivary complex, retrotrapezoid and magnus raphe nuclei in the caudal pons
- Hypoglossus, dorsal vagal motor, ambiguous, pre-Bötzinger, inferior olivary, arcuate, obscurus and pallidus raphe nuclei in the medulla oblongata.

All the above-listed nuclei and structures have been identified in accordance with the human brainstem atlas of Olszewski and Baxter [17]. A diagnosis of 'hypoplasia,' which represents the most frequent structural alteration observed in this study, was formulated according to the following method.

Diagnosis of hypoplasia of a given nucleus

The features of a given nucleus detected in a single section are always compared with those observable in the section at the same level of the brainstem in a group of age-matched stillbirths that are always used in the authors' studies as controls, as they agree with the atlas of reference [17]. Whenever a significantly decreased number of neurons and/or decreased area of a nucleus is found even in a single histological section (and therefore a hypoplasia of the nucleus is suspected), the protocol continues not only with the morphological examination but also with the morphometric evaluation (by using an Image-Pro Plus Image Analyzer, Media Cybernetics, MD, USA), in serial histological sections throughout its full extension.

To confirm a diagnosis of hypoplasia, the examinations are always carried out by two independent and blinded observers and the results are compared to evaluate the inter-observer reproducibility employing the Kappa Index (KI) [18], where values 0 – 0.2 mean 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). The application of this method for the diagnosis of hypoplasia in the present study revealed in all cases very satisfactory KI values (from 0.85 to 0.92).

Physico-chemical investigations

10 μm thickness sections from the same brainstem paraffin-embedded samples were prepared with a Leica RM2125RT microtome (Nussloch, Germany). The sections were layered on polymeric slides, deparaffinized with a drop of xylol and washed with double-distilled water. The samples were mounted on aluminum scanning electron microscope (SEM) stubs and examined with an FEGESEM (Quanta 650, Thermo Fisher Analytics, TX, USA) coupled to EDS (Thermo Fisher Analytics), in order to verify the cell morphology and the presence of inorganic contaminants. The analysis was performed according to a protocol developed within the EU project 'Nanopathology: the health impact of nanoparticles' (QOL-FP5-2002-05-147) [19]. The elemental chemical composition of

Table 2. Neuropathological findings in sudden infant death syndrome, sudden intrauterine unexplained death syndrome and control cases.

Cases	Brain histology
SIDS 1 (snud)	Raphe nuclei hypoplasia
SIDS 2	Pre-Bötzinger nucleus hypoplasia, area postrema erosion, negative orexin expression
SIDS 3	Facial/parafacial complex and pre-Bötzinger nucleus hypoplasia, ependyma desquamation
SIDS 4	Facial/parafacial complex hypoplasia, increased nicotinic acetylcholine receptors immunoexpression
SIDS 5	Area postrema and ependyma desquamation, decreased serotonin immunoexpression
SIUDS 1	Facial/parafacial complex hypoplasia, cerebral and cerebellar cortex delayed maturation
SIUDS 2	Facial/parafacial complex hypoplasia, pre-Bötzinger nucleus hypoplasia, negative serotonine immunoexpression
SIUDS 3	Facial/parafacial complex and pre-Bötzinger nucleus hypoplasia, delayed maturation of cerebral and cerebellar cortex, ependyma desquamation
SIUDS 4	Facial/parafacial complex hypoplasia, delayed maturation of cerebral and cerebellar cortex
SIUDS 5	Kölliker-Fuse nucleus, facial/parafacial complex and pre-Bötzinger nucleus hypoplasia, ependyma desquamation
Ctrl SIDS 1	No brain alteration
Ctrl SIDS 2	No brain alteration
Ctrl SIDS 3	Arcuate nucleus hypoplasia
Ctrl SIDS 4	No brain alteration
Ctrl SIDS 5	No brain alteration
Ctrl SIUDS 1	No brain alteration
Ctrl SIUDS 2	No brain alteration
Ctrl SIUDS 3	No brain alteration
Ctrl SIUDS 4	No brain alteration
Ctrl SIUDS 5	Obscurus raphe nucleus hypoplasia, mild arcuate nucleus hypoplasia

Ctrl: Control; SIDS: Sudden infant death syndrome; SIUDS: Sudden intrauterine unexplained death syndrome; snud: Sudden neonatal unexplained death.

the identified foreign bodies were obtained through the x-ray microprobe of the EDS. In this setup, the authors were able to work with a nonconductive sample at low vacuum, significantly reducing artifact signals. The events were observed using secondary-electron (SE) detectors and backscattered-electron (BSE) detectors at medium–low vacuum, at acceleration voltages from 10 to 30 kV. The authors considered single particles (10 µm–10 nm) or organic–inorganic composite aggregates as valid results.

Once identified, the particles were photographed, morphologically identified (micro- and nano-sized particles, clusters of particles or particle aggregates embedded in organic substrate), counted and square density calculated.

Statistics

For each sample's tissue section, the area and the particle density were calculated. Statistics were performed with the non-parametric Mann–Whitney test, adopting a significance level of $p < 0.05$ (IBM SPSS Statistics 25 software).

