

BASIC SCIENCE ARTICLE



Mitochondrial DNA content: a new potential biomarker for Sudden Infant Death Syndrome

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BACKGROUND: Sudden Infant Death Syndrome (SIDS) occurs in apparently healthy infants and is unpredictable and unexplained despite thorough investigations and enormous research efforts. The hypothesis tested in this case–control study concerns mitochondrial involvement in SIDS occurrence.

METHODS: Mitochondrial DNA content (MtDNAcn) was measured in 24 SIDS cerebral cortex samples and 18 controls using real-time PCR.

RESULTS: The median (interquartile range) mtDNAcn in SIDS and controls was 2578 (2224–3838) and 1452 (724–2517) copies per nuclear DNA, respectively ($P = 0.0001$). MtDNAcn values were higher in SIDS victims born to non-smoking parents ($n = 7$) 4984 (2832–6908) compared to the controls ($n = 5$) 2020 (478–2386) ($P = 0.006$). Increased levels of mtDNAcn have been observed in the SIDS cases with mild defects in nuclei not essential for life compared to those found in SIDS cases with severe alterations of respiratory function ($P = 0.034$) 3571 (2568–5053) ($n = 14$) 2356 (1909–3132) ($n = 8$), respectively.

CONCLUSIONS: Our study revealed for the first time higher mtDNAcn in the cerebral cortex of the SIDS cases than the controls, indicating metabolic alterations. MtDNAcn plays an important role in compensatory mechanisms against environmental factors affecting human health. Despite the small sample size, mtDNA may prove to be a potential forensic biomarker for autopsied SIDS victims for gaining new insights into the etiology of SIDS.

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IMPACT:

- Mitochondrial DNA content evaluated in cerebral cortex samples is higher in SIDS victims than controls.
- These results represent a novel line of investigation for the etiology of SIDS and could have a significant role in the compensatory mechanism due to environmental factors affecting human health.
- These findings suggest that the mitochondria are involved in SIDS: mtDNA content may represent a biomarker of this syndrome.

INTRODUCTION

SIDS is the leading cause of postnatal infant mortality and accounts for 38% of all sudden unexpected infant deaths (SUIDS).¹ Despite the large number (12,517) of studies conducted on SIDS, its pathogenesis remains unclear. SIDS is generally considered to be a multifactorial disorder and its exact cause is unknown even after thorough case investigations have been conducted, such as a complete forensic autopsy, examination of the death scene, and gathering information acquired from medical history.² As suggested by Filliano and Kinney in their “Triple Risk Hypothesis”, SIDS can be considered the fatal outcome of various contributory causes such as intrinsic child vulnerability, a critical period in the development of the autonomic regulation of both the respiratory and the cardiovascular systems and exogenous triggers.³

Developmental alterations of the nervous system, such as hypoplasia and agenesis of brainstem nuclei that are essential for

life, especially those that control breathing or disorders of the chemoreceptor system, have often been found in SIDS victims in the past.^{4–8} These defects have frequently been attributed to risk factors, such as perinatal exposure to tobacco smoke, environmental pollution, and drug and alcohol use in pregnancy. These toxic agents can cause severe alterations to the blood–brain barrier (BBB), which allows toxins to cross the BBB and enter the brain parenchyma. Consequently, toxins act directly not only on the neurotransmitter systems (essential for homeostatic control of a developing human brain), but can also affect the number of mitochondria, that are known to be sensitive markers of cellular damage.⁹ Increases in mtDNA levels have recently been observed in sudden intrauterine unexplained deaths (SIUDS).¹⁰

The aim of this study was to determine whether these mitochondrial alterations can serve as biomarkers for both sudden infant and early childhood deaths, since two similar events such as

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Table 1. Main characteristics of the studied groups.

Characteristics	SIDS (<i>n</i> = 24)	Controls (<i>n</i> = 18)	<i>P</i>
Age (months at death)	1.417 (0.042–3.000)	0.550 (0.030–3.978)	0.408
Weight at death (g)	4040 (2913–7000)	3670 (1605–4200)	0.156
Parents smoke	7 on 24	2 on 9	
Sex			0.332
Female	13	7	
Male	11	11	
Gestational age at birth	39 (38–40)	37 (29–39)	0.013
Weight at birth	3130 (2245–3390)	1883 (1520–3140)	0.041

Data are expressed as median (IQR).

