Title:	**GLA Gene- Fabry Disease Association and Curation**
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1. Purpose and Objective

This Standard Operating Procedure (SOP) describes the process of association of GLA genetic variants, related biochemical results (enzymatic activity and biomarker level), and the provided clinical information for analyzed patients at Centogene AG with different forms of Fabry disease.

2. Area of Application

This SOP applies to Reporting and Curation departments at Centogene.

3. Terms and Abbreviations

GLA: alpha-galactosidase A
FD: Fabry disease
CuRepo: Curation repository
Lyso-Gb3: globotriaosylsphingosine
HPO: Human Phenotype Ontology
VUS: Variant of Uncertain Significance
WES: Whole Exome Sequencing
DBS: dried blood spot
G2P: Genotype – to – Phenotype
IOs: Internal Observations
ERT: enzyme replacement therapy
LIMS: Laboratory information management system
OMIM:Online Mendelian Inheritance in Man
TIA: transient ischemic attack

4. Applicable Documents

SOPeIT-82 GLA genetic variants classification
SOPeIT-86 GLA variant curation
SOPeIT-36 Adding new genes, transcripts and diseases in Curation Repository
SOPeIT-79 Curation of GLA cases

5. Responsibilities

This SOP applies to all employees responsible for curating gene- disease associations.

6. Reagents, materials and devices

Software:

- UniDB: http://ts0001.russ.CENTOGENE.internal/unidbweb/variantsearch
- CentoMD®: www.centomd.com
- Curation Repository: https://srv-centomd.CENTOGENE.internal/curation-repo
- OMIM:https://www.omim.org
- Gepado: https://gepado-prod.centogene.internal/Xpro/
- CentoLSD: https://www.centogene.com/centolsd.html

Other websites often used during gene disease association and curation:

- Pubmed: https://www.ncbi.nlm.nih.gov/pubmed/
- GeneReviews: https://www.ncbi.nlm.nih.gov/books/NBK1116/
- Orphanet: https://www.orpha.net/consor/cgi-bin/index.php

7. Procedure

Before proceeding

A. Background:

GLA gene: is located on chromosome X (Xq22.1) and encodes for alpha-galactosidase A, and is a Lysosomal enzyme that hydrolyzes ceramide trihexoside, and it can catalyze the hydrolysis of melibiose into galactose and glucose.
Fabry disease (FD) is an X-linked inborn error of glycosphingolipid catabolism resulting from deficient or absent activity of the Lysosomal enzyme alpha-galactosidase A. The disorder is a systemic disease, manifest with progressive renal failure, cardiac disease, cerebrovascular disease, small-fiber peripheral neuropathy, and skin lesions, among other abnormalities.
Despite being X-linked, heterozygous females can suffer from symptoms of similar severity to males due to X-inactivation. Females heterozygous for FD show a wide variety of clinical symptoms ranging from GLA levels within normal range without clinical symptoms or disease severity similar to hemizygous males (Metha et al., 2010).
There are two forms of FD:

- Fabry disease OMIM 301500
- Fabry disease, cardiac variant OMIM 301500

B. Workflow description

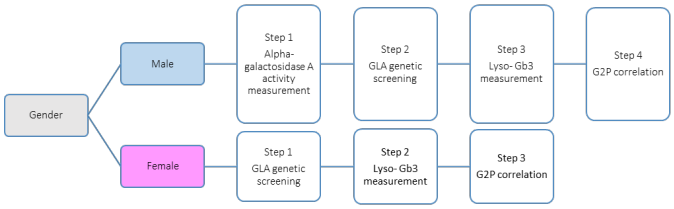
The gene- disease association and curation process implies the review of evidences from internal databases (UniDB, CuRepo, CentoMD®) and the external OMIM database for identification of appropriate FD form.
The internal evidences are collected from internally analyzed patients. These patients were referred at Centogene as:

i. primary request to confirm the clinical suspicion of FD (targeted diagnostics)

