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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 produce protein with 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unique mutations in the *GALNS* gene believed to negatively impact GALNS protein function, of which 39 are previously unpublished, together with 26 single-nucleotide polymorphisms.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.

## Keywords

MPS IVA; Morquio A; Mucopolysaccharidosis type IVA; GALNS; Lysosomal storage disorder; Mutation

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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 11 different lysosomal enzymes required for the degradation of mucopolysaccharides or glycosaminoglycans (GAGs). Morquio A is caused by deficiency of galactosamine-6-sulfatase (*N*-acetyl-galactosamine-6-sulfate sulfatase; GALNS). GALNS deficiency leads to the accumulation of the GAGs keratan sulfate (KS) and chondroitin-6-sulfate (C6S) in lysosomes and results in prominent skeletal and connective tissue abnormalities. In addition to skeletal and connective tissue abnormalities, patients also experience joint hypermobility (Aslam et al., 2013), muscle weakness, 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 (Solanki et al., 2013), 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 (Harmatz et al., 2013; Hendriksz et al., 2013; Montano et al., 2007b; Tomatsu et al., 2011).

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 (Hendriksz et al., 2013). 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 (Meikle et al., 1999; Nelson, 1997; Nelson et al., 2003; Pinto et al., 2003; Poorthuis et al., 1999). 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 disease (Labrousse et al., 2010; Nakamura et al., 2011; Scott et al., 2013).

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 (Wood et al., 2013); diagnosis of Morquio A can be supported by molecular analysis of the *GALNS* gene (Wood et al., 2013). 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 (Tomatsu et al., 2004d; Whitley et al., 1989a; Whitley et al., 1989b; Wood et al., 2013). Enzyme assays performed on dried blood spot samples are an alternative screening tool (Camelier et al., 2011) but are not recommended for Morquio A diagnosis where alternatives exist, since assay robustness and sample quality are potential concerns (Wood et al., 2013). 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 to measure KS both quantitatively and qualitatively at the same time (Hintze et al., 2011; Martell et al., 2011; Oguma et al., 2007; Tomatsu et al., 2010; Tomatsu et al., 2013; Oguma et al., 2007). A diagnosis of Morquio A is established if GALNS enzyme activity is markedly decreased in fibroblasts or leukocytes and control enzymes display wild-type activity (Wood et al., 2013). Additional reference enzyme measurements are critical to confirm sample integrity and exclude other disorders, such as MPS VI (caused by loss of arylsulfatase B activity; patients with Morquio A have been misdiagnosed with MPS VI), Morquio B (caused by deficiency of  $\beta$ -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 mucopolidoses types II/III (leads to mislocalization of GALNS and other lysosomal enzymes in some tissues).

The *GALNS* gene is approximately 50kb long and contains 14 exons, producing a 2339-bp mRNA that encodes a 522-amino acid protein (Nakashima et al., 1994; Tomatsu et al.,

1991). The protein structure of the human GALNS protein has recently been solved (Rivera-Colón et al., 2012). The GALNS active site is a large trench containing a catalytic formylglycine aldehyde, derived from a cysteine residue by action of the formylglycine-generating enzyme (FGE) (Cosma et al., 2003; Dierks et al., 1997; Dierks et al., 2003; Rivera-Colón et al., 2012). The GALNS protein is found as a homodimer (Pshezhetsky and Potier, 1996) and is described as occurring in a multiprotein complex with other lysosomal enzymes (Adzhubei et al., 2010; Pshezhetsky and Potier, 1996).

The *GALNS* mutations that cause Morquio A are very heterogeneous and are detected throughout the gene (Tomatsu et al., 2005). Even the most frequently detected mutations are relatively uncommon (Tomatsu et al., 2005); however, founder effects can greatly alter *GALNS* allele frequencies in individual populations (Kato et al., 1997; Wood et al., 2013; Yamada et al., 1998). DNA methylation at CpG sites occurs in every exon but one and inappropriate repair is thought to lead to transition mutations at these sites (Tomatsu et al., 2004c). 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; Meyer et al., 2013). 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 (Landsverk et al., 2012). Parental testing can confirm biallelic inheritance and indicate cases where deletion/duplication testing is necessary or uniparental isodisomy (UPD) may be a possibility (Catarzi et al., 2012). 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 Clínic, Barcelona, Spain; and Hospital de Clínicas 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). 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 (Zhao et al., 1990) or radiolabelled substrates (Hopwood and Elliott, 1983); assays were performed according to the manufacturer’s protocols.