SIUDS and SIDS in terms of their risk factors differ greatly as functions considered to be indispensable after birth like respiration, are not important in the intrauterine environment.

MATERIALS AND METHODS

Subjects

This case–control study was performed at the “Lino Rossi” Research Center at Milan University. All of the selected cases (24 SIDS and 18 controls), which occurred between 2007 and 2020, followed the guiding principles laid down in the ethical code by the Ministry of Health in accordance with Italian Law n.31/2006 “Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Foetal Death”.² This law decrees that all infants suspected of SIDS who died suddenly in Italian regions within the first year of age, as well as all fetuses who died without any apparent cause, must be submitted to a thorough diagnostic post-mortem investigation. The anatomo-pathological protocol includes the in-depth examination of the autonomic nervous system, especially of the brainstem centers checking the vital functions. The neuronal centers that preside over the respiratory function, namely the Kölliker–Fusé 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, which are all components of the so-called “respiratory network” (RN), were considered essential for maintaining life. These centers are linked together and can coordinate each other via excitatory and/or inhibitory connections, in relation to the need, to control breathing. The morphological study must be completed by investigations of molecular genetics.

In order to obtain high-quality samples, this study considered cases with proven anamnestic information, including detailed medical records, details on parents’ health and lifestyles (tobacco, alcohol, and drug use habits). Following a thorough autopsy, in 24 cases (age months median: 1.42, IQR: 0.04–3.00) the cause of death remained undetermined and SIDS was diagnosed. For the remaining 18 infants (age months median: 0.550, IQR: 0.030–3.978), the cause of death was established at autopsy (5 severe pneumonia, 1 Dandy–Walker syndrome, 1 high-grade pulmonary edema, 1 fibromuscular hyperplasia of the pulmonary arteries in a newborn with ascites, 1 malaria, 1 acute renal tubular necrosis, 1 hepato-renal polycystosis and pneumonia, 1 pneumonia and meningitis, 1 meningitis and sepsis, 1 cardiomyopathy, 1 bronchopneumonia, and Wilson’s Disease, 1 cystic lymphangioma, 1 hemolytic uremic syndrome and 1 cystic teratoma of the oral cavity. These cases were used as “controls” (Table 1).

MtDNA content evaluated by real-time quantitative PCR analysis

Formalin-fixed, paraffin-embedded cerebral cortex tissues were cut into 6 slices of 5- μ m sections. Total DNA was extracted from the cerebral cortex using ReliaPrep™ FFPE gDNA Miniprep System (Promega, Milan, Italy). MtDNA content was measured using real-time PCR. ABI Prism 7900 Fast sequence detection system was used for Real-time quantitative PCR analysis in accordance with a method published in a previous study.¹¹ Real-time PCR was performed using the RNase P gene, as an endogenous

control (cat. no. 4316844, Applied Biosystems, Waltham, MA) and AB Mitochondrial Gene 7S, encoding D-loop, as target gene (Hs02596861_s1, Assays-on-Demand Gene Expression Products, Applied Biosystems, Waltham, MA). RNase P is a single-copy nuclear gene that encodes for the RNase P enzyme. D-Loop is a mtDNA replication initiation site. Target and reference genes were amplified in separate wells in duplicate. Reaction conditions included 10 μ l of 2 \times TaqMan Fast Universal PCR Master MIX, 1 μ l of primers and probes mixture, 150 ng of template DNA, and nuclease-free water in a 96-well reaction plate. The total reaction volume was 20 μ l. The cycling conditions were as follows: 20 s at 95 °C and 40 cycles of 3 s at 95 °C followed by 30 s at 60 °C. The $2^{-\Delta\Delta C_t}$ was calculated for each experimental sample and data were presented graphically as relative quantification.