Results

Brain abnormalities present in all brainstem tissue samples

Many brainstem developmental alterations have been detected in SIDS and SIUDS cases. Sometimes, these changes were associated with an abnormal expression of neurochemicals (serotonin, orexin and nicotinic acetylcholine receptors). On the contrary, rare and minor brainstem abnormalities were found in the control group. Table 2 summarizes the main neuropathological findings in the three groups.

Abnormality in two structures of the brainstem involved in intrauterine breathing control were the most frequent findings in test groups: hypoplasia of the facial/parafacial complex in the caudal pons and hypoplasia of the pre-Bötzinger nucleus in the medulla oblongata (Figure 1A–D). Interesting was the frequent observation of erosion of both the ependyma and area postrema, which are brain protective structures whose integrity can be compromised by neurotoxic substances (Figure 1E–H).

Foreign particles present in all brainstem tissue samples

With the FEGESEM, the authors identified the presence of many micro- and nano-sized foreign bodies in both the test and control groups, and their elemental chemical composition was evaluated. As carbon and oxygen elements

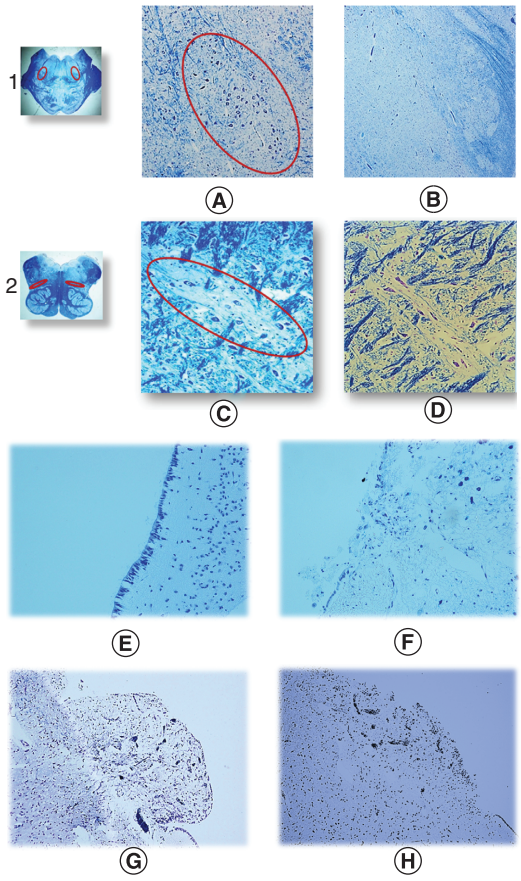

Figure 1. Neuropathological main findings. (1) Transversal histological section of caudal pons showing in the circled areas the location of facial-parafacial complex (F/PFc). **(A)** Normal cytoarchitecture of the F/PFc in a newborn of the control group (male, 1 month old). **(B)** Hypoplasia of the F/PFc in a male, age-matched Sudden Infant Death Syndrome (SIDS) case. **(2)** Transversal histological section of medulla oblongata showing in the circled areas the location of the pre-Bötzinger nucleus (pBn). **(C)** Normal cytoarchitecture of the pBn in a fetus of the control group (male, 37 gestational weeks). **(D)** Hypoplasia of the pBn in a male, age-matched Sudden Intrauterine Unexplained Death Syndrome (SIUDS) case. **(E)** Normal structure of the ependyma (EP) covering the fourth ventricle in a control case (female, 1 month old). **(F)** EP desquamation in a male age-matched SIDS case. **(G)** Normal structure of the area postrema (AP) in the medulla oblongata of a control case (female, 1 month old). **(H)** AP erosion in a SIDS case (male, 1 month old). Kluver–Barrera stain; magnification (1) and (2) 0.5×; **(A & B)** 10×; **(C & D)** 20×; **(E–H)** 10×.

are omnipresent in biological samples, the authors could not distinguish between elemental and oxidation state (i.e., magnesium and magnesium carbonate); nevertheless, they were focused on environmental rather than biologically present elements. Due to the higher atomic density of foreign particles, they appear brighter when compared with organic tissue.

At first, the EDS spectra of the normal brain tissue were recorded. Depending on the sampling site, there were small compositional differences, reflecting tissue specificities (rostral pons, caudal pons and medulla oblongata). After fixation, the tissues contained carbon, oxygen, sodium, phosphorus and chlorine. This spectrum (i.e., blank spectrum) was subtracted from the spectra obtained when the x-ray microprobe was focused on the foreign particles.

In Figure 2, in both the test and control samples, the authors identified metallic debris made primarily of iron: iron-chromium-nickel, iron-copper-silicon and iron-chromium particles. The lower part of Figure 2E is of particular interest, as it shows metallic particles inside the blood vessel, which subsequently could enter brain tissues.

