Research Article

Relation of Macrophage Migration Inhibitory Factor to Pulmonary Hemodynamics and Vascular Structure and Carbamyl-Phosphate Synthetase I Genetic Variations in Pediatric Patients with Congenital Cardiac Shunts

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Macrophage migration inhibitory factor (MIF) plays an important pathophysiological role in pulmonary hypertension (PHT). Previously, we demonstrated that serum MIF is increased in pediatric PHT associated with congenital heart disease (CHD). In the present study, we determined possible associations between MIF levels, hemodynamic and histological parameters, and mitochondrial carbamyl-phosphate synthetase I (CPSI) T1405N polymorphism in a similar population. The asparagine 1405 variant (related to A alleles in the C-to-A transversion) has been shown to be advantageous in pediatric PHT compared to the threonine 1405 variant (C alleles). Forty-one patients were enrolled (aged 2-36 months) and subsequently divided into 2 groups after diagnostic evaluation: the high-pulmonary blood flow (high PBF) group (pulmonary-to-systemic blood flow ratio 2.58 (2.21-3.01), geometric mean with 95% CI) and the high-pulmonary vascular resistance (high PVR) group (pulmonary vascular resistance 6.12 (4.78-7.89) Wood units x m²). Serum MIF was measured using a chemiluminescence assay. The CPSI polymorphism was analyzed by polymerase chain reaction followed by high-resolution melting analysis. Medial hypertrophy of pulmonary arteries was assessed by the histological examination of biopsy specimens. Serum MIF was measured using a chemiluminescence assay. The CPSI polymorphism was analyzed by polymerase chain reaction followed by high-resolution melting analysis. Medial hypertrophy of pulmonary arteries was assessed by the histological examination of biopsy specimens. Serum MIF was measured in patients compared to controls (p = 0.045), particularly in the high-PVR group (n = 16) (p = 0.022) and in subjects with the AC CPSI T1405N genotype (n = 16) compared to those with the CC genotype (n = 25) (p = 0.017). Patients with high-PVR/AC-genotype profile (n = 9) had the highest MIF levels (p = 0.030 compared with the high-PBF/CC-genotype subgroup, n = 18). In high-PVR/AC-genotype patients, the medial wall thickness of intra-acinar pulmonary arteries was directly related to MIF levels (p = 0.033). There were no patients with the relatively rare AA genotype in the study population. Thus, in the advantageous scenario of the asparagine 1405 variant (AC heterozygosity in this study), heightened pulmonary vascular resistance in CHD-PHT is associated with medial hypertrophy of pulmonary arteries where MIF chemokine very likely plays a biological role.

1. Introduction

Inflammation and immunity play a central role in the pathogenesis of pulmonary vascular disorders. A number of inflammatory mediators, such as cytokines, chemokines and their receptors, adhesion molecules and their ligands, components of the complement system, and proteases, were shown to play a pivotal role in the initiation and progression of pulmonary vascular abnormalities [1]. In pediatric pulmonary hypertension, mediators of inflammation were
investigated in the human clinical setting and experimental models [2–4]. Studies have focused on specific etiologies of pulmonary arterial hypertension, as for the case of congenital heart disease [5, 6].

For chemokines, a role for the macrophage migration inhibitory factor (MIF) in clinical and experimental pulmonary vascular disease was demonstrated [1]. MIF is a noncanonical ligand of CXC chemokine receptors, leading to the activation of several signaling pathways that results in the inhibition of endothelial cell apoptosis and induction of smooth muscle cell proliferation. It is also associated with the exaggerated recruitment of peripheral blood mononuclear cells and proinflammatory endothelial cell behavior [1, 7–9]. In a recent study of ours involving pediatric patients with congenital cardiac communications, heightened serum MIF was observed in a specific subgroup of subjects with elevated pulmonary vascular resistance [10]. This finding suggests the possible involvement of this chemokine in the early development of pulmonary vasculopathy in congenital heart disease.

Another interesting observation in the pediatric population is the association of a functional polymorphism of the mitochondrial enzyme carbamyl-phosphate synthetase I (CPSI) with the development of pulmonary hypertension. A C-to-A nucleotide transversion in the exon 36 of the gene that encodes CPSI results in the substitution of threonine for a C allele (asparagine 1405 variant) were less likely to develop neonatal pulmonary hypertension [12]. Furthermore, the CPSI T1405N polymorphism was shown to be associated with postoperative pulmonary hypertension in children undergoing repair for congenital cardiac anomalies [12–14].

