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***The role of DNA Methylation in Anderson Fabry Disease
pathogenesis: novel nutrigenomic frontier in a CRISPR/cas9-
based cellular model***

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ABSTRACT

Anderson Fabry disease (AFD) is a rare X-linked lysosomal storage disorder caused by partial or total deficiency of the enzyme α -galactosidase A (α -GAL A), resulting in the accumulation of globotriaosylceramide (GB3) in lysosomes. Although research has mainly focused on genetic mutations of the *GLA* gene, growing evidence suggests that epigenetic mechanisms, particularly DNA methylation, may also contribute to disease heterogeneity, especially among female carriers.

This study investigated the role of DNA methylation in AFD pathogenesis by analyzing global and gene-specific methylation profiles in peripheral blood mononuclear cells (PBMCs) from female AFD patients and healthy controls. The results revealed significant global DNA hypomethylation in AFD patients, indicative of genomic instability and chronic inflammation, together with hypermethylation of the *GLA* promoter region, potentially responsible for further gene silencing and reduced α -GAL A expression. These findings highlight a dual mechanism, genetic and epigenetic, underlying disease severity and variability.

To explore these mechanisms in vitro, a CRISPR/Cas9-engineered cellular model of AFD, called HEK-GLAKD, was developed, reproducing the characteristic hypomethylated and inflammatory profile of the disease. This model was subsequently used to assess the effects of plant-derived extracellular vesicles (FicoVes), previously reported to possess anti-inflammatory properties. Treatment with FicoVes restored global methylation levels, reduced Interleukin-6 (*IL-6*) and Interleukin-8 (*IL-8*) gene expression and secretion and decreased lipid accumulation, indicating an overall anti-inflammatory and epigenetically stabilizing effect. Importantly, these findings suggest that FicoVes may represent a valuable adjuvant co-treatment to current enzyme replacement therapy (ERT), acting through complementary epigenetic and anti-inflammatory pathways.

Collectively, this work demonstrates that DNA methylation plays a critical role in AFD pathophysiology and highlights the therapeutic potential of epigenetic modulation. Moreover, the established AFD cellular model provides a robust platform for future mechanistic studies and for the development of innovative adjuvant strategies targeting both genetic and epigenetic aspects of the disease. Moreover, by modulating DNA methylation and cytokine expression, FicoVes could enhance the efficacy of existing therapeutic approaches and contribute to improved clinical outcomes in AFD patients.

INTRODUCTION

Lysosomes are intracellular membrane-bound organelles that perform multiple functions essential for cellular homeostasis. They contain a variety of digestive enzymes and that are involved in several cellular processes, including macromolecular catabolism and recycling (Luzio et al. 2007). The activity of the lysosomal system is highly regulated in response to environmental changes in order to provide appropriate responses to diverse types of stimuli (Maegawa 2019).

Lysosomal storage disease (LSD) is a disorder caused by a deficiency of enzymes in lysosomes, leading to the accumulation of undigested or partially digested macromolecules (Fig. 1) (Sun 2018; Platt et al. 2018). The clinical manifestations of Lysosomal Storage Disorders (LSDs) may affect different tissues, especially liver, heart, muscle, and brain. Neurologic damage is almost always present and is usually progressive (Burlina et al, 2024). Lysosomal diseases are rare and their onset may occur at any age, but they rarely present at birth. Most LSDs are typically present in early or late infancy, but juvenile and adult forms also exist and are associated with predominantly neuropsychiatric and behavioral symptoms. Early childhood onset is generally associated with a poorer prognosis (Maegawa 2019).

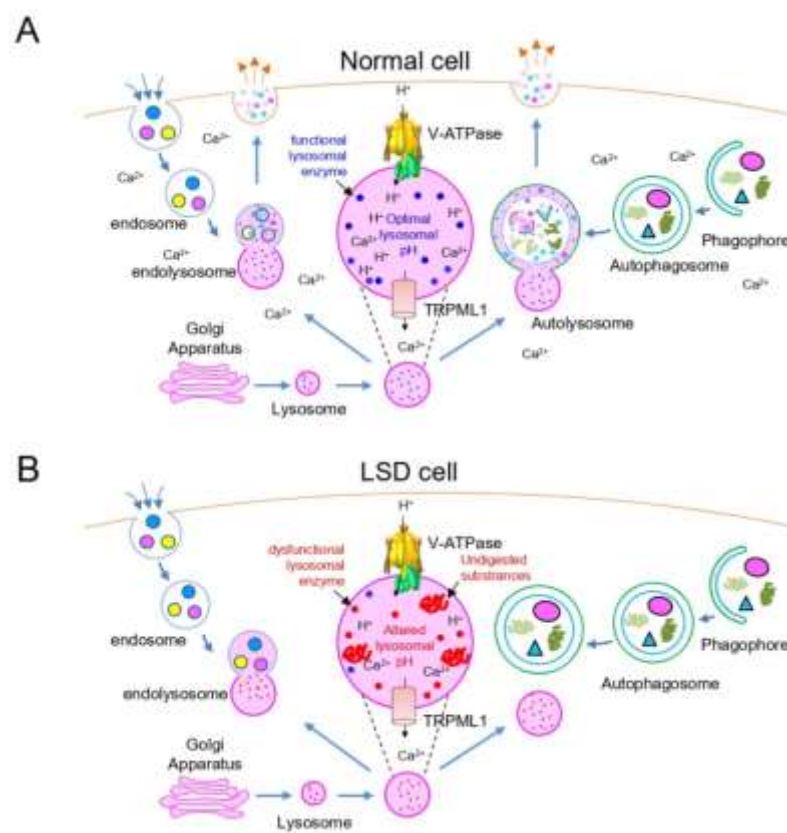


Fig. 1 Schematic representation of the lysosome and autophagy pathway in normal cells (A) and lysosomal storage disease (LSD) cells (B). (A) Lysosomes fuse with autophagosomes to form autolysosomes. Lysosomes also fuse with endosomes to form highly dynamic membrane structures called endolysosomes. (B) Lysosomes loaded with undigested substances lead to defective lysosomal fusion with autophagosomes or endosomes (Kuk et al. 2021).

Specific treatment options are available for some LSDs. Enzyme replacement therapy (ERT) is approved for Pompe Disease (PD), Anderson Fabry Disease (AFD), alpha-mannosidosis, and mucopolisaccharidosis (MPS). ERT is not able to cross the blood–brain barrier (BBB), therefore, it has no effect on neurologic complications. Intraventricular ERT has been approved for neuronal ceroid lipofuscinosis type II (CLN2). Preclinical and clinical trials of ERT, hematopoietic stem cells transplantation (HSCT), substrate reduction therapy (SRT), or gene therapy have been reported for Krabbe Disease (KD), metachromatic leukodystrophy (MLD), MPS IIIA and B, GM1, and GM2 gangliosidosis, fucosidosis, and some ceroid lipofuscinoses (Platt et al. 2018; Specchio and Curatolo 2021). Recently, oral chaperone therapy has been approved for Fabry disease in patients with an amenable genetic variant (Germain et al. 2016).

To date, it is well known that, despite the increasing number of promising therapies for the treatment of lysosomal storage disorders, a definitive gold standard capable of producing a significant reduction in symptoms has yet to be established. These disorders still warrant further investigation and clarification, with the aim of expanding current knowledge and, where possible, designing even more effective therapies. These new treatments could complement existing ones, enhancing their efficacy while minimizing associated risks.

1. ANDERSON FABRY DISEASE

Lysosomal diseases are congenital enzymopathies caused by genetic mutations that alters the function of enzymes responsible for the degradation of proteins or macromolecular complexes. This leads to the accumulation of undegraded substrates in various cells and tissues (Brady et al. 1967; Mehta et al. 2006). In this context, AFD is a rare and progressive X-linked genetic lysosomal storage disorder resulting from mutations in the *GLA* gene, a 14kb housekeeping gene that is located on the long arm of #X chromosome (Xq22). *GLA* gene encodes for the α -Galactosidase A (α -GAL A) an ubiquitous lysosomal acid hydrolase (Echevarria et al. 2016; Bernardes et al. 2020). A quantitative or functional deficiency of α -GAL A leads to the progressive accumulation of glycosphingolipids, mainly GB3 in lysosomes of various cell types, mainly vascular endothelial cells (Germain et al. 2021; Di

Risi et al. 2021). The marked increase of water-soluble deacylated GB3, also known as globotriaosyl-sphingosine (lysoGB3) is a hallmark of AFD (Aerts et al. 2008). LysoGB3 can leave cells and reach the bloodstream leading to plasma levels that are elevated by more than a hundredfold (Ferraz et al. 2016). Furthermore, the GB3 accumulation can then cause multisystem disorders including an alteration of cellular homeostasis, neuropathic pain, gastrointestinal disorders, angiokeratoma, fatigue, impaired sweating, hot/cold intolerance and respiratory involvement (Fig. 2).

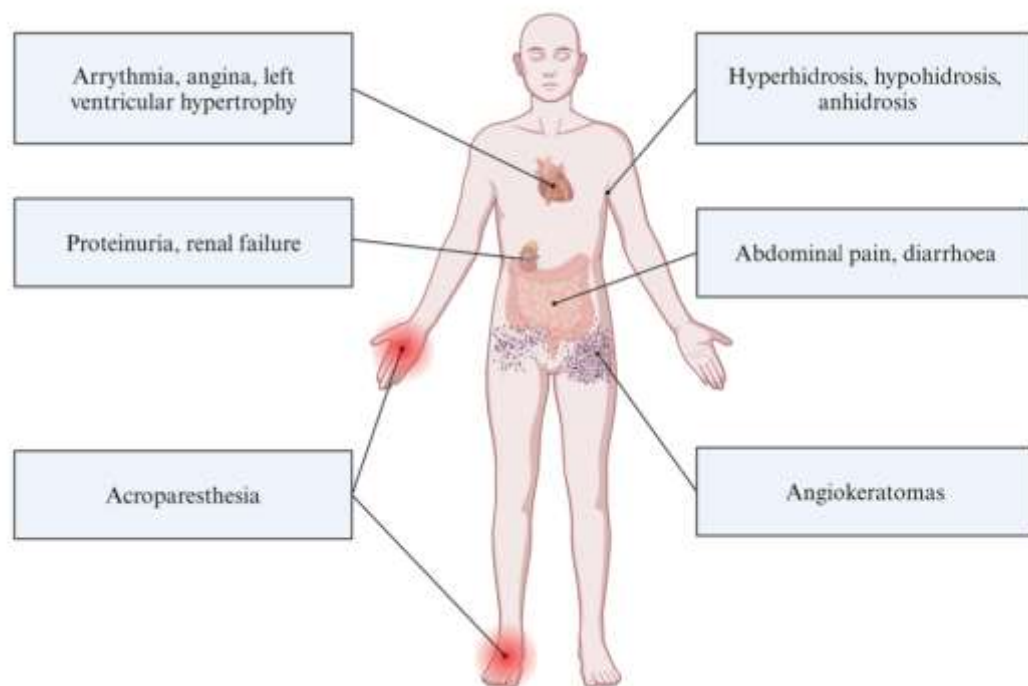


Fig. 2 Main symptoms of Fabry disease (Borisch et al. 2024).

1.1. THE PHYSIOPATHOLOGY OF AFD LINKED TO X CHROMOSOME INACTIVATION

Since the disease has a X-linked heredity schema, males may be hemizygous and, depending on the inherited mutation, they will develop the classic or the late-onset variant (Spada et al. 2006). Even though, females may be also affected and potentially may develop different clinical features. The severity and organ involvement varies depending on the number of cells in which the mutated gene is expressed through random X inactivation (Wilcox et al. 2008). Females heterozygous for X-linked diseases have body cells that express the normal

gene (wild type) and cells that express the mutant gene (mutant cells) (Mehta et al. 2004). The disease manifests in a highly variable manner in women, in fact clinical phenotypes ranging from asymptomatic individuals to women presenting severity comparable to classically affected men. This is because males carrying the mutation typically also express severe symptoms due to the presence of only one X chromosome, which lacks the normal copy of the gene to compensate (Izhar et al. 2023). The variability of symptoms extends not only to the intensity of symptoms but also to the range of affected tissues and the onset of signs. Notably, the onset of initial symptoms and complications in adulthood tends to occur later in females compared to males (Wang et al. 2007). The dramatic influence of *GLA* gene was demonstrated by a group of monozygotic twins with the gene mutated. Analysis of the twins' fibroblasts revealed that one of them had a preferentially active maternally derived X chromosome and she was asymptomatic. The other twin had a AFD phenotype and a preferentially active paternally-derived X chromosome, indicating that the mutation was located on the paternal X chromosome and that the differences in X inactivation were responsible for the symptomatology (Echevarria et al. 2016). The frequent appearance of clinical disease in heterozygous females also indicates a lack of enzymatic complementation (Mehta et al. 2004). It is therefore hypothesized that “oblique” X inactivation is at the basis of the variability of the phenotype in women (Arends et al. 2017). In women with random inactivation of the X chromosome, AFD worsens inexorably with age. Young patients are generally mildly affected, while older women show progression of left ventricular hypertrophy and deterioration of renal function over time. This supports the hypothesis of inefficient cross-correction between healthy and diseased cells in heterozygotes (Echevarria et al. 2016). Patients with asymmetric XCI profiles have a different course of the disease, which depends on the predominantly expressed allele. Minor expression of the mutant *GLA* allele is associated with a mild phenotype and progression over 3 years. In contrast, women where the non-mutant allele was preferentially repressed exhibit greater disease severity, earlier onset and more rapid progression with age, requiring closer monitoring and early therapeutic intervention (Bernardes et al. 2020; Di Risi et al. 2021). Until now Enzyme Replacement Therapy (ERT) with recombinant human α -Gal A is used as clinical practice for AFD (Eng et al. 2001a, 2001b; Banikazemi et al. 2007; Schiffmann et al. 2001).

2. THE EPIGENETICS

Epigenetics is the branch of genetics that studies heritable modifications of gene expression that lead to phenotypic alterations without changing the nucleotide sequence of DNA. The term “epigenetic” was first introduced by Waddington in 1939, who coined the concept of the “epigenetic landscape” to describe the molecular and biological mechanisms that transform a genetic trait into a visualized phenotype. This concept encompasses the relationship between genotype and phenotype (Esteller 2008). The dynamic structure of chromatin is closely linked to gene regulation and many other biological functions; therefore, understanding the factors that regulate chromatin dynamics is essential (Tolsma and Hansen 2019). Epigenetic mechanisms allow our cells to inherit changes in gene expression and phenotype without altering the underlying DNA sequence. These processes can occur at various levels of the genome's nuclear organization, from chromosome positioning during interphase in the nucleus to the molecular level through post-translational modifications (PTMs) of histones (Nadal et al. 2018). Emerging epigenetic tools can be used as preventive, diagnostic, and therapeutic biomarkers (Zhang et al. 2020). Recent investigations into the etiology of cancer have highlighted a key role played by epigenetics. In particular, aberrant DNA and histone modifications have been shown to silence tumor suppressor genes or activate oncogenes in multiple cancer models. Typically, the genome of a cancer cells is globally hypomethylated and locally hypermethylated. In this regard, it has also been demonstrated that many nutritional factors can influence gene expression through epigenetic changes (Wu et al. 2015), making nutrigenomic research particularly promising. Epigenetic mechanisms include histone post-translational modifications, RNA interference, and DNA hypermethylation and demethylation (Fig. 3). Compared to the numerous studies in the field of genetics, research in epigenetics is relatively recent. Unlike genetic changes, which are difficult to reverse, epigenetic modifications can be pharmacologically reversible.

With regard to lysosomal storage disorders, the role of epigenetics remains a relatively unexplored field and it is still not well understood, as stated by Hassan et al. 2017, they want to encourage researchers to consider epigenetics as a potential mechanism to explain the complexities of biological functions and the pathogenesis of these rare disorders. In particular, concerning AFD, Di Risi et al. 2021 highlighted that relatively few studies have addressed the complexity of the DNA methylation landscape in AFD, which therefore remains poorly investigated. The hope for the future is that ad hoc and ultra-deep methylation analyses of the *GLA* gene will provide epigenetic signatures capable of predicting whether

pre-symptomatic female carriers will develop symptoms, thus supporting timely clinical interventions.

