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Molecular testing of 163 patients with Morquio A (Mucopolysaccharidosis IVA) identifies 39 novel GALNS mutations

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ABSTRACT

Morquio A (Mucopolysaccharidosis IVA; MPS IVA) is an autosomal recessive lysosomal storage disorder caused by partial or total deficiency of the enzyme galactosamine-6-sulfate sulfatase (GALNS; also known as N-acetylgalactosamine-6-sulfate sulfatase) encoded by the GALNS gene. Patients who inherit two mutated GALNS gene alleles have a decreased ability to degrade the glycosaminoglycans (GAGs) keratan sulfate and chondroitin 6-sulfate, thereby causing GAG accumulation within lysosomes and consequently pleiotropic disease. GALNS mutations occur throughout the gene and many mutations are identified only in single patients or families, causing difficulties both in mutation detection and interpretation. In this study, molecular analysis of 163 patients with Morquio A identified 99 unique mutations in the GALNS gene believed to negatively impact GALNS protein function, of which 39 are previously unpublished, together with 26 single-nucleotide polymorphisms. Recommendations for the molecular testing of patients, clear reporting of sequence findings, and interpretation of sequencing data are provided.

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1. Introduction

Morquio A syndrome (also known as mucopolysaccharidosis type IV A or MPS IVA) is a member of a group of inherited metabolic disorders collectively termed mucopolysaccharidoses (MPSs). An MPS disorder is caused by a deficiency of one of the 11 different lysosomal enzymes required for the degradation of mucopolysaccharides or glycosaminoglycans (GAGs). Morquio A is caused by a deficiency of galactosamine-6-sulfatase (N-acetylgalactosamine-6-sulfate sulfatase; GALNS). GALNS deficiency leads to the accumulation of the GAGs keratan sulfate (KS) and chondroitin-6-sulfate (CS6) in lysosomes and results in prominent skeletal and connective tissue abnormalities. In addition to skeletal and connective tissue abnormalities, patients also experience joint hypermobility [2], muscle weakness, and pulmonary and cardiac manifestations of the disease, all of which can result in reduced endurance and impact both quality of life and mortality. Compared with patients with other MPS disorders, those with Morquio A often show joint hypermobility in contrast to joint stiffness. Morquio A often shows prominent spine involvement [50], but patients are typically reported to be intellectually normal. The clinical presentation of Morquio A varies among patients in both the specific features observed and their severity, making any single metric an incomplete description of disease burden [17,18,34,57].

Morquio A is a rare disorder, with incidence estimated to range from 1 in 76,000 to 1 in 640,000 live births in different populations [18]. The
incidence has been reported as one in 76,000 in Northern Ireland, one in 640,000 in Western Australia, one in 450,000 in the Netherlands, and one in 450,000 in Portugal [30,37,38,43,44]. More accurate data about the incidence of Morquio A can be obtained in the future if screening of newborns is introduced, as this occurs for other lysosomal storage disorders such as Pompe and Fabry diseases [25,35,49].

Diagnosis of Morquio A begins with clinical suspicion, followed by screening tests (which are sometimes omitted if there is a known family history). The Morquio A diagnostic algorithm recommends a GALNS enzyme activity assay performed in leukocytes or fibroblasts as the gold standard for diagnosis of Morquio A [65]; diagnosis of Morquio A can be supported by molecular analysis of the GALNS gene [65]. Screening tests that may also be used for Morquio A are urinary GAG analysis and/or enzyme activity analysis performed on dried blood spots. Urinary GAG analysis measures either the total accumulation of all urinary GAGs (quantitative assay) or the relative abundance of each of the GAGs (qualitative assay). It is recommended to perform both quantitative and qualitative urinary GAG analyses in parallel, because quantitative GAGs are not always elevated in Morquio A patients and both tests are susceptible to false-negative results due to low KS excretion (relative to other GAGs) in teenagers and adults [59,63–65]. Enzyme assays performed on dried blood spot samples are an alternative screening tool [7] but are not recommended for Morquio A diagnosis where alternatives exist, since assay robustness and sample quality are potential concerns [65]. A liquid chromatography/tandem mass spectrometry-based approach may also be used to measure levels of keratanase (relative to other GAGs) in teenagers and adults [59,63–65]. Enzyme assays performed on dried blood spot samples are an alternative screening tool [7] but are not recommended for Morquio A diagnosis where alternatives exist, since assay robustness and sample quality are potential concerns [65]. A liquid chromatography/tandem mass spectrometry-based approach may also be used to measure levels of keratanase II-digested mono- and di-sulfated KS disaccharides, providing a means try-based approach may also be used to measure levels of keratanase II-digested mono- and di-sulfated KS disaccharides, providing a means.