Because of the functional role of the CPSI T1405N polymorphism in the pediatric population, particularly with regard to pulmonary circulation, we hypothesized that circulating levels of MIF may correlate with CPSI genotypes in young patients with congenital cardiac communications and altered pulmonary hemodynamics. Therefore, we examined possible correlations between serum MIF levels, patterns of clinical and hemodynamic presentation, and T1405N genotypes in these patients. A morphometric analysis of pulmonary vessels was performed to demonstrate the presence of arterial remodeling. We also examined the possible influence of Down syndrome.

2. Methods

2.1. Study Population. The study population consisted of pediatric patients (up to 3 years of age) who were admitted to the Heart Institute (InCor), University of São Paulo School of Medicine, Brazil, for repair of congenital cardiac communications. The presence of relatively simple cardiac anomalies with unrestricted communication between the cardiac chambers and/or the great arteries (in the absence of pulmonary stenosis) was required for inclusion. Therefore, all patients had clinical features suggestive of elevated pulmonary arterial pressure. Patients with complex anomalies, including those with univentricular physiology, were not included. Neonates, patients under intensive care, and those with relevant comorbidities or extracardiac syndromes other than Down syndrome were not included. Having met the inclusion criteria, with an informed consent signed by family members, patients entered the study consecutively. After inclusion, patients were divided into 2 groups. Those with clinical features suggestive of pulmonary overcirculation (congestive heart failure with failure to thrive and an enlarged heart with pulmonary congestion) were considered for surgical treatment with no need for cardiac catheterization. Patients not presenting these features were assumed to have inappropriately elevated pulmonary vascular resistance and were assigned to cardiac catheterization to confirm this preliminary assumption. Mildly decreased peripheral oxygen saturation was observed in some of these patients, compatible with bidirectional shunting across the cardiac septal defects. Pediatric controls were recruited from the same geographic area and had the same distribution of ethnic backgrounds as the patients in the study. The control group contained subjects with Down syndrome as well, but with no signs of heart disease or pulmonary hypertension. They were screened at the Heart Institute. The study protocol was approved by the Institutional Scientific and Ethics Committee (CAPPesq no. 0502.11).

2.2. Echocardiography and Cardiac Catheterization. In addition to providing detailed anatomical data, transthoracic echocardiography was used for estimating the pulmonary-to-systemic blood flow ratio according to a previously reported methodology [15, 16]. The direction of flow across the cardiac defects and the size of the left cardiac chambers were also considered when assigning patients to one of the clinical groups. Left-to-right shunting (exclusive or predominant) in the presence of an enlarged left heart was considered as indicative of increased pulmonary blood flow. Bidirectional shunting with a relatively small-sized left heart was suggestive of heightened pulmonary vascular resistance. Cardiac catheterization was considered only for patients who were suspected to have higher levels of pulmonary vascular resistance based on clinical data and echocardiographic evaluation. Catheterization was performed under general anesthesia and mechanical ventilation. Pulmonary and systemic blood flow were determined using the Fick principle and used for the assessment of pulmonary and systemic vascular resistance [17]. The parameters were determined at baseline and during nitric oxide inhalation (40 ppm, 10 min).

2.3. Serum Levels of MIF. Peripheral venous blood was collected from patients and controls, and serum samples were obtained and stored at -80°C until use. After protein immobilization on nitrocellulose membranes, processing (immunoblotting) was performed as previously described [18], and
MIF was detected using a human cytokine detection kit (R&D Systems, Minneapolis, MN, USA). Chemiluminescence was used for the semiquantitative assessment of MIF level in serum. The average signal of a pair of duplicate spots representing the MIF protein was normalized using internal controls, and the results are expressed as units of pixel intensity (upi).

2.4. CPSI T1405N Polymorphism. Genomic DNA from patients was extracted from peripheral blood using the salting-out procedure. Genotypes for the CPSI T1405N polymorphism were detected by polymerase chain reaction (PCR) followed by high-resolution melting (HRM) analysis with the Rotor Gene 6000® instrument (Qiagen, Courtaboeuf, France). The amplification of the fragments was performed using the following primers: sense 5′- AGCCAC ATCAGACTGGCTCA -3′ and antisense 5′- CTTCTTGA GACGGCCATGC -3′ (68 pairs base). A 35-cycle PCR was performed with an annealing temperature of 53.4°C. PCR was performed using a 10 μL of reaction solution with the addition of fluorescent DNA-intercalating SYTO9® (1.5 μM; Invitrogen, Carlsbad, CA, USA). In the HRM phase, the Rotor Gene 6000® measured the fluorescence for each 0.1°C temperature increase in the range of 68-80°C. The melting curve was generated by the decrease in fluorescence with the increase in temperature, and for the analysis, nucleotide changes result in different curve patterns.

