Identification of 19-nor-5,7,9(10)-cholestatrien-3β-ol in patients with Smith-Lemli-Opitz syndrome

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Abstract We have identified the third unknown sterol in the plasma and tissues of Smith-Lemli-Opitz homozygotes as 19-nor-5,7,9(10)-cholestatrien-3β-ol. The structure was established from capillary gas–liquid chromatography retention index and characteristic fragmentation pattern by mass spectrometry that were identical to a synthetic reference standard. Evidence is presented that 19-nor-5,7,9(10)-cholestatrien-3β-ol is not an artifact formed during the chemical isolation of the relatively unstable 7-dehydrocholesterol. It is possible that 19-nor-5,7,9(10)-cholestatrien-3β-ol may contribute to the clinical abnormalities in patients with Smith-Lemli-Opitz syndrome.—**Batta, A. K., G. Salen, G. S. Tint, and S. Shefer.** Identification of 19-nor-5,7,9(10)-cholestatrien-3β-ol in patients with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* 1995. **36:** 2413–2418.

Supplementary key words 19-nor-5,7,9(10)-cholestatrien-3β-ol • 7-dehydrocholesterol • 8-dehydrocholesterol • cholesterol • mass spectrometry

We recently described a severe defect in late cholesterol biosynthesis in Smith-Lemli-Opitz syndrome (1–4). Homozygotes with this recessive inherited birth defect manifest mental retardation with multiple organ congenital organ anomalies (5–9) due to a block in the penultimate reaction in the cholesterol biosynthetic pathway that involves the incomplete conversion of 7-dehydrocholesterol to cholesterol (2). As a consequence, plasma and tissue cholesterol concentrations are reduced and the precursor, 7-dehydrocholesterol, accumulates markedly (2–4). However, in the plasma and tissues of all homozygotes, two additional sterols besides 7-dehydrocholesterol were detected in quantity (2). The second most abundant sterol was conclusively identified as 8-dehydrocholesterol (5,8-cholestadien-3β-ol, **Fig. 1**) and although it produces an almost identical mass spectrum as 7-dehydrocholesterol, it could be easily distinguished from 7-dehydrocholesterol by different retention index on capillary gas–liquid chromatography and by R_f value on argentation thin-layer chromatography (10). Most importantly, nuclear magnetic resonance spectrometry shows a spectrum specific for 8-dehydrocholesterol (11).

The third unknown sterol was different. The mass spectrum of its trimethylsilyl ether suggested the presence of three nuclear double bonds and one less methyl group in the ring system. This paper describes the structural identification of this compound.

EXPERIMENTAL

Reference standards of cholesterol and 7-dehydrocholesterol were purchased from Steraloids, Inc. (Wilton, NH). 7-Dehydrocholesterol was crystallized from methanol before use. 7-Dehydrocholesterol acetate was prepared by reacting 7-dehydrocholesterol with acetic anhydride–pyridine overnight and then precipitating the product into water. The precipitate was collected, washed with water, dried, and crystallized from methanol. A reference standard of 19-nor-5,7,9(10)-cholestatrien-3β-ol was synthesized via irradiation of 7-dehydrocholesterol with ultraviolet light (5.5 watt mercury lamp, 254 nm wave length; Aldrich Chemical Co., Milwaukee, WI) for 2 h in the presence of eosin yellow as sensitizer (12). The 7-7′-dimer obtained was boiled with 2-ethoxyethanediol (12) and the major product obtained was isolated pure by preparative thin-layer chromatography. The ultraviolet absorption pattern of the compound and the mass spectral fragmentation pattern of its TMS ether were identical with those reported for 19-nor-5,7,9(10)-cholestatrien-3β-ol (13). All reagents and sol-

The following trivial names and abbreviations were used: cholesterol, 5-cholesten-3β-ol; 7-dehydrocholesterol, 5,7-cholestadien-3β-ol; 8-dehydrocholesterol, 5,8-cholestadien-3β-ol; GLC, gas–liquid chromatography; TMS, trimethylsilyl; GC–MS, gas chromatography–mass spectrometry.

