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Analyses of IL-6, IL-10, IL-17A, INF-γ and TNF-α Cytokines in Diseases of Mucopolysaccharidosis Type I and Mucopolysaccharidoses Type VI

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Abstract Background: Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases, which are caused by the deficiency of one of the enzymes essential for the catabolism of Glycosaminoglycans. Seven different types of MPS have already been identified, and some are classified into subtypes depending on which enzyme is deficient as correlated to clinical findings. This article aims to evaluate some cytokines in order to understand the immunological relationship in plasma samples of patients with MPS type I (MPS I) and in leukocyte samples of patients with MPS type VI (MPS VI) by comparing healthy individual controls. Methods: 28 subjects (MPS I = 7, healthy controls = 7 and MPS VI = 7; healthy controls = 7) as well as dosages of IL-6, IL-10, IL-17A and TNF-α which were determined by ELISA. Results: In both comparisons that were performed, TNF-α levels were different among MPS I and MPS VI individuals when compared to healthy controls, suggesting that this cytokine could be a potential inflammatory marker for disease monitoring. On the other hand, IL-17A showed a negative correlation with IL-6 only in individuals with MPS VI, confirming the variability of multi-systemic symptoms of this disease. Conclusions: The results presented in this article show a relationship between human genetics and immunology, trying to monitor different stages of life of patients with these diseases, in order to improve their quality of life. The analysis of the cytokine profile is very important in the evaluation of the relation between diseases and their systemic effects.

Keywords: *Mucopolysaccharidoses, Lysosomal storage diseases, Inborn errors of metabolism*

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1. Introduction

Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases caused by deficiency of one of the enzymes that are essential for the catabolism of glycosaminoglycans (GAGs). The accumulation of non-degraded GAGs results in multiple organ dysfunction, resulting in distinct clinical manifestations, with an incidence of 1 in every 25,000 live births [1,2,3]. Seven different types of MPS have already been identified, and some are classified into subtypes depending on which enzyme is deficient as correlated to clinical findings. Although each MPS is clinically different, most patients usually have a normal developmental period, followed by a decline in physical and/or mental functions. MPS I (1: 35,700 live births) is divided into three subtypes based on the severity of symptoms: Hurler (severe), Hurler-Sheie (medium) and Sheie (mild). All three subtypes result from a lack or insufficiency of the alpha-L-iduronidase enzyme, whereas patients with MPS VI (1: 207,000-2,000,000 live births), are characterized by the deficiency of arylsulphatase B-enzymes enzyme, with a variable spectrum of symptoms [3].

The most common clinical manifestations of MPS I include hearing loss, hydrocephalus, cardiopathy, respiratory problems, hepatosplenomegaly, inguinal and umbilical hernia, multiplex dysostosis, limited joint mobility and cognitive impairment [4]. When they have MPS VI, patients present the first signs of the disease in childhood, characterized by hepatosplenomegaly, an infiltrated face and alterations in the spine. During their evolution, they present changes in various organs and tissues, including growth retardation; flexural contractures of fingers, knees or shoulders; and there are also cardiovascular changes, ocular changes, neurological changes, and obstructive sleep apnea syndrome [5,6,7].

Alterations in the inflammatory profile are linked to the development of several rare diseases; however, their relation to MPS are still not totally clear. Cytokine profile analysis is very important in relation to the evaluation of the diseases their systemic effects, and studies in animal models with MPS have reported high levels of various inflammatory cytokines [7,8]. Furthermore, since most of these patients report chronic pain, some studies that used anti-inflammatories as a treatment showed an improvement in mobility, tolerance to physical exercise and an increase in bone length [9,10]. According to Polgreen et al., in 2016, tumor necrosis factor alpha (TNF-α) is directly associated with pain and physical incapacity in MPS, thus an analysis of pro-inflammatory and anti-inflammatory cytokines is of great importance to understand this pathology better [11].