Additional reference enzyme measurements are critical to confirm sample integrity and exclude other disorders, such as MPS VI (caused by loss of β-galactosidase due to mutations in GLB1; patients with Morquio B have been misdiagnosed with MPS VI), Morquio B (caused by a deficiency of β-galactosidase due to mutations in GLB1; patients with Morquio B have been misdiagnosed with Morquio A), multiple sulfatase deficiency (mutations in the SUMF1 gene result in reduced activity of multiple sulfatases, including GALNS), and mucolipidoses types II/III (leads to mislocalization of GALNS and other lysosomal enzymes in some tissues).

The GALNS gene is approximately 50 kb long and contains 14 exons, producing a 2339-bp mRNA that encodes a 522-amino acid protein [36, 55]. The protein structure of the human GALNS protein has recently been solved [47]. The GALNS active site is a large trench containing the catalytic formylglycine aldehyde, derived from a cysteine residue by action of the formylglycine-generating enzyme (FGE) [9,12,13,47]. The GALNS protein is found as a homodimer [45] and is described as occurring in a multiprotein complex with other lysosomal enzymes [1,45].

The GALNS mutations that cause Morquio A are very heterogeneous and are detected throughout the gene [60]. Even the most frequently detected mutations are relatively uncommon [60]; however, founder effects can greatly alter GALNS allele frequencies in individual populations [24,65,66]. DNA methylation at CpG sites occurs in every exon but one, and inappropriate repair is thought to lead to transition mutations at these sites [58]. Multiple introns contain Alu repetitive elements, which can undergo recombination and lead to large deletions and/or rearrangements [http://genome.ucsc.edu/; February 2009 assembly; [31]]. This mutational heterogeneity can lead to difficulties in interpretation of molecular testing results, as novel mutations/variants of unknown significance may be detected relatively frequently.

Molecular analysis can confirm the Morquio A diagnosis and aid in genetic counseling by detecting causative mutations in the GALNS gene. Morquio A is an autosomal recessive disorder, so for disease to occur, both GALNS alleles must carry mutations that decrease or eliminate GALNS enzyme activity. In standard DNA sequencing approaches, separate PCR reactions amplify the GALNS exons and short stretches of the adjacent sequence, followed by sequencing of the individual PCR products. However, standard sequencing approaches can yield misleading results when one patient allele contains deletions or point mutations that eliminate PCR primer binding sites, resulting in apparent homozygosity due to allele dropout [27]. Parental testing can confirm biallelic inheritance and indicate cases where deletion/duplication testing is necessary or uniparental isodisomy (UPD) may be a possibility [8]. However, not all mutations can be detected by standard molecular approaches, and sometimes the clinical significance of newly detected sequence alterations will not be clear. For example, standard GALNS sequencing approaches do not sequence deep within most introns, and the functional consequences of deep intronic mutations can be difficult to assess. Molecular analysis reports should clearly state which conclusions are possible for any detected sequence alterations.

In this study, we report the molecular testing that led to the identification of genetic lesions in GALNS in 163 patients with Morquio A. We identified 99 mutations believed to be disease associated or that are likely disease associated, of which 39 are previously unpublished. Of the 39 novel mutations, 25 are missense mutations, six are nonsense mutations, five are small deletions, two are intronic mutations that likely affect splicing sites, and one is a large deletion-duplication. We also propose guidelines for the interpretation and reporting of GALNS mutations based on the supporting information available. Using these classification guidelines, 16 of the 39 novel changes are “disease associated” and 23 are “likely disease associated”. In addition to the 39 novel changes believed to be disease associated or that are likely disease associated, two novel changes were detected that are classified as “variants of unknown significance”. We also identified 26 separate single-nucleotide polymorphisms (SNPs). Finally, we provide guidelines and recommendations for molecular testing and highlight situations where inaccurate genotyping conclusions may occur.

2. Materials and methods

Individuals genotyped in this study had received a diagnosis of Morquio A prior to and independent of any molecular testing results; and thus, no power calculations were performed or required for the purposes of this study. All detected mutations in the GALNS gene were checked against reported SNPs. At three of the six institutions reporting novel alterations in GALNS, novel alterations were shown to be absent from the GALNS sequences of at least 100 unaffected individuals; at the remaining three institutions, this was not routinely performed for GALNS alterations identified in patients with a diagnosis of Morquio A. The results reported here 1) have been reviewed and approved by a duly constituted ethics committee (Hospital Clinic, Barcelona, Spain; and Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil), 2) are from samples referred as diagnostic samples, which do not require ethical approval at this institution (Willink Biochemical Genetics Unit, Manchester, United Kingdom), or 3) are from retrospective case reports that do not require ethics committee approval at these institutions (Unidade de Bioquímica Genética, Porto, Portugal; Children’s Hospital of Orange County, Orange, California, United States; King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; and SA Pathology, North Adelaide, South Australia, Australia).

