

REVIEW ARTICLE

Genetics and Gene Therapy of Anderson-Fabry Disease

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Abstract: Fabry's disease is a genetic disorder of X-linked inheritance caused by mutations in the alpha galactosidase A gene resulting in deficiency of this lysosomal enzyme. The progressive accumulation of glycosphingolipids, caused by the inadequate enzymatic activity, is responsible of organ dysfunction and thus of clinical manifestations. In the presence of a high clinical suspicion, a careful physical examination and specific laboratory tests are required, finally diagnosis of Fabry's disease is confirmed by the demonstration of absence or reduced alpha-galactosidase A enzyme activity in hemizygous men and gene typing in heterozygous females; in fact the performance of enzymatic activity assay alone in women is inconclusive. Measurement of the biomarkers Gb3 and Lyso Gb3 in biological specimens may facilitate diagnosis. Because of its multisystemic involvement Fabry's disease may present a large spectrum of clinical manifestations as acroparesthesias, hypohidrosis, angiokeratomas, signs and symptoms of cardiac, renal, cerebrovascular involvement (renal insufficiency, proteinuria, left ventricular hypertrophy, strokes). Enzyme replacement therapy with recombinant α -galactosidase A is actually the specific therapy for Fabry disease. Early beginning of this treatment has shown beneficial effects in particular in cardiac and renal disease, a less efficacy it has been reported in central nervous system involvement. ERT has shown to be associated to a significant reduction of Gb3 accumulation in several tissues, in particular heart and kidney; moreover it improves pain related quality of life. Next generation lysosomal storage disorder treatment is based on new strategic approaches as stem cell based therapy, pharmacological chaperones, viral gene therapy; concerning Fabry's disease, it has been recently addressed to great interest this last innovative method, that is to say viral gene therapy, for delivering recombination enzyme into main involved tissues; promising results have been reported in animal models. Great efforts have been made and are still required in this field in order to make available a more effective, safer, advantageous therapeutic strategy for patients with Fabry's disease.

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1. INTRODUCTION

Lysosomal storage disorders comprise a group of rare genetic disorders caused by mutations in genes encoding lysosomal enzymes, resulting in accumulation of several substrates (glycolipids, glycoproteins, oligosaccharides) in different tissues thus causing the clinical manifestation of the diseases [1]. Anderson-Fabry disease is an X-linked lysosomal storage disorder caused by a deficiency of enzymatic

activity of α -galactosidase A, a lysosomal enzyme involved in the metabolism of glycosphingolipids [2].

Storage glycolipid accumulation appears the main cause of clinical manifestations and finally organ failure in Fabry disease. Because of multisystemic involvement, Fabry disease is characterized by phenotypic heterogeneity which is inter and intra familial [3]. Common symptoms are angiokeratomas, acroparesthesia, neuropathic pain, cerebrovascular disease, renal failure, cardiomyopathy. Although Fabry disease is an X linked disorder it does not affect only men; indeed females may develop signs and symptoms because of the random inactivation of the X chromosomes (*Lyonization*) [4]. This process could be responsible for mosaicism which

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characterized females resulting in the escaping of GLA gene from inactivation in the germ or somatic cells [5].

Definitive diagnosis requires genetic testing, biochemical dosage of enzyme activity, tissue exam demonstrating intracellular Gb3 accumulation. If a known, established, pathologic mutation is detected it is sufficient for the diagnosis. On the other hand, for novel mutations or atypical variants, *in silico* analysis might contribute. However *in silico* analysis is not definitive, for example the mutation Asn215Ser is benign *in silico* but it has been found to be associated with severe Gb3 accumulation. Another mutation Asp313Tyr is harmful *in silico* and benign or of unknown significance as a genetic variant. The substitution Arg118Cys is another example of these conflicting results; in fact it was first interpreted as pathologic, later it has been described as a not causing a disease phenotype. In the regard of these examples and to other questioned mutations (Ala143Thr, Ser126Gly, Arg112His), it is necessary a complete detailed precise interpretation of genetic data in association with histological studies demonstrating Gb3 storage [6]. The pathologic study is essential to confirm disease-causing mutations and to disclose and better interpreted the role of novel variants.

It is desirable a wide screening of high risk population from different specialty settings (cardiology, nephrology, neurology, internal medicine, dermatology, ophthalmology) to increase and extend the yield of diagnosis of Fabry disease.

Before the introduction of ERT, Fabry disease treatment was symptomatic consisting of analgesic, anti-inflammatory, opioids for neuropathic pain, ACE inhibitors as nephroprotective, diuretics, antiarrhythmics for cardiac disease [7]. ERT provides an exogenous source of deficient enzyme in patients with this progressive disorder.

Two recombinant enzyme preparations are available: agalsidase alpha and agalsidase beta. The first one is produced in a human cell line, the second one derives from the expression of human GLA gene into CHO cells. Both are administered by intravenous infusion bi-weekly; agalsidase- α at a dose of 0,2 mg/kg, agalsidase β at a dose of 1 mg/kg. Both treatments have been resulted associated with good outcomes: reduction in plasma and urinary Gb3 levels, decreased glycosphingolipid storage in several cells (endothelium, glomerular cells, tubular epithelial cells.) [8-12].

The estimated incidence of Fabry disease is between 1 in 40000 to 1 in 117000 live male births. Fabry disease shortens life expectancy by approximately 15 years in females and 20 years in males. The gene involved in the pathogenesis of Fabry disease encodes a 429 amino acid polypeptide, it is located on the long arm of X chromosome in region q21,22. The enzymatic defect is heterogeneous [13-15].

Diagnosis of Fabry disease is difficult; it requires a high clinical suspicion, good physical examination, specific tests in order to detect premature target organ involvement. Finally confirmation is based on plasma alpha galactosidase assay demonstrating low enzyme levels in affected males and genetic analysis in female carriers who express variable enzyme activity, which is often normal [13].

Biochemical tests include the measurement of enzyme activity in plasma, in leukocytes from peripheral blood, cul-

tured fibroblasts, in samples extracted from filtered paper blood spots. Another biochemical test consists of measuring globotriaosylceramide in 24 hours urinary collection, it is mainly performed in female subjects [16].

However, biochemical tests have a low sensitivity in females and in those males with residual activity of alpha galactosidase A.

2. GENETICS OF FABRY DISEASE

The GLA gene is a 12kb gene with 7 exons, situated in the long arm of the X chromosome. The protein encoded by this gene, firstly constituted of 429 amino acids, is afterward processed to a 370 amino acid glycoprotein. It exists as a homodimer in lysosomes.

The identified mutations in Fabry patients are numerous, the **principals** are missense/nonsense mutation, followed by small deletions, small insertions, small indels, large deletions, splice defects, complex rearrangements and finally large insertion [16]. These genetic **mutations** are related to many different abnormalities such as irregular intracellular trafficking, altered protein folding, reduced activity of the active site, and less affinity to substrates. However, a lower residual enzyme activity can **occur** in the late onset variants [16]. This large and heterogeneous pattern of possible mutations known in the GLA gene, furthermore associated with predictable numerous other possible de novo mutations, suggest that a suggestive clinical pattern, even in the absence of a positive family history, does not exclude Fabry disease.

The reduced activity of alpha galactosidase A results in an accumulation of glycosphingolipids, that the cells are unable to catabolize, determining the **increase** of the storage, especially of Gb3, in all organs causing a **multisystemic** dysfunction in classic forms [17].

A lot of genetic variants have been identified, thanks to the spread of screening for Fabry disease; numerous variants are of unknown significance (GVUS), infact most of these subjects does not manifest typical features of Fabry disease. Since the approbation of ERT, numerous screening studies have been performed in high-risk population and in newborns. In high-risk population, the prevalence of individuals with a *gla* variant was 0,6%, in newborn screening was 0,04%. Several studies show that a significant number of male patients with GVUS presented residual enzyme activity. On the other hand, affected males with classical mutation do not express enzyme activity or show near absent *gla* activity. Among variants frequently described in the *gla* gene there are p.D313Y, p.E66Q, p.A143T, p.R118C, p.N215S, p.M296I, p.R112H [18, 19].