All samples used for this study are from the prefrontal cortex, a region that in humans occupies the largest part of the frontal lobes and that, in addition to a specific involvement in the control of the pre-motor activity, plays an important role in the coordination of autonomic functions, such as in maintenance of ventilation, with important pathophysiological implications in prenatal life. In fact, as demonstrated in humans through *in vivo* neuronal stimulation and magnetic resonance imaging (MRI) scanning techniques, the prefrontal cortex controls the periodic fetal respiratory activity, which is essential for lung development. We have specifically considered the prefrontal cortex also for its implication in the pathogenetic mechanism of SIUDS and SIDS.¹²

Statistical analysis

Data are expressed as a median value (interquartile range–IQR). The difference between groups was compared using the unpaired Student’s *t*-test or unpaired Wilcoxon test as appropriate. Probability <0.05 was considered statistically significant.

To our knowledge, there is no available data regarding the statistical distribution of mitochondrial DNA in the brain. It was impossible to draw firm conclusions in this regard due to our small sample size, therefore nonparametric tests were adopted when dealing with this variable.

Linear regression analysis was performed using the least-squares method. The correlation between values was performed using the Spearman correlation. Statistical analysis was performed using SPSS software (v.23).

A receiver-operating characteristic (ROC) analysis was performed to test the accuracy of mtDNAcn in predicting SIDS using cerebral cortex tissues. The ability of mtDNAcn from cerebral cortex tissues in predicting SIDS was considered excellent if the area under the curve (AUC) was >0.90, good if it was between 0.80 and 0.90, fair if it was between 0.70 and 0.80, and poor if it was between 0.60 and 0.70.

RESULTS

mtDNAcn in cerebral cortex tissues of SIDS and controls

Mitochondrial DNA content (mtDNAcn) was analyzed in the neonatal cerebral cortex of SIDS and controls. MtDNAcn was higher in the SIDS cases (*n* = 24, median: 2578, IQR: 2224–3838) than in the controls (*n* = 18, median: 1452, IQR: 724–2517) (*P* = 0.0001) (Fig. 1). All samples were analyzed using real-time quantitative PCR and statistical analysis was performed using nonparametric tests.

mtDNA levels in cerebral cortex tissues of SIDS cases with mild and severe neuronal alterations

Developmental alterations in specific brainstem centers of the nervous system are commonly found in SIDS victims. Many of these defects are deemed to be “severe” as they affect the nerve centers that control vital body functions like breathing and therefore they are known to be incompatible with life and therefore can explain the pathogenetic mechanism of SIDS. Other alterations deemed as “mild” since they have been found in adults who have died from proven causes and therefore are not incompatible with neonatal life and would not cause a SIDS death. In these cases, there are certainly other factors and mechanisms that can cause death that are currently unknown.

MtDNAcn was higher (*P* = 0.034) in SIDS cases with mild nuclei defects that were not deemed to be essential for life, such as hypoplasia of the arcuate nucleus, 3571 (2568–5053) (*n* = 14), than