The workflow to diagnose patients with clinical suspicion of FD is impacted by the gender of the analyzed individuals.
In case of **male patients**, the corresponding workflow at CENTOGENE starts with a blood-based enzymatic test, which measures the activity of the GLA-encoded enzyme, alpha-galactosidase A. If this test is positive (with pathologically reduced enzyme activity), a GLA-specific next generation sequencing (NGS)-based assay and quantification of a FD-specific biomarker in dried blood spot (DBS)-derived samples are initiated in parallel. Identification of no hemizygous GLA variant despite high biomarker values entails copy-number analysis using multiplex ligation-dependent probe amplification (MLPA). The genetic test is followed by measurements of FD- specific biomarker, Lyso-Gb3. The positive test results (disease- causing GLA variant, pathologically low enzyme activity and pathological high biomarker level) are correlated with clinical symptomatology, establishing the genetic diagnosis (section 7A) for final documentation (including reporting of genetic diagnosis).
In case of **female patients**, the corresponding workflow at CENTOGENE starts with GLA-specific next generation sequencing (NGS)-based assay and if the case (i.e. suggestive clinical picture, positive family history, hints for gross / gene rearrangements), multiplex ligation-dependent probe amplification (MLPA), followed by quantification of a FD-specific biomarker in dried blood spot (DBS)-derived samples. Identification of at least one GLA disease-causing variant

is correlated with the Lyso-Gb3 biomarker levels. The positive test results (disease-causing GLA variant and pathological high biomarker level) are correlated with clinical symptomatology, establishing the genetic diagnosis (section 7A) for final documentation (including reporting of genetic diagnosis). NOTE: Measurement of alpha- galactosidase A enzyme activity is unreliable for identification of heterozygous FD females.
In case of G2P mismatch or non- informative clinical information, the lack of clinical correlation is stated in the case documentation.

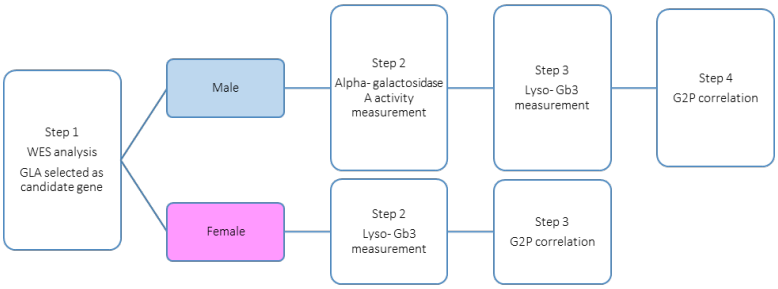
Figure: workflow representation for patients with clinical suspicion of FD disease



ii) primary request was to genetically diagnose a patient for which no clinical suspicion of FD had been raised (screening diagnostics)

At CENTOGENE, the current stand-alone approach in these instances is whole exome sequencing (WES). If any FD relevant GLA variant is identified, clarification of variant clinical class (classification according to ACMG guidelines for novel variants) and G2P correlations implies the measurements of alpha- galactosidase A (for males) and Lyso-Gb3 biomarker (for males and females).

Figure: workflow representation for patients with no FD clinical suspicion



7.1 Review the enzymatic evidences

- The internal alpha-galactosidase A enzymatic results are stored in Gepado, UniDB and CuRepo.
- At CENTOGENE the screening method for alpha- galactosidase A deficiency is based on the quantitative determination of alpha- galactosidase A activity in DBS from male patients. The quantitation is performed by fluorimetry. The current reference for normal values is >=15.3 µmol/l/h.
- The enzymatic levels below the 15.3 µmol/l/h are interpreted as pathological and supportive evidence for presence of FD.

7.2 Genetic evidences

- Results from genetic analyses are stored in UniDB and CuRepo
- In order to associate GLA gene with FD, the patients must present clinically relevant variants (likely pathogenic and pathogenic variants) in hemizygous state for males, or heterozygous / homozygous / compound heterozygous state in females.
- The GLA gene is analyzed by an amplicon based next-generation sequencing approach. The amplicons cover the entire coding region and the highly conserved exon-intron splice junctions.
- To detect gross rearrangements within GLA gene, quantitative PCR assay (qPCR) or multiplex ligation-dependent probe amplification (MLPA) is performed

7.3 Biomarker evidences

- The concentration of the biomarker Lyso-Gb3 in dried blood spot is measured using tandem mass spectrometry.
- Lyso-Gb3 (sphingolipid globotriaosylceramide) is a reliable marker in FD. Lyso-Gb3 levels correlate with disease severity and to decline with enzyme replacement therapy (Aerts et al., 2008)
- The Lyso-Gb3 levels >=2.14 ng/ml (normal reference values <=1.8 ng/ml) are evaluated as very strong pathological and supportive evidence for presence of FD under the PVS2- CENTOGENE criterion (see SOPeIT-82 Classification of GLA variants and SOPeIT- 86 GLA variant curation).
- The Lyso-Gb3 levels >= 1.82 ng/ml (reference <=1.8 ng/ml) but below the PVS2 cutoff (i.e < 2.14 ng/ml) are assessed as pathogenic strong evidence and supportive evidence for presence of FD under the PS3- ACMG criterion (see details of how this observation is applied during GLA variant classification and curation process under PS3 type of evidence in SOPeIT- 82 Classification of GLA variants and SOPeIT- 86 GLA variant curation)

