ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Polymorphism in cytolytic pathway genes in patients with pediatric inflammatory multisystemic syndrome temporally associated with SARS-CoV-2

Polimorfismos em genes da via citolítica em pacientes com síndrome inflamatória multissistêmica pediátrica temporariamente associada ao SARS-CoV-2

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ABSTRACT

Introduction: Pediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 (PIMS-TS) is a systemic hyperinflammatory disease that occurs in a small number of children after being infected with SARS-CoV-2. Macrophage activation syndrome, an aggressive condition characterized by the excessive inflammation and activation of well-differentiated macrophages, has been shown to occur in patients infected by SARS-CoV-2. Considering the clinical and pathophysiological similarities between these diseases, our main objective was to determine whether gene polymorphisms associated with macrophage activation syndrome were also present in patients with PIMS-TS. **Methods:** DNA from 10 pediatric patients with PIMS-TS (case group) and ten COVID-19 patients without PIMS-TS (control group) were genotyped by Real-time PCR analysis (TaqMan®) for single nucleotide polymorphisms (SNP) in four genes associated with macrophage activation syndrome: *perforin 1 (PRF1)*, *granzyme B (GZMB)*, *syntaxin 11 (STX11)*, and *syntaxin binding protein 2 (STXBP2)*. The SNP analysis was performed using the additive, dominant, and recessive models. **Results:** A significantly higher frequency of an SNP (C wild allele in rs6573910) in the *GZMB* gene was observed in both the additive and dominant models in the PIMS-TS group than controls. A borderline significant difference was also observed for the G allele in rs7764017 of the *STX11* gene in the PIMS-TS

RESUMO

Introdução: A síndrome multissistêmica inflamatória pediátrica temporariamente associada ao SARS-CoV-2 (SIMP-TS) é uma doença hiperinflamatória sistêmica que ocorre em um pequeno número de crianças após serem infectadas pelo SARS-CoV-2. A síndrome de ativação de macrófagos (SAM), uma condição agressiva caracterizada pela inflamação excessiva e ativação de macrófagos bem diferenciados, demonstrou ocorrer em pacientes infectados por SARS-CoV-2. Considerando as semelhanças clínicas e fisiopatológicas entre essas doenças, neste estudo o nosso principal objetivo foi determinar se polimorfismos gênicos associados à SAM também estavam presentes em pacientes com SIMP-TS. **Métodos:** DNA de dez pacientes pediátricos com SIMP (grupo caso) e dez pacientes COVID-19 sem SIMP (grupo controle) foram genotipados por análise de PCR em tempo real (tecnologia TaqMan®) para polimorfismos de nucleotídeo único (SNPs) em quatro genes selecionados associados com SAM: perforina 1 (*PRF1*), granzima B (*GZMB*), sintaxina 11 (*STX11*) e proteína de ligação de sintaxina 2 (*STXBP2*). A análise dos SNPs foi realizada utilizando o modelo aditivo, dominante e recessivo. **Resultados:** Uma frequência significativamente maior de um SNP (alelo selvagem C em rs6573910) no gene *GZMB* foi observada pelos modelos aditivo e dominante no grupo SIMP quando comparado aos controles. Além disso, uma significância limítrofe foi observada para o alelo G em rs7764017 do gene *STX11* no grupo

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group in the additive model. **Conclusions:** This study indicated the presence of two polymorphisms in genes associated with macrophage activation syndrome (*GZMB* and *STX11*) in patients who developed PIMS-TS. If the presence of these SNPs is validated in a larger number of PIMS-TS cases, they can be used as potential biomarkers for early identification of pediatric patients with a higher probability of developing PIMS-TS associated with SARS-CoV-2 infection.

Keywords: COVID-19, pediatric inflammatory multisystem syndrome, macrophage activation syndrome, hemophagocytic lymphohistiocytosis, single nucleotide polymorphism, polymorphism.

SIMP pelo modelo aditivo. **Conclusões:** Nosso estudo indicou a presença de dois polimorfismos em genes associados à SAM (*GZMB* e *STX11*) em pacientes que desenvolveram SIMP-TS. Uma vez validada a presença desses SNPs em um número maior de casos de SIMP-TS, eles podem ser usados como potenciais biomarcadores para a identificação precoce de pacientes pediátricos com maior probabilidade de desenvolver SIMP-TS associado à infecção por SARS-CoV-2.

Descritores: COVID-19, síndrome multissistêmica inflamatória pediátrica, síndrome de ativação macrofágica, linfo-histiocitose hemofagocítica, polimorfismo de nucleotídeo único, polimorfismos.