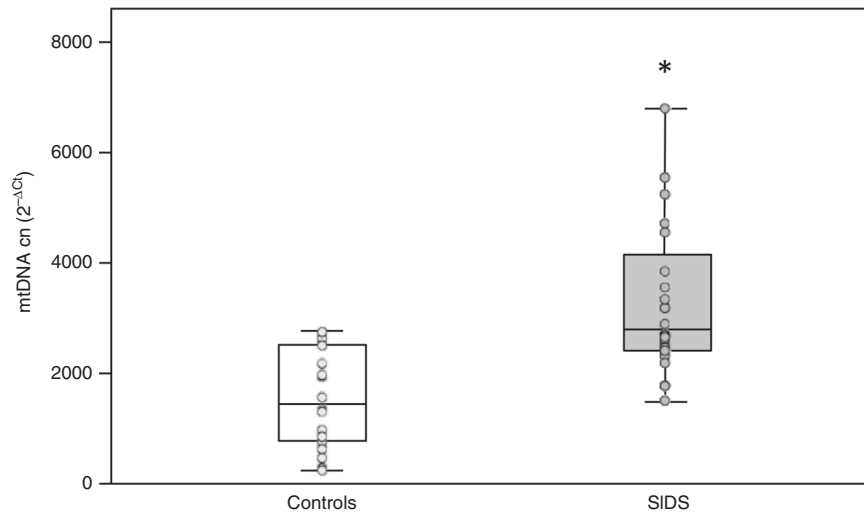


Fig. 1 Relative quantification of mtDNAcn in the cerebral cortex of control (controls $n = 18$) and SIDS (cases $n = 24$) groups. Total DNA isolated from tissues was quantified via Real-time quantitative PCR analysis using the RNAse P as an endogenous control. Data were analyzed by the comparative Ct method and are expressed as median and interquartile range of D-loop relative quantification, which corresponds to the $2^{-\Delta C_t}$. * $P < 0.05$ vs control group.

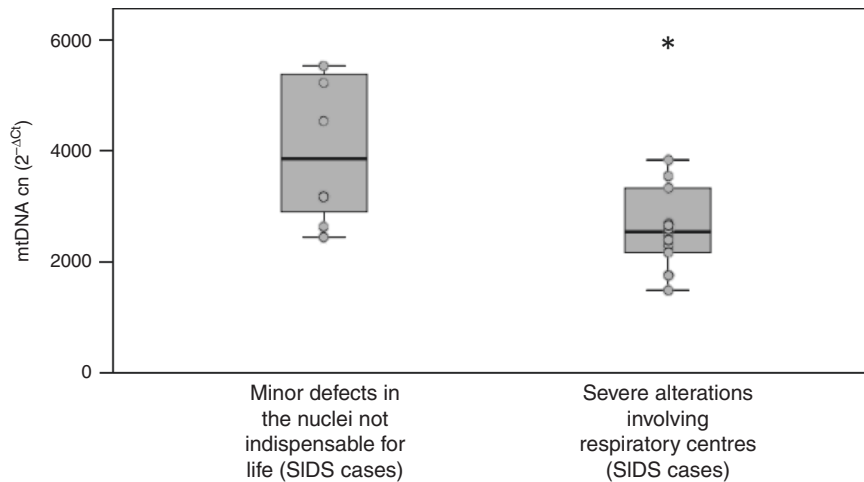


Fig. 2 Relative quantification of mtDNAcn in the cerebral cortex of SIDS cases showing minor defects in nuclei not essential for life ($n = 14$) and of SIDS cases with severe alterations in respiratory function ($n = 8$). Data were analyzed by the comparative Ct method and are expressed as median and interquartile range of D-loop relative quantification, which corresponds to the $2^{-\Delta C_t}$. * $P < 0.05$ vs severe alterations in SIDS group.

mtDNAcn detected in SIDS cases with severe alterations (hypoplasia and/or agenesis) involving the respiratory centers, 2356 (1909–3132) ($n = 8$) (Fig. 2).

mtDNA content in cerebral cortex tissues of SIDS and controls born to smoking or non-smoking parents

MtDNAcn proved to be higher in the cerebral cortex samples of controls born to smoking parents, 2444 (1777–1889) ($n = 2$), than in cases born to non-smoking parents, 2020 (478–2386) ($n = 5$), ($P = 0.12$). MtDNAcn was reduced in SIDS victims born to smoking parents, 2661 (2314–3939) ($n = 7$) than those born to non-smoking parents, 4984 (2832–6908) ($n = 6$), ($P = 0.046$). MtDNAcn was higher in SIDS victims born to non-smoking parents, ($n = 6$, median: 4984, IQR: 2832–6908) than in the controls born to non-smoking parents, ($n = 5$, median: 2020, IQR: 478–2386) ($P = 0.006$) (Fig. 3).

ROC curve

The areas under the ROC curves are shown in Fig. 4.

The area under the ROC curve was 0.86 [95% CI: 0.75–0.97], $P = 0.0001$, for all cases (ctr, $n = 18$; SIDS $n = 24$) (Fig. 4a). Considering only cases with non-smoking parents (ctr, $n = 5$; SIDS $n = 7$), the area under the ROC curves was 0.86 [95% CI: 0.64–1.00], $P = 0.042$, as cigarette smoking is a confounding factor (Fig. 4b).