7.4 G2P correlation

- Genotype-to-phenotype (G2P) correlations in FD is challenging, due to “private” pathogenic variants in the many families with FD history and due to significant phenotypic variability carrying the same genetic variant and even within the same family.
- Some individuals present later-onset with atypical variants (Rolfs et al., 2005); see Table 1
- Physician must provide sufficient clinical information, which are stored in internal database (UniDB, CuRepo) following translation into HPO terminology to support diagnosis of FD forms.
- Additionally, use informative families for segregation observations to determine the G2P correlation. We consider families informative with at least three affected family members

Table 1: Phenotypic description of major symptoms for the most important clinical forms of FD

Age	Form /OMIM	Specific Symptoms	Description

Age	Form /OMIM	Specific Symptoms	Description
Childhood Adolescence	Classic/ 301500	Angiokeratoma Acroparesthesia Hypohidrosis/ Anhidrosis TIA/ Stroke Left ventricular hypertrophy	Onset of symptoms usually occurs in childhood or adolescence with the appearance of angiokeratomas, periodic crises of severe pain in the extremities (acroparesthesia), hypohidrosis, and the characteristic corneal and lenticular opacities. Although proteinuria may be detected early, renal insufficiency usually occurs in the third to fifth decade of life. Death occurs from complications of renal disease, cardiac involvement, and/or cerebrovascular disease.
6^{th} decade of life	Atypical/ 301500	Cardiomyopathy Proteinuria	The atypical variant of FD has been reported in which cardiac disease, specifically left ventricular hypertrophy, with or without renal failure, develops in the sixth decade of life. These patients have residual GLA activity

7.5 Validity of GLA – FD association

For a valid GLA-FD association, the curator must agree with biochemical-genotype-phenotype correlation

- Thus, in male patients, pathological alpha-galactosidase A levels must associate with pathological GLA genotype (hemizygote), pathological Lyso-Gb3 (>1.8 ng/ml; reference <=1.8 ng/ml; PVS2 and/ or PS3) levels and suggestive FD phenotype.
- In female patients pathological GLA phenotype (heterozygote or homozygote) must associate with pathological Lyso-Gb3 levels /PVS2 and/ or PS3) and suggestive FD symptomatology
- When no clinical information is provided, but enzyme activity, genotype and Lyso-Gb3 are correlated, GLA-FD association is still valid, however the unknown form of FD is documented as such
- Validity of GLA-FD association is provided below:

7.5.1 Summary of GLA-FD classical form association

Pathogenic variants in GLA gene are associated with Fabry disease, which is an X-linked disorder. The classic form, occurring in hemizygote males with less than 1% alpha-galactosidase A activity usually has its onset in childhood or adolescence with periodic crises of acroparesthesia, the appearance of angiokeratomas, anhidrosis, hypohidrosis, and rarely hyperhidrosis, characteristic corneal and lenticular opacities, and proteinuria. Gradual deterioration of renal function to end-stage renal disease (ESRD) usually occurs in men in the third to fifth decade and additionally cardiac and/or cerebrovascular disease may develop. Heterozygous females typically have milder symptoms at a later age of

onset than males. However, heterozygous females with severe symptoms similarly to the classic phenotype have been reported. Lyso-Gb3 levels are slightly higher in affected males than affected females

The alpha-galactosidase A activity are lower than 15.3 µmol/l/h (IOs 1.2 +/-2.6 µmol/l/h; n=488 Fabry male patients) and levels of Lyso- Gb3 biomarker >1.8 ng/ml (IOs 27.0 +/- 34.5 ng/ ml, n= 664 Fabry male patients; 25.2+/-32.8 ng/ml, n= 597 Fabry female patients).

The age at diagnosis of classical Fabry patients is 35.3 +/-18.8 yrs for male patients (n= 1,258) and 38.0 +/-19.7 yrs for female patients (n=1,027).

Over 99% of the FD male patients with classical form are hemizygote for disease causing GLA variants; less than 1% have more complex genotypes like for example two hemizygous GLA disease causing variants. Over 97% of the FD female patients with classical phenotype are heterozygote for GLA disease causing variants, less than 1% are homozygote or carry at least two GLA disease causing variants with an unknown phase (trans or cis).

