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ORIGINAL ARTICLE

PHOX2B polyalanine repeat length is associated with sudden infant death syndrome and unclassified sudden infant death in the Dutch population

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Abstract Unclassified sudden infant death (USID) is the sudden and unexpected death of an infant that remains unexplained after thorough case investigation including performance of a complete autopsy and review of the circumstances of death and the clinical history. When the infant is below 1 year of age and with onset of the fatal episode apparently occurring during sleep, this is referred to as sudden infant death syndrome (SIDS). USID and SIDS remain poorly understood despite the identification of several environmental and some genetic risk factors. In this study, we investigated genetic risk factors involved in the autonomous nervous system in 195 Dutch USID/SIDS cases and 846 Dutch, age-matched healthy controls. Twenty-five DNA variants from 11 genes previously implicated in the serotonin household or in the congenital central hypoventilation syndrome, of which some have been associated with SIDS before, were tested. Of all DNA variants considered, only the length variation of the polyalanine repeat in exon 3 of the *PHOX2B* gene was found to be statistically significantly associated with USID/SIDS in the Dutch population after multiple test correction. Interestingly, our data suggest that contraction of the *PHOX2B* exon 3 polyalanine repeat that we found in six of 160 SIDS and USID cases and in six of 814 controls serves as a probable genetic risk factor for USID/SIDS at least in the Dutch population. Future studies are needed to confirm this finding and to understand the functional effect of the polyalanine repeat length variation, in particular contraction, in exon 3 of the *PHOX2B* gene.

Keywords Sudden infant death syndrome, SIDS · Unexplained sudden infant death, USID · Congenital central hypoventilation syndrome, CCHS · Autonomic nervous system, ANS · Genetic association · SNPs, single nucleotide polymorphisms · *PHOX2B* polyalanine repeat length

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Introduction

Sudden and unexpected death in infancy (SUDI) includes every infant that dies suddenly and unexpectedly, even when no investigations are done. If investigations, including performance of a complete autopsy and review of the circumstances of death and the clinical history, do not lead to a diagnosis, the case is considered unclassified sudden infant death (USID). Cases of sudden and unexpected death of an infant below 1 year that remain unexplained, with onset of the fatal episode apparently occurring during sleep, are referred to as sudden infant death syndrome (SIDS) [1]. The SIDS incidence rate in the Netherlands is ~0.08 per 1,000 live births [2]. This value is considerably lower than in other countries such as the USA, where it is reported to be around 0.57 per 1,000 living births [3]. Furthermore, given that the practised definition of SIDS in the Netherlands comprises infant cases from birth up to 3 years of age, the reported Dutch SIDS incidence must be considered as over-rated. Accordingly, several cases classified in the Netherlands as SIDS are in fact USID considering the internationally accepted definition [1]. The relatively low USID/SIDS rate in the Netherlands might be partly explained by the success of a parental education system that is based on the findings of the Working Group on SIDS of the Dutch Paediatric Society (Landelijke Werkgroep Wiegendood, LWW).

Previous studies have identified several environmental risk factors for SIDS, considering sleeping manners, smoking habits of the parents, socioeconomic trades and few intrinsic factors such as premature birth [3, 4, 5]. Notably, the physiological mechanisms on how these factors contribute to SIDS remain hypothetical. Furthermore, not all SIDS cases are, and many healthy adults during their infancy have been, subject to one or more of the known risk factors. Therefore, additional environmental as well as non-environmental factors must exist. Non-environmental factors could be of particular relevance in the Dutch population, where the main environmental factors associated to USID/SIDS are somewhat controlled due to the LWW education program. Several genetic factors that could explain USID/SIDS have been suggested [3, 6, 7] are mainly from three categories: long QT syndrome, immune system and autonomous nervous system (ANS) [3, 6, 7]. The latter is the focus of the present study.

