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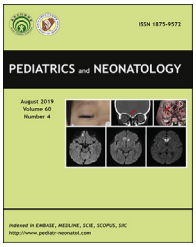

Original Article

Chemokine levels predict progressive liver disease in Down syndrome patients with transient abnormal myelopoiesis

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Background: Transient abnormal myelopoiesis (TAM) is a neonatal preleukemic syndrome that occurs exclusively in neonates with Down syndrome (DS). Most affected infants spontaneously resolve, although some patients culminate in hepatic failure despite the hematological remission. It is impossible to determine the patients who are at high risk of progressive liver disease and leukemic transformation. The objective is to search for biomarkers predicting the development of hepatic failure in DS infants with TAM.

Methods: Among 60 newborn infants with DS consecutively admitted to our institutions from 2003 to 2016, 41 infants with or without TAM were enrolled for the study. Twenty-two TAM-patients were classified into "progression group" (n = 7) that required any therapy and "spontaneous resolution group" (n = 15). Serum concentrations of chemokines (CXCL8, CXCL9, CXCL10, CCL2 and CCL5) and transforming growth factor (TGF)-β1 were measured at diagnosis of TAM for assessing the outcome of progressive disease.

Results: Three patients developed leukemia during the study period (median, 1147 days; range, 33–3753). Three died of hepatic failure. All patients in the progression group were preterm birth <37 weeks of gestational age and were earlier than those in the spontaneous resolution group (median, 34.7 *vs.* 37.0 weeks, $p < 0.01$). The leukocyte counts and CXCL8 and CCL2 levels at diagnosis in the progression group were higher than those in the spontaneous resolution group (leukocyte: median, 81.60 *vs.* 27.30 $\times 10^9$/L, $p = 0.01$; CXCL8: 173.8 *vs.* 34.3 pg/ml, $p < 0.01$; CCL2: 790.3 *vs.* 209.8 pg/mL, $p < 0.01$). Multivariate analyses indicated that an increased CCL2 value was independently associated with the progression and CXCL8

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with the death of liver failure, respectively (CCL2: standardized coefficient [sc], 0.43, $p < 0.01$; CXCL8: sc $= -0.46$, $p = 0.02$).
Conclusion: High levels of circulating CXCL8 and CCL2 at diagnosis of TAM may predict progressive hepatic failure in DS infants.

1. Introduction

Transient abnormal myelopoiesis (TAM) is an almost unique hematological disorder that occurs in 5–10% of newborn infants with Down syndrome (DS).[1] TAM is considered a self-limiting disease during the first few months after birth; however, approximately 20% of TAM patients die in infancy. Previous studies have reported that the following five risk factors were associated with death by 9 months of age: preterm birth at <37 weeks of gestational age (GA), a high leukocyte count ($>100 \times 10^9$/L), severe liver failure manifesting as increasing jaundice with a direct bilirubin (DB) level >5 mg/dl, bleeding diatheses, and ascites.[2–4] Hepatic involvement, which is represented by liver fibrosis, has been recognized as a critical risk factor for early mortality.[5]

Several cytokines and chemokines have been identified as regulators of inflammation or fibrosis in chronic liver diseases.[6] Platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β1 were found to be highly expressed in megakaryoblasts, which might account for the liver fibrosis in DS patients with TAM.[7,8] Hyaluronic acid (HA) and Krebs von den Lungen-6 (KL-6) have been reported to be noninvasive indicators of liver fibrosis and hepatic cancer.[9,10] However, these fibrotic markers may represent the ongoing process, without predicting the imminent progression or outcome of hepatic fibrosis in DS infants with TAM. Furthermore, TAM-associated liver disease deteriorates even after hematological remission.[4,11–14] The biomarkers for predicting disease progression and the pathophysiology of liver disease in DS patients with TAM remain elusive.

In the present study, we prospectively measured the serum levels of major chemokines at the time of diagnosis of TAM in DS infants and assessed the levels for the development and outcome of progressive liver disease. The biological and clinical significance of CXCL8 and CCL2 levels at the diagnosis are discussed in relation to both TAM and liver failure.