7.5.2 Summary of GLA- FD, atypical cardiac variant association

Pathogenic variants in GLA gene are associated with Fabry disease, which is an X-linked disorder. Hemizygous males with greater than 1% alpha-galactosidase activity may develop an atypical Fabry disease, a cardiac variant phenotype that usually presents in the sixth to eighth decade with left ventricular hypertrophy, cardiomyopathy and arrhythmia, and proteinuria, but without end-stage renal disease (ESRD). Female with cardiac disease are asymptomatic during most of their lives and typically present, as male patients, in the sixth to eight decade of life left ventricular hypertrophy, hypertrophic cardiomyopathy (HCM), conduction disturbances and arrhythmias. There are no specific genotypes reported / known for the atypical cardiac variant of FD.
The alpha-galactosidase A activity are lower than 15.3 µmol/l/h (IOs 2.4+/-3.4 µmol/l/h; n= 37 Fabry male- atypical cardiac form patients) and levels of Lyso- Gb3 biomarker > 1.8 ng/ml (IOs 24.9+/-38.1 ng/ ml, n= 57 Fabry male- atypical cardiac form patients; 26.1+/-34.8 ng/ml, n= 51 Fabry female- atypical cardiac form patients).
The internal age at diagnosis of classical Fabry - atypical cardiac form patients is 48.6 +/-15.34 yrs for male patients (n= 148) and 51.8 +/-18.3 yrs for female patients (n=114).
Over 99% of the FD male patients with atypical cardiac form are hemizygote for disease causing GLA variants; less than 1% are carrying two hemizygous GLA disease causing variants. Over 94% of the FD female patients with atypical cardiac form are heterozygote for GLA disease causing variants; roughly 2% are homozygote and the rest carry at least two GLA disease causing variants with an unknown phase (trans or cis).

7.5.3 Summary of GLA- FD, atypical renal variant association

Pathogenic variants in GLA gene are associated with Fabry disease, which is an X-linked disorder. Hemizygous males with greater than 1% alpha-galactosidase activity may develop a renal variant phenotype, associated with end-stage renal disease (ESRD) but without the skin lesions or pain. The early symptoms of classic Fabry disease may not occur in individuals with the renal variant who develop renal insufficiency, and therefore the renal variant may be underdiagnosed. There are no genotypes reported/known for the atypical renal variant of FD.
The alpha-galactosidase A activity are lower than 15.3 µmol/l/h (IOs 5.3 +/-5.0 µmol/l/h; n= 30 Fabry male- atypical renal form patients) and levels of Lyso- Gb3 biomarker >1.8 ng/ml (IOs 22.2 +/-30.5 ng/ml, n=30 Fabry male- atypical renal form patients; 36.1 +/-30.6 ng/ml ; n=11 Fabry female- atypical renal form patients).
The internal age at diagnosis of classical Fabry - atypical renal form patients is 48.3 +/-14.1 yrs for male patients (n= 174) and 49.1 +/-18.8 yrs; for female patients (n=56).
Almost 99% of the FD male patients with atypical renal form are hemizygote for disease causing GLA variants; roughly 1% are carrying two hemizygous GLA disease causing variants. Close to 95% of the FD female patients with atypical renal form are heterozygote for GLA disease causing variants, roughly 2% are homozygote and the rest carry at least two

GLA disease causing variants with an unknown phase (trans or cis).

7.6. Inconclusive data:

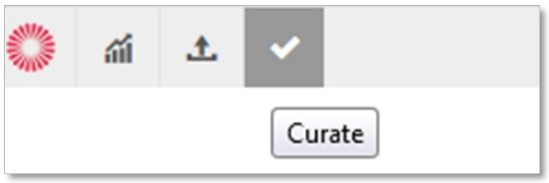
During GLA – FD association and curation, unclear or contradictory observations can be identified. In these situations GLA and FD association remains inconclusive.
Examples of inconclusive or unresolved GLA -FD associations