2.5. Assessment of Medial Hypertrophy of Pulmonary Arteries. Lung biopsy specimens were collected in selected cases during cardiac surgery for the repair of heart lesions. Only patients with clinical features suggestive of inappropriately elevated pulmonary vascular resistance were considered for lung biopsy. The surgeon was asked to determine whether the procedure would be low risk on an individual basis. Specimens were collected with the airways distended, fixed in formalin, and subjected to histological processing. Four-micrometer-thick sections were obtained and subjected to hematoxylin-eosin stain and Miller micrometer. The wall thickness was measured from the external to internal elastic lamina and across the shorter axis of the vessel. Wall thickness was measured from the external to internal elastic lamina and computed as a percentage of the external diameter as follows:

\[
\text{Percent wall thickness} = \frac{2 \times \text{wall thickness}}{\text{external diameter}} \times 100. \tag{1}
\]

In each patient, a mean value was calculated for each artery category, and the final result was obtained after a Z-score transformation. For this purpose, normal values for age were obtained from reference [19]. In average, 4 precapillary and 12 intra-acinar arteries were examined per patient.

2.6. Statistical Analysis. Results involving numeric variables are presented as geometric means with 95% confidence intervals (95% CI) or estimated marginal (adjusted) means with standard error (SE). For the categorical variables, data are presented as the number of patients and percentages or proportions. For most of the numeric variables corresponding to demographic and diagnostic findings, a normal distribution was not observed. Therefore, the differences between two groups were analyzed using the Mann-Whitney test. For categorical variables, the differences were analyzed using the chi-square family of tests. In all groups and subgroups, the MIF concentration distribution was sufficiently close to the normal distribution. Initially, the difference between patients and controls was tested using Student’s t-test. However, MIF levels were influenced by age (linear regression analysis with calculation of Pearson’s coefficient). For this reason, all subsequent analyses of MIF concentrations involving comparisons between groups were performed using the general linear model (analysis of covariance), including the age as a covariate, and the results are expressed as the adjusted means with SE. The analysis of covariance and regression analysis were used to test for possible associations between MIF levels and medial hypertrophy of pulmonary arteries and hemodynamic parameters. For all statistical procedures, 0.05 was adopted as the significance level. All tests were performed using the IBM SPSS statistical software (version 25, Armonk, NY, USA).

3. Results

Forty-one patients were enrolled, with ages ranging from 2 to 36 months. For the entire cohort, pulmonary-to-systemic blood flow ratio (echocardiography) was 2.29 (1.99-2.64) (geometric mean with 95% CI), and peripheral oxygen saturation was 94% (93%-96%). Twenty-seven patients (65.9%) had Down syndrome. Cardiac catheterization was considered unnecessary (high-pulmonary blood flow (PBF) group, n = 25) had a higher pulmonary-to-systemic blood flow ratio than did the high-PVR group (2.58 (2.21-3.01) and 1.88 (1.43-2.48), respectively, p = 0.024). Furthermore, they were younger (respective ages, 8.5 (6.5-11.1) mos. and 15.1 (10.8-21.2) mos., p = 0.009) and had higher peripheral oxygen saturation (96% (94%-97%) and 93% (91%-95%), respectively, p = 0.018). Thus, high-PBF and high-PVR groups were clearly distinct on the basis of clinical and hemodynamic parameters.

| Study population, n (%) (n = 41) | 25 (61.0) | 16 (39.0) | 0 (0.0) |
|----------------------------------|----------|----------|--------|

*According to reference [12]. CC denotes homozygosity for the C-encoded Thr 1405 variant; AA, homozygosity for the A-encoded Asn 1405 variant; and AC, heterozygosity for this polymorphism at position 1405.
Table 2: Demographic, diagnostic, and hemodynamic variables in groups defined according to the CPSI T1405N polymorphism.

| Variable                               | CC (n = 25) | AC (n = 16) | P     |
|----------------------------------------|------------|------------|-------|
| Age, months                            | 9.9 (7.2-13.6) | 10.4 (7.2-15.0) | 0.726† |
| Gender, M:F                            | 6:19       | 7:9        | 0.326‡ |
| Down syndrome, n (%)                   | 16 (64.0)  | 11 (68.8)  | 0.754‡ |
| Peripheral oxygen saturation (%)       | 95 (94-97) | 93 (91-95) | 0.089† |
| Pulmonary-to-systemic blood flow ratio§| 2.43 (2.01-2.94) | 2.10 (1.65-2.66) | 0.341† |
| High-PBF patients : high-PVR patients  | 18:7       | 7:9        | 0.070‡ |

A: pretricuspid defects, B: posttricuspid defects except for atrioventricular septal defects, C: atrioventricular septal defects, D: conotruncal defects.