Fabry mutations are transmitted by X-linked inheritance; it means that the gene causing the trait or the disorder is located on the X chromosome; this causes the phenotype to be expressed in males who are necessarily hemizygous for the gene mutation. Males inherit mutations from their carrier mother. Carrier females, who have only one copy of the mutation, do not usually express the phenotype, although differences in X chromosome inactivation can lead to varying degrees of clinical expression in carrier females, since some cells will express one X allele and some will express the other. Males have almost 100% penetrance, on the other

hand, females have about 70% penetrance. In regard of this high penetrance it is not perfectly correct to define females as healthy carriers; indeed clinical manifestations are present in two-thirds of women, which usually manifest mild late-onset form of the disease. However, severe forms with organ damage are also described in females; this is explained with Lyon hypothesis according to which one of X chromosome is inactivated because of a process involving repetitive sequences of DNA, the other active chromosome gives all the genetic information. Inactivation is random so females are also "mosaic" for any genes that are present in different alleles on their two X chromosomes.

Lyonization consists of a random X chromosome inactivation in the different tissues and organs in the female's body; for example in one organ may occur in the 70% of the cells the expression of the healthy gene and respectively in the 30% of the expression of the defective gene. This phenomenon is the basis of the fact that the X-inactivation pattern in an organ is related to a different clinical manifestation, the severity of the symptoms, the severity of organs involvement.

Heterozygous female may express a great broad clinical variability. It is very likely that other factors influence the genotype-phenotype correlation.

Studying the phenotypic expression of a given mutation and its pathogenesis requires a careful homogeneous multidisciplinary evaluation both of the proband and his collaterals [20, 21].

The isolation and sequencing of the entire genomic sequence encoding alpha galactosidase A have allowed detection and characterization of the mutations causing Fabry disease.

The genotype of Fabry disease is heterogeneous with more than 800 mutations of GLA gene identified so far. Newborn screening has allowed to report a high incidence of disease. A wide spectrum of mutations has been detected: missense, nonsense mutations, splice-site mutations, insertion-deletion mutations, gene rearrangements. About 60% are missense mutations causing single amino acid substitutions in alpha galactosidase A [22, 23].

The amino acid residues in which the mutations are most frequently localized are the follows:

TRYPTOPHANE. It has the highest frequency of mutations between the amino acids of the α GAL A. Tryptophanes are usually targets of nonsense mutations. It contributes to the formation of the hydrophobic core of the protein, so point mutations of this residue may negatively affect the proper polypeptide's internal folding.

CYSTEINE. It is the second most commonly mutated residue. All mutant-affected cysteines are involved in disulfide bonds; those bonds, present in number 5 in the protein, confer stability to folded protein. Mutations in this residue are associated with severe rather than moderate clinical forms of the disease.

GLYCINE. Mutations affecting this amino acid residue alter the protein's stability.

ARGININE and LISINA. The arginine side chain has both an aliphatic portion and a charged planar guanidine group; Arginine can therefore, make hydrophobic and hydrophilic interactions. The preponderance of mutations affecting arginine contrasts with the shortage of lysine mutations that is chemically similar, underlining the importance of arginine in protein folding. Lysine is the only amino acid which until now has not been found mutated in mAF patients.

CHARGED RESIDUES. Mutations that affect charged residues are common. Point mutations that produce a spilled charge or out of place destabilize the protein's conformation.

HIDDEN RESIDUES. These mutations create defective folding polypeptides, in which the hydrophobic core is destroyed and the enzyme is not able to fold or remain conformed to the acidic environment of the lysosome.

CARBOHYDRATE BINDING SITES. A GAL A contains 4 potential attack sites for N-linked carbohydrates (N139, N192, N215, N408) one of which (N408) is not used. The 3 sites are located in domain 1. N215 is the only attack site affected by mutations that produce a moderate variation of the disease.

MUTATION NEXT TO THE ACTIVE WEB SITE. The highest frequency of mutations affects the region included within 10 angstroms from the galactose binding site.

The active site is highly sensitive to mutations, even conservative substitutions (D92N) can lead to complete loss of enzymatic activity.

INTERFACE BETWEEN THE TWO DOMAINS. A GAL A acts as a homodimer with a wide interface between the two monomers. The interface includes 54 residues, 27 for each domain; It maintains the orientation of the 2 domains. Domain 2, although having a non-known function, is required for the stability of the enzyme, the mutations that affect it may, in fact, lead to complete loss of enzymatic activity.

HOT SPOTS. In the linear amino acid sequence, 3 segments have a higher frequency of mutations: the portions between amino acids 162-172; 215-231; 258-269. The first segment encloses *pleated sheet* β 4, the second includes α 5 helix and β 6 *pleated sheet*, the third includes β 7 *pleated sheet*.

STOP MUTATIONS. They produce a poorly folded protein with a shorter polypeptide chain; Sometimes no protein is produced because of the early degradation of the mRNA. Regardless of the domain concerned, they are associated with a severe phenotype.

1989 Bernstein *et al.* identified the first exonic point mutation of codon 356 causing abnormalities of kinetics and physical properties of the enzyme [24].

1990: Hitoshi Sakuraba *et al.* reported two point mutations in two Japanese families. One proband was a hemizygous man affected by classical form of the disease caused by a substitution resulting in early truncation of the polypeptide chain. The second proband was affected by an atypical variant of Fabry disease caused by a substitution of one amino acid residue 301 associated with residual enzyme

activity. The substitutions in codons 301, 356 involve CpG dinucleotide sites, hotspots for point mutations [25].

1993 Christine M Eng *et al.* carried out a study in 148 unrelated families; they described 20 mutations, of which 17 were reported as novel mutations in subjects with classical phenotype: N34S, C56G, W162R, R227Q, R227X, D264V, D266V, S297F, D313Y, G328A, W340X, E398X, IVS2+2, IVS5 Δ-2,3, 773 Δ2, 954Δ5, 1016Δ11, 1123Δ53. This study showed codon 227 as a hotspot point. They reported 10 missense mutations, 3 nonsense mutations, 2 splice site mutations, 4 **deletion**, 1 insertion. Each mutation was described in unrelated patients, thus highlighting the genetic heterogeneity of molecular lesions [25].

2001 Grace A Ashley *et al.*, reported novel mutations and 20 novel mutations (M51K, D92N, D136H, F169S, C172F, L191Q, S247P, Q250X, P259R, G261D, T282N, R301P, W349X, T410K, 124delAT, 842delTAA, 1033delTC, 82insG, 893insG, 903insG) in 40 unrelated families with classical phenotype of the disease. Among novel mutations, fourteen were missense or nonsense mutations, 3 microdeletions, 3 microinsertions; among these mutations one was particularly interesting: it was an **insertion** g82insG causing a frameshift mutation in codon 30. This results in the production of a truncated protein which is degraded by proteasome system.

2002 Garman and Garboczi distinguished two main classes of alterations of the protein on the basis of the site of point mutations: mutations in the active site and those causing errors in protein folding. The authors put together about 245 mutations of GLA gene including 171 point mutations, 20 non sense mutations, 12 splice site mutations, 42 deletions and/or insertions. All cases of Fabry's disease are characterized by a single mutation, except two individuals that presented double mutations (E66Q/R112C e L45R/H46S). Mutations that overall cause severe phenotype are located at the hidden site of the protein, on the other hand, mutations which are associated with moderate phenotype are usually less destructive of the hydrophobic core [27].

2002 Satoshi Ishi detected a rare alternative splicing introducing an intron sequence (57 nucleotides) into the intron 4 of the gene; moreover, they identified a novel substitution resulting in an increased alternative splicing in a patient with a cardiac variant of the disease. The sequence of the intron 4 includes a GA transversion at nucleotide position 9331 (IVS4+919G-A) that is placed at 3'terminal intron insertion (nucleotides 9278-9334). This mutation occurring in intron 4 was found in 6 patients affected from cardiac variant of the disease and is associated with residual enzyme activity of 10% leading to cardiac phenotype. All splicing **mutations** have been reported in flanking regions of exons resulting in exon **slippage** or in abnormal splice site recognition [28].

2003 Makiko Yasuda *et al.* identified two novel frameshift mutations (1277delAA (del2) and 1284delACTT (del4)) in two men with classic form. This is the first description of mutations concerning the formation of 3'terminal. These two mutations generate multiple transcripts characterized by various **lengths** of 3'terminal end sequence.

