Abstract. – OBJECTIVE: Hunter syndrome, or mucopolysaccharidosis type II (MPS II), is caused by deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS), which is responsible for degrading heparan and dermatan sulfate. The IDS gene is located on chromosome Xq28; pathological variants in this gene mostly consist of missense mutations and small and larger deletions, which produce different phenotypes. However, there is only one record in our population concerning the molecular mechanism of this disease; a genotype–phenotype description is not available.

PATIENTS AND METHODS: There were included 24 unrelated male patients; clinical features were recorded at a database, fluorometric IDS enzyme activity testing was done for each individual, followed by Sanger sequencing to identify mutations.

RESULTS: The mutational spectrum was found in 16 out of 24 Mexican patients with MPS II, and its range of phenotypes was described. The most frequent variants were of the missense type. The most affected exons were exon 3 (c.275T>G, c.284_287del, c.325T>C), exon 8 (c.1035G>C, c.550G>A), exon 9 (c.1229_1229del), and exon 7 (c.979A>C; this variant has not been previously reported). Exon 5 (c.438C>T, a non-pathogenic variant) was the least frequent. It was also found that the most severely affected patients were those with large deletions (2 out of 24) [rsalIDS: IDSP1 (P164)x0, FMR1, AFF2 (P164)x2] involving genes and pseudogenes. We found 2 patients with a synonymous mutation in exon 4.

CONCLUSIONS: Our results confirmed reports in the literature, since the most frequent variants were reported in exons 3 and 8. However, this result varies from one previous report in our population, which mentions large deletions and rearrangements as the most frequent alterations, since complex rearrangements were not found. According to what has been previously found, the most severely affected patients are those in which a whole gene has been deleted.

Key Words: Mucopolysaccharidoses, Iduronate-2-sulfatase, Hunter syndrome, MPS II, Mutations.

Introduction

Mucopolysaccharidoses (MPSs) are a heterogeneous group of lysosomal diseases that are innate errors of metabolism. These MPSs are
caused by defects in lysosomal enzymes, causing lysosomal accumulation of glycosaminoglycans (GAGs), which are important components of the extracellular matrix, in various tissues. Muco-poly saccharidosis type II (MPS II), also known as Hunter syndrome (OMIM #309900), is a genetic condition with an X-linked recessive inherited pattern, commonly present in male patients and rarely observed in females. MPS II has a global incidence of around 0.68/100,000 newborn, although this varies in Brazil and the USA, where the incidence is lower (<0.4/100,000 newborn). This syndrome is classified into two phenotypes depending on central nervous system (CNS) involvement: as severe, with mental retardation, developmental and language delay, seizures, and cognitive and behavioral problems; or as mild, with no involvement of CNS. However, all patients display multiple features, such as coarse facies, macrocephaly or dolichocephaly, recurrent airway infection, cardiomyopathy, joint stiffness, claw hands, and short stature. MPS II patients show a neurological affection and 82% present cardiovascular alterations.

MPS II is caused by a deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS) (UniProtKB - P22304) required for desulfation of iduronic acid-2-sulfate in heparan sulfate and dermatan sulfate for the first step in its degradation. It is formed by a 550-amino acid (aa) chain that contains two domains: “heavy chain” or SD1 and “light chain” or SD2. The first 33 amino acids correspond to the propeptide and signal peptide. The N-terminal SD1 domain corresponds to amino acids 34 to 443; this domain is important since it contains the catalytic site at C84. The SD2 C-terminal domain corresponds to amino acids 455 to 550 and provides stability to the protein. Although it does not have a catalytic site, it is important for the activity of the enzyme. The catalytic site is conformed by nine residues, D45, D46, C84, R88, K135, H138, H229, D334, and K347, of which the most important is C84 due to its posttranslational modification in C-formylglycin (3-oxoalanine, FGly). To maintain protein stability, there are two important components of IDS. The first are the disulfide bonds between Cys171 and Cys184 and between Cys422 and Cys432; the second are the Ca\(^{2+}\) ligands, which confer thermostability upon the protein.