Total urinary GAGs were quantified using the Alcian blue or dimethylmethylene blue (DMB) method (Panin et al., 1986). 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 (Hopwood and Harrison, 1982; Humbel et al., 1972).

### 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 sequence), one complex large deletion-duplication, and two intronic changes affecting consensus splice sites (Fig 1, Fig 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.602G>A (p.Gly201Glu, 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 (Hendriksz et al., 2013; Tomatsu et al., 2005; 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-multiethnic' 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 (Dung et al., 2013; Rivera-Colón et al., 2012; Tomatsu et al., 2004c; Tomatsu et al., 2005). 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.1259C>G (p.Pro420Arg) also affect amino acid residues where other missense mutations have previously been reported (Tomatsu et al., 1997; Tomatsu et al., 2005; Wang et al., 2010; Ye et al., 2013).

Three novel missense changes that directly impact the active site of the GALNS enzyme (Rivera-Colón et al., 2012) were identified: c.242C>T (p.Pro81Leu), c.118G>A (p.Asp40Asn) 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 Ca<sup>2+</sup> ion coordinated by oxygen atoms from five residues (Rivera-Colón et al., 2012). The overall negative charge coordinating the calcium ion would change with c.118G>A (p.Asp40Asn) and c.865A>G (p.Asn289Asp), likely with deleterious effects on Ca<sup>2+</sup> binding. Additionally, the primary active site residue Arg83 (Rivera-Colón et al., 2012) 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 c.251C>A (p.Ala84Glu) had 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) (Ye et al., 2013) 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 (Rivera-Colón et al., 2012).

**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, with no detectable GALNS enzyme activity. A patient found to be a compound heterozygote for the deletion c.1275delA (p.Val427SerfsTer13) and the missense mutation c.850T>G (p.Phe284Val) was diagnosed with Morquio A at five years of age with very low GALNS enzyme activity. The deletion c.405\_422+1del removes sequence from the end of exon four and the beginning of intron four and may affect splicing in addition to deleting amino acid residues. A large deletion-duplication was detected by comparative genome hybridization in one patient: the apparent duplication encompassed exons 6 to exon 9 with approximate breakpoints at nucleotide position g.87,424,250 in intron 5 and g.87,430,150 in intron 9, and the apparent deletion was within intron 10 with approximate break points at nucleotides g.87,418,915 and g.87,419,462. This complex rearrangement is predicted to result in duplication of sequences between introns 5 and 9 and deletion of sequences within intron 10.

**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 (Laradi et al., 2006; Romdhane et al., 2012).

## 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.602G>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.1429\_1455del127 (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” (Rivera-Colón et al., 2012) 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.[Thr313Ala; 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.Gly247Asp)) 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 (Tomatsu et al., 2005), p.Gly116Val (p.Gly116Ser (Dung et al., 2013; Tomatsu et al., 2004a)), p.Phe156Leu (p.Phe156Cys and p.Phe156Ser (Bunge et al., 1997; Tomatsu et al., 2005; Yamada et al., 1998)), p.His166Arg (p.His166Gln (Tomatsu et al., 2004c; Tomatsu et al., 2005)), p.Asn289Asp (p.Asn289Ser (Wang et al., 2010)), p.Arg380Gly (p.Arg380Ser and p.Arg380Thr (Montano et al., 2007a; Tomatsu et al., 2004a; Tomatsu et al., 2005)), and p.Pro420Arg (p.Pro420Ser (Ye et al., 2013)). These previously published missense changes at the same amino acid residues may strengthen the link between these novel changes and disease.

