The role of endothelium-derived hyperpolarizing factor in children with familial mediterranean fever

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ABSTRACT

Objectives: Familial Mediterranean Fever is an auto-inflammatory disease characterized by inflammatory attacks in serous tissues often accompanied by endothelial dysfunction. This study aimed to evaluate the effect of endothelium-derived hyperpolarizing factor, which is an indicator of endothelial dysfunction in children with familial Mediterranean fever

Methods: This study include 57 children with familial Mediterranean fever and 31 children as healthy controls. Blood samples were collected from all participants to measure their endothelium-derived hyperpolarizing factor, complete blood count and C-reactive protein. In addition, inflammatory markers, mutation analyses, and microalbuminuria were examined only in the patient group.

Results: The mean age of the patient group was 9.8 ± 4.0 (2.5-18) years, while the mean age of control group was 9.5 ± 3.9 (2.5-16) years (p=0.808). Study group had significantly higher C-reactive protein levels and systolic and diastolic blood pressures and lower endothelium-derived hyperpolarizing factor values than the control group (p=0.0001, p=0.002, p=0.035 and p=0.009, respectively).

Conclusion: Low levels of endothelium-derived hyperpolarizing factor, high levels C-reactive protein and high blood pressure in patients with familial Mediterranean fever can be attributed to the changes in the endothelium resulting from subacute inflammation.

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Keywords: Blood pressure; Children; Endothelium-derived hyperpolarizing factor; Familial mediterranean fever; Inflammation; Endothelial dysfunction.

INTRODUCTION

Familial Mediterranean fever (FMF) is an auto-inflammatory disease characterized by self-limiting attacks that cause peritonitis, arthritis, and sterile serositis. Developing in childhood and continuing through adulthood, this disease is seen in all around the world, but more commonly occurs in Turkish, Arabic, Armenian and Sephardic Jewish populations inhabiting in the eastern Mediterranean region[1-4]. For these patients, amyloid protein can accumulate in various body organs, especially in the kidneys, resulting in kidney failure, the most feared complication of the disease[2,3].

The Mediterranean fever gene (MEFV) is localized on the chromosome 16p13.3 and is responsible for FMF[4,5]. As a result of a defect in this gene, the synthesis of pyrin (also known as marenostrin), which regulates neutrophil activity, is disrupted, causing inflammation in serous tissues through a disruption in leukocyte apoptosis and secretion of IL-1 [6]. Even though the disease is characterized by attacks, low degree of inflammation persist in patients t during attack-free periods[2-4,7]. Low levels of chronic inflammation has been observed in diabetes mellitus, cardiac diseases, rheumatoid arthritis, lupus, and renal failure[8-11]. Chronic inflammation has also been reported to cause endothelial dysfunction. Endothelial dysfunction is defined as a loss of normal vascular homeostatic features and has been shown to cause vascular pathologies such as atherosclerosis and hypertension[11]. More recent research has demonstrated the presence of atherosclerosis and impaired cardiac functions in patients with FMF[12,13]. However, hypertension is not observed in

children and adolescents with FMF despite the presence of inflammation[14,15].

Biological biomarkers secreted from the vascular endothelium play a major role in regulating vascular tone. Physiological and chemical stimulation could lead to release of vasoconstrictors such as angiotensin-II and endothelin-1 and vasodilator mediators such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF)[16-19]. NO and prostacyclin are the best known vasodilator mediators that are inhibited by ATP sensitive potassium channels. EDHF, on the other hand, performs vasodilation effect by opening calcium channels in vascular smooth muscles and cannot easily be inactivated[19]. Through such effects, EDHF contributes to hypotension or helps maintain normal blood pressure.

The early detection of endothelial dysfunction in these patients could play a vital role in identifying any dysfunction of the renal and/or cardiovascular systems before the development of serious complications. Therefore, this study aimed to determine the role of EDHF in early detection of possible complications in children with FMF.

