Interstitial Deletion of 2q22.2q22.3 Involving the Entire *ZEB2* Gene in a Case of Mowat-Wilson Syndrome

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Keywords

Mowat-Wilson syndrome · *ZEB2* · Array CGH · Syndromic congenital heart disease · Hirschsprung disease

Abstract

Mowat-Wilson syndrome (MWS) is a rare autosomal dominant syndrome characterized by dysmorphic features, mental retardation, and congenital heart disease (CHD). MWS results from microdeletions of chromosome 2q23 or de novo SNVs involving the *ZEB2* gene. Here, we report on an Egyptian MWS patient diagnosed by chromosomal microarray (CMA). A 1-year-old male child was referred to the CHD clinic, National Research Centre, presenting with dysmorphic features and CHD. The patient was referred to the human cytogenetics department for cytogenetic analysis and for screening of subtelomere rearrangements and microdeletion loci, using MLPA, and all revealed normal results. CMA revealed an interstitial 2.27-Mb microdeletion in chromosome 2q, involving the entire *ZEB2* gene and other genes. This study emphasizes the significance of CMA in the detection of microdeletions/microduplications and as a screening

tool in cases presenting with CHD and extracardiac manifestations. MWS should be suspected in patients presenting with the characteristic facial dysmorphism, developmental delay, seizures, Hirschsprung disease, and congenital heart anomalies, especially those involving the pulmonary arteries or pulmonary valves. It is recommended to include the *ZEB2* locus in the MLPA microdeletions probes.

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Introduction

Mowat-Wilson syndrome (MWS; OMIM 235730), a rare autosomal dominant multiple congenital anomaly syndrome, is present in 1: 50,000–70,000 individuals [Mowat and Wilson, 2010]. It is characterized by intellectual disability (ID), facial dysmorphism in addition to congenital anomalies [Mowat et al., 1998]. Developmental disorder is the main presenting feature with a wide spectrum of clinically heterogeneous features. Characteristic dysmorphic facial features include microcephaly, square-shaped face, a prominent narrow triangular chin,

hypertelorism with deep-set large eyes, broad nasal bridge, saddle nose with a prominent rounded nasal tip, and open mouth with everted or full lower lip, and posteriorly rotated ears and big uplifted ear lobes with a central depression [Garavelli and Mainardi, 2007].

The patients manifest moderate to severe ID, epilepsy and congenital malformations that could include Hirschsprung disease (HSCR) and genital anomalies, specifically hypospadias in males [Garavelli and Mainardi, 2007].

More than half of the patients with MWS are born with Hirschsprung disease that mainly causes the patients to suffer from severe constipation, intestinal blockage, and enlargement of the colon [Ivanovski et al., 2018]. Also chronic constipation is a frequent manifestation seen in patients not diagnosed with Hirschsprung disease [Ishihara et al., 2005].

Congenital heart disease (CHD) has been documented in 45% of the cases in the form of patent ductus arteriosus (PDA), ventricular septal defect (VSD), atrial septal defect (ASD), Fallot tetralogy, pulmonary atresia with or without stenosis, aortic coarctation, bicuspid aortic valve, and aortic valve stenosis [Amiel et al., 2002]. Agenesis of the corpus callosum (ACC) and eye defects were also reported [Yamada et al., 2001]. Mowat et al. [1998] proposed that MWS results from microdeletion in 2q22q23 or due to a de novo mutation of a gene present in this region.

Through studies of de novo translocations involving a breakpoint at 2q22 in individuals affected by the syndrome, 2 study groups identified a gene, *SIP1*, which is deleted by chromosomal rearrangement (abbreviated now as *ZEB2*; OMIM# 605802). Further analysis of other patients revealed heterozygous frameshift mutations affecting the *ZEB2* gene causing early truncation of the protein [Cacheux et al., 2001; Wakamatsu et al., 2001].

The actual prevalence of MWS is undetermined, but it seems that it is underestimated. MWS is a relatively newly described syndrome with a wide phenotypic variability, which makes the diagnosis difficult, specifically in patients without HSCR [Zweier et al., 2002]. Since the first delineation by Mowat et al. [1998], more than 300 cases with *ZEB2* mutations, deletions or cytogenetic abnormalities have been stated mainly from Northern Europe, Italy, Australia, and the United States, and more than 100 mutations were reported [Wakamatsu et al., 2001; Ghoumid et al., 2013; Paz et al., 2015; Ivanovski et al., 2018].