2.1. Patient material and molecular analysis

Genomic DNA was isolated from fibroblasts or peripheral blood cells using standard protocols. GALNS exons and adjacent intron regions were amplified by PCR reactions and then sequenced; in some cases, a single-strand conformation polymorphism assay was also performed, all using standard protocols (see Supplementary methods in Appendix A). Molecular testing was performed at the laboratories included in this publication, and no data from BioMarin Pharmaceutical-sponsored studies are reported.
The DNA and protein sequence numbering was based on the GALNS cDNA sequence (GenBank entry NM_000512.4), with the sequence position +1 corresponding to the A of the initial ATG in the reference sequence. Novel mutations are defined as nucleotide changes in the GALNS gene that have not previously been described and published; they are classified by the supporting evidence available per Table 1.

2.2. GALNS enzyme assay and urinary GAG testing

GALNS enzyme activity was measured in leukocytes or cultured fibroblasts using either fluorogenic [69] or radiolabeled substrates [20]; assays were performed according to the manufacturer’s protocols.

Total urinary GAGs were quantified using the Alcian blue or dimethylmethylene blue (DMB) method [42]. Qualitative GAG analysis for the detection of KS was carried out by one dimensional or two dimensional low voltage electrophoresis of extracted GAGs, or by thin layer chromatography [21,22].

3. Results

3.1. Detected alterations in GALNS

In this study, molecular testing was performed on 163 patients with Morquio A (Supp. Table S1). A total of 99 predicted sequence alterations suspected of negatively impacting GALNS function were identified, of which 39 are previously unpublished: 25 missense changes, six nonsense changes, five small deletions (one in-frame deletion, three frameshifts, and one deletion that encompasses exonic and intronic sequences), one complex large deletion-duplication, and two intronic changes affecting consensus splice sites (Figs. 1, 2, Table 2, Supp. Table S2; information from all patients in Supp. Table S1). Six of these novel changes were detected in more than one patient: c.347G>T (p.Gly116Val, found in seven patients), c.107T>G (p.Leu36Arg, found in three patients), c.802G>A (p.Gly267Glu, found in three patients, of whom two are siblings), c.422G>A (p.Trp141Ter, found in two patients), c.242C>T (p.Pro81Leu, found in two patients), and c.1155C>A (p.Tyr385Ter, found in two patients); all other novel mutations were only detected in a single patient. In addition to the 39 novel GALNS alterations associated with Morquio A, we also detected two missense variants of unknown significance (Table 2, Supp. Table S1, Supp. Table S2) and 26 separate SNPs (Supp. Table S3).

The sequence alterations in GALNS associated with Morquio A are numerous, heterogeneous, and are mostly missense mutations [118, 60; unpublished results]. While some sequence alterations have clear-cut impacts on GALNS enzyme function (e.g., nonsense mutations, large deletions, or frameshifts), the interpretation of detected missense mutations can be more difficult. This poses a challenge to those who interpret sequence alterations in GALNS, since the detection of novel or poorly characterized missense mutations is a relatively common occurrence in the molecular analysis of patients with Morquio A. Here, we have created and used a GALNS sequence alteration categorization and reporting system consistent with guidelines from the Human Genome Variation Society and Emory Genetics Laboratory (Table 1). This reporting system aims to communicate succinctly what can reasonably be concluded about a detected GALNS sequence alteration. For example, reasonably confident statements can be made about sequence alterations that have clear-cut predicted effects on the GALNS protein and alterations that have been detected multiple times in independent families, while more cautious statements are appropriate for missense alterations detected in a single patient. We believe that this reporting system is useful both in publishing GALNS alterations and in communicating GALNS sequence results to clinicians.

3.1.1. Missense alleles

Among the 25 novel missense changes detected, the most frequently detected novel allele was the missense change c.347G>T (p.Gly116Val), detected 14 times in seven patients. In every case, molecular testing indicated that patients were homozygous for the c.347G>T (p.Gly116Val) allele; of these seven patients, the ethnicity of six was ‘Asian-multiplicift’ and one was Norwegian (but possibly of Asian origin). Where assayed, patients homozygous for the c.347G>T (p.Gly116Val) change were diagnosed with Morquio A at three years of age or younger and had substantial reductions in GALNS enzyme activity (6% or less of wild-type). While the specific c.347G>T (p.Gly116Val) change has not previously been published, c.346G>A (p.Gly116Ser) is a previously reported mutation associated with a severe growth phenotype [14,47,58,60]. In addition to c.347G>T (p.Gly116Val), the novel alterations c.107T>G (p.Leu36Arg), c.497A>G (p.His166Arg), c.865A>G (p.Asn289Asp), c.1138A>G (p.Arg380Gly), and c.1295C>G (p.Pro428Arg) also affect amino acid residues where other missense mutations have previously been reported [54,60,62,67].