2. Materials and methods

2.1. Patient enrollment

Sixty DS infants admitted to the tertiary neonatal intensive care unit (NICU) at Kyushu University Hospital between January 1st, 2003 and December 31st, 2016, and observed until May 31st, 2018 were enrolled for the study. One DS patient was excluded because he was born at 26 weeks of gestational age (GA) (body weight: 360 g) and died within 24 h after birth. In 18 DS infants, the serum samples were of insufficient volume for the measurement of the chemokine concentrations. The clinical diagnosis of trisomy 21 was determined based on a cytogenetic study, and all of the participants had simple trisomy 21. The diagnosis of TAM was made within a few days after birth based on the hematological findings, including an increased number of abnormal blast cells in the peripheral blood. In total, 41 DS infants were enrolled in the study (Supplementary Fig. 1). The demographics and clinical findings of the 41 patients who were enrolled and the 18 patients who were excluded did not differ to a statistically significant extent (data not shown). The Ethics Committee of Kyushu University approved the study. Informed consent was obtained from all parents.

2.2. Measurement of serum chemokine concentrations

The biochemical and hematological findings of all study subjects were assessed by the results of routine blood tests on admission to the NICU. The residual serum samples were stored at −30 °C until the following analysis. The concentrations of chemokines (CXCL8, CXCL9, CXCL10, CCL2 and CCL5) were determined by a cytometric bead array (Becton Dickinson, San Jose, CA, USA). The TGF-β1 level was determined by an enzyme-linked immunosorbent assay (Becton Dickinson, San Jose, CA, USA). The HA and KL-6 levels were measured by a latex agglutination-turbidimetric immunoassay (Fujirebio Inc., Tokyo, Japan) and chemiluminescent enzyme immunoassay (Eisai, Tokyo, Japan), respectively.[15,16] The detection limits of each chemokine and TGF-β1 were 4 pg/ml and 100 pg/ml, respectively.

2.3. Statistical analysis

The differences in the group means and the categorical distributions were compared using Wilcoxon's rank-sum test, Kruskal–Wallis's test and chi-squared/Fisher's exact test, respectively. The associations among parameters were analyzed using Spearman's correlation coefficient. To identify the independent predictors for the development of progressive disease or death, stepwise multiple regression analyses and a multiple linear regression analysis were performed. A p-value of <0.05 was the criterion for entry into the model; variables with p values of >0.05 were eliminated. All of the analyses were performed using the STATA Statistical Software (version 10.0; Stata Corporation, College Station, TX, USA). P-values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. The clinical course of the study population

A total of 22 infants developed TAM at birth. The clinical profiles and early outcomes of the DS infants did not differ between the TAM patients and the non-TAM infants (Table 1). Three TAM patients died of hepatic failure due to TAM at 42, 73 and 232 days after birth. One patient with TAM died of acute megakaryoblastic leukemia (AMKL) at age 2 years 6 months. One TAM patient died of congenital heart disease at age 11 months. One non-TAM infant died of hydrops fetalis due to chylothorax and chylous ascites at 33 days after birth. Seven of 22 TAM neonates required therapy for progressive liver disease, even after hematological remission, and were classified into *"progression group"* (Table S1). In the remaining 15 TAM patients, the hematological and hepatological condition resolved spontaneously; these patients were classified into *"spontaneous resolution group"*. During the observation period, three of four patients in progression group died of liver failure and the other one died of AMKL (Table 2). Two patients in progression group and one in resolution group developed AMKL. Among the five reported risk factors for early death in TAM patients, preterm birth, a high frequency of hyperbilirubinemia (DB $\geq$ 5 mg/dl) and a prolonged

Table 1 Clinical profiles and the early outcome of patients with or without TAM.