The characterization of the above-mentioned mutations allowed the identification of a novel molecular mechanism

causing the disease. **The polyadenylation** signal is found within coding sequence; polyadenylation code likely is important for translation and stability of mRNA. Moreover, it helps to the nuclear export. In this regard, defects of the 3'-terminal end may induce deep effects of genetic expression, thus leading to disease. Numerous mutations involving polyadenylation signal have been identified as responsible of Fabry disease. Human GLA gene is the only one of eukaryotes genes lacking of a 3' untranslated region except for a rare variant with a short 3' UTR. The discovery of these defects (del2 and del4) was thus interesting; subsequent studies have been carried out in order to help characterize these molecular lesions [29].

2010 Dominique P. Germain and co-workers identified novel mutations as Met42Arg, Gly43Ser, Gly132Glu, Lys168Asn, Gln212Stop, Phe295Cys, Leu300Pro, Gly328Glu [30].

These studies and others show the wide heterogeneity of GLA mutations. All members of a family which is suspected to have Fabry disease, should be subjected to genetic analysis because the detection of a mutations allows more accurate detection of disease carriers and offers the possibility of doing prenatal diagnosis. Besides, the discovery of novel mutations is useful to evaluate genotype-phenotype correlation and provides a better interpretation of structure-function relationships.

Mutations are responsible of altered synthesis, trafficking, folding, degradation of the enzyme and reduced enzymatic activity [5].

Diagnostic work up includes genetic, biochemical, histological testing.

Biochemical assay evaluates the enzymatic activity in the blood or white blood cells; it is also used to measure plasma levels of Lyso Gb3, globotriasylsphingosine. Genetic testing is commonly performed with **the** biochemical study [5].

Gb3 and Lyso Gb3 represent sensitive biomarkers, their measurement could facilitate diagnosis in females and in the early stages of Fabry disease; moreover they could be useful for establishing effectiveness of ERT. Alpha Gal A activity is considered pathogenetic if it is reduced of at least one third of the mean normal value. In reality, numerous studies suggest that there is not a true threshold, each variant may be associated with biological, histological clinical alterations [5].

A linear relationship between enzyme activity and sphingolipids accumulation in a target organ may lead to the hypothesis that the phenotypic expression of the disease is a continuum, so that complete clinical manifestations reflect the extreme end of this spectrum [5].

In the presence of a strong clinical suspect, genetic screening by DNA sequencing is the first approach so that you can identify pathogenetic variants. GLA gene is also carrier of several missense mutation and rare variants that have been detected in general populations and which should not be considered pathogenetic variants. For example, the p.Asp313Tyr variant has been described as causal variant of Fabry disease; however it has been reported to show a frequency of 0,4% in the non -Finnish European population,

this value is greater than prevalence of Fabry disease in that population, so it is not considered a disease causing variant, it has often reported as deleterious or probably damaging [31].

The genetic landscape of Fabry disease is complicated by some factors (genetic background, epigenetics, probably environmental) that influence functional and phenotypic consequences of a specific variant so that variant results pathogenic in some genetic background but not in other ones.

The recognition of numerous variants of GLA genes has brought to define criteria by which a mildly symptomatic or asymptomatic patient, that is carrier of a specific variant would be considered to develop disease-related complications. Additional factors could influence the relationship between genotype and phenotype of Fabry disease. They include genetic, epigenetic, environmental factors.

Some polymorphisms have been associated with some clinical manifestations. For example, G-174C of interleukin 6, G894T of endothelial nitric oxide synthase, the A13G and G79A of the protein Z seem to be correlated to the presence of ischemic cerebral lesions [32].

Three groups of alpha galactosidase A residual activity have been described: 0 to approximately 10% of normal activity in males; the second group includes subjects with residual enzyme activity in the range 15 to 30% of normal; enzyme activity is above 35-40% of mean normal activity.

The first group has been associated with nonsense and certain missense mutations. Moreover, this group seems less influenced by factors as environmental and or epigenetic. The second group has been associated with missense and some splice GLA mutations and the phenotype could be importantly influenced by genetic and epigenetic factors. In the last group, clinical manifestations don't seem to be related to the level of enzyme activity.

A controversial group exists: it consists of likely non-pathogenic mutations. An example of these insidious mutations is the mutation D313Y. First it was reported as pathogenic mutation associated with cerebrovascular involvement. On the other hand, other works reported normal or minimally decreased enzyme activity in white blood cells [33-36].

In the regard of the above mentioned variation it is not simple to establish the pathogenicity of a GLA variant. To date, the best method to assess whether the deficient alpha galactosidase A activity is clinically important is the demonstration of accumulation of Gb3 in tissue specimens [37-39].

Our group has described a novel mutation of α galactosidase A gene: c.614delC [40].

Recently, we have reported some clinical cases of a Sicilian family to express phenotypical variability of Fabry disease in subjects with the same genetic mutation. In all members direct sequencing of α galactosidase A gene revealed a polymorphism -10C>T; IVS 2-76_80del5; IVS 4-16 A>G; IVS6-22 C>T. Intronic mutations and polymorphisms of single nucleotide have a role which is not clear yet. A better acknowledgment of these genetic alterations is required in order to explain the variability of clinical expression and disease severity also in the males. The significance of this

haplotype in Fabry disease remains unknown. Bono *et al.* have recently described milder or asymptomatic patients with residual alpha galactosidase A activity, of these subjects 12% showed polymorphism in the promoter region of the GLA gene; in particular 99% expressed simultaneous polymorphisms spread throughout the gene. The -10C>T; IVS 2-76_80del5; IVS 4-16 A>G; IVS6-22 C>T and -12G>A, IVS4+68 A>G, IVS 6-22C>T occurred simultaneously in 8,9% and 3,7% of the subjects. Although some individuals with intronic mutations or single nucleotide polymorphism a careful clinical follow up before to start enzyme replacement therapy, avoiding ERT in all these subjects could result in the eventual progression of the disease [41].

3. GENE THERAPY

Since its introduction, ERT has shown to be effective in slowing down disease progression or in stabilizing Fabry disease, above all if it is started at early age. Enzyme replacement has the inconvenience of biweekly intravenous infusion throughout life.

To date, enzyme replacement therapy is the only available specific treatment for Fabry disease. Two recombinant enzyme preparations are available since 2001 when they were approved by European Medicines Agency: both are administered biweekly, agalsidase alfa is administered at a recommended dose of 0.2 mg/kg and agalsidase beta at a dose of 1 mg/kg, the latter was also approved by FDA [2]. It has been shown that ERT importantly attenuates pain and ameliorate quality of life [42]. Moreover, it causes an important reduction of tissue globotriaosylceramide storage in heart and kidney [1].

Ramaswami and other researchers reported the improvement of kidney and heart pathology at an early phase of the disease after ERT [43].

However, ERT is not likewise effective once targets organs are damaged severely [44]. Enzyme replacement therapy has other drawbacks. It usually triggers the production of anti- α galactosidase A antibodies, representing a limitation of the treatment and thus leading to unsatisfactory results [45]. Another disadvantage is the annual cost of this therapy. In recent years, new expression systems have been developed in order to intensify the production of recombinant enzyme and reduce costs of enzyme replacement therapy.

Recently, in order to obviate the negative aspects of ERT, novel approaches aim to increase bioavailability and to reduce immunological response. In mice models and on cultured fibroblasts some researchers had reported the efficacy of a molecule of modifier α -N-acetylgalactosaminidase with α -galactosidase A like substrate [46].

Gene therapy and chaperons are new approaches on development. The latter could be used in conjunction with ERT [7].

Among these systems Calhoun developed a promising one, based on Baculovirus expression system [47, 48].

In the light of these limitations, new therapeutic strategies are actually investigated and pursued. Successful ERT may be impeded by immune reactions, miss-targeting of recombinant enzymes, inadequate level of the drug in impor-

tant tissues as brain and bone. Brain is affected in the most severe phenotypes. Stroke is one of the most significant manifestation of Fabry disease and represents an important risk factor for premature death. Hence the need to develop new therapeutic strategies to target the brain [1]. In order to overcome these difficulties it comes the need of further research to improve therapeutic strategies. Future alternatives include pharmacological chaperones, gene therapy, stem cell based therapy [1].