Currently, the following 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.His145Tyr), 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>T, c.791G>C (p.Ser264Thr), c.865A>G (p.Asn289Asp), c.1138A>G (p.Arg380Gly), c.1244G>T (p.Gly415Val), 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 (pVal16Glu) 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  $\beta$ -galactosidase,  $\alpha$ -iduronidase, arylsulfatase B, iduronate sulfatase, and  $\beta$ -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% (Ng and Henikoff, 2002). 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% (Adzhubei et al., 2010). 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—where it is common to identify both causative mutations in *IDUA* (up to 95% of the time in one study of 85 MPS I families (Beesley et al., 2001))—in Morquio A, a second mutation is not identified in up to 15% of patients (Tomatsu et al., 2005). The lower mutation detection rate in patients with Morquio A compared to 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 (Villani et al., 2010), cystic fibrosis (Diana et al., 2012; Hantash et al., 2009), and familial hypercholesterolemia (Laios and Glynnou, 2008). Even in patients who are genuinely homozygous for a mutation that affects *GALNS* function, testing of the patient alone can still result in molecular underdiagnosis since cases of UPD will not be detected; a case of Morquio A resulting from UPD has been reported (Catarzi et al., 2012).

The ideal molecular testing approach outlined in Figure 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 (Karageorgos et al., 2007). 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 (Landsverk et al., 2012). Additionally, parental genotyping can aid in differentiating alleles that are genuinely associated with disease from benign polymorphisms, as recently shown in MPS VI (Zanetti et al., 2009). 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 (Harmatz et al., 2013; Montano et al., 2007b; Tomatsu et al., 2011; Montano et al., 2008; Tomatsu et al., 2011). A single, multivariate metric of disease severity does not currently exist, but weight, height, relative growth rate, and age at diagnosis are key markers (Hendriksz et al., 2013). Concentrations of KS measured in blood and urine, particularly in younger than pre-adolescent patients, have been found to correlate with Morquio A clinical status, suggesting their use in assessing disease severity and prognosis (Dung et al., 2013; Harmatz et al., 2013; Martell et al., 2011; Tomatsu et al., 2004d; Tomatsu et al., 2005; Tomatsu et al., 2010; Harmatz et al., 2013), and additional Morquio A biomarkers have also been proposed (Martell et al., 2011).

Furthermore, we believe that the possibility exists for unexpected interactions between alleles, particularly between missense alleles. Most mutations in *GALNS* are missense mutations (Tomatsu et al., 2005), with consequences that can potentially include reduction in *GALNS* enzyme activity, disruption of protein-protein interactions with other lysosomal

hydrolases (Pshezhetsky and Potier, 1996), or GALNS protein destabilization and degradation. Because the GALNS enzyme is found as a homodimer (Pshezhetsky and Potier, 1996; Rivera-Colón et al., 2012), 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 (Caciotti et al., 2005; Gieselmann et al., 1991; Thomas, 1994; Zlotogora and Bach, 1983). 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* (Caciotti et al., 2003). 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 heterozygosity, 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.