MATERIAL AND METHODS

STUDY POPULATION

This prospective cross-sectional study was carried out between February 2019 and July 2019 at the Pediatrics and Pediatric Nephrology outpatient clinics of the Health Sciences University, Okmeydani Training and Research Hospital. Prior to any research protocols, written approval was obtained from the hospital's local ethics committee (08.01.2019/1089). Before to the study, in accordance with the principles of the World Medical Association Declaration of Helsinki, a detailed explanation about the objectives and scope of the study was given, written informed consent was obtained from the parents and also from the patient if patient is over nine years old. Patients who applied between the specified dates and provided consent were randomly selected. Healthy volunteers who applied for school or sports participation were also randomly selected and gave their consent. Children with no complaints, no known chronic disease and normal physical examination findings were accepted as the control group. The study sample consisted of 36 healthy controls and 64 children with FMF aged between 2.5 and 18 who were followed up in the Pediatric Nephrology outpatient clinic. Pediatric FMF criteria were used in the diagnosis of FMF during patient screening[20] Tel-Hashomer clinical criteria were used to confirm the diagnosis of FMF in patients receiving colchicine response[2-4,21]. Detailed histories, including surgical, procedural, and family histories, and examinations of all patients were recorded, and body mass indexes (BMI) were calculated by measuring each child's weight and height. Blood pressure in both groups were measured at least three times and then their mean value was calculated. These calculated values were < 90 p according to the American Academy of Pediatrics (AAP) guidelines and were within normal limits[22] .The patients' daily doses of colchicine were recorded. In this cohort, none of the patients received any treatments using biological agent. In addition, genetic analysis results of patients with FMF were evaluated. Children with known cronic diseases such as cardiac, neurological were not included in the study. Patients who were over the age of 18 years, obese (BMI > 95 p), and receiving anti-inflammatory medications and/or anticoagulants were excluded from the study. Children under 2.5 years of age and those with a BMI > 95 p were excluded from the control group in order to match the age range of the FMF group. A total of 12 children (7 patients and 5 healthy controls) were excluded from the study because, some registration information or laboratory test results were unavailable. As a result, the study sample included a total of 88 participants: 57 FMF patients and 31 healthy controls.

LABORATORY TESTS

At the time that whole blood, erythrocyte sedimentation rate (ESR), and CRP samples were taken from the healthy controls who applied for school or sports participation, blood samples were also taken for EDHF. In addition, serum amyloid A (SAA), fibrinogen, mutation analysis, complete urinalysis, microalbumin and creatinine were measured in spot urine samples from the FMF group. Also, 5 mL of blood samples were collected from all participants for EDHF, and they were spun in a 4000-RPM centrifuge for 10 minutes to obtain serum samples, which were stored until the time of the study at -80ºC. The frozen samples were thawed at room temperature. For the detection of EDHF, the samples were analyzed using commercially available human ELISA kits (SUNRED human EDHF ELISA kit Shanghai, China, 2019), following the manufacturer's instructions, and the results were recorded in ng/mL.

STATISTICAL ANALYSIS

Statistical analysis was performed using the statistical package SPSS software (Version 17.0, SPSS Inc., Chicago, IL, USA). When continuous variables were normal, they were describle as the mean ± standard deviation (p>0.05 in Kolmogorov-Smirnov test or Shapira-Wilk (n<30)), and when the continuous variables were not normal, they were described as the median.

Comparisons between groups were performed using the Student's t-test (group: patient& control) or one-Way ANOVA (group: patient&other&control) for normally distrubited data and Mann Whitney U test or Kruscall Wallis test abnormally for the data. The catagorical variables between the groups were analyzed using the Chi-square test or Fisher Exact test. Receiver operating characteristic (ROC) curves were constructed and the areas under curve (AUC), as the sensitivity (sen.), and the specificity (spe.) were calculated. A multiple logistic regression analysis was used to determine associations between measurements with groups as dependent variables, and values of p<0.05 were considered statistically significant.

RESULTS

The demographic data of the participants and certain laboratory findings are presented in Table I. A total of 57 FMF patients and 31 healthy controls were included in the study. The mean ages of the study and control groups were 9.8 ± 4.0 (2.5-18) years and 9.5 ± 3.9 (2.5--16) years, respectively, and there was no statistically significant difference between the groups in terms of age (p=0.808). In the study group, there were 31 (54.4%) male patients, and the control group included 13 (41.9%) males, with no statistically significant difference between the groups in terms of gender (p=0.372). The mean age at the time of diagnosis was 6.05 (1.5-8.9) years, with a mean follow-up duration of 3.77 (0.7-10) years, and all FMF patients received colchicine therapy. The dose of colchicine administered to the patients was 0.84 (0.3–1.53) mg/m2/day, with an average of 0.92 ± 0.4 mg/day. Urea and creatinine levels were in the normal limits for the FMF patients, in 9 (15.8%) of the patients had a microalbuminuria level above 30 mg/day. In the FMF group, fibrinogen, SAA, mutation analysis, urine microalbumin, urine creatinine and urine microalbumin/creatinine values were also examined.

