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# Soluble Fas (CD95/Apo-1), Soluble Fas Ligand, and Activated Caspase 3 in the Cerebrospinal Fluid of Infants with Posthemorrhagic and Nonhemorrhagic Hydrocephalus

URSULA FELDERHOFF-MUESER, CHRISTOPH BÜHRER, PETER GRONECK, MICHAEL OBLADEN, PETER BARTMANN, AND AXEL HEEP

*Department of Neonatology, Charité Children's Hospital, Campus Virchow Klinikum, D-13353 Berlin [U.F.-M., C.B., M.O.], Department of Neonatology, Cologne Children's Hospital, 50735 Cologne [P.G.], and Department of Neonatology, Friedrich-Wilhelms University, 53113 Bonn [P.B., A.H.], Germany*

## ABSTRACT

Hydrocephalus may result in loss of tissue associated with neuronal degeneration, axonal damage, and reactive gliosis. The soluble form of the anti-apoptotic regulator Fas (sFas) and the pro-apoptotic factors soluble FasL (sFasL) and activated caspase 3 were studied in the cerebrospinal fluid of infants with hydrocephalus. Fifteen preterm infants with posthemorrhagic hydrocephalus undergoing serial reservoir puncture and seven term or near-term infants with nonhemorrhagic hydrocephalus and shunt surgery were included in the study. Twenty-four age-matched patients with lumbar puncture for the exclusion of meningitis served as controls. Elevated levels of sFas were observed in infants with posthemorrhagic hydrocephalus [median (range), 131 ng/mL (51–279 ng/mL)] and in nonhemorrhagic hydrocephalus [127 ng/mL (35–165 ng/mL)]. sFas concentrations were highest in a subgroup of eight patients with posthemorrhagic hydrocephalus developing periventricular leukomalacia [164 ng/mL (76–227 ng/mL)]. In contrast, in 24 control infants, sFas was low, in 15 cases below detection limit (0.5 ng/mL) and in nine cases, 24 ng/mL (20–43 ng/mL). sFasL and activated caspase 3 did not differ from control infants in all groups of patients. Increased intrathecal release of sFas in the cerebrospinal fluid of infants with hydrocephalus may serve as an indicator of brain injury from progressive ventricular dilatation. **(*Pediatr Res* 54: 659–664, 2003)**

**Abbreviations**

**IVH,** intraventricular hemorrhage
**CSF,** cerebrospinal fluid
**sFas,** soluble Fas
**sFasL,** soluble Fas ligand
**VLBW,** very low birth weight
**ICP,** intracranial pressure
**PVL,** periventricular leukomalacia

IVH in preterm infants remains the most important prognostic factor in neonatal intensive care for adverse long-term neurodevelopmental outcome. In 35% of cases IVH causes slowly progressive ventricular enlargement as a result of impaired circulation of CSF. Blood clots obstruct circulation and absorption sites of CSF such as basal cisternae, the fourth ventricular foramina, and the aqueduct of Sylvius (1). Both hemorrhage and posthemorrhagic hydrocephalus may cause parenchymal damage associated with hemorrhagic infarction, atrophy of the periventricular white, and, to some extent, gray matter damage (2–4). The neuropathologic consequences associated with hydrocephalus are moderate in the adult, but severe in the developing brain, as previously shown in ultrastructural studies. The mechanisms by which cell death occurs are controversially discussed, and only a few experimental reports exist. Miyan and coworkers (4a) attribute cortical damage to be the result of impaired development of glial cells, loss of myelination, and also neuronal death by necrosis. Animal studies by Del Bigio and Zhang (5) of the immature brain (3-wk-old rats) reveal that damage of cortical neurons (layers II and IV), axons, and oligodendrocytes caused by hydrocephalus are associated with apoptotic cell death. Unfortunately, there is a lack of animal data addressing earlier developmental stages.

The apoptotic cell death program is characterized by a concert of extracellular and intracellular signaling pathways requiring *de novo* transcription and translation. Important molecules for the initiation and execution of the apoptotic program

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Correspondence: Ursula Felderhoff-Mueser, M.D., Department of Neonatology, Charité, Children's Hospital, Campus Virchow, Augustenburger Platz 1, D-13353 Berlin, Germany; e-mail: ursula.felderhoff@charite.de
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are the death receptor Fas (CD95/Apo-1) and a group of intracellular cysteine proteases, the caspases, thought of as central initiators and executioners of cell death.