Among therapeutic options, pharmacological chaperons appear an encouraging treatment for specific variants of the disease. This approach induce and facilitates the correct folding of mutated enzymes. A pharmacological chaperone is a small molecular inhibitor that corrects the folding of a mutated enzyme, stabilizing the tertiary structure of the enzyme; thus preventing its degradation in the endoplasmic reticulum, this also results in enhanced enzymatic activity [1]. One of the newest therapies for Anderson-Fabry disease is represented by the utilization of pharmacological chaperones or substrate reduction and, between these, the 1-deoxygalactonojirimycin which is currently evaluated in a phase 3 clinical trial, is the most advance for clinical use. These molecules act binding the mutant α -galactosidase A, improving, in this way, its stability and favoring its transfer to the lysosomes. The patients who can have the best benefits with this approach are those with non-classical phenotype, that are usually characterized by missense mutations and so could be more sensible to this chaperoning effect [49].

Some studies have reported positive results with the compound 1-deoxygalactonojirimycin. DGJ is actually administered orally in a phase III clinical trial (Amigal, Amicus Therapeutics, Cranberry, NJ, USA). Phase II trials indicated that DGJ was safe and well tolerated. The handicap of this therapy is the possibility to use chaperone therapy only in those patients with mutations causing expression of misfolded proteins [50]. Migalastat (1-deoxygalactonojirimycin) binds active site of specific mutant enzyme resulting in the stabilization of the protein, regulation of their trafficking to lysosomes, finally allowing the enzyme to catabolize its substrates [50]. Previous studies have employed an *in vitro* assay in order to measure the response of α Gal A mutant forms to migalastat; this approach could allow the detection of patients to be submitted to the treatment with pharmacological chaperons [51].

Thanks to its mechanism of action migalastat seems to increase the levels of α galactosidase A more significantly than ERT, which is administered every other week. Moreover migalastat may possess few disadvantages compared to ERT that could be associated to immunogenicity and infusion-associated reactions [50].

It has been observed that DGJ increases and facilitates the activity of lymphocytes from patients with Fabry disease, the transport of the altered enzyme to the lysosomes and the metabolism of the accumulated Gb3 *in vivo* [52-54]. Many other amino sugars analogues have been developed and tested on patient's derived cells in particular in subjects affected from other LSDs, as Gaucher disease, [55-58].

Numerous mutations cause the expression of enzyme with residual enzyme activity but very instable because of their misfolded structure. Asano *et al.* investigated the opportunity of using specific chemical chaperones in order to avoid that mutant enzymes are degraded in ER, allowing them to display their residual activity [59]. However the predictability of efficacy in these patients requires a direct experimental demonstration. The response to DGJ is indeed variable as reported by several studies [60, 61].

The main limit of chaperone treatment is that it is indicated only for those patients with missense mutations causing the production of misfolded GLA proteins.

In responder patients chaperone therapy is associated to improved enzyme activity and GLA levels, decreased levels of Gb3 accumulation demonstrated with kidney biopsies and in urine exams [7].

In many studies, it has been demonstrated that some mutant α -galactosidase A proteins are unable to express their catalytic activities because of their instability [62-64]. Galactose analog compound 1-deoxygalactonojirimycin A and galactose itself are able to restore the activity of mutant α -galactosidase A in cells and tissues of Fabry's patients.

It is possible to classify the molecular pathology of inherited metabolic diseases into three groups according to the structure and function of mutant proteins:

Biosynthetic deficit

Biological activity deficit

Unstable protein with normal or almost-normal activity: in its normal mature folded form the mutant protein preserves its biological function.

In the last of those three classes, it is possible to expect that if something could preserve the stability and the transfer to the correct cellular compartment (the lysosomes in this case) the protein should maintain its biological activity; and this represents the principle of action of the chaperone therapy. This kind of therapy is particularly important in the patient with a brain involvement in which those compound are delivered through the blood brain barrier to the central nervous system.

The kinetic properties of the wild type and the p.Q279E mutant enzymes, transiently expressed in COS-1 cells, are almost the same with the exceptions that the PQ279E proteins are unstable at neutral pH in the ER/Golgi apparatus in which are rapidly degraded, and they have a low catalytic activity due to their molecular instability. A study demonstrated that a treatment with intravenous of galactose had beneficial effect in a Fabry patient with hypertrophic cardiomyopathy [65], however, a long term treatment with high dose of galactose in human cannot be realistic, so the 1-deoxygalactonojirimycin, which has the capacity to guarantee the stabilization especially on mutant α -galactosidase A and 50-fold lower on β -galactosidase, has been tested and could represent a new molecular therapy in Fabry disease as chemical chaperone.

The utilization of pharmacological chaperones has been proposed as a new way of therapy in lysosomal diseases, especially in those with an involvement of the CNS. Today,

enzyme replacement therapy is largely used, with successful results, for extraneural pathology [66], results that unfortunately has not been confirmed for neural tissue damage instead and another disadvantage could be the intravenous administration for life.

The other clinical approach that has been tested is the inhibition of glucosyl-transferase with the purpose to preserve the storage of substrates, however this approach, depriving cells of the biological activity of glycosphingolipids could generate various dysfunctions in the somatic cells and side effects such as diarrhea and headache.

Chemical chaperone therapy could restore the missing enzymes activity in somatic cells and tissues and has the advantage of a non invasive administration and, even though is a mutation specific drug therapy, at least one-third to half of patients with Fabry disease can be candidates of this therapeutic trial [67].

FUTURE THERAPEUTIC PERSPECTIVES

The pseudoexon activation is an important disease mechanism which has been studied and progressively clarified during the last years. Pseudoexons are vulnerable portions of DNA sequence and several single nucleotide variations could be enough to modify the fine balance between positive and negative splicing regulatory factors, thus resulting in pseudoexon inclusion and disease. In this regard, it is desirable a therapeutic approach which should act upon pseudoexon activation. Mutations that cause pseudoexon activation are likely underreported because intronic sequences are not evaluated during routine diagnostic approach.

Palhais *et al.* investigated the splicing regulatory mechanism, which leads to the activation of GLA pseudoexon. In order to clarify this interest, it is a need to refer to a deep intronic mutation c.639+919 G>A that was described as the most prevalent mutation in the Taiwanese population. This substitution causes inclusion of a 57 bp pseudoexon in the GLA product, causing an in frame TGA premature stop codon and production of a truncated protein [68, 69].

Recently, splice switching oligonucleotides (SSO) have been described as a promising potential therapeutic alternative for diseases caused by aberrant splicing [29-31 di 6]. SSOs recover the correct splicing pattern by impeding the access of SRE or spliceosome components to their binding sequences, thus regulating pre mRNA splicing. In this context, pseudoexon activating mutations are potentially responsive to SSO based therapy because the original splicing signals are maintained thus splicing correction could result in expression of wild type mRNA. The authors studied the possible response to SSO based therapy in those variants of Fabry disease caused by the c.936+919G>A because the main organs affected in this variant (heart and kidney) are the same organs in which SSO reach high concentrations according to their biodistribution. In particular, they reported that specific SSOs may induce pseudo exon skipping and restore physiological splicing of normal GLA transcription products [70].

In the GLA gene, the pseudoexon activating mutation is located near the 5' ss, the researchers designed three different

SSOs for matching the mutant sequence c.639+919G>A overlapping the terminal 5'ss and one which overlaps the 3'ss.

SSOs can be modified in order to target specific mutation. This is a potential action reinstating the full length protein. This study is an interesting demonstration of the potential role of SSOs as effective therapeutic alternative in those patients that are carriers of mutations causing pseudoexon inclusion as c.639+919G>A, which has a high frequency [68, 71, 72].

Gene sequencing has become an important clinical test, which is carried out in the context of the diagnostic assessment of several diseases; the wide application of this procedure has been leading to the availability of personalized therapies for specific genetic mutations. Moreover, the reduction of costs for genetic analysis has allowed its use more routine in numerous contexts. Fabry disease is a good example demonstrating this notable evolution [73].

During the last decades, an array of different viral vectors and viral serotypes has been investigated for performing gene therapy in the treatment of lysosomal storage disorders. Successful results have been obtained in animal models; also clinical trials reported promising results on metachromatic leukodystrophy [74, 75].