	TAM	non-TAM	*p*-value
Number of patients, male: female	22, 14: 8	19, 12: 7	>0.99
Gestational age, weeks	35.4 (30.1–39.3)	37.6 (28.3–40.1)	0.12
Birth weight, gram	2328 (1326–3100)	2395 (491–4014)	0.28
Maternal age, years	36 (24–44)	34 (27–43)	0.53
Days at diagnosis of TAM	0 (0–8)	0[a](0–5)	0.26
Clinical manifestation/anomaly			
Gastrointestinal atresia	1, 4.5%	1, 5.2%	>0.99
Congenital heart disease	14, 63.6%	10, 52.6%	0.54
Hypothyroidism	11, 50%	6, 31.6%	0.34
Others	3, 13.6%[b]	4, 21.1%[c]	0.68
Outcome			
Observed period, days	1010 (43–3753)	1654 (33–3412)	0.66
Death	5, 22.7%[d]	1, 5.3%[e]	0.19
AMKL	3, 13.6%	0	0.24

Each number represents the median and the ranges in parentheses.
TAM: transient abnormal myelopoiesis, AMKL: acute megakaryoblastic leukemia.
[a] Days at admission.
[b] One case of hydronephrosis, one of chylothorax and one of chylous ascites.
[c] Two cases of chylothorax, one of chylous ascites, and one of hydrops fetalis due to chylothorax and chylous ascites.
[d] Three patients died of liver failure due to TAM. One patient died of AMKL, and one congenital heart disease.
[e] This patient died of hydrops fetalis due to chylothorax and chylous ascites.

Table 2 Clinical profiles and laboratory findings during the course of TAM.

	Progression	Spontaneous resolution	*p*-value
Number of patients, male: female	7, 5: 2	15, 9: 6	>0.99
Gestational age, weeks	34.7 (30.1–35.4)	37.0 (34.4–39.3)	<0.01
Birth weight, gram	1792 (1502–2630)	2455 (1326–3100)	0.07
Maternal age, years	39 (24–40)	35 (26–44)	0.55
Days at diagnosis of TAM	0 (0–2)	0 (0–8)	0.64
Outcome			
Observed period, days	795 (43–3135)	1392 (54–3753)	0.27
Death	4, 57.1%	1, 6.7%[b]	0.02
AMKL	2, 28.6%	1, 6.7%	0.23
Laboratory findings at diagnosis			
Leukocyte, $\times 10^9$/L	81.60 (34.13–331.05)	27.30 (9.82–434.80)	0.01
Blast, $\times 10^9$/L	56.55 (21.67–297.95)	3.18 (0.19–418.71)	0.01
	63.5% (49.5–90.0)	28% (1.0–96.3)	
Hemoglobin, g/dL	10.6 (7.3–15.9)	15.7 (9.5–26)	0.05
Platelet, $\times 10^9$/L	324 (138–1208)	154 (33–1508)	0.08
Direct bilirubin, mg/dL	1.3 (0.2–1.6)	0.7 (0.1–1.9)	0.16
ALT, IU/L	70 (24–550)	11 (6–228)	<0.01
LDH, IU/L	4913 (1231–9353)	864 (367–4187)	<0.01
PT-INR[a]	3.29 (2.19–7.43)	1.60 (1.06–2.42)	<0.01
Hyaluronic acid, ng/mL[a]	3175 (706–22800)	518 (112–1500)	<0.01
KL-6, IU/mL[a]	317 (252–481)	207 (93–439)	0.46

Each number represents the median and the ranges in parentheses.
TAM: transient abnormal myelopoiesis, AMKL: acute megakaryoblastic leukemia, ALT: alanine transaminase, LDH: lactate dehydrogenase, PT-INR: prothrombin time-international normalized ratio, KL: Krebs von den lungen.
Progression *vs.* Spontaneous resolution; PT-INR 6 *vs.*12, Hyaluronic acid 6 *vs.*12, KL-6 5 *vs.*6.
[a] Comparative analyses were completed in patients with data available for analyses.
[b] This patient died of congenital heart failure.

prothrombin time-international normalized ratio (PT-INR) were observed in progression group (Table S2).[2–4] All of the infants in progression group had three or more risk factors for early death (Table S1). The cause of ascites in one patient in spontaneous resolution group was due to chylous ascites. Although the incidence of ascites did not differ between the two groups, three early deceased patients in progression group had intractable ascites of hepatic failure due to TAM (Tables S1 and S2).

3.2. Laboratory findings of the DS infants developing TAM

We characterized the laboratory findings of patients in the progression and spontaneous resolution groups (Table 2). All patients in progression group were preterm birth <37 weeks of GA. At the diagnosis of TAM, the leukocyte and the blast counts, alanine transaminase (ALT), lactate dehydrogenase (LDH), PT-INR and HA values in progression group were higher than those in spontaneous resolution group.