ANS includes genes involved in the congenital central hypoventilation syndrome (CCHS). CCHS patients do not react properly on elevated CO_2 levels in the blood, which may be of particular relevance in USID/SIDS since several environmental risk factors, such as laying in the face-down position, are expected to result in elevated CO_2 levels. The expansion of the polyalanine repeat in exon 3 of the paired-like homeobox 2B (*PHOX2B*) gene is reported in 96 % of known CCHS patients [8, 9]. Other genes have also been associated with CCHS, such as the rearranged during transfection (*RET*) gene [10–13], the glial-derived neurotrophic factor (*GDNF*) gene [10–13], the brain-derived neurotrophic factor (*BDNF*) gene [11], the human achaete–scute homolog-1 (*HASH1*) gene [11], the paired-like homeobox-2A (*PHOX2A*) gene [10–13], the endothelin-1 (*EDN1*) gene [11, 13], the bone morphogenic protein-2 (*BMP2*) gene [13] and the endothelin converting enzyme-1 (*ECE1*) gene [10, 11, 13]. All of these genes play a role in embryologic brain development. Notably, rare DNA variants in the *ECE1*, *RET*, *PHOX2A*, *BMP2* and *EDN1* genes have previously been found in single SIDS cases [13].

Furthermore, ANS includes genes involved in the serotonin household. Serotonin influences cardiovascular control, temperature control, sleep–wake cycle and breathing control. The serotonin transporter (*5HTT*) serves as a bottleneck in regulation of serotonin release and reuptake. Previous genetic associations of DNA polymorphisms of the *5HTT* gene and its promoter region (*5HTTLPR*) with SIDS have been inconclusive [7], with studies reporting statistically significant associations in certain populations [14–18], but no association in other [14, 15, 19, 20]. The latter study showed some evidence that structural differences in the serotonergic brainstem may contribute to the risk of SIDS [20].

Notably, most DNA variants previously found to be statistically significantly associated with SIDS are rare so that large studies are required in order to achieve statistical power. Several environmental risk factors are suspected to trigger SIDS through low CO2-levels, to which the ANS should respond. Thus, the combination of such an environmental risk factor with a genetic abnormality in one of the ANS genes could potentially explain a subset of the SIDS cases. Because the incidence of SIDS in the Netherlands is very low due to the attempted control of environmental factors, the percentage of genetic factors that underlie SIDS could be relatively enriched in the Dutch SIDS population. We therefore studied 25 DNA variants from 11 genes (*RET*, *PHOX2B*, *PHOX2A*, *HASH1*, *EDN1*, *5HTTLPR*, *ECE1*, *GDNF*, *BDNF*, *5HTT* and *BMP2*) previously involved in ANS particularly the serotonin pathway and CCHS, in the Dutch USID/SIDS population via a retrospective case–control study to investigate their association with USID/SIDS using overall 195 cases and 846 healthy age-matched controls.

Patients and methods

Study design and samples

We designed a retrospective case–control study to test for the presence of genetic risk factors with SIDS in the Netherlands. Details of case collection and the ethical approval have been documented previously [5]. In summary, a total of 200 USID/SIDS cases collected across the Netherlands from

1984 to 2005 were gathered. Of those, 167 involved infants below 1 year of age (SIDS), whereas 33 were older (USID). After histology review [21], cases were categorised as either genuine USID/SIDS: no findings at histology, or (A) USID/SIDS with incidental findings, but most likely not related to death, or (B) USID/SIDS with more extensive findings that might have contributed to the demise but probably are not a cause of death, or (C) probable cause of death. The latter cases were excluded from the current study except for the severe infectious cases such as bronchopneumonia because of the ongoing discussion if these can cause death on its own or are activators of other fatal predispositions [1]. This resulted in five cases (four SIDS and one USID) that were excluded from the current study because of clear signs of storage disease or fatal aspiration indicating a putative cause of death. In the current study, we re-categorised the remaining cases as either (1) SIDS category I according to the San Diego criteria/USID (SIDS category I according to the San Diego criteria; n=124), or (2) SIDS with minor infections most likely not contributing to the demise (previously A/B, SIDS category I according to the San Diego criteria; n=40), or (3) USID/SIDS with incidental findings most likely not related to death and not infectious (previously A/B, SIDS category II according to the San Diego criteria; n=4), or (4) severe infectious cases such as bronchopneumonia (previously C, SIDS category II according to the San Diego criteria; n=14). Of 13 cases, there were too little histology slides to review [21]. Age-matched healthy infants were collected from the Generation R Study [22]. The DNA was isolated from cord blood, and interviews for environmental factors were taken during pregnancy and after birth at 2, 6, 8 and 12 months of age. Forty-eight percent was female. All controls were of Dutch European origin. Finally, 195 cases and 846 controls all from the Netherlands were included in this study.