The IDS (OMIM *300823, HGNC ID:5389) gene is located at Xq28 locus; it has approximately 28 kb and contains 9 exons that code for the active IDS enzyme. 80 kb towards the telomeric region, there is a pseudogene, IDSPI (HGNC ID:5390), which is about 14 kb long; it has a homologous sequence between IDS that includes exon 3 (99.9% identical) and exon 2, as well as introns 2, 3, and 7 (96% identical). 3% of patients present recombination between these regions, leading to complex rearrangements. Until now, more than 300 mutations have been related to this syndrome. The most common are missense mutations, followed by nonsense, large or small deletions, and rearrangements. So far, it has not been possible to establish a genotype-phenotype correlation; however, those patients with large deletions involving the gene and the pseudogene or chromosomal rearrangements are known to have more severe phenotypes. In Mexico, Alcántara et al. identified in a sample of 25 patients complex chromosomal rearrangements were present in 36% of these patients, which differs from the rate reported in world literature (18-30%).

The most frequent type of pathogenic variant associated with MPS II is the missense type, which causes an amino acid substitution, leading to misfolding proteins, catalytic inactivation, or premature degradation. The endoplasmic reticulum (ER) functions as a quality control mechanism and retains proteins, with folding defects affecting traffic through the Golgi apparatus and proteins further translocated to the lysosomes for their function, resulting in lysosomal accumulation of GAGs.

Due to the genetic and clinical heterogeneity of MPS II patients, the aim of this study is to further expand knowledge of the mutational spectrum and to describe the clinical phenotype not only at the neurological level, but also throughout other systems, in MPS II from the Mexican population.

**Patients and Methods**

We included 24 non-related male patients with confirmed biochemical diagnoses of MPS II. Complete medical histories were available for all patients. Genomic DNA was isolated from peripheral blood samples by the Miller method. IDS enzymatic activity assay from the leukocytes was analyzed in 12 MPS II patients, while the remaining 12 patients had been previously studied for IDS enzymatic assay in dried blood spots (DBSs) by Centogene. All procedures performed in this study were in accordance with the...
Declaration of Helsinki and its modifications. This study was approved by Local Ethics and Research Committees (1305, CIBO, IMSS); written informed consent was obtained from parents or legal tutors of the patients. Clinical and demographic data, such as age, weight, height, age of onset, age of diagnosis, and main symptoms, were obtained from clinical history.

**IDS Enzymatic Activity by Leukocytes**

An enzymatic-activity measurement according to the method of Chamoles et al\(^{20}\) was performed using fluorescence with 4MU-alpha-L-iduronide-2-sulphate (code M2, Moscaldam Substrates\(^R\)). This technique consists of two steps: first, the IDS enzyme present in the sample removes the sulfate group from 4-methylumbelliferyl-iduronate-2-sulfate substrate. Then, another purified lysosomal enzyme (alpha-iduronidase) is added, which cleaves 4MU from the substrate, generating free 4MU, which is measured by fluorometry (Turner 450, Sunnyvale, CA, USA) and is directly proportional to the amount of IDS enzyme. The normal range for the enzymatic assay in leukocytes was 27-103 nMol/mg protein (prot)/h. For DBS carried out by Centogene\(^R\), the reported normal range was >5.6 µmol/L/h.

**Detection of Deletions by MLPA**

Once the DNA was obtained, multiplex ligation-dependent probe amplification (MLPA) was carried out in 21/24 patients according to the MLPA\(^R\) DNA Protocol version MDP-005, 2017 (MRC Holland\(^R\)). Salsa P164-B2 probemix was used to detect deletions; this method is based on the amplification of multiple probes using a pair of primers fluorescently labeled to detect a specific DNA sequence. Once obtained, samples were analyzed by capillary electrophoresis using an ABI-PRISM\(^R\) 310 genetic analyzer (Applied Biosystems\(^R\), Foster City, CA, USA). Subsequently, the results were interpreted using Chromas\(^R\) software (Technelysium\(^R\)), yielding ratio charts that indicated the presence or absence of the probes, thus confirming deletion of one or more probes of the *IDS* gene or the *IDSP1* pseudogene. The reference values for an adequate gene dose were considered to range from 0.7 to 1.3; however, in these patients, a deletion should have a ratio value of 0 given that the probes belong to the X chromosome and all subjects were male.