Cytokines are glycoproteins, which generally have low molecular weight [4] and play a central role in mediating and regulating immune responses [5]. They act as messengers among the cells of the immune, hematopoietic and neuroendocrine systems [6]. Cytokines are stored as preformed molecules that act especially by paracrine (in neighboring cells) and autocrine (in the producer cells themselves) mechanisms. Different types of cells secrete the same cytokine, and a single cytokine can act on several cell types, which is a phenomenon called pleiotropy [12]. Cytokines have been classified as pro-inflammatory or anti-inflammatory, according to the functions performed. In this work, we analyzed the anti-inflammatory cytokine interleukin-10 (IL-10), which may, among other functions, inhibit the production of pro-inflammatory cytokines [13]. Among the pro-inflammatory cytokines we used iInterleukin 6 (IL-6), Interleukin 17 (IL-17A), Interferon gamma (IFN-γ) and TNF-α. It is known that the production of anti-inflammatory cytokines is regulated by a variety of factors [13].

This article aims to evaluate some cytokines in order to understand the immunological relationship in plasma samples of patients with MPS I and in leukocyte samples from patients with MPS VI by comparing them to healthy controls.

2. Materials and Methods

2.1. Patients and Controls

This study was characterized by a prospective study. The diagnosis of MPS I and MPS VI in patients occurred at the Federal University of Rio Grande do Sul (Porto Alegre, RS, Brazil). Plasma and leukocyte analyses were performed on 28 individuals (MPS I = 7, healthy controls = 7 and MPS VI = 7, healthy controls = 7) as well as dosages of Interleukin-6 (IL-6, catalog number 900-K16), IL-10 (IL-10, catalog number 900-K21), IL-17A (IL17A, catalog number 900-K84), INF-γ (INF-γ catalog number 300-02) and tumoral necrosis factor-alpha (TNF-α, catalog number 900-K25) -Linked Immunosorbent Assay (ELISA) with commercial reagents (Peprotech Inc., NJ, USA).The Research Ethics Committee of the Methodist University Center - IPA (nº 1.290.503/2015), approved this study.

2.2. Assessment of Lysosomal Acid Hydrolases

Analyses of human lysosomal acid hydrolases were performed in plasma (MPS I) and leukocyte (MPS VI). Activities of enzyme α-iduronidase [14] were estimated by incubation with 4- methylumbelliferyl-α-L-iduronide (Sigma) as the substrate, under standard conditions (pH 2.8, at 37°C). Fluorescence was evaluated by spectrofluorometry (365 nm excitation and 450 nm emission). Data are expressed as nmol/h/mg of protein [15]. Activities of enzyme Arylsulphatase B were estimed by incubation with 4-Nitrocatecol-sulfate (Sigma) as substrate, under standart conditions (pH 6.0, at 37ºC) [16].

2.3. Systemic Cytokines

In order to evaluate the serum concentrations of IL-6, IL-10, IL-17A, INF-γ and TNF-α cytokines, a volume of 8 mL of blood was collected in tubes without any anti-coagulant so as to obtain serum, and the samples were separated by centrifugation for 10 minutes at 1048*g*, aliquoted and frozen at -20°C for further analysis. Cytokines were determined by enzyme-linked immunosorbent assay (ELISA), using specific kits (Mini ELISA Development Kit, Peprotech Inc., New Jersey, USA) and following the manufacturer's recommendations.

2.4. Statistical Analysis

The data were evaluated through the Student's t-test, followed by the Levene's test, which were used to compare results of the analysis of the plasma, serum and leukocytes groups (MPSI, MPSVI and CS), Correlations among variables were performed through the Spearman Test. Was used ROC curves evaluated the specificity and sensitivity of the determination of TNFα in the discrimination between mucopolysaccharidosis I and VI. The statistical significance value was of $p<0.05$. All the analyses were made with SPSS® 22.0 for Windows® (IBM Inc., Armonk, NY).

3. Results

The enzyme deficiency was determined by measuring the activity of α-iduronidase enzymes in plasma samples in the diagnosis of MPS I and arylsulphatase B in leukocytes in the diagnosis of MPS VI in a comparison with samples from normal individuals (Table 1).

Table 1. Enzymatic values of the MPSI (nmol/h/mL), MPSVI (nmol/h/mg protein) patients and Control Samples

	n	Patients	CS	p(value)
Plasma				
MPS I		-0.05 ± 0.49	9.48 ± 3.20	0.001*
α-iduronidase	**7**			
RI	**7**	**0.07 ± 0.17**	**10.74 ± 3.20**	
Leukocytes				
MPS VI		35.16 ± 20.39*	99.05 ± 12.47	0.019*
Arylsulphatase B	**7**			
RI	**7**	**25,5 ± 23,7**	**112.9 ± 19.4**	

Abbreviations: MPSI: Mucopolysaccharidose Tipo I; MPSVI: Mucopolysaccharidose Type VI; CS: Control Samples; RI: Reference Intervals. Data are expressed in mean ± SD. Statistical p-values were obtained with the Student t test, *p-value ≤ 0.05 is significant.