Ethical approval

The project protocol was approved by the medical ethical committees of the Erasmus MC, Rotterdam and three collaborating hospitals. All committees agreed that whenever the parents had given approval for investigation into the cause of death of their child (an autopsy), they had implicitly agreed to participate in this study, as this study is a further investigation into the cause of death. Because forensic paediatric autopsies are done without parental consent, inclusion of the four forensic cases in this study was done after parental approval, and the approval of the public prosecutor or the Attorney General involved in the respective cases was obtained.

DNA isolation

Case DNA samples were isolated from paraffin-embedded tissue samples (see supplementary methods part 1). DNA concentrations of case samples were estimated using the nanodrop ND-1000 spectrometer, and the quality was tested with an amelogenin-PCR [23]. When the amelogenin-PCR identified gender matched the record gender information, a PCR-based DNA quantification was carried out using the Quantifyler Realtime PCR-kit (Applied Biosystems Inc., Carlsbad, CA, USA) applying the manufacturer's protocol. Control DNA samples from the Generation R Study were isolated as described previously [22], were provided to this study with a random identification number and were quantified using PicoGreen® based on the manufacturer's protocol.

DNA variants and genotyping

Twenty-two DNA variants from 11 genes (*RET*, *PHOX2B*, *PHOX2A*, *HASH1*, *EDN1*, *5HTTLPR*, *ECE1*, *GDNF*, *BDNF*, *5HTT* and *BMP2*) were ascertained from the literature in 2006 using PubMed (www.ncbi.nlm.nih.gov) based on their reported involvement in ANS [6, 8–16]. Furthermore, three tagging SNPs from the exons of the *RET*, *EDN1* and *ECE1* genes were selected using the HapMap CEU data release #20 [24]. The 25 DNA variants were genotyped using various technologies depending on marker type and technology constrains such as fluorescence-based fragment length analysis, SNaPshot® single-base primer extension multiplexing (AB), Sequenom iPLEX mass spectroscopy multiplexing (Sequenom Inc., San Diego, CA, USA) and TaqMan® (AB) analysis. Multiplex genotyping strategies were employed unless the genotyping method of choice did not allow to such as with TaqMan. For further details, see supplementary methods 2–4. The impact of expected DNA fragmentation due to paraffin embedding of the case materials was aimed to be minimized by designing genotyping assays targeting short DNA fragments. Amplicon fragment length across the 25 loci ranged between 71 and 231 bp with an average length of 117 bp. Due to expected low DNA yields from the paraffin-embedded case samples, DNA quantity of case samples was measured using a highly sensitive PCR-based approach. All cases and/or controls that in the first analysis showed the minor allele and thus influenced the statistical significance of the association were retyped in singleplex using the same primers as used in the multiplex analysis. Only confirmed findings are reported.

Blinding of genotype collection and testing for DNA contaminations

PCRs were carried out in MicroAmp optical 348 well reaction plates (AB) containing 1 ng DNA per well of either case or control sample. The person who ran and analysed the plates was blinded for the case and control locations at the time of genotyping. To control for DNA and PCR-product contamination, each plate had two rows with eight wells of aquadest

representing no DNA (negative controls 1); four empty wells to serve as negative control in the subsequent second step of genotyping, such as *SNaPshot* primer extension reaction, where they were filled with the respective reaction mix (negative controls 2); 12 DNA control samples of known genotypes (positive controls) and eight duplicates of another plate, to check for reproducibility between plates. The locations of the 12 DNA controls were known to the person who ran and analysed the plates. All plates were independently scored by two experienced analysts. In cases where there was a discrepancy between the analysts, the specific test was rerun. After all genotypes were scored and collected, the locations of cases and controls were released to the analyst, and statistical data analyses were performed. Because discordance was sporadically found between the duplicates, DNA plates produced from the same mother plates as used for producing the DNA plates for the present study were used for genotyping of three polymorphic miniSTR loci (D10S1248, D14S1434 and D22S1045, see supplementary methods part 5) for contamination detection. Only samples that showed no indication of the presence of more than one individual in the miniSTR results were finally used in the USID/SIDS marker genotype analyses.