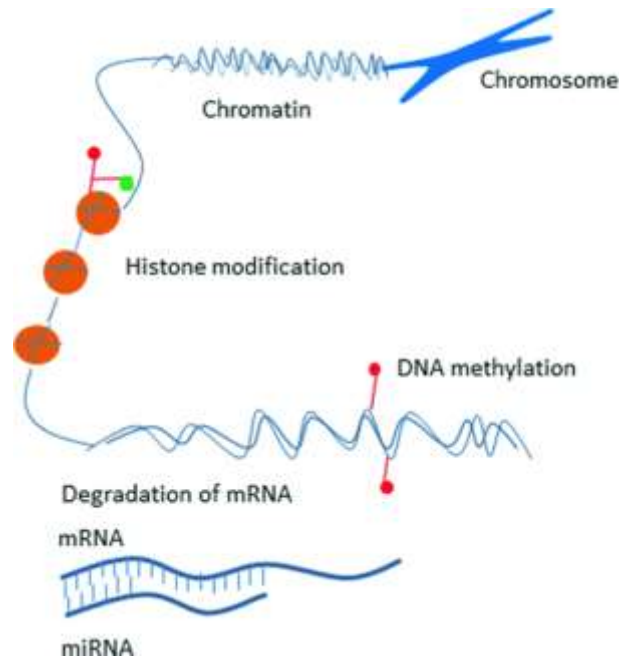


Fig. 3 The primary mechanisms of epigenetic modifications include DNA methylation, histone modifications, miRNAs (Esteller 2008).

2.1 DNA METHYLATION

DNA methylation is one of the most extensively studied epigenetic modifications in mammals. In normal cells, it ensures proper regulation of gene expression and stable gene silencing. DNA methylation is closely associated with histone modifications and the interplay between these epigenetic marks is crucial for regulating genome function by altering chromatin architecture. The covalent addition of a methyl group typically occurs at cytosine residues within CpG dinucleotides, which are concentrated in large clusters known as CpG islands (Li et al. 2025). Aberrant DNA methylation profiles have been detected in several diseases, including cancer and genetic disorders (Mahmood and Rabbani 2019; Robertson 2005). DNA methylation analysis gave an essential contribution to shed light on the complex epigenetic regulation of X chromosome genes (Sharp et al. 2011; Duncan et al. 2018). It is widely described that the inactive X chromosome in female mammalian cells is highly methylated (Fang et al. 2019; Disteche and Berletch 2015) while the active one is not. However, the X chromosome epigenetic landscape may be more complex than previously

believed being the gene bodies and intergenic regions often highly methylated on Xa (Cotton et al. 2015).

2.1.1 *TET1 and TET2 enzymes*

DNA oxidation, mediated by the Ten-Eleven Translocation (TET) family, represents an epigenetic modification capable of converting 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC), which regulates various biological processes (Yang et al. 2018). Currently, growing evidence indicates that TET proteins are involved in the control of active or passive DNA demethylation through multiple mechanisms (Fig. 4); moreover, their activity can be regulated by certain cellular factors (Jia et al. 2015). Over time, it has been demonstrated that the demethylation of 5-mC, promoted by the conversion of 5-mC to 5-hmC mediated by TET1 and TET2, occurs in a range of physiological processes such as zygotic epigenetic reprogramming, somatic cell reprogramming, removal of genomic imprinting, early embryonic development, and primordial germ cell development (Wu and Zhang 2011). Mutations in TET1 and TET2 drive tumorigenesis in various hematological malignancies and solid tumors (Jiang 2020).

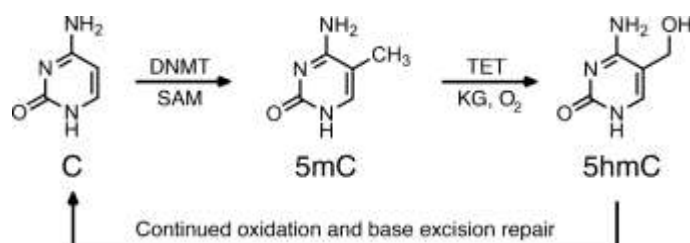


Fig. 4 DNA Methylation and Active Demethylation: DNA methylation is dynamically regulated by methyl-“writer” enzymes (DNMTs) and methyl-“eraser” enzymes (TETs), allowing this mechanism to govern local gene expression, a process crucial for cellular function and identity (Chua et al. 2019).

2.1.2 *DNA methyltransferases: key enzymes in DNA methylation*

DNA methyltransferases are responsible for the establishment and maintenance of DNA methylation patterns. It is well known that the inactivation of certain tumor suppressor genes occurs as a consequence of hypermethylation within promoter regions. Indeed, numerous

studies have demonstrated a wide range of genes silenced by DNA methylation in various types of cancer. On the other hand, global genomic hypomethylation, which induces genomic instability, also contributes to cellular transformation (Kulis and Esteller 2010). DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from S-adenosyl-L-methionine to the C5 position of cytosine residues, primarily within 5'-cytosine-phosphate-guanine-3' (CpG) islands (Castillo-Aguilera et al. 2017). This enzyme family, responsible for DNA methylation in mammals, includes DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT1 is the most abundant and important maintenance methyltransferase, responsible for propagating pre-existing DNA methylation patterns (Fernandes et al. 2017). Generally, the lack of DNA methylation in gene promoter regions is often associated with transcriptional activation and gene expression, whereas promoter methylation can lead to gene silencing (Castillo-Aguilera et al. 2017). CpG island hypermethylation also plays a significant role in the epigenetic silencing of genes involved in cell cycle regulation, receptor signaling, DNA repair, and apoptosis (Fang et al. 2003). Therefore, regulating DNMT activity through specific inhibitors represents a promising therapeutic strategy for halting abnormal cellular processes (Fernandes et al. 2017).

3. THE EPIGENETIC SIGNATURE IN AFD

In recent years, the concept of epigenetics, particularly DNA methylation, has gained significant attention in the scientific community. It has been observed that global or gene-specific methylation patterns can vary in the onset of various diseases. As a result, DNA methylation has, in some cases, become a prognostic marker for the onset and progression of several conditions, such as lupus erythematosus, atherosclerotic disease, other autoimmune diseases, and neurodegenerative diseases (Fuss et al. 2009). DNA methylation analysis gave an essential contribution to shed light on the complex epigenetic regulation of X chromosome genes (Sharp et al. 2011; Li and Zhang 2014; Duncan et al. 2018). It is widely described that the inactive X chromosome in female mammalian cells is highly methylated (Fang et al. 2019; Disteche and Berletch 2015) while the active one is not. The results from recent studies pointed out how the analysis of DNA methylation may help to explain the variability of clinical phenotype observed in AFD. DNA methylation occurs at both X and autosomal genes, prevalently at cytosine residues of CpG sites, and has important regulatory effects on gene expression (Suzuki and Bird 2008).

4. THE INFLAMMATORY SIGNATURE IN AFD

Inflammatory activation plays an important role in AFD pathogenesis. Glycolipids, especially GB3, can bind to toll-like receptor 4 and trigger Notch1 signaling, which in turn activates the nuclear factor kappa B signaling cascade. This process results in the production of pro-inflammatory cytokines, leading to a chronic inflammatory state and associated vasculopathy (Yuan et al. 2024).

4.1 PRO AND ANTI-INFLAMMATORY CYTOKINES IN AFD

It is known until now that pro-inflammatory interleukins expression were upregulated in AFD patients, indicating the role of the innate immune process in AFD etiology, and the expression is positively correlated with disease burden and clinical markers, these information are enough to consider inflammatory pathway a promising therapeutic target for AFD (Yuan et al. 2024).

4.1.1 *Interleukin-6 (IL-6)*

IL-6 is a pleiotropic cytokine involved in several physiological and pathological conditions. Its action is mediated by two central signaling systems: classical IL-6 signaling acts through the binding of IL-6 to the membrane-bound IL-6 receptor (IL-6R) α -subunit and the glycoprotein 130 (gp130) signal-transducing subunit. At the same time, IL-6 trans-signaling is activated in the presence of complexes of IL-6 and the soluble form of the IL-6 receptor (sIL-6R) linked to the membrane-bound gp130 (Schumertl et al. 2022). Given this information, it is known that IL-6 has a dual role, acting as both a pro-inflammatory and an anti-inflammatory cytokine. The pro-inflammatory effects of IL-6 are primarily mediated through classical signaling via the membrane-bound IL-6 receptor (mIL-6R), which leads to the activation of inflammatory pathways. Conversely, IL-6 can also exhibit anti-inflammatory properties through trans-signaling *via* sIL-6R, promoting the production of anti-inflammatory molecules (Opal and DePalo 2000; Scheller et al. 2011). Neto et al. 2021 reported higher serum IL-6 and TNF- α levels in patients treated with ERT than those not treated, so it is possible that the massive GB3 released from cells into circulation on the initiation of ERT would lead to the transient upregulation of immune status. With the continued ERT and residual GB3, the inflammatory response tends to stabilize (Yuan et al. 2024).

4.1.2 Interleukin-8 (IL-8)

IL-8, also known as C-X-C motif chemokine ligand 8 (CXCL8), is a pro-inflammatory chemokine, a member of the CXC family of chemokines, produced under inflammatory conditions by immune and other cell types (Matsushima et al. 2022). The most prominent role of IL-8 is the attraction of neutrophils to the sites of inflammation, but also the promotion of monocyte-macrophage growth and differentiation (Corre et al. 1999), endothelial cell survival, proliferation and angiogenesis (Li et al. 2003). IL-8, similarly to other pro-inflammatory cytokines, is known to be overexpressed in patients affected by Fabry disease, and it has been particularly observed to be elevated in female patients, especially in relation to higher clinical burden as measured by the Mainz Severity Score Index (MSSI) (Yuan et al. 2024). Notably, skin fibroblasts derived from female Fabry patients display significantly increased IL-8 expression, suggesting a potential sex-specific contribution to the pathophysiology of pain in women with this condition (Hofmann et al. 2024).

4.1.3 Interleukin-10 (IL-10)

IL-10, a cytokine with pleiotropic immunosuppressive functions, is also the founding member of the IL-10 family of cytokines. It asserts different anti-inflammatory functions, it is not surprising that IL-10 exerts essential regulatory roles in many human autoimmune diseases (Wang et al. 2019). It was already demonstrated that IL-10 is usually significantly higher in the AFD cohort than in the healthy controls (Ivanova et al. 2025).

5. THE BIOLOGICAL MATERIALS USED FOR STUDYING AFD AND THE ABSENCE OF A PRECLINICAL MODEL

Until now different rare diseases are studied through patients' samples such as blood samples, biopsy, biological fluids, but the lack of adequate preclinical models to study represents the more intricate counterpart to the study of rare diseases. In fact, according to Anthony J Burand Jr 1, Cheryl L Stucky one of the limitations in AFD research is the lack of a stabilized cellular model (Burand and Stucky, 2021). This lack leads to a slowdown in obtaining results as much of the work is based on the study of samples taken from affected patients but the obtaining is not always rapid and periodic.

5.1 CRISPR/Cas9 METHODOLOGY

The CRISPR/Cas9 technique was developed in 2012 (Jinek et al. 2012).

Specifically, Emmanuelle Charpentier and Jennifer Doudna published a groundbreaking study in June 2012, where they showed that the CRISPR/Cas9 system, originally a natural defense mechanism used by bacteria against viruses, could be programmed to cut DNA at specific locations. This discovery marked the beginning of modern gene editing.

Currently, CRISPR/Cas9 emerges as a powerful genome editing technique, providing the opportunity to efficiently delete genes to establish isogenic cells (Larson et al. 2013; Qi et al. 2013; Cong et al. 2013). The CRISPR/Cas system was first described as an adaptive immune system in bacteria and archaeons, and has now been engineered as RNA-guided endonucleases (RGENs) for genome editing. Typically, a type II CRISPR system functions by the CRISPR RNA (crRNA) interacting with a *trans*-activating crRNA (tracrRNA) to form a crRNA·tracrRNA duplex [this RNA duplex could be replaced with a single guide RNA (sgRNA) Fig. 4], which directs *Streptococcus pyogenes* Cas9 (SpyCas9) to specific sites, thereby generating a DNA strand break (Jinek et al. 2012). The gene editing process involves the generation of a double-stranded break (DSB) at the targeted DNA sequence. This DSB subsequently triggers two competing DNA repair systems which are homology-directed repair (HDR) or non-homologous end-joining (NHEJ). NHEJ is an error-prone process in mammalian cells and can give rise to insertions or deletions (termed INDELs), either of which could change the protein coding sequences. In contrast, HDR involves homologous recombination with a donor DNA sequence to then introduce precise DNA mutations or the insertion of specific sequences in the targeted locus, such as the insertion of the DNA sequence (Cai et al. 2016).

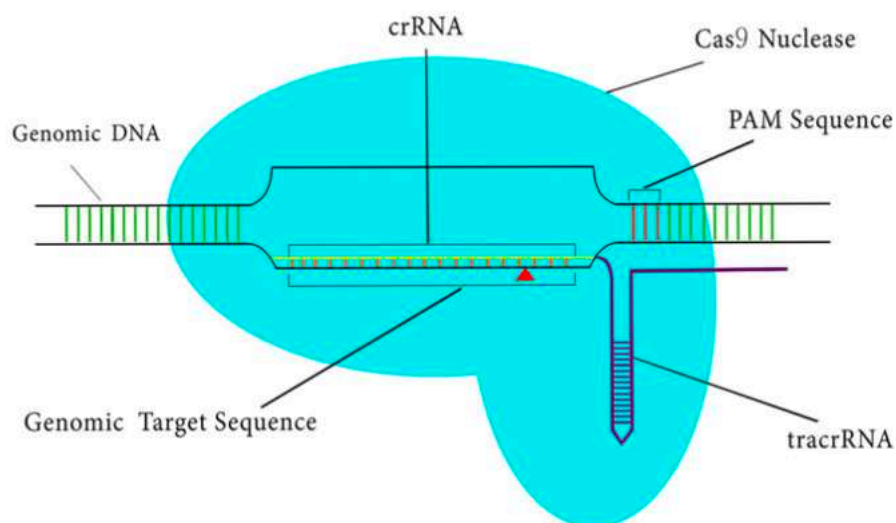


Fig. 4 Schematic representation of the molecular events involved in genome editing via CRISPR/Cas9. The sgRNA molecule consists of two parts: (1) CRISPR RNA (crRNA) (yellow) and (2) trans-activating CRISPR RNA (tracrRNA) (purple). TracrRNA forms a complex with Cas9 nuclease (blue). Cas9 is guided toward the target sequence based on the specificity determined by crRNA. It is only in the presence of a PAM that Cas9 can engage in cleaving activity. Cleavage occurs at approximately 3–4 bp upstream of the PAM sequence. The cleavage site is distinguished by a red triangle (Zarei et al. 2019).

5.1.1 CRISPR/Cas9 in the modern science

Genome engineering has introduced a new way of clarifying the function of genes involved in disease. There are numerous methods for editing genes and studying their functioning, most of which are based on nuclease enzymes. By making an incision at a specific location, these enzymes trigger an immune response in that location (Zarei et al. 2019).

To date, it is one of the most impactful methods on modern science, widely used for gene therapy. The strategies for gene therapy are diverse, enabling not only the correction, replacement, or removal of genetic mutations but also the creation of inactive mutations in pathogen genomes to combat infectious diseases or induce somatic mutations for therapeutic and protective purposes (Xiao-Jie et al. 2015). Gene therapy is now considered to be applicable to treat various genetic disorders, including blood diseases, cancer, AIDS, diabetes, cardiovascular diseases, and neurodegenerative disorders (Xu and Li 2020). Among the various gene therapies, including somatic cell gene therapy, germline gene therapy, and gene therapy vaccines, CRISPR/Cas9 technology, which modifies DNA through gene editing, has attracted much attention due to its potential to revolutionize the approach to genetic diseases and other challenging health conditions (Cring and Sheffield 2022). CRISPR/Cas9 even offers potential for effectively correcting numerous genetic diseases.