In the following figures are shown the presence of metallic particles of aluminum or aluminum-copper alloy (Figure 3) and of silver (Figure 4). Other debris of gold, titanium-silicon, nickel, zinc-copper alloy was also identified. In addition, the authors detected submicron- or nano-sized debris embedded in organic structure. These aggregates were made of biologically normal chemical constituents, only in abnormal amounts or with unusual metallic elements. The authors also noted that in the same areas where foreign bodies were identified, calcium and calcium-phosphate debris was always present. This is the 'normal' calcification of the brain tissue that follows inflammatory processes (as in brain metastatic calcification).

Another unexplained finding is shown in Figure 5. The pictures represent biological morphologies, but the EDS spectrum identifies a non-biological content. In fact, these structures are made of silicon. The enrichment in silicon implies a biological mechanism never described in the literature, especially not in the brain.

For a better overview, the identified foreign bodies are divided into 9 classes, according to their elemental chemical composition classification used in material science (i.e., iron-chromium and iron-chromium-nickel are two different formulations of stainless steel).

These are the classes the authors identified:

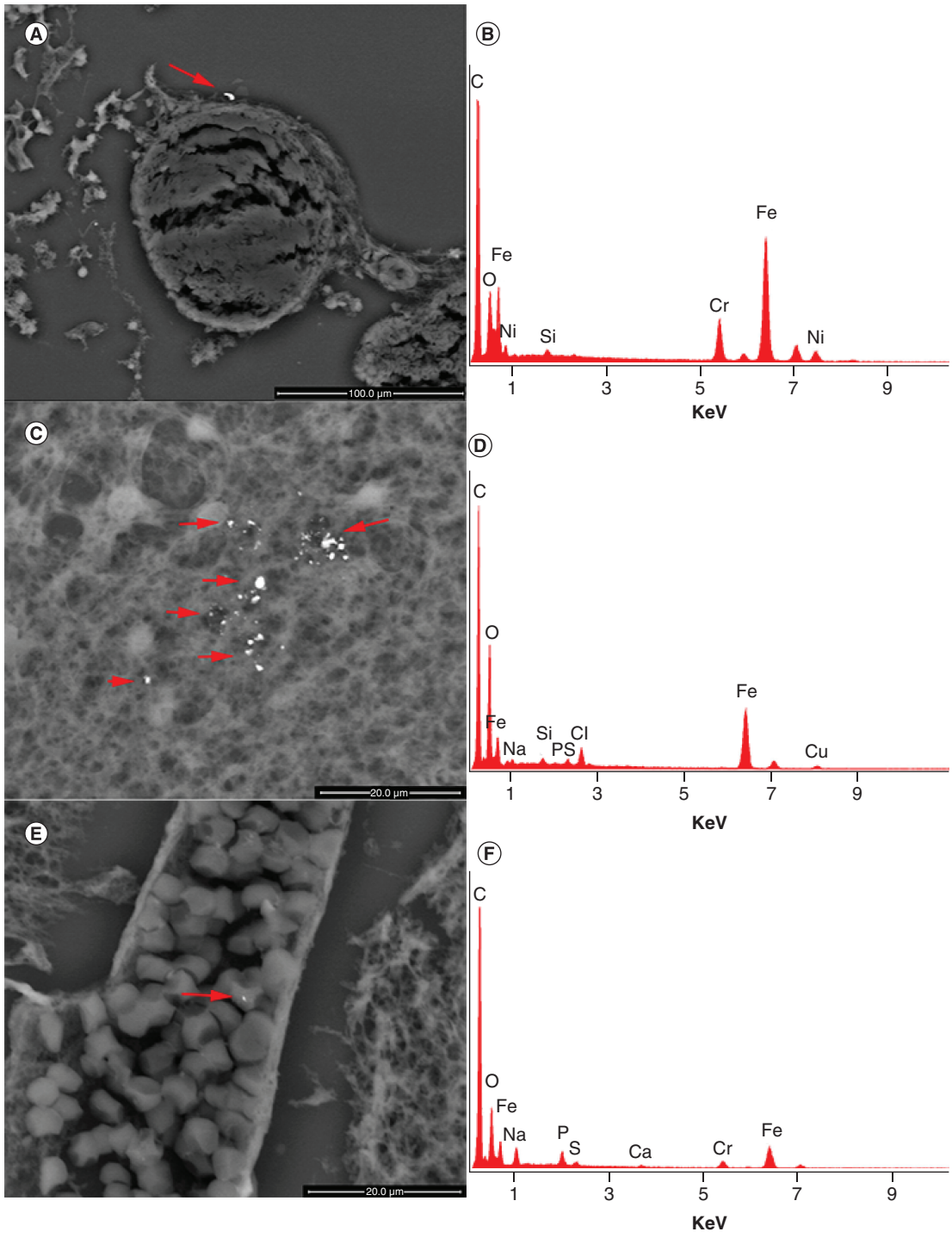

Figure 2. Iron-enriched particles are found in brain tissue. (A & B) Represent approximately 7 µm–sized stainless steel microparticles composed of iron-chromium-nickel alloy, found in a sudden infant death syndrome (SIDS) brainstem sample. **(C & D)** Iron-copper nanoparticles in a control SIDS sample. **(E & F)** An iron-chromium nanoparticle found in a brain–blood vessel of a SIDS control.