**Sanger Sequencing**

Sanger sequencing was performed for 18 out of 24 patients, searching for mutations in exons 2-9 of the *IDS* gene using the primers and protocol for DNA amplification reported by Zhang et al\(^{21}\), since that is where most pathogenic variants have been reported (HGMD, 2021).

First, PCR was carried out to obtain the specific DNA fragments for each exon to be studied. Once the amplicons were obtained, they were purified with ExoSAP-IT™ (Applied Biosystems\(^R\), Foster City, CA, USA) and sequencing PCR was performed with BigDye\(^R\) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems\(^R\), Foster City, CA, USA). Afterwards, all fragments were re-purified with Sephadex\(^R\) G-50 and suspended in Hi-Di™ formamide for capillary electrophoresis with ABI-PRISM® 310 equipment (Applied Biosystems™ Foster City, CA, USA). The obtained results were interpreted using Chromas\(^R\) software (Technelysium\(^R\)) for the identification of pathogenic, rare, or novel *IDS* gene variants.

In four of the 24 MPS II patients included in the study, three mutations were identified by Centogene\(^R\), and those too were detected by us.

For pathogenic variant analysis, a bioinformatic assay was performed using the Swiss Institute of Bioinformatics (SIB)’s software for modeling proteins. To predict cryptic splicing sites, Splice Site Prediction Software (SSPS) based on a neural network (BDGP) was used. The pathogenicity of missense variants was evaluated via PROVEAN software (J. Craig Venter Institute\(^R\)) and Polyphen-2 software (Biobyte Solutions GmbH\(^R\)).

**Statistical Analysis**

Descriptive statistics were used to estimate the prevalence of specific symptoms and patient signs, (dichotomous outcome: absent or present) and the results of laboratory analyzes. For the analysis of the prevalence of the data studied, only those patients for whom data of these particular characteristics were available were included.

**Clinical Description**

Sociodemographic analysis was carried out for all 24 male patients. The mean age was 9, with a range of 2-19 years. The mean weight was 29.8 (range: 15.2-50) kg and mean height was 115.6 (range: 91-154) cm. The mean age of symptom onset was 20 (range: 6-48) months old and the mean age of diagnosis by enzymatic assay was 5 (range: 1-14) years old.

Of the 24 patients, 24 (100%) manifested symptoms due to organ and system involvement in the head and neck, 22 (91.6%) manifested them in the osteoarticular system, 21 (87.5%) manifested...
neurological symptoms or symptoms in the integumentary system or oral cavity, 20 (83.3%) manifested otorhinological symptoms, 19 (79.1%) in the respiratory system, and 18 (75%) in the gastrointestinal system. The least frequent were symptoms in the cardiovascular (10; 41.6%), ocular (6; 25%), and genitourinary (3; 12.5%) symptoms.

Five out of 24 patients were classified as having a mild phenotype, of whom three belonged to the 6 to 10-year-old group and 2 belonged to the 11 to 20-year-old group. Nineteen out of 24 were classified as having a severe phenotype, of whom 6 were from the 0–5-year-old group, 7 were from the 6–10-year-old group, and 6 were from the 11 to 20-year-old group (Supplementary Table I).

Biochemical Description

Two methods were used for enzymatic assay. First, enzymatic assay on leukocytes was performed in 12 out of 24 patients with a mean of 1.38 (range: 0-4.27) nMol/mg prot/h (normal range: 27-103 nMol/mg prot/h). Mild phenotype patients (2) showed a mean value of 0.2325 (0.1-0.365) nMol/mg prot/h, whereas patients with a severe phenotype (10) had a mean value of 1.85 (0-4.27) nMol/mg prot/h (Supplementary Table I).

The second method was enzymatic assay in DBS, performed in 12 out of 24 patients by Centogene®, reporting a value of <0.8 µmol/L/h (normal range: >5.6 µmol/L/h) in patients with mild and severe phenotype (Supplementary Table I).