Furthermore, there is a significant proportion of cases (up to 15%) in which the second causative mutation is not detected by standard Sanger sequencing (Tomatsu et al., 2005). 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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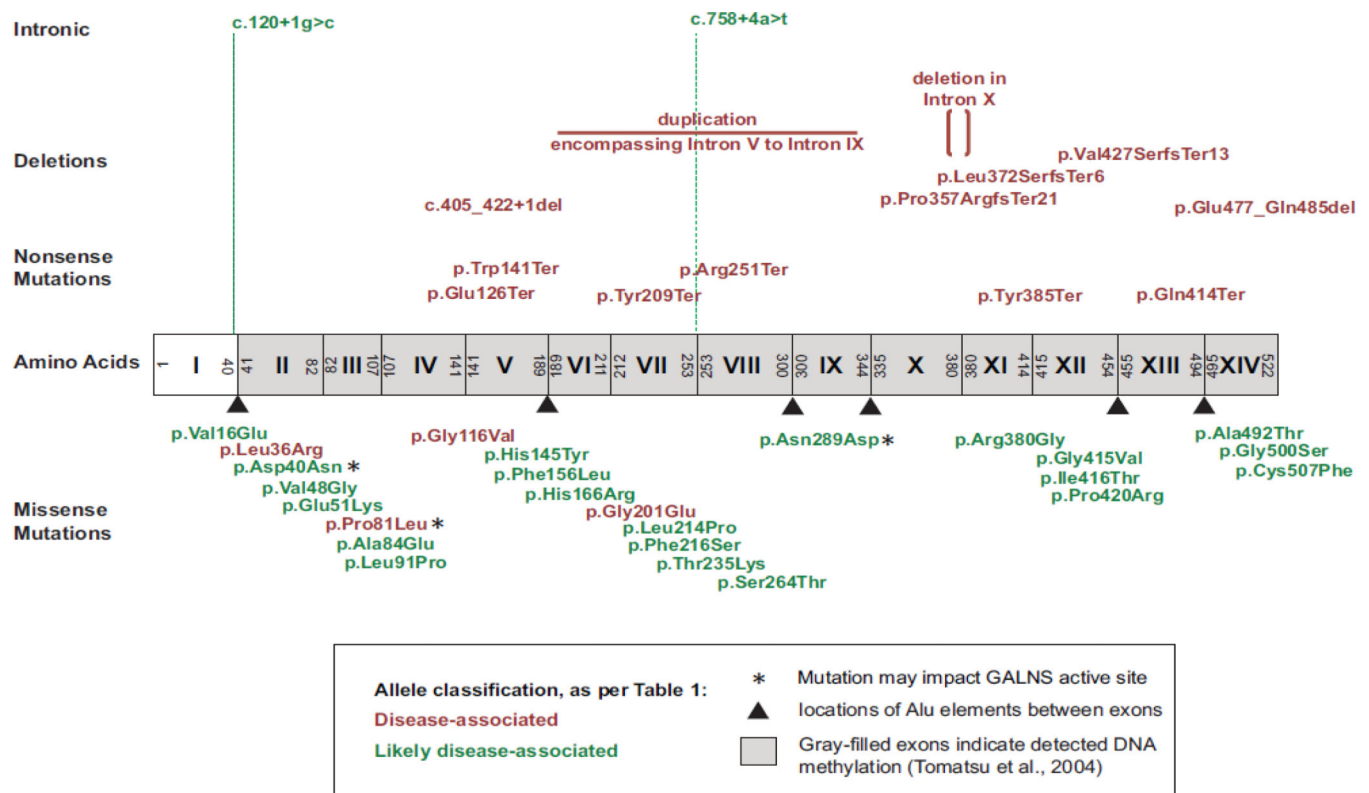
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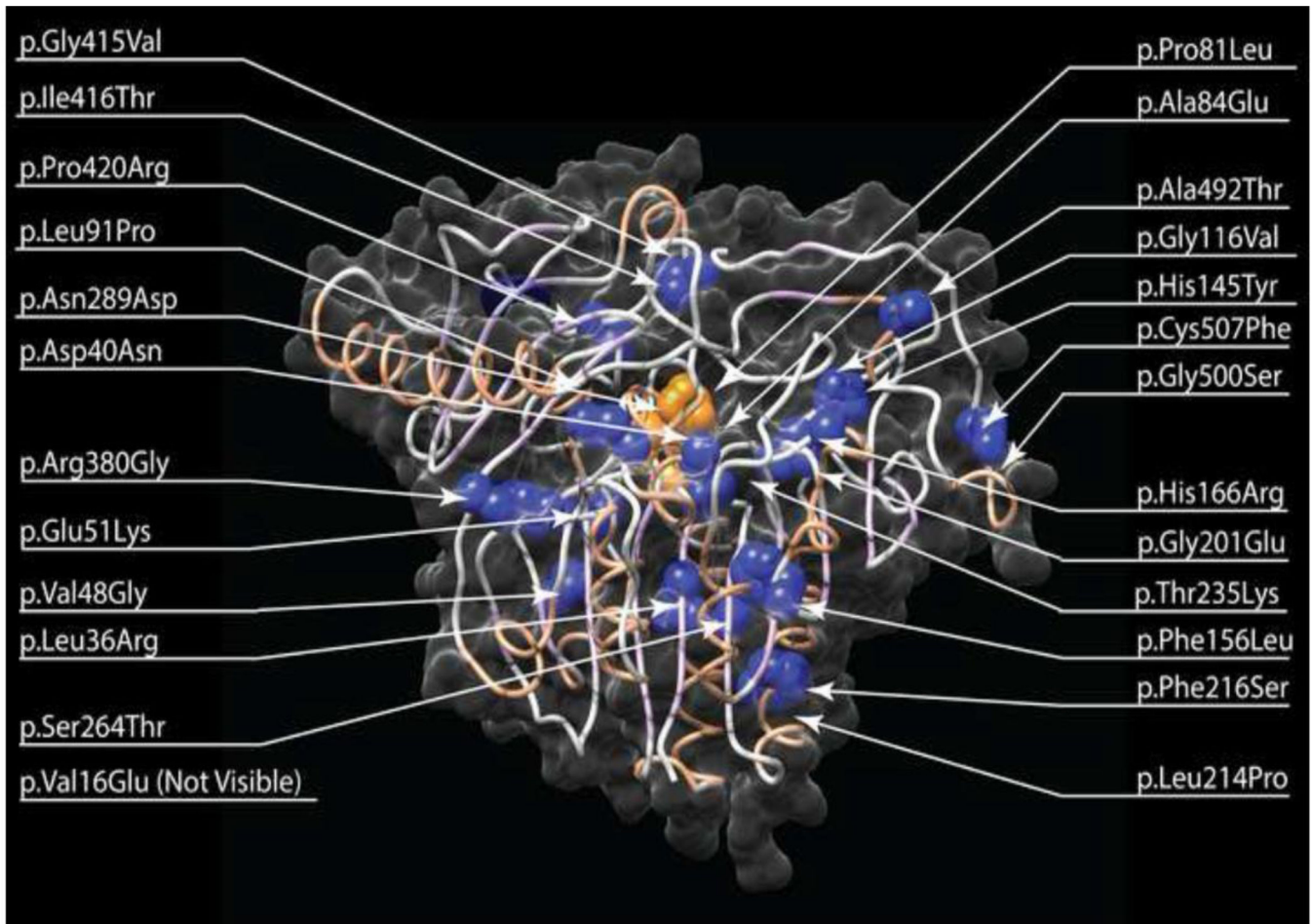
### Highlights