- Alpha- galactosidase A and / or Lyso-Gb3 are within pathological range, but genetic GLA screening revealed no GLA clinically relevant variant. These cases are labelled as “FD without genetic confirmation” and monitored regularly (quarterly) against new knowledge (DNA changes newly associated with FD including deep intronic, promotor and other regulatory regions)
- Alpha- galactosidase A is altered due to pre-analytical failures. The activities of Alpha- galactosidase A and the internal control enzymes were decreased. Thus, the result of the decreased measurement of enzyme activity cannot be considered as diagnostically valid: These cases are labelled as “pre-analytical failure”, and a recommendation to provide another sample for measurement repetition is communicated to the sender physician.
- Pathogenic genotypes in GLA have been detected in clinically suspected patients, however no Alpha-galactosidase A and / or Lyso-Gb3 could be measured due to insufficient material, or due to inappropriate sample type (DNA only). These cases are labelled as “FD without biochemical confirmation” and recommendation to provide an appropriate sample type (i.e. DBS filtercard) for biochemical confirmation is communicated to the sender physician.
- Alpha- galactosidase A is and / or Lyso-Gb3 are within normal range despite identification of pathological GLA variants. In this situation, clarification if patient is already on ERT is required. In this situation, clarification if patient is already on ERT is required. These cases are not processed for CentoLSD unless clarified.
- The clinical information (healthy, asymptomatic) does not correlate with biochemical and genetic results. In this situation, clarification with physician is initiated. These cases are not processed for CentoLSD unless clarified.
- Patient with positive diagnostic evidence for FD (see above) is suspected having FD and patient age is not provided: when physician does not provide the CI of the patient or no information on the age, then FD classical form (as being most frequent) is used by default. These cases are labelled as “FD without clinical details”.

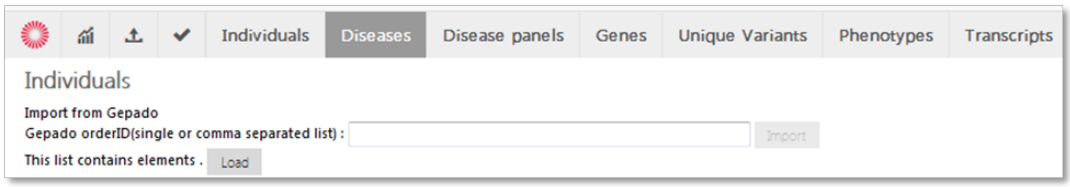
7.7. Storage and management of GLA-FD associations

- GLA gene is associated with different types of FD in CuRepo system as master data under ***Diseases module***. A detailed description of how genes and diseases are submitted and edited in CuRepo is indicated in SOPeIT- 36 Adding new genes, transcripts and diseases in Curation Repository.
- Under ***Diseasesmodule***, the gene and disease associations are documented into a structured format, and only the approved associations are used for curation by case processes (see SOP Case curation: Curation of GLA screened individuals).
- Only curators responsible for gene-disease curation can approve Gene-Disease-MOI associations. Once associations approved, the responsible curators can add changes/ updates
- CuRepo system tracks automatically all applied changes, and display them under the ***History*** option.
- To review the current status of the GLA-FD types associations go under https://srv-centomd.centogene.internal/curation-repo/ and log in

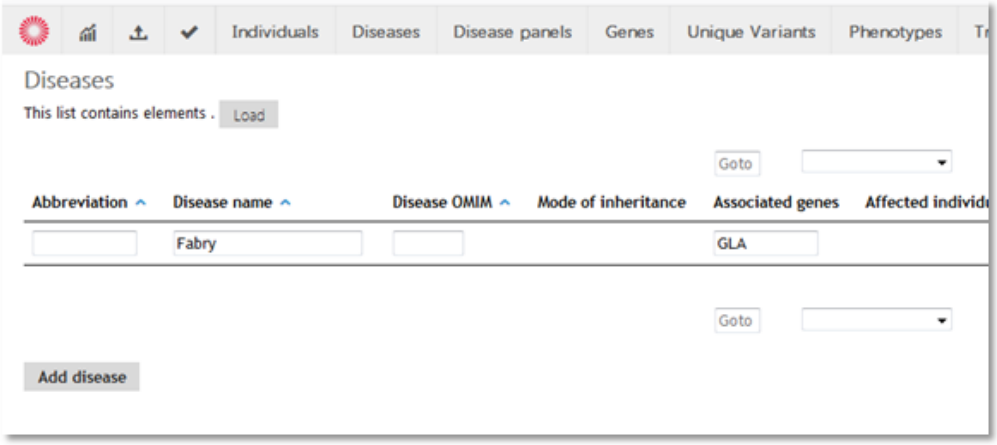
- Select the curation symbol (dark grey below):



- Select ***Diseases*** Module (dark grey below):