CPSI: carbamyl-phosphate synthetase I.

†Numeric variables are expressed as geometric mean (95% CI).
††Mann-Whitney test; †‡Chi-square test; †¶likelihood ratio; †§estimated by echocardiography.
*A: pretricuspid defects, B: posttricuspid defects except for atrioventricular septal defects, C: atrioventricular septal defects, D: conotruncal defects.

For the CPSI T1405N genotype distribution, only the genotypes CC and AC were present in the cohort (Table 1). The distribution of genotypes was skewed from the expected distribution within the general population (p = 0.010 compared with the Hardy-Weinberg equilibrium) [12]. However, it was not significantly different from the distributions reported in two studies involving children with postcardiac surgery pulmonary hypertension (p = 0.065 and p = 0.082 compared with the data reported in references [13] and [14], respectively).

Demographic, diagnostic, and hemodynamic variables in groups defined according to the CPSI T1405N genotypes present in the study population are depicted in Table 2. Lower levels of pulmonary arterial pressure and pulmonary vascular resistance were observed in patients with the AC genotype compared to those with the CC genotype. However, the differences must be interpreted with caution, as both variables are known to correlate directly with the patient's age. In the specific subgroup of patients subjected to cardiac catheterization (n = 16), subjects with the AC T1405N genotype were younger than those with the CC genotype (10.9 (7.1-16.6) and 21.1 (11.7-37.9) months of age, respectively, p = 0.051). No other differences were observed. In particular, there was no association of CPSI T1405N genotypes with the presence of Down syndrome.

Serum MIF levels were influenced by the age in patients and pediatric controls (Figure 1). Levels were elevated in patients compared to controls even after adjustment for age. Figure 1 shows that the highest MIF levels were detected in young patients. In the figure, no controls are observed above the level of 10000 upi. MIF levels were not influenced by the presence or absence of Down syndrome (p = 0.180 for the entire study population, n = 66 and p = 0.581 for the patient population, n = 41).

The analysis of serum MIF levels in patient groups according to the clinical and hemodynamic profiles and CPSI T1405N genotypes is shown in Figure 2. The highest levels were observed in the high-PVR group and in patients with the AC genotype compared to those with the CC genotype and controls. Figure 2 also shows that the pattern of MIF concentrations in controls and patient groups according to the CPSI genotypes did not seem to be influenced by the presence or absence of Down syndrome, acknowledging that there was a restricted power for subgroup analyses.

Figure 3 shows the levels of the MIF chemokine when the two factors (clinical/hemodynamic profile and CPSI T1405N genotypes) were analyzed in combination. Because of the small number of cases per subgroup, only one difference was tested. High-PVR/AC-genotype subjects had higher levels of MIF in the serum than those in the high-PBF/CC-genotype subgroup.

Lung biopsy material was available for the analysis of arterial wall thickness in 10 of 16 patients of the high-PVR group. Medial hypertrophy was present in arteries accompanying the terminal bronchioles, respiratory bronchioles, and alveolar ducts (Figure 4). The respective Z-scores were 7.28 (3.96-13.39), 3.53 (1.28-9.68), and 2.73 (0.91-8.19). A positive association was observed between serum MIF levels and the magnitude of medial hypertrophy of pulmonary arteries in 6 patients with the AC CPSI T1405N genotype (they were, in fact, high-PVR/AC-genotype subjects).
However, the association was significant only for the arteries accompanying respiratory bronchioles and alveolar ducts (Figure 5). There was no similar trend in the high-PVR/CC-genotype patients (n = 4, data not shown). Pulmonary vascular occlusive lesions were not observed in any patients of the high-PVR/AC-genotype subgroup. Lesions were observed in 2 of 4 individuals in the high-PVR/CC genotype subgroup. Consistent with the association between MIF levels and pulmonary arteriolar hypertrophy was the observation that serum MIF correlated with pulmonary vascular response to inhaled nitric oxide. Again, this association was observed only in subjects with the AC genotype (Figure 5). Patients with high levels of MIF had a greater response, that is, low levels of PVR during nitric oxide inhalation.