- Morquio A is an autosomal recessive disorder caused by mutations in the *GALNS* gene
- *GALNS* mutations are heterogeneous and many are rare or poorly characterized
- 39 novel *GALNS* mutations were identified in study of 163 patients with Morquio A
- Recommendations for molecular testing, reporting, and interpretation are provided



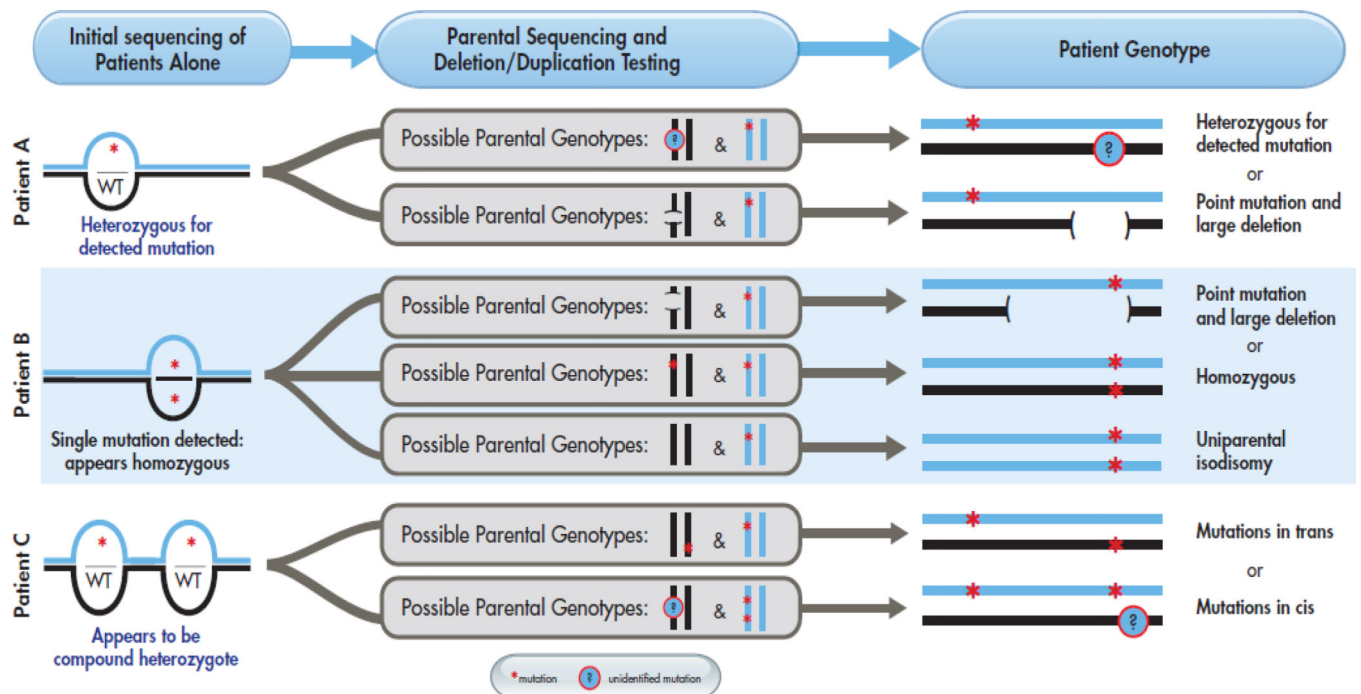
**Figure 1. Location of 39 novel mutations detected in the *GALNS* gene**  
 Exons are depicted as boxes and labeled with roman numerals. Novel alleles are color coded by their classification per Table 1.