Fas, a member of the tumor necrosis factor/nerve growth factor superfamily, is located on the cell surface and has been implicated in initiating the apoptotic program in a variety of disease models (6–9) and also in the regulation of immunologic responses (10, 11). Cells expressing Fas on their cell surface can be induced to undergo apoptosis on binding to its endogenous ligand, FasL (12). Both Fas and FasL exist in membrane-bound and soluble forms. On cellular activation, the soluble form of the Fas receptor, sFas, is generated by alternative splicing of the full-length Fas mRNA (13). It regulates cell death by inhibiting the binding between Fas and FasL on the cell surface (14, 15). sFasL, a type II membrane protein, is expressed on activated T cells and released by cleavage through metalloproteinases. It serves as a death-inducing element, but under experimental conditions is less potent than FasL itself (16).

Within the apoptotic signal transduction pathway, the intracellular cysteine protease caspase 3 plays a pivotal role as a regulator and executioner of acute cell death. The presence of the activated form of caspase 3 marks the point of no return within the complex apoptotic signaling cascade (17, 18). Under apoptosis-inducing conditions triggered by Fas receptor activation, Jurkat cells, murine fibrosarcoma cells, and murine microglial cells have been shown to have the ability to release activated caspase 3 into the cytoplasm (19).

Both components of the apoptotic cell death program, the Fas/FasL system and caspase 3, have not yet been investigated in hydrocephalic brains. We have determined the levels of sFas, sFasL, and activated caspase 3 in the CSF of preterm infants with posthemorrhagic hydrocephalus. Results were compared with infants with nonhemorrhagic hydrocephalus and CSF of control subjects undergoing lumbar puncture for the exclusion of meningitis.

## METHODS

### Patient Selection

CSF was obtained prospectively when clinically indicated after obtaining parental consent from three groups of infants treated at the Departments of Neonatology of the Friedrich-Wilhelm University in Bonn, the Cologne Children's Hospital, and the Charité, Campus Virchow Klinikum, Humboldt University in Berlin, Germany, between March 1999 and October 2001: *1*) VLBW (birth weight < 1500 g) and preterm infants with progressive posthemorrhagic hydrocephalus (maximum birth weight, 1685 g), *2*) term or near-term infants with congenital hydrocephalus, and *3*) preterm, near-term, and term infants undergoing lumbar puncture for septic workup. The study was approved according to the guidelines of the Ethical Committee of the Friedrich-Wilhelm University in Bonn.

***VLBW with progressive posthemorrhagic hydrocephalus.*** Ventricular CSF was obtained from 15 VLBW infants admitted for surgical treatment of posthemorrhagic hydrocephalus after IVH (grades III/IV). Clinical details are summarized in Table 1. Eight infants later developed signs of cystic PVL on ultrasound (Table 2).

**Table 1.** *Clinical data of infants with posthemorrhagic hydrocephalus*

| Neonatal profile ($n$ = 15) | Data | (%) |
|---|---|---|
| Birth weight (g) | 810 (470–1 685)* | |
| Gestational age at birth (wk + d) | 25 + 3 (23 + 3–3 + 4)* | |
| Number of boys | 8 | 53 |
| Respiratory distress syndrome (grades III/IV) | 13 | 87 |
| Seizures | 9 | 60 |
| Apnea-bradycardia syndrome | 14 | 93 |
| Necrotizing enterocolitis grade III | 1 | 7 |
| Retinopathy of prematurity (> grade II) | 3 | 20 |
| IVH grades III/IV | 15 | 100 |
| Bilateral | 8 | 53 |
| Unilateral | 7 | 47 |
| Cystic PVL | 8 | 53 |

* Median (range).

In all patients ventricular dilatation and increased ICP were present as confirmed by physical examination and ultrasound. CSF drainage by surgical ventriculostomy and implantation of a reservoir (Rickham) was performed on median day 21 (range, 18–25) when at least one of the following criteria was met: *1*) signs of IVH and progressive ventricular dilatation identified by ultrasound, (ventricular width > 97% percentile) (20), *2*) excessive enlargement of head circumference (>2 cm/wk, daily measurement), *3*) bulging fontanel with widening of the sagittal suture, and *4*) clinical signs of increased ICP (seizures, apnea-bradycardia, and hypoventilation) (21, 22). All patients later required shunt surgery for symptomatic posthemorrhagic hydrocephalus.