3.3. The levels of chemokines and TGF-β1 in all of the DS infants

Table 3 shows the serum levels of chemokines and TGF-β1 in all of the DS infants. The CXCL8 and CCL2 levels in progression group were significantly elevated in comparison to spontaneous resolution group and non-TAM group. Among the TAM patients, the serum CXCL8 and CCL2 levels in progression group were higher than those in spontaneous resolution group (CXCL8: $p < 0.01$; CCL2: $p < 0.01$). The CXCL8 and CCL2 levels at the time of diagnosis were positively correlated in the TAM infants (correlation coefficient [cc] = 0.77, $p < 0.01$). The serum levels of TGF-β1 at the times of diagnosis were positively correlated with the CCL2 levels in the TAM patients (cc = 0.46, $p = 0.04$).

Table 3 Serum chemokine levels at the time of diagnosis of TAM.

	Progression n = 7	Spontaneous resolution n = 15	Non-TAM n = 19	*p*-value[a]
CXCL8, pg/ml	173.8 (29.3 −>100000)	34.3 (<4 −847.3)	23.5 (<4 −2531.8)	0.01
CXCL9, pg/ml	476.4 (<4 −1101.5)	23.1 (<4 −1400.9)	6.8 (<4 −7110.3)	0.96
CXCL10, pg/ml	1618.2 (64.3 −3117.4)	237.3 (44.8 −3369.6)	491.6 (6.5 −5049.0)	0.19
CCL2, pg/ml	790.3 (250.8 −4599.0)	209.8 (<4 −1203.2)	86.0 (<4 −911.8)	<0.01
CCL5, pg/ml	6416.8 (1365.8 −93311.0)	3195.0 (147.6 −8315.7)	8257.7 (1200.3 −47436.0)	0.04
TGF-β1, pg/ml	3283.3 (2325.0 −26560.0)	3575.0 (1250.0 −15520.0)	7746.0 (1466.7 −31133.3)	0.10

TAM: transient abnormal myelopoiesis, TGF: transforming growth factor.
Each number represents the median and the ranges in parentheses.
[a] Kruskal–Wallis test was performed in the three groups.

3.4. The predictive factors for early death

We employed stepwise multiple regression analyses to determine the clinical variables, chemokines and TGF-β1 levels at the diagnosis that predicted progressive liver disease. The multivariate analysis revealed that GA and the CCL2 level at diagnosis were independently associated with progression (GA: standardized coefficient [sc]; −0.64, $p < 0.01$; CCL2: sc = 0.43, $p < 0.01$). Furthermore, GA, the leukocyte count and CXCL8 or CCL2 level were included in a multiple linear regression analysis in order to assess the risk factors for early death in TAM patients. The CXCL8 level at diagnosis and GA were found to be an independent predictor of early death (Table 4; CXCL8: sc = 0.46, $p = 0.02$; GA: sc = −0.41, $p = 0.04$). In the TAM patients, the peak DB value was positively correlated with the CXCL8 (Fig. 1a) and CCL2 (**b**) levels. All patients in progression group showed a peak DB value of >5 mg/dl (Fig. 1, closed triangles and closed circles). Six of the 7 patients in progression group showed a CXCL8 level of >90 pg/ml and a CCL2 level of >450 pg/ml at diagnosis, respectively. All patients with fatal liver disease showed a peak DB value of >10 mg/dl.

4. Discussion

The notable finding of the present study was that serum levels of CXCL8 and CCL2 at diagnosis were significantly elevated in DS patients who developed TAM and progressive liver disease. The CCL2 levels were found to be independently associated with the development of hepatic failure, and the CXCL8 level was also found to be independently associated with early death from TAM. High chemokine levels at diagnosis of TAM and increasing bilirubin levels during the disease course may indicate the need for an intensified therapeutic strategy for the control of TAM-associated liver disease.

CXCL8, also referred to as IL-8, is a chemokine which plays a role as a neutrophil-activating factor in inflammation.[17] CXCL8 is one of the key molecules to attract neutrophils, mainly via its receptors CXCR1 and CXCR2, which release reactive oxygen species as well as proteases, and thereby evoke hepatocyte necrosis.[6] The increased expression of CXCL8 in liver cells, resident hepatic macrophages and T cells, along with circulating

Table 4 Multivariate analysis for association variables at diagnosis of TAM discriminating deceased patients from surviving patients.

