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ORIGINAL ARTICLE

Association of dopamine transporter and monoamine oxidase molecular polymorphisms with sudden infant death syndrome and stillbirth: new insights into the serotonin hypothesis

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Abstract Recent findings demonstrated the role of neurotransmitters in the aetiopathogenesis of sudden unexpected deaths in infancy. Although genes involved in serotonin metabolism have been proposed as risk factors for sudden infant death syndrome (SIDS), the contribution of additional neurotransmitters and genes different from the serotonin transporter (*SLC6A4*, *5-HTT*) has not been investigated. Considering the common metabolic pathway and synergism between dopamine and serotonin, the role of dopamine transporter (*SLC6A3*, *DAT*) and monoamine oxidase A (*MAOA*) genes in SIDS and stillbirth (sudden intrauterine unexplained death, SIUD) was investigated. Genotypes and allelic frequencies of *DAT* and *MAOA* were determined in 20 SIDS and five stillbirth cases and compared with 150 controls. No association was found between *DAT* polymorphisms and SIDS either at genotype (P=0.64) or allelic (P=0.86) level; however, a highly significant association was found between *MAOA* genotypes (P=0.047) and alleles (P=0.002) regulating different expression patterns (3R/3R vs 3.5R/3.5R+4R/4R) in SIDS + SIUD and controls. Analysis of combined *5-HTTLPR* (serotonin transporter linked polymorphic region)/*MAOA* genotypes revealed that frequency of L/L-4R/4R genotype combination was eightfold higher in SIDS + SIUD than in controls (P<0.001). Findings are discussed considering the metabolic association among *DAT*, *5-HTT* and *MAOA* with special emphasis on the linked action of *5-HTT/MAOA* in regulating serotonin metabolism of SIDS and SIUD infants.

Keywords *DAT* · *5-HTT* · *MAOA* · Neurotransmission · SIDS

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Introduction

Sudden infant death syndrome (SIDS) is defined as the sudden death of an infant within 1 year of age, that is, unexpected by medical history and for which a full postmortem examination fails to identify a possible cause of death [1]. Even though the SIDS rate in developed countries decreased more than 50% during the last decade, the syndrome is still the most frequent cause of postneonatal infant death and can be considered responsible of about 25% of deaths in children between 1 month and

1 year of age [2]. The incidence of SIDS in Italy is supposed to be 0.70 in 1,000 live births each year; however, only a marginal number of these deaths are diagnosed and fully investigated. In fact, the majority of them do not undergo a complete diagnostic procedure to avoid additional sorrow to the parents [3]. A comparable incidence has been determined in the USA with a rate in the range 0.67–0.80 in 1,000 births [4, 5]. A much lower incidence has been reported in Japan where the rate is of 0.24 in 1,000 births [6]. Interestingly, the decrease in SIDS in Asian countries compared to western countries is in agreement with different population frequencies of serotonin transporter genotypes.

Similarly to SIDS, the sudden intrauterine unexplained death (SIUD), also referred to as stillbirth, is defined as delivery of a dead fetus occurring after 22 completed weeks of gestation with no evident pathologic cause of death [7]. Although not properly estimated, its incidence in Italy is about six to eightfold greater than that of SIDS and has remained unchanged during the last 20 years [8].

Although aetiopathogenesis of SIDS and SIUD is still obscure [2], they are frequently hypothesised as different expressions of the same pathogenic mechanism based on cytoarchitectural modifications of brainstem nuclei [9]. Recent advances in molecular genetics have opened new perspectives for the assessment of molecular mechanisms altered in SIDS and SIUD. Among the genes analysed during the last decade, several studies identified that polymorphisms in the serotonin transporter gene *SLC6A4* (also called *SERT* or *5-HTT*) are a predisposing factor in infant death [3, 10, 11]. Noteworthy is that the increased frequency of the long allele (L) of the promoter region of *SLC6A4* gene (*5-HTTLPR*) and allele 12 of intron 2 (*Stin*2) of the same gene in specific populations is in agreement with the patterns of SIDS prevalence in different ethnic groups [3, 12, 13].

Recently, genetic and physiology studies of human biogenic amine transporters demonstrated strict correlations between the dopamine and the serotonin systems and provided insights into how genomic variation in their transporter genes influences brain physiology [14]. In particular, similarly to *5-HTT*, the dopamine transporter (DAT) plays a pivotal role in dopaminergic neurotransmission, influencing dopaminergic activity in the synaptic space through reuptake of released dopamine into the presynaptic terminal.