CSF samples were obtained depending on clinical criteria of elevated ICP with therapeutic intention. Sterile reservoir puncture was performed using a 27-gauge butterfly needle, and CSF was withdrawn during a time period of 2–5 min. Samples were collected until the time of permanent shunt placement when total CSF protein content was normalized [median postnatal age day 70 (41–123)]. Three samples per patient were analyzed in a time period between reservoir implantation and shunt surgery: sample 1 at the time near first ventriculostomy [postnatal day 30 (10–35)], sample 2 [postnatal day 43 (21–44)], and sample 3 shortly before permanent shunt implantation [postnatal day 64 (41–117)].

**Table 2.** *Ultrasound findings in PVL patients*

| PVL patient no. | IVH (left/right) | Localization of cystic defects on ultrasound |
|---|---|---|
| 1 | IV/II | Large cystic parietal periventricular white matter defect on the left |
| 2 | IV/0 | Multiple frontal white matter cysts on the left |
| 3 | I/III | Frontal white matter cyst on the right |
| 4 | IV/IV | Frontal white matter cyst on the right |
| 5 | III/IV | Bilateral frontal white matter cysts |
| 6 | IV/− | Large cystic periventricular white matter defect on the left |
| 7 | IV/II | Bilateral multiple frontal cystic lesions |
| 8 | II/IV | Large hemorrhagic infarct in the frontal and parietal periventricular white matter |

Fourteen ICP measurements could be obtained from six infants during CSF reservoir puncture. ICP was measured (normal, 40–50 cm $H_2O$) via a tube connected to the butterfly puncture needle before and after tapping. In the remaining infants this procedure was impossible to perform during s.c. reservoir puncture as infants were not sedated. Spontaneous movements and crying may cause incorrect results. In addition, protein clotting can obstruct the manometry device (1).

***Newborn infants with congenital hydrocephalus.*** Ventricular CSF samples were taken from seven patients with a median gestational age of 38 wk 0 d (31 wk 4 d–38 wk 4 d) and a birth weight of 2750 g (2070–3900 g) undergoing surgery for congenital nonhemorrhagic hydrocephalus at the time of first shunt implantation [postnatal day 5 (2–45)]. Patients suffered from hydrocephalus associated with spinal dysraphism with Arnold Chiari malformation (one of seven patients), aqueduct stenosis (two of seven patients), and congenital nonhemorrhagic hydrocephalus of unknown origin (four of seven patients). In all patients hydrocephalus and increased ICP were present as confirmed by clinical criteria and ventricular enlargement on ultrasound.

***Preterm, near-term, and term infants undergoing lumbar puncture for septic workup.*** Lumbar CSF samples were obtained from 11 preterm infants [gestational age 27 wk 5 d (25 wk 5 d–32 wk 5 d); birth weight 835 g (550–1560 g)] and 13 term or near-term infants [gestational age 38 wk 0 d (32 wk 0 d–40 wk 0 d); birth weight 2890 g (1990–3450 g)] who underwent lumbar puncture for septic workup and exclusion of meningitis. All control infants showed no neurologic deficits on clinical and ultrasound examination.

In all samples ventricular CSF leukocyte counts, CSF protein concentrations, and bacterial cultures were routinely obtained. None of the patient or control group had a history of autoimmune or progressive malignant disease. CSF samples were immediately centrifuged; the supernatant was separated from the pellet and stored at −40°C until analysis.

### sFas, sFasL, and Activated Caspase 3 Assays

sFas concentrations were determined with a commercial second-generation sandwich ELISA (R&D Systems, Wiesbaden, Germany) using a polyclonal coating antibody, binding to sFas amino acid residues 305–319, and a monoclonal detecting antibody, binding to residues 110–120 of spliced sFas at a detection limit of 0.5 ng/mL. FasL concentrations were determined with a sandwich ELISA (R&D Systems) using MAb for coating and binding (clones 4H9 and 4A5) at a detection limit of 0.5 ng/mL.