Molecular Diagnosis

MLPA

The MLPA technique was performed on 21 out of 24 patients, two of whom (9.5%; patients 4 and 5) presented a large deletion involving the IDS gene and the IDSP1 pseudogene. It comprises about 48 kb and affects all probes of every exon of IDS (1 to 9) and the 3 probes from the IDSP1. Probes from contiguous genes (AFF2, FMR1) were intact (Figure 1).

Figure 1. Ratio charts of patients with gene-pseudogene (IDS-IDSP1) deletion. Reference values are 0.7 to 1.3, indicating an adequate gene dose of each probe. A, Ratio chart of a patient without deletion. B, Schematic representation of IDS-IDSP1 deletion on the X chromosome. The red box indicates the chromosomal location of gene–pseudogene deletion; outlined in positions 5’-3’ are the FRM1 and AFF2 genes. C-D, Ratio charts of patients 4 and 5, referred to in Supplementary Table I, respectively. The red box frames the deleted probes, the blue arrow indicates the absence of a probe signal corresponding to IDS-IDSP1. Adjacent to these are the reference probes for the FMR1 and AFF2 genes, which are at their normal gene doses.
**Clinical Manifestations in Patients with Large Deletions**

Both patients (4 and 5) have severe disease phenotypes, with enzymatic activities of 0 nMol/mg prot/h (Supplementary Table I) and significant neurological involvement. Both have experienced developmental and language delays and need support from others to perform daily activities, as they neither walk nor communicate. Patient 4 was the most deeply affected; he presented seizures, hydrocephaly, swallowing and feeding problems, hypotony, heart murmur and cardiomiopathy, glaucoma, and frequent upper airway infection. Osteoarticular pain was not assessable. Patient 5 also showed decreased visual acuity and more affection in the oral cavity due to delayed tooth eruption and chronic cough. Both showed similar affectation in the otorhinological and integumentary systems.

**Sanger Sequencing**

Exons 2-9 were analyzed by Sanger sequencing in 18 out of 22 patients without detected deletion by MLPA. Seven pathogenic variants were found in 10 patients, and three additional pathogenic variants reported from 4 patients by Centogene® were considered to analyze our sample, yielding a total of 10 pathogenic variants in 14 out of 22 patients (64%). The most frequent variant was the missense type in [9 of 22 patients (41%)], followed by intronic variants in 3 patients (14%), and finally frameshift by small deletions (<20 bp) in 2 patients (9%).

The most heavily affected exons were exons 3 and 8 (both 4 out 22 patients, 18%), followed by exons 9 (2 out of 22 patients, 9%) and 7 (1 out of 22 patients, 5%). In 3 out of 22 patients, an intronic variant was found, 1 on intron 2 (5%) and 2 on intron 7 (9%). All are described on Table I. In 8 out of 22 patients, no pathogenic variant was found.

**Pathogenic Variant Analysis**

Description and bioinformatic assay were performed for ten variants to determine their pathogenicities and to establish genotype–phenotype descriptions. The proteins were modeled with Swiss-model software to compare the normal structure of the IDS protein with that modified by the observed variants.

For intronic variants, SSPS was used to predict a new acceptor site for cryptic splicing for posterior analysis of how these affected the amino acid chain of the IDS protein.

**Pathogenic Variant Description**

**NM_000202.8:c.241-1G>C**

Intronic variant CS06628, located at X: 149503490, found in intron 2, upstream of the limit of exon 3 (1 base prior to the exon), was caused by a G>C change resulting in an alteration of an acceptor site (AG) for the splicing zone. Analysis with SSPS yielded a new acceptor site for cryptic splicing at nucleotide 280 from the coding sequence (CDS), triggering a frameshift with a premature termination codon, generating a transcript that codes for a non-functional protein of 82 aa. No previous report of this variant was found.

**NM_000202.8:c.1009del**

Intronic variant CS128213 was found in two patients, located at X: 149487100 and corresponding to a deletion of a nucleotide (A) in intron 7 at the splicing acceptor site (AG). It causes an alteration of alternative splicing. A new acceptor site for cryptic splicing at nucleotide 280 from the coding sequence (CDS), triggering a frameshift with a premature termination codon, generating a transcript that codes for a non-functional protein of 82 aa. No previous report of this variant was found.