1. Silicon [Si]
2. Silicon-based compounds [COSiAlCaKFe]
3. Calcium-based compounds [COCaSiAlKMgFe]
4. Calcium compounds (as a possible result of an inflammation mechanism) [CaO, Ca–P]

5. Al-based compound [Al, AlP, AlCu] (Figure 3)
6. Fe compounds [Fe, FeO, FeCr, FeCrNi] (Figure 2)
7. Silver or gold compounds [Ag, Au] (Figure 4)
8. Titanium compounds [Ti, TiFeNiCu]
9. Na-P-Mg compound

Actual & trending differences between SIDS/SIUDS & controls

First, despite the low number of samples, the authors were surprised to have found particles present in all the samples analyzed, including control samples (Table 3). When the data from Table 3 (number of micro- and nano-sized

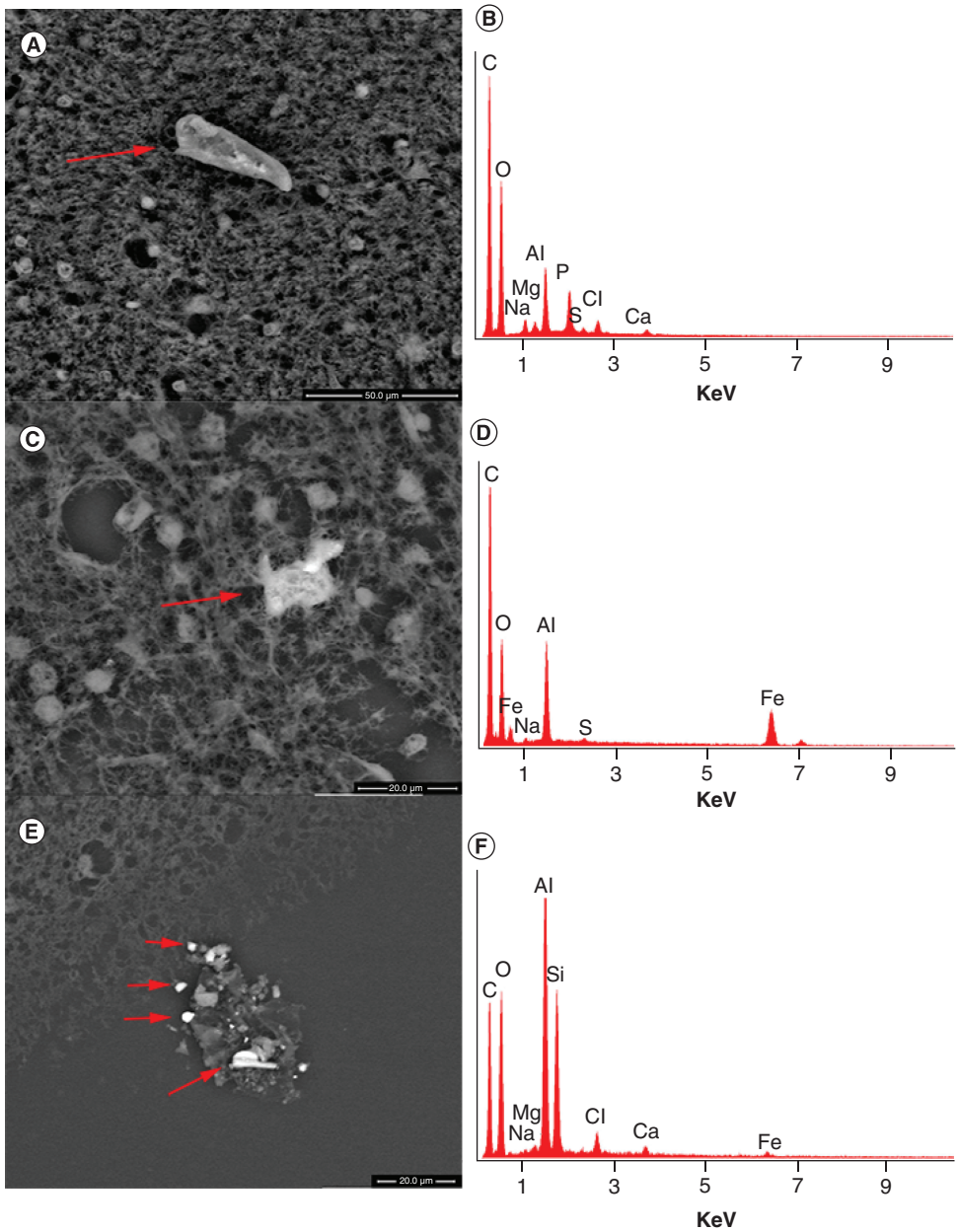

Figure 3. Aluminum-enriched particles found in brain tissues. (A & B) Microparticle composed mainly of aluminum and phosphorus, found in a control Sudden Infant Death Syndrome (SIDS) case. **(C & D)** Microparticle made of aluminum and iron in a Sudden Intrauterine Unexplained Death Syndrome (SIUDS) sample. **(E & F)** Cluster of microparticles made by aluminum and silicon-chlorine found in a control SIDS case.