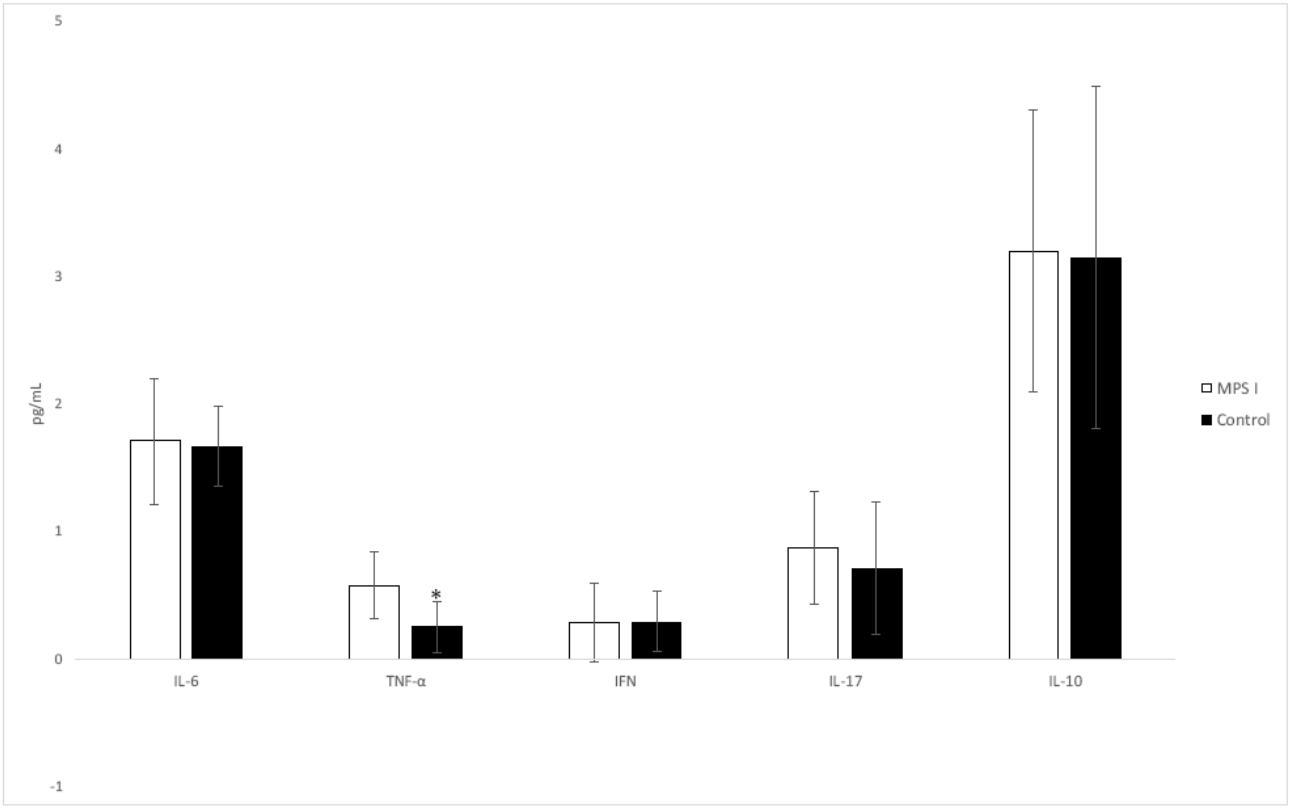

Figure 1. Inflammatory mediators: IL6, TNFα, IFN, IL17 and IL10. Immunohistochemical staining in the serum (pg/mL) of Mucopolysaccharidose Type I (MPSI) and Control samples (Controls). Data are expressed in mean ± SD. Statistical *p* values were obtained with the Student's t-test. * Statistical differences according to Levene's test ($p < 0.05$)