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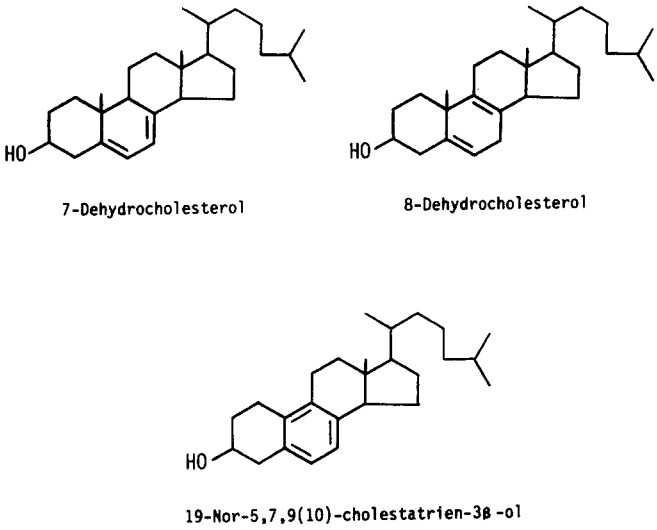

Fig. 1. Structures of 7-dehydrocholesterol, 8-dehydrocholesterol, and 19-nor-5,7,9(10)-cholestatrien-3β-ol.

vents were reagent grade and were purchased from Aldrich Chemical Co.

Methods

Gas–liquid chromatography (GLC). A Hewlett-Packard model 5890A gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused silica CP-Sil-19 CB (stationary phase, 85% dimethyl, 7% cyanopropyl, 7% phenyl, and 1% vinylsiloxane) or CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m × 0.22 mm I.D.) (Chrompack, Inc., Raritan, NJ) and helium was used as the carrier gas. The GLC operating conditions were as follows. Injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 265°C when using a CP-Sil-19 CB column and 278°C when using a CP-Sil-5 CB column (14). Flow rate of the carrier gas was maintained at 1 ml/min for both columns. The sterols (5–10 μg) were reacted with 100 μl of Sil-Prep (hexamethyldisilazane–trimethylchlorosilane–pyridine, 3:1:9; Alltech Associates, Inc., Deerfield, IL) for 20 min at 55°C. Solvents were evaporated at 55°C under N_2 and the trimethylsilyl (TMS) ether derivative formed was taken in 100 μl of hexane. One microliter was injected into the GLC column simultaneously with 5α-cholestane used as the internal standard.

Gas chromatography–mass spectrometry (GC–MS). Mass spectra of the various sterols were carried out on a Hewlett-Packard Model 5988 gas chromatograph–mass spectrometer using a 25 m CP-Sil 5CB capillary column.

Clinical

The patients were homozygotes who exhibited the phenotype typical of the Smith-Lemli-Opitz syndrome. All were mentally retarded. Case histories have been reported previously (4). The research protocol was reviewed and approved by the Human Research Committee at the University of Medicine and Dentistry of New Jersey, Newark, NJ.

Sample collection

The blood was collected in EDTA and plasma was recovered after centrifugation at 5,000 *g* for 10 min to remove the erythrocytes. The plasma was stored in the dark at -20°C until used. Stool samples were frozen and stored at -20°C.

Statistics

Unpaired Student's *t*-test was used to calculate the significance (*P*) values.

Sterol analysis

Plasma (0.1–0.5 ml) was added to 30 ml 95% ethanol containing 2 ml of 10 N sodium hydroxide and refluxed for 1 h. After cooling, the solution was diluted with 30 ml water and extracted with hexane (4 × 50 ml) (2, 10). The combined hexane extract was evaporated to dryness and an aliquot was silylated with Sil-prep and subjected to GLC or GC–MS.

The lyophylized feces (1 g) were exhaustively extracted with ethanol containing 0.5% ammonium hydroxide in a Soxhlet extractor and solvent was removed under reduced pressure (10). The residue was taken in 10 ml 0.5 N NaOH and extracted with n-hexane (10 ml × 4). The hexane layer was washed once with water (10 ml) and evaporated to dryness under nitrogen. The residue containing the fecal neutral sterols was dissolved in chloroform (10 ml) and aliquots were used for GLC or GC–MS.