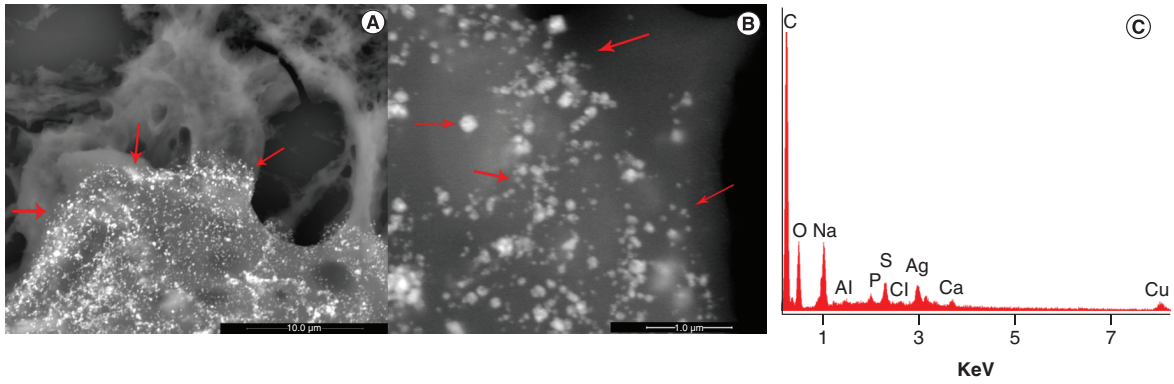

Figure 4. An example of an aggregate containing numerous nanoparticles in a Sudden Intrauterine Unexplained Death Syndrome sample. The nanoparticles are made of several elements: sodium, silver, sulfur, chlorine, copper and potassium.

Table 3. List of the microparticles and nano-sized particles identified in the sudden infant death syndrome/sudden intrauterine unexplained death syndrome/control samples and their density.

Cases	n (μPs)	n (NPs)	Sample area (cm^2)	n (total)	Particles' total density (n/cm^2)	μPs density (n/cm^2)	NP density (n/cm^2)	Death time
SIDS								
1 (snud)	90	367	6.24	457	73.23	14.42	58.81	1 pn h
2	56	179	5.67	235	41.44	9.87	31.56	1 pn month
3	3305	3246	3.5	6551	1871.71	944.28	927.42	1 pn month
4	394	285	2.25	679	301.77	175.1	126.66	2 pn months
5	338	266	3.15	604	191.74	107.3	84.44	6 pn months
SIUDS								
1	1584	1249	5.25	2833	539.62	301.71	237.9	36 gw
2	707	602	5.36	1309	244.21	131.9	112.31	37 gw
3	1088	299	4.9	1387	283.06	222.04	61.02	38 gw
4	315	147	5.8	462	79.65	54.31	25.34	38 gw
5	1015	935	4.6	1950	423.91	220.65	203.26	40 gw
Ctrl SIDS								
1	115	151	1.326	258	194.57	86.72	113.87	1 pn day
2	143	147	2.38	290	121.85	60.08	61.76	2 pn day
3	152	215	4.75	367	77.26	32	45.26	1 pn month
4	350	147	1.766	497	281.43	198.19	83.24	1 pn month
5	52	62	1.13	114	100.84	46.01	54.87	11 pn months
Ctrl SIUDS								
1	2267	1901	1.65	4168	2526.06	1373.94	1152.12	34 gw
2	459	308	3.96	767	193.69	115.9	77.78	37 gw
3	162	66	2.268	228	100.53	71.43	29.1	37 gw
4	763	615	3.52	1378	391.48	216.76	174.72	39 gw
5	2655	2554	1.766	5209	2949.6	1503.4	1446.21	41 gw

μP: Microparticle; Ctrl: Control; gw: Gestational week; NP: Nanoparticle; pn: Postnatal; SIDS: Sudden infant death syndrome; SIUDS: Sudden intrauterine unexplained death syndrome; snud: Sudden neonatal unexplained death.

particles, density and age of death) were analyzed with an independent, non-parametric test, the authors confirmed that there is a trend of SIDS samples containing more microparticles than their corresponding controls ($p = 0.056$); SIDS controls have a generally lower number of microparticles than those found in SIUDS (cases or controls, $p = 0.008$); and the numbers of micro- (but not nano-) particles and their densities are statistically higher in total (case + control) SIUDS than in total SIDS ($p = 0.005$). In general, the authors were intrigued by the high amounts