5.1.2 The importance of CRISPR-based cellular models in studies of human diseases

CRISPR/Cas9 technology has been recently applied to disease-focused research through the production and characterization of patient-derived iPS cells from individuals with specific genetic diseases. Genome editing in the form of Zinc-finger nuclease (ZFNs), Transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 has been a tremendous leap forward for the direct manipulation of the human genome, and has already changed the way

many labs understand gene function in normal and transformed cultures (Dow, 2015). Compared to ZFNs and TALENs, other genome editing techniques, CRISPR/Cas9 provides the possibility of inducing multiple mutations simultaneously, it is a unique advantage of this method compared to others (Horii et al. 2013; Wang et al. 2013a). In this regard, multiple gene targeting via multiple sgRNAs and Cas9 nucleases has been studied in the cell line of organisms such as rats, mice and zebrafish (Wang et al. 2013a; Jao et al. 2013; Li et al. 2013). The targeting efficiency of CRISPR/Cas9 is significantly higher than ZFNs and TALENs depending on the species and the delivery method. For example, the efficiency of inducing allelic mutations at six similar genomic sites in human stem cells via TALENs and CRISPR/Cas9 was 0–34% and 51– 79%, respectively, which means that it is more efficient to replace TALENs with CRISPR/Cas9 in the production of mutated colonies (Ding et al. 2013b, 2013a). The reason that causes the different efficiency of CRISPR/Cas9 in creating mutations, is the result of sgRNAs different specificity for their target sites in the genome, because however the sgRNA has a high on-target score, it is always possible to obtain some off target, reducing the success in achieving the desired genetic modification (Bortesi et al. 2016).

A method of CRISPR/Cas9 strategies is the use of purified Cas9 proteins in combination with gRNA, which are called Cas9/gRNA ribonucleoprotein complexes (Cas9-RNPs). Technically, by eliminating the Cas9 transcription and translation stages, this method increases the efficiency and speed of the genome editing process to a significant degree, at the same time as reducing the off-target effects, the production of mosaic founder animals, triggered immune responses and toxicity. Finally, using Cas9-RNPs, no insertional mutagenesis will occur. In addition, for genome editing in cells that lack strong translational machinery, such as stem cells, the use of the protein form of Cas9 is preferred over the plasmid or mRNA forms (Seki and Rutz 2018; Kouranova et al. 2016; Auer et al. 2014).

6. EXTRACELLULAR VESICLES

Exosomes are extracellular vesicles (EVs) secreted by various cell types, including animal, plant, and bacterial cells. They play a crucial role in intercellular communication and facilitate interspecies and interkingdom communication by delivering a variety of bioactive molecules (Liang et al. 2015; Raimondo et al. 2015). Interest in exosomes as carriers has surged in recent years (Sil et al. 2020; H. Rashed et al. 2017), due to their advantages in efficient and safe biomolecule delivery. Mammalian EVs have been successfully used to

deliver siRNAs (Shtam et al. 2013), miRNAs (Li et al. 2019), proteins (Haney et al. 2015), small-molecule drugs (Tian et al. 2014) and CRISPR/Cas9 components (Chen et al. 2019), aiming to develop novel therapeutics (Meng et al. 2020).

6.1 PLANT-DERIVED EXTRACELLULAR VESICLES AS PROMISING ADJUNCTS IN THE TREATMENT OF HUMAN DISEASES

Plant cells have been observed to secrete vesicles that are biogenically and morphologically comparable to mammalian-derived EVs (An et al. 2007). Plant-derived nanovesicles (PDNVs), with diameters ranging from 30 to 150 nm and a phospholipid bilayer structure, are gaining attention as eco-friendly, sustainable, and biocompatible delivery platforms (Lu et al. 2023; Bokka et al. 2020). PDNVs naturally contain plant-origin bioactive compounds (phytocompounds) widely used for therapeutic, cosmetic, and nutraceutical purposes, varying by source and extraction method (Naselli et al. 2023). Using vesicles naturally produced by plant cells as delivery agents could overcome issues, like cytotoxicity, oxidative stress, inflammation and bioaccumulation, faced by existing nanodelivery systems such as lipidic nanoparticles, metallic nanoparticles, synthetic polymers and so on. Furthermore, the natural origin of PDNVs from affordable edible plants, alleviating toxicity concerns (Raimondo et al. 2015; Wang et al. 2013b; Zhuang et al. 2015). PDVs have been shown to be vital in the regulation of physiological processes via intercellular transport of bioactive cargos (Cui et al. 2020; Woith et al. 2019). For instance, proteomic studies have demonstrated that PDNVs are abundant in enzymes that are essential for cell wall remodelling as well as proteins with antifungal and antimicrobial properties (De La Canal and Pinedo 2018; Liu et al. 2021; Regente et al. 2017; Rutter and Innes 2018). In addition, PDVs have also been found to transfer small non-coding RNAs (sRNAs) between cells, suggesting a role in gene regulation that can potentially alter cell proliferation and differentiation (Cui et al. 2020; Urzì et al. 2021). Furthermore, it has been postulated that the contents of PDNVs contribute to their intrinsic therapeutic activities as well. As most reported PDNVs are isolated from edible plants, they are expected to be safe for human consumption. In vitro studies have also demonstrated the non-toxicity of PDNVs (Naselli et al. 2024).

7. INTERPLAY BETWEEN EPIGENETIC MECHANISMS AND THE EFFECTS OF NATURAL COMPOUNDS

Given that epigenetic modifications are reversible, there is growing research investment in developing drugs that can modulate these mechanisms. A promising approach involves the use of functional foods or dietary supplements as natural epigenetic modulators for disease treatment (Farheen et al. 2024; Zhang and Tang 2023). In recent years, the study of PDEV as potential modulators of gene expression has gradually increased. In particular, these nanoscale vesicles, naturally secreted by various plant species, are now being explored not only as bioactive dietary components but also as novel delivery systems with the potential to modulate epigenetic mechanisms in the human body. As the field continues to evolve, understanding the molecular interactions between PDEVs and host cells, particularly at the level of gene regulation and chromatin remodeling, will be essential for unlocking their full potential in nutrigenomic strategies aimed at promoting health and preventing disease.

7.1 FicoVes AS ANTI-INFLAMMATORY AND ANTIOXIDANT AGENTS

Recently, Naselli et al. (2024) characterized vesicles derived from *Opuntia ficus-indica*, named FicoVes Fig. 5 and demonstrated their antioxidant, anti-inflammatory, and pro-healing properties, suggesting their promising application in biomedical fields. *Opuntia ficus-indica*, commonly known as the prickly pear cactus, is a versatile and nutritionally rich plant, highly valued in both medicinal and nutritional contexts. It thrives in arid environments and is widely cultivated in Mediterranean regions, where it is an iconic symbol of Southern Italy (La Scala et al. 2024). Prickly pear fruit has also been studied for its diuretic, hypoglycemic, analgesic, and antimicrobial effects (Murthy and Paek 2020). Prickly pear extracts are renowned for their antioxidant capability and anti-inflammatory properties (Butera et al. 2002; Aragona et al. 2018). As asserted by Naselli et al. (2024) FicoVes properties, along with their biocompatibility and ability to modulate gene expression, make FicoVes a promising candidate in the field of nutrigenomics for future therapeutic applications, in particular applying this interesting properties to disease characterized by inflammation and oxidation.

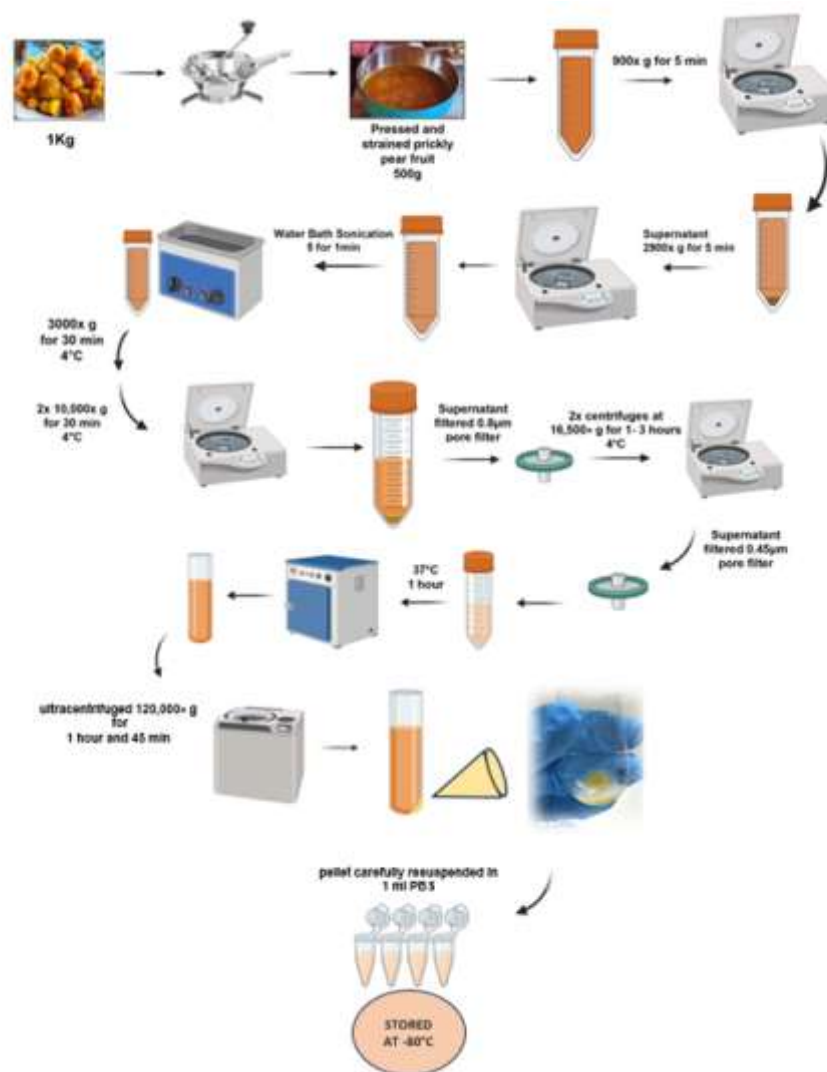


Fig. 5 Schematic of the workflow used for the isolation and purification of vesicles from *Opuntia ficus-indica*. Differential centrifugation was used to prepare samples enriched in vesicles. Further separation and purification of isolated samples were performed by ultracentrifugation (UC).

AIMS

This study primarily aims to explore how epigenetic regulation influences the onset and progression of AFD and to identify specific DNA methylation changes underlying these pathogenic mechanisms. This was approached by assessing whether affected individuals exhibit a distinct genomic DNA methylation profile compared to healthy controls and by investigating the potential utility of DNA methylation as epigenetic diagnostic biomarkers. Additionally, the study explored whether promoter methylation of the *GLA* gene is associated with the interindividual variability in symptom severity observed particularly in female patients, thereby assessing its potential role as a prognostic marker.

Given the current lack of a specific experimental model to investigate AFD and explore potential therapeutic strategies, the second objective of this study is to develop an *in vitro* cellular model of AFD to study the disease under controlled conditions. For this purpose, it was applied CRISPR/Cas9-mediated gene editing to induce a knockdown of *GLA* expression, mimicking the residual enzyme activity typically found in heterozygous female affected by AFD.

Once the validity of the cellular model was confirmed, the third aim of this thesis was to explore a potential therapeutic strategy based on the modulation of DNA methylation patterns through plant-derived extracellular vesicles (PDEV), specifically FicoVes. These nanovesicles, naturally secreted by *Opuntia ficus-indica* fruits, have already been shown to possess anti-inflammatory and antioxidant properties. In this context, the study aimed to investigate whether FicoVes could exert additional epigenetic effects by modulating gene-specific or global DNA methylation profiles and the consequent effects in the AFD cellular model, with the long-term goal of evaluating their potential as adjunctive therapeutic agents in the treatment of AFD.

MATERIALS AND METHODS

1. BLOOD SAMPLING

In collaboration with Center for Research and Diagnosis of Lysosomal Storage Disorders of IRIB-CNR in Palermo (Italy), peripheral blood samples were collected from female patients (Tab. 1) and female healthy subjects and experimental plans were approved by the Hospital Ethics Committee of the University of Palermo; signed informed consent was obtained from all participants. Peripheral blood was collected, using EDTA as an anticoagulant, and dried on absorbent paper (dried blood spot, DBS).

PBMCs from each blood sample were collected using ficoll density gradient separation (Ficoll® Paque Plus- Cytiva 17-1440-02) as previously described (Fuss et al. 2009).

Tab. 1 Clinical and genetic data of patients included in the study: mutation category, mutation details, age at diagnosis and clinical manifestations.

<i>PATIENTS</i>	<i>MUTATION</i>	<i>MUTATION</i>	<i>AGE</i>	<i>SYMPTOMS</i>
<i>CLASS</i>				
AT590	CLASSIC	G85D c.254G>A	33	Proteinuria, albuminuria, cornea verticillata, TIA, left ventricular hypertrophy
AT591	CLASSIC	G85D c.254G>A	57	Proteinuria, albuminuria, cornea verticillata, TIA, left ventricular hypertrophy, acroparesthesias, angiokeratomas
AT707	LATE-ONSET	N215S c.644A>G	20	Childhood acroparesthesias, heat/cold intolerance, recurrent abdominal
AT799	CLASSIC	G85D c.254G>A	53	Cornea verticillata
AT800	CLASSIC	G85D c.254G>A	38	Albuminuria
AT801	CLASSIC	G85D c.254G>A	29	No symptoms
AZ196	CLASSIC	c.62T>G L21R	74	Cardiomyopathy, left ventricular hypertrophy, arrhythmia
AZ237	LATE-ONSET	G395A c.1184G>C	60	Acroparesthesias, cerebral ischemia, heat/cold intolerance, hypoanhidrosis, albuminuria, arterial hypertension, gastrointestinal manifestations
AZ288	LATE-ONSET	F113L c.337T>C	36	Arrhythmia, recurrent fever

AZ289	LATE-ONSET	F113L c.337T>C	30	Arrhythmia
AZ552	LATE-ONSET	R301G	51	Acroparesthesias, abdominal pain, gastrointestinal manifestations, ear tinnitus, hypertension, recurrent headaches
BA868	CLASSIC	L414Cfs*3	59	Hypoanhidrosis, fatigability, cardiomyopathies, left ventricular hypertrophy
BA869	CLASSIC	G361R c1081	63	No symptoms
		G>A		
BC176	LATE-ONSET	G395A c.1184G>C	52	Acroparesthesias, proteinuria, TIA
BC375	LATE-ONSET	G395A c.1184G>C	30	No symptoms
BC376	CLASSIC	C.718_719delAA	60	Acroparesthesias, heat/cold intolerance, hypoanhidrosis, left ventricular hypertrophy, albuminuria, cornea verticillata
BC468	LATE-ONSET	M51I	62	Acroparesthesias, heat/cold intolerance, left ventricular hypertrophy
BF568	LATE-ONSET	C174W	69	Acroparesthesias, left ventricular hypertrophy

2. GENOMIC DNA ISOLATION

Isolation of genomic DNA from cells was carried out with the PureLink Genomic DNA Kit (Invitrogen, UK). Cells harvested from the culture, and PBMCs isolated by Ficoll-Paque™ density gradient centrifugation of whole blood were transferred into 1.5 ml Eppendorf tubes. To each sample, 40 µl of Proteinase K (20mg/mL) and 40 µl of RNase A (1.33 mg/ml) were added and incubated at room temperature for 2 minutes. Subsequently, the sample was resuspended and incubated with 200 µl of Buffer AL (lysis buffer) at 55 °C for 10 minutes. Following lysis, 400 µl of absolute ethanol were added, and the mixture was transferred into the spin column supplied with the kit, containing a cationic resin that binds DNA and enables its separation from other cellular components. The column was centrifuged for 1 minute at 12,000 rpm, followed by two wash steps with 500 µl of Buffer AW1 (wash buffer 1) and

500 µl of Buffer AW2 (wash buffer 2), each step including an additional centrifugation. Finally, DNA bound to the resin was eluted in Tris-HCl pH 8.0 (elution buffer). DNA quantification was performed using the NanoDrop® ND-1000 spectrophotometer, which allows for rapid and accurate measurement of DNA concentration. The principle underlying this method is absorbance detection, based on the fact that the maximum absorbance wavelength of DNA is 260 nm. According to the Lambert-Beer law, the following relation holds:

$$50 \text{ ng/}\mu\text{l}:1 \text{ OD} = x: \text{measured OD}$$

Thus, at a wavelength of 260 nm, an absorbance value of 1 corresponds to a concentration of 50 ng/µl for double-stranded DNA.

3. GENETIC ANALYSIS

Genomic DNA was isolated from dried blood spot using silica-coated magnetic particles in a robotic workstation designed for automated purification of nucleic acids. DNA concentrations were estimated using a biophotometer (Eppendorf, Hamburg, Germany). The search for mutations in the *GLA* gene was performed via Sanger sequencing. Eight pairs of primers were designed to analyze eight target regions containing the seven exons of the *GLA* gene, including the flanking regulatory sequences, and the cryptic exon. PCR products were purified and sequenced at Eurofins Genomics (Ebersberg, Germany) (Duro et al. 2024).