Statistical analyses including confounding factor consideration

Potential deviation from the Hardy–Weinberg equilibrium (HWE) was tested for every DNA variant used by applying the bi-allelic chi-square test with 1 degree of freedom in controls. As the 25 DNA variants tested are located in 11 genes, the number of independent multiple tests was set to 11. The association between each DNA variant and USID/SIDS was examined by cross-tabulation of the case–control status stratified by genotypes, and the Fishers exact test was used to estimate the statistical significance of the association. Multiple testing problem was corrected for by using the Bonferroni method [25]. Haplotype association was examined for all chromosomal regions by considering sliding windows of two DNA variants using R library haplo.stats v1.3.8 [26]. Genetic interactions between DNA variants were tested in pair-wise fashion by comparing deviances of logistic models with and without a multiplicative interaction term. The difference in model deviances follows chi-square distribution with 1 degree of freedom under the null hypothesis of no interaction. Suggestive interactions were further investigated using cross-tabulates stratified by genotypes.

For the SIDS subjects used here for genetic analysis, prone sleeping (odds ratio (OR)=21.3, 95 % confidence interval (CI)=[7.4, 61.1], $P<0.005$), pre- and postnatal parental smoking (smoking by both parents (OR=7.3, 95 % CI=[1.8, 30.7], $P=0.006$), smoking of one parent (OR=3.1, 95 % CI=[1.1, 8.8], $P=0.04$)) and premature birth (OR=3.2, 95 % CI=[1.2, 8.3], $P=0.02$) were previously found to be significant environmental risk factors, while controlled for socioeconomic status [5]. To exclude the potential confounding effects of environmental and intrinsic factors on genetic association, we repeated the multivariate logistic regression analysis with the current case population, including USID cases and current control group, testing for gender, prone sleeping, pre- and postnatal parental smoking, premature birth, breastfeeding and bed sharing. To investigate if genetic factors are specifically associated with certain USID/SIDS sub-categories that we previously determined via histological re-examination of the USID/SIDS subjects used here [21], we repeated the genetic association testing for each of the four USID/SIDS subgroups. All statistical analyses were performed using R v2.9.2 and SPSS v20.0 (SPSS Inc, Chicago, IL, USA).

Results

As DNA analysis from paraffin-embedded materials used here for the case samples is known to be challenging and working with DNA plates produced via robotic means in large cohort studies used here for the control samples may provide risks for minor DNA contaminations, we took extra attention on assuring data authenticity. Most notably, we tested for DNA contamination by use of highly polymorphic miniSTR systems (see "Patients and methods" sections and supplementary method 5). Thirty-two case (24 SIDS and 7 USID) and 21 control samples revealed STR profiles that could not be explained by the presence of DNA from a single individual per sample alone. As these results can be interpreted as potential contaminations and thus could (but not necessarily will) impact on the association results, we took a conservative approach and excluded these 53 samples from further analyses. This leaves 163 cases (138 SIDS and 25 USID) and 825 healthy age-matched controls for statistical analyses. Of the SIDS cases, 37 % were females (51 females, 86 males, one unknown), the mean age was 140 days (approximately 4.5 months; range 17 to 338 days) and ethnicities were European ($n=124$; 89.9 %), African ($n=4$; 2.9 %), Arabic ($n=2$; 1.4 %), Asian ($n=1$; 0.7 %), Latin American ($n=1$; 0.7 %), mixed European-African ($n=3$; 2.2 %), mixed European–Arabic ($n=1$; 0.7 %), mixed European–Latin American ($n=1$; 0.7 %) and mixed Arabic–Latin American ($n=1$; 0.7 %). Of the USID cases, 44 % were females (11 females, 14 males), the mean age was 525 days (approximately 1 year and 5 months, range from 371 to 977 days) and ethnicities were European ($n=22$, 88 %), Arabic ($n=1$; 4 %), Asian ($n=1$; 4 %) and mixed European–Latin American ($n=1$; 4 %).