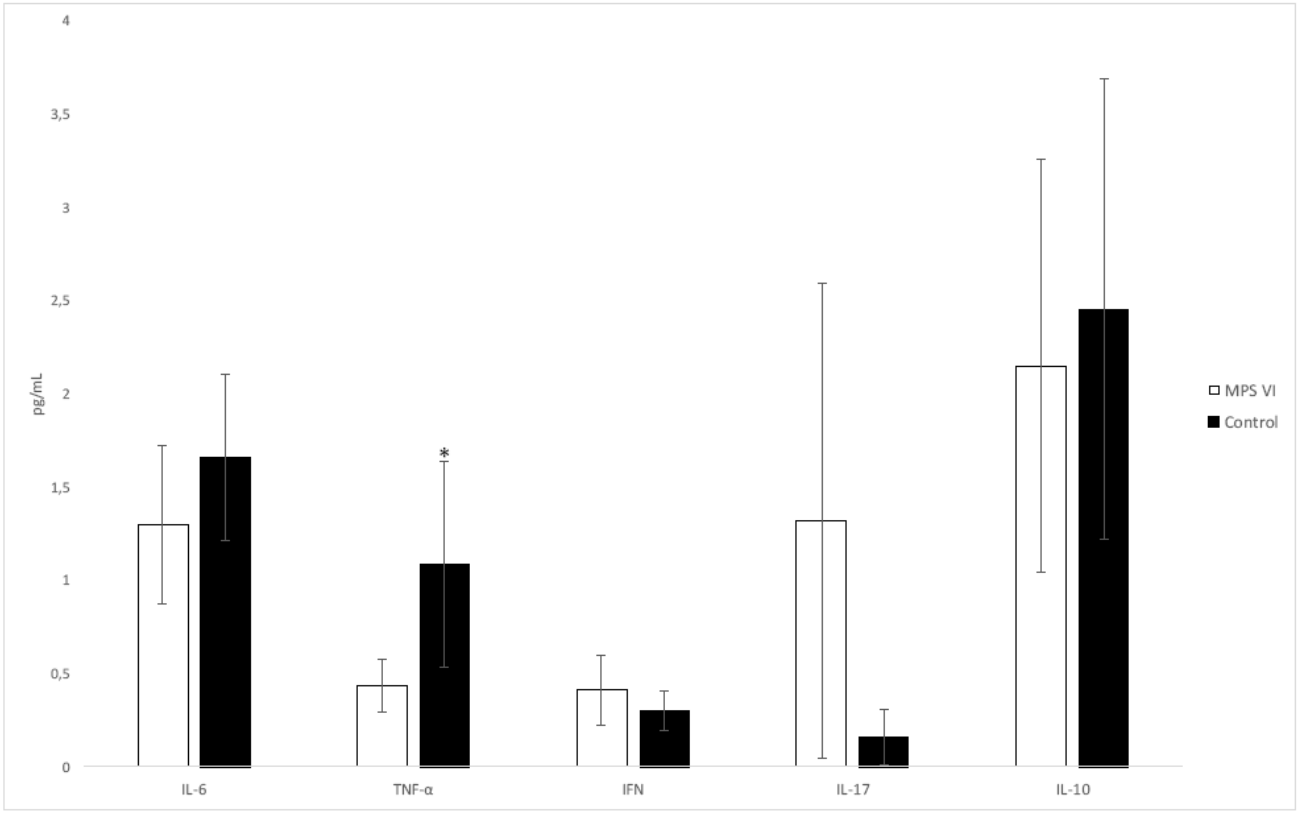

Figure 2. Inflammatory mediators: IL6, TNFα, IFN, IL17 and IL10. Immunohistochemical staining in the serum (pg/mL) of Mucopolysaccharidose Type VI (MPSVI) and Control samples (Controls). Data are expressed in mean ± SD. Statistical *p* values were obtained with the Student's t-test. * Statistical differences according to Levene's test ($p < 0.05$)