Activated caspase 3 was measured by quantitative sandwich ELISA (R&D Systems). The ELISA uses 96-well microtiter plates precoated with an MAb specific for caspase 3 and measures the relative amount of caspase 3 large subunit modified with biotin-ZVKD-fmk (fluoromethylketone).

Protein concentrations in CSF were determined by ELISA technique (Pierce, Perbio Science, Bonn, Germany). All assays were performed at least in duplicate. A microplate photometer (Dynatech MR5000, Denkendorf, Germany) was used for analysis of 96-well microtiter plates.

Statistical analyses and graphs were produced with SPSS 10.7 software (SPSS Inc., Chicago, IL, U.S.A.). The Mann-Whitney *U* test for comparing distributions, the Friedman test for related samples, and a Kruskal-Wallis/ANOVA were applied. All data are given as median (range) unless indicated otherwise.

## RESULTS

From a total number of 15 patients with posthemorrhagic hydrocephalus, three repeated samples were analyzed per patient: sample 1 at the time near first ventriculostomy [postnatal day 30 (10–35)], sample 2 [postnatal day 42 (21–44)], and sample 3 shortly before permanent shunt implantation [postnatal day 64 (41–117)]. In patients with nonhemorrhagic hydrocephalus samples were analyzed from postnatal day 5 (2–45). Leukocyte counts and bacterial cultures did not reveal any evidence of inflammation.

In six infants a total of 14 ICP measurements were obtained during CSF reservoir puncture. ICP was 130 cm $H_2O$ (50–180 mm $H_2O$; normal value, 40–50 mm $H_2O$) (1).

sFas concentrations were well above the detection limit in all samples taken from infants with posthemorrhagic hydrocephalus. sFas concentrations were 131 ng/mL (51–279 ng/mL; $n = 45$, three repeated samples per patient) without significant difference among samples 1, 2, and 3 ($p = 0{,}247$; Fig. 1). All infants with posthemorrhagic hydrocephalus suffered from IVH grade III or IV. There was no correlation between classified severity of IVH on ultrasound and sFas levels. In addition, sFas levels were not higher in the group with bilateral higher grade (III/IV) IVH.

In the group of patients with nonhemorrhagic hydrocephalus, sFas was 127 ng/mL (35–165 ng/mL; $n = 7$), without significant difference ($p = 0.09$) compared with the posthemorrhagic group (Fig. 1). Analysis of single samples per patient or day when the Rickham puncture was performed did not show results essentially different from those obtained when analyzing all samples.

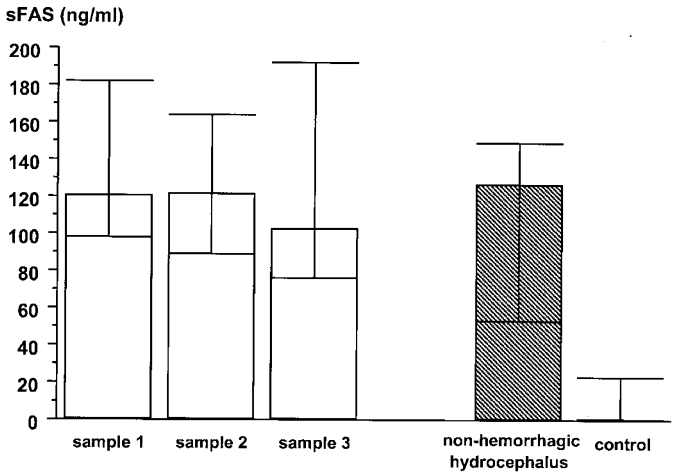


**Figure 1.** sFas levels in the CSF of infants with posthemorrhagic hydrocephalus in samples taken at several time points after the bleeding (samples 1, 2, and 3) and of infants with nonhemorrhagic hydrocephalus compared with lumbar CSF obtained in preterm and term infants for the exclusion of meningitis. Values are plotted as median (interquartile range).

sFas concentrations were highest in the subgroup of eight patients with posthemorrhagic hydrocephalus developing PVL [164 ng/mL (76–227 ng/mL)]. In contrast, in 24 control infants, sFas was low, in 15 cases below detection limit (0.5 ng/mL) and in nine cases, 24 ng/mL (20–43 ng/mL).