Herein, we describe the first Egyptian MWS patient diagnosed by chromosomal microarray (CMA).

Fig. 1. Facial features of our Mowat-Wilson syndrome case.

Clinical Report

The proband was a 1-year-old male child, referred to the CHD clinic, centre of scientific excellence, National Research Centre, due to respiratory distress and cyanosis. The parents reported that their child had delayed motor and mental milestones. Pedigree analysis showed negative parental consanguinity with no family history of congenital anomalies or similarly affected family members.

The child is the second of a healthy young couple, delivered by caesarean section. At birth, his weight was normal (2.750 kg), and cyanosis was noticed since birth. Full history was taken from the parents including developmental milestones, detailed history of cardiac symptoms, convulsions and/or other relevant symptoms.

On clinical examination the child was in respiratory distress with cyanosis, but with a pleasant smile. Full detailed examination was carried out with special emphasis on cardiac examination, craniofacial dysmorphic features, ears, nose and throat. He had peculiar dysmorphic facies in form of a long face, frontal bossing, sparse hair, microcephaly, scanty medially flared broad eyebrows, downward prominent columella with short philtrum, deep-set wide eyes, mild epicanthic fold, hypertelorism, low-set ears with everted (uplifted) lobules, pointed chin, retro micrognathia, and short neck as shown in Figure 1.

Limb examination revealed a right clenched fist with overriding fingers. Anthropometric measurements showed normal length (on mean) and failure to gain weight, his weight was 7 kg (−3.1 SD). The patient also showed microcephaly (−3.2 SD).

Neurological examination revealed hypotonia with hyporeflexia. Brain MRI showed ACC and white matter degeneration as shown in Figure 2. EEG was normal and no epileptic focus or hyperactive spikes could be detected. Echocardiography confirmed VSD, ASD, and moderate valvular pulmonary stenosis. Pelviabdominal ultrasonography was normal.

Clinical reevaluation of the proband at age 3 years revealed the same clinical features, and his face became more elongated. Hypotonia persisted with a wide-based, unsteady gait. No convulsions were detected by the parents, and the patient had a normal bowel habit. Hearing test was normal and no eye defects could be detected. Neurodevelopmental delay, severe ID, and severe speech delay became evident clinically and by specific investigations. The patient was diagnosed with multiple congenital anomaly/intellectual disability syndrome.

Fig. 2. Brain MRI showing agenesis of corpus callosum.

Materials and Methods

Conventional cytogenetic analysis by GTG-banding was used [Verma and Babu, 1995], and karyotype description followed the International System for Human Cytogenomic Nomenclature recommendations [ISCN, 2016].

DNA was extracted from 5 mL of venous blood collected in PAXgene tube using PAXgene kit (PreAnalytix, Hiden, Germany). Measurements of quality and quantity of DNA were done using the NanoDrop spectrophotometer.

MLPA assay was used as a screening test for patients with dysmorphic features, developmental delay, and CHD. It was done using 2 commercial kits: SALSA MLPA P245 microdeletion Kit and MLPA P070 subtelomere Kit.

Copy number variants (deletions, duplications) were detected by CMA over the genome using HD microchips (Affymetrix, Santa Clara, CA, USA). Through 4 days' workflow, DNA digestion, ligation, amplification, purification, fragmentation, labeling, and then loading to the microchips was performed. Hybridization was done for 16 h in Gene chip hybridization oven 645 (Affymetrix). The microchips were then washed and stained in fluidic station 450 (Affymetrix). Thereafter, microchips were scanned in Gene chip scanner 3000 (Affymetrix) and analyzed by the chromosome analysis suit (CHAS) software.

Results

Conventional cytogenetic analysis revealed a 46,XY normal male karyotype. MLPA revealed normal copy numbers in all subtelomeres and microdeletion loci (which does not involve the MWS locus) (Fig. 3, 4).

CMA showed an interstitial 2.27-Mb deletion of chromosome 2q22 including the entire *ZEB2* gene: arr[hg19] 2q22.2q22.3(143375948_145653287)×1 as seen in Figure

5. Other genes were also found to be deleted in this region including *HSR*, *VCRL2*, *TSE2*, *CMD1H*, *COPD*, *CHDS2*, *GTDC1*, *ARHGAP15*, and *MAFD5*.