The *SLC6A3* gene (commonly referred as *DAT*), codes for the dopamine transporter and has a 40-bp VNTR polymorphism in the 3′ untranslated region of exon 15 [15]. This VNTR copy number varies between 3 and 11, but the 9 and 10 repeat alleles are the most common in the general population [16]. Compared with the 9-repeat allele, the 10-repeat allele has been associated with increased gene expression both in vitro [17] and in vivo [18], even though results are still contradictory and some studies reported an opposite allelic associations [19].

Intracellular levels of serotonin and dopamine are regulated by the mitochondrial enzyme monoamine oxidase A (MAOA) which regulates serotoninergic and dopaminergic signals through catabolism of vesicular serotonin and dopamine in the presynaptic region (Fig. 1). Sabol et al. [20] described an upstream variable number of tandem repeats (R) within the *MAOA* promoter region. This polymorphism consists of a 30-bp repeated sequence present in three, 3.5, four or five copies. Several studies indicated that the number of repeats is related to the transcriptional efficiency of the gene with the 3R allele associated with reduced transcription activity compared to other alleles [21, 22].

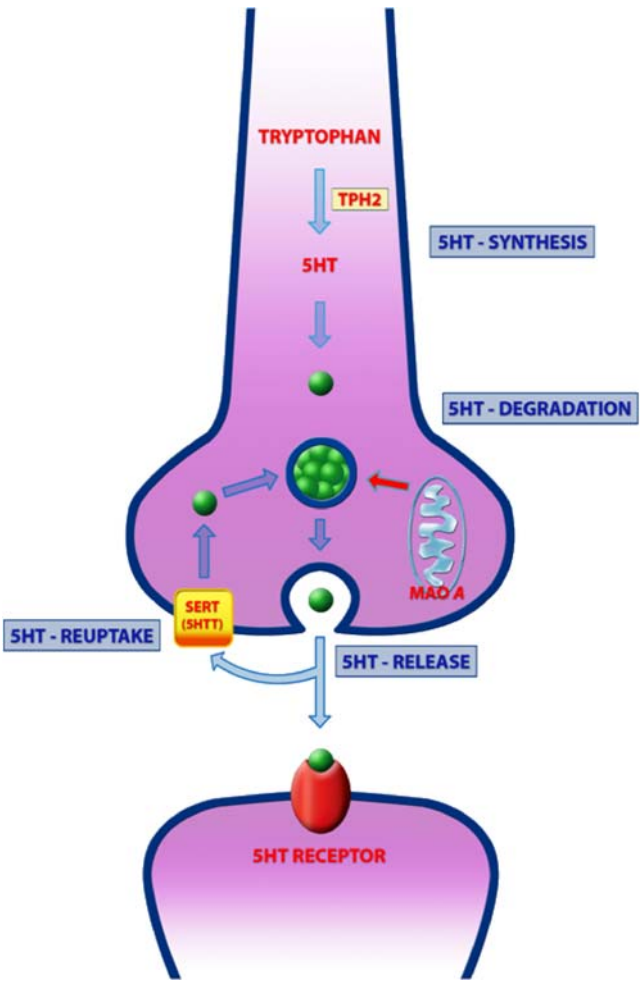

Fig. 1 Serotonin metabolism at presynaptic and synaptic level influenced by mitochondrial monoamine oxidase A and 5-HT transporter. *5-HTT* mediates serotonin reuptake, while *MAOA* reduces vesicular recaptured serotonin. *MAOA* is the prevailing monoamine oxidase isoform and mainly expressed in the nervous system. Dopamine and serotonin pathways are strictly homologous

In spite of increasing literature on the role of DAT and MAOA in different pathologies [22–24], there are no published data on the potential role of these two systems in SIDS and SIUD. Therefore, considering the functional involvement of serotonin metabolism in SIDS [3], an investigation was carried out in an Italian SIDS and stillbirth cohort to assess whether or not the dopamine transporter gene *DAT* and *MAOA* could be implicated in sudden unexpected fetal and infant deaths. Results are discussed considering recent findings on the role of *5-HTT* polymorphisms in SIDS infants and linked function of dopamine and monoamine oxidase. To demonstrate the possible association between different systems involved in serotonin neurotransmission metabolism, our data are compared with the ones obtained from Nonnis Marzano et al. [3]. Conclusions provide additional knowledge on the serotonin hypothesis on the basis of newly discovered association between SIDS/stillbirth and enzymes regulating the neurotransmission pathway.