Three novel missense changes that directly impact the active site of the GALNS enzyme [47] were identified: c.242C>T (p.Pro81Leu), c.1138A>G (p.Asn289Asp) and c.865A>G (p.Asn289Asp) (Fig. 2). The change c.242C>T (p.Pro81Leu) alters the middle residue of the FGE recognition motif and may reduce or eliminate the FGE-dependent posttranslational generation of the GALNS catalytic residue. Consistent with this interpretation, a patient homozygous for the change c.242C>T (p.Pro81Leu) had substantially reduced levels of GALNS enzyme activity. The GALNS active site contains a Ca2+ ion coordinated by oxygen atoms from five residues [47]. The overall negative charge coordinating the calcium ion would change with c.1138A>G (p.Asp40Asn) and c.865A>G (p.Asn289Asp), likely with deleterious effects on Ca2+ binding. Additionally, the primary active site residue Arg83 [47] is adjacent to an amino acid affected by the novel missense change c.251C>A (p.Ala84Glu), which would substitute a bulky charged residue for a small nonpolar residue and may impact the structure and charge of the enzyme active site. A patient homozygous for the change

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**Table 1** Suggested GALNS mutation reporting guidelines.

<table>
<thead>
<tr>
<th>Mutation disease associated</th>
<th>Mutation likely disease associated</th>
<th>Variant of unknown significance (VUS)</th>
<th>Likely benign variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known to cause disease and found in more than one family in association with enzyme defect</td>
<td>Disease diagnosis confirmed and unlikely to be benign variant and one of below:</td>
<td>Ambiguous/unclear effect on protein</td>
<td>Nucleotide change present at high frequency in population</td>
</tr>
<tr>
<td>or clear-cut effect of mutation on protein;</td>
<td>• Likely to effect splicing or • Nucleotide change in trans to known mutation or mutation that is disease associated or • Reported in homozygous state in single individual/family or • Significantly alters characterized active site residue</td>
<td>Rare in population with inadequate clinical or segregation information</td>
<td>Or accurate published evidence characterizes as benign polymorphism</td>
</tr>
<tr>
<td>• Frameshift</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Nonsense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Catalytic residue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Large deletions/insertions</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>• Initiation/termination codon changed</td>
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</tbody>
</table>

Mutation reporting based on recommendations of Emory Genetics Laboratory [40,46] and the Human Genome Variation Society [10] (http://www.hgvs.org/mutnomen).
c.251C>A (p.Ala84Glu) had a rapidly progressing disease, as characterized by a severe growth phenotype, and no detectable GALNS enzyme activity.

### 3.1.2. Nonsense alleles

Six novel nonsense mutations were identified: c.376G>T (p.Glu126Ter), c.422G>A (p.Trp141Ter), c.627C>G (p.Tyr209Ter), c.751C>T (p.Arg251Ter), c.1155C>A (p.Tyr385Ter), and c.1240C>T (p.Gln414Ter). These mutations would be expected to severely affect the GALNS protein by introducing early stop codons. Additionally, the previously reported nonsense mutation c.1559G>A (Trp520Ter) [67] was detected. This mutation is of interest because it removes only the final three C-terminal amino acids but is associated with Morquio A, consistent with the role of the GALNS C-terminus in forming the enzyme’s active site pocket [47].

### 3.1.3. Deletions

Five novel small deletions were identified, all of which are currently private mutations since they have been detected on only one occasion to date. Both the novel in-frame deletions and frameshift mutations detected here are associated with rapidly progressing disease, as characterized by a severe growth phenotype. Homozygosity for the frameshift mutation c.1114delC (p.Leu372SerfsTer6) was detected in a patient with rapidly progressing disease, as characterized by a severe growth phenotype, and no detectable GALNS enzyme activity. Interestingly, a different mutation at the same nucleotide has previously been reported (c.120+1G>C) and is also associated with early-onset Morquio A [28,48].

### 3.1.4. Alterations affecting splice sites

Two novel changes affecting intronic consensus splice sites were identified: c.120+1G>C and c.758+4A>T. While the exact consequences of these changes on the mature GALNS mRNA sequence cannot easily be predicted, a patient homozygous for the change c.120+1G>C was diagnosed with Morquio A at 6 months of age and had very low GALNS enzyme activity. Interestingly, a different mutation at the same nucleotide has previously been reported (c.120+1G>A) and is also associated with early-onset Morquio A [28,48].

### 4. Discussion

#### 4.1. Detected alterations

In this study, molecular testing of 163 patients with Morquio A (Supp. Table S1) identified 99 separate changes, of which 39 are previously unpublished (Supp. Table S2), together with 26 separate SNPs (Supp. Table S3). Of the novel changes in GALNS identified in this study, the novel changes c.107T>G (p.Leu36Arg), c.242C>T (p.Pro81Leu), c.422G>A (p.Trp141Ter), and c.682G>A (p.Gly201Glu) were found in more than one family. While the novel allele c.347G>T (p.Gly116Val) was detected in seven patients, we currently lack information on any potential familial relationships between the patients; the remaining novel alleles were only detected in a single individual or family (Supp. Table S2). The alleles c.376G>T (p.Glu126Ter), c.422G>A (p.Trp141Ter), c.627C>G (p.Tyr209Ter), c.751C>T (p.Arg251Ter), c.1070delC (p.Pro357ArgfsTer21), c.1114delC (p.Leu372SerfsTer6), c.1155C>A (p.Tyr385Ter), c.1240C>T (p.Gln414Ter), c.1428_1455del27 (p.Glu477_Gln485del), and the complex deletion-duplication can confidently be predicted to affect protein function and have not been previously described. Further, the missense changes c.242C>T...
(p.Pro81Leu), c.118G>A (p.Asp40Asn) and c.865A>G (p.Asn289Asp) are all predicted to greatly alter amino acid residues characterized as “primary active site residues” [47] and so can also be predicted to affect protein function.