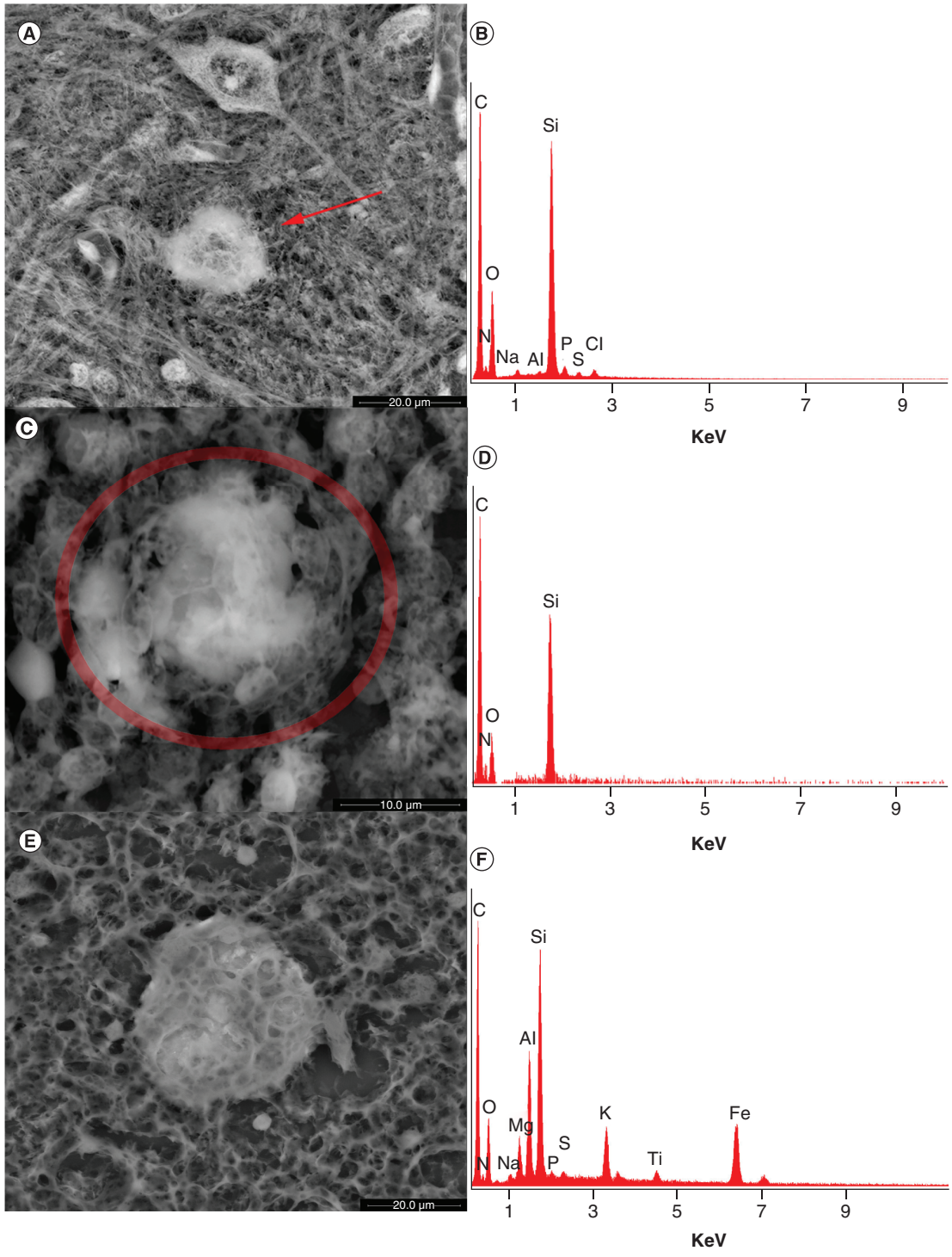

Figure 5. Morphologies of biological structures enriched with silicon, in a sudden infant death syndrome control case (A & B). In a sudden intrauterine unexplained death syndrome (SIUDS) case **(C & D)** and in a sudden infant death syndrome (SIDS) case **(E & F).** The organic structures are enriched with **(D)** silicon and **(F)** silicon-aluminum-iron-potassium-magnesium, with traces of titanium.

Table 4. List of classes of identified materials in the brain samples with their frequency.