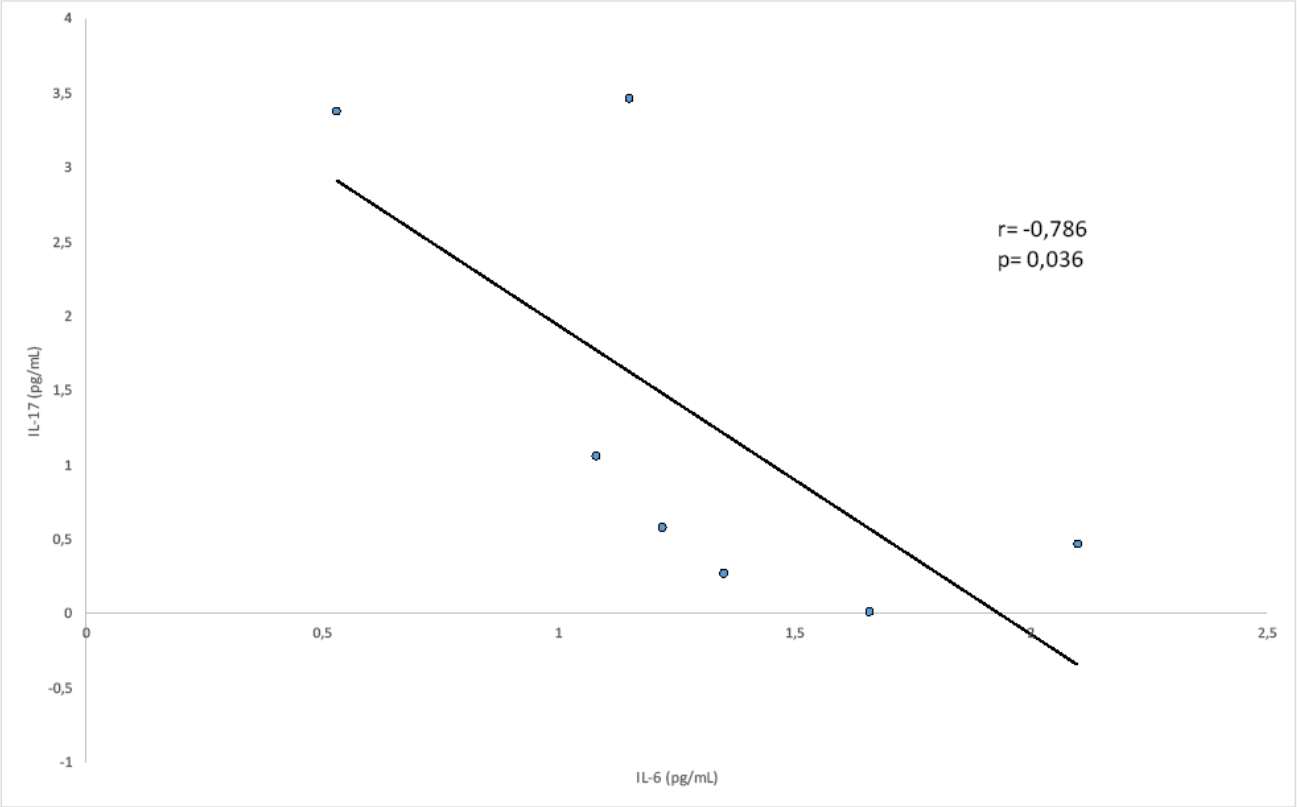

Figure 3. Serum correlation between MPSVI patients and Control samples: IL-6 e IL-17A (r= -0,786; p= 0,036). Correlations between variables were performed through Spearman Test