Next, we inspected the complete locus drop-out rates across the 25 DNA variants tested. For the *5HTTLPR* fragment length, 57.1 % of the cases and 21.3 % of controls had no

genotype results. We therefore excluded this DNA variant from further analyses. The drop-out rates for the remaining 24 DNA variants considering the 163 case samples after quality control ranged from 0 % for 12 loci (*RET* P1039L, *RET* R114H, *RET* L56M, *RET* G691S, *RET* D489N, *BDNF* rs8192466, *GDNF* R93W, *EDN1* rs1476046, *5HTT* rs2020939, *PHOX2B* 618ins, *ECE1* rs1076669 and *BMP2* L292F) to 4.3 % for *RET* T706A and *ECE1* rs6665399 with an average drop-out rate for the cases across all 24 loci of 1.04 % (supplementary table 3). Drop-out rates for control samples were found to be lower with an average rate across all 24 loci of 0.34 % and a range from 0 % for 13 loci (*RET* P1039L, *RET* R114H, *RET* R418Q, *RET* G691S, *RET* D489N, *BDNF* rs8192466, *GDNF* R93W, *EDN1* rs5370, *5HTT* rs2020939, *PHOX2B* 618ins, *ECE1* rs1076669, *ECE1* T354A and *BMP2* L292F) to 2.3 % for *BMP2* rs2273073 (supplementary table 3). All 24 DNA variants considered followed HWE ($P>0.05$) in the controls, and no genetic interactions were found among DNA variants of different genes.

Of the 24 DNA variants tested, only the polyalanine repeat length variation in exon 3 of the *PHOX2B* gene was found to be statistically significantly associated with USID/SIDS in the tested Dutch samples (Table 1; for non-significant variants, see Table 2). The normal length of the *PHOX2B* polyalanine repeat as established with the method used was 112 bp (representing 20 alanine repeats), and most cases and controls (98.3 %) were found to be homozygous for this normal length allele. However, contractions of the alanine repeat array (varying from 3 bp or one alanine to 12 bp or four alanines) were statistically significantly more often found in USID/SIDS cases compared to healthy age-matched controls (OR=6.05, 95 % CI=[2.02, 18.1], $P<0.001$). Notably, the USID/SIDS association effect of the contracted repeat remained statistically significant after Bonferroni multiple test correction. This effect was also seen at the homozygote stage of the contracted allele (OR=infinitive, $P<0.001$), albeit only with one SIDS case sample (no USID case, hence one in 160 cases in total) with a contraction of three repeats (9 bp or three alanines) in both alleles, while none of the 814 control samples showed the homozygote stage of this repeat contraction. Repeat expansions (varying from 3 bp or one alanine to 15 bp or five alanines) also occurred in our case and control samples. A statistically significant USID/SIDS association of the expanded allele was only seen for the homozygote stage (OR=infinitive; $P<0.001$) also after multiple test correction. This effect was caused by a single control sample (both alleles expanded by 12 bp or four alanines). However, when considering the presence of at least one expanded allele, no statistically significant USID/SIDS effect of the expanded repeat was seen (OR=1.27, 95 % CI=[0.14, 11.4], $P=0.83$). The genotype combination of one expanded and one contracted allele present within the same individual was not found among the case and control samples tested, which may simply be a sample size effect or may have an underlying unknown biological reason. Noteworthy, three of the six cases that carried repeat contractions were older than 1 year and therefore fall under the USID definition, while the other three cases were under 1 year of age and classified as SIDS category I according to the San Diego criteria. If one applies the <1-year definition of SIDS to the cases and controls used in this study, the association of the *PHOX2B* exon 3 repeat contraction remains significantly associated for the homozygous state (OR not applicable/infinitive and $P<0.001$) and the <112 allele frequency (OR=3.97, 95 % CI=[1.1–14.17], $P=0.034$). The heterozygous state is not statistically significantly SIDS associated any longer (OR=1.97, 95 % CI=[0.39–9.87], $P=0.33$), which could be due to lack of statistical power. However, the heterozygous state remains significantly associated with the USID cases considering the <3-year age criterion (OR=18.4, 95 % CI=[4.32–78.38], $P<0.001$), as does the <112 allele frequency (OR=17.24, 95 % CI=[4.19–71.04], $P<0.001$).