4. Discussion

In this study, the elevated serum levels of MIF chemokine were observed in patients with congenital cardiac shunts and altered pulmonary hemodynamics compared to pediatric controls. The highest MIF levels were observed in younger patients, particularly those with a high-PVR presentation and were associated with the AC CPSI T1405N genotype. MIF was shown to stimulate pulmonary artery smooth muscle cell proliferation in hypoxic pulmonary hypertensive rats, an effect that involves ERK 1/2 and JNK phosphorylation [7]. In isolated pulmonary artery rings, MIF enhanced constriction in response to hypoxia and potentiated constrictions that were preevoked by agonists through the PKC, p38, and ERK 1/2 signaling pathways [20]. In another study,
increased MIF in pulmonary arteries was associated with cyclin D1 upregulation via the ERK signaling pathway in pulmonary hypertensive broilers [21]. Considering that MIF is very likely involved in pulmonary vascular smooth muscle cell growth in human disease, the closeness between its levels and the CPSI T1405N polymorphism observed in this study suggests the existence of a phenotype in which some patients with heightened pulmonary vascular resistance (i.e., those with the asparagine 1405 variant) remain relatively protected from developing the most severe forms of the disease while sustaining a medial hypertrophy pattern of vascular remodeling. The association we observed between circulating MIF levels and pulmonary arterial wall thickness in high-PVR/AC-genotype patients is consistent with this hypothesis, recognizing the limitation of having a small number of lung biopsies for analysis in this study. Furthermore, heightened serum MIF correlated with the magnitude of pulmonary vasodilation in response to nitric oxide administration during cardiac catheterization.

We have only preliminary hypotheses about the possible relationships between MIF, the CPSI T1405N polymorphism, and pulmonary vasoreactivity in pediatric subjects. In the studies by Summar et al. [13] and Canter et al. [14], it was suggested that the postoperative elevation of pulmonary arterial pressure (i.e., patients with the CC CPSI genotype compared to those with the AA genotype) was due to increased pulmonary vascular tone. However, there were no specific data indicating an increased pulmonary vascular response to stimuli frequently present in the early postoperative period, such as changes in alveolar oxygen tension, pH, and pCO2. In the study by Pearson and coworkers involving neonates with pulmonary hypertension [12], vasoreactivity was not tested either. Obviously, patients in these studies had elevated pulmonary artery pressure, but it is not known whether they were vasoreactive. It is important to consider that pulmonary vasoreactivity and the pulmonary vascular response to vasodilators, for example, inhaled nitric oxide during preoperative cardiac

Figure 2: Serum MIF levels in patient groups according to the clinical/hemodynamic presentation (a) and carbamyl-phosphate synthetase I (CPSI) T1405N polymorphism (b, c, d). Despite a relatively small number of subjects for the subgroup analysis, specific data for the subgroups with (d) and without Down syndrome (c) are presented. In all graphs, the bars represent the estimated marginal means with SE after adjustment for age. Means not sharing the same letter were significantly different by post hoc multiple comparison tests. PBF and PVR, pulmonary blood flow and pulmonary vascular resistance, respectively. CC and AC, CPSI T1405N genotypes present in the study population.
Figure 3: Serum MIF levels in patient subgroups according to the clinical/hemodynamic profile and CPSI T1405N genotypes analyzed in combination. All results are presented as estimated marginal means with SE after adjustment for age. PBF and PVR, pulmonary blood flow and pulmonary vascular resistance, respectively. CC and AC, CPSI T1405N genotypes present in the study population.

![Graph showing MIF levels](image1)

**Figure 3: Serum MIF levels in patient subgroups according to the clinical/hemodynamic profile and CPSI T1405N genotypes analyzed in combination.**

(a) High PBF/CC gen. (n = 18)
(b) High PBF/AC gen. (n = 7)
(c) High PVR/CC gen. (n = 7)
(d) High PVR/AC gen. (n = 9)

![Graph showing MIF levels](image2)

**Figure 3: Serum MIF levels in patient subgroups according to the clinical/hemodynamic profile and CPSI T1405N genotypes analyzed in combination.**

(a) High PBF/CC gen. (n = 18)
(b) High PVR/AC gen. (n = 9)

*p = 0.030

Figure 4: Photomicrographs of arteries accompanying terminal bronchioles showing severe hypertrophy of the medial layer and absence of intimal lesions. Miller’s elastic stain, objective magnification 20x for both panels. (a) 9-month-old patient with Down syndrome, with atrioventricular septal defect. Peripheral oxygen saturation was 88%, and pulmonary vascular resistance was 5.3 Wood units × m² decreasing to 1.8 units × m² during nitric oxide inhalation. Z-score transformed pulmonary artery wall thickness was 8.1, and serum MIF level was 12305 upi by chemiluminescence. (b) 4-month-old patient, with ventricular septal defect. Peripheral oxygen saturation was 95%, and pulmonary vascular resistance was 4.0 Wood units × m² decreasing to 1.3 units × m² while on nitric oxide. Pulmonary artery wall thickness was 21.2 (Z-score), and serum MIF level corresponded to 11014 upi. Both patients had AC CPSI T1405N genotype.