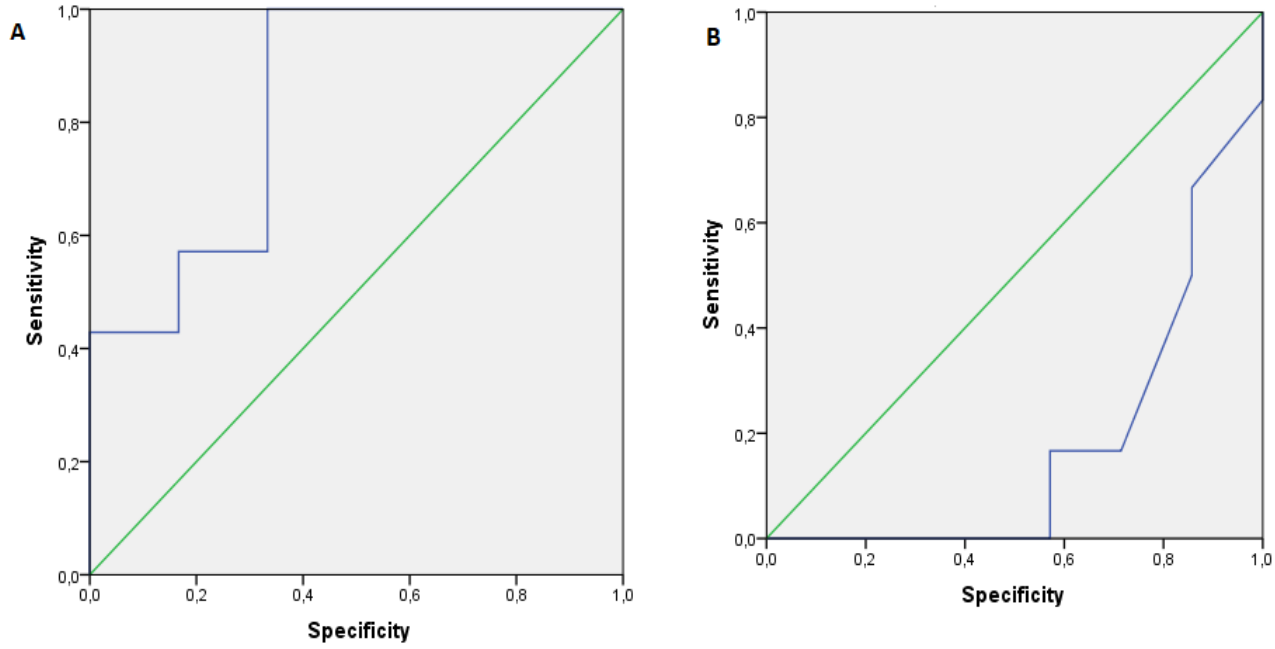

Figure 4. ROC curves evaluated the specificity and sensitivity of the determination of TNFα in the discrimination between MPS I (A) and MPS VI (B).

Regarding the levels of TNF-α in MPS I, the TNF-α values showed a significant increase (p=0,04) in patients with MPS I disease (0.5764 ± 0.288236 pg/mL) compared to Controls (0.2441 ± 0.22053 pg/mL) (Figure 1). However, in relation to TNF-α levels, the values of patients with MPS VI (0.4296 ± 0.17036 pg/mL) were lower (p = 0.02) when compared to controls (1.0854 ± 0.59787 pg/mL) (Figure 2).

As Figure 1 and Figure 2 show, we found no significant differences in IL-6, IL-10, IL-17A and IFN-γ in MPS I and MPS VI in comparison with the Controls.

After carrying out the general non-parametric correlation between the groups in MPS VI, we found a negative correlation between IL-6 and IL-17 (r= -0,786; p= 0,036) (Figure 3).

To emphasize the specificity and sensitivity of TNFα between MPS I and MPS VI, we used ROC curves and calculating the area under the curve with CI 95% (Figure 4). We obtained the area under the curve of 0.83 (p = 0.04) for MPS I and 0.18 (p = 0.05) for MPS VI.

4. Discussion

MPS are considered Lysosomal Storage Disorders (LSD), which are genetic diseases characterized by the accumulation of specific biological material, such as proteolypsid or metabolic intermediates present within the lysosomes. Lysosomes are important for the normal functioning of the immune system, as they are involved in controlling the expression of cell membrane receptors that are responsible for signal transduction, being essential for the processing and presentation of antigens, cytokine secretion and molecules, and in the process of phagocytosis. There is a strong correlation between abnormal lysosome functioning and a probable impaired immune response in individuals affected by MPS, since patients have recurrent respiratory infections. In the literature, there are few descriptions of isolated cases of immune evaluation in those affected by this disease.

In view of the above, and considering the complexity attributed to MPS because they are chronic, systemic and evolutionary, in which life expectancy is correlated with the severity of clinical symptoms, whose heart disease, repeated respiratory infections and upper airway obstructions are the main causes of death, we decided to evaluate in this work the inflammatory profile of patients diagnosed with two MPS [17].

In our study, 5 cytokines were evaluated and it was found that TNF-α is significantly different in MPS I and MPS VI when compared to controls. We considered that these differences in serum levels could be a reflection of important pathways in the pathophysiology of MPS I and MPS VI, contributing to the severity of the disease. In view of the biological effects of overlapping cytokines and their simultaneous production in peripheral blood mononuclear cells (PBMCs) in some pathological states, it becomes increasingly important to specifically determine their concentrations *in vitro* and *in vivo*. However, these assays may be influenced by different cytokines and/or inhibitory substances present in human body fluids, or produced from cells in culture. Furthermore, some pharmacological agents, which are possibly present in samples from patients with MPS, may also influence this bioassay [18,19]. To better characterize the sensitivity and specificity of our study, we performed the ROC curve for TNFα (Figure 4). That confirmed an excellent sensitivity in MPS I, as demonstrated in the results among patients, contrary to the results obtained in MPS VI that demonstrated a greater specificity with higher levels between the controls.