Materials and methods

Twenty SIDS victims (ten females and ten males, 2 days to 7 months old) and five stillbirth fetus (two females and three males, 34th–40th gestation week) were selected for this study after a complete postmortem investigation performed according to pathologic and forensic criteria [8] which did not reveal a possible cause of death. Seventeen infants died within the third month of age, while only three passed between 5 and 7 months of age. Five SIDS were less than 1 month of age. The five stillbirths were AGA (appropriately grown for gestational age). All infants and fetuses belonged to Caucasian ethnicity except than one Afro-Italian SIDS infant. Brainstem tissues of SIDS and SIUD victims were obtained during necroscopy and conserved either as fresh ethanol-fixed or paraffin-embedded tissues. Brainstem tissue was sectioned at the level of the arcuate nucleus, and anatomical/histological investigations found hypoplasia of this nucleus in some cases. Besides brainstem sections from the deaths, whole blood or urine samples were obtained from 150 Caucasian healthy subjects (80 females and 70 males) older than 1 year of age to be used as controls. Most of controls were university students living in good health conditions and without any sleeping problem. Ten controls were children under 10 years.

Genomic DNA was extracted from autopsy fresh tissues using the Aqua Pure Genomic-DNA Kit (Bio-Rad Laboratories) following the producer's instructions. Paraffined tissues were heated and treated with xylol to eliminate paraffin, according to the optimised protocol proposed by Shi et al. [25]. In particular, deparaffination was carried out on five slices of 10-μm thickness obtained from each paraffin block. After deparaffination, DNA was extracted and purified using a standard proteinase K/SDS–phenol/chloroform method [26]. Extraction yield ranged between approximately 1,000 and 10,000 ng.

DNA was extracted from 200 μl of whole-blood samples of healthy controls using the Charge Switch gDNA blood kit (Invitrogen). Urine samples were used to avoid blood collection from children. About 100 ml of first morning urine was collected and centrifuged at 15,000 rpm. Sedimented cells were then pipetted and submitted to DNA extraction carried out by means of QIAmp DNA blood mini kit (Qiagen).

The *SLC6A3* VNTR polymorphism was amplified by polymerase chain reaction (PCR) (thermal cycler MJ Research PTC100) using specific primers suggested by Gerra et al. [24]: forward, 5′-TGTGGTGTAGG-GAACGGCCT-3′; reverse, 5′-CTTCCTGGAGGT-CACGGCTCAAGG-3′. PCR amplification was performed in a final volume of 20 μl consisting of 30 ng of genomic DNA, dNTPs 0.2 mM, 10 pmol of forward and reverse primers, 1× reaction buffer and 1 U of GoTaq DNA Polymerase (Promega, Madison WI, USA). Amplification conditions were the following: initial denaturation step at 94°C for 10 min, 45 cycles including 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. PCR products were separated by 2.5% agarose gel electrophoresis followed by ethidium bromide staining and visualised under UV light. The different alleles were determined using the GeneRuler 100 bp DNA ladder (Fermentas, Hanover, MD, USA).

The *MAOA* VNTR was typed using primers suggested by Cohen et al. [27]: forward, 5′-ACAGCCTGACCGTGGA-GAAG-3′; reverse, 5′-GAACGGACGCTCCATTCGGA-3′. PCR amplification was conducted in a final volume of 25 μl consisting of 30 ng of genomic DNA, dNTPs 0.2 mM, 10 pmol of forward and reverse primers, 1× reaction buffer and 1 U of GoTaq DNA Polymerase (Promega). Amplification conditions were: 5 min at 95°C for initial denaturation, followed by 40 cycles of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C and a final extension at 72°C for 10 min. PCR products were separated by 2.5% agarose gel electrophoresis, stained with ethidium bromide and UV-revealed. Allele repeats 3, 3.5, 4 and 5, corresponding, respectively, to 320, 335, 350 and 380 bp, were identified using the GeneRuler 100 bp DNA ladder (Fermentas) and are herein referred as 3R, 3.5R, 4R and 5R, respectively.

Methodological procedures to detect *5-HTTLPR* polymorphisms were described in detail in a previously published paper [3].