Analysis of the correlation between PVL and sFas levels in patients with posthemorrhagic hydrocephalus showed that sFas was higher in patients with PVL, although significance was only reached in analysis of sample 3 (sample 1, $p = 0.052$; sample 2, $p = 0.054$; sample 3, $p = 0.019$). Mean sFas concentrations were higher in the subgroup of eight patients with PVL ($p = 0.014$; Fig. 2). Restricting the analysis to the third sample taken near the time period when PVL was diagnosed on ultrasound did not yield different results. In this limited number of patients the localization and severity of parenchymal involvement on ultrasound did not correlate with sFas levels. In addition, sFas did not correlate with postnatal age in all CSF samples, and there was no difference in sFas levels in VLBW control infants and term or near-term infants.

Protein concentrations in patients with posthemorrhagic hydrocephalus were 2.43 mg/dL (0.76–10.61 mg/dL) in first samples, in second samples 2.02 mg/dL (0.72–7.86 mg/dL), and in third samples 1.76 mg/dL (0.75–3.58 mg/dL), owing to ongoing resorption of blood. sFas levels did not correlate with protein contents (Spearman). In infants with nonhemorrhagic hydrocephalus and in control infants protein levels were low [0.73 mg/dL (0.32 - 1.86 mg/dL)].

To identify early markers of acute apoptotic cell death, sFasL and activated caspase 3 were also measured. Both sFasL and activated caspase 3 remained below the detection limit in all samples of hydrocephalic patients and control infants (data not shown).

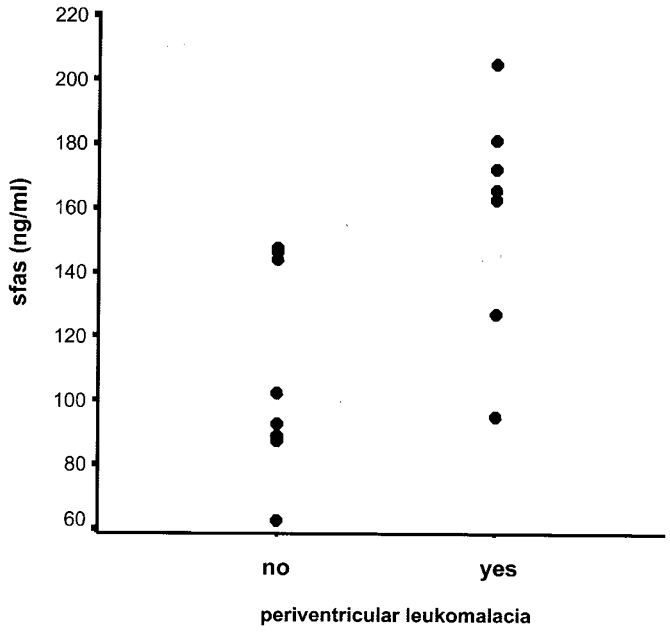


**Figure 2.** Mean values of sFas (samples 1, 2, and 3) in a subgroup of patients with posthemorrhagic hydrocephalus developing PVL ($n = 8$) compared with preterm infants without PVL ($n = 7$; $p = 0.014$).

## DISCUSSION

High concentrations of sFas are found in ventricular CSF of preterm infants with posthemorrhagic hydrocephalus and infants with congenital nonhemorrhagic hydrocephalus. Furthermore, in the small group of patients analyzed, sFas levels were higher in infants developing PVL. The pro-apoptotic markers sFasL and activated caspase 3 were found to be below detection limits. Our findings may indicate the involvement of apoptotic processes in the brain of these infants. The involvement of the Fas cell death system has experimentally been shown in several animal models of infant hypoxic-ischemic injury (9), trauma to the developing brain (23), and intracerebral hemorrhage (24) and in newborns with pontosubicular neuron necrosis after asphyxia (25).

Sampling CSF has been frequently used as a minimally invasive method to obtain markers of predictive or diagnostic value. Besides markers of neuronal degeneration and astrogliosis (26), neurotrophic factors have been detected in the CSF and also suggested to be prognostic markers in hydrocephalus in adults and older children (27, 28). Elevated concentrations of sFas in the CSF have been identified in several acute and chronic neurologic disease states, such as head injury (29), cerebral HIV infection (30), stroke (31), multiple sclerosis (32), and Parkinson's disease (33). In older children undergoing shunt surgery for increased ICP, sFas elevation has been suggested as a regulating element possibly antagonizing ongoing apoptotic processes (34). The truncated form of the Fas receptor, sFas, may indicate activation of the Fas/FasL system, but its definite role in this pathway remains controversial (35).