Discussion

MWS has wide variations of symptoms, ranging from typical dysmorphic features and delayed developmental milestones to that with HSCR, CHD, and a range of other congenital anomalies [Garavelli and Mainardi, 2007]. Abdalla and Zayed [2014] reported the first Egyptian MWS case conceived by intracytoplasmic sperm injection (ICSI). The patient was diagnosed clinically according to the characteristic facial gestalt of MWS, global developmental delay, severe ID with impaired speech, epilepsy, ACC, and bilateral sensorineural hearing loss as a new manifestation. However, molecular testing for *ZEB2* was recommended for proper diagnosis, as it was not available according to Abdalla and Zayed [2014].

The distinctive facial features are considered the most clinically reliable feature for diagnosing those patients, the rarity of this syndrome and the phenotypic variability may seldom make it difficult to diagnose. Moreover, a change in some of the facial features tends to occur with age. In any case, the gestalt is typical at all ages [Garavelli et al., 2009]. The clinical suspect must be confirmed with molecular testing, beginning with the analysis of the *ZEB2* gene, and if a variant is not detected, MLPA analysis/array CGH for microdeletions should be carried out.

Fig. 3. MLPA ratio chart using MLPA microdeletion kit showing no copy number variation.

Fig. 4. MLPA ratio chart using MLPA subtelomere kit showing no copy number variation.

Fig. 5. Chromosomal microarray showing interstitial deletion of 2q22.2q22.3 (indicated by the red bar at the top), the CN state = 1, log 2 = -0.45, allele difference = 2.

In the present study, our patient exhibited distinctive dysmorphic facial features; however, clinical presentation was at an early age and lacked HSCR, which is a common finding. He also had some atypical features, such as a clenched fist with overriding fingers, normal stature, and a normal EEG. The diagnosis was established after CMA that showed a microdeletion involving the whole *ZEB2* gene.

HSCR usually manifests as severe constipation, intestinal blockage, and enlargement of the colon. It was considered the main diagnostic feature of MWS before delineation of the recognizable facial features which led to a high proportion of MWS patients with HSCR, representing 44.2% of the patients [Ivanovski et al., 2018]. Nonetheless, it is possible that the presence of HSCR is underestimated in many patients who had severe constipation, not diagnosed by rectal biopsy due to fear of complications or absence of compliance.

The variation in severity of HSCR may be caused by both variants in *ZEB2* and epigenetic factors [Ishihara et al., 2005]. Yoneda et al. [2002] reported a woman, who developed a late-infantile-onset of ID, severe constipation, and megacolon in adulthood without typical clinical features of MWS. The authors detected a deletion in exon 3 of the *ZEB2* gene and suggested that the patient could represent an intermediate stage of severity due to partial deletion of the gene.

Although our patient had constipation upon first evaluation, normal bowel habit was documented by his parents in the following reevaluation, which excluded HSCR clinically. Similar to our patient, many other MWS patients without HSCR but with constipation were reported constituting 26–29% of all cases [Yamada et al., 2001; Wilson et al., 2003; Dastot-Le Moal et al., 2007; Ivanovski et al., 2018].

With the development of advanced molecular and high-throughput diagnostic techniques, more HSCR-free patients were diagnosed, and the frequency of HSCR began to decline, reflecting the importance of recognizable facial features in diagnosing those patients rather than the presence of HSCR. Ivanovski et al. [2018] reported a frequency of 30.6% of HSCR among a cohort of 87 MWS patients, while the combined frequencies of HSCR and constipation were 72%.

In addition to the distinctive facial features, our patient exhibited other features consistent with loss of function of *ZEB2* [Mowat et al., 2003] including severe ID, microcephaly, hypotonia, hyporeflexia, ACC, and severe congenital heart defects (Table 1).