Numerous advantages characterized gene therapy compared to enzyme replacement treatment for example, it is associated with long term therapeutic effect, it is not risky in regard to parental administration, moreover, it is less expensive than ERT. In the field of Fabry disease, gene therapy should require the transfer of GLA gene into mutated cells.

In mice models, it has been observed that the therapeutic effects of this approach are influenced by several factors as the sex of mice, the type of vectors, the choice of promoter, the method of administration of the vectors.

One method consisted of the administration of rAAV-GLA (adeno-associated virus carrying human GLA) *via* the hepatic portal vein [76]. Authors reported that GLA activity in the livers of transfected mice was 20-35% of that of the normal animals two weeks after the injection. In addition to increase enzyme levels, they observed a relevant reduction in Gb3 levels almost to the point of normalization. The lowering of Gb3 concentrations was described up to 25 weeks after treatment in liver, heart, spleen, also they did not report notable immune response to the virus or GLA. Nevertheless the portal vein delivery is clinically disadvantageous if the procedure should be performed many times [7].

Adeno-associated virus vector including GLA gene driven by chicken actin promoter and CMV enhancer, could be injected into muscle. Takahashi *et al.* investigated this route of administration showing a significant increase of plasmatic GLA activity up to 30 weeks. Moreover, they reported the complete elimination of Gb3 from tissues and improvement of Fabry related cardiomyopathy [77]. On the other hand, Park and colleagues did not show positive results performing this same route of application, however they described satisfactory results by using intravenous administration. Another *via* of introduction in lung for its function of secretions of proteins into the blood vessels [78, 79].

Over recent years, in order to reduce Gb3 concentrations in the kidneys, which are common target organs in Fabry disease, it has been developed a strategy consisting of administration of a solution into the left kidney of Fabry mice. This preparation contained naked plasmid DNA encoding GLA gene that is introduced through hydrodynamics based transfection. These authors reported good outcomes as increased enzyme activity in the treated kidney and in other organs (liver, heart) and reduced tissutal Gb3 levels [80]. Other experimental studies have been conducted in neonatal period. The effects of gene therapy were good. They were obtained with the introduction of AAV vector or lentiviral vector. This allowed a good GLA expression preventing also GB3 storage [81]. Fabry disease is a good example of disorder that could be treated with gene therapy. Cross-correction mechanisms give the opportunity to transfect a small number of cells resulting in the potential correction of distant cells too [82].

Gene transference is usually performed through two strategies: *ex vivo* gene therapy and *in vivo* therapy. The first one consists of an *in vitro* cultures of cells extracted from the patient; after this step they are reimplanted. *In vivo* gene therapy consists of a direct administration of the genetic material which could be systemic or in local site. Actually, several types of vectors are available for their application for Fabry gene therapy: retroviral vectors; lentiviral vectors, adenoviral vectors, adenoassociated viral vectors, non-viral vectors [2].

In 2000, a clinical trial was approved in the USA for Fabry gene therapy using a strategy with a retroviral vector; however its protocol was later withdrawn [83]. Passineau *et al.* have recently tried to treat Fabry disease with adenoviral vectors. In this animal model, adenoviral vector expressing GLA gene was administered *via* oral cannulation of the submandibular duct. Researchers measured enzyme activity after 4 days finding a significant increase levels in the serum and in the liver. However they did not evaluate the effects of this increased enzyme activity on the Gb3 accumulation [84].

In regard to the limited application of retroviral and adenoviral vectors, Jung and colleagues conducted an experiment using a recombinant adenoassociated viral vector encoding human alpha galactosidase A and it was injected into the hepatic portal vein. Two weeks later, beyond the observation of increased enzyme activity in the livers of treated mice, authors reported a reduction of Gb3 accumulation too. At a later time, it was reported the opportunity to obtain a more stable expression of the enzyme by administering recombinant AAV driven by modified chicken β -actin promoter. Over the years, AAV vectors have improved. Cheng and co-workers constructed an AAV vector with a liver restricted promoter. This promoter induced higher levels of expression that those observed with CMV promoter. Recently, Scheule and co-workers have reported promising results with AAV. They tested AAV8-hepatocyte specific vector in non human primates [85]. Other authors administered vectors in neonatal mice showing that an early treatment could facilitate an immune tolerance to the transgene products and prevent Gb3 accumulation thus reducing organ damage [86].

In order to overcome some limits of viral vectors as inflammatory response and mutagenesis, more safe non viral systems have been constructed. Novo was the first one to use a non viral vector in Fabry disease and specifically a plasmid with a muscle specific promoter; thus they transfected myogenic cells, 7 days later, they reported a relevant increased enzyme activity in the muscular site of injection [87]. In this setting, the first most important study demonstrating the role of non viral vectors was carried out by Przybylska. They injected intravenously a cationic lipid-pDNA complex. This study has highlighted the ability of non viral constructs to determine normal enzyme levels in knockout model mouse. They choose lung as an organ of administration. However, this delivery system did not reach a significant level of transgene expression which could permit to use it as stand alone treatment [88].

Recently Nakamura carried out a study with naked plasmid encoding alpha galactosidase A which was administered in the left kidney of the mice. This method showed a longer expression of the transgene up to 8 weeks in the treated kidney [89]. Ariz Perez Ruiz de Garibay and co workers have reported interesting results with non viral vectors constructed with solid lipid nanoparticles. These systems increased enzyme levels in HepG2 cells. Previously *in vivo* experiment in mice used SLN vectors encoding the green fluorescent protein to transfect liver, spleen, lung. The same group of researchers also worked on an *in vitro* model IMFE1, which consisted of immortalized microvascular endothelial cells obtained from Fabry patients [90].

A good and efficient vector should be realized in order to satisfy some features as accessing to target cells, adequate gene delivery, long-term expression and it has to be without side effects. Moreover, a vector should be customized for each different setting [2]. Retroviruses are suitable for performing an *ex vivo* strategy and obtaining a long term expression in dividing cells; lentiviral vectors are effective in transfecting non dividing cells, they have a wide tissue tropism [2].

Recently adenoviral vectors seem to improve regarding Fabry gene therapy in fact some researches have shown promising results, the main risks are linked to the immune response requiring challenging regimes of immunosuppression [91].

Further research is needed to demonstrate safety and efficacy of gene therapy approach.

CONCLUSION

Anderson-Fabry disease is a rare X-linked lysosomal storage disorder caused by a deficiency of the enzyme α -galactosidase A. MAF causes glycolipids, such as globotriaosylceramide (Gb3), to accumulate in the vascular endothelium of several organs, including the skin, kidneys, nervous system, and heart, thereby triggering inflammation and fibrosis. These processes generally result in organ dysfunction, which is usually the first clinical evidence of mAF [92, 93]. Patients with classic mAF have various symptoms, eg, acroparesthesias, hypohidrosis, angiokeratomas, corneal opacities, cerebrovascular lesions, cardiac disorders, adrenal dysfunction. However, evolving knowledge about the

natural course of disease suggests that it is more appropriate to describe mAF as a disease with a wide spectrum of heterogeneously progressive clinical phenotypes [94, 95].

Patients with suspected or documented mAF should be followed in centers with proven experience in diagnosis and treatment of lysosomal disease, with the organization of multidisciplinary teams. The present and future challenge is to improve the timing and accuracy of the diagnosis, to better define the ERT start and stop time, and to evaluate the sustainability of the treatment.

New therapeutic tools that use small molecules, called chaperons, that are linked to the enzyme's active site in a reversible way, can increase the enzymatic activity in patients with specific genetic mutations, if still present, by stabilizing and improving the efficiency of the enzyme.

Gene therapy, that is the introduction of a working copy of the gene encoding α galactosidase A in the patient's cells (fibroblasts, lymphocytes B, hepatocytes, hematopoietic cells) through retrovirus, adenovirus and adeno-associated viruses, has shown encouraging results in some experimental animals with an increased enzymatic activity after a 6-month period. While promising to offer permanent MAF cure, gene therapy is still far from being able to enter in a short term in daily clinical practice.

Among viral vectors to date AAVs have reported the best application in Fabry disease. numerous studies have shown promising successful results concerning the use of AAVs, however the main limit of AAVs is the difficulty of a large scale production [92].

Emerging approaches concern non viral vectors: biocompatibility and facilitating large scale production are important features of these constructs giving good prospects for future application in Fabry treatment [1].