**NM_000202.8:c.275T>G p.[Leu92Arg]**

Missense variant located at X: 149503455 at nucleotide 275 of CDS on exon 3, caused by a T>G change and generating a substitution from Leucine (L), an aliphatic chain amino acid, to Arginine (R), a basic amino acid, at position 92. The PROVEAN score of -5.511 and Polyphen-2 score of 1.0 predict a deleterious effect. 3D modeling of the protein displays a protein conformational change generating a non-functional enzyme. No previous report of this variant was found.
Missense variant rs113993946 located at exon 9, caused by a G>C substitution at nucleotide 1403 generating a substitution of 468 amino acid, which in turn causes a change from the basic amino acid Arginine (R) to the secondary amine Proline (P). This previously de-
scribed variant has a worldwide frequency of <0.01 (gnomAD, 2021)\(^\text{37-38}\). The PROVEAN score of -6.5 and Polyphen-2 score of 1 indicate that it has a deleterious effect. A conformational change of the protein was observed by 3D modeling as well as the ligands for the calcium ion, which interact with the enzyme's catalytic site.

**NM_000202.8:c.550G>A p.[Gly340Asp]**  
(Mutational Spectrum of the IDS gene in Mexican Patients with Hunter Syndrome)

Missense variant CM981048\(^\text{22}\) was identified in two patients. Located at exon 8, it was caused by a G>A substitution, producing a change in an aliphatic chain amino acid glycine (G) for dicarboxylic aspartic acid (D) at position 340 of the amino acid chain. The PROVEAN score of -6.6 and the Polyphen-2 score of 1 predicted a detrimental effect. In protein 3D modeling, a conformational change can be observed, mainly affecting two important ligands for the calcium ion. This variant was previously reported and associated with a mild-phenotype patient with MPSII by Karsten et al\(^\text{29}\).

**NM_000202.8:c.325T>C p.[Trp109Arg]**  
(Mutational Spectrum of the IDS gene in Mexican Patients with Hunter Syndrome)

Missense variant CM128183\(^\text{22}\) was also found, located at exon 3 with a T>C substitution in codon 109 of CDS, leading to a change of the aromatic amino acid tryptophan (W) to the basic amino acid arginine (R). The PROVEAN score of -13.034 and Polyphen-2 score of 1 indicate a detrimental effect due to this change. Protein 3D modeling showed a conformational change. This variant has been previously reported\(^\text{30-32}\) in association with MPS II.

**NM_000202.8:c.1035G>C p.[Trp345Cys]**  
(Mutational Spectrum of the IDS gene in Mexican Patients with Hunter Syndrome)

Missense variant CM128176\(^\text{22}\) was found in 2 patients. Due to a G>C substitution on exon 8 at CDS position 1035, an aromatic amino acid, tryptophan (W), was changed to the thioamino acid cystein (C) on position 345 of the amino acid chain. The PROVEAN score of -11.6 and Polyphen-2 score of 1 indicate that it has a detrimental effect. 3D modeling was carried out, and a conformational change could be observed. This variant was previously reported in association with MPS II by Popowska et al\(^\text{33}\).

**NM_000202.8:c.979A>C p.[Thr327Pro]**  
(Mutational Spectrum of the IDS gene in Mexican Patients with Hunter Syndrome)

Missense variant caused by a change of A>C, leading to a substitution at position 327 of an hydroxyamino, threonine (T), for a secondary amine, proline (P). A PROVEAN score of -5.74 and Polyphen-2 score of 1 indicate a detrimental effect. 3D modeling was carried out, showing a conformational change. This is a new variant not previously reported.

**NM_000202.8:c.284_287del p.[Arg95Asn fs*34]**  
(Mutational Spectrum of the IDS gene in Mexican Patients with Hunter Syndrome)

A frameshift variant located at X: 149503443-149503446 corresponds to a three-nucleotide deletion, causing a change in the reading frame by losing nucleotides in positions 284–287 of exon 3. This leads to a change from the basic amino acid arginine (R) to dicarboxylic asparagine (N) at position 95 and a premature termination codon producing a protein length of 126 aa instead of 550 aa. No previous report was found.