The mechanism for elevated levels of TNF-α in MPS is probably related to this excess of GAGs, which stimulates macrophages through type 4 receptor (TLR-4), causing multiple inflammatory effects. The exacerbated and persistent Th1 cytokine response may contribute to organ damage, leading to multiple organ failure and death, contributing to the high morbidity of MPS I [13,20]. In addition, Dermatan Sulfate (DS) is endotoxin-like, which triggers the proinflammatory response through TNF-α, promoting cellular apoptosis of the chondrocytes. Thus, DS accumulation causes tissue and cell injury [21,22]. Our results demonstrated an increase of TNF-α in MPS I, which corroborates with the results of Polgree et al. (2016), in which TNF-α was shown to be significantly elevated in children with the disease, associated with pain and physical incapacity, when compared with healthy children [11]. On the other hand, the results of TNF-α in MPS VI were expressed as a decrease, corraborating with a study [9], In which rats with MPS VI had their levels of TNF-α inhibited, showing an improvement of physical and skeletal functions when compared to rats with elevated levels of TNF-α.

Another pro-inflammatory cytokine, the major activator of macrophages that participates in both the innate immunity and also in acquired immunity, is IFN-γ. It was demonstrated in an animal model in a study with MPS IIIB, and values increased in patients as compared to controls [23]. Killedar et al (2010) suggest that T cells are activated *in vivo* during MPS IIIB progression by developing a predominantly Th1 cytokine profile, demonstrating increased IL-2 and INF-y [23]. However, our study found no significant difference in any of the types of MPS that were analyzed for this parameter, as opposed to these surveys.

IL-6 was surprising, since we did not obtain any significant difference in this parameter in the groups that were analyzed. The literature shows that IL-6 acts both in innate and also in adaptive immune response, and it is a pleiotropic cytokine that influences immune responses and inflammatory responses, being one of the major mediators of the acute phase of inflammation [24]. In the study by Donida et al. (2015), plasma levels of IL-6 in samples from patients with MPS IVA were shown to be increased in comparison to controls, proving that the pro-inflammatory state persists in these patients [25]. According to Petersen et al. (2005), IL-6 produced in muscles is synthesized in a TNF-α-independent pathway, and its anti-inflammatory action stimulates the production of other anti-inflammatory cytokines such as Interleukin-10 (IL-10), which inhibits the production of TNF-α, and of the antagonist receptor of Interleukin-1 (IL-1ra), thereby regulating tissue homeostasis, besides stimulating lipolysis as well as oxidation of fats [18,26]. Our studies did not demonstrate alteration in IL-10, leading to the belief that patients follow an adaptive response to the disease.

IL-17 is a potent pro-inflammatory cytokine that amplifies ongoing inflammation, inducing the expression of TNF-α [27]; however, in our results, we did not obtain alterations in this cytokine in the two diseases that were investigated. IL-17A plays an important role in protecting against extracellular bacteria and fungi, as it recruits neutrophils into infected areas. In pro-inflammatory micro-environments, human TReg cells can be induced to produce the pro-inflammatory cytokine IL-17 [28]. However, IL-17 levels showed a negative correlation with IL-6 in MPS VI, showing that the two variables presented opposite inflammatory profiles for this disease, contrary to the literature in which IL-17A is described as having its biological function, albeit a yet little known function, but which appears to be associated with autoimmune diseases that are capable of inducing a strong inflammatory response, in addition to stimulating the secretion of IL-6 and IL-8 by human fibroblasts [28].

5. Conclusion

Our study carried out an analysis of the cytokine profile, showing the importance of the evaluation of the relation

between diseases and their systemic effects. The results presented in this article show a relationship between human genetics and immunology, trying to monitor different stages of life of patients with MPS I and MPS VI, in order to improve their quality of life.

6. Data Availability

The Cytokines data used to support the findings of this study were supplied by ELISA test under license and so cannot be made freely available. Requests for access to these data should be made to: Prof. Dr. Alexandre Silva de Mello; e-mail:melloas@gmail.com.

Conflicts of Interest

The authors declare that they have no competing interests.

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