4. EPIGENOMIC ASSESSMENT OF DNA METHYLATION

To evaluate the effects of different molecules on DNA methylation in treated and untreated cells, it was employed the Methylation Sensitive Arbitrarily Primed – Polymerase Chain Reaction (MeSAP-PCR) (Volpes et al. 2023) performed in triplicate. This method involves methylation-sensitive restriction of genomic DNA, followed by two consecutive PCR reactions to identify altered methylation patterns at multiple genomic sites, with a preference for GC-rich regions. This approach provides a qualitative and semi-quantitative estimate of global DNA methylation levels and enables the detection of methylation differences between genomes.

4.1 FIRST STEP: GENOMIC DNA RESTRICTION

Genomic DNA (2 µg) from each sample was digested for 16 h at 37 °C with 10 units of **RsaI**, a methylation-insensitive restriction endonuclease recognizing the site *GTAC*, yielding monodigested DNA (MD). Half of the *RsaI*-digested DNA (1 µg) was further treated with 5 units of **HpaII**, a methylation-sensitive restriction endonuclease that does not cleave DNA when the internal cytosine of its recognition site (*CCGG*) is methylated, thus generating bidigested DNA (BD). Both digestions were processes adhered to the 3% threshold of glycerol, to ensure optimal reaction conditions.

4.2 SECOND STEP: AMPLIFICATION OF DIGESTED DNA

Both mono- and bidigested DNA were subsequently used as templates for **Arbitrarily Primed-PCR (AP-PCR)** (Fig. 12), conducted under two different amplification programs: **AP1-M** and **AP2-M**.

In both reactions, an arbitrary primer with high GC content (>50%) and a CGCG tail at the 3' end (to target CpG regions) was used, with the sequence:

5'- AACTGAAGCAGTGGCCTCGCG -3'.

- **AP1-M program (4 cycles):** featured a low annealing temperature, allowing primer elongation from multiple sites across the DNA template. This aspecific amplification ensures genome-wide representation with preference for CpG islands. The primer anneals where interactions are moderately stable, favored by conditions such as high oligonucleotide concentration, elevated MgCl₂, and low annealing temperature.
- **AP2-M program (30 cycles):** immediately followed AP1-M and employed a higher annealing temperature to increase specificity and yield of fragments generated in the initial reaction.

For each sample, 150 ng of DNA was amplified in an **AP1-M reaction mix** containing 4 mM MgCl₂, 0.2 mM dNTPs, 10 µM primer, and 0.8 U of Taq DNA polymerase in a final volume of 50 µl.

AP1-M thermal profile:

1. 94 °C for 5 min

2. 94 °C for 30 s
 3. 40 °C for 60 s
 4. 72 °C for 90 s
- } ×4 cycles

For **AP2-M**, the reaction mix included 4 mM MgCl₂, 0.2 mM dNTPs, and 2.5 U of Taq polymerase in a final volume of 50 µl.

AP2-M thermal profile:

1. 94 °C for 60 s
 2. 60 °C for 60 s
 3. 72 °C for 120 s
- } x 30 cycles

4.3 THIRD STEP: ELECTROPHORESIS AND DETECTION

Amplified DNA was separated on 6% polyacrylamide gels for ~2 h 30 min at 200 V. The resulting DNA fingerprint for each lane was visualized by staining with **GelRed** (Biotium) for 20 min and detected with the **Chemidoc System (Bio-Rad)**, followed by destaining in distilled H₂O under gentle agitation. Comparisons between monodigested and boubledigested DNA revealed bands corresponding to co-migration of fragments with identical molecular weight but different sequences, consequently, differences such as band appearance/disappearance and intensity variations (increase/decrease) were observed.

4.4 FOURTH STEP: DENSITOMETRIC SCANNING AND IMAGE ANALYSIS

The banding patterns were analyzed by densitometric scanning using **Adobe Photoshop** and **ImageJ** software. ImageJ generates graphs plotting band intensity (y-axis) against molecular weight (x-axis) for each sample, allowing comparison of MD and BD profiles and highlighting differences such as band gain/loss or changes in intensity.

Profiles were normalized using the densitometric scan of a molecular weight marker (100 bp ladder, Invitrogen). According to the principle of methylation-sensitive restriction, if CpG-rich regions are hypermethylated, the banding pattern of bidigested DNA mirrors that of monodigested DNA. Conversely, under hypomethylated conditions, distinct patterns emerge. Thus, band appearance/disappearance or intensity variations reflect differential DNA methylation states. All variations were recorded and tabulated for each sample. Finally, statistical analysis was performed using the one-way analysis of variance (ANOVA)

and Tukey's multiple comparisons test to assess the significance of differences in band appearance/disappearance and intensity variations between treated and control cultures. For the semi-quantitative evaluation, since a band in the fingerprinting profile may represent the optical result of co-migration of different amplicons sharing the same molecular weight but differing in sequence, it was calculated the differences in banding patterns between MD and DD, assigning double weight to band appearances/disappearances.

5. EPIGENETIC STUDIES OF *GLA* GENE: MSRE-PCR

Methylation Sensitive Restriction Endonuclease-PCR (MSRE-PCR) (Naselli et al. 2025) was performed in order to determine the methylation status of the CpG sites, present in the 5' *flanking region* of the *GLA* gene. In the region upstream the starting site (approximately about 687pb), the investigation was focused on only one CpG site whose methylation could be revealed by MSRE-PCR, using only the HpaII enzyme for digestion, considering this site as a reference about methylation status of the promoter. This site "62" was named since the cutting site for the methylation sensitive enzyme is located between the 62nd and 63rd nucleotide from the start of the analyzed sequence. For each sample one digestion were carried out over night at 37 °C; typically, 150 ng of DNA and 1 U of the enzyme were used. Digestions were processes adhered to the 3% threshold of glycerol, to ensure optimal reaction conditions. At the end of the incubation, the digested products were amplified using 0.5 M each primer (5'-CCGTGGTTTTCAAACGTTTTTCG-3' sense and 5'-GTGGACGCTCATGACTGTGTAT-3' antisense for 1M), 0.2 mM each dNTP, 1.5 mM MgCl₂ and 0.05 U/μl of Taq (Invitrogen, USA) using program indicated in Tab. 2.

The thermal profile is:

Tab. 2 Thermal profile indicating MSRE-PCR for GLA gene.

STAGE	TEMPERATURE	DURATION
Initial melting (hot start)	94°	5'
Melting	94°	30 sec
Annealing	61°	30 sec
Extension	72°	1'
Repetition of the Melting, Annealing and Extension phases for 30 cycles		
Last Extension	72°	6'
Soak file	4°	∞

6. α -GALACTOSIDASE A ACTIVITY ASSAY

α -GAL A enzymatic activity assay performed using the dried-blood filter paper (DBFP) test described by Chamoles et al. (2001) with some modifications [unpublished data]. A spot of 10 μ L of blood in a circle of paper 6 mm in diameter was placed into a 96-well plate, suitable for fluorometric assays, and incubated for 18 h at 37 °C in a thermomixer; the reaction was terminated by the addition of 250 μ L of 0.1 mol/L ethylenediamine (pH 11.4). The background fluorescence, i.e., fluorescence which was not due to the specific enzyme activity, was determined for each sample, conducting another reaction in the presence of 0.14 mmol/L 1-deoxygalactonojirimycin (DGJ, the inhibitor of alpha galactosidase A) in citrate phosphate buffer (pH 4.5). This background was subtracted from the fluorescence of the sample. In each assay, it was added positive and negative controls and a calibration curve with 4-methylumbelliferone.

7. LysoGB3 DETERMINATION

The determination of lysoGB3 in blood was performed via tandem mass spectrometry (MS/MS) methodology, as previously described by Polo et al. (2017).

8. STUDY OF *GLA* GENE SEQUENCE AND sgRNA DESIGN

The preliminary phases to perform the KO were based on the design of single guide-RNAs (sgRNA), which must be appropriately conjugated with the protein system to favor a particular specificity towards the sequence that has to be engineered. It was decided to focus on a specific portion of the gene that concerns the first exon and a part of the intronic portion between the first and second exon, resulting in a big deletion of a 240bp sequence with the aim to eliminate the first splicing site (Fig. 6).

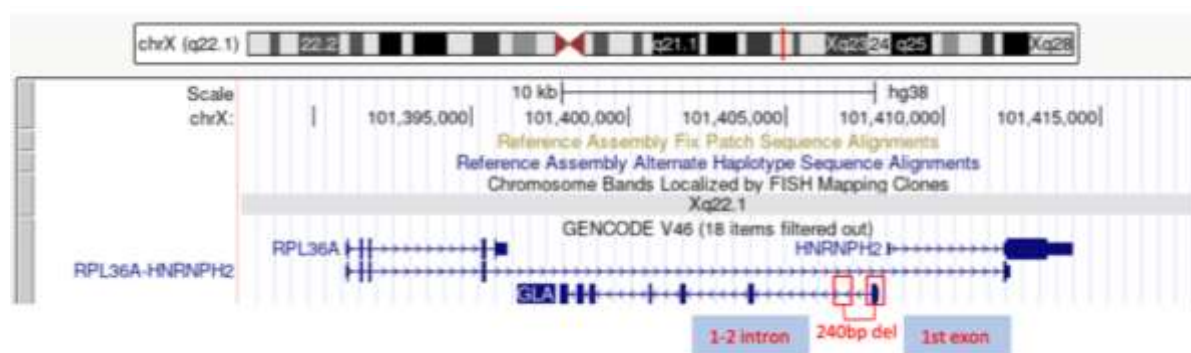


Fig. 6 Representation of the sgRNA position to make the big deletion in *GLA* gene.

The appropriate guides (Tab. 3) were designed using CHOP-CHOP and IDT software and obtained best sequences are:

Tab. 3 sgRNA sequences for GLA gene KO.

BINDING SITE	SEQUENCE	NOMENCLATURE	ON	OFF
			TARGET SCORE	TARGET SCORE
<i>1st EXON</i>	GAGGAACCCAGAACTACATCTGG	GLA-KDe	71	53
<i>1-2 INTRON</i>	GGGAACTGGAGAGTCTCAACGGG	GLA-KDi	82	59

The selection of sgRNAs was based not only on the target site, but also on their on target and off-target scores. It is well established that higher values for both parameters increase the likelihood of accurate and specific targeting of the intend genomic site.

9. PRIMERS DESIGN FOR DELETION EVALUATION BY PCR

To firstly evaluate if the right genomic deletion occurred, a couple of primers was designed. The couple presented in Tab. 4 amplify for a region that overlap the programmed deletion sequence, producing a WT amplicon of 756bp and in case of correct deletion, an amplicon of 516bp.

Tab. 4 Primers sequence to evaluate GLA gene deletion.

PRIMERS	SEQUENCE	WT	KD
		PRODUCT	PRODUCT
<i>F-KD GLA</i>	AGTCATCGGTGATTGGTCCG	756bp	516bp
<i>R-KD GLA</i>	CGAGAAATCCACGACCCCAA		

10. CELL CULTURE AND CAS9-WT PROTEIN TREATMENT

All normaloid cell lines were used for the experiments, were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with

10% fetal bovine serum (Gibco Life Technologies), 2 mmol/L L-glutamine, 1% non-essential amino acids, 10 mM HEPES, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin and were maintained at 37 °C in 5% CO₂, and 95% humidity. The medium was changed 2-3 times per week. Cell lines used for gene engineering were: HEK293T and AC16. HEK293T cells were selected as a model system for this study due to their high transfection efficiency, which makes them particularly suitable for optimizing genome editing protocols. Their ease of handling and robust response to transfection procedures rendered them a promising platform for the development and refinement of the experimental workflow. AC16 cells, a human cardiomyocyte cell line derived from fusion of primary ventricular heart cells with SV40-transformed fibroblasts, were included in the study due to their relevance as a cardiac model, and this cell line has been gently provided by Prof M.C. Cerra and Prof T. Angelone from Dept of Biology, Ecology and Earth Science, University of Calabria, Rende, CS, Italy. Given that AFD is characterized by significant cardiac involvement, the use of AC16 cells provided a biologically relevant system for disease modeling. AC16 cells retain several cardiomyocyte-like features, including expression of cardiac-specific markers and partial metabolic functionality. Therefore, achieving a stable knockout in this cell line would represent a significant step toward the establishment of a reliable in vitro model for studying AFD-related cardiac pathology. For genome editing experiments, wild-type Cas9 (Cas9-WT) protein was used at a final concentration of 30 nM, corresponding to 75 pmol per reaction. A total of 500k cells were treated with Cas9-WT in complex with the appropriate single-guide RNA (sgRNA) for 24 hours under standard culture conditions. The Cas9-sgRNA ribonucleoprotein (RNP) complex was prepared immediately prior to transfection to ensure maximal activity and specificity.

11. LIPOFECTION, RNPs COMPLEX DELIVERY SYSTEM

Nucleic acid insertion into a variety of cell types has been important since the 1980s (Baeshen et al. 2014) and the outcomes are often beneficial for advancing basic scientific research, the pharmaceutical industry, and medical uses. Lipid based transfection, also known as lipofection, is another form of chemical based transfection. Lipofection requires the formation of lipoplexes followed by incubation with target cells when the lipoplexes may fuse with the plasma membrane or are endocytosed by the target cells (Balazs and Godbey 2011) (Fig. 7). As previously mentioned, 500k cells were used for each treatment. To prepare the treatment, sgRNAs (IDT) were first solubilized in IDT nuclease-free duplex buffer (IDT)

to obtain a 100 μM stock concentration. Cas9-WT protein was diluted in OPTI-MEM to a final concentration of 2 μM . The crRNA (CRISPR RNA) with a tracrRNA (*trans*-activating RNA labeled with the fluorophore ATTO550) duplex for each previously designed guide was then prepared at a final concentration of 2 μM . Duplex formation was achieved by incubation at 95 $^{\circ}\text{C}$ for 5 minutes, followed by 10 minutes at room temperature (RT). Once the duplex was formed, RNP complex assembly was performed by combining Cas9-WT with the sgRNAs in OPTI-MEM. The mixture was incubated for 10 minutes at RT, and RNP solutions corresponding to guides designed to act together were subsequently mixed. Following RNP assembly, liposome assembly was carried out. Briefly, 10 μL of LipofectamineTM RNAiMAX was incubated with 250 μL of OPTI-MEM for each treatment (corresponding to 500k cells) to allow liposome swelling. After 5 minutes at RT, the liposomes were co-incubated with the preassembled RNPs. The mixture was then incubated for 20 minutes at RT prior to treatment of the cells, resulting in a final RNP concentration in culture of 20 nM.

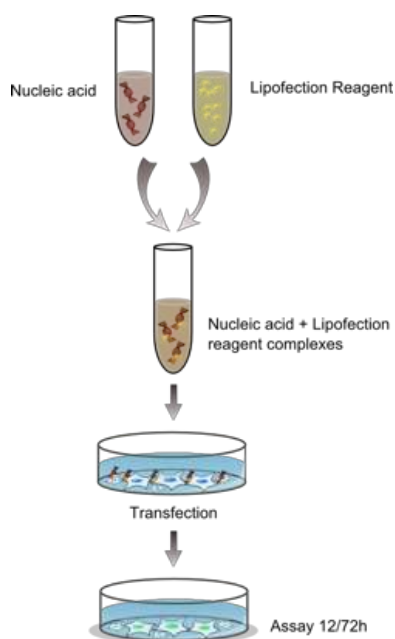


Fig. 7 Schematic representation of lipofection procedures.

12. FACS SORTING: PURIFICATION OF POSITIVE CELL POPULATION

Fluorescence-Activated Cell Sorting FACS is a highly sophisticated technique for purifying cell populations of interest, in which a very high purity (95-100%) of the sorted population

can be obtained. For flow cytometry analysis or cell sorting, cells were washed once with $1\times$ PBS and detached using reagents appropriate for the specific cell type. After a 5-minute incubation at 37°C , the reaction was stopped by adding twice the volume of culture medium, and cells were collected in 15 mL tubes. Samples were centrifuged at $300\times g$ for 5 minutes, the supernatant was removed, and cell pellets were resuspended in 1 mL of $1\times$ PBS. The suspension was passed through a $40\text{ }\mu\text{m}$ filter into FACS tubes. Cell sorting was performed on a FACSaria III™ (Becton Dickinson) at a flow rate of 1 (arbitrary units), following the manufacturer's instructions. Fluorescence intensity of the ATTO550-labeled tracrRNA was used as the gating parameter, with mock-transfected or untreated cells serving as controls. A total of 400k HEK293T cells and the maximum achievable number of AC16 cells were sorted into 1.5 mL tubes for downstream DNA isolation and reseeded for clonal selection.