Gene therapy represents a promising safe alternative to enzyme replacement treatment. Considerable progress has been obtained in the setting of gene therapy, viral vectors, genetic transference. The most important goal of this emerging strategy is to reach a safer, easily feasible, clinically available and more effective therapeutic approach for Fabry patients [1].

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

- [1] Saida Ortolano, Irene Vieitez, Carmen Navarro, Carlo Spuch. Treatment of lysosomal storage diseases: recent patents and future strategies. Revent patent on endocrine, metabolic and immune drug discovery 2014,8,9-25.
- [2] Aritz Perez Ruiz de Garibay, Mari'a A'ngeles Solini's, Alicia Rodri'guez-Gascòn. Gene Therapy for Fabry Disease: A Review of the Literature. *BioDrugs* (2013) 27:237-246.
- [3] A. Tuttolomondo, I. Simonetta, G. Duro, R. Pecoraro, S. Miceli, P. Colomba *et al.* Inter-familial and intra-familial phenotypic variability in three Sicilian families with Anderson-Fabry disease. *Oncotarget* 2017 Sep 5; 8(37): 61415-61424
- [4] Eng CM, Fletcher J, Wilcox WR, Waldek S, Scott CR, Sillence DO, *et al.* Fabry disease: Baseline medical characteristics of a cohort of 1765 males and females in the Fabry Registry. *J Inher Metab Dis* 2007; 30(2): 184-92.
- [5] Marian AJ. Challenges in the Diagnosis of Anderson-Fabry Disease: A Deceptively Simple and Yet Complicated Genetic Disease.. *J Am Coll Cardiol.* 2016 Sep 6;68(10):1051-3.
- [6] Favalli V, Disabella E, Molinaro M, Tagliani M, Scarabotto A, Serio A *et al.* Genetic Screening of Anderson-Fabry Disease in Probands Referred From Multispecialty Clinics. *J Am Coll Cardiol.* 2016 Sep 6;68(10):1037-50.
- [7] Rozenfeld PA. Fabry disease: treatment and diagnosis. *IUBMB Life.* 2009 Nov;61(11):1043-50
- [8] Eng CM, Guffon N, Wilcox WR, Germain DP, Lee P, Waldek S, *et al.* International Collaborative Fabry Disease Study Group. Safety and efficacy of recombinant human alpha-galactosidase A replacement therapy in Fabry's disease. *N Engl J Med.* 2001 Jul 5;345(1):9-16.
- [9] Schiffmann R, Kopp JB, Austin HA 3rd, Sabnis S, Moore DF, Weibel T *et al.* Enzyme replacement therapy in Fabry disease: a randomized controlled trial. *JAMA.* 2001 Jun 6;285(21):2743-9.
- [10] Thurberg BL, Randolph Byers H, Granter SR, Phelps RG, Gordon RE, O'Callaghan M. Monitoring the 3-year efficacy of enzyme replacement therapy in fabry disease by repeated skin biopsies. *J Invest Dermatol.* 2004 Apr;122(4):900-8.
- [11] Thurberg BL, Rennke H, Colvin RB, Dikman S, Gordon RE, Collins AB, *et al* ; Globotriaosylceramide accumulation in the Fabry kidney is cleared from multiple cell types after enzyme replacement therapy. *Kidney Int.* 2002 Dec;62(6):1933-46.
- [12] William R. Wilcox, Maryam Banikazemi, Nathalie Guffon, Stephen Waldek, Philip Lee, Gabor E *et al* ; Safety and Efficacy of Enzyme Replacement Therapy for Fabry Disease. *The American Journal of Human Genetics* Volume 75, Issue 1, p65-74, July 2004
- [13] Mahmud HM. Fabry's disease- a comprehensive review on pathogenesis, diagnosis and treatment. *J Pak Med Assoc.* 2014 Feb; 64(2):189-94.
- [14] Mac Dermot KD, Holmes A, Miners AH. Anderson- Fabry disease: Clinical manifestation and impact of disease in a cohort of 60 obligate carrier females. *J Med Genet* 2001; 38: 769-75. 4.
- [15] Mac Dermot KD, Holmes A, Miners AH. Anderson- Fabry disease: Clinical manifestation and impact of disease in a cohort of 98 hemizygous males. *J Med Genet* 2001; 38: 750-760.
- [16] Bela Kertesz, Attila; Edes, Istvan Fabry Disease Cardiomyopathy: from Genes to Clinical Manifestations. *Current Pharmaceutical Biotechnology*, Volume 13, Number 13, October 2012, pp.2477-2484(8)
- [17] Schiffmann R, Fuller M, Clarke LA, Aerts JM. Is it Fabry disease? *Genet Med.* 2016 Dec;18(12):1181-1185
- [18] L van der Tol, B E Smid, B J H M Poorthuis, M Biegstraaten, R H Lekanne Deprez, G E Linthorst *et al* ; A systematic review on screening for Fabry disease: prevalence of individuals with genetic variants of unknown significance. *Journal of Medical Genetics* 2014 Jan; 51(1):1-9
- [19] Exome variant server. 2013. <http://evs.gs.washington.edu/>. 19-2-2013
- [20] Lyon MF.: X-chromosome inactivation: a repeat hypothesis. *Cytogenet Cell Genet* 1998; 133
- [21] Wang R ,Lelis A, Mirocha J, Wilcox WR.: Heterozygous Fabry women are not just carriers, but have a significant burden of disease and impaired quality of life. *Genet Med* 2007; 34-45
- [22] Germain D.: Fabry disease. *Orphanet J Rare Dis* 2010; 5 – 30
- [23] Scriba K. Zur: Pathogenese des Angiokeratoma corporis diffusum Fabry mit cardio-vasorenalem Symptomen-komplex. *Verh Dtsch Ges Pathol* 1950; 221