To investigate possible associations between genotypes/alleles and experimental groups, a likelihood ratio (LR) χ^2 test was performed. It is a more robust test than the classical Pearson χ^2 to deal with low-number categories such as genotype and allele groups. Statistical analyses were

Table 1 Dopamine transporter VNTR genotypes and allele frequencies (%) in SIDS + SIUD and in healthy controls

DAT	Controls (n=150)		SIDS + SIUD (n=25)		LR χ^2 test	P value
	Number	Percentage	Number	Percentage		
Genotypes					0.22	0.64
9/9	0	0.0	1	4.0		
9/10	82	54.7	10	40.0		
9/11	0	0.0	0	0.0		
10/10	54	36.0	14	56.0		
10/11	14	9.3	0	0.0		
11/11	0	0.0	0	0.0		
Alleles					0.03	0.86
9	82	27.3	12	24.0		
10	204	68.0	38	76.0		
11	14	4.7	0	0.0		

performed using Statistica software (version 7.1). Values of P were considered highly significant when lower than 0.01 and significant in the range $0.01<P<0.05$. In addition, values in the range $0.05<P<0.10$ were considered as a trend of significance. Degrees of freedom were equal to one less than the number of alleles/genotypes.

Results

Dopamine transporter

Dopamine transporter genotypes and allelic frequencies are shown in Table 1. Although VNTR alleles with 9, 10 and 11 repeats [16] were detected in all considered samples, a different distribution was observed between pathologic and non-pathologic samples. Noteworthy is that the 11-repeat allele was not found in SIDS and SIUD samples (0%), and its frequency in healthy controls (4.7%) was limited only to a few heterozygotes 10/11 (9.3%). The allele with three repeats was never detected. The 9 and 10 repeats were the most common forms in both SIDS + SIUD and controls, with the 10-repeat allele being the highest in both classes (SIDS + SIUD 76% vs. controls 68%).

Four different genotypes were detected, and the heterozygotes 9/10 (SIDS + SIUD 40% vs. controls 54.7%) and homozygotes 10/10 (SIDS + SIUD 56% vs. controls 36%) were the most represented allelic combinations.

Considering overall data of dopamine transporter polymorphisms, no statistical significance emerged between pathologic and control samples both at genotype (χ^2=0.22, P=0.64) and allele (χ^2=0.03, P=0.86) level.

Table 2 Frequency of *MAOA* VNTR genotypes and alleles determined in SIDS infants and controls

MAOA	Controls (n=150)		SIDS + SIUD (n=25)		LR χ^2 test	P value
	Number	Percentage	Number	Percentage		
Genotypes					3.94	0.047
3/3	39	26.0	4	16.0		
3/3.5	0	0.0	0	0.0		
3/4	43	28.7	6	24.0		
3/5	0	0.0	0	0.0		
3.5/3.5	0	0.0	0	0.0		
3.5/4	4	2.6	1	4.0		
3.5/5	0	0.0	0	0.0		
4/4	64	42.7	14	56.0		
4/5	0	0.0	0	0.0		
5/5	0	0.0	0	0.0		
Alleles					9.60	0.002
3	121	40.3	14	28.0		
3.5	4	1.3	1	2.0		
4	175	58.4	35	70.0		
5	0	0.0	0	0.0		

Monoamine oxidase A

Three variants of the *MAOA* VNTR (30-bp repeated sequence) polymorphisms were detected and the results are reported in Table 2. Alleles defined by three (320 bp), 3.5 (335 bp) and four (350 bp) repeats within the promoter region were identified in 25 pathologic samples and 150 controls. The 2.5R and 5R alleles were absent in both groups. Our data are in agreement with varying number of allele repeats originally defined by Sabol et al. [20, 28].

Frequency of genotype 3R/3R was about twice in the controls (26%) compared to SIDS + SIUD infants (16%). Some difference also emerged for genotype 3.5R/4R, even though this genotype was poorly represented in our investigation (controls 2.6% vs. SIDS + SIUD 5%). Genotype 3R/4R were similar in the two groups, whilst 4R/4R was higher in pathologic samples (56%) than in controls (42.7%).

Considering allelic frequencies of three, 3.5 and four repeats, opposite trends were observed for 3R and 4R alleles (respectively, SIDS + SIUD 28% vs. controls 40.3% and SIDS + SIUD 70% vs. controls 58.4%). Comparisons between pathologic and non-pathologic samples were statistically significant for frequency of genotype 3/3 vs. 3/4+3.5/4+4/4 (LR χ^2=3.94, P=0.047) and highly significant for distribution of allele 3 vs. 3.5+4 (LR χ^2=9.60, P=0.002).