13. SINGLE CELL CLONING WITH LIMITING DILUTION

A portion of the transfected and sorted positive cells was subjected to manual single-cell cloning (Fig. 8) in order to isolate individual cellular colonies, thereby generating monoclonal populations potentially carrying the targeted deletion and consequent gene knockdown. Briefly, the procedure was performed by diluting the cell suspension to a final concentration of 1 cell/100 μL in culture medium, and subsequently seeding 100 μL per well into a 96-well plate. The plate was incubated at 37°C and 5% CO_2 for approximately 4–7 days without moving the plate. Once colonies became distinguishable under the microscope ($100\times$ magnification), wells containing a single colony were selected, while those containing two or more colonies were discarded. Culture medium was replaced every 3–4 days, and plates were incubated under the same conditions for an additional two weeks, or until wells reached $\sim 50\%$ confluency. Single-cell colonies were individually expanded by detachment with trypsinization and transfer into 24-well plates containing 500 μL of culture medium per well. Cells were maintained at 37°C and 5% CO_2 until reaching 50–70% confluency, then transferred into P6 plates for further expansion. Once complete confluency was achieved, cells were harvested by trypsinization and collected for downstream analyses, including DNA and RNA extraction.

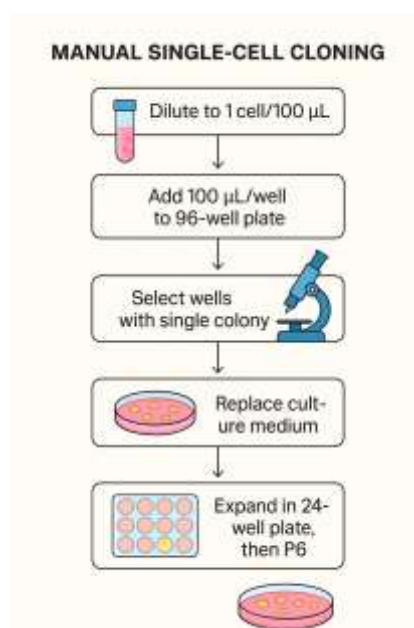


Fig. 8 Schematic representation of the single-cell cloning process.

14. QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR): GENE EXPRESSION ANALYSIS OF *GLA* IN DELETION-POSITIVE COLONIES

Total RNA was extracted from cells using PureLink™ RNA Mini Kit according to the manufacturer's protocols (Invitrogen); the RNA was eluted in RNase free water and stored at -80°C until qRT-PCR analysis. Nucleic acid concentrations were measured by spectrophotometry (NanoDrop 1000 Spectrophotometer). First-strand cDNA was synthesized from total RNA (100 ng) using a SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. mRNA expression levels were determined using first strand cDNA as the template by quantitative real-time PCR (qPCR), with Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen) in a 7300 Real Time PCR system (Applied Biosystem, Philadelphia, PA, USA) using the primers sequences in Tab. 5 and Tab. 6.

The sequences of the *GLA* primers used were:

Tab. 5 Primers sequence for GLA qPCR.

GLA Forward	TCCTGCATCAGTGAGAAGCTC
GLA Reverse	CTGATTGATGGCAATTACGTCC

GAPDH was used as an internal control with the following primers:

Tab. 6 Primers sequence for GAPDH qPCR.

GAPDH Forward	AGCCACATCGCTCAGACAC
GAPDH Reverse	GCCCAATACGACCAAATCC

PCR data obtained by the instrument software were automatically analysed by the Relative Quantification Study Software (Applied Biosystem) and expressed as target/reference ratio.

15. SDS-PAGE AND WESTERN BLOTTING ANALYSIS

After identifying the most suitable clone for subsequent experiments, in order to complete the expression study following engineering, both CL2 OLD and HEK293T wild-type cells were detached and processed for protein extraction. Cell lysates were centrifuged at $13,000 \times g$ for 5 min, and the resulting supernatants (cytosolic fractions) were aliquoted and stored at -80°C for up to two weeks. Protein concentration was quantified using the Bradford protein assay reagent (Bio-Rad, Milan, Italy). Subsequently, 30 μg of total protein was resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad) with the “STANDARD SD” protocol (30 min at 400 mA). Membranes were blocked for 1 h at room temperature in 5% BSA (in TBS 1×), and then incubated overnight at 4°C with the following primary antibodies diluted in 5% BSA/TTBS: mouse monoclonal anti- α -galactosidase A (1:250; Cat. No. MABS1935, Sigma-Aldrich/Merck, St. Louis, USA) and rabbit monoclonal anti- β -actin (1:10,000; Cat. No. ab8227, Abcam). After two washes with TTBS, membranes were incubated for 1 h at room temperature with Alexa Fluor™-conjugated secondary antibodies diluted 1:10,000 in 5% BSA/TTBS: Goat anti-Rabbit IgG (H+L), Alexa Fluor™ 790, and Goat anti-Mouse IgG (H+L), Alexa Fluor™ 680 (Thermo

Fisher). Blots were then washed five times with TTBS and visualized using the Odyssey® Imaging System (LI-COR Biosciences). Densitometric analysis of immunoblots was performed using ImageJ software.

16. CELL VIABILITY ASSAY - MTT FOR HEK-GLAKD TREATMENT WITH FicoVes

HEK-GLAKD cells were seeded onto 96-well plates at concentrations of 7kcells/well, respectively, and treated after 24-48-72h with increasing concentrations of FicoVes for 24 h in a humidified incubator (5% CO₂ in air at 37 °C) (Ragusa et al. 2023). Cell viability was assessed with MTT (Thiazolyl Blue Tetrazolium Bromide (MTT) BioChemica, A2231, Panreac AppliChem) assay, as previously performed (Bellavia et al. 2024). The absorbance was measured at 595 nm. Three independent experiments were performed to generate the percentage of growth versus control (untreated cells).

17. TREATMENT OF HEK-GLAKD CELLS WITH FicoVes

HEK293T-wt and HEK-GLAKD cells were seeded at a density of 6×10^5 cells per T25 flask. HEK-GLAKD cells were subsequently treated with FicoVes at concentrations of 5, 25, and 50 µg/mL for 24 h. As positive controls, cells were treated with 10 µM 5-Azacytidine, a well-known demethylating agent, and with BOBCAT339 (33 and 75 µM). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 2 mmol/L L-glutamine, 1% non-essential amino acids, 10 mM HEPES, 50 U/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin, and maintained at 37 °C in a humidified atmosphere with 5% CO₂. After 24 h of treatment, conditioned media were collected, and supernatants were stored at -80 °C for future experiments.

18. EVALUATION OF ATTO-LABELED FicoVes UPTAKE

FicoVes, kindly labeled with the *ATTO633 NHS ESTER* fluorophore by the group of Prof. Fabio Palumbo (University of Palermo, Italy), were used to treat HEK-GLAKD cells seeded

in 96-well plates suitable for fluorescence microscopy analysis. Cells were exposed to FicoVes at concentrations of 5, 25, and 50 µg/mL for 24 h and maintained at 37 °C in a humidified atmosphere containing 5% CO₂, in culture medium appropriate for the cell line. After 24 h of incubation, conditioned media were transferred to empty wells of the same plate, and cells were washed twice with PBS 1× supplemented with calcium and magnesium to remove excess non-internalized FicoVes while preventing cell detachment. Both treated cells and conditioned media were analyzed using the GloMax system. Subsequently, PBS was removed, and cells were fixed by adding 100 µL of 4% paraformaldehyde per well for 20 min at room temperature. After two washes with PBS 1×, nuclei were stained with 100 µL per well of Hoechst solution (1:1000 dilution) for 10 min at room temperature. Finally, two additional washes with PBS 1× (5 min each) were performed before fluorescence microscopy imaging.

19. GENE PROMOTER METHYLATION STATUS ASSESSMENT BY METHYLATION-SENSITIVE RESTRICTION ENDONUCLEASE-PCR (MSRE-PCR) OF PRO/ANTI INFLAMMATORY GENES

For the analysis of gene promoter methylation status, MSRE-PCR was performed as described by Bellavia et al., 2016. Genomic DNA from differentiated cells was separately digested with HpaII/MspI and HhaI methylation-sensitive restriction endonucleases (MSREs) and amplified by PCR in the presence of specific primers for the cg sites of *IL-6*, *IL-8* and *IL-10* genes, important for their expression (Larsson et al. 2012; Nile et al. 2008; Takahashi et al. 2015). We studied six sites for *IL-6* gene (three for HhaI MSRE and three for HpaII MSRE), two sites for *IL-8* gene (two for HpaII MSRE) and one sites for *IL-10* gene (one for HhaI MSRE).

Primers were designed using the bioinformatics software BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Primers sequences and PCR settings are listed in files Tab. 7-14.

Tab. 7 Primer couples used for MSRE-PCR on the IL-6 gene promoter.

SITE	PRIMER	SEQUENCE
IL-6/1	FORWARD	5'-GCGATGGAGTCAGAGGAAAC-3'
	REVERSE	5'-TTGTGGAGAAGGAGTTCATAGC-3'

IL-6/2	FORWARD	5'-GCGATGGAGTCAGAGGAAAC-3'
	REVERSE	5'-TTGTGGAGAAGGAGTTCATAGC-3'
IL-6/3	FORWARD	5'-GAACTCCTTCTCCACAAGTAAGTGC-3'
	REVERSE	5'-AGCCTACCCACCTCCTTTCTC-3'
IL-6/4	FORWARD	5'-GAACTCCTTCTCCACAAGTAAGTGC-3'
	REVERSE	5'-AGCCTACCCACCTCCTTTCTC-3'
IL-6/5	FORWARD	5'-CTTCGGTCCAGTTGCCTTCTCCCT-3'
	REVERSE	5'-CAGGGCAAGGGGAACGCAT-3'
IL-6/6	FORWARD	5'-CTTCGGTCCAGTTGCCTTCTCCCT-3'
	REVERSE	5'-CAGGGCAAGGGGAACGCAT-3'

Tab. 8 Primer couples used for MSRE-PCR on the IL-8 gene promoter.

SITE	PRIMER	SEQUENCE
IL-8/1	FORWARD	5'-TAAAATGATTGGCTGGCTTATCTT-3'
	REVERSE	5'-GTTTCTTCTGGCTCTTGTCTTA-3'
IL-8/2	FORWARD	5'-ATAAGGCACAACTTTCAGAGAC-3'
	REVERSE	5'-GTTACCAAAGCATCAAGAATAGC-3'

Tab. 9 Primer couples used for MSRE-PCR on the IL-10 gene promoter.

SITE	PRIMER	SEQUENCE
IL-10	FORWARD	5'-GGAACACGCGAATGAGAACCC-3'
	REVERSE	5'-TGGTTTTGCAAGAGCAAGCCC-3'

The thermal cycles are:

Tab. 10 PCR program used for IL-6/1, IL-6/2 and IL-8/2 sites digested with HpaII/MspI.

STAGE	TEMPERATURE	DURATION
Initial melting (hot start)	95°	5'
Melting	95°	1'
Annealing	57,2°	1'

Extension	72°	50''
Repetition of the Melting, Annealing and Extension phases for 30 cycles		
Last Extension	72°	6'
Soak file	4°	∞

Tab. 11 PCR program used for IL-6/3, IL-6/4 sites digested with HpaII/MspI.

STAGE	TEMPERATURE	DURATION
Initial melting (hot start)	95°	5'
Melting	95°	1'
Annealing	60,5°	1'
Extension	72°	50''
Repetition of the Melting, Annealing and Extension phases for 30 cycles		
Last Extension	72°	6'
Soak file	4°	∞

Tab. 12 PCR program used for IL-6/5 and IL-6/6 sites digested with HpaII/MspI.

STAGE	TEMPERATURE	DURATION
Initial melting (hot start)	95°	5'
Melting	95°	1'
Annealing	63,5°	1'
Extension	72°	50''
Repetition of the Melting, Annealing and Extension phases for 30 cycles		
Last Extension	72°	6'
Soak file	4°	∞

Tab. 13 PCR program used for the IL-8/1 site digested with HpaII/MspI.

STAGE	TEMPERATURE	DURATION
Initial melting (hot start)	94°	5'
Melting	94°	1'
Annealing	59°	1'
Extension	72°	1'
Repetition of the Melting, Annealing and Extension phases for 30 cycles		
Last Extension	72°	7'
Soak file	4°	∞

Tab. 14 PCR program used for the site IL-10 digested with HhaI.

STAGE	TEMPERATURE	DURATION
Initial melting (hot start)	94°	5'
Melting	94°	1'
Annealing	56°	1'
Extension	72°	1'
Repetition of the Melting, Annealing and Extension phases for 30 cycles		
Last Extension	72°	7'
Soak file	4°	∞

20. INTERLEUKIN 6 AND INTERLEUKIN 8 GENE EXPRESSION ANALYSIS BY REAL-TIME PCR

IL-6 and IL-8 qRT-PCR was performed in 96-well plates using the Step-One Plus Real-Time PCR system (Applied Biosystem). For quantitative SYBR® Green real-time PCR, using Quantinova SYBR® Green PCR Kit, Quantitect® Primer Assay was purchased from Qiagen, 70ng cDNA were used. Relative changes in gene expression between control and treated samples were determined with the $\Delta\Delta C_t$ method. Final values were expressed as fold change.

21. INTERLEUKIN 6 AND INTERLEUKIN 8 QUANTIFICATION IN CONTIDIONATED MEDIUM BY ELISA ASSAY

ELISA is an immunological biochemical assay used to detect and measure antibodies, antigens, peptides, proteins, glycoproteins, and hormones in biological samples. The method is based on the principle of detecting the antigen-antibody interaction and the enzymatic activity linked to the antibodies (Aydin 2015).

For the enzyme-linked immunosorbent assay (ELISA) Human IL-6 ELISA Kit (*Antibodies*) and Human IL-8 ELISA Kit (*biorbyt*) were used. Briefly, the 15x wash buffer was diluted as recommended, and both the biotinylated detection antibody and the protein standard were reconstituted according to the kit instructions. The protein standard was serially diluted, and conditioned media were prepared following the guidelines of kit instructions. Subsequently, 100 µl of either protein standard, sample, or control were added to each well and incubated

at room temperature for 2 hours. After incubation, wells were aspirated and washed four times. The diluted biotinylated detection antibody was then added at 100 µl per well and incubated for an additional 2 hours at room temperature. Following four further washes, 100 µl of streptavidin–HRP solution, prepared at a 1:400 dilution, was added to each well and incubated at room temperature for 30 minutes. Wells were then aspirated and washed four times. For color development, 100 µl of ready-to-use substrate solution were added to each well and incubated at room temperature until sufficient color intensity was observed. Finally, 100 µl of stop solution were added to terminate the reaction, and absorbance was measured at 450 nm.

22. OIL RED-O STAINING

Oil Red-O (ORO) is a fat-soluble dye used to stain neutral lipids, cholesteryl esters, and lipoproteins (Bharati et al. 2022). It has been widely used for intracellular lipid staining and tissue staining due to its cellular permeability, neutral fats, mainly triglycerides, can be stained with an orange-red tint (Mehlem et al. 2013; Koopman et al. 2001; Levene et al. 2012; Catta-Preta et al. 2011).

The procedures conducted for this technique are the following:

cells were seeded in a P6 well plate with a density of 80k cells/well, cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 2 mmol/L L-glutamine, 1% non-essential amino acids, 10 mM HEPES, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin and were maintained at 37 °C in 5% CO₂, and 95% humidity for 24h. To prepare the Oil Red-O stock solution, 0.35 g of Oil Red-O powder was dissolved in 100 ml of 99% isopropanol. The solution was stirred overnight, filtered through a 0.22 µm filter, and stored at 4 °C for a maximum of six months. The working solution was freshly prepared by mixing 60% of the stock solution with 40% distilled water. For staining, the culture medium was first removed from the wells. Cells were washed once with 1 ml of PBS, which was then aspirated, and subsequently fixed with 1 ml of 4% paraformaldehyde (PFA) for 20 minutes at room temperature. After fixation, PFA was discarded and cells were washed twice with 1 ml of PBS for a total of 5 minutes. The wells were then rinsed with 1 ml of 60% isopropanol (diluted in distilled water) for no longer than 5 minutes and aspirated. Complete drying of the cells was ensured, either by blotting the plate on absorbent paper and using warm air. Cells were then incubated with 1.5 ml of the working Oil Red-O solution for 20 minutes at room temperature. Following incubation, the

stain was carefully removed (one well at a time), and the cells were washed four times with distilled water to eliminate excess dye. Finally, wells were filled with distilled water, and images were acquired using a microscope at three different magnifications (10x, 20x, 40x). Three images were taken per well, and quantitative image analysis was performed using ImageJ software. For lipid quantification, the water was discarded, and the wells were allowed to dry completely. Lipid-bound Oil Red-O was then eluted by adding 500 μ l of 100% isopropanol per well and incubating for 10 minutes at room temperature. The solution was homogenized by gentle pipetting (up-and-down movements). From each well, 140 μ l of the eluted solution was transferred into a 96-well plate. Three blank wells containing only 100% isopropanol were included as controls. Absorbance was measured at 492 nm and 600 nm using a spectrophotometer.