Based on literature reviewing, all patients with MWS have moderate to severe ID and delayed developmental milestones. Hypotonia is a frequent sign detected in about 79.1% of the patients in their first years of life [Ivanovski

Table 1. Clinical features of Mowat-Wilson syndrome

	Lurie et al., 1994 (n = 1)	Amiel et al., 2002 (n = 8)	Yamada et al., 2002 (n = 10)	Zweier et al., 2002 (n = 4)	Garavelli et al., 2003 (n = 1)	Mowat et al., 2003 (n = 21)	Ivanovski et al., 2018 (n = 344)	Present case (n = 1)	Total cases (n = 345)
Male/female	M	3/5	7/3	4/0	M	15/6	183/161	M	184/161
Typical face	1/1	5/5	5/5	3/3	1/1	21/21	100%	1/1	100%
Broad eyebrows	+	+	+	+	+	+		–	
Deep-set eyes	+	+	+	+	+	+		+	
Fleshy upturned lobules	+	+	+	+	+	+		+	
Pointed chin	+	+	+	+	+	+		+	
Moderate to severe ID	1/1	8/8	10/10	4/4	1/1	21	100%	1/1	100%
Seizures/abnormal EEG	1/1	6/7	8/10	3/4	1/1	15/3	241/307 (78.5%)	0/1	241/308 (78.2%)
Agenesis of corpus callosum	Not stated	6/8	3/4	1/3	Not stated	5/16	Not stated	1/1	
Congenital heart anomaly ASD VSD PS Pulmonary artery sling	1/1	5/8	3/10	¾	0/1	9/21	193/332 (58.1%)	1/1 + + + –	194/332 (58.4%)
Hirschsprung disease	1/1	7/8	4/10	2/4	1/1	13/21	148/335 (44.2%)	0/1	148/336 (44%)
Microcephaly	1/1	7/7	10/10	2/4	0/1	16/20	244/314 (77.7%)	1/1	245/315 (77.8%)
Renal anomaly	0/1	2/4	Not stated	Not stated	Not stated	6/16	59/233 (25.3%)	0/1	59/234 (25.2%)
Short stature (<3rd centile)	1/1	Not stated	Not shown	3/4	0/1	8/18	70/151 (46.4%)	1/1	71/151 (47%)
Genital anomalies	0/1	2/4	Not shown	1/4	1/1	11/15	105/244 (43%)	0/1	105/245 (42.8%)
Molecular cytogenetic defect	Interstitial deletion of 2q22q23 including the entire <i>ZEB2</i> gene	Largescale <i>SMAD1</i> deletions or truncating [Lurie, 1994; Garavelli et al., 2003] mutations in 8 of 19 patients	<i>ZEB2</i> mutation in 6 patients without Hirschsprung disease	Direct sequencing demonstrated truncating <i>ZFH1B</i> mutations in all 4 patients	Direct sequencing of <i>ZFH1B</i> revealed de novo heterozygous frameshift mutation nt901delC in exon 7	Not identified	Not stated	2.27-Mb deletion at 2q22 including the entire <i>ZEB2</i> gene	

ID, intellectual disability; ASD, atrial septal defect; VSD, ventricular septal defect; PS, pulmonary stenosis.

et al., 2018]. About 96% of MWS patients showed abnormal MRI results including abnormalities of the corpus callosum (79.6%) as the most frequently reported [Garavelli et al., 2017]. Epilepsy, which is considered as one of the most frequent features of MWS occurring in more than 80% of reported patients, was not a feature in our proband who did not exhibit epileptic fits or abnormal EEG features till he completed his third year of age. The mean onset of epilepsy was reported to be at 27.5 months of age; however, the onset may be delayed to 11 years of age [Ivanovski et al., 2018].

The present patient exhibited severe congenital heart defect in the form of ASD, VSD and pulmonary stenosis. CHD was reported to occur in about 60% of the patients and most commonly include PDA, VSD and ASD, while pulmonary stenosis occurs in about 20%. Complex cardiac anomalies, however, are much less frequently reported [Adam et al., 2006, Dastot-Le Moal et al., 2007; Ivanovski et al., 2018].

ZEB2, a zinc finger E-box-binding homeobox 2 gene also known as *SIP1* (Smad interacting protein 1) or *ZF-HX1b* (zinc finger homeobox gene 1b), is located in cytogenetic band 2q22.3 and is expressed mainly in the development of the nervous system. To date, *ZEB2* has been shown to control the formation of the neocortex, hippocampus, corpus callosum, and spinal cord, as well as autonomic and enteric nervous systems [Hegarty et al., 2015].

Gliogenesis and myelinogenesis are also controlled by *ZEB2*. The various roles of *ZEB2* in both neurogenic and gliogenic developments reflect the broad range of target genes and binding partners of *ZEB2* as well as the complex regulation of *ZEB2* at the genetic level [Cerruti-Mainardi et al., 2004]. *ZEB2* mRNA is found in almost every part of the human tissues [Cacheux et al., 2001], which may point to its role in congenital heart defects observed.