Group	Elements / Case no	Si	Si-Al-Ca-Fe	Ca-Mg-Si-Al	Ca	Al	Fe-(Cr-Ni)	Ag-Cu	Au	Ti-Fe-Ni-Cu	Cu-Zn	Na-P-Mg-Ca
SIDS	1 (snud)	11	4	0	2	10	3	1†	1	1	0	1
	2	8	7	5	10	5	6	1†	0	1	2	0
	3	5	12	1	7	0	11	3†	0	0	0	2
	4	7	6	2	5	2	8	0	1	1	1	0
	5	3	7	17	11	0	10	**0**	0	1	0	0
Total		**34**	**36**	**25**	**35**	**17**	**38**	**0**	**2**	**4**	**3**	**3**
Ctrl SIDS	1	15	7	0	3	11	7	2†	1	1	0	0
	2	1	5	4	8	3	4	0	0	1	0	4
	3	13	9	0	4	4	8	2	1	0	0	1
	4	10	7	2	40	8	17	0	0	1	2	0
	5	3	5	3	5	3	7	1†	0	0	0	0
Total		**42**	**33**	**9**	**60**	**29**	**43**	**2**	**2**	**3**	**2**	**5**
SIUDS	1	11	4	3	11	2	5	**0**	0	5	1†	0
	2	10	5	2	5	4	3	**0**	1†	4	0	4
	3	1	1	0	13	0	5	0	0	1	0	2
	4	5	4	2	7	6	6	0	2	4	0	1
	5	8	10	5	7	0	4	2†	0	4	2	2
Total		**35**	**24**	**12**	**43**	**12**	**23**	**0**	**2**	**18**	**2**	**9**
Ctrl SIUDS	1	5	5	5	8†	1	6	2†	0	2	2	0
	2	4	7	2	2	4	3	0	0	0	1	0
	3	9	9	4	3	4	3	0	0	2	0	0
	4	5	5	3	8	1	5	0	1	1	0	1
	5	7	13	2	2	2	11	2†	0	2	1	1
Total		**30**	**39**	**16**	**15**	**12**	**28**	**0**	**1**	**7**	**4**	**2**

†Presence of high-density groups of nanoparticles.
Ctrl: Control; SIDS: Sudden infant death syndrome; SIUDS: Sudden intrauterine unexplained death syndrome; snud: Sudden neonatal unexplained death.

of particles found in SIUDS controls, meaning that the fetal tissue contained a higher microparticle concentration than fully grown brain tissues in already born babies.

The data from Table 4 on the element's frequency suggests that SIUDS samples contained more Ti–Fe–Ni–Cu particles than their control ($p = 0.053$) or SIDS samples ($p = 0.018$). A similar trend was observed for calcium, Na–P–Mg–Ca and Si–Al–Ca–Fe particles. In SIDS samples, there was a certain enrichment of aluminum ($p = 0.248$). Of note, the small sample size was the reason why the authors could not draw clear conclusions and stronger claims.

Discussion

Perinatal autopsy is a standard procedure applied in search of a fetal or newborn cause of death. In SIDS/SIUDS cases, it is crucial to perform an accurate autopsy and a thorough neuropathological examination. Previous studies on SIDS and SIUDS demonstrated developmental brain alterations that are incompatible with a healthy life. Recently, the authors reported, for the first time, the surprising presence of silver nanoparticles in the brain of a fetus which died unexpectedly at the end of a regular pregnancy [20]. This finding led the authors to perform the present study.

Organic (soluble) metal ions are essential components of multiple enzymes involved in oxidation-reduction processes, both in anabolism and in catabolism [21]. However, metal-based implants enter miscommunication with immune cells, resulting in oxidation-mediated corrosion [22]. If a permanent metallic implant can provoke chronic inflammation, then any other tissue-delivered metallic deposits, even when surrounded by fibrotic tissue, can become a source of complication in susceptible individuals.

Some of the particles identified showed surprising amounts of aluminum alloys (Figure 3C & E) or aluminum and phosphorous (Figure 3A). This is at least intriguing, as today it is known that aluminum has no known role in biological systems [23]. The authors speculate that its sources could be aluminum cutlery, aluminum-containing pharmacy products or immunotherapy vaccines containing aluminum derivatives dissolved in Na–Cl,

whose crystals are seen enriched with aluminum. Since the mother can transfer blood contaminants to her fetus, aluminum particles, known to be able to travel long distances [24], could have reached the brain tissues, as aluminum has a keen 'tropism' toward complex fatty tissues [25]. Furthermore, samples contained metallic deposits made of iron (Figure 2), silver (Figure 4), gold or titanium. Considering blood as a carrier of environmental pollution nanoparticles that cross vascular epithelia into the tissues [26–28], and the presence of metallic debris inside fetal blood vessels (Figure 2E), it seems that the particles reach their destination via bypassing the blood–brain barrier [29].

Normally, one could expect to see $CaCO_3$, $CaPO_4$ or Na–P–Ca deposits in samples coming from adult brain tissue (with unknown mechanism), but there is no explanation for how and why it would happen in an early stage of human development. Even more puzzling was the presence of silicon particles or silicon-impregnated tissue (Figure 5), since a physiological role (to eliminate aluminum [30]) is known only for silicic acid, but not for any other form. Because silicon is not a metabolic element, the authors hypothesize that foreign microsensors might have been injected.

The changed structures were not stained by hematoxylin & eosin or other dyes, which can be the reason for the lack of anatomical parts. Based on the results of this study, the authors believe that toxic substances are able to alter the blood–brain membrane (known as a barrier in the sense of preventing unwanted guests' entry) during pregnancy, facilitating their entry into the brain parenchyma and thus interfering with the development of important nerve centers used to control vital functions, as demonstrated by the frequent presence of hypoplasia of the facial/parafacial complex and the pre-Bötzinger nucleus in SIDS and SIUDS cases (Figure 1). The authors' hypothesis finds support in many articles of the literature demonstrating the mechanisms of toxicity exerted by nicotine, metals and air pollution in general on the developing nervous system [31–35].