When the study and control groups were compared, there was no statistically significant differences in terms of height, weight, BMI, leukocyte count, hemoglobin, hematocrit and platelet count. Blood pressure values were < 90 p according to the AAP guidelines and were within normal limits for patients and controls Systolic and diastolic blood pressures were higher in the study group than in the control group, while EDHF values were lower in the study group than in the control group (p=0.002; p=0.035 and p=0.009; p=0.0001, respectively) (Table II). The EDHF results of the study and control groups are shown in Figure 1.

In our study, we examined the relationship between EDHF and some laboratory findings (Table III). According to these findings, a negative correlation was found between EDHF and ESR (r=-0.279, p=0.035). However, there was no statistically significant relationship between EDHF and SAA, CRP, fibrinogen and leukocyte count.

The ROC curve was also plotted for EDHF and when

TABLE I. CLINICAL FEATURES AND LABORATORY FINDINGS OF PATIENT GROUP

Variable	Results
The number of patients	57
Sex, boys/girls (n)	31/26
Clinical features	**n (%)**
Family history	30 (52.6)
Abdominal pain	55 (96.5)
Fever	34 (59.6)
Myalgia	18 (31.5)
Arthralgia	31 (54.4)
Erysipelas-like erythema	8 (14.0)
Splenomegaly	5 (8.8)
Peritonitis	3 (5.3)
Pleuritis	2 (3.5)
Laboratory results	**Median (Min-Max)**
ESR (mm/h)	26.49 (3-116)
Fibrinogen (g/L)	2.81 (1.66-5.35)
SAA (mg/dL)	11.78 (0.06-138)
Urine microalbumin (mg/dL)	10.22 (0.25-55.14)
Urine creatinine (mg/dL)	184.16 (30-478)
uMA/uCr (mg/mg)	0.04 (0.0-0.39)

ESR: Erythrocyte sedimentation rate, SAA: Serum amyloid A, SD: Standart deviation, uMA/uCr: Urine microalbumin/urine creatinine ratio

TABLE II. DEMOGRAPHIC CHARACTERISTICS AND LABORATORY DATA OF PATIENTS AND CONTROLS

	Patients (n=57) Mean ± SD	Controls (n=31) Mean ± SD	p
Age(years)	9.8 ± 4.10	9.5 ± 3.9	0.808
BMI (kg/m2)	18.3 ± 3.3	17.8 ± 3.5	0.539
Systolic BP (mmHg)	110.9 ± 10.99	103.9 ± 8.	0.002*
Diastolic BP (mmHg)	65.8 ± 7.4	61.9 ± 8.4	0.035*
Hemoglobin (g/dL)	12.4 ± 1.4	12.6 ± 1.1	0.396
Hematocrit (%)	37.2 ± 3.5	37.1 ± 3.0	0.930
Platelet count (10^9/L)	298.9 ± 68.0	294.8 ± 51.9	0.772
Parameters	Median(min-max)	Median(min-max)	
Weight (kg)	31(13-68)	32 (13-73)	0.938
Height (cm)	135 (86-170)	135 (89-170)	0.985
Leucocyte count (10^9/L)	8200 (4000-18500)	7260 (4800-15800)	0.129
CRP (mg/L)	3.6 (0.13-157.5)	0.4 (0.2-3.7)	0.0001*
EDHF (ng/mL)	4.3 (2.9-8.9)	4.9 (3.1-59.9)	0.009*

*p<0.05, BP:Blood pressure; BMI: Body mass index; CRP: C-reactive protein, EDHF: Endothelium-derived hyperpolarizing factor; SD: Standart deviation