Introduction

In the second half of April 2020, a new syndrome potentially associated with SARS-CoV-2 infection that affected children and adolescents was described in the United Kingdom,[1] Italy,[2] and New York,[3] followed by reports worldwide. This syndrome, called pediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 (PIMS-TS), was shown by epidemiological studies to occur approximately 1 month after peak COVID-19 incidence. The clinical and serological characteristics of this syndrome are similar to diseases such as Kawasaki-like disease, toxic shock syndrome, and macrophage activation syndrome (MAS).[4-6]

Although the pathophysiology of PIMS-TS is not well understood and the clinical symptoms are heterogeneous at presentation, it has been characterized as a delayed immune phenomenon associated with a hyperinflammatory state, a common feature of the diseases mentioned above.

MAS involves aggressive and life-threatening conditions; immune system activation can be triggered by several types of infectious and/or rheumatological conditions and can also occur in patients with Kawasaki syndrome. Interestingly, patients with COVID-19 show similar inflammatory symptoms to secondary hemophagocytic lymphohistiocytosis (sHLH)/MAS, particularly those associated with specific rheumatological conditions, and isolated cases of COVID-19 have been reported to develop MAS. In addition, specific polymorphisms associated with MAS, such as those in the *pentraxin 3* gene, have been associated with respiratory complications, including those in adults with COVID-19. Taken together, these data provide strong evidence that SARS-CoV-2 infection can lead to MAS. Hence, understanding of the clinical and biological similarities between patients with PIMS-TS and sHLH/MAS could help clarify the intricate immunoinflammatory response involved in these diseases.

In the present study, we tested whether polymorphisms in four genes, *PRF1*, *GZMB*, *STX11*, and *STXBP2*, which are associated with sHLH/MAS, occurred in patients with PIMS-TS. Detecting these polymorphisms could help identify pediatric patients susceptible to PIMS-during the early phase of the disease.

Materials and methods

Study population

Peripheral blood samples from 10 pediatric patients (aged 2-12 years) with confirmed PIMS-TS (case group) were collected from the pediatric Hospital Pequeno Príncipe (Curitiba, PR, Brazil) between October 2020 and April 2021. The samples were collected during hospitalization after obtaining informed parental consent. This study was approved by the National Research Ethics Committee (4.572.430/2020). The definition of PIMS-TS was based on the WHO criteria.[7] A real-time reverse transcriptase-polymerase chain reaction assay for SARS-CoV-2 (nasopharyngeal swabs) was performed for all 10 patients during ICU hospitalization and all were negative. *Post-mortem* lung biopsies of 10 adults who died from severe COVID-19 were used as a control group (non-PIMS-TS COVID-19 group). These patients were part of a previous study (National Research Ethics Committee approval 3.944.734/2020) conducted by our group.[8] The clinical and laboratory data for the PIMS-TS and non-PIMS-TS COVID-19 patients are presented in Table 1.

DNA isolation and genotyping

Biopur Mini Spin Plus Kit (Mobius Life Science, Curitiba, PR, Brazil) was used to isolate DNA from peripheral blood samples of the case group. In the control group, DNA was isolated from formalin-fixed paraffin-embedded tissue using a commercially available DNA isolation kit (Qiagen, Venlo, Netherlands). Single nucleotide polymorphism (SNP) selection was based on reported associations with sHLH/MAS[9,10] and was further verified using the SNPinfo Web Server.[11] Four genes and 8 SNPs were selected: *perforin 1* (*PRF1* - rs10999426, rs885821, rs885822, and rs35947132); *granzyme B* (*GZMB* - rs6573910); *syntaxin 11* (*STX11* - rs7764017), and *syntaxin binding protein 2* (*STXBP2* - rs6791; rs2303115). The patients' purified DNA was amplified by real-time reverse transcriptase-polymerase chain reaction using a TaqMan fluorescence system (Applied Biosystems, Waltham, MA, USA) according to manufacturer instructions. Family genetic analysis was used to visualize the results. Using an additive model made it possible to observe the frequency distribution between the groups. Specific groupings were also organized into dominant and recessive family grouping models.

Statistical analysis

Nominal and non-nominal variables (genotyping) were expressed as number and frequency/percentages and by median and interquartile range, respectively.