This study identified 25 novel changes predicted to result in missense mutations in the GALNS protein either associated or likely to be associated with Morquio A disease; additionally, the novel missense changes c.[937A>G; 977G>C] (p.[Thr313 Ala; Trp326Ser]) were found in cis in one allele of a patient with Morquio A (both in trans with the known allele c.740G>A (p.Gly247 Asp)) and are both currently classified as “variants of unknown significance”. For a subset of these novel missense changes, previous studies have identified Morquio A patients with different missense changes at the same amino acid residue: p.Leu36Arg is a novel change, but p.Leu36Pro has been described [60], p.Gly51Lys (p.Gly51Ser [14,52]), p.Phe156Leu (p.Phe156Cys and p.Phe156Ser [4,60,66]), p.His166Arg (p.His166Gln [58,60]), p.Asn289Asp (p.Asn289Ser [62]), p.Arg380Gly (p.Arg380Ser and p.Arg380Thr [32,52,60]), and p.Pro420Arg (p.Pro420Ser [67]). These previously published missense changes at the same amino acid residues may strengthen the link between these novel changes and disease.

Currently, the following missense mutations have only been described in one family and changes at these amino acids have not been previously noted but are associated with a GALNS enzyme defect in the patient: c.47T>A (p.Val16Glu), c.120 + 1G>C, c.143T>G (p.Val48Gly), c.151G>A (p.Glu51Lys), c.251C>A (p.Ala84Glu), c.272T>C (p.Leu91Pro), c.433C>T (p.His145 Tyr), c.466T>C (p.Phe156Leu), c.497A>G (p.His166Arg), c.641T>C (p.Leu214Pro), c.647T>C (p.Phe216Ser), c.704C>A (p.Thr235Lys), c.758+4A>C (p.Glu48Val), c.865A>G (p.Asn289Asp), c.1138A>G (p.Arg380Gly), c.1244G>T (p.Gly416Thr), c.1247T>C (p.Ile416Thr), c.1259C>G (p.Pro420Arg), c.1474G>A (p.Ala492Thr), c.1498G>A (p.Gly500Ser), and c.1520G>T (p.Cys507Phe); these changes are classified as “likely disease associated,” and it is recommended that when found in a second family, patients should be verified to have a GALNS enzyme activity defect to confirm a diagnosis of Morquio A. Of particular note is the novel change c.47T>A (p.Val16Glu), which mutation predicting programs do not strongly associate with disease. The c.47T>A (p.Val16Glu) allele was documented in a patient with a less severe Morquio A growth phenotype, but with accompanying detection of KS and elevation of urinary GAGs, together with a demonstrated GALNS enzyme activity defect in both dried blood spots and leukocytes and normal activity of the control enzymes β-galactosidase, α-iduronidase, arylsulfatase B, iduronate sulfate, and β-glucuronidase; further, this patient had a pedigree indicating closely related parents. The novel splice site alterations reported here are c.120+1G>C and c.758+4A>T. While the c.120 + 1G>C mutation is predicted to be a clear splicing defect, the c.758+4A>T change is less well characterized as a splicing defect and we recommend, as for all changes categorized as “likely disease associated”, that if it should be identified in a second family, a defect in GALNS enzyme activity be demonstrated.

4.2. Challenges in interpretation

The high proportion of detected novel changes emphasizes the heterogeneity in GALNS mutations. This heterogeneity creates challenges in the interpretation of patient genotypes as many patients will carry novel or poorly characterized mutations. For example, some patients with identified deleterious mutations in both GALNS alleles also had additional GALNS sequence variants detected (Supp. Table S3). These variants have been reported as SNPs even if an eventual role in modulating phenotype (i.e. aberrant splicing induction) cannot be excluded. Given the potential complexity and ambiguity in interpreting the functional consequences of a mutation, we recommend standardized molecular testing reports that clearly state what conclusions can be drawn (Table 1). It is recommended that physicians collect as much as possible of a patient’s clinical phenotype data and laboratory screening results together with the age of diagnosis; however, as evidenced by our experience (see Supp. Table S1), this is often not practically possible. To aid in the interpretation of sequencing data, it is important for detected mutations to be reported to available mutation databases, such as the Human
Gene Mutation Database, because independent reports that associate a particular GALNS variant with Morquio A increase confidence in its association with disease.