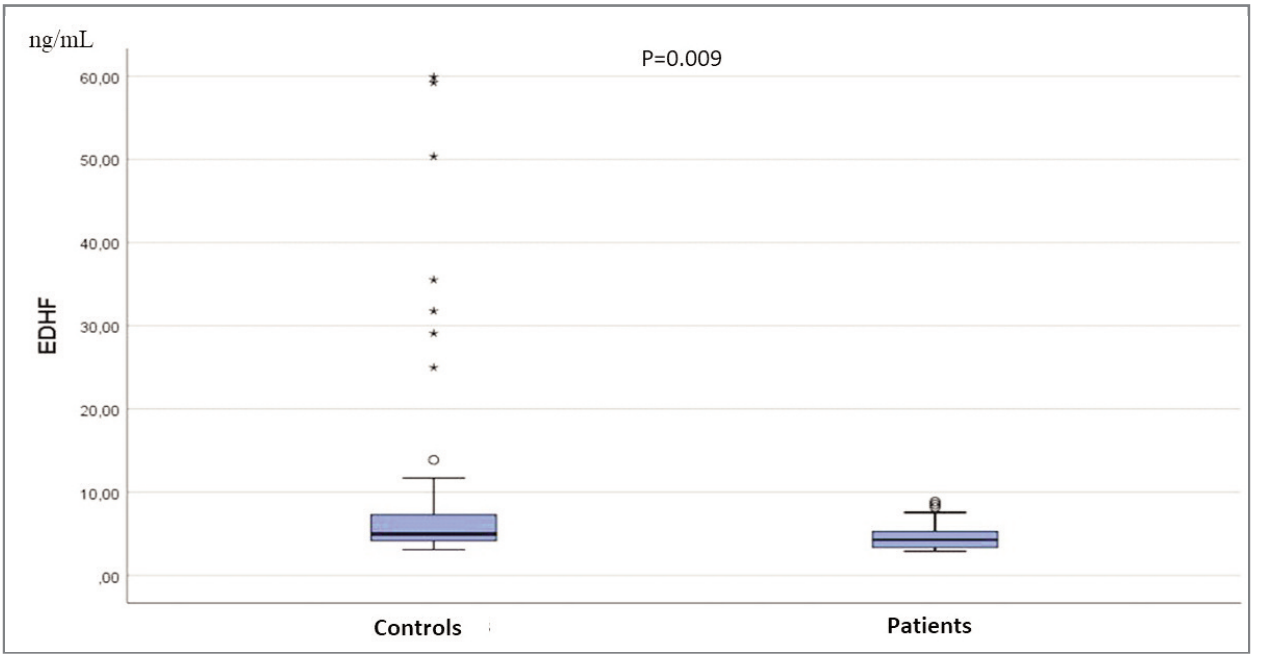

FIGURE 1. EDHF results of patient and control groups

a cut off value for the EDHF was being determined, the AUc was found to be 0.67 (95% CI, 0.55-0.79) (Figure 2).

The *MEFV* gene analysis results of the study group were evaluated. The most common gene mutation was *M694V* gene mutation, which occurred in 36 (63.2%) patients, and the *R202Q* polymorphism was the second most common genetic anomaly in the exon 10 region of the *MEFV* gene, which was detected in 26 (45.6%) patients (homozygous in 4 patients). A homozygous

TABLE III. PEARSON CORRELATION BETWEEN EDHF AND OTHERS ACUTE PHASE REACTANCE

		SAA (mg/dL)	ESR (mm/h)	CRP (mg/L)	Fibrinogen (g/L)	Leucocyte count (10^9/L)
EDHF (ng/mL)	r	-0.122	-0.279	-0.134	-0.236	-0.054
	p	0.365	0.035*	0.321	0.077	0.691

*p<0.05, CRP: C-reactive protein; EDHF: Endothelium-derived hyperpolarizing factor; SAA: Serum amyloid A, ESR: Erythrocyte sedimentation rate