Table 1
Clinical and laboratory baseline characteristics of the PIMS-TS COVID-19 (case) and non-PIMS-TS COVID-19 (control) groups

Variables	PIMS-TS (n=10)	non-PIMS-TS COVID-19 (n=10)
Age [a]	9.5 (2.0-12.0)	80.5 (46.0-87.0)
Sex [b]		
Male	5 (50.0)	6 (60.0)
Female	5 (50.0)	4 (40.0)
Clinical aspects (symptoms)		
Fever	7/10	1/10
Abdominal pain	4/10	–
Skin rash	3/10	–
Vomiting	3/10	0/10
Bipalpebral edema	2/10	–
Myocarditis	2/10	–
High blood pressure	–	7/10
Obesity (BMI>30)	–	1/10
Laboratory test results [a]		
CRP (mg/L)	222.500 (9.200-281.000)	126.800 (52.000-171.400)
D-Dimer (ng/mL)	1187.05 (93.00-2230.67)	705.00 (425.00-6544.00)
Ferritin (ng/mL)	133.300 (13.9-133.5)	–
Troponin (pg/ml)	5.0 (5.0-90.0)	15200 (9.000-24.000)
Platelets (mm^3)	141000 (66000-314000)	121500 (111000-311000)
Lymphocytes (mm^3)	6349.50 (510.00-30110.00)	718.50 (423.00-990.00)

BMI = Body mass index; PIMS-TS = Pediatric Inflammatory Multisystem Syndrome Temporally Associated with SARS-CoV-2.
[a] Median/Interquartile range (Q1-Q3); [b] Absolute number (percentage); – Not available or detected.

In the additive model, significance was determined by logistic regression analysis, while Fisher's exact test or Pearson's chi-square test was used in the dominant and recessive models. Bonferroni correction was used for multiple testing, with $p < 0.002$ considered significant. The data were analyzed using IBM SPSS Statistics 20.0 (IBM, Armonk, NY, USA).

Results

Ten cases and 10 controls were genotyped for polymorphisms in the 4 selected genes associated with sHLH/MAS. In the additive model, only the polymorphism for *GZMB*, a wild C allele in rs6573910 (C/T), was significantly ($p = 0.028$) more frequent in the PIMS-TS group (Table 2). For the *STX11* gene in the additive model, the polymorphic GG genotype in rs7764017 SNP was present only in the PIMS-TS group, with borderline significance ($p = 0.076$). The G allele of the same gene was more frequently in the PIMS COVID-19 group by the dominant model (Table 3), however also with no statistical significance ($p = 0.087$; OR = 1.6 and CI = 1.0-1.6). Of relevance to note, that after Bonferroni correction, all the significant p-values observed in this initial analysis were lost, as the adjusted significant p-value for these genotypic associations were set as < 0.002.

Table 2
Genotypic SNP analysis of the *PRF1*, *GZMB*, *STX11* and *STXBP2* genes in 2 groups of patients using the additive model

Gene - reference SNP [a] Allele variation [1/2]	Homozygous 1/1	Heterozygous 1/2	Homozygous 2/2	p-value
PRF1 – rs10999426 A/G	AA	AG	GG	
PIMS-TS	0 (0.0)	6 (60.0)	4 (40.0)	0.303
non-PIMS-TS COVID-19	0 (0.0)	9 (90.0)	1 (10.0)	
PRF1 – rs885821 A/G	AA	AG	GG	
PIMS-TS	0 (0.0)	1 (10.0)	9 (90.0)	0.273
non-PIMS-TS COVID-19	1 (10.0)	3 (30.0)	6 (60.0)	
PRF1 - rs885822 A/G	AA	AG	GG	
PIMS-TS	3 (30.0)	7 (70.0)	0 (0.0)	0.356
non-PIMS-TS COVID-19	1 (10.0)	8 (80.0)	1(10.0)	
PRF1 - rs35947132 A/G	AA	AG	GG	
PIMS-TS	0 (0.0)	0 (0.0)	10 (100.0)	0.474
non-PIMS-TS COVID-19	0 (0.0)	2 (20.0)	8 (80.0)	
GZMB - rs6573910 C/T	CC	CT	TT	
PIMS-TS	6 (60.0)	3 (30.0)	1 (10.0)	0.028
non-PIMS-TS COVID-19	1 (10.0)	3 (30.0)	6 (60.0)	
STX11 - rs7764017 A/G	AA	AG	GG	
PIMS-TS	3 (30.0)	3 (30.0)	4 (40.0)	0.076
non-PIMS-TS COVID-19	6 (60.0)	4 (40.0)	0 (0.0)	
STXBP2 - rs6791 A/G	AA	AG	GG	
PIMS-TS	2 (20.0)	3 (30.0)	5 (50.0)	0.400
non-PIMS-TS COVID-19	1 (10.0)	6 (60.0)	3 (30.0)	
STXBP2 - rs2303115 A/G	AA	AG	GG	
PIMS-TS	4 (40.0)	4 (40.0)	2 (20.0)	0.214
non-PIMS-TS COVID-19	1 (10.0)	4 (40.0)	5 (50.0)	