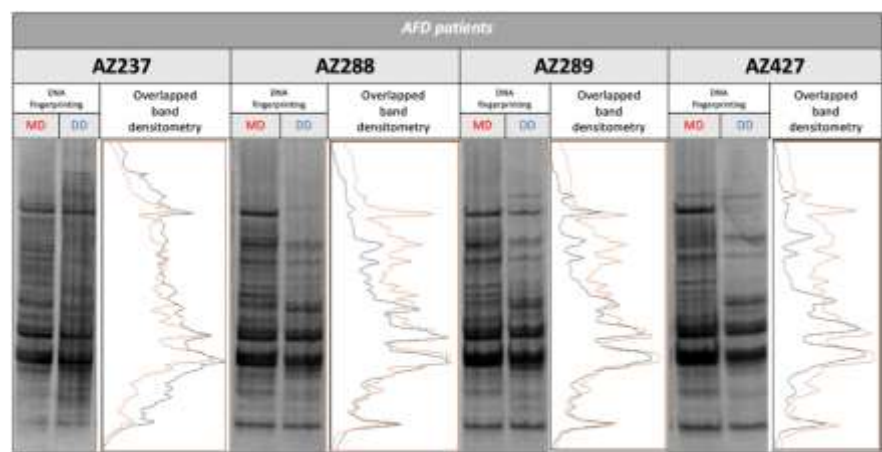
RESULTS

1. DNA METHYLATION STUDIES IN PBMCS SAMPLES FROM PATIENTS

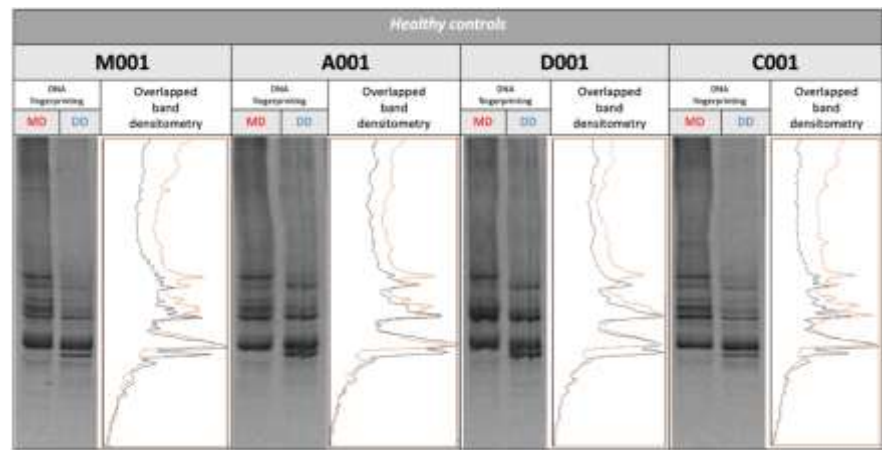
1.1 WIDESPREAD DNA HYPOMETHYLATION CHARACTERIZES FABRY DISEASE GENOMES

To assess DNA methylation in patients with AFD and compare it with that of healthy individuals, it was performed MeSAP-PCR. The differences in band patterns generated after digestion with methylation-sensitive (RSAI) and non-sensitive enzymes (HpaII) indicate the degree of global DNA methylation. The comparison between the band patterns of AFD patients and healthy controls revealed a higher number of bands in affected individuals, (Tab. 15) suggesting a state of DNA hypomethylation with respect to healthy subjects (Fig. 9).

A



B



C

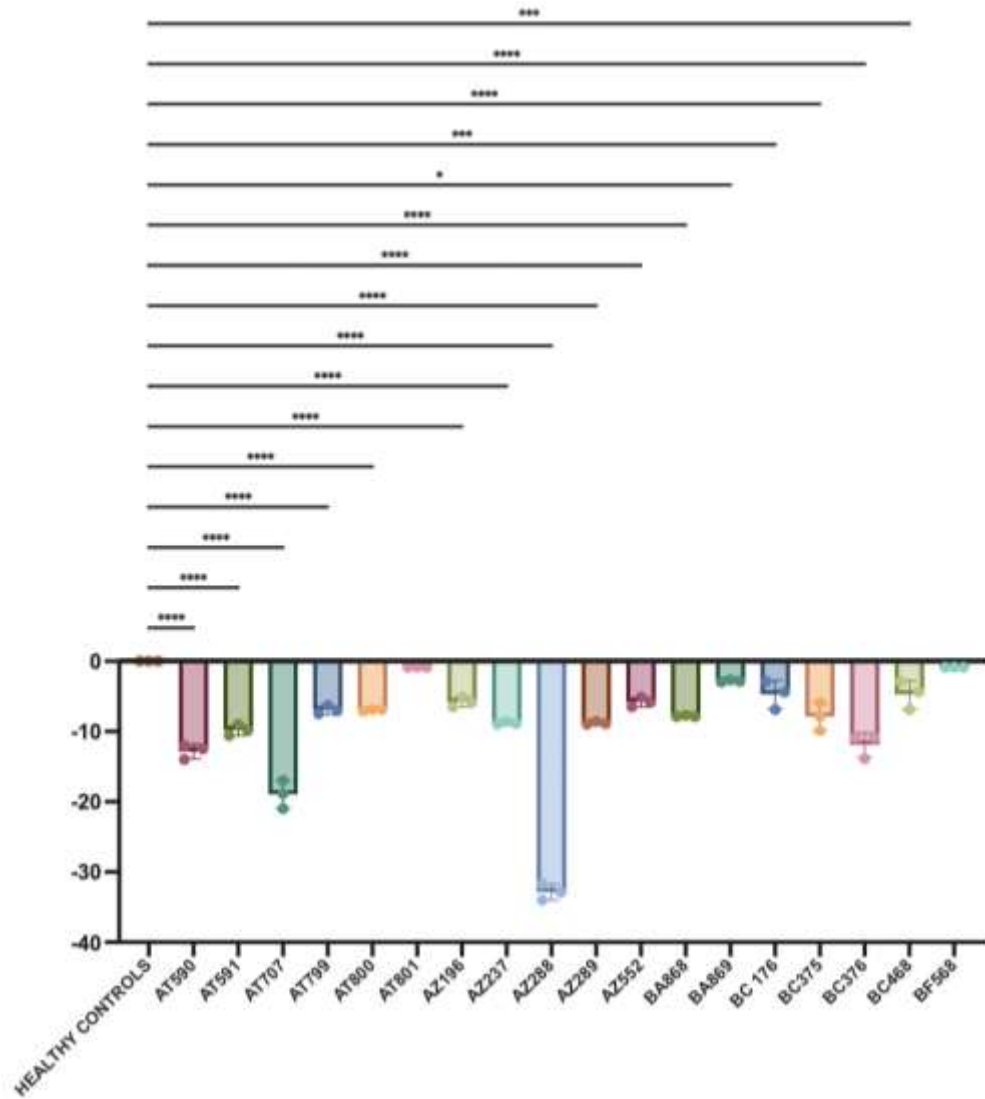


Fig. 9 Representative MeSAP-DNA fingerprinting and relative scanning densitometry (A-B) indicating genomic DNA methylation of (A) AFD affected patients and (B) healthy controls; (C) Graphic representation of the changes in DNA methylation in patients net of the average data of healthy subjects (data shown in Table 1). Band pattern variation, in terms of intensification/attenuation and appearance/disappearance was evaluated by densitometer scanning of mono- digested DNA (MD) in comparison with double-digested DNA (DD). Every single value obtained for the patients is reported here after subtracting the average control value in order to highlight the real difference. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $**p<0.005$, $***p<0.001$, $****p<0.0001$.

Tab. 15 Genomewide DNA methylation study in 18 patients in relation to the average of the variations in healthy subjects and relative significance analysis. Intensifications/attenuations (I/A) and appearances/disappearances (A/D).

	SAMPLES	I/A MEAN	A/D MEAN	Tot. variations	Numerical distance	Significance
	CTR MEAN	3.556	2.278	8.112		
1	AT590	9	6	21.000	-12.888	$p < 0.001$
2	AT591	12	3	18.000	-9.888	$p < 0.001$
3	AT707	3	12	27.000	-18.888	$p < 0.001$
4	AT799	7	4	15.000	-6.888	$0.02 < p < 0.001$
5	AT800	9	3	15.000	-6.888	$0.02 < p < 0.001$
6	AT801	7	1	9.000	-0.888	NS
7	AZ196	6	4	14.000	-5.888	NS
8	AZ237	11	3	17.000	-8.888	$0.0025 < p < 0.001$
9	AZ288	11	15	41.000	-32.888	$p < 0.001$
10	AZ289	11	3	17.000	-8.888	$0.0025 < p < 0.001$
11	AZ552	4	5	14.000	-5.888	NS
12	BA868	12	2	16.000	-7.888	$0.005 < p < 0.0025$
13	BA869	3	4	11.000	-2.888	NS
14	BC176	3	7	17.000	-8.888	NS
15	BC375	4	5.5	15.000	-6.888	$0.005 < p < 0.0025$
16	BC376	6	7	20.000	-11.888	$p < 0.001$
17	BC468	4	4.5	13.000	-4.888	NS
18	BF568	5	2	9.000	-0.888	NS

1.2 COMPARISON BETWEEN GENOMIC DNA METHYLATION AND BIOCHEMICAL DATA

It was analyzed biochemical data regarding lysoGB3 accumulation and α -GAL A enzymatic activity (Tab. 16) to correlate them with the previously obtained methylation data.

It was arbitrarily set a cutoff based on the number of band variations observed in the DNA methylation pattern of patients. This cutoff was set at 6 variations, allowing us to identify two distinct populations within the cohort based on the degree of global DNA methylation. In Fig. 10, it is shown that 67% of the patients exhibit more than 6 variations in methylation towards demethylation, while 33% show fewer than 6 variations. Among those with more than 6 variations, 58% are characterized by lysoGB3 accumulation >2.3 nmol/L (pathological), whereas 42% have lysoGB3 accumulation <2.3 nmol/L (normal). Regarding enzymatic activity, 75% of patients with more than 6 variations have α -GAL A activity >3 nmol/L/h (normal), while 25% have α -GAL A activity <3 nmol/L/h (pathological). In contrast, the category with fewer than 6 variations is characterized by 67% lysoGB3 accumulation >2.3 nmol/L (pathological) and 33% of this category have lysoGB3 accumulation <2.3 nmol/L (normal). Indeed, for the enzymatic activity, 33% have α -GAL A activity <3 nmol/L/h (pathological), while 67% have α -GAL A activity >3 nmol/L/h (normal).

Tab. 16 Comparison between the genomic methylation data obtained by MeSAP-PCR and the biochemical data, in particular the values of α -GAL A activity and lysoGB3. Considered as -6 the cut-off value of the genomic methylation variation below which the patients are categorized in orange those with values lower than -6, while in purple those with values higher than -6. Furthermore, the respective values of enzymatic activity and lysosomal accumulation are tabulated, dividing them into pathological and non-pathological (red and green).

PATIENTS	GENOMIC METHYLATION	α -GAL A activity (pat<3nmol/mL/h)	lysoGB3 (pat>2.3nmol/L)	MUTATION CLASS	MUTATION
AT590	-12.888	9	5.73	CLASSIC	G85D c.254G>A
AT591	-9.888	14.8	9.42	CLASSIC	G85D c.254G>A
AT707	-18.888	3	2.8	LATE- ONSET	N215S c.644A>G
AT799	-6.888	6.2	4.83	CLASSIC	G85D c.254G>A
AT800	-6.888	8.1	5.85	CLASSIC	G85D c.254G>A
AT801	-0.888	2.6	10.82	CLASSIC	G85D c.254G>A
AZ196	-5.888	6.5	6.23	CLASSIC	c.62T>G L21R
AZ237	-8.888	1.3	1.27	LATE- ONSET	G395A c.1184G>C

AZ288	-32.888	3.2	1.65	LATE- ONSET	F113L c.337T>C
AZ289	-8.888	1.8	1.53	LATE- ONSET	F113L c.337T>C
AZ552	-5.888	5.8	1.78	LATE- ONSET	R301G
BA868	-7.888	14.3	12.98	CLASSIC	L414Cfs*3
BA869	-2.888	2.4	3.06	CLASSIC	G361R c1081 G>A
BC176	-8.888	4.7	1.56	LATE- ONSET	G395A c.1184G>C
BC375	-6.888	11.9	1.53	LATE- ONSET	G395A c.1184G>C
BC376	-11.888	8.5	8.65	CLASSIC	C.718_719delAA
BC468	-4.888	9.2	1.4	LATE- ONSET	M51I
BF568	-0.888	5.3	2.93	LATE- ONSET	C174W

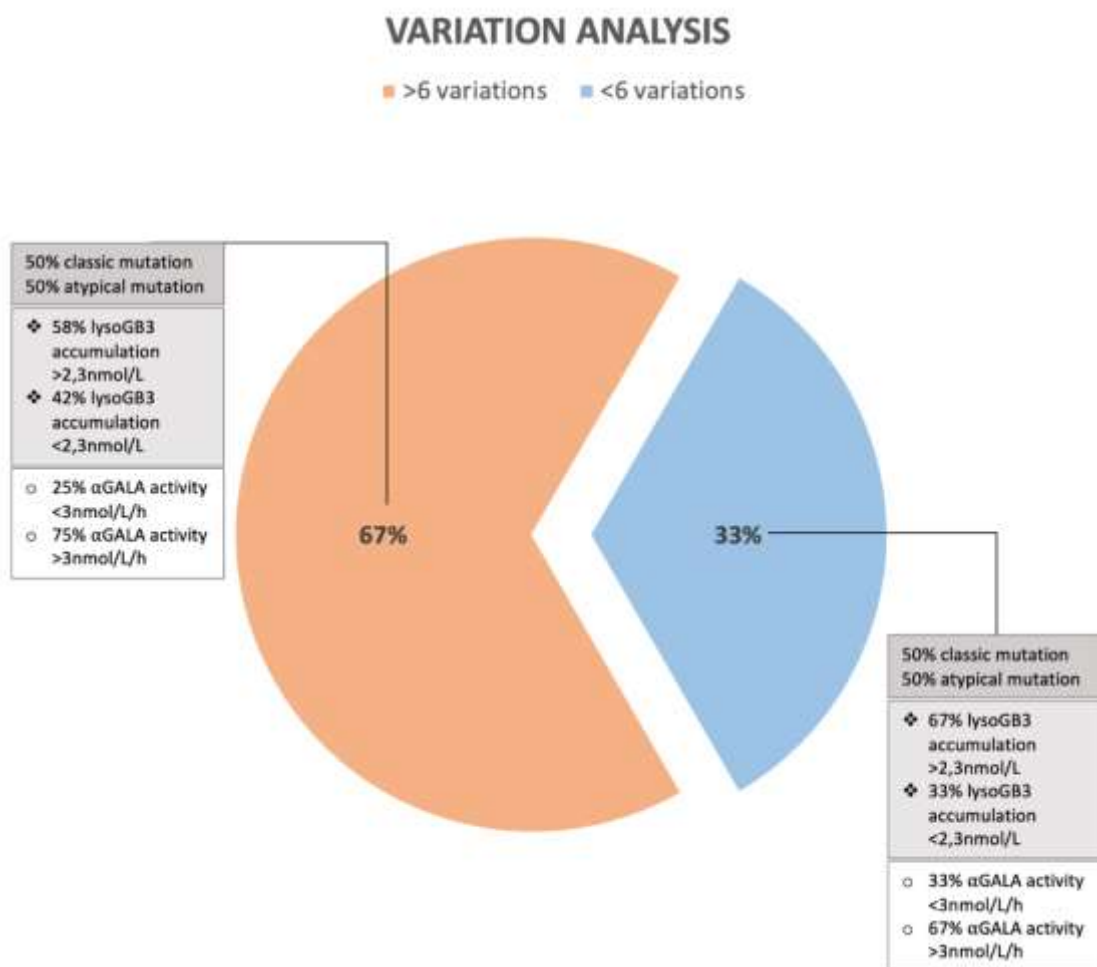


Fig. 10 Pie chart representing the categorization of patients analyzed in the work. Patients' data divided according to the number of methylation variations and then analyzed in percentage according to mutation class, lysosomal accumulation and enzymatic activity.