The most common molecular defects reported in MWS patients are de novo heterozygous pathogenic

Table 2. Deletion and duplication involving *ZEB2*

	Yuan et al., 2015 (male)	Mak et al., 2016 (female)	Baxter et al., 2017 (female)	Present case (male)
Typical face		Dysmorphic with hyper nasal voice but not fitting Mowat-Wilson syndrome features		+
Hypertelorism	–		+	+
Long face	–		–	+
eye anomalies	Small eyes		Medial flare brows	Deep-set wide eyes
Low set ears	+		–	+
Auricle dysplasia	+		+	+
Short philtrum	+		+	+
Pointed chin	–		–	+
Micrognathia	+		–	+
Clenched fist	–		–	+
Mild to moderate ID	Positive	Positive	Positive	Positive
Seizures/abn EEG	Negative	Negative	Positive	Negative
Hypotonia	Positive	Negative	Negative	Positive
Agenesis of corpus callosum	Negative	Negative	Positive	Positive
Congenital heart anomaly	ASD	TOF and PFO	ASD,PDA, bicuspid aortic valve	ASD, VSD, PS
Hirschsprung disease	Negative	Negative	Positive	Negative
Skull anomalies	Scaphocephaly	Negative	Microcephaly	Microcephaly
Short stature	Negative	Negative	Positive	Negative
Genitourinary anomalies	Small testes	Dysfunctional uterine bleeding	Neurogenic bladder, kidney reflux, incontinence	Negative
Molecular cytogenetic defect	Triplication/duplication at 2q22.2q22.3	2.1-Mb duplication at 2q22.3	69-kb duplication at 2q22.3	2.27-Mb deletion at 2q22
Genes involved	<i>ARHGAP15</i> , <i>LOC101928386</i> , <i>GTDC1</i> , <i>ZEB2</i> , <i>ZEB2-AS1</i> , <i>LINC01412</i> , <i>LOC105373656</i> , <i>TEX41</i>	<i>ARHGAP15</i> , <i>LOC101928386</i> , <i>GTDC1</i> , <i>ZEB2</i> , <i>ZEB2-AS1</i> , <i>LINC01412</i> , <i>LOC105373656</i> , <i>TEX41</i>	<i>ZEB2</i> , <i>ZEB2-AS1</i> , <i>LINC01412</i> , <i>LOC105373656</i>	<i>ZEB2</i> , <i>HSR</i> , <i>VCRL2</i> , <i>TSE2</i> , <i>CMD1H</i> , <i>COPD</i> , <i>CHDS2</i> , <i>GTDC1</i> , <i>ARHGAP15</i> , <i>MAFD5</i>

ASD, atrial septal defect; VSD, ventricular septal defect; PS, pulmonary stenosis; TOF, tetralogy of Fallot; PFO, patent foramen ovale; PDA, patent ductus arteriosus; ID, intellectual disability.

changes in the *ZEB2* gene including deletions and other mutations [Ghoumid et al., 2013]. Rarely affected sibs were suggested to result from germline somatic mosaicism [McGaughan et al., 2005; Cecconi et al., 2008].

More than 80% of the cases with MWS are mainly due to *ZEB2* gene mutations. Up till now, 180 distinctive SNVs in the *ZEB2* gene have been published including nonsense mutations which account for about 41% and were mainly present in exon 8 [Dastot-Le Moal et al., 2007]. Small insertions/deletions were detected in 46% of the patients reported by Ivanovski et al. [2018].

The majority of *ZEB2* SNVs cause loss of function by premature termination or large gene deletions [Wakamatsu et al., 2001; Ghoumid et al., 2013; Ivanovski et al., 2018]. A number of cytogenetic abnormalities have been

reported in association with MWS including chromosome deletions or translocations involving the breakpoint 2q22.

Patients with normal karyotypes have been also diagnosed during systematic CMA screening due to dysmorphic features and ID or epilepsy and brain malformations. Most reported chromosomal rearrangements resulted in complete *ZEB2* deletion; however, intronic disruption by the translocation breakpoint without presence of a deletion were sporadically reported [Cacheux et al., 2001; Engenheiro et al., 2008].