Variables	Standard coefficient	*p*-value
CXCL8, pg/mL	0.46	0.02
Gestational age, weeks	−0.41	0.04
Leukocyte, $\times 10^9$/L	−0.08	0.66

TAM: transient abnormal myelopoiesis.

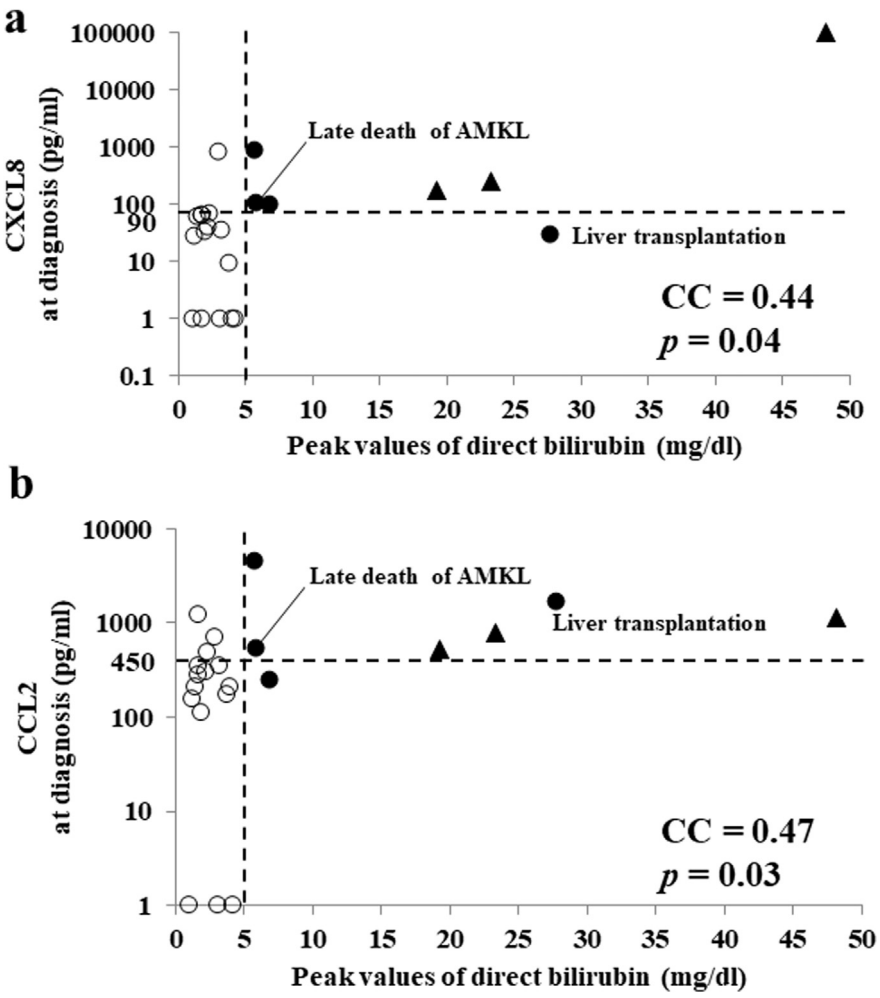

Figure 1 Scatter plots of the associations between chemokines and the peak DB values in DS neonates with TAM. The peak DB values were significantly correlated with the levels of CXCL8 (**a**) and CCL2 (**b**). In 7 patients of progression group, closed circles (●) represent the surviving cases, while closed triangles (▲) represent cases with a fatal outcome due to TAM. Open circles (○) represent the infants in spontaneous remission group. All of the patients in progression group showed a peak DB value of >5 mg/dl, and 6 of them showed a CXCL8 level of >90 pg/ml and a CCL2 levels of >450 pg/mL at diagnosis, respectively. All of the infants in spontaneous resolution group showed a peak DB value of <5 mg/dl. All patients with fatal liver disease showed a peak DB value of >10 mg/dl. DB: direct bilirubin, DS: Down syndrome, TAM: transient abnormal myelopoiesis, CC: correlation coefficient.