Noteworthy is the fact that in all samples next to the dominant element mentioned earlier, there were always accompanying amounts of other elements, which differed from those shown in Figure 3.

The babies can have been exposed to micro- and nano-sized contamination either before and after birth. In the first case, food-borne inorganic contamination [36,37] and esthetic implants [38] are the suspected sources of many of the particles we have found, as there is no mechanism by which the placental barrier prevents the entry of contaminants from the mother's blood. In the second case, contaminants are found inside ingested food: mother's milk, indicating that the mother's system is partially clearing/storing contaminants in the milk, and infant formula, as contamination has already been confirmed [39].

Finally, particles of unusual composition can be produced during the process of uncontrolled combustion during waste incineration, or simply by tobacco smoking [40], as inhaled airborne particles can enter easily enough human body liquids [41].

Toward foreign, non-degradable entities, the human body reacts with oxidation [42], and inflammation [43]. If it becomes chronic, inflamed sites are a perpetual source of cytokines, chemokines, danger signals and oxidized molecules. It is well known that these factors are at the bottom of many diseases [44]. Considering the data presented, although the authors cannot be sure that the body's reaction to different metallic categories is the same, their opinion is that after toxic metallic particles have crossed the blood–placental barrier, fetal brain microglia [45] or other fetal or maternal immune cells [46] are engaging microparticles and nanoparticles in a way that could lead to overproduction of inflammatory molecules. Instead of being eliminated, the particles trigger chronic inflammatory reaction(s), which could be connected to abnormal brain development. A likely example of this can be seen in Figure 1, where the protective area postrema and ependymal linings are severely damaged, allowing unrestricted access into the brain parenchyma.

In summary, the main finding is that the authors have identified a comparable number of metallic particles in both SIDS/SIUDS brains and respective control samples (Tables 3 & 4 & Figure 5). To the authors' knowledge, for healthy brain development, no foreign inorganic particles are needed, nor have a recognized physiological role. Therefore, it can be assumed that despite control samples not being identified as SIDS/SIUDS, the presence of foreign particles is not a normal situation and would not contribute to the newborn's well-being – quite the opposite. In formulating a diagnosis of SIDS or SIUDS it is important to check for possible nanoparticle contamination in any essential organ as, once the threshold is reached, it could play a significant role in the event of death.

The present research provided something previously not described in the literature. The authors observed fetal bloodborne micro- and nanoparticle contamination, their precipitation in the brain tissue (due to extravasation) and tissue physical change, due to impregnation with metallic particles. Any alteration of nerve centers important for the control of vital functions is already highlighted in the literature on SIDS/SIUDS. The results of the

present study could have implications for understanding the pathogenic mechanism of SIUDS and SIDS and their prevention.

Conclusion

Nanotechnology products are used in a growing number of items marketed for human health, beauty and well-being. Even today, the scientific world is aware of the neurotoxic effects of certain engineered nanoparticles, with an inverse relation to the stage of nervous system development. Our study highlights new evidence of nano-sized foreign bodies' presence in brain tissue during perinatal life. Based on the findings, the authors propose that the current autopsy protocol of brain tissue analysis of perinatal deaths is supplemented with FEGESEM coupled with EDS analysis. That way, one could assess the presence of inorganic entities in abnormally changed brain tissues, as this can be a factor or a co-factor of lethality.

Identification of similar entities in other neurological diseases such as Parkinson's or Alzheimer's could open new investigations on these inorganic–organic mechanisms. It is therefore important, given the widespread use of nanomaterials, that during a brain autopsy the pathologist consider the role of toxic nanoparticles capable of altering the cytoarchitecture and functionality of important brain structures.

Future perspective

The new physico-chemical approach proposed represents an innovation in the field of medicine – in particular, in that of pathology and forensic medicine. The ultrastructural methodology can identify micro- and nano-sized foreign bodies in tissues and inside cells and open a new scenario for understanding nanotoxicity. The possibility of determining their chemical composition can allow researchers to trace this exposure in a patient's life. This new investigative approach can highlight the role of environmental contamination in perinatal deaths and allows for the discovery of new mechanisms of body uptake and internal dissemination. The discovery of some patho-mechanisms and NEP kinetics can help not only in diagnosis but also in the development of a new therapeutic approach and new mechanisms of 'cleaning' the body from this contamination.

Summary points

- In sudden infant death syndrome and sudden intrauterine unexplained death syndrome, the underlying cause of death is unknown.
- In this study, in-depth anatomopathological procedures were supplemented with the use of field emission gun environmental scanning electron microscopy and energy dispersive spectroscopy in search of possible causes of death.
- Nano-sized foreign particles, particle clusters and organic–inorganic structures have been found in altered brain tissues of fetuses and newborns who died suddenly without any apparent cause of death.
- The presence of inorganic entities could be considered a possible cofactor of lethality in perinatal life.
- The search for toxic nanoparticles can provide valuable additional clues in cases of idiopathic death.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2021-0203

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