**NM_000202.8:c.1229_1229delT p.[Leu410Arg fs*30]**  
(Mutational Spectrum of the IDS gene in Mexican Patients with Hunter Syndrome)

Frameshift variant located at position X:149483170, corresponding to a single nucleotide deletion of T on exon 9 and causing a change in the reading frame from an aliphatic chain amino acid, leucine (L), to a basic arginine (R) at position 410 and a premature termination codon giving a protein length of 438 aa instead of 550 aa. No previous report was found.

**Identified Non-Pathogenic Variants**

In Patients 12 and 18, a synonymous variant was observed: NM_000202.8:c.438C>T p.[Thr146=] (rs1141608)\(^\text{22, 34}\), classified as benign. Located in exon 4, it was caused by a C>T substitution in codon 146, whereby the amino acid threonine (Thr) was encoded without clinical significance. It was considered important for the present population description to include them. This variant presents a frequency of 0.157 in the Mexican population\(^\text{22, 34}\).

**Clinical Manifestations in Patients with Pathogenic Variants**

All patients have macrocephaly and coarse facies, although these characteristics were not marked in all patients; so, these features would be omitted at the next descriptions.
c.241-1G>C: Patient 11, a 9-year-old with a severe phenotype. Age of onset was 18 months and age of diagnosis was 3 years. The most affected system was osteoarticular, while the ocular, gastrointestinal, and genitourinary systems were less affected.

c.1090del: Patients 2 and 6 were carriers for this variant. The first patient had a mild phenotype; the age of onset was 2 years, and the age of diagnosis was 8 years. The most affected system was osteoarticular and the least were the ocular, gastrointestinal and cardiovascular systems. The second patient had a severe phenotype. The age of onset was 2 years and the age of diagnosis was 8 years. The most affected system was also osteoarticular, but to a greater extent than in patient 2; the least affected systems were gastrointestinal and genitourinary.

c.275T>G: Two patients were found with this variant: patients 15 and 19, both with severe phenotypes. The age of onset for patient 15 was at 6 months; that for patient 19 was 2 years. The age of diagnosis for patient 15 was 2 years and that for patient 19 was 7 years. Both were mainly affected on their osteoarticular systems and least affected on their cardiovascular, ocular, gastrointestinal, and genitourinary systems.

c.1403G>C: Patient 16, a 5-year-old with a severe phenotype. The age of onset was 2 years and the age of diagnosis was 3 years. The most affected systems in this patient were osteoarticular and neurological; the least affected systems were cardiovascular, ocular, and genitourinary.

c.550G>A: Two patients were found with this variant: patient 8, who had a severe phenotype, and patient 14, who had a mild phenotype. Both had a similar age of onset at 2 years old and similar ages of diagnosis at 5 and 4 years old, respectively. The most affected systems in patient 8 were the osteoarticular and neurological; in patient 14, only the osteoarticular system was affected. The least affected systems for both patients were the genitourinary and gastrointestinal systems.

c.325T>C: Patient 20 (2 years old), with a severe phenotype. The age of onset was 6 months and the age of diagnosis was 1 year. The patient’s most affected systems were osteoarticular and neurological, while the least affected were the genitourinary, ocular, and gastrointestinal systems.

c.1035G>C: Two patients with this variant were found: patient 21 (6 years old), with a mild phenotype, and patient 23, (14 years old) with a severe phenotype. Patient 21 had an age of onset of 9 months and was diagnosed at age 9; this patient was mildly affected on every system evaluated and was the least of all the analyzed patients. Meanwhile, patient 23 had severely compromised osteoarticular and respiratory systems; the ocular, genitourinary, and gastrointestinal systems were less affected.

c.979A>C: Patient 24 (8 years old), with a mild phenotype. The age of onset was 6 months and the age of diagnosis was 7 years. The most affected system on this patient was osteoarticular and the least affected were the genitourinary, gastrointestinal, and ocular systems.

c.284_287del: Patient 17 (7 years old) with a severe phenotype. The age of onset was 3 years old and the age of diagnosis was 5 years old. The most affected systems on this patient were neurological and osteoarticular, and the least affected were genitourinary, cardiovascular, and ocular.

c.1229_1229del: Patient 7 (15 years old) with a severe phenotype. The age of onset was 6 months and the age of diagnosis was 7 years. The most affected system in this patient was osteoarticular and the least affected were genitourinary, cardiovascular, ocular and gastrointestinal.