[a] SNP identifier based on NCBI; 1 = wild type allele; 2 = mutated allele. PIMS-TS: Pediatric Inflammatory Multisystem Syndrome Temporally Associated with SARS-CoV-2. Genotypes expressed as number (%); Logistic regression, $p < 0.05$ = significant. After the Bonferroni test, $p < 0.002$ = significant.

Discussion

PIMS-TS is a serious disease that affects pediatric patients who manifest, among other symptoms, hyperinflammatory shock that can involve several organs.[12] Specifically, children present with high fever, rash, conjunctivitis, peripheral edema, and gastrointestinal symptoms. The latest bulletin from the Brazilian Ministry of Health[13] reported 319 cases of PIMS-TS (aged 0-19 years), with 23 deaths recorded. Specifically, 15 cases of PIMS-TS and 3 deaths were reported at Hospital Pequeno Principe between March 2020 and June 2021 (6 boys and 9 girls; mean age 9.7 years). In our sample of 10 PIMS-TS cases, the sex distribution was equal and the mean patient age was 9.5 years. The patients developed the commonly reported symptoms, with fever, abdominal pain, vomiting, and skin rash being the most frequent (30-70% of the patients).

MAS is a severe, potentially life-threatening condition characterized by excessive activation of well-differentiated macrophages. It results in fever, hepatosplenomegaly, lymphadenopathy, severe cytopenia, serious liver disease, coagulopathy, and neurologic involvement.[14,15] This condition is usually seen in systemic-onset juvenile idiopathic arthritis and rarely manifests with other subtypes of the disease.[16] A variety of triggering factors, including viral infections, have been implicated in the pathogenesis of MAS associated with systemic-onset juvenile idiopathic arthritis.[17] MAS has also been associated with cytokine storm in COVID-19 patients, which supports its association with sHLH in PIMS-TS. In about 50% of patients with sHLH, the underlying genetic defect is caused by mutations in genes of the apoptotic program (such as those assessed in the present study), which leads to death in target cells.[18] The similar clinical symptoms and the physiological processes among these diseases and PIMS-TS, as well as the presence of polymorphisms in genes associated with sHLH/MAS, provided the rationale for our study. Among polymorphisms in the 4 investigated genes, 2 were identified more frequently or only in the PIMS-TS group: rs6573910 (C/T) in *GZMB* and rs7764017 (A/G) in *STX11*.

The *GZMB* gene is located in the 14q12 chromosome region and encodes a member of the granzyme subfamily of proteins, part of the peptidase S1 family of serine proteases.[19] The encoded preproprotein is secreted by natural killer cells and cytotoxic T lymphocytes, which proteolytically process it to generate the active protease (granzyme B) that induces apoptosis. The granzyme B also processes cytokines and degrades extracellular matrix proteins, playing an essential role in chronic inflammation and wound healing. Expression of this gene, which occurs mostly in the bone marrow, has been reported as elevated in patients with cardiac fibrosis and pulmonary cystic fibrosis.[19-22] Interestingly, Ramcharan et al. (2020) described cardiac findings and short-term outcomes in children with PIMS-TS that may be due to post-viral immune reactions[22]. Furthermore, pulmonary fibrosis is a post-COVID-19 event related to remodeling and inflammatory processes during the course of the disease.[23,24] Hence, these findings reinforce the involvement of granzyme B in inflammatory pathways and fibrosis. Granzyme B has also been associated with sHLH/MAS. Based on this evidence, this protease could be involved in PIMS-TS due to its similarities to MAS. Rs6573910 (C/T) is a 2KB upstream intronic variant in *GZMB*. The reported minimum allele frequency in the 1000G Database is 0.3017 for the T allele and 0.6983 for the C allele.[23] In our PIMS-TS sample, the frequency detected for this allele was 0.2500 for the T allele and 0.7500 for the C allele. According to the additive model, the wild C allele in rs6573910 (C/T) in *GZMB* was also more frequent in the PIMS-TS group than the control group ($p = 0.028$). Therefore, even considering that the initial significance of this association was not maintained after Bonferroni correction, the C allele for this SNP could be a risk factor for PIMS-TS.