All six SIDS/USID cases with the *PHOX2B* polyalanine repeat contraction were of Dutch European bio-geographic origin as reported by the parents during interviews, and their USID/SIDS status was previously categorised based on histology re-review as SIDS category I according to the San

Table 1 Evidence for statistically significant USID/SIDS association of the *PHOX2B* exon 3 polyalanine repeat in the Dutch population considering a total of 163 cases and 825 healthy age-matched controls

Genotypes (bp)	# Cases	# Controls	OR	95 % CI		*P* value
				Low	High	
112/112	153	805				
<112/<112	1	0	Not applicable (infinitive)			<0.001[a]
112/<112	5	6	43.5	13.11	282.9	<0.001
Alleles <112	7	6	6.05	2.02	18.1	<0.001
>112/>112	0	1	Not applicable (infinitive)			<0.001[a]
112/>112	1	2	1.004	0.05	37.8	1.0
Alleles>112	1	4	1.27	0.14	11.4	0.83
Dropouts	3	11				

[a] Fisher exact test for association

Table 2 Results for DNA variants tested without finding statistically significant USID/SIDS association in the Dutch population studied

Gene	RS number	Nucleotide change	Amino acid change	Chromosome	Position	Controls			Cases			*P* value USID/SIDS association
						AA	AB	BB	AA	AB	BB	
ECE1	rs6665399	C8234021T	Intron region	1	21426520	167	414	241	33	74	49	0.84
ECE1	rs1076669	C1022T	T341I	1	21446404	686	135	3	139	24	0	0.47
ECE1		A1060G	T354A	1	21446441	824	0	0	163	0	0	–
PHOX2B		618/619insC	722–759del37nt	4	41442961	824	0	0	163	0	0	–
GDNF		C277T	R93W	5	37851912	811	13	0	161	2	0	0.74
EDN1	rs1476046	G7639A	Intron region	6	12401207	43	295	482	2	74	87	0.78
EDN1	rs5370	G594T	K198N	6	12404241	512	271	41	102	56	3	0.38
RET		G35A	R12H	10	42892747	814	0	0	162	0	0	–
RET		C166A	L56M	10	42916005	815	7	0	160	3	0	0.25
RET		C1157T	A386V	10	42924578	822	0	0	161	0	0	–
RET		G1253A	R418Q	10	42924674	814	10	0	162	0	0	0.16
RET		G1463A	D489N	10	42926862	822	1	1	163	0	0	0.55
RET	rs1799939	G2071A	G691S	10	42930125	570	233	21	112	48	3	0.96
RET		A2116G	T706A	10	42932086	823	0	0	156	0	0	–
RET		C3116T	P1039L	10	42942105	822	1	1	161	2	0	0.23
RET		G341A	R114H	10	42917799	824	0	0	163	0	0	–
BDNF		C289T	I2T	11	27636715	807	17	0	161	2	0	0.48
HTT	rs2020939	C17223T	Intron region	17	25574858	233	423	168	49	81	33	0.75
BMP2	rs2273037	T570A	S190R	20	6698882	775	29	1	152	6	0	0.97
BMP2	rs235768	T109G	S37A	20	6707115	280	401	142	53	86	23	0.77
BMP2		C874T	L292F	20	6707419	824	0	0	163	0	0	–
PHOX2A		C287A	T96K	11	71629959	819	0	0	162	0	0	–
					Allele frequency	155/158	<155	>158	155/158	<155	>158	>158=0.32
HASH1		108–131del	A36–A43	12		1,547	21	62	307	6	9	<155=0.42

A common allele, *B* rare allele

Diego criteria/USID (cases with no pathological findings whatsoever) or SIDS/USID with signs of minor infection [21] (for further case description, see Table 3 and supplementary material 6).

Data on the environmental factors that are involved in some of cases used here have been published before [5]. Although in that previous study [5] a different control group was applied and only the cases under 1 year of age were used, similar results are obtained when testing the complete SIDS/USID case group as used here and the control group as used here for which environmental data were also available to us. Smoking by both parents (OR=129.8, 95 % CI=[23.6, 715.1], $P<0.0005$), smoking of one parent (OR=13.4, 95 % CI=[5.2, 34.4], $P<0.005$) and prone sleeping (OR=39.6, 95 % CI=[15.6, 100.9], $P<0.005$) were all identified as statistically significant environmental risk factors for USID/SIDS when controlled for socioeconomic status. Preterm birth did not reach statistical significance (OR=2.6, 95 % CI=[0.8, 8.4], $P=0.11$]) in contrast to our previous study [5]. However, none of the highlighted statistically significant environmental risk factors showed up as statistically significantly associated confounding factors for the *PHOX2B* exon 3 alanine repeat contraction. No association was found between any of the 24 tested DNA variants and any of the four USID/SIDS sub-category as previously determined by histological re-evaluation [21] (data not shown).