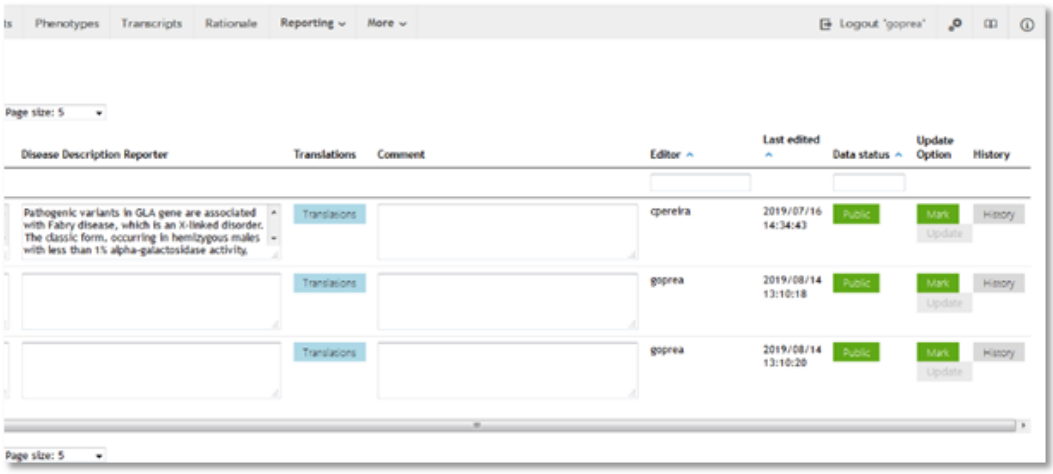
- By default, the structure of this module is indicated as following: ***Abbreviation, Disease name, Disease OMIM, Mode of inheritance, Associated genes, Affected individuals, Disease description, Disease description reporter, Translations, Comment, Editor, Last edited, Data Status, Update option, History.*** The option to add a new disease (***Add disease***) is available
- To initiate a search, add under Disease name ***Fabry***; under Associated genes add ***GLA,*** and press ***Load*** *(see* the screenshot below)



- All GLA- FD associated types are displayed. Each GLA- FD form is represented by one row in the database, and is linked with its own status. Thus during curation by case process, only pre-linked and approved associations are available for selection.

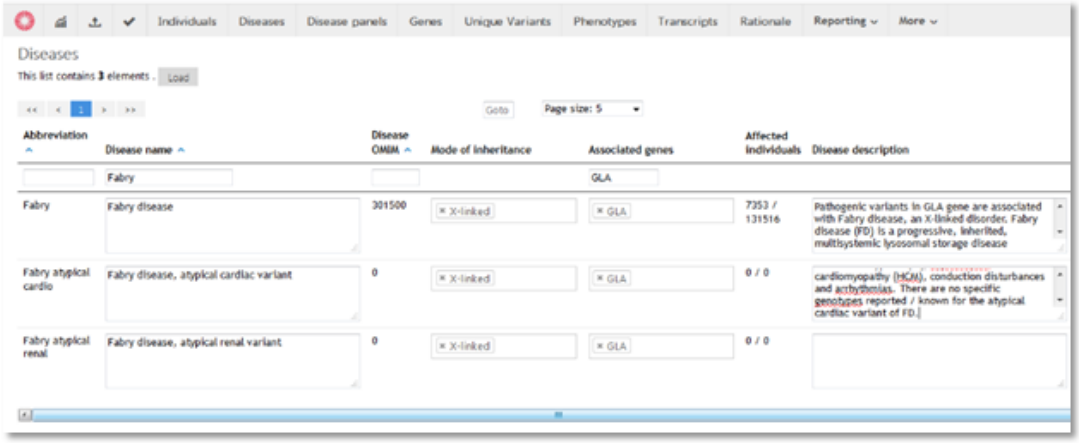
Left side of the screenshot (represented are: ***Abbreviation, Disease name, Disease OMIM, Mode of inheritance, Associated genes, Affected individuals, Disease description)***
Note that current OMIM database (v August 13, 2019) provides the same OMIM ID for all types of FD. CuRepo does not supports multiple disease forms under the same OMIM ID (i.e. it is required an unique ID for each disease name, form or type). Therefore, the two atypical FD forms are at database level linked to OMIM "0".