Stability of 7-dehydrocholesterol toward heat and alkali

In order to prove that 7-dehydrocholesterol was not partially converted into 19-nor-5,7,9(10)-cholestatrien-3β-ol under the conditions of plasma workup, the following experiments were performed. Thus, in a set of experiments, 7-dehydrocholesterol or its acetate (100 μg in 50 μl acetone) was added to 0.5 ml plasma from healthy control subjects and saponified in 1 N sodium hydroxide for 1 h. The sterols were isolated as described above. In a second set of experiments, aliquots of 7-dehydrocholesterol (100 μg in 50 μl acetone) were added directly to 30 ml 95% ethanol containing 2 ml of 10 N sodium hydroxide and saponified and sterols were isolated as described above. In other experiments, aliquots

of 7-dehydrocholesterol were added to ethanol and sodium hydroxide and saponified as above. The contents were then allowed to stand at room temperature for 3 h and again saponified and then sterols were extracted. In still other experiments, aliquots of 7-dehydrocholesterol were saponified three times in 1 N sodium hydroxide and after the first and second saponifications, the mixtures were allowed to stand at room temperature for 3 h. After the third saponification, sterols were extracted. In parallel experiments, sets 2–4 were repeated but no sodium hydroxide was added. All experiments were performed in duplicate.

RESULTS

A typical capillary GLC chromatogram of the plasma sterols from a homozygote with the Smith-Lemli-Opitz syndrome is shown in **Fig. 2.** As can be seen, three peaks in addition to the peak due to cholesterol (peak a) are observed. Peak b has been conclusively characterized as due to 8-dehydrocholesterol (10) and peak c, the most abundant, is for 7-dehydrocholesterol (2). A detailed examination of the mass spectral fragmentation pattern of the trimethylsilyl ether of the third unknown compound (GLC peak d) suggests that it contains an additional double bond and one less carbon atom than 7-dehydrocholesterol consistent with 19-nor-5,7,9(10)-cholestatrien-3β-ol (**Fig. 3**). This structure was suggested by the molecular ion peak at *m/z* 440 that indicated a molecular formula of $C_{26}H_{40}O$ for the parent sterol. Loss of a trimethylsilyl group resulted in the base ion peak at *m/z* 350. Further elimination of 113 mass units (C_8H_{17}; side chain) caused a fragment ion at *m/z* 237 that corresponds with an ion formula of $C_{18}H_{21}^+$ (ion fragment a, **Fig. 4**) while the additional loss of ring D resulted in strong ion fragment at *m/z* 195 [M^+–(side chain + 42)] (ion fragment b). Both fragments a and b are characteristic of sterols and strongly suggest that the sterol contains 26 carbons instead of 27 carbons and has the complete iso-octane side chain and three nuclear double bonds. The ion fragment at *m/z* 183 resulted from loss of ring A due to retro Diels-Alder rearrangement in fragment a (ion fragment c). The mass spectral fragmentation pattern was identical to that reported for the trimethylsilyl ether of a synthetic nor-sterol, 19-nor-5,7,9(10)-cholestatrien-3β-ol (13). This reference standard was synthesized following the method reported in the literature (12) and the trimethylsilyl ether of the synthetic compound showed identical GLC retention indices on two capillary columns (**Table 1,** Fig. 2) and identical mass spectral fragmentation pattern (Fig. 3) as that of unknown compound corresponding to GLC peak d (Fig. 2) in the plasma of patients with Smith-Lemli-Opitz syndrome. Thus, the structure of the third unusual compound in the plasma of these patients was identified as 19-nor-5,7,9(10)-cholestatrien-3β-ol.