5-HTTLPR/*MAOA* combined systems

Results obtained by combination of *5-HTTLPR*, a known risk factor for SIDS and *MAOA* genotypes and allelic data, are herein reported in Table 3. Results concerning polymorphisms of promoter region of serotonin transporter were obtained by Nonnis Marzano et al. [3] where it was reported that L/L genotype contributed to 60% of SIDS cases with respect to 14% of controls (P=0.001), and L allele was twofold greater in SIDS than in controls (P< 0.001). In this work, 48% of pathologic samples belonged to genotype L/L-4R/4R. This value was about eightfold higher the one determined in non-pathologic samples (6%) and was the most represented data considering all different groups (both pathologic and non-pathologic). Genotype L/L-3R/4R was twofold in SIDS + SIUD (12%) compared to controls (6%). Comparison between *5-HTTLPR*/*MAOA* genotypes L/L-4R/4R and L/L-3R/3R was statistically highly significant (LR χ^2=16.89, P=0.00001). The major contribution of *MAOA* allele 4R in pathologic samples also emerged during analysis of allelic combinations. In fact, L-3.5R + 4R frequency (3.5R and 4R have enhanced metabolic activity compared to 3R and can therefore be considered together) in pathologic samples was the highest among all groups. Its frequency was more than twofold in SIDS+SIUD (58.7%) than in controls (25.7%). Once again, statistical comparison between *MAOA* alleles associated to *5-HTT* long allele was highly significant

Table 3 Combination of *5-HTTLPR* and *MAOA* data in pathologic and non-pathologic samples

5-HTTLPR	MAOA	Controls (n=150)		SIDS + SIUD (n=25)		LR χ^2 test	P value
		Number	Percentage	Number	Percentage		
Genotypes							
S/S	3/3	13	8.7	0	0.0		
S/S	3/4	13	8.7	0	0.0		
S/S	3.5/4	0	0.0	0	0.0		
S/S	4/4	17	11.3	0	0.0		
L/S	3/3	26	17.3	4	16.0		
L/S	3/4	21	14.0	3	12.0		
L/S	3.5/4	4	2.7	1	4.0		
L/S	4/4	34	22.6	2	8.0		
L/L	3/3	4	2.7	0	0.0	16.89	0.00001
L/L	3/4	9	6.0	3	12.0		
L/L	3.5/4	0	0.0	0	0.0		
L/L	4/4	9	6.0	12	48.0		
Haplotypes							
S	3	86	24.6	7	12.0		
S	3.5–4	110	31.4	7	12.0		
L	3	64	18.3	10	17.3	15.64	0.0001
L	3.5–4	90	25.7	34	58.7		

Statistical significance was assessed between combinations having the L/L genotype and L allele

(LR χ^2=15.64, P=0.0001). A representation of combination of genotypes and alleles in SIDS + SIUD versus controls is illustrated in Fig. 2. Differences between genotypes and alleles data having different functional activities are clearly evident.

Discussion

Although recent literature described the role of *DAT* and *MAOA* polymorphisms in different pathologies [22–24, 29] and the evidence that different genotypes can moderate children sensitivity to environmental insults [24, 30], no data on their potential role in unexpected fetal and infant deaths were ever investigated. The two systems appear of particular interest in the field of unexpected deaths considering their functional link with the serotonin transporter and the previously defined involvement of serotonin in SIDS [3, 11, 31].

The *DAT* gene, coding for the dopamine transporter, has a 40-bp VNTR polymorphism in the 3′ untranslated region of exon 15, associated to differential expression activity. Contrary to our hypotheses, *DAT* genotypes did not display

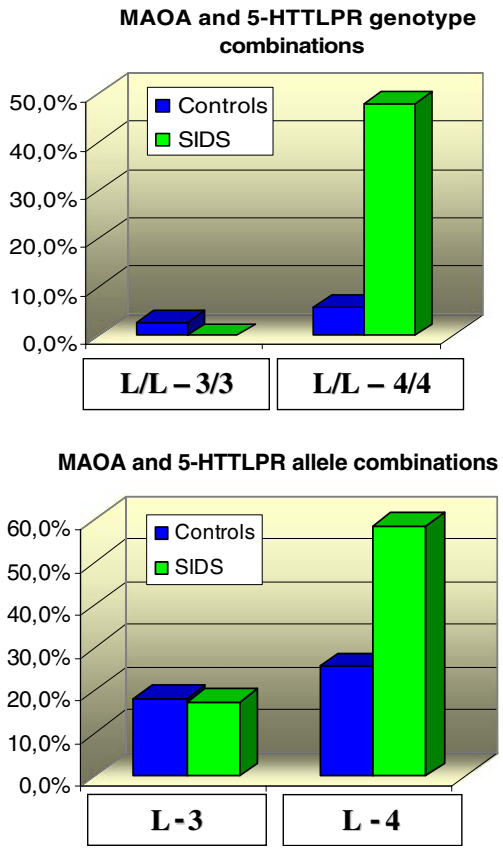

Fig. 2 Frequency of combined *5-HTTLPR/MAOA* genotypes (P=0.00001) and alleles (P=0.0001) in SIDS + SIUD and controls relevant for differential functional activity