![Photomicrograph](image3)

**Figure 4: Photomicrographs of arteries accompanying terminal bronchioles showing severe hypertrophy of the medial layer and absence of intimal lesions.**

(a) 9-month-old patient with Down syndrome, with atrioventricular septal defect. Peripheral oxygen saturation was 88%, and pulmonary vascular resistance was 5.3 Wood units × m² decreasing to 1.8 units × m² during nitric oxide inhalation. Z-score transformed pulmonary artery wall thickness was 8.1, and serum MIF level was 12305 upi by chemiluminescence. (b) 4-month-old patient, with ventricular septal defect. Peripheral oxygen saturation was 95%, and pulmonary vascular resistance was 4.0 Wood units × m² decreasing to 1.3 units × m² while on nitric oxide. Pulmonary artery wall thickness was 21.2 (Z-score), and serum MIF level corresponded to 11014 upi. Both patients had AC CPSI T1405N genotype.

![Photomicrograph](image4)
catheterization in congenital heart disease, are not necessarily related phenomena. Furthermore, preoperative and postoperative contexts are considerably different and not comparable to the neonatal condition. The present study corresponds to preoperative observations. The data presented in Table 2 show that patients of both groups (CC and AC genotypes) had pulmonary vasodilation in response to inhaled nitric oxide. In particular, patients with the AC genotype had a more pronounced response (\( y = 0.92 - 6.98E^{-5}x \) \( r = -0.90 \), \( p = 0.014 \)). No significant correlation was seen in patients with the CC genotype.

In vascular and inflammatory processes, the relation of cytokine expression to arginine availability and nitric oxide production cannot be easily anticipated. As a rule of thumb, L-arginine and nitric oxide have important anti-inflammatory, antithrombotic, and antiproliferative properties [22]. However, in addition to being a substrate for nitric oxide production by nitric oxide synthetases, L-arginine can be metabolized to urea and L-ornithine by arginases. In fact, arginases and nitric oxide synthetases effectively compete for L-arginine. The product of arginase activity, L-ornithine, is a precursor for the production of polyamines and proline, which controls cell proliferation and collagen synthesis, respectively [23]. In experimental models, increased arginase I expression was shown to be associated with increased aortic smooth muscle cell and endothelial cell proliferation [24, 25]. In pediatric pulmonary hypertension, the CPSI T1405N polymorphism was studied with a focus on arginine and nitric oxide metabolism [12–14], and not on the activation of arginase pathway and subsequent events. For nitric oxide itself, patients with more effective production (i.e., those with the asparagine 1405 CPSI variant) would be expected to be relatively protected from inflammatory...
insults. However, there have been conflicting data about the role of nitric oxide in the regulation of cytokine expression. Some studies indicate a proinflammatory role of nitric oxide [26, 27]. The immunoregulatory effects of nitric oxide seem to be determined by its levels, although the type of responder cell, the cytokine, and the stimulus also play a role in different experiments [28]. Nitric oxide was shown to induce MIF mRNA and protein release in human fetal membranes [29]. Additional observations may have implications for the results of the present study. For example, MIF can elicit the Th2-type immune response [30], shown to be involved in experimental pulmonary vascular remodeling [31], and arginase I is strongly induced by Th2 cytokines [32, 33].

In conclusion, data from the present study indicate the existence of a subset of young patients with congenital cardiac shunts in whom heightened circulating levels of MIF chemokine are associated with medial hypertrophy of small pulmonary arteries and elevated pulmonary vascular resistance. Some of our findings suggest that pulmonary vascular resistance is dynamic rather than fixed in these patients. In our study population, this phenotype was associated with AC heterozygosity for the CPSI T1405N polymorphism, consistent with previous observations that the presence of at least one copy of the A allele is protective against aggressive forms of pulmonary vascular disease. Our data do not provide evidence for any causal relationship between CPSI T1405N polymorphism and cytokine (MIF) expression. However, in theory, the existence of interrelated biological pathways is possible. Further studies are warranted for a better understanding of these interrelationships in the pediatric population.