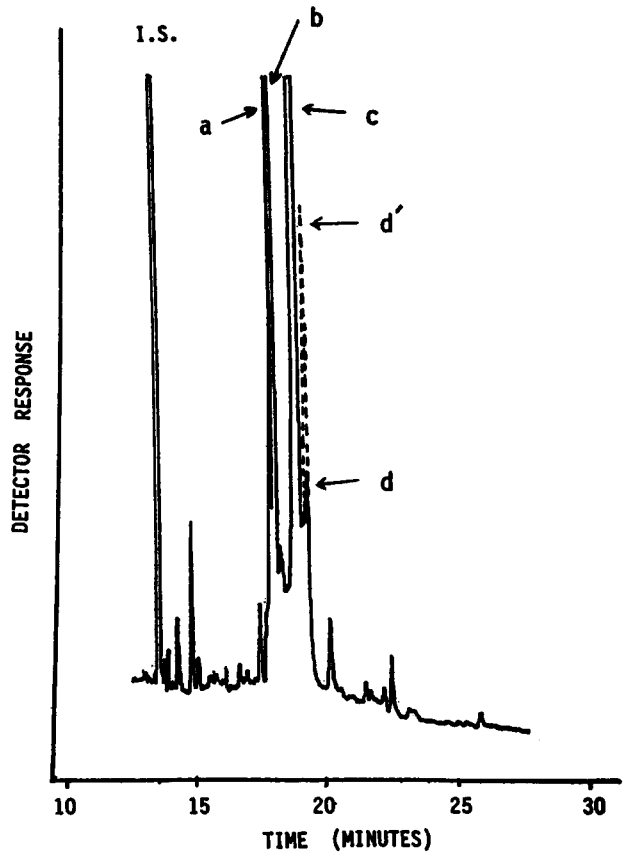

Fig. 2. Capillary GLC profile of the trimethylsilyl ethers of plasma sterols in a patient with Smith-Lemli-Opitz syndrome. Column: CP-Sil-19 CB. See Experimental Section for conditions of chromatography. Peak identification: a, cholesterol; b, 8-dehydrocholesterol; c, 7-dehydrocholesterol; d, 19-nor-5,7,9(10)-cholestatrien-3β-ol; d', 19-nor-5,7,9(10)-cholestatrien-3β-ol after the plasma was spiked with a reference standard of the compound. It should be noted that the column was overloaded with the sample in order to accentuate the level of 19-nor-5,7,9(10)-cholestatrien-3β-ol.

Because patients with Smith-Lemli-Opitz syndrome have markedly increased proportions of 7-dehydrocholesterol in the plasma, and as 7-dehydrocholesterol is relatively unstable when exposed to light, we attempted to test whether this nor-sterol could be formed artificially during workup of the plasma or stool samples of the patients. Commercial 7-dehydrocholesterol or its acetate was crystallized and used to spike control plasma before alkaline hydrolysis and sterol isolation or simply added to ethanol and refluxed for 1 h. Sterols were extracted and examined by capillary GLC and GC–MS. It was found that both the commercial 7-dehydrocholesterol and its acetate contained approximately 4.5% of 19-nor-5,7,9(10)-cholestatrien-3β-ol. The proportion of 19-nor-5,7,9(10)-cholestatrien-3β-ol remained