significant differences between SIDS + SIUD and controls. Three alleles (9, 10, 11 repeats) were present in both pathologic and control samples, with 9 and 10 repeats being the most common forms. The frequency of the 10-repeat allele resulted the highest both in SIDS + SIUD (76%) and in controls (68%), and they were in agreement with previously published data by Persico et al. [32] for Italian (63%) and Caucasian-American populations (71%). The 11-repeat allele was not found in SIDS; however, its frequency was also very low in healthy controls (4.7%). Considering experimental results and statistical elaborations, dopamine did not seem to be involved in SIDS and stillbirths. A possible synergic action between serotonin and dopamine in etiopathogenesis mechanisms cannot be demonstrated in our study, although it must be remarked that one limitation of this investigation is the low number of patients and additional observations will have to be carried out in a larger sample set.

Novel important results emerged by analyses of an upstream VNTR region within the *MAOA* promoter [20] that contains a 30-bp repeat sequence consisting of three, 3.5, four or five copies. Expression studies indicated that the number of repeats directly correlates to the transcriptional efficiency of the gene, with the 3-repeat allele having lower gene expression, and hence reduced activity, relative to the alleles 3.5R and particularly 4R [21, 27].

In this investigation, a statistically significant difference in genotype 3R/3R and 4R/4R distribution between pathologic and control samples was observed. In particular, a lack of genotype 3R/3R was observed in SIDS + SIUD with a higher recurrence of genotype 4R/4R in this group with respect to controls. Moreover, differences also emerged at allelic level with allele 4R in SIDS + SIUD showing higher frequency (70%) than the controls (58.4%). An opposite trend was observed for allele 3R which was more frequent in healthy (40.3%) than pathologic samples (28%). Interestingly, allelic frequencies of healthy control samples were in strict agreement with the ones reported by Deckert et al. [33] for the general Italian population (allele 4R, 57%; allele 3R, 40%). Therefore, differences observed between experimental pathologic and non-pathologic samples (P=0.002) were supported by literature data [33] and confirmed the involvement of the *MAOA* system in SIDS and SIUD. Apparently, no significant differences were attributed to one of the two subgroups of deaths; however, stillbirths will have to be investigated in a larger sample. It must be remarked that allele 4R is responsible of enhanced protein expression and therefore greater catabolic action on recaptured serotonin. Hence, its high frequency in pathologic samples may act synergically with serotonin transporter to influence SIDS and stillbirth pathogenesis.

This was confirmed by the analysis of combined *5-HTTLPR/MAOA* genotypes that demonstrated a major

incidence of L/L-4R/4R group in SIDS + SIUD (48%) with respect to data detected in the other pathologic (4–16%) and healthy groups (2.7–22.6%). Differences were supported by highly significant statistics ($P<0.001$). Noteworthy is that the frequency of L/L-4R/4R genotype combination was eightfold greater in SIDS + SIUD than in controls. Considering the supposed putative role of *5-HTTLPR* long allele as a risk factor for SIDS [3, 12, 13], the combination L-4R was assessed to verify a possible synergism of both alleles in the syndrome pathogenesis. The L-4R allele combination was three times more frequent than the L-3R in pathologic samples, and differences were statistically significant ($P<0.001$).

In conclusion, the present study does not demonstrate any apparent involvement of dopamine transporter in the etiopathogenesis of SIDS and stillbirth. However, our results do confirm the role of highly expressed *MAOA* 4R allele which can be considered an additional predisposing factor that synergically acts with L allele of serotonin transporter in moderating children's sensitivity to environmental insults. Considering serotonin metabolism, it can be hypothesised that enhanced serotonin reuptake driven by L/L genotype is followed by increased vesicular serotonin catabolism caused by *MAOA* 4R/4R genotype. A lack of interneuronal serotonin release could consequently happen whenever original production from tryptophan was lacking due to environmental or metabolic factors.

The limitations of this study mainly referred to a low number of available SIDS, and SIUD samples do not diminish the potential importance of results. Starting from our data obtained in a homogeneous ethnic group, additional investigations will have to be carried out in other populations to assess ethnicity differences.

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