We recommend caution in using software programs that predict the consequences of amino acid substitutions on protein structure. Two of the more well-known software algorithms for predicting the effects of nonsynonymous SNPs are SIFT and PolyPhen-2. SIFT assumes that changes in conserved regions of a protein are more deleterious than in other regions so the availability of suitable homologs is particularly important. SIFT has a reported true-positive prediction rate of 69% using the Swiss-Prot database, with a false-positive rate of 20% [39]. PolyPhen-2 makes a prediction based on 11 assessments (eight based on sequence and three on structure), with reported true-positive prediction rates of 92% and 73% on HumDiv and HumVar datasets, respectively, and a false-positive rate of 20% [1]. As with all statistical probabilities, the predictions are more likely to be true for the entirety of a dataset than for any specific mutation, warranting further caution in their use for the interpretation of individual novel mutations in a diagnostic setting.

Molecular testing approaches have limitations in mutation detection in patients with Morquio A. In contrast to MPS I—for which it is common to identify both causative mutations in IDUA (up to 95% of the time in one study of 85 MPS I families [3])—in Morquio A, a second mutation is not identified in up to 15% of patients [60]. The lower mutation detection rate in patients with Morquio A compared with those with MPS I may be due to a greater frequency of large deletions, duplications, or other rearrangements in GALNS than in IDUA, where large deletions have yet to be reported.

In addition to limitations in mutation detection, there are limitations in what can be concluded from molecular testing of the patient alone. For example, an inaccurate finding of homozygosity can occur when one patient allele lacks a PCR primer binding site due to deletions, point mutations, or SNPs, causing that exon to “drop out” from PCR amplification; since only one patient exon will produce a PCR product and yield sequence data, any mutations present in that exon’s PCR product will falsely appear to be homozygous. Apparent homozygosity due to allele dropout has been reported in patients with MPS VI [61], cystic fibrosis [11,16], and familial hypercholesterolemia [26]. Even in patients who are genuinely homozygous for a mutation that affects GALNS function, testing of the patient alone can still result in molecular under-diagnosis since cases of UPD will not be detected; a case of Morquio A resulting from UPD has been reported [8].

The ideal molecular testing approach outlined in Fig. 3 aims to address potential errors when genotyping patients with Morquio A. When possible, both parents should be genotyped to confirm biallelic inheritance of the causative mutations, allowing detection of situations where (1) deletions or polymorphisms at PCR primer sites could result in allele dropout and misinterpretation of patient sequencing results, (2) multiple alterations may be present in cis, or (3) homozygosity results from UPD (Fig. 3). Inaccurate and incomplete molecular diagnoses resulting from the absence of parental genotyping are potential concerns for many autosomal recessive disorders. In patients with MPS VI, multiple examples of patients with two deleterious mutations in cis have been reported [23]. At one molecular testing center, parental genotyping of patients with 40 different autosomal recessive disorders revealed that of 75 apparently homozygous patients, four were incorrectly assessed as homozygotes due to allele dropout and two were genuine homozygotes but resulted from UPD [27]. Additionally, parental genotyping can aid in differentiating alleles that are genuinely associated with disease from benign polymorphisms, as recently shown in MPS VI [68]. However, we recognize that circumstances do not always allow for parental testing to be performed. In this study, relatively few patients had parental testing performed and we recognize that this is the challenging reality we face when molecularly testing patients: it is not always feasible to test other family members or perform additional analyses.

4.3. Challenges in genotype/phenotype correlations

It is often desirable to make specific genotype/phenotype correlations between specific alleles and disease severity in Morquio A patients, but we believe that this practice contains two potential sources of error: first, the arbitrary classification of a complex disease state based on a few metrics (or even a single metric) and second, the consideration of a single mutation divorced from its context in the patient’s genotype. Morquio A manifests along a broad spectrum of clinical features and severity due to both the pleiotropic nature of the disease and the varied molecular consequences of its many distinct mutations; this can create difficulties in succinctly and accurately describing the disease state. The metrics most commonly used to classify Morquio A severity are patient height and growth rates. However, a patient of a given height can present with other symptoms (e.g., pulmonary or cardiac manifestations) of greater or lesser severity than might be expected; consequently, the level of dysmorphism does not fully define the disease severity [17,33,34,57]. A single, multivariate metric of disease severity does not currently exist but, weight, height, relative growth rate, and age at diagnosis are key markers [18]. Concentrations of KS measured in blood and urine, particularly in younger than preadolescent patients, have been found to correlate with Morquio A clinical status, suggesting their use in assessing disease severity and prognosis [14,17,17,29,56,59,60], and additional Morquio A biomarkers have also been proposed [29].