Right side of the screenshot (represented are: ***Disease description reporter, Translations, Comment, Editor, Last edited, Data Status, Update option, History***). Note that all associations are approved (Data status is ***Public;*** see the screenshot below)

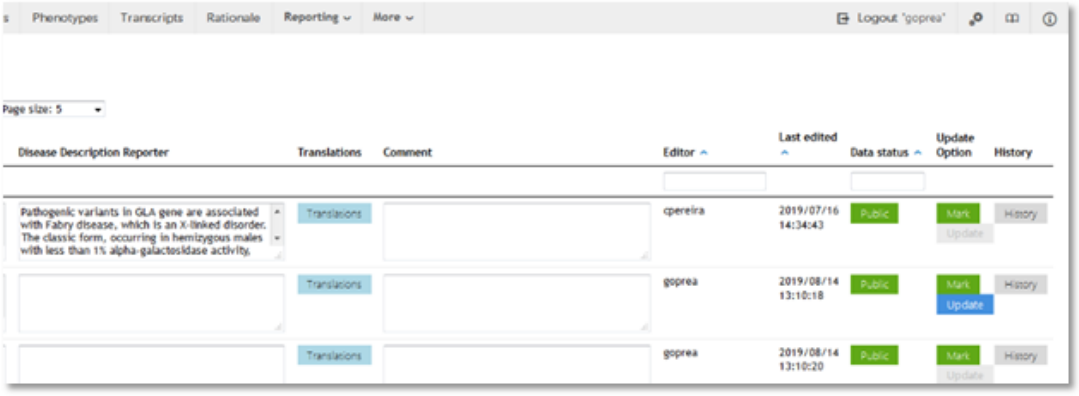


- Curator can edit associations on approved (***Public***) status. Any item subjected to change (i.e. ***Disease name, Mode of inheritance, Associated genes, Disease descriptions, Disease Description Reporter, Comment***) leads to activation of ***Update*** option (by default inactive, grey color; see the screenshot above). Only by pressing ***Update*** option, changes are saved by the system and used downstream (for example during GLA case curation)

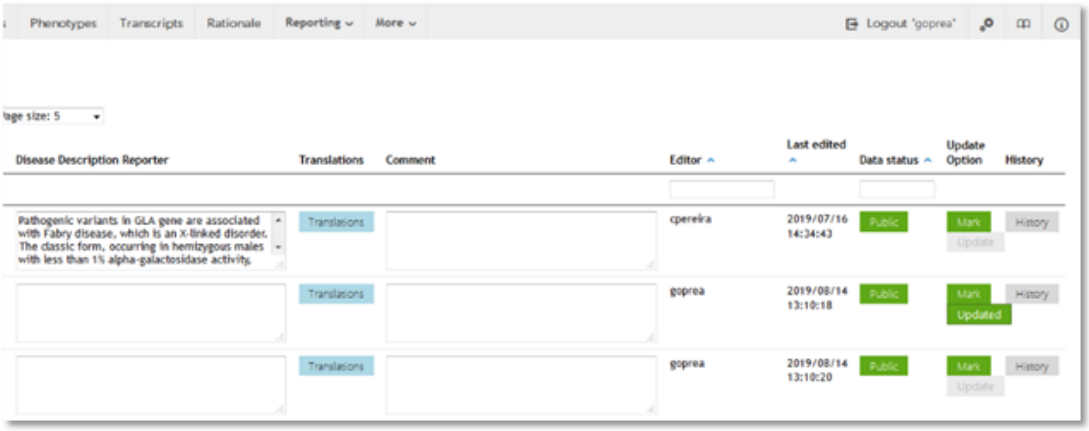
Example of change: Add disease description of FD, atypical cardiac variant
Left side of the screenshot: curator adds the description under Disease description for FD, atypical cardiac variant (see the screen shot below)



Right side of the screenshot: Once Disease description field processed, ***Update*** option becomes automatically active (blue color; screenshot below).