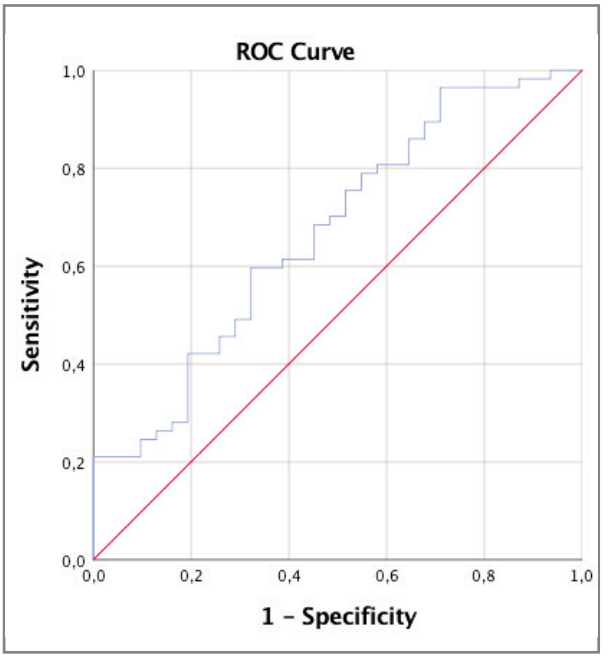

FIGURE 2. ROC curve for the EDHF values

M694V mutation was detected in 10 patients (17.5%) and a heterozygous *M694V* mutation was detected in 26 (45.6%) patients. Only 2 patients (3.5%) had no genetic mutations. No statistical relationship was found between mutation results and EDHF levels (p= 0.692). All mutations detected in other patients are shown in Table IV.

TABLE IV. GENETIC RESULTS OF PATIENTS WITH FAMILIAL MEDITERRANEAN FEVER

MEFV gene mutations	Patients (n=57)*	%
Homozygous	10	17.6
Compound heterozygous	31	54.4
Heterozygous	14	24.6
No identifiable mutation	2	3.5
Positivity of each mutation		
p.M694V	35	61.4
p.R202Q polymorphism (homozygous)	26(4)	45.6
p.E148Q	8	14.0
p.M680I	5	8.8
p.V726A	4	7.0
p.RS224205	2	3.5
p.RS28940579	1	1.8
p.RS61752717	1	1.8
p.E167D	1	1.8
p.R761H	1	1.8
p.E148V	1	1.8
p.M694I	1	1.8
p.F479L	1	1.8

n (%): number of patients (% ratio of patients). *total number is more than 57 and total percentage is more than 100 because some patients had more than one mutation

DISCUSSION

This study revealed that EDHF levels, a known marker of endothelial functions, were higher in FMF patients than in healthy controls. Our study was the first to investigate EDHF levels in patients with FMF, although other studies have examined endothelial functions. Various studies have shown the presence of endothelial dysfunction in FMF patients by measuring asymmetric dimethyl arginine levels[23-26] . Some research has suggested that there are more endothelial microparticles in patients with FMF, which is indicative of endothelial dysfunction[27]. It has been shown that procalcitonin levels increase during FMF attacks[28]. Procalcitonin has also been shown to reduce blood pressure by disrupting vascular endothelial functions[29]. We believe that EDHF can also be a mediator with an effect similar to that of procalcitonin. EDHF levels in patients with FMF could be recognized as another indicator of endotheli-

al dysfunction.

It has been shown to increase in some acute phase reactants in patients with FMF[30]. In this study, C-reactive protein levels were higher in the patient group than in the control group. These results may indicate an ongoing subacute inflammation in patients with FMF. This suggestion could be supported using SAA and fibrinogen levels, but we did not study these parameters in the control group in this study. Lower EDHF levels could be result from this subacute inflammation and endothelial damage. Unfortunately, human EDHF studies are limited, and children have not been studied; therefore, EDHF levels by age group are not known.NO and prostacyclin released from the endothelium are the best known vasodilator mediators, and they appear to be inhibited by ATP- sensitive potassium channels; however, EDHF, which is active in coronary, mesenchymal and renal arteries and activated by calcium-dependent potassium channels, also plays an important role in local vasodilatation[31]. EDHF's vasodilating effect work in the same way as NO to prevent hypertension in the body[16-18]. EDHF acts as a vasodilator by opening calcium channels in vascular smooth muscles and small arteries, although it cannot be easily inactivated like NO or prostacyclin[19,31].