- [24] Bernstein HS, Bishop DF, Astrin KH, Kornreich R, Eng CM, Sakuraba H, *et al* ; Fabry disease: six gene rearrangements and an exonic point mutation in the α galactosidase gene. *J Clin Invest* 1989 ; 1390-1399
- [25] Hitoshi Sakuraba, Akihiro Oshima, Yukiko Fukuhara, Michie Shimmoto, Yoshiro Nagao, David F. Bishopt *et al* : Identification of Point Mutations in the α -Galactosidase A Gene in Classical and Atypical Hemizygoties with Fabry Disease. *Am. J. Hum. Genet* 1990; 784-789
- [26] Christine M. Eng, Lois A. Resnick-Silverman, Dana J. Niehaus, Kenneth H. Astrin, and Robert J. Desnick: Nature and Frequency of Mutations in the α -Galactosidase A Gene That Cause Fabry Disease. *Am. J. Hum. Gene* 1993;1186- 1197
- [27] Scott C. Garman and David N. Garboczi : Structural basis of Fabry disease. *Molecular Genetics and Metabolism* 77 2002; 3-11
- [28] Satoshi Ishii, Shoichiro Nakao, Reiko Minamikawa-Tachino, Robert J. Desnick, Jian-Qiang Fan: Alternative Splicing in the α -Galactosidase A Gene: Increased Exon Inclusion Results in the Fabry Cardiac Phenotype *Am. J. Hum. Genet.* 2002; 994-1002
- [29] Makiko Yasuda, Junaid Shabbeer, Makiko Osawa, Robert J. Desnick: Fabry Disease: Novel α -Galactosidase A 3-Terminal Mutations Result in Multiple Transcripts Due to Aberrant 3-End Formation *Am. J. Hum Genet* 2003; 162-173
- [30] Dominique P Germain Fabry disease Germain Orphanet Journal of Rare Diseases 2010, 5- 30
- [31] Daniel Oder, Nurcan Üçeyler, Dan Liu,Kai Hu,Bernhard Petritsch,Claudia Sommer *et al* ; Organ manifestations and long-term outcome of Fabry disease in patients with the GLA haplotype D313Y.*BMJ Open.* 2016; 6(4): e010422.
- [32] Altarescu G,Moore DF,Schiffmann R. Effect of genetic modifiers on cerebral lesions in Fabry disease.*Neurology.*2005 Jun 28; 64(12):2148-50.
- [33] Malte Lenders,Thomas Duning ,Michael Schelleckes,Boris Schmitz,Sonja Stander,Arndt Rolfs *et al* ;Multifocal White Matter Lesions Associated with the D313Y Mutation of the α -Galactosidase A Gene. *Plosone* 2013;8:e55565
- [34] Yasuda M,Shabbeer J,Benson SD,Maire I,Burnett RM,Desnick RJ. Fabry disease: characterization of alpha-galactosidase A double mutations and the D313Y plasma enzyme pseudodeficiency allele.*Hum Mutat.*2003 Dec;22(6):486-92.
- [35] Froissart R, Guffon N, Vanier MT, Desnick RJ, Maire I. Fabry disease: D313Y is an alpha-galactosidase A sequence variant that causes pseudodeficient activity in plasma.*Mol Genet Metab.* 2003 Nov;80(3):307-14.
- [36] Eng CM, Resnick-Silverman LA, Niehaus DJ, Astrin KH, Desnick RJ. Nature and frequency of mutations in the alpha-galactosidase A gene that cause Fabry disease.*Am J Hum Genet.* 1993 Dec;53(6):1186-97.
- [37] Askari H, Kaneski CR, Semino-Mora C, Desai P, Ang A, Kleiner DE, *et al.* Cellular and tissue localization of globotriaosylceramide in Fabry disease.*Virchows Arch.*2007 Oct;451(4):823-34. Epub 2007 Aug 3.
- [38] Su Xu,Yi Lun,Nastry Brignol,Rick Hamler,Adriane Schilling,Michelle Frascella, *et al* ; Coformulation of a Novel Human α -Galactosidase A With the Pharmacological Chaperone AT1001 Leads to Improved Substrate Reduction in Fabry Mice*Mol Ther.* 2015 Jul; 23(7): 1169-1181.
- [39] Roy S,Touboul D,Brunelle A,Germain DP,Prognon P,Laprèvote O *et al* ; [Imaging mass spectrometry: a new tool for the analysis of skin biopsy. Application in Fabry's disease]. *Ann Pharm Fr.*2006 Sep;64(5):328-34.
- [40] P.Colomba, A.Nucera, C.Zizzo, G.Albeggiani, D.Francofonte, A.Tuttolomondo *et al* ; Identification of a novel mutation in the alpha-galactosidase A gene in patients with Fabry disease. *Clinical Biochemistry* 45 (2012) 839-841.
- [41] Tuttolomondo A,Duro G,Pecoraro R,Simonetta I,Miceli S,Colomba P, *et al* ; A family with various symptomatology suggestive of Anderson-Fabry disease and a genetic polymorphism of alpha galactosidase A gene.*Clin Biochem.* 2015 Jan;48(1-2):55-62. doi: 10.1016/j.clinbiochem.2014.09.018. Epub 2014 Oct 2.
- [42] Hoffmann B,Garcia de Lorenzo A,Mehta A,Beck M,Widmer U,Ricci R;FOS European Investigators. Effects of enzyme replacement therapy on pain and health related quality of life in patients with Fabry disease: data from FOS (Fabry Outcome Survey).*J Med Genet.*2005 Mar;42(3):247-52.
- [43] Uma Ramaswami Update on role of agalsidase alfa in management of Fabry disease. *Drug Des Devel Ther.* 2011; 5: 155-173.
- [44] Weidemann F, Niemann M, Breunig F, *et al.* Long-term effects of enzyme replacement therapy on Fabry cardiomyopathy: evidence for a better outcome with early treatment. *Circulation.* 2009; 119(4):524-9.
- [45] Wilcox WR, Linthorst GE, Germain DP, *et al.* Anti-alpha-galactosidase A antibody response to agalsidase beta treatment: data from the Fabry Registry. *Mol Genet Metab.* 2012;105(3):443-9.
- [46] Tajima Y,Kawashima I,Tsukimura T,Sugawara K,Kuroda M,Suzuki T, *et al* ; Use of a modified alpha-N-acetylgalactosaminidase in the development of enzyme replacement therapy for Fabry disease.*Am J Hum Genet.*2009 Nov;85(5):569-80.
- [47] Desnick RJ,Schuchman EH. Enzyme replacement therapy for lysosomal diseases: lessons from 20 years of experience and remaining challenges.*Annu Rev Genomics Hum Genet.*2012;13:307-35.
- [48] David Hudgins Calhoun,George Coppola Recombinant α -galactosidase a therapy for Fabry disease US 6461609 (2002)
- [49] Hollak CE,Weinreb NJ. The attenuated/late onset lysosomal storage disorders: Therapeutic goals and indications for enzyme replacement treatment in Gaucher and Fabry disease.*Best Pract Res Clin Endocrinol Metab.*2015 Mar;29(2):205-18.
- [50] Benjamin ER,Della Valle MC,Wu X,Katz E,Pruthi F,Bond S *et al* ; The validation of pharmacogenetics for the identification of Fabry patients to be treated with migalstat.*Genet Med.*2017 Apr; 19(4):430-438
- [51] Wu X, Katz E,Della Valle MC,Mascioli K,Flanagan JJ, Castelli JP, *et al* ; A pharmacogenetic approach to identify mutant forms of α -galactosidase A that respond to a pharmacological chaperone for Fabry disease.*Hum Mutat.*2011 Aug;32(8):965-77
- [52] Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal alpha-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med.* 1999;5(1):112-5.
- [53] Hamanaka R, Shinohara T, Yano S, *et al.* Rescue of mutant alpha-galactosidase A in the endoplasmic reticulum by 1-deoxygalactonojirimycin leads to trafficking to lysosomes. *Biochim Biophys Acta.* 2008;1782(6):408-13.
- [54] Shiozuka C, Taguchi A, Matsuda J, *et al.* Increased globotriaosylceramide levels in a transgenic mouse expressing human alpha1,4-galactosyltransferase and a mouse model for treating Fabry disease. *J Biochem.* 2011;149(2):161-70.
- [55] Chang HH, Asano N, Ishii S, Ichikawa Y, Fan JQ. Hydrophilic iminosugar active-site-specific chaperones increase residual glucocerebrosidase activity in fibroblasts from Gaucher patients.*FEBS J* 2006; 273(17): 4082-92.
- [56] Fan, J.Q., Ishii, S., Asano, N., Desnick, R.J. Method for enhancing mutant enzyme activity in Gaucher disease. US0119874 2003
- [57] Lieberman RL, D'Aquino JA, Ringe D, Petsko GA. Effects of pH and iminosugar pharmacological chaperones on lysosomal glycosidase structure and stability. *Biochemistry* 2009; 48(22): 4816-27.
- [58] Maegawa GH, Tropak MB, Buttner JD, Rigat BA, Fuller M, Pandit D. *et al.* Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. *J Biol Chem* 2009;284(35): 23502-16.
- [59] Asano N, Ishii S, Kizu H, Ikeda K, Yasuda K, Kato A *et al.* *In vitro* inhibition and intracellular enhancement of lysosomal alpha-galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin and its derivatives. *Eur J Biochem.* 2000 Jul;267(13):4179-86.
- [60] Benjamin ER, Flanagan JJ, Schilling A, Chang HH, Agarwal L, Katz E, Wu X *et al.* The pharmacological chaperone 1-deoxygalactonojirimycin increases alpha-galactosidase A levels in Fabry patient cell lines. *J Inherit Metab Dis.* 2009 Jun;32(3):424-40
- [61] Park JY, Kim GH, Kim SS, Ko JM, Lee JJ, Yoo HW. Effects of a chemical chaperone on genetic mutations in alpha-galactosidase A