DISCUSSION

To our knowledge, this is the first time that mtDNAcn has been evaluated in formalin-fixed cerebral cortex samples from SIDS cases. The results show a significant increase of mtDNA content in SIDS victims compared to controls, which supports our hypothesis of the involvement of this biomarker in SIDS pathogenesis.

To date few authors have focused on the role of mtDNA in SIDS, performing all analyses on fresh tissue (usually blood and spleen), and have obtained some controversial results. Divne et al.¹³ did not find any specific mtDNA mutations or polymorphisms in association with SIDS. In a study on mtDNA polymorphisms, Laer et al.¹⁴ highlighted its role in a subset of SIDS cases with regard to

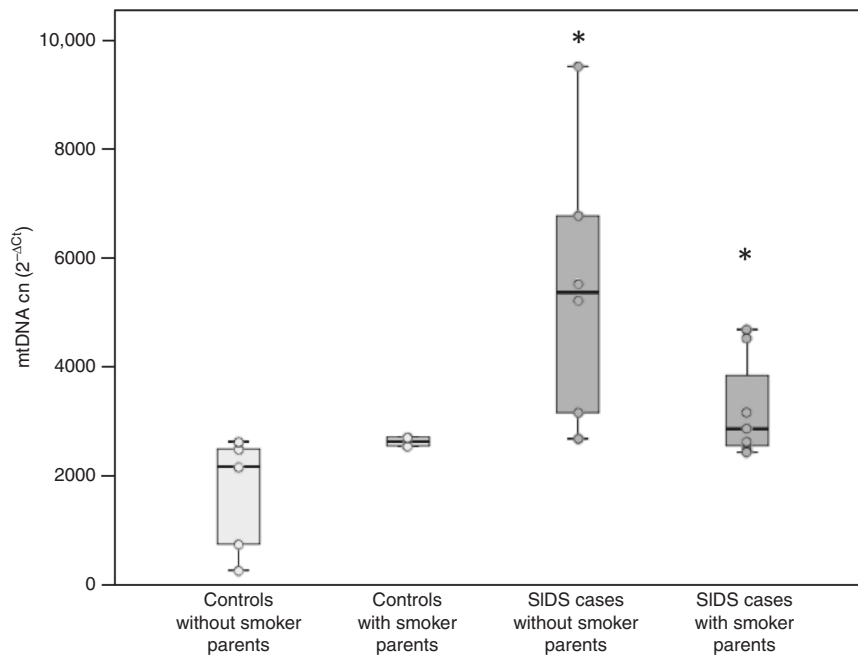


Fig. 3 Relative quantification mtDNAcn in the cerebral cortex of control and SIDS infants born to smoking and non-smoking parents. The bars represent the median and interquartile range. Nonparametric Mann–Whitney *U* test was used to determine the differences in mtDNAcn between the groups. MtDNAcn proved to be higher for the smoking parents control group ($n = 5$) than for the non-smoking parents control group ($n = 2$). MtDNAcn was lower in SIDS smoking parents group ($n = 7$) than for the non-smoking parents control group ($n = 6$) ($P = 0.046$). When only analyzing non-smoking parents group, mtDNAcn proved to be higher in the SIDS group ($n = 6$) than in the control group ($n = 5$) ($P = 0.006$).