In an experimental study, the presence of both NO and EDHF in the afferent arterioles of the kidneys was shown to be important in vasodilatation, and therefore might play a role in regulating blood pressure[32]. In patients with FMF, despite the presence of inflammation, hypertension is not an expected finding. In a study of patients with FMF, systolic and diastolic blood pressures and BMI were lower in adolescents than in the healthy control group, but diastolic blood pressure was higher in adults[14]. Evrengül *et al.*[15] reported that diastolic blood pressure was lower in children with FMF. In this study, the study group was selected from children and adolescents; adult patients were excluded from the study. There was no difference in the BMI between the patients and controls. In our study, systolic and diastolic blood pressures were within the normal range in patients and controls; however, when the patient and control groups were compared, the FMF group had significantly higher blood pressure than healthy controls. These results may be due to endothelial damage and lower EDHF levels in the patient group.

Colchicine therapy has an anti-inflammatory effect as well as a protective effect on cardiac functions[33]. In a large study of adults, 0.5 mg of colchicine daily was shown to reduce myocardial infarction rates in individuals with chronic cardiovascular disease compared to the placebo group[34] . One study showed that colchicine protects endothelial functions[35]. All of our patients received regular colchicine treatment and none used biological agents. EDHF levels were not tested before colchicine treatment in our patients. Although EDHF levels were lower in the study patients compared to the control group, we did not detect significant hypertension in any of our patients. Therefore, colchicine could reduce chronic inflammation and endothelial dysfunction. Colchicine may have played a protective role in endothelial function, but it is not known whether the difference in the FMF group was due to the use of colchicine or a direct effect of EDHF.

It has been suggested that microalbuminuria could be an indicator of endothelial dysfunction. Evidence demonstrates a relationship between endothelial dysfunction and microalbuminuria due to subclinical inflammation. Some studies also have reported that endothelial dysfunction increases intracapillary glomerular pressure and causes mesenchymal proliferation and ultimately microalbuminuria[9,36]. Increased vascular permeability and proinflammatory cytokines have been suggested to increase the synthesis of NO, which is caused by endothelial dysfunction. Testing for microalbuminuria is recommended for early diagnosis of renal amyloidosis in FMF patients[37]. Microalbuminuria is defined as excretion of 30–300 mg of albumin per day (30-300 mg/day) in urine[38]. In a study by Kukuy *et al.*[39] of 15 adult FMF patients with moderate proteinuria (0.5 g/day) and amyloidosis and 10 adult FMF patients with moderate proteinuria and no amiloidosis amyloidosis (all detected by biopsy), 73.3% of patients had hypertension in the amiloidosis positive group, while only 1 in 10 patients without amyloidosis had hypertension. In our FMF patients, blood pressure levels were higher than those in the control group. All the patients received colchicine treatment, and none of the patients had proteinuria levels that qualified as nephrotic syndrome; however 15.8% of the patients had microalbuminuria levels above 30 mg/day. All patients had urine albumin levels under 300 mg/day, which had no relationship with inflammatory markers and EDHF. This could be attributed to our relatively small sample size or to the fact that none of our patients showed high levels of microalbuminuria.

The distribution of *MEFV* gene mutation in our patients is shown in Table IV. The most well-known mutations related to FMF according to different geographical distribution include *M694V, E148Q, M680I,*

R202Q, V726A, F479L, R761H, L110P, P369S, R408Q. It has been reported that *M694V* mutation is detected in the most severe forms of the disease[2-4,40]. In our study group, 10 patients had *M694V* homozygous mutations, 26 patients had *M694V* heterozygous mutations, and 26 patients had *R202Q* polymorphism. A previous study suggested that R202Q is not a mutation, but a genetic polymorphism[40]. It has been suggested that the M694V mutation has a more severe course, and that the R202Q homozygous polymorphism affects recurrent febrile episodes[41]. However, the relationship between these mutations and EDHF could not be evaluated because we did not have a sufficient number of patients.

Certain limitations should be recognized when considering the findings of this study. First, the study sample consisted of a rather limited number of cases, so larger case series are needed to generalize our results to a wider population. Second, variation in EDHF levels according to age groups in children and adults is not exactly known.

CONCLUSION

In conclusion, our study shows that children with FMF have lower levels of EDHF than healthy children. These lower EDHF levels and higher blood pressures in patients with FMF compared to the control group may suggest that subacute inflammation decreases. EDHF levels in these patients and can cause an increase in blood pressure. According to this result, children with FMF may be expected to be more hypertensive than the normal population in adulthood.

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