Discussion

Our study provides evidence for USID/SIDS association of the *PHOX2B* gene in the Dutch population. The *PHOX2B* polyalanine repeat had not been associated with SIDS in previous studies, although expansion of its exon 3 polyalanine repeat had been identified in a large proportion of CCHS patients [7–9]. In fact, the *PHOX2B* exon 3 polyalanine repeat expansion was described as characteristic genetic element of CCHS [7–9] and additionally has been proven causative in a mouse model [27]. Here, we found one SIDS category I according to the San Diego criteria case with one allele

Table 3 Particulars of Dutch USID/SIDS cases carrying the contracted allele of the *PHOX2B* exon 3 polyalanine repeat

Contracted PHOX2B alleles	Sex	Age (days)	Pregnancy	Sleeping position	Parental smoking	Microbiology	Autopsy
Both	Male	140	Dizygotic twin, 3,600 g at birth	Supine	No	Negative	No abnormalities
One	Female	18	Unremarkable	Supine	Maternal pre- and postnatal	Negative	No abnormalities
One	Male	126	Unremarkable	Supine	Maternal pre- and postnatal	Echovirus 18	Laryngitis
One	Male	405	Unremarkable	Prone	Paternal and maternal pre- and postnatal	Negative	Tonsillitis and laryngitis
One	Female	545	Dizygotic twin, 2,010 g at birth	Prone	Maternal postnatal	*Staphylococcus aureus* in nose and throat	No abnormalities
One	Male	977	Unremarkable	Supine	No	Negative	Meckel's diverticulum with ectopic pancreatic tissue

expanded (by one repeat) but also noted repeat expansions among three healthy controls, two with one allele expanded (one repeat and five repeats, respectively) and one with both alleles expanded (by four repeats). Although we did find a statistically significant association with USID/SIDS when the expanded allele occurred in the homozygous stage, no association was seen when considering both homozygote and heterozygote carriers of the expanded allele together. Because the significant finding came from one homozygous control individual only and carrying only one copy of the expanded allele did not provide any USID/SIDS association, it is difficult to conclude based on our current data whether the repeat expansion is involved in USID/SIDS and more data are needed.

However, our study provides evidence for a statistically significant USID/SIDS association with the contraction of the *PHOX2B* exon 3 polyalanine repeat, which remained significant after correction for multiple testing. As with the expanded allele, we also see for the contracted allele a significant SIDS association in the homozygote stage (again only one sample, but a case instead of a control sample as for the expanded repeat). However, in contrast to the repeat expansion, we see a statistically significant USID/SIDS association when considering homo- and heterozygote carriers of the contracted allele together. This provides more convincing evidence for USID/SIDS involvement of the repeat contraction than what we obtained for the repeat expansion. Exon 3 polyalanine repeat contractions have previously been found in healthy control populations of CCHS and SIDS studies, but not in SIDS cases [9, 10, 13, 28–30]. A recent study by Jennings et al. [31] reports four phenotypic CCHS cases that were all negative for the *PHOX2B* polyalanine repeat expansion but had heterogenic deletion in or of the *PHOX2B* gene, all including exon 3 where the polyalanine repeat is located. No CCHS case or control individual has been previously reported carrying both alleles in the contracted repeat length fashion, such as one of the USID/SIDS cases observed here.