Discussion

Phenotype Analysis

The main manifestations in our sample group were thick facies (24/24, 100%) and affectations in the osteoarticular (22/24, 91.6%) and neurological (20/24, 83.3%) systems. These are the first manifestations of this syndrome to appear, as was reported in previous studies. This can guide us to adopt an early approach in the study of patients with these characteristics, coupled with the developmental delay milestones that they generally present. Cardiovascular affectations are very important, since they constitute the first cause of mortality in these patients and are frequently observed at around 5 years of age with a prevalence of 62%; however, in our study, only 41.6% of the patients had such affectations.

Regarding phenotypes, no mild-phenotype patients were found in our group under 6 years of age; however, severe phenotype patients were
found in all age groups, suggesting that patients with severe phenotypes are diagnosed earlier (around 3-4 years old), but not patients with mild phenotypes. This would be due to the delay in the appearance of symptoms.

**Biochemical Analysis**

Once MPSII is clinically suspected, the diagnosis must be confirmed by measuring enzyme activity in leukocytes, fibroblasts, or plasma. In our patients, values far below normal for the enzymatic assay – even as low as 0 nMol/mg prot/h – can be observed, giving us diagnostic confirmation of MPS II. Although patients with a mild phenotype show lower values for the enzyme assay, there are probably other factors that contribute to the expression of the disease. This confirms the difficulty in making direct genotype–enzymatic activity–phenotype correlations in these patients, as reported by Holt et al.

**Molecular Analysis (MLPA and Sanger Sequencing)**

Eleven pathogenic variants were found in 16 patients. In MLPA analysis, two out of 22 patients with severe phenotypes presented a large deletion (>20 bp); this represents a frequency of 9.5%, similar to previous reports in the literature, which range from 4 at 10%37. It encompasses the IDS/IDSP1 region and does not involve contiguous genes, leading to non-transcription for proteins, since all exons are lost.

Sanger sequencing identified 10 pathogenic variants in 14 patients, 4 of them with mild phenotypes and 10 of them with severe phenotypes. The most affected nucleotide was guanine, and the most frequent transition (44.4% of the sample) was G>C; this is a little a higher than was found in previous reports. The most affected amino acids were leucine and tryptophan with a frequency of 27.3% and the least affected was threonine with a frequency of 9%. The most frequent variant type was missense with a frequency of 95%, and the least affected was threonine with a frequency of 9%. The most frequent variant type was missense with a frequency of 95%, and the least affected was threonine with a frequency of 9%; both of these values are a little higher than what was found in previous reports. The most affected amino acids were leucine and tryptophan with a frequency of 27.3% and the least affected was threonine with a frequency of 9%.

**Genotype-Phenotype Description and Molecular Analysis**

**IDS/IDSP1 Deletion Phenotype**

A complete deletion of IDS/IDSP1 was present in the two patients with severe disease phenotypes who showed the most significant neurological involvement of all patients. This is consistent with the literature. Both patients have an enzymatic activity of 0.0 nMol/mg prot/h, contributing greatly to their phenotypes.

**Missense Variant Phenotypes**

Missense variants have been correlated with severe and mild disease phenotypes; this may be because these types of alterations cause misfolding of the protein and could trigger a premature destruction of the enzyme by ER degradation, leading to minimal or no enzyme activity and thus accumulation of GAGs in the lysosomes, yielding the clinical phenotype of MPS II.

However, this does not mean that it leads to total catalytic inactivation. Some studies have found some IDS missense variants with residual enzyme activity; this could explain why some patients showed a minimum activity in the enzymatic assay. This correlates with what we found in our patients, since two missense variants (i.e., 550G>A and 1035G>C) were associated with either mild or severe phenotypes. Both variants caused an alteration in the binding ligands for calcium ions important for thermostability of the protein, leading to a catalytic inactivation of the enzyme. Mild forms of the disease could be explained by some modifier genes.