- in Korean patients with Fabry disease. *Exp Mol Med.* 2009 Jan 31;41(1):1-7.
- [62] Okumiya, T., Ishii, S., Kase, R., Kamei, S., Sakuraba, H., Suzuki, Y. α -Galactosidase gene mutations in Fabry disease: heterogeneous expressions of mutant enzyme proteins. *Hum Genet.* 1995;95:557-561.
- [63] Okumiya, T., Ishii, S., Takenaka, T., Kase, R., Kamei, S., Sakuraba, H. *et al.* Galactose stabilizes various missense mutants of α -galactosidase in Fabry disease. *Biochem Biophys Res Commun.* 1995; 214:1219-1224.
- [64] Ishii, S., Kase, R., Sakuraba, H., Suzuki, Y. Characterization of a mutant α -galactosidase gene product for the late-onset cardiac form of Fabry disease. *Biochem Biophys Res Commun.* 1993;197:1585-1589.
- [65] Frustaci, A., Chimenti, C., Ricci, R., Natale, L., Russo, M.A., Pieroni, M. *et al.* Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. *N Engl J Med.* 2001;345:25-32
- [66] Lachmann, R.H. Enzyme replacement therapy for lysosomal storage diseases. *Curr Opin Pediatr.* 2011;23:588-593.
- [67] Yoshiyuki Suzuki. Chaperone therapy update: Fabry disease, GM1-gangliosidosis and Gaucher disease. *Brain Dev.* 2013 Jun;35(6):515-23
- [68] Cartegni, L., Chew, S.L., Krainer, A.R. Listening to silence and understanding nonsense: exonic mutations that affect splicing nature reviews. *Genetics.* 2002;3:285-298
- [69] Ishii, S., Nakao, S., Minamikawa-Tachino, R., Desnick, R.J., Fan, J.Q. Alternative splicing in the alpha-galactosidase a gene: increased exon inclusion results in the Fabry cardiac phenotype. *Am. J. Hum. Genet.* 2002;70:994-1002
- [70] Palhais B, Dembic M, Sabaratnam R, Nielsen KS, Doktor TK, Bruun GH, Andresen BS. The prevalent deep intronic c. 639+919 G>A GLA mutation causes pseudoexon activation and Fabry disease by abolishing the binding of hnRNP1 and hnRNP A2/B1 to a splicing silencer. *Mol Genet Metab.* 2016 Nov;119(3):258-269.
- [71] Doktor, T.K., Hua, Y., Andersen, H.S., Brøner, S., Liu, Y.H., Wieckowska, A., *et al.* RNA sequencing of a mouse-model of spinal muscular atrophy reveals tissue-wide changes in splicing of U12-dependent introns. *Nucleic Acids Res.* 2016;
- [72] Kole, R., Krainer, A.R., Altman, S. RNA therapeutics: beyond RNA interference and antisense oligonucleotides nature reviews. *Drug Discov.* 2012;11:125-140.
- [73] Daniel P. Judge, Ike S. Okwuosa and Albert A. Hagège. GLA-Ring Opportunities and Challenges for Fabry Disease. *Journal of the American College of Cardiology*, Volume 68, Issue 23, December 2016
- [74] Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, *et al.* Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science.* 2013 Aug 23; 341(6148): 1233158.
- [75] Biffi A., Gentner B.R., Naldini L. Gene vector WO 2010125471 (2010)
- [76] Sung-Chul Jung, Ina P. Han, Advait Limaye, Ruian Xu, Kamala Tirumalai, Gary J. Murray, *et al.* Adeno-associated viral vector-mediated gene transfer results in long-term enzymatic and functional correction in multiple organs of Fabry mice. *Proc Natl Acad Sci U S A.* 2001 Feb 27; 98(5): 2676-2681.
- [77] Hiroshi Takahashi, Yukihiko Hirai, Makoto Migita, Yoshihiko Seino, Yuh Fukuda, Hitoshi Sakuraba. *et al.* Long-term systemic therapy of Fabry disease in a knockout mouse by adeno-associated virus-mediated muscle-directed gene transfer. *Proc Natl Acad Sci U S A.* 2002 Oct 15; 99(21): 13777-13782.
- [78] Park J, Murray GJ, Limaye A, Quirk JM, Gelderman MP, Brady RO, *et al.* Long-term correction of globotriaosylceramide storage in Fabry mice by recombinant adeno-associated virus-mediated gene transfer. *Proc Natl Acad Sci USA.* 2003 Mar 18; 100(6): 3450-4.
- [79] Chester Li Robin J, Ziegler^a Maribeth Cherry^a Michael Lukason^a Robert J. Desnick^b Nelson S. Yew^a *et al.* Adenovirus-Transduced Lung as a Portal for Delivering α -Galactosidase A into Systemic Circulation for Fabry Disease. *Mol. Ther.* Volume 5, Issue 6, June 2002, Pages 745-754.
- [80] Gen Nakamura, Hiroki Maruyama, Satoshi Ishii, Masaaki Shimotori, Shigemi Kameda, Toru Kono *et al.* Naked Plasmid DNA-Based α -Galactosidase A Gene Transfer Partially Reduces Systemic Accumulation of Globotriaosylceramide in Fabry Mice. *Mol. Biotechnol.* 2008, 38(2), 109-119
- [81] Ogawa K, Hirai Y, Ishizaki M, Takahashi H, Hanawa H, Fukunaga Y, *et al.* Long-term inhibition of glycosphingolipid accumulation in Fabry model mice by a single systemic injection of AAV1 vector in the neonatal period. *Mol Genet Metab.* 2009 Mar;96(3):91-6.
- [82] Sands MS, Davidson BL. Gene therapy for lysosomal storage diseases. *Mol Ther.* 2006;13(5):839-49.
- [83] Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, Oxford. 2012. <http://www.wiley.co.uk/genmed/clinical>. Accessed 02 Nov 2012.
- [84] Passineau MJ, Fahrenholz T, Machen L, *et al.* alpha-Galactosidase A expressed in the salivary glands partially corrects organ biochemical deficits in the Fabry mouse through endocrine trafficking. *Hum Gene Ther.* 2011;22(3):293-301.
- [85] Ziegler RJ, Cherry M, Barbon CM, *et al.* Correction of the biochemical and functional deficits in Fabry mice following AAV8-mediated hepatic expression of alpha-galactosidase A. *Mol Ther.* 2007;15(3):492-500.
- [86] Sabatino DE, Mackenzie TC, Peranteau W, *et al.* Persistent expression of hF. IX After tolerance induction by in utero or neonatal administration of AAV-1-F.IX in hemophilia B mice. *Mol Ther.* 2007;15(9):1677-85.
- [87] Novo FJ, Gorecki DC, Goldspink G, *et al.* Gene transfer and expression of human alpha-galactosidase from mouse muscle *in vitro* and *in vivo*. *Gene Ther.* 1997;4(5):488-92.
- [88] Przybylska M, Wu IH, Zhao H, *et al.* Partial correction of the alpha-galactosidase A deficiency and reduction of glycolipid storage in Fabry mice using synthetic vectors. *J Gene Med.* 2004;6(1):85-92.
- [89] Nakamura G, Maruyama H, Ishii S, *et al.* Naked plasmid DNA-based alpha-galactosidase A gene transfer partially reduces systemic accumulation of globotriaosylceramide in Fabry mice. *Mol Biotechnol.* 2008;38(2):109-19.
- [90] Shen JS, Meng XL, Schiffmann R, *et al.* Establishment and characterization of Fabry disease endothelial cells with an extended lifespan. *Mol Genet Metab.* 2007;92(1-2):137-44.
- [91] Li C, Ziegler RJ, Cherry M, *et al.* Adenovirus-transduced lung as a portal for delivering alpha-galactosidase A into systemic circulation for Fabry disease. *Mol Ther.* 2002;5(6):745-54.
- [92] Tuttolomondo A, Pecoraro R, Simonetta I, Miceli S, Pinto A, Licata G. Anderson-Fabry disease: a multiorgan disease. *Curr Pharm Des.* 2013;19(33):5974-96. Review
- [93] Tuttolomondo A, Pecoraro R, Simonetta I, Miceli S, Arnao V, Licata G, Pinto A. Neurological complications of Anderson-Fabry disease. *Curr Pharm Des.* 2013;19(33):6014-30. Review.
- [94] Rigoldi M, Concolino D, Morrone A, Pieruzzi F, Ravaglia R, Furlan F, Santus F, Strisciuglio P, Torti G, Parini R. Intrafamilial phenotypic variability in four families with Anderson-Fabry disease. *Clin Genet.* 2014 Sep;86(3):258-63.
- [95] Messina S, Tortorella G, Concolino D, Spanò M, D'Amico A, Bruno C, Santorelli FM, Mercuri E, Bertini E. Congenital muscular dystrophy with defective alpha-dystroglycan, cerebellar hypoplasia, and epilepsy. *Neurology.* 2009 Nov 10;73(19):1599-601.
- [96] Grieger JC, Samulski RJ. Adeno-associated virus vectorology, manufacturing, and clinical applications. *Methods Enzymol.* 2012; 507: 229-54.