CXCL8, might contribute to the progression of hepatic disease and fibrosis in adult chronic liver diseases including viral, autoimmune and alcoholic hepatitis.[6,18–20] Biliary atresia (BA) is the most common cause of neonatal cholestasis. The high expression of CXCL8 in the liver and circulating CXCL8 might play an important role in the inflammation and fibrosis of the liver in patients with BA.[21] CCL2 (monocyte chemoattractant protein-1, MCP-1) is one of the pivotal chemokines during hepatic fibrogenesis.[6] In the chronically inflamed liver, CCL2 is secreted by hepatocytes, biliary epithelial cells, Kupper cells, and hepatic stellate cells (HSCs).[22–25] In one patient with TAM who developed late-onset severe cholestasis, the histopathological examination of affected liver specimens revealed diffuse pericellular fibrosis without megakaryoblast invasion and the distinct CCL2 expressions in the infiltrating interstitial cells consisting of HSCs, portal fibroblasts, and endothelial cells.[11] Chemokines have been identified as central regulators of liver fibrosis.[6] In patients with TAM, PDGF and TGF-β were reportedly produced by blast cells, and they may accelerate cholestasis.[7,8,26] In this study, the serum levels of TGF-β1 were positively correlated with the CCL2 levels at diagnosis. The infiltration of megakaryoblasts was histologically evident in the liver fibrosis of TAM patients.[2,27] On the other hand, the fibrotic liver of several patients with TAM contained no apparent blast cells.[2,11–14] The liver dysfunction progressed, even after megakaryoblasts disappeared from the peripheral blood.[4,11–14] Taken together, these findings suggest the possibility that megakaryoblast-secreted cytokines/chemokines may recruit HSCs and hepatic macrophages, which sustain the persistent inflammation and tissue damage, leading to liver fibrosis.

The treatment strategy for TAM-associated liver disease has not yet been established. Low-dose cytarabine therapy, exchange transfusion, and corticosteroid therapy may have beneficial effects on the outcome of TAM patients with a high risk of early death.[2–5,13,28–30] On the other hand, the

treatment effects of low-dose cytarabine on the damaged liver in TAM patients after the megakaryoblasts disappear from circulation remain unclear. Exchange transfusions may rescue patients from organ failure.[28–30] In the present study, one patient in progression group showed a reduced leukocyte count and reduced serum levels of CXCL8 and CCL2 after three exchange transfusions (leukocyte count: 331.05 to 57.51 $\times$ 10^9/L, CXCL8: 892.6 to 48.6 pg/dl, CCL2: 4599 to 351.6 pg/ml). Repeated exchange transfusions might control both the circulating blast cells and hypercytokinemia before serious hepatic damage can occur. No effective therapies have yet been found for controlling progressive liver disease in patients with TAM. Recently, a living-donor liver transplantation was successfully performed in a patient with TAM and liver failure.[14] Both preventive and curative measures for progressive cholestasis are needed for patients with high CXCL8 and CCL2 at diagnosis of TAM.

The present study has limitations, including the small study population and the lack of a direct analysis of blast cells and liver tissue specimens. However, the multivariate analysis indicated that preterm birth at $<$37 weeks of GA, one of the risk factors for early death, was an independent risk factor for both progressive liver disease and death of liver failure. The peak DB value was positively correlated with the levels of CXCL8 or CCL2. A peak DB level of $>$5 mg/dl was also one of the risk factors for early death in TAM patients.[4,13] In progression group, all 7 patients with high levels of CXCL8 at diagnosis showed peak DB values of $>$5 mg/dl. A more detailed analysis of the chemokine kinetics may clarify the disease process of cholestasis in patients with TAM.

In conclusion, circulating CXCL8 and CCL2 were associated with the development of liver fibrosis in DS neonates with TAM. Further prospective studies are expected to reveal the clinical and biological roles of chemokines in TAM-associated liver disease.

Conflict of interest statement

The authors declare no conflicts of interests in association with this study. This study was supported in part by the Japan Society for the Promotion of Science KAKENHI: #15K09717, #16K19688 and #17K16300.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pedneo.2018.09.005.