As already mentioned, the binding ligands for calcium are of the utmost importance, so if any related residue undergoes modification, the protein stability will be affected. This is the case for the 979A>C variant, in which there is also a loss of N-linked glycosylation at N325, so catalytic inactivation of the enzyme might be expected. However, the patient (24) with this variant shows a mild phenotype. To date, only one case has been reported in which a mild phenotype is associated with MPSII due to a pathogenic variant of N11543. It is known that 8 asparagine residues (N) in IDS are N-glycosylation sites (N31, 115, 144, 246, 280, 325, 513 and 537), which are very important steps, since oligosaccharides intervene with the folding, transport, stability, and function of proteins; this serves as a quality control for the correct folding of proteins. However, expression studies have shown that none of these glycosyla-
tion sites are essential save N280, as it serves to direct the enzyme to the lysosome. The protein transport could be replaced if needed by M6P-dependent by a bypass mechanism; all of these could explain the residual enzyme activity that is associated with the patient’s phenotype.

On the other hand, we know that alterations in the conformation of proteins, such as folding defects, activate the quality control mechanisms in the ER (ERQC) and the unfolded protein response (UPR); if this misfolding is not corrected, the protein will be retained in the ER and degraded by the ubiquitin-proteasome system (UPS). This mechanism may explain what happens in the 1403G>C and 325T>C variants, the former of which involves the gain of a loop rather than a strand in SD1, triggering a loss of interaction with the pyrrolidone carboxylic acid at position Q465, and the latter of which involves a helix gain and loop loss, producing a protein misfolding. This triggers the rescue processes already mentioned, producing severe disease phenotypes in patients 16 (for the 1403G>C variant) and 20 (for the 325T>C variant).

Finally, there are missense variants that also affect the enzyme’s active site, reducing or abolishing its catalytic activity, either by alterations to the main residues of the active site or by affecting the calcium ion binding ligands. This results in a severe disease phenotype in patients 12 (for the 241-1G>C variant) and 6 (1009del).

Frameshift Variant Phenotypes

The deletions of <20 bp analyzed in this study are considered small. Two deletions are analyzed, each resulting in frameshift variants: 284_287del, resulting in the loss of 4 pb, and 1229_1229del, resulting in a single nucleotide loss. The former generates a premature termination codon, resulting in a 126 aa protein with misfolding and alteration in the structure of its catalytic site, leading to a premature degradation. The latter causes a premature termination codon at 438 aa, so the SD2 domain is lost, and structural modifications probably lead to premature degradation at ER. Both variants correlate with the severe phenotypes of patients 17 (284_287del) and 7 (1229_1229del).

Conclusions

This is the first report of genotype–phenotype association in our population that includes a complete analysis of the clinical characteristics of these patients, rather than just analyzing them by their severity. The main manifestations in our sample group were thick facies and affectations in osteoarticular and nervous systems. Biochemical assay helped us to confirm the diagnoses of these patients. Unfortunately, however, it does not help us to predict disease severity, nor the pathogenic variants in which we can observe different phenotypes associated with the same molecular variant. The exception to this is that complete IDS/IDSP1 deletion is associated with a severe phenotype. Something very similar occurs with missense-type variants, for which most patients present this same phenotype. The data obtained broadens our knowledge of the heterogeneous panorama of the mutational spectrum of IDS in our population and its implications for phenotype. It is very important to continue with molecular characterization to better understand our patients and offer them more adequate treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.
Mutational spectrum of the IDS gene in Mexican patients with Hunter syndrome

Ethics Approval
This study was approved by Local Ethics and Research Committees (1305, CIBO, IMSS); written informed consent was obtained from parents or legal tutors of the patients. The integrity of the patients was respected in accordance with good ethical practices and the Declaration of Helsinki and their modifications.

Availability of Data and Materials
Data and materials are available upon reasonable request to the Corresponding Author.

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Authors’ Contribution
The authors who contributed to the manuscript were: RH, MA, for analysis, experimentation and data collection; RTLC and MTMT for experimentation and analysis; MRSC for design, experimentation, analysis, and financing support.

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