Furthermore, we believe that the possibility exists for unexpected interactions between alleles, particularly between missense alleles. Most mutations in GALNS are missense mutations [60], with consequences that can potentially include reduction in GALNS enzyme activity, disruption of protein–protein interactions with other lysosomal hydrolases [45], or GALNS protein destabilization and degradation. Because the GALNS enzyme is found as a homodimer [45,58], a Morquio A patient expressing two missense mutations could have three distinct GALNS dimers (aa, ab, and bb), each potentially with distinct enzymatic activity levels and protein interactions. Moreover, there are examples of pseudodeficiency alleles from other lysosomal storage disorders; these are ordinarily benign partial loss-of-function mutations that can potentially cause disease when combined with other loss-of-function mutations [6,15,51,70]. A complex example from the lysosomal storage disorder GM1 gangliosidosis describes an ordinarily benign polymorphism that increases the strength of a deleterious partial loss-of-function allele when combined in cis [5]. While no examples of pseudodeficiency alleles or complex allelic interactions are currently described in Morquio A, we believe that the possibility warrants caution and the analysis of mutations in the context of complete and accurate patient genotypes.

4.4. Implications for molecular testing in clinical practice

From a clinical and counseling perspective, the precise definition of both causative mutations for Morquio A is particularly important. In addition to assisting in the prediction of phenotype, the detection of both mutations is required for molecular prenatal diagnosis as well as carrier testing for family members. Therefore, in patients heterozygous for two known or probable mutations, it is very important to obtain parental DNA samples to confirm biallelic inheritance of these mutations. For patients appearing homozygous for a mutation, parental samples are also necessary to show that both parents do indeed carry the mutation, confirming homozygosity, or to indicate cases where further deletion/duplication or UPD studies are required. Unfortunately, the precise definition of large deletions or duplications using standard Sanger sequencing can often prove particularly difficult, especially when the deletion spans either end of the GALNS gene. Therefore, these studies are rarely performed as part of the initial sequence studies and in many cases will not be completed once a diagnosis has been “confirmed” by enzyme studies and the detection of a single mutation.
### Genotypes of patients with Morquio A carrying novel GALNS mutations identified in this study.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Classification of novel allele(s) per Table 1</th>
<th>Allele 2</th>
<th>Predicted effect on protein</th>
<th>Reported country/ethnicity</th>
<th>M/F</th>
<th>Age at diagnosis (years)</th>
<th>Reported as low?</th>
<th>% Wild-type</th>
<th>Reporting institute</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>c.107T&gt;G</td>
<td>p.Leu36Arg</td>
<td>Disease associated</td>
<td>c.107T&gt;G</td>
<td>p.Leu36Arg</td>
<td>Asian-multiethnic</td>
<td>F</td>
<td>2</td>
<td>Yes</td>
<td>6</td>
<td>Willink</td>
</tr>
<tr>
<td>3</td>
<td>c.107T&gt;G</td>
<td>p.Leu36Arg</td>
<td>Disease associated</td>
<td>c.107T&gt;G</td>
<td>p.Leu36Arg</td>
<td>Asian-multiethnic</td>
<td>F</td>
<td>12</td>
<td>Yes</td>
<td>5</td>
<td>Willink</td>
</tr>
<tr>
<td>5</td>
<td>c.120+1g&gt;c</td>
<td>-</td>
<td>Likely disease associated</td>
<td>c.120+1g&gt;c</td>
<td>-</td>
<td>Middle Eastern</td>
<td>M</td>
<td>0.5</td>
<td>Yes</td>
<td>3</td>
<td>Willink</td>
</tr>
<tr>
<td>7</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>Disease associated</td>
<td>c.242C&gt;T</td>
<td>p.Pro81Leu</td>
<td>Turkish</td>
<td>M</td>
<td>1</td>
<td>Yes</td>
<td>6</td>
<td>Willink</td>
</tr>
<tr>
<td>8</td>
<td>c.251C&gt;A</td>
<td>p.Ala84Glu</td>
<td>Likely disease associated</td>
<td>c.251C&gt;A</td>
<td>p.Ala84Glu</td>
<td>Portuguese</td>
<td>M</td>
<td>4</td>
<td>Yes</td>
<td>0</td>
<td>CGMJM</td>
</tr>
<tr>
<td>9</td>
<td>c.272T&gt;C</td>
<td>p.Leu91Pro</td>
<td>Likely disease associated</td>
<td>c.272T&gt;C</td>
<td>p.Leu91Pro</td>
<td>Portuguese</td>
<td>F</td>
<td>3</td>
<td>Yes</td>
<td>0</td>
<td>CGMJM</td>
</tr>
<tr>
<td>15</td>
<td>c.347C&gt;T</td>
<td>p.Gly116Val</td>
<td>Disease associated</td>
<td>c.347C&gt;T</td>
<td>p.Gly116Val</td>
<td>Middle Eastern</td>
<td>F</td>
<td>0.8</td>
<td>Yes</td>
<td>2</td>
<td>Willink</td>
</tr>
<tr>
<td>16</td>
<td>c.347G&gt;T</td>
<td>p.Gly116Val</td>
<td>Disease associated</td>
<td>c.347G&gt;T</td>
<td>p.Gly116Val</td>
<td>Middle Eastern</td>
<td>F</td>
<td>0.8</td>
<td>Yes</td>
<td>2</td>
<td>Willink</td>
</tr>
<tr>
<td>17</td>
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<td>p.Gly116Val</td>
<td>Disease associated</td>
<td>c.347C&gt;T</td>
<td>p.Gly116Val</td>
<td>Middle Eastern</td>
<td>F</td>
<td>0.8</td>
<td>Yes</td>
<td>2</td>
<td>Willink</td>
</tr>
<tr>
<td>18</td>
<td>c.376G&gt;T</td>
<td>p.Glu126Ter</td>
<td>Disease associated</td>
<td>c.376G&gt;T</td>
<td>p.Glu126Ter</td>
<td>Australian</td>
<td>4.4</td>
<td>Yes</td>
<td>1.7</td>
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<td></td>
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<tr>
<td>19</td>
<td>c.422G&gt;A</td>
<td>p.Trp141Ter</td>
<td>Disease associated</td>
<td>c.422G&gt;A</td>
<td>p.Trp141Ter</td>
<td>UK</td>
<td>8</td>
<td>Yes</td>
<td>6</td>
<td>Willink</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>c.433C&gt;T</td>
<td>p.His145 Tyr</td>
<td>Likely disease associated</td>
<td>c.433C&gt;T</td>
<td>p.His145 Tyr</td>
<td>Canadian</td>
<td>4.7</td>
<td>Yes</td>
<td>4</td>
<td>SA Path</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>c.466C&gt;T</td>
<td>p.Phe156Leu</td>
<td>Likely disease associated</td>
<td>c.466C&gt;T</td>
<td>p.Phe156Leu</td>
<td>Middle Eastern</td>
<td>F</td>
<td>2</td>
<td>Yes</td>
<td>4</td>
<td>SA Path</td>
</tr>
<tr>
<td>22</td>
<td>c.497A&gt;G</td>
<td>p.His166Arg</td>
<td>Likely disease associated</td>
<td>c.497A&gt;G</td>
<td>p.His166Arg</td>
<td>UK</td>
<td>F</td>
<td>10</td>
<td>Yes</td>
<td>10</td>
<td>Willink</td>
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<tr>
<td>26</td>
<td>c.627C&gt;G</td>
<td>p.Tyr209Ter</td>
<td>Disease associated</td>
<td>c.627C&gt;G</td>
<td>p.Tyr209Ter</td>
<td>Middle Eastern</td>
<td>F</td>
<td>2</td>
<td>Yes</td>
<td>4</td>
<td>SA Path</td>
</tr>
</tbody>
</table>
Novel alleles in **bold**. Where data were not provided to the lab for the given patient, the field is left blank. The field "Classification of novel allele(s) per Table 1" refers to the novel allele(s), if any; when the novel alleles differ, in this study both happened to share the same classification.