**Figure 2. Location of novel Morquio A missense mutations mapped on the protein structure of the human GALNS protein**

Novel missense mutations identified in this study are mapped on the human GALNS protein structure (pdb:4FDI). Mutations identified were mapped to the structure and visualized using UCSF Chimera (<http://www.cgl.ucsf.edu/chimera>). The protein chain is represented as a ribbon showing beta sheets as light purple and alpha helices as light red, surrounded by a transparent solvent excluded surface. Mutation positions are represented as spheres for the wild type side chains. Note that position 16 is in the N-terminal disordered region. Mutations colored in orange are predicted to affect GALNS active site primary residues; all others are colored in blue.



**Figure 3. Potential complexity of patient genotypes**

For three example initial genotyping results (apparently heterozygous, apparently homozygous, and apparently a compound heterozygote), illustrates how parental genotyping results can confirm the initial genotype or reveal unanticipated complexity.

**Table 1**Suggested *GALNS* Mutation Reporting Guidelines

Mutation disease associated	Mutation likely disease associated	Variant of unknown significance (VUS)	Likely benign variant
<p>Known to cause disease and found in more than one family in association with enzyme defect</p> <p>OR</p> <p>Clear-cut effect of mutation on protein:</p> <ul style="list-style-type: none"> <li>• Frameshift</li> <li>• Nonsense</li> <li>• Catalytic residue</li> <li>• Large deletions/insertions</li> <li>• Initiation/termination codon changed</li> </ul>	<p>Disease diagnosis confirmed</p> <p>AND</p> <p>Unlikely to be benign variant</p> <p>AND <u>ONE OF BELOW</u>:</p> <ul style="list-style-type: none"> <li>• Likely to effect splicing</li> </ul> <p>OR</p> <ul style="list-style-type: none"> <li>• Nucleotide change in trans to known mutation or mutation that is disease associated</li> </ul> <p>OR</p> <ul style="list-style-type: none"> <li>• Reported in homozygous state in single individual/family</li> </ul> <p>OR</p> <ul style="list-style-type: none"> <li>• Significantly alters characterized active site residue</li> </ul>	<p>Ambiguous/unclear effect on protein</p> <p>Rare in population</p> <p>Reported in single individual/family with inadequate clinical or segregation information</p>	<p>Nucleotide change present at high frequency in population</p> <p>OR</p> <p>Accurate published evidence characterizes as benign polymorphism</p>

Mutation reporting based on recommendations of Emory Genetics Laboratory (Richards et al., 2008; Ogino et al., 2007) and the Human Genome Variation Society (den Dunnen and Antonarakis, 2000)(<http://www.hgvs.org/mutnomen/>).