1.3 LOCAL DNA HYPERMETHYLATION OF THE *GLA* GENE IN AFD PATIENTS

To evaluate the methylation of the *GLA* gene promoter, DNA samples from both healthy individuals and patients with AFD were treated with a methylation-sensitive endonuclease using the MSRE-PCR technique. The results, shown in Fig. 13A, indicate that in healthy individuals, the *GLA* gene promoter is methylated ranging from 50% to 70%. The results obtained from MSRE-PCR (Fig. 13B) for the 11 samples from AFD patients showed an average methylation level of the *GLA* gene site ranging from 60% to 90%.

There is an increased percentage of methylation in AFD patients with respect to healthy subjects. Fig. 12B summarize the variations in methylation percentages for each examined sample.

Healthy controls			
S001		M001	
UN	HpaII	UN	HpaII

Fig. 11 Healthy controls MSRE-PCR. Representative MSRE-PCR on GLA gene of 2 healthy subjects (UN: undigested sample; HpaII: sample digested with HpaII)

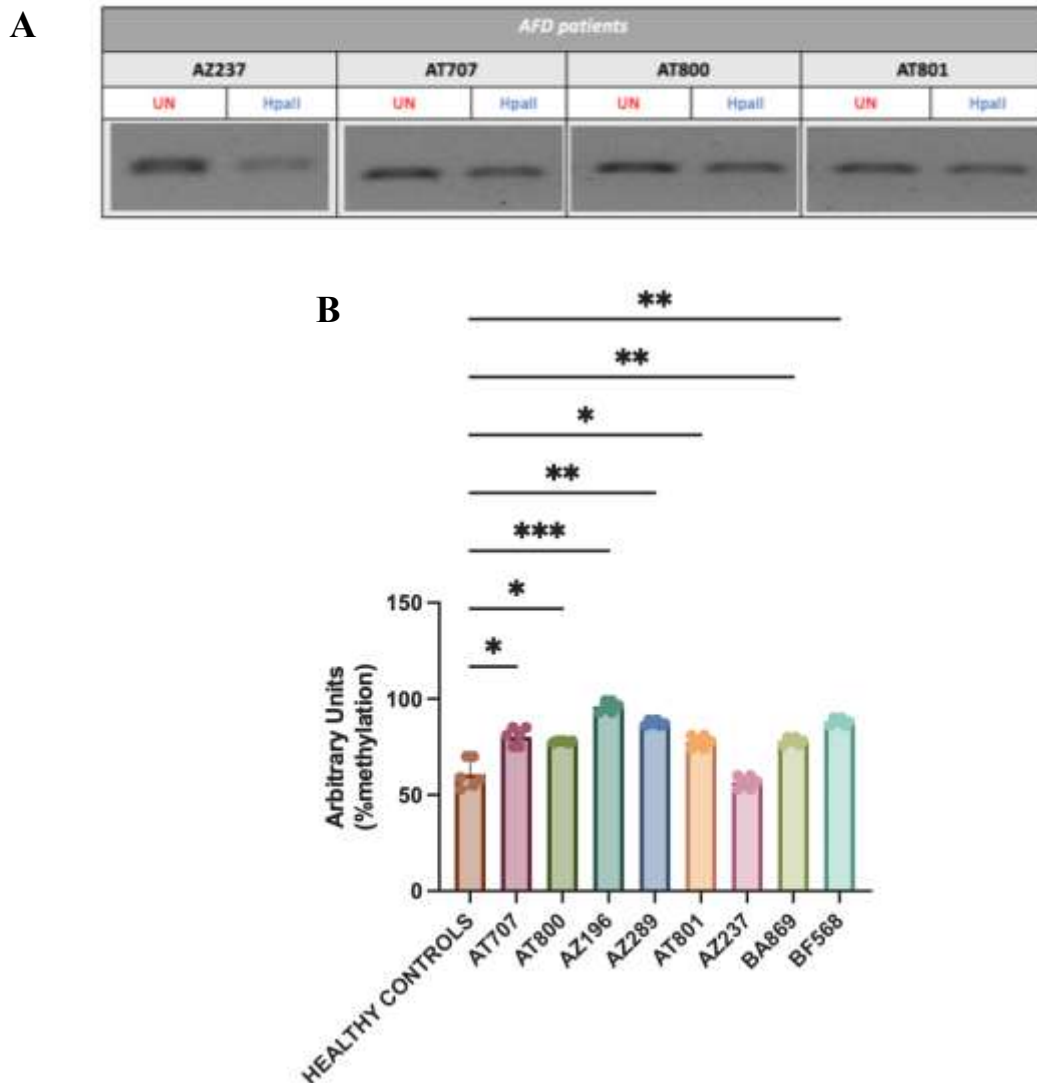


Fig. 12 AFD patients MSRE-PCR. (A) Representative MSRE-PCR on GLA gene of 4 AFD patients (UN: undigested sample; HpaII: sample digested with HpaII). (B) MSRE-PCR revealing methylation status of 62 site in the promoter region of GLA gene. Densitometry of the bands is showed by histograms, which represent the mean of three experiments. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.0005$.

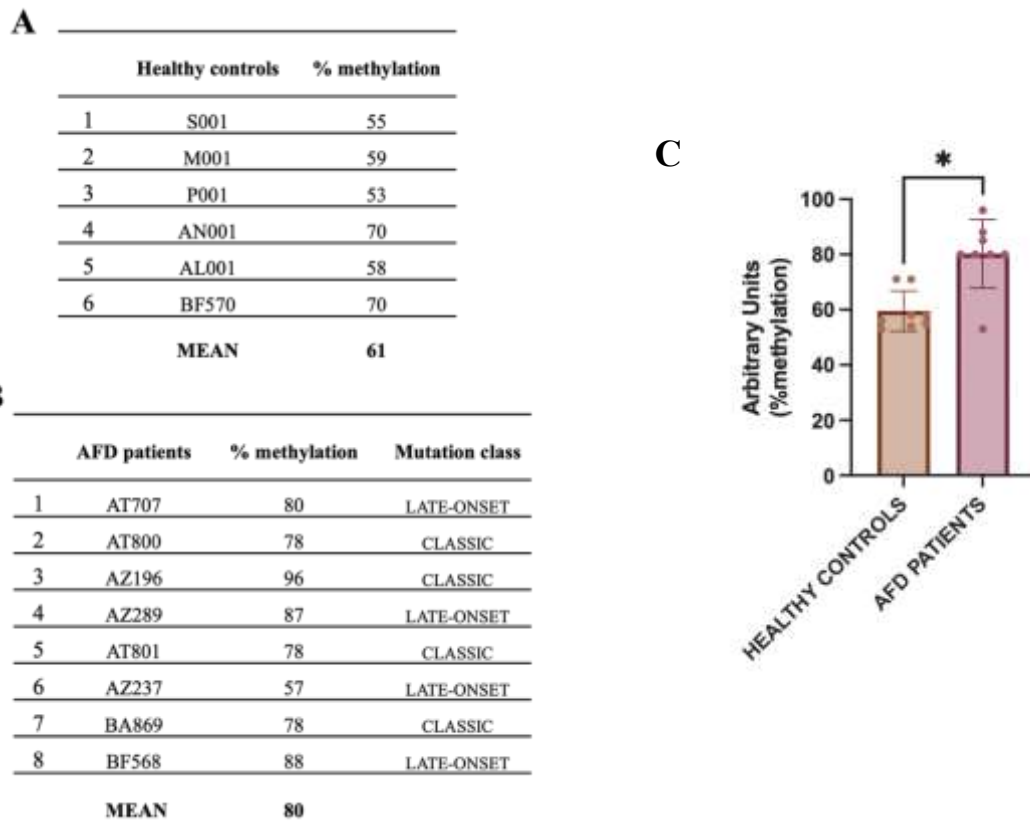


Fig. 13 Methylation percentages of pathological subjects, set the undigested genomic DNA to 100%. (A) *GLA* promoter methylation status of healthy controls; (B) *GLA* promoter methylation status of AFD patients object of the study; (C) Histogram showing data means of methylation analysis, healthy controls vs AFD patients. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (*) $p < 0.0025$.

1.4 COMPARISON BETWEEN *GLA* GENE METHYLATION AND BIOCHEMICAL DATA

One aim was to compare and associate the site-specific methylation data of the *GLA* gene with the enzymatic activity of α -GAL A and lysosomal accumulation lysoGB3, which, as a reminder, result from the lack of expression or activity of the *GLA* gene.

Considering a state of hypermethylation for *GLA* gene promoter of AFD patients as a methylation level greater than 60%, Tab. 17 shows data on the analyzed patients, including *GLA* gene promoter methylation levels, enzymatic activity, and lysosomal accumulation. The 85% of patients exhibiting *GLA* gene hypermethylation also had lysoGB3 accumulation > 2.3 nmol/L, indicating a pathological condition. Regarding α -GAL A enzymatic activity, 57% of the patients with hypermethylated promoter also showed reduced enzymatic activity.

However, 43% of patients with *GLA* gene hypermethylation still exhibited normal enzymatic activity (Fig. 14).

Tab. 17 Comparison between gene methylation data of the GLA gene promoter and the values of α -GAL A activity and lysoGB3. In orange the abnormal gene methylation values and in light blue the normal values. In green are indicated the normal values of α -GAL A activity and lysoGB3 while in red the pathological ones.

<i>AFD</i> <i>patients</i>	<i>Promoter</i> <i>methylation</i> <i>status</i>	<i>α-GAL A activity</i> <i>(pat<3nmol/mL/h)</i>	<i>lysoGB3</i> <i>(pat>2.3nmol/L)</i>	<i>MUTATION</i> <i>CLASS</i>	<i>MUTATION</i>
AT707	80	3	2.8	LATE-ONSET	N215S c.644A>G
AT800	78	8.1	5.85	CLASSIC	G85D c.254G>A
AZ196	96	6.5	6.23	CLASSIC	c.62T>G L21R
AZ289	87	1.8	1.53	LATE-ONSET	F113L c.337T>C
AT801	78	2.6	10.82	CLASSIC	G85D c.254G>A
AZ237	57	1.3	1.27	LATE-ONSET	G395A c.1184G>C
BA869	78	2.4	3.06	CLASSIC	G361R c.1081 G>A
BF568	88	5.3	2.93	LATE-ONSET	C174W

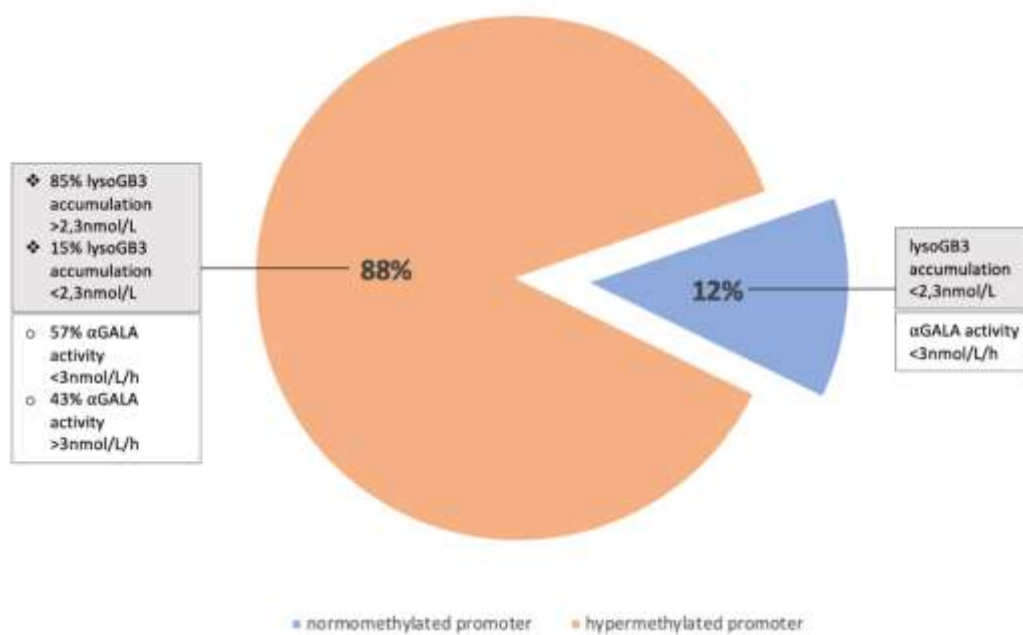


Fig. 14 Pie chart representing the categorization of patients analyzed in the work. Patients divided according to methylation status of GLA gene promoter then analyzed in percentage according to lysosomal accumulation and enzymatic activity.

2. CREATION OF AFD CELL MODEL THROUGH CRISPR/Cas9

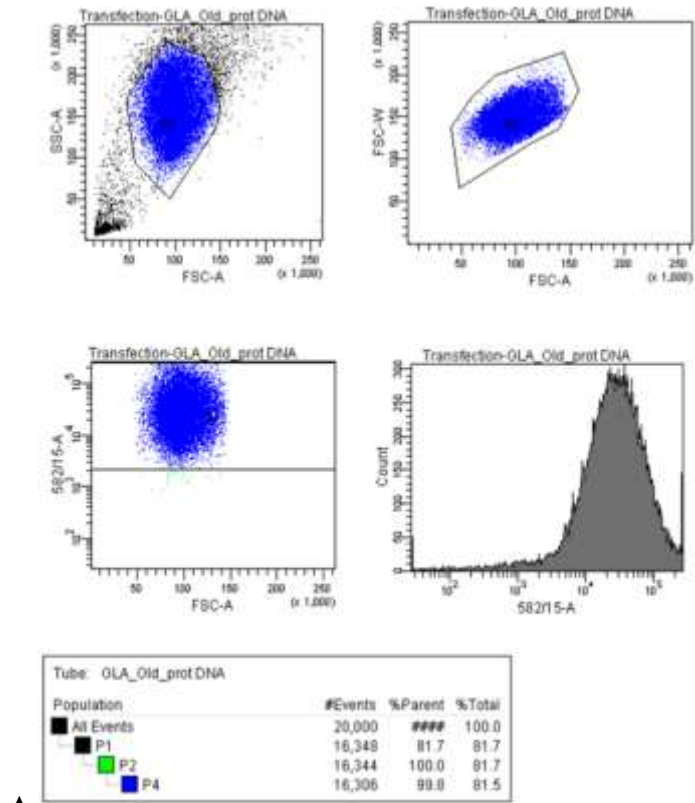
In the present project, the second objective, as previously stated, was the establishment of a cellular model of AFD, specifically through the knockdown of the *GLA* gene. This objective was pursued using the HEK293T cell line, which is highly amenable to transfection, and AC16 cells, a line of normal human cardiomyocytes.

2.1 EFFICIENT CELL FACS-SORTING FOR POSITIVE HEK293T AND AC16 CELLS AFTER LIPOFECTION

FACS sorting was performed after lipofection to select the cells positive for transfection, based on the fluorescence signal from ATTO550. As shown in Tab. 18, approximately 400,000 transfected cells were sorted for each condition. The transfection efficiency was remarkably high, approaching nearly 100%. Fig. 15 shows a representative FACS plot, where an efficiency of 99.8% can be observed.

Tab. 18 Representation of the sorted cell populations (HEK293T, AC16) and the corresponding lipofection efficiency.

<i>SAMPLES</i>	<i>SORTED HEK293T CELLS</i>	<i>TRANFECTION EFFICIENCY</i>	<i>SORTED AC16 CELLS</i>	<i>TRANFECTION EFFICIENCY</i>
MOCK	400.000	-	200.000	-
-CTR	400.000	-	200.000	-
OLD PROT	400.000	99.7%	125.000	38.3%
NEW PROT	400.000	99.5%	110.000	26.1%



A.