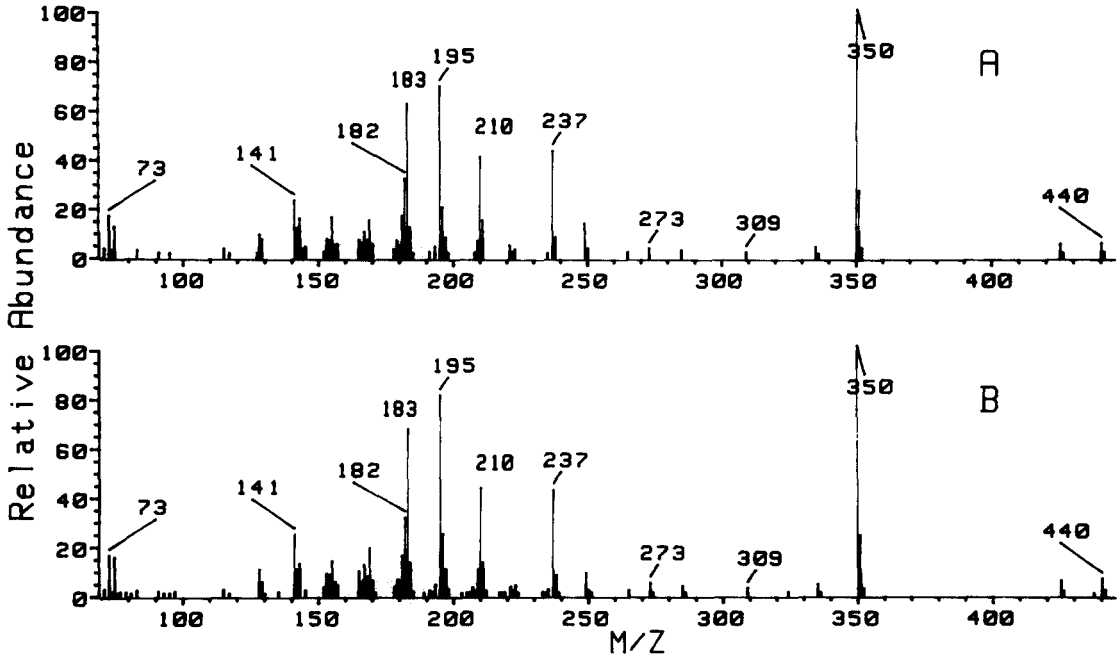

Fig. 3. Mass spectrum of the trimethylsilyl ether of 19-nor-5,7,9(10)-cholestatrien-3β-ol: A: synthetic; B: present in plasma of a patient with Smith-Lemli-Opitz syndrome.

virtually unchanged after refluxing 7-dehydrocholesterol (or its acetate) in alcohol in presence or absence of sodium hydroxide, although prolonged treatment with sodium hydroxide resulted in some loss of 7-dehydrocholesterol (data not reported).

7-Dehydrocholesterol and 8-dehydrocholesterol were present in plasma from all 44 patients we have studied so far, and 19-nor-5,7,9(10)-cholestatrien-3β-ol was present in 35 subjects and accounted for up to 10% of the plasma sterols (**Table 2**). Although the relative proportions of 7- and 8-dehydrocholesterol were significantly higher in the more severely affected patients, 19-nor-5,7,9(10)-cholestatrien-3β-ol was similarly proportioned in all patients (data not shown). Stool from four patients also contained small amounts (1.8% of fecal sterols) of 19-nor-5,7,9(10)-cholestatrien-3β-ol. Thus, 19-nor-5,7,9(10)-cholestatrien-3β-ol is an abnormal sterol present in the plasma and tissues of most subjects with Smith-Lemli-Opitz syndrome.

DISCUSSION

We have now characterized a third aberrant sterol in the plasma of homozygotes with Smith-Lemli-Opitz syndrome (2). Quantitatively, 7-dehydrocholesterol is most abundant, and along with a deficiency of cholesterol points to a defect in the last enzyme in the cholesterol biosynthetic pathway, 7-dehydrocholesterol-Δ^7-reductase as the inherited abnormality responsible for the biochemical and clinical features in Smith-Lemli-Opitz syndrome. As a result of impaired synthesis, tissue and plasma cholesterol concentrations are quite low and 7-dehydrocholesterol accumulates. However, two additional sterols were also detected in quantity. 8-Dehydrocholesterol is the second most abundant sterol. Although, this compound is not a precursor of cholesterol, it is apparently formed enzymatically by a reversible isomerase located in hepatic microsomes in patients with Smith-Lemli-Opitz syndrome that transforms the double bond at C-7(8) to C-8(9). This new 5,8-

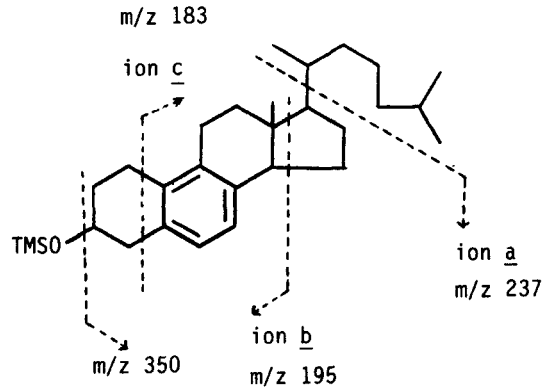

Fig. 4. Mass fragmentations in trimethylsilyl ether of 19-nor-5,7,9(10)-cholestatrien-3β-ol.