Notably, *PHOX2B* knockout mice have been made, but homozygous knockouts were never born alive, while heterozygous knockouts had an altered response to hypoxia and hypercapnia at birth resulting in longer posthypoxic apnoeas that recovered during maturity [32]. Furthermore, relative to normal *PHOX2B*+/+ mice, these *PHOX2B*−/+ mice had a longer apnoea time during regular sleep, while during the wake period there was no breathing difference [33]. Furthermore, birth weight of *PHOX2B*−/+ mice was reported to be lower than that of their *PHOX2B*+/+ siblings [33]. These findings in mice are in line with our results of the association between USID/SIDS and the contraction of the *PHOX2B* exon 3 polyalanine repeat. By definition SIDS occurs during sleep [1], and one of the risk factors is low birth weight [3]. Furthermore, many USID/SIDS cases are reported with a probable internal or external cause of hypoxia, and many of the well-known risk factors can be linked with breathing such as prone sleeping [3–5], soft bedding [3], parental smoking [3, 5] and minor infection [3, 6, 21]. Four out of the six USID/SIDS cases for which we identified *PHOX2B* exon 3 repeat contractions had one or more of these environmental risk factors (Table 2). Furthermore, Lavezzi et al. [28] found a decreased amount of neurons that stained positively with *PHOX2B* immunohistochemistry in the retrotrapezoid nucleus in the medulla oblongata in 12 out of 22 SIDS cases (55 %) compared to none out of 11 age-matched deceased controls. This finding corresponds to a hypoplastic retrotrapezoid nucleus that is toughed to be responsible for the "drive to breath" [28]. All the cases tested in their studies had a normal 20/20 *PHOX2B* polyalanine repeat, but they did hypothesise that part of the SIDS cases are due to an unknown *PHOX2B* mutation or mutation in the *PHOX2B* pathway. Other studies have also associated the *PHOX2B* gene to SIDS; Rand et al. [29] did previously find an association between Caucasian American SIDS cases and a common polymorphism in intron 2 (IVS2+101>G; g.1364A>G) of the *PHOX2B* gene; there was no association in African American SIDS cases since the incidence of this polymorphism is very high in African

Americans (frequency of the G allele was 0.52 in the matched controls) [29].

Besides *PHOX2B*, no DNA variant from any other gene investigated here showed statistically significant association with USID/SIDS in our dataset. Previous studies carried out in other populations found SIDS associations for ten of the 24 DNA variants tested here in the Dutch population (i.e. *5HTT* rs202939, *RET* R12H, *RET* L56M, *RET* A386V, *RET* R418Q, *RET* G691S, *EDN1* rs5370, *PHOX2A* T96K, *ECE1* rs1076669 and *BMP2* rs235768) [13, 14]. That none of these DNA variants revealed a statically significant association with USID/SIDS in our study may be explained by the rare variant nature of these DNA polymorphisms, perhaps in combination with population effects. Previously reported SIDS associations for all these genetic loci were caused by single individual findings only [13, 14].

This study is not without caveats. Firstly, autopsy material is usually not prepared and handled in DNA-free environment so that DNA contamination can potentially occur at this level already. Secondly, DNA fragmentation during paraffin embedding, release and/or autolysis of the tissue prior to collection causes low DNA yields, which can lead to increased risk of detecting minor DNA contaminations and to allelic or complete locus dropout [34]. Therefore, using paraffin-embedded materials as DNA source causes problems in genetic analysis such as the high dropout rates and contamination encountered in this study. Using robotics for extracting and handling DNA samples in large cohort studies, such as used here for the control samples, is not free of risk, either as it potentially can introduce minor DNA contamination. A conservative approach towards data cleaning was taken by excluding all samples that showed even the smallest signs of potential DNA contamination during highly sensitive miniSTR profiling without testing and knowing if such effects would impact on the genetic associations and by excluding *5HTTLPR* because of an usually high drop-out rate. By doing so, the statistical power to detect statistically significant genetic associations was reduced, and the risk of false negative findings was increased. The very high dropout of the *5HTTLPR* fragment length analysis might be explained by the large number of repeats in the sequence and the large amounts of guanine and cytosine, although a 70 % GC nucleotide mixture was used. To control for the difference in DNA quantity and quality between the paraffin-extracted case DNA and cord blood-extracted control DNA, a PCR-based DNA quantification method was used for the cases, and PCR primers were designed for short amplicons (see supplementary method sections 2 and 3 for details).

In conclusion, of the 24 DNA variants tested from 11 genes involved in the autonomous nervous system, only the length variation of the polyalanine repeat in exon 3 of the *PHOX2B* gene was found to be statistically significantly associated with USID/SIDS in the Dutch population after multiple test correction. Our data enforce the previous hypothesis that there is an association between *PHOX2B* and SIDS and suggest that contractions of the *PHOX2B* exon 3 polyalanine repeat probably serve as genetic risk factor for USID/SIDS at least in the Dutch population. Future studies and larger study populations are needed to confirm this and to understand the functional effect of the polyalanine repeat length variation, in particular its contraction, in exon 3 of the *PHOX2B* gene.

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