**Table 2**  
Genotypes of Patients With Morquio A Carrying Novel *GALNS* Mutations Identified in This Study

Case number	Allele 1		Classification of novel allele(s) per Table 1	Allele 2		Reported country/ethnicity	M/F	Age at diagnosis (years)	GALNS enzyme activity		Reporting institute
	Nucleotide change	Predicted effect on protein		Nucleotide change	Predicted effect on protein				Reported as low?	% Wild-type	
1	c.47T>A	p.Val116Glu	Likely disease associated	c.47T>A	p.Val116Glu	Brazilian	F	10	Yes	9	HCPA
2	c.107T>G	p.Leu36Arg	Disease associated	c.107T>G	p.Leu36Arg	Asian-multieθνic	F	2	Yes	6	Willink
3	c.107T>G	p.Leu36Arg	Disease associated	c.107T>G	p.Leu36Arg	Asian-multieθνic	M		Yes	4	Willink
4	c.107T>G	p.Leu36Arg	Disease associated	c.107T>G	p.Leu36Arg	Asian-multieθνic	F	12	Yes	5	Willink
5	c.118G>A	p.Asp40Asn	Likely disease associated	c.118G>A	p.Asp40Asn	Asian-multieθνic	F				Willink
6	c.120+1g>c	-	Likely disease associated	c.120+1g>c	-	Middle Eastern	M	0.5	Yes	3	Willink
7	c.151G>A	p.Glu51Lys	Likely disease associated	c.151G>A	p.Glu51Lys	Brazilian	F	4	Yes	0.6	HCPA
8	c.242C>T	p.Pro81Leu	Disease associated	c.242C>T	p.Pro81Leu	Turkish	M	13	Yes	6	Willink
9	c.251C>A	p.Ala84Glu	Likely disease associated	c.251C>A	p.Ala84Glu	Portuguese	M	4	Yes	0	CGMIM
10	c.272T>C	p.Leu91Pro	Likely disease associated	c.272T>C	p.Leu91Pro	Portuguese	F	3	Yes	0	CGMIM
11	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Asian-multieθνic					Willink
12	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Asian-multieθνic	F	3	Yes	3	Willink
13	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Asian-multieθνic	M	3	Yes	2	Willink
14	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Asian-multieθνic	F				Willink
15	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Asian-multieθνic	F	0.08	Yes	2	Willink
16	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Asian-multieθνic	M	1	Yes	5	Willink
17	c.347G>T	p.Gly116Val	Disease associated	c.347G>T	p.Gly116Val	Norwegian	M		Yes		Willink
18	c.376G>T	p.Glu126Ter	Disease associated	c.865A>G	p.Asn289Asp	Australian		4.4	Yes	1.7	SA Path
19	c.422G>A	p.Trp141Ter	Disease associated	c.422G>A	p.Trp141Ter	UK	M	3	Yes	6	Willink
20	c.433C>T	p.His145Tyr	Likely disease associated	c.433C>T	p.His145Tyr	Canadian		4.7	Yes		SA Path