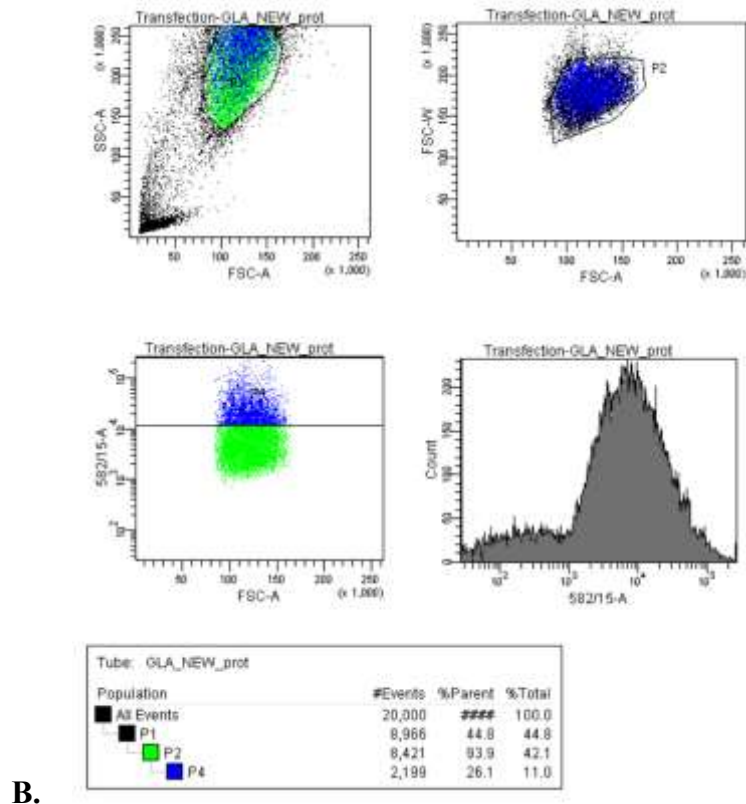


Fig. 15 Representative FACS sorting plot of HEK293T cells (A) and AC16 cells (B) after lipofection.

2.2 DNA EXTRACTION AND PCR FROM SORTED HEK2937 CELLS

After selecting the transfected cells, DNA was extracted from the sorted fraction, yielding a good amount of DNA from each sample (Tab. 19). A control PCR was then performed using the primers listed in Tab. 4 to assess whether the sorted population carried the intended deletion, even if only in a heterogeneous manner. As shown, both populations manipulated with CAS9-WT displayed a band at the expected molecular weight corresponding to the induced deletion (Fig. 16).

Tab. 19 DNA extracted from sorted cells population.

<i>SAMPLES</i>	<i>HEK293T DNA</i>	<i>AC16 DNA</i>
	<i>CONCENTRATION</i>	<i>CONCENTRATION</i>
MOCK	281.85 ng/ μ L	51 ng/ μ L
-CTR	171.05 ng/ μ L	40.75 ng/ μ L
OLD PROT	162.45 ng/ μ L	38.1 ng/ μ L
NEW PROT	209.3 ng/ μ L	26.9 ng/ μ L

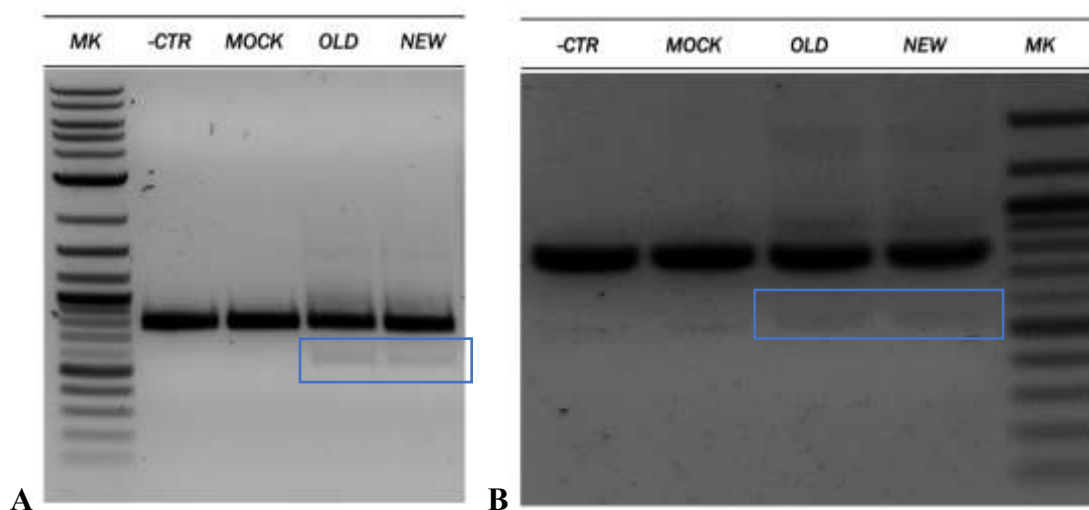


Fig. 16 Agarose gel showing the PCR results used to assess the presence of the programmed deletion in both HEK293T cells (A) and AC16 cells (B). Both the population treated with the old batch of CAS9-WT and the one with the new batch display the expected band shift (516 bp vs 756 bp).

2.3 CLONAL SELECTION HEK293T AND AC16 CELLS, DNA AND RNA

EXTRACTION

After obtaining a band at the expected molecular weight, but still detecting the WT band, clonal selection was carried out to isolate an isogenic *GLA* KD clone (Fig. 17). After the appropriate culture period, 7 clones derived from treatment with the old protein batch and 7 clones from treatment with the new batch were selected (Tab. 20). DNA was then extracted from these clones for PCR analysis.

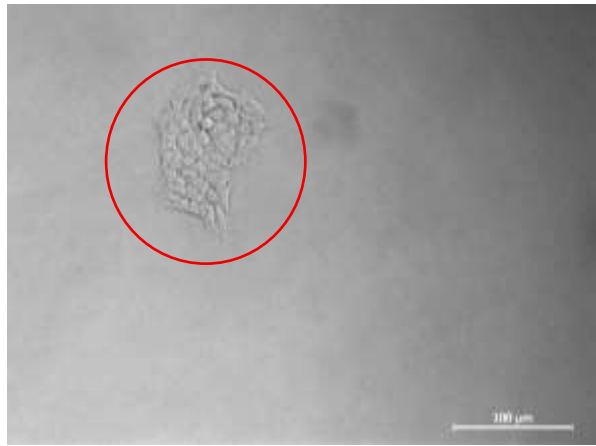


Fig. 17 Representative image of a single clonal population.

Tab. 20 Selected clones and corresponding DNA quantification.

<i>CLONES FROM OLD PROTEIN</i>	<i>DNA concentration (ng/μL)</i>	<i>CLONES FROM NEW PROTEIN</i>	<i>DNA concentration (ng/μL)</i>
1 OLD	217.25	1 NEW	170.65
2 OLD	431.05	2 NEW	176.4
4 OLD	193.2	4 NEW	122.85
5 OLD	116.95	5 NEW	152.55
6 OLD	145.15	6 NEW	220.6
7 OLD	209.75	7 NEW	190

2.4 PCR AND qRT-PCR HEK293T SELECTED CLONES

PCR analysis performed on the monoclonal cell population confirmed the presence of the 516 bp amplicon, indicative of the successful gene deletion. Clones C2 OLD, C7 OLD, and C1 NEW tested positive for the deletion while still retaining the wild-type amplicon (Fig. 18

A, B). In order to evaluate *GLA* gene residual expression Real Time PCR was carried out, this technique utilises the fluorophore, Sybr Green, which intercalates in a nonspecific manner into the DNA double helix, that is formed during the PCR. Sybr Green when excited emits a green light whose intensity is proportional to the concentration of amplified DNA. In particular the number of the PCR cycle when sybr green starts to emit fluorescence is proportional to the original amount of cDNA in each sample. High-quality RNA was subsequently extracted from these clones (Tab. 21) and used for q-RT-PCR analysis. Gene expression quantification revealed residual transcript levels of 9.4% in clone C2 OLD, 18% in clone C7 OLD, and 32.6% in clone C1 NEW, compared to the non-deleted control (Fig. 19).



Fig. 18 (A, B) Agarose gel electrophoresis showing PCR results for the detection of the targeted gene deletion in monoclonal cell populations from HEK293T cells.

Tab. 21 RNA concentration (ng/ μ L) extracted from the analyzed monoclonal clones.

SELECTED HEK293T CLONES	RNA concentration (ng/μL)
C2 OLD	58.48
C7 OLD	67.72
C1 NEW	57.56

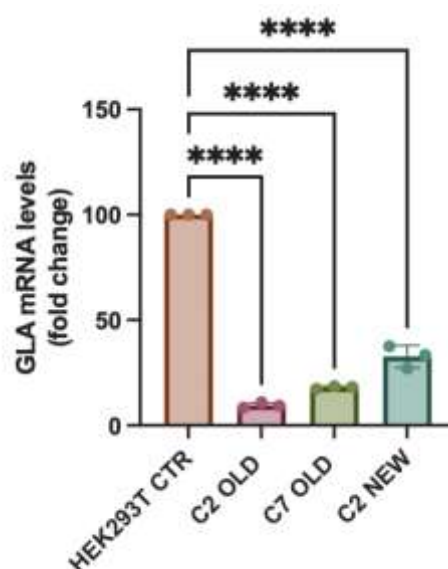


Fig. 19 Histogram showing relative gene expression levels measured by qRT-PCR in the HEK293T selected monoclonal cell populations. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (***) $p < 0.0001$.

2.5 qRT-PCR IN AC16 SELECTED CLONES

In order to evaluate in AC16 selected clones directly *GLA* gene residual expression Real Time PCR was carried out, this technique utilises the fluorophore, Sybr Green, which intercalates in a nonspecific manner into the DNA double helix, that is formed during the PCR. Sybr Green when excited emits a green light whose intensity is proportional to the concentration of amplified DNA. In particular the number of the PCR cycle when sybr green

starts to emit fluorescence is proportional to the original amount of cDNA in each sample. High-quality RNA was subsequently extracted from these clones (Tab. 22) and used for q-RT-PCR analysis. Gene expression quantification revealed residual transcript levels of 56,7% in clone C3 OLD, compared to the non-deleted control (Fig. 20).

Tab. 22 RNA concentration (ng/μL) extracted from the analyzed monoclonal clones.

SELECTED AC16 CLONES	RNA concentration (ng/μL)
C2O	34.56
C3O	74.32
C4O	36.24
C5O	29.56
C8O	36.32
C2N	69.64
C5N	65.96

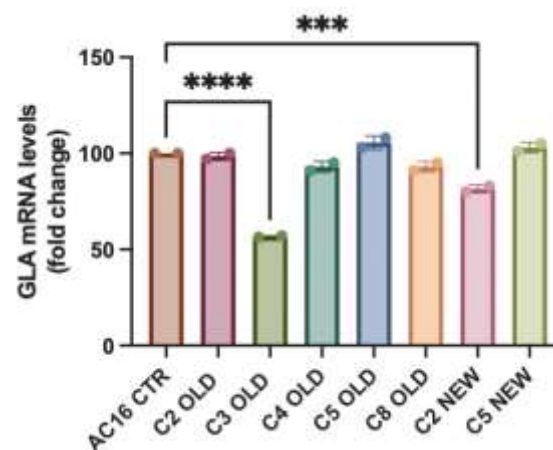


Fig. 20 Histogram showing relative gene expression levels measured by q-RT-PCR in the selected monoclonal AC16 cell populations. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (***) $p < 0.0005$, (****) $p < 0.0001$.

2.6 RESIDUAL PROTEIN EXPRESSION ANALYSIS BY WESTERN BLOT

Since among the analyzed clones in HEK293T cells, CL2 OLD is the most favorable data in terms of KD value and residual *GLA* gene expression, this clone was selected as the optimal experimental system for subsequent expression analysis and from now it will be called *HEK-GLAKD*.

2.6.1 *Impact of programmed deletion on α -galactosidase A protein expression*

In order to assess whether the programmed gene deletion affects protein expression in addition to gene expression, a Western blot analysis was performed.

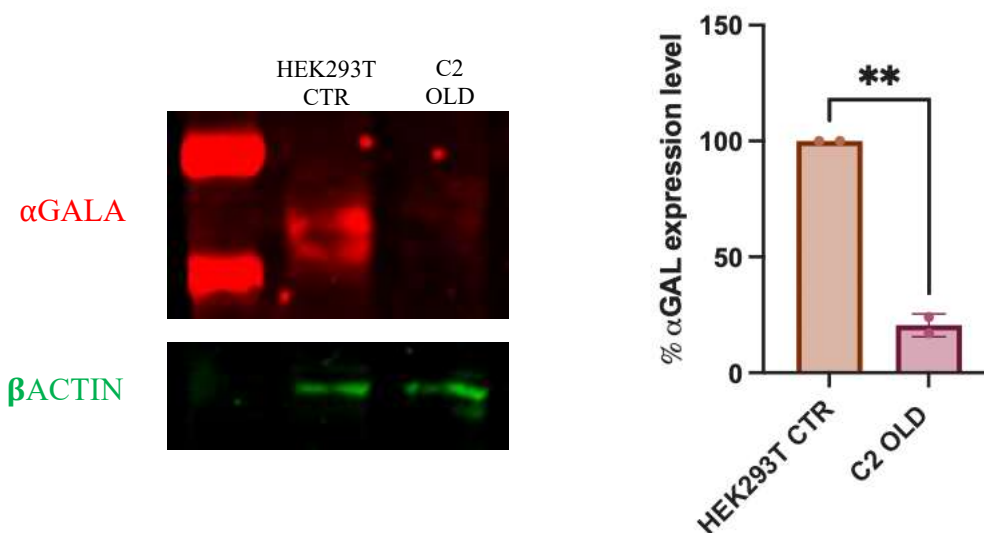


*Fig. 21 α GAL protein expression by Western blot analysis. Those are representative of three experiments with similar results. Statistical analysis performed with t-student test. Differences were considered significant at (**) $p < 0.005$.*

As shown in the Fig. 21, engineering of the cell line resulted in an 81% reduction in expression in cells positive for the deletion and genetic knockdown. From now, the cellular model is designated as HEK-GLAKD.

3. FicoVes EXHIBIT NO CYTOTOXIC EFFECTS ON HEK-GLAKD CELLS UNDER ANY CONDITION TESTED

To assess the cytocompatibility of FicoVes for HEK-GLAKD cells, cell lines were treated with FicoVes 5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ for 24, 48 and 72h. As shown in Fig. 22 However FicoVes significantly alter the viability of cells at some concentration and time of treatment, the viability levels were above 70% in all times and concentration tested, so it is possible to identify FicoVes no toxic treatment, as suggested by the guidelines (Standard, I. ANSI/AAMI/ISO 10993-5:2009/(R)2014).

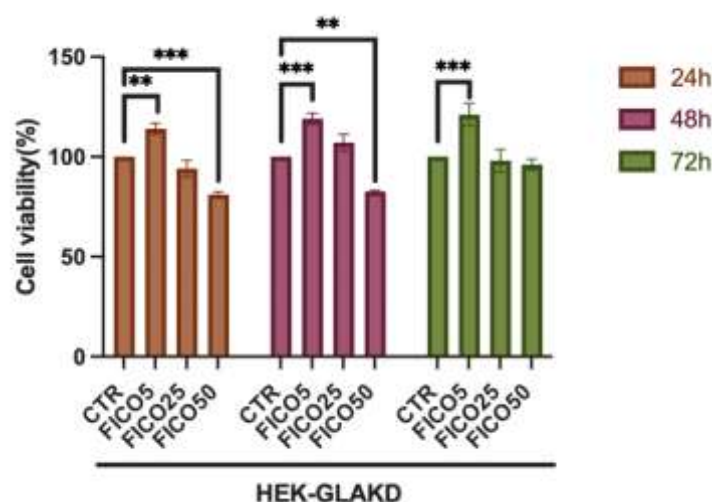


Fig. 22 Effects of FicoVes (5–50 $\mu\text{g/mL}$) on cell viability tested for 24h-48h-72h. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (**) $p < 0.05$, (***) $p < 0.001$.

4. EFFECTIVE INTERNALIZATION OF FicoVes IN HEK-GLAKD CELLS

To assess the localization of FicoVes in HEK-GLAKD cells after 24 h of treatment, an uptake assay was performed using *ATTO633 NHS ESTER* labeling, allowing visualization of their cellular distribution by fluorescence microscopy. The results indicate that FicoVes primarily localize in the perinuclear cytoplasm (Fig. 23), and that the uptake level is dose-dependent, as shown in Fig. 24, after *GloMax®* analysis.