Data Availability

The data used to support the conclusions of the present study correspond to the projects FAPESP #2011/09341-0 and 2015/21587-5 and are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] M. Rabinovitch, C. Guignabert, M. Humbert, and M. R. Nicolls, “Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension,” Circulation Research, vol. 115, no. 1, pp. 165–175, 2014.

[2] M. Duncan, B. D. Wagner, K. Murray et al., “Circulating cytokines and growth factors in pediatric pulmonary hypertension,” Mediators of Inflammation, vol. 2012, Article ID 143428, 7 pages, 2012.

[3] S. Fleck, G. Bautista, S. M. Keating et al., “Fetal production of growth factors and inflammatory mediators predicts pulmonary hypertension in congenital diaphragmatic hernia,” Pediatric Research, vol. 74, no. 3, pp. 290–298, 2013.

[4] J. Costa, Y. Zhu, T. Cox, P. Fawcett, T. Shaffer, and D. Alapati, “Inflammatory response of pulmonary artery smooth muscle cells exposed to oxidative and biophysical stress,” Inflammation, vol. 41, no. 4, pp. 1250–1258, 2018.

[5] R. F. A. Pinto, M. de Lourdes Higuichi, and V. D. Aiello, “Decreased numbers of T-lymphocytes and predominance of recently recruited macrophages in the walls of peripheral pulmonary arteries from 26 patients with pulmonary hypertension secondary to congenital cardiac shunts,” Cardiovascular Pathology, vol. 13, no. 5, pp. 268–275, 2004.

[6] K. Sungprem, A. Khongphathanayothin, P. Kiettisanpipop, P. Chotivitayatarkorn, Y. Poovorawan, and P. Lertsapcharoen, “Serum level of soluble intercellular adhesion molecule-1 correlates with pulmonary arterial pressure in children with congenital heart disease,” Pediatric Cardiology, vol. 30, no. 4, pp. 472–476, 2009.

[7] B. Zhang, M. Shen, M. Xu et al., “Role of macrophage migration inhibitory factor in the proliferation of smooth muscle cell in pulmonary hypertension,” Mediators of Inflammation, vol. 2012, Article ID 840737, 10 pages, 2012.

[8] L. Tu, A. Huertas, M. Le Hiress et al., “MIF/CD74-dependent interleukin-6 and monocyte chemoattractant protein-1 secretion by pulmonary endothelial cells in idiopathic pulmonary hypertension,” American Journal of Respiratory and Critical Care Medicine, vol. 187, article A1739, 2013.

[9] M. Le Hiress, L. Tu, N. Ricard et al., “Proinflammatory signature of the dysfunctional endothelium in pulmonary hypertension. Role of the macrophage migration inhibitory factor/CD74 complex,” American Journal of Respiratory and Critical Care Medicine, vol. 192, no. 8, pp. 983–997, 2015.

[10] L. Zorzanelli, N. Y. Maeda, M. M. Clavé, V. D. Aiello, M. Rabinovitch, and A. A. Lopes, “Serum cytokines in young pediatric patients with congenital cardiac shunts and altered pulmonary hemodynamics,” Mediators of Inflammation, vol. 2016, Article ID 7672048, 9 pages, 2016.

[11] M. L. Summar, N. Scott, E. Cummings, H. Hutcheson, S. Dawling, and B. Christman, “Analysis of 200 patients undergoing bone marrow transplant shows allelic disequilibrium between drug related toxicity and a common exonic polymorphism in the CPSI gene and correlates with disruption of urea cycle intermediates,” American Journal of Human Genetics, vol. 65, 1999.

[12] D. L. L. Pearson, S. Dawling, W. F. Walsh et al., “Neonatal pulmonary hypertension – urea-cycle intermediates, nitric oxide production, and carbamyl-phosphate synthetase function,” The New England Journal of Medicine, vol. 344, no. 24, pp. 1832–1838, 2001.

[13] M. L. Summar, I. Hall, B. Christman et al., “Environmentally determined genetic expression: clinical correlates with molecular variants of carbamyl-phosphate synthetase I,” Molecular Genetics and Metabolism, vol. 81, pp. 12–19, 2004.

[14] J. A. Canter, M. L. Summar, H. B. Smith et al., “Genetic variation in the mitochondrial enzyme carbamyl-phosphate synthetase I predisposes children to increased pulmonary
artery pressure following surgical repair of congenital heart defects: a validated genetic association study," *Mitochondrion*, vol. 7, no. 3, pp. 204–210, 2007.