The *STX11* gene is located at 6q24.2 and consists of 9 exons. Like *GZMB*, the translated protein, syntaxin, is highly expressed in the bone marrow. This protein has been implicated in intracellular transport vesicles and may regulate protein transport in the endosome and trans-Golgi network. Mutations in this gene have been associated with primary hemophagocytic lymphohistiocytosis.[25] In this gene, we observed a polymorphism in rs7764017 (A/G) in *STX11*. In the 1000G database, the minimum allele frequency reported in rs7764017 (A/G) is 0.3842 for the wild A allele and 0.6158 for the polymorphic G allele.[26] In our patients, the observed frequency was 0.4500 for the A allele and 0.5500 for the G allele. In the additive model, the GG genotype of this SNP was observed only in the PIMS-TS group ($p = 0.076$). Because rs7764017 is located in the intron and is classified as a non-coding transcript variant,[26] this SNP may not impact the translation process of the *STTX11* gene. However, it cannot be ruled out that it

Table 3
Genotypic SNP analysis of the *PRF1*, *GZMB*, *STX11* and *STXBP2* genes in 2 groups of patients using the dominant and recessive models

Gene - reference SNP [a] and allele variation	Models		PIMS-TS (n=10)	non-PIMS-TS COVID-19 (n=10)	p-value
PRF1	Dom A	AA+AG	0 (0.0)	0 (0.0)	N/A
rs10999426 A/G		GG	0 (0.0)	0 (0.0)	
	Rec A	GG+AG	0 (0.0)	0 (0.0)	N/A
		AA	0 (0.0)	0 (0.0)	
PRF1	Dom A	AA+AG	1 (10.0)	4 (40.0)	0.303[b]
rs885821 A/G		GG	9 (90.0)	6 (60.0)	
	Rec A	GG+AG	10 (100.0)	9 (90.0)	1.000[c]
		AA	0 (0.0)	1 (10.0)	
PRF1	Dom A	AA+AG	10 (100.0)	9 (90.0)	1.000[c]
rs885822 A/G		GG	0 (0.0)	1 (10.0)	
	Rec A	GG+AG	7 (70.0)	9 (90.0)	0.582[b]
		AA	3 (30.0)	1 (10.0)	
PRF1	Dom A	AA+AG	0 (0.0)	0 (0.0)	N/A
rs35947132 A/G		GG	0 (0.0)	0 (0.0)	
	Rec A	GG+AG	0 (0.0)	0 (0.0)	N/A
		AA	0 (0.0)	0 (0.0)	
GZMB	Dom C	CC+CT	9 (90.0)	4 (40.0)	0.057[c]
rs6573910 C/T		TT	1 (10.0)	6 (60.0)	
	Rec C	TT+CT	4 (40.0)	9 (90.0)	0.057[c]
		CC	6 (60.0)	1 (10.0)	
STX11	Dom A	AA+AG	6 (60.0)	10 (100.0)	0.087[c]
rs7764017 A/G		GG	4 (40.0)	0 (0.0)	
	Rec A	GG+AG	7 (70.0)	4 (40.0)	0.370[b]
		AA	3 (30.0)	6 (60.0)	
STXBP2	Dom A	AA+AG	5 (50.0)	7 (70.0)	0.650[b]
rs6791 A/G		GG	5 (50.0)	3 (30.0)	
	Rec A	GG+AG	8 (80.0)	9 (90.0)	1.000[b]
		AA	2 (20.0)	1 (10.0)	
STXBP2	Dom A	AA+AG	8 (80.0)	5 (50.0)	0.214[b]
rs2303115 A/G		GG	2 (20.0)	5 (50.0)	
	Rec A	GG+AG	6 (60.0)	9 (90.0)	0.350[b]
		AA	4 (40.0)	1 (10.0)	

[a] SNP identifier based on NCBI dbSNP; Dom: Dominant model; Rec: Recessive model; Genotypes expressed as number (%); N/A: not applicable; [b] Pearson chi-square. [c] Fisher's exact test. After the Bonferroni test, $p < 0.002$ = significant.

influences the splicing process of syntaxin mRNA and affects its translation.

Taken together, this preliminary study found 2 SNPs that are associated with sHLH/MAS, are present in PIMS-TS patients, and may have an etiologic role in the syndrome. Additional studies with a larger number of PIMS-TS cases and pediatric controls without PIMS-TS are necessary for independent validation of these findings and to determine these SNPs' potential as biomarkers for PIMS-TS.

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