Similar to our patient, Engenheiro et al. [2008] reported a patient with a normal karyotype diagnosed as MWS by array CGH. The patient revealed 0.6-Mb interstitial microdeletion at chromosome 2q22. The deleted region

included the entire *ZEB2* gene and the authors suggested that MWS may be underdiagnosed and should be considered in patients with ID, dysmorphic features, epilepsy and CHD, especially with pulmonary valve stenosis or pulmonary artery sling.

In a large cohort reported by Ivanovski et al. [2018], chromosome deletions were reported in 11.5% of the patients ranging in length from few kilobases to 16.7 Mb, mostly involving the entire *ZEB2* gene or its first exons. Others encompassed exons 5–8, leading to an altered reading frame. Rare variants included 3' UTR region deletion, possibly altering protein synthesis or regulation, and an intronic change resulting in altered splice acceptor site of exon 2 and expected to produce a smaller protein with abnormal function [Zweier et al., 2006].

Using CMA, a de novo novel duplication of 69 kb encompassing exons 1 and 2 of the *ZEB2* gene was first reported by Baxter et al. [2017] in a 14-year-old female clinically diagnosed to have MWS. She suffered from microcephaly, seizures, distinctive facies, and HSCR. Yuan et al. [2015] also reported a boy showing a de novo 2.9-Mb triplication/duplication encompassing chromosome 2q22.3 that covered the entire *ZEB2* gene in addition to *GTDC1*, *TEX41*, and part of *ARHGAP15*. The patient presented with hypotonia, dysmorphic facial features in form of small eyes, flat nasal bridge and flat facial profile that were considerably different than those present in MWS, as well as ASD.

Similarly, Mak et al. [2016] reported an overlapping 2.1-Mb region duplication that fully comprised *ZEB2* as well as *GTDC1*, *TEX4* in addition to part of *ARHGAP15*. The patient suffered from mild ID, distinctive hyper nasal voice, and dysmorphic facial features that also did not fit those of MWS patients.

The studies conducted by Baxter et al. [2017], Yuan et al. [2015], and Mak et al. [2016] indicate that duplication of the *ZEB2* is of clinical significance and may result in MWS or other pathogenic consequences, and that the *ZEB2* gene is likely to be a dosage-sensitive gene, and its duplication/triplication may cause gene disruption or even novel function due to genetic fusion as shown in Table 2.

The deleted region in our patient included, in addition to the *ZEB2* gene, *GTDC1* and *ARHGAP15* which were duplicated in both patients of Yuan et al. [2015] and Mak et al. [2016]. The presence of CHD in both reports and the severe degree of cardiac anomalies in our patient indicate the crucial role of *ZEB2* dosage in cardiac development and the possible role of other associated gene abnormalities in augmenting this effect.

Other genes found to be deleted by CMA in the 2q22.2q22.3 region included *HSR*, *VCRL2*, *TSE2*, *CMD1H*, *COPD*, *CHDS2*, *GTDC1*, and *ARHGAP15*. Abnormalities in the *GTDC1*, *ARHGAP15*, *TSE2*, and *MAFD5* genes showed no significant phenotype correlation, which proves that the *ZEB2* gene is the main gene responsible for the patient's phenotype.

In conclusion, we report the first MWS patient in Egypt diagnosed using CMA which highlights the significance of molecular cytogenetic techniques in detection of microdeletion/microduplication syndromes and its importance as a screening test in cases presenting with CHD and extracardiac manifestations with facial dysmorphism. It is also recommended to include the *ZEB2* locus in the MLPA microdeletion probes.

MWS is a rare syndrome showing a wide spectrum of dysmorphic features and few atypical presentations making it a rather challenging diagnosis for clinical geneticists; however it should be suspected in patients presenting with distinctive facial dysmorphism, severely impaired speech, developmental delay, ID, seizures or brain malformations, HSCR, in addition to congenital heart anomalies, especially those involving the pulmonary arteries or pulmonary valves.

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Statement of Ethics

The study was conducted according to the guidelines of the Medical Research Ethics Committee of the National Research Centre based on the World Medical Association Declaration of Helsinki, and an informed consent has been taken from the patient's guardians.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors have made substantial contributions to the conception of the work, analysis and interpretation of data, participated

in revising the work, responsible for all aspects of the work in ensuring that questions related to the accuracy of the work are appropriately investigated, and they all approved the final version to be published.

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