[15] T. H. Laird, S. A. Stayer, S. M. Rivenes et al., "Pulmonary-to-systemic blood flow ratio effects of sevoflurane, isoflurane, halothane, and fentanyl/midazolam with 100% oxygen in children with congenital heart disease," *Anesthesia & Analgesia*, vol. 95, no. 5, pp. 1200–1206, 2002.

[16] J. X. Liu, J. H. Wang, S. R. Yang et al., "Clinical utility of the ventricular septal defect diameter to aorta root diameter ratio to predict early childhood developmental defects or lung infections in patients with perimembranous ventricular septal defect," *Journal of Thoracic Disease*, vol. 5, no. 5, pp. 600–604, 2013.

[17] J. L. Wilkinson, "Haemodynamic calculations in the catheter laboratory," *Heart*, vol. 85, no. 1, pp. 113–120, 2001.

[18] T. Kiss, K. Kovacs, A. Komocsi et al., "Novel mechanisms of sildenafil in pulmonary hypertension involving cytokines/chemokines, MAP kinases and Akt," *PLoS One*, vol. 9, no. 8, article e104890, 2014.

[19] S. G. Haworth and A. A. Hislop, "Pulmonary vascular development: normal values of peripheral vascular structure," *The American Journal of Cardiology*, vol. 52, no. 5, pp. 578–583, 1983.

[20] B. Zhang, Y. Luo, M. L. Liu et al., "Macrophage migration inhibitory factor contributes to hypoxic pulmonary vasoconstriction in rats," *Microvascular Research*, vol. 83, no. 2, pp. 205–212, 2012.

[21] H. Li, Y. Wang, L. Chen et al., "The role of MIF, cyclinD1 and ERK in the development of pulmonary hypertension in broilers," *Avian Pathology*, vol. 46, no. 2, pp. 202–208, 2017.

[22] N. N. Huynh and J. Chin-Dusting, "Amino acids, arginase and nitric oxide in vascular health," *Clinical and Experimental Pharmacology & Physiology*, vol. 33, no. 1-2, pp. 1–8, 2006, Review.

[23] N. E. King, M. E. Rothenberg, and N. Zimmermann, "Arginine in asthma and lung inflammation," *The Journal of Nutrition*, vol. 134, no. 10, pp. 2830S–2836S, 2004.

[24] L. H. Wei, G. Wu, S. M. Morris, and L. J. Ignarro, "Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation," *Proceedings of the National Academy of Sciences*, vol. 98, no. 16, pp. 9260–9264, 2001.

[25] H. Li, C. J. Meiningier, K. A. Kelly, J. R. Hawker Jr, S. M. Morris Jr, and G. Wu, "Activities of arginase I and II are limiting for endothelial cell proliferation," *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, vol. 282, no. 1, pp. R64–R69, 2002.

[26] A. D. Ormerod, P. Copeland, I. Hay, A. Husain, and S. W. B. Ewen, "The inflammatory and cytotoxic effects of a nitric oxide releasing cream on normal skin," *Journal of Investigative Dermatology*, vol. 113, no. 3, pp. 392–397, 1999.

[27] L. J. Hofseth, S. Saito, S. P. Hussain et al., "Nitric oxide-induced cellular stress and p53 activation in chronic inflammation," *Proceedings of the National Academy of Sciences*, vol. 100, no. 1, pp. 143–148, 2003.

[28] Y. Kobayashi, "The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation," *Journal of Leukocyte Biology*, vol. 88, no. 6, pp. 1157–1162, 2010.

[29] A. Zicari, C. Ticconi, F. Ietta et al., "Macrophage migration inhibitory factor-nitric oxide interaction in human fetal membranes at term pregnancy," *Journal of the Society for Gynecologic Investigation*, vol. 13, no. 4, pp. 263–270, 2006.

[30] R. Das, J. E. Moss, E. Robinson et al., "Role of macrophage migration inhibitory factor in the Th2 immune response to epicutaneous sensitization," *Journal of Clinical Immunology*, vol. 31, no. 4, pp. 666–680, 2011.

[31] E. Daley, C. Emson, C. Guignabert et al., "Pulmonary arterial remodeling induced by a Th2 immune response," *The Journal of Experimental Medicine*, vol. 205, no. 2, pp. 361–372, 2008.

[32] C. A. Louis, V. Mody, W. L. Henry Jr, J. S. Reichner, and J. E. Albina, "Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages," *The American Journal of Physiology*, vol. 276, no. 1, Part 2, pp. R237–R242, 1999.

[33] M. Modolell, I. M. Corraliza, F. Link, G. Soler, and K. Eichmann, "Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines," *European Journal of Immunology*, vol. 25, no. 4, pp. 1101–1104, 1995.