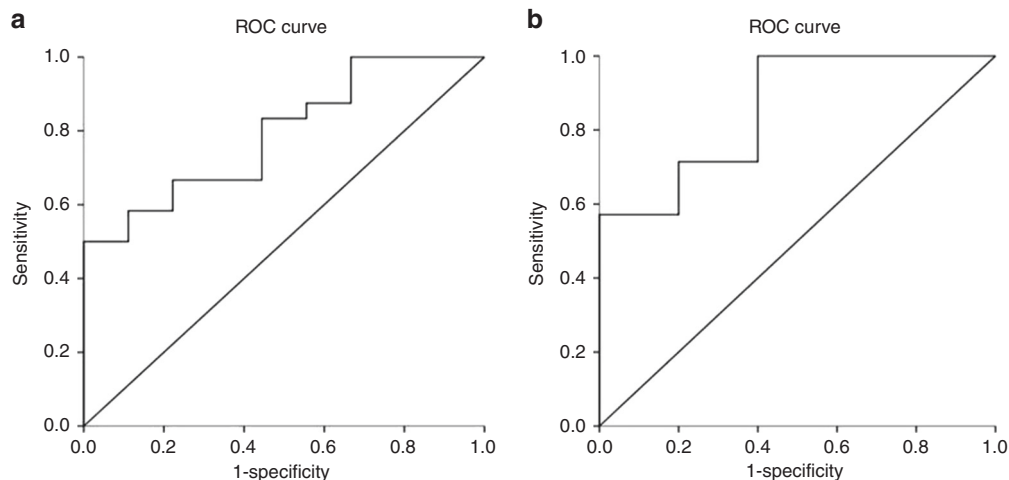


Fig. 4 ROC curves for Sudden Infant Death Syndrome cases. **a** This figure shows the area under the ROC curves for all cases of Sudden Infant Death Syndrome (AUC 0.86 [95% CI: 0.75–0.97], $P = 0.0001$) Ctr ($n = 18$); SIDS ($n = 24$). **b** This figure shows the area under the ROC curves for Sudden Infant Death Syndrome occurring in infants born to non-smoking parents (AUC 0.86 [95% CI: 0.64–1.00], $P = 0.042$). Ctr ($n = 5$); SIDS ($n = 7$). ROC receiver–operator curve.

sex and age. In accordance with our assumption, Opdal et al.^{15,16} interpreted the mtDNA instability found in some SIDS cases to be a genetic predisposition and not the cause of death.

The mtDNA mutations and variations observed in SIDS cases,^{15,17} the apneic pauses during sleep of infants who later succumb to SIDS¹⁸ and ATP deficiency suggest that mitochondrial activity may play a role in the etiology of SIDS. The number of copies and mitochondrial genome integrity are essential for proper cellular functioning.

Following severe mitochondrial dysfunction, a compensatory response occurs which leads to increased mitochondrial biogenesis and respiratory function, resulting in increased mtDNAcn.^{11,19}

Reduced mitochondrial function leads to an increased number of mtDNA copies due to a compensatory mechanism.^{20,21}

Although significant differences were observed between cases and controls when comparing gestational age and birth weight, the differences observed in mtDNA copies at death did not depend on these differences. As regards the controls and SIDS cases born at 33 gestational weeks or born weighing 1000 g or more, the amount of mtDNA in SIDS cases proved to be higher than in the controls ($P = 0.01$, $P = 0.001$ respectively) (data not shown).

Variability in SIDS compensatory mechanisms was observed in relation to the type of nerve structure involved in defective

development. Moreover, in SIDS, developmental defects of the nervous system and more specifically of brainstem centers, are very frequent findings. In this study, these alterations were divided into “mild” and “severe” categories. “Severe” defects were those involving brainstem centers that control breathing and which are therefore considered to be incompatible with life already in the perinatal period. These structures are essentially: the Kölliker–Fuse nucleus, the facial/parafacial complex, the pre-Bötzinger nucleus in the pons/medulla oblongata, and the intermedialateral nucleus in the rostral spinal cord. These nervous centers are linked together via interneuronal synapses in a “respiratory network (RN)” and are able to modulate one another when necessary. Contrastingly, mild alterations are those involving brainstem structures that control functions that are not essential for life, such as hypoplasia of the arcuate nucleus which can also be found in adults.

A noteworthy finding of this study is that mtDNA_{cn} were higher in SIDS victims with mild defects than those with severe defects. Given that respiratory activity must respond to harmful stimuli in order to maintain O₂ and CO₂ homeostasis in blood and tissues, we believe that the presence of developmental alterations of one or more RN components reduces the ability to react and compensate for mitochondrial dysfunction. This means that if mitochondrial dysfunction is associated with alterations in vitally important centers, the compensatory capacity for SIDS is less than that of cases with minor brainstem alterations.