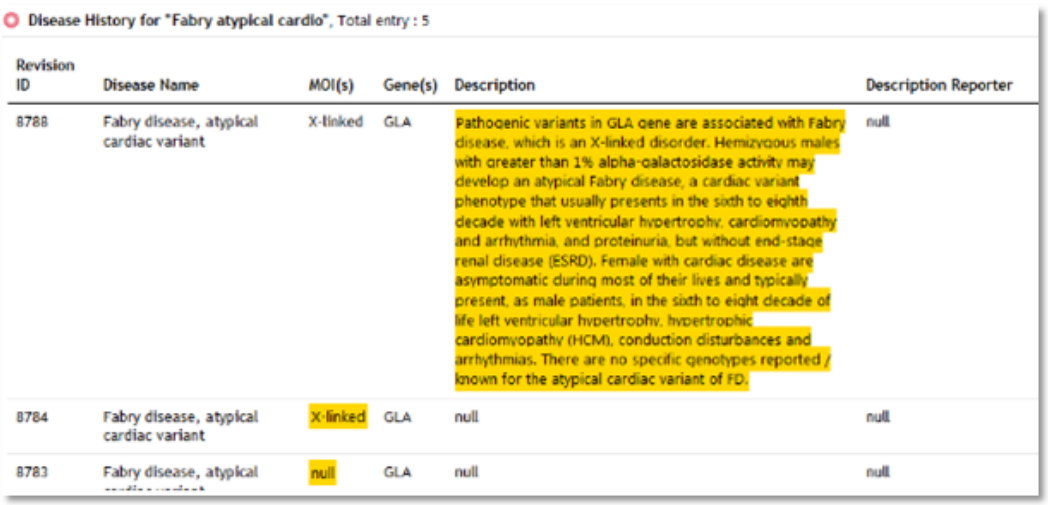


- Press ***Update*** (the blue Update option in the screen shot above turns into green- see the screenshot below):

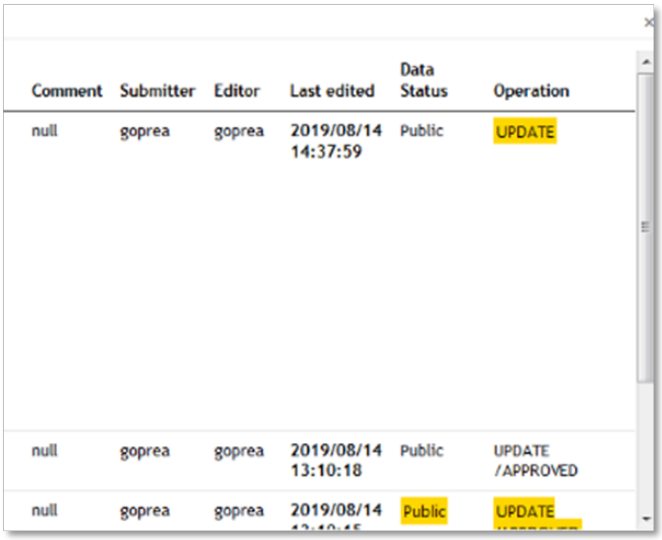


- Under ***History*** all applied changes to an GLA- FD form associations are indicated (see screenshot above). The changes are highlighted (yellow color). Under History window the following details are indicated: ***Revision ID*** (unique number, automatically generated by the system for every saved change); ***Disease name, MOI(s), Gene(s), Description, Description Reporter, Comment, Submitter, Editor, Last edited, Data status, Operation.***

Example of *History* window using the example above, i.e. tracking of changes under GLA- FD, atypical cardiac variant associations.
Left side of the screenshot (including: ***Revision ID, Disease name, MOI(s), Gene(s), Description, Description Reporter***). The revisions display the most up to date one on the top. Note that changes are highlighted under Disease name and Description, respectively).



Right side of the screenshot (including: ***Comment, Submitter, Editor, Last edited, Data status, Operation).*** System indicates for each revision the editor (i.e. who performed the change), when (expressed as date- year/month/day and time- hours:minutes:seconds)



Comment	Submitter	Editor	Last edited	Data Status	Operation
null	goprea	goprea	2019/08/14 14:37:59	Public	UPDATE
null	goprea	goprea	2019/08/14 13:10:18	Public	UPDATE /APPROVED
null	goprea	goprea	2019/08/14	Public	UPDATE

- The GLA- FD associations are used for the following processes:
  - Curation by case
  - Reporting
  - Data transfer for digital products (like CentoLSD, CentoMD®)

8. References

Mehta A, Beck M, Eyskens F, et al. Fabry disease: a review of current management strategies. QJM. 2010;103:641–659.

Aerts JM, Groener JE, Kuiper S, et al. Elevated globotriaosylsphingosine is a hallmark of Fabry disease. Proc Natl Acad Sci U S A. 2008;105(8):2812–2817. doi:10.1073/pnas.0712309105

Rolfs A, Bottcher T, Zschiesche M, Morris P, Winchester B, Bauer P, Walter U, Mix E, Lohr M, Harzer K, Strauss U, Pahnke J, Grossmann A, Benecke R. Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study. Lancet. 2005;366:1794–6.

9. Appendices

1. SOPeIT-77 APPX1_Training module GLA gene Fabry disease curation pba.xlsx