The soluble form of Fas ligand, a molecule with pro-apoptotic properties, and the activated form of caspase 3 have also been investigated in this study. sFasL has been attributed to inflammatory processes and is predominantly expressed on the surface of activated T cells. On activation its is released through cleavage by metalloproteinases (36). An increased amount of FasL is produced in the CSF concomitantly with Fas receptor up-regulation caused by acute disease states such as in infectious diseases (*i.e.* AIDS) correlating with high viral load (30). Ertel and coworkers (37) have previously shown that CSF from patients with severe brain trauma contained high concentrations of sFasL, possibly causing edema and local tissue destruction. In this study we were unable to detect activated FasL, confirming our previous findings in older children with symptomatic hydrocephalus (34). Hydrocephalus and also PVL do not represent acute insults, in which brain cells possibly develop adaptive mechanisms and do not produce acute phase molecules, possibly explaining the absence of FasL in the CSF in these patients.

Tissue expression of Fas and caspase 3 has been broadly investigated in animal models of traumatic brain injury (8, 23, 38). In recent studies caspase 3 activity and sFasL were determined in various extracellular fluids including the CSF of adults with severe traumatic brain injury (19, 37, 39). However, in addition to the absence of sFasL, we were unable to detect activated caspase 3 in the CSF investigated. In contrast to brain trauma, in which widespread tissue loss is present, hydrocephalus is a more chronic event with, in most cases,

immediate clinical relief of symptoms after puncture. It may well be possible that tissue destruction caused by hydrocephalus is more subtle. If only small amounts of FasL or activated caspase 3 are released into the CSF, they may not be detected by the ELISA test systems used. Furthermore, inflammation after trauma perpetuates apoptotic processes whereas in hydrocephalus inflammatory processes do not play a role.

Cell death mechanisms in hydrocephalus are generally unclear. There is an unfortunate lack of neuropathologic evidence, because of the fact that patients with this disorder can be successfully treated and survive for a very long time. There are also a limited number of animal studies in this field, but some evidence points toward apoptotic mechanisms contributing to the damage. Del Bigio and Zhang (5) found in a rat model that hydrocephalus causes, in addition to axonal injury, gradual cell death in the cerebrum, particularly in the white matter with apoptosis of oligodendrocytes. Fas has been reported to be physiologically expressed on developing neurons (9) and astrocytes (40). Under pathologic conditions of the CNS, many different cell types are capable of expressing Fas, *i.e.* neurons (9), oligodendrocytes (7), microglial cells (41), and astrocytes (42). The soluble form of Fas is produced by alternative splicing of full-length Fas mRNA on stimulation of the receptor and is regarded as a regulator of apoptosis. Therefore, high levels of sFas in the CSF of hydrocephalic infants indicate an involvement of the Fas/FasL system, a major component of the apoptotic cell death machinery. Animal studies will be needed to exactly determine the exact role of Fas/FasL activation caused by hydrocephalus. However, in this clinical study we were unable to correlate either the amount of tissue involvement or the magnitude of ICP with sFas levels. Unfortunately ICP could be reliably measured only in a small group of patients. This procedure has been shown to be difficult to perform during s.c. reservoir puncture as infants are usually not sedated, and crying and spontaneous movements may cause incorrect results. In addition, protein clotting can obstruct the manometry device (1). The median ICP detected in this study was 130 cm $H_2O$, well above normal values.

## CONCLUSIONS

The present study extends previous observations and confirms the presence of sFas as a potentially apoptosis-regulating element in infant posthemorrhagic and nonhemorrhagic hydrocephalus. This may indicate a propensity for the involvement of the Fas/FasL pathway, which is known as a key modulator of apoptotic processes. Although the number of patients without and with PVL is very limited in this study, seven and eight patients per group, respectively, higher amounts of sFas were detected in the PVL group, suggesting sFas also may be a possible predictive marker for ongoing tissue loss. Further multicenter studies in a larger group of patients are needed to confirm these findings.

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