Moreover, the relationship between passive cigarette exposure during infancy and SIDS risk was evaluated. Firstly, the mtDNA content in controls not exposed to smoke was compared with controls exposed to smoke. It was observed that smoke exposure increased the number of mitochondria in controls probably due to respiratory compensation as previously described.²² Contrastingly, lower mtDNA_{cn} were observed in SIDS cases exposed to cigarette smoke than in the SIDS cases not exposed to cigarette smoke.

Although mtDNA_{cn} increases in healthy people and infants when exposed to cigarette smoke, in SIDS cases mtDNA_{cn} decreases regardless of the severity. A decrease in the mtDNA copy number was observed in all SIDS cases regardless of the severity of the abnormalities in brain stem respiratory nuclei.

This phenomenon supports the “Triple Risk Hypothesis”. According to this theory, SIDS occurs due to a combination of three factors: a vulnerable infant, a delicate phase of development, and an exogenous stressor occurring at a critical time. An infant is considered vulnerable when it has morphological abnormalities and biochemical defects of neurotransmission, particularly serotonergic, in the brainstem, according to the unified neuropathological theories.^{23,24} There are some regions in the brain, such as the main nuclei and structures that regulate vital cardiac and respiratory functions that are active both before and after birth.

The risk factors associated with SIDS may be a combination of specific molecular mechanisms, neurotransmitters, and pathways.

It was observed that smoke exposure causes a decrease in mtDNA_{cn}, therefore only SIDS cases that had not been exposed to cigarette smoke were analyzed, which made the difference between the SIDS cases and controls even clearer.

Indeed, the receiver–operator curve (ROC) analysis provided the result of 0.86 considering all the samples and 0.86 considering only the SIDS cases and the controls not exposed to cigarette smoke.

A reliable biomarker for SIDS would enable us to identify high-risk populations that can be monitored by clinicians in order to prevent SIDS deaths from occurring. Moreover, when a fatal outcome cannot be avoided, a reliable biomarker can relieve the emotional burden to the family and facilitate the diagnosis of the medical examiner determining the cause of death.

All these considerations suggest that mtDNA content could be a new biomarker for SIDS. Some papers propose other biomarkers for SIDS, such as orexin, serotonin (5-HT), hypoxanthine, and fetal

hemoglobin,²⁵ which are all associated with mitochondrial activity. Unfortunately, there are no known biochemical biomarkers that can be used to identify SIDS victims at autopsy. Finding a reliable biomarker proves to be a difficult task due to the relatively low incidence of SIDS and the scarce availability of SIDS and non-SIDS autopsy tissue. Despite the limited number of samples used in this study, mtDNA_{cn} is potentially a promising forensic biomarker for SIDS cases, however additional steps should be taken, such as additional validation and larger datasets are required to determine the reliability, sensitivity, and specificity of the biomarker.

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AUTHOR CONTRIBUTIONS

D.L. conceived the study, analyzed, interpreted the data, and organized the collaboration. G.A. performed formalin-fixed, paraffin-embedded cerebral cortex tissues cut. D.L. and R.D. performed DNA extraction, RT-qPCR. D.L. and R.D. wrote the manuscript. A.M.L. oversaw forensic autopsies, provided case review, and revised the manuscript. S.F. revised the manuscript. All authors contributed to the revision of the manuscript and approved the final draft.

COMPETING INTERESTS

The authors declare no competing interests.

CONSENT STATEMENT

This is a case-control study performed at Research Center "Lino Rossi", University of Milan. All the selected cases (24 SIDS and 18 controls), arrived from 2007 since 2020, have followed the ethical measures guaranteed by the Ministry of Health in accordance with the Italian Law n.31/2006 "Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Foetal Death".² This law decrees that all infants suspected of SIDS who died suddenly in Italian regions within the first year of age, as well as all fetuses who died without any apparent cause, must be submitted to a thorough diagnostic post-mortem investigation.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Debora Lattuada.

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