Case number	Allele 1		Classification of novel allele(s) per Table 1	Allele 2		Reported country/ethnicity	M/F	Age at diagnosis (years)	GALNS enzyme activity		Reporting institute
	Nucleotide change	Predicted effect on protein		Nucleotide change	Predicted effect on protein				Reported as low?	% Wild-type	
21	c.466T>C	p.Phe156Leu	Likely disease associated	c.466T>C	p.Phe156Leu	Middle Eastern	F	2	Yes		KFSH&RC
22	c.497A>G	p.His166Arg	Likely disease associated	c.497A>G	p.His166Arg	UK	F		Yes		Willink
23	c.602G>A	p.Gly201Glu	Disease associated	c.602G>A	p.Gly201Glu	Middle Eastern			Yes		Willink
24	c.602G>A	p.Gly201Glu	Disease associated	c.602G>A	p.Gly201Glu	Middle Eastern		5.2			SA Path
25	c.602G>A	p.Gly201Glu	Disease associated	c.602G>A	p.Gly201Glu	Middle Eastern			Yes		SA Path
26	c.627C>G	p.Tyr209Ter	Disease associated	c.627C>G	p.Tyr209Ter		F	4	Yes		Willink
27	c.641T>C	p.Leu214Pro	Likely disease associated	c.641T>C	p.Leu214Pro	Turkish			Yes	4	Willink
28	c.647T>C	p.Phe216Ser	Likely disease associated	c.647T>C	p.Phe216Ser	Indian			Yes	8	Willink
29	c.791G>C	p.Ser264Thr	Likely disease associated	c.791G>C	p.Ser264Thr	UK	M	2	Yes	4	Willink
30	c.1070delC	p.Pro357ArgfsTer21	Disease associated	c.1070delC	p.Pro357ArgfsTer21	Middle Eastern	M	1			KFSH&RC
31	c.1114delC	p.Leu372SerfsTer6	Disease associated	c.1114delC	p.Leu372SerfsTer6	Portuguese	M	10	Yes	0	CGMIM
32	c.1138A>G	p.Arg380Gly	Likely disease associated	c.1138A>G	p.Arg380Gly	Cape Verdean	M	3	Yes	0	CGMIM
33	c.1244G>T	p.Gly415Val	Likely disease associated	c.1244G>T	p.Gly415Val	Portuguese	M	15	Yes	0	CGMIM
34	c.1247T>C	p.Ile416Thr	Likely disease associated	c.1247T>C	p.Ile416Thr	Asian-multiethnic	M		Yes		Willink
35	c.1259C>G	p.Pro420Arg	Likely disease associated	c.1259C>G	p.Pro420Arg	Asian-multiethnic	F		Yes		Willink
36	c.1429 1455del27	p.Glu477 Gln485del	Disease associated	c.1429 1455del27	p.Glu477 Gln485del	Middle Eastern	F	13			KFSH&RC
37	c.1474G>A	p.Ala492Thr	Likely disease associated	c.1474G>A	p.Ala492Thr	Middle Eastern	M	9	Yes		KFSH&RC
38	c.1498G>A	p.Gly500Ser	Likely disease associated	c.1498G>A	p.Gly500Ser	Moroccan	F	11	Yes	0	CGMIM
39	c.143T>G	p.Val48Gly	Likely disease associated	c.604delG	p.Glu202LysfsTer115	UK	M		Yes		Willink
40	c.242C>T	p.Pro81Leu	Disease associated	c.1156C>T	p.Arg386Cys	Other	M		Yes		Willink

Case number	Allele 1		Classification of novel allele(s) per Table 1	Allele 2		Reported country/ethnicity	M/F	Age at diagnosis (years)	GALNS enzyme activity		Reporting institute
	Nucleotide change	Predicted effect on protein		Nucleotide change	Predicted effect on protein				Reported as low?	% Wild-type	
41	c.[566+? 1003-?dup; 1139+? 1140-?del]	complex del-dup: duplication from intron 5 to intron 9, deletion in intron 10	Disease associated	NF	NF	Middle Eastern	F	12			KFSH&RC
42	c.751C>T	p.Arg251Ter	Disease associated	c.268C>T	p.Arg90Trp	UK	F	4	Yes	6	Willink
43	c.1171A>G	p.Met391Val	Disease associated	c.405 422+1del	-	French Canadian		48			SA Path
44	c.925G>A	p.Gly309Arg	Disease associated	c.422G>A	p.Trp141Ter	UK	M				Willink
45	c.1171A>G	p.Met391Val	Likely disease associated	c.704C>A	p.Thr235Lys	French Canadian		8.9	Yes		SA Path
46	c.860C>T	p.Ser287Leu	Likely disease associated	c.758+4a>t	-	Greek	M		Yes		Willink
47	c.740G>A	p.Glu247Asp	VUS, VUS	c.[937A>G; 977G>C]	p.[Thr313Ala; Trp326Ser]	Australian		1.1	Yes	0.8	SA Path
48	c.752G>A	p.Arg251Gln	Disease associated	c.1155C>A	p.Tyr385Ter	UK	F		Yes		Willink
49	c.752G>A	p.Arg251Gln	Disease associated	c.1155C>A	p.Tyr385Ter	UK	M		Yes		Willink
50	c.776G>A	p.Arg259Gln	Likely disease associated	c.1520G>T	p.Cys507Phe	Hispanic	F		Yes	7	CHOC
51	c.850T>G	p.Phe284Val	Disease associated	c.1275delA	p.Val427SerfsTer13	UK	M	5	Yes	4	Willink
52	c.860C>T	p.Ser287Leu	Disease associated	c.1240C>T	p.Gln414Ter	New Zealander		1.8	Yes	0	SA Path

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 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.

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