TABLE 1. Capillary GLC retention indices of the trimethylsilyl ethers of 7-dehydrocholesterol, 8-dehydrocholesterol, and 19-nor-5,7,9(10)-cholestatrien-3β-ol

Sterol	Relative Retention Time[a]		Retention Index (Kovats)[b]	
	CP-Sil-5 CB	CP-Sil-19 CB	CP-Sil-5 CB[c]	CP-Sil-19 CB[d]
Cholesterol	1.39	1.33	3188	3206
7-Dehydrocholesterol	1.46	1.41	3226	3255
8-Dehydrocholesterol	1.40	1.34	3194	3217
19-Nor-5,7,9(10)-cholestatrien-3β-ol	1.42	1.44	3206	3271
GLC peak d[e]	1.42	1.44	3206	3270

[a]Retention times are expressed relative to that of 5α-cholestane. Retention time of 5α-cholestane was 13.20 min on CP-Sil-5 CB column and 11.65 min on CP-Sil-19 CB column.
[b]Retention indices (Kovats values) were determined by previous injection of a hydrocarbon mixture C_{31}–C_{33} under identical GLC conditions.
[c]The retention times of the various n-alkanes on CP-Sil-5 CB column were as follows: C_{31}, 16.49 min; C_{32}, 18.61 min; C_{33}, 21.24 min.
[d]The retention times of the various n-alkanes on CP-Sil-19 CB column were as follows: C_{31}, 13.52 min; C_{32}, 15.26 min; C_{33}, 17.46 min.
[e]GLC of plasma sterol fraction from a homozygote, retention time and Kovats values calculated for peak d (Fig. 2).

cholestadien-3b-ol, being a 1,õable than 7-deçnd will not fragment into vitamin D when exposed to ultraviolet light. It shows a very similar mass spectrum to 7-dehydrocholesterol but can be distinguished by capillary GLC, argentation thin-layer chromatography, and NMR spectrometry.

The third unknown compound did not show a similar mass spectrum but rather indicated a lower molecular weight than either 7-dehydrocholesterol or 8-dehydrocholesterol and contained 26 carbons and three nuclear double bonds. The mass spectral fragmentation pattern of its trimethylsilyl ether was found to be identical with that reported for 19-nor-5,7,9(10)-cholestatrien-3β-ol (13). A reference standard of this compound was synthesized by thermal decomposition of the dimer of 7-dehydrocholesterol acetate obtained via UV irradiation (12). Both the GLC retention indices of the trimethylsilyl ethers of the synthetic and natural compounds on two different capillary columns and their mass spectral fragmentation patterns were identical. Moreover, 19-nor-5,7,9(10)-cholestatrien-3β-ol is not an artifact formed during the chemical isolation of sterols from plasma or tissues. The origin of this sterol is not yet known. This sterol has an aromatic ring B and thus has structural similarity to anabolic steroids or estrogens that have aromatic ring A and are much more metabolically active. It is tempting to speculate that some of the developmental abnormalities in homozygotes with Smith-Lemli-Opitz syndrome may relate not only to the deficiency of cholesterol but to the presence of the ring-B aromatic 19-nor-5,7,9(10)-cholestatrien-3β-ol.

In summary, we have conclusively identified three aberrant sterols in the plasma of patients with Smith-Lemli-Opitz syndrome. These sterols are also excreted in the stool and found in tissues of these subjects. 19-Nor-5,7,9(10)-cholestatrien-3β-ol is not an artifact formed from 7-dehydrocholesterol during the chemical workup of plasma samples.

TABLE 2. Plasma and fecal concentrations of 7-dehydrocholesterol, 8-dehydrocholesterol, and 19-nor-5,7,9(10)-cholestatrien-3β-ol in patients with Smith-Lemli-Opitz syndrome

Sterol	Plasma[a]	Stool[b]
	mg/dl	*mg/g dry stool*
Cholesterol	49 ±42[c]	2.9 ±2.3
	(3.7–190)[d]	(1.3–6.3)
7-Dehydrocholesterol	16 ±10	2.7 ±3.1
	(0.2–61)	(0.8–7.4)
8-Dehydrocholesterol	13 ±6	0.9 ±1.1
	(1–26)	(0.03–2.4)
19-Nor-5,7,9(10)-cholestatrien-3β-ol	2.8 ±2.1	0.09 ±0.05
	(0–9.6)	(0.04–0.15)

[a]n = 44; females = 21, mean age = 8.6 years (range = 1 day–31 years); males = 23, mean age = 10.3 years (range = 6 days–35 years).
[b]n = 4; females = 3, mean age = 4.7 years (range = 1-14 years); male = 1, age = 13 years.
[c]Mean ± standard deviation.
[d]Range.

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