CGMJ, Unidade de Bioquímica Genética, Centro de Genética Médica Jacinto Magalhães (CGMJ), do Centro Hospitalar do Porto (CHP), Porto, Portugal; CHOC, Children's Hospital of Orange County, Orange, California, United States; HCPA, Laboratório de Genética Molecular, Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil; KFSH&RC, Department of Medical Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; SA Path, SA Pathology, Women's and Children's Hospital, North Adelaide, South Australia, Australia; Willink, Willink Biochemical Genetics Unit, Department of Genetic Medicine, Saint Mary's Hospital, Manchester, United Kingdom.
Furthermore, there is a significant proportion of cases (up to 15%) in which the second causative mutation is not detected by standard Sanger sequencing [60]. Many of these may be due to heterozygous deletions/duplications that are masked by a normal sequence in the other allele. Therefore, more extensive mutation studies are required in an attempt to define the second mutation. As for cases where deletions cause apparent homozygosity, further analysis is labor intensive and may rarely be carried out. In these cases, prenatal diagnosis using mutation studies is not feasible and must rely on enzyme studies, which are currently available in only a limited number of centers and can potentially prove problematic in cases with considerable residual GALNS enzyme activity. Also, the lack of detection of the second mutation means that conclusive carrier testing is not available for all family members. All of these are reminders that while molecular testing provides diagnostic and genetic counseling information, enzyme activity testing of GALNS, along with other enzymes, remains the standard for diagnosis of Morquio A.

Conflicts of interest statement

Dr Fietz has received travel support and honoraria from BioMarin Pharmaceutical. Drs Church, Morrone, and Tylee have received consultant fees and limited travel support from BioMarin Pharmaceutical and provide a diagnostic service for MPS for samples from Turkey that is funded by BioMarin. Dr Pollard is a paid employee of the Greenwood Genetic Center, which has contracts with BioMarin. Dr Wang holds a financial interest in BioMarin. Dr Mooney serves as a consultant for BioMarin Pharmaceutical. Mrs Davidson and Dr Miller are employees of BioMarin. Drs Al-Sayed, Brusius-Facchin, Caciotti, Coll, Gort, Kubaski, Lacerda, Laranjeira, Leistnner-Segal, Pajares, and Ribeiro declare no potential conflicts of interest.

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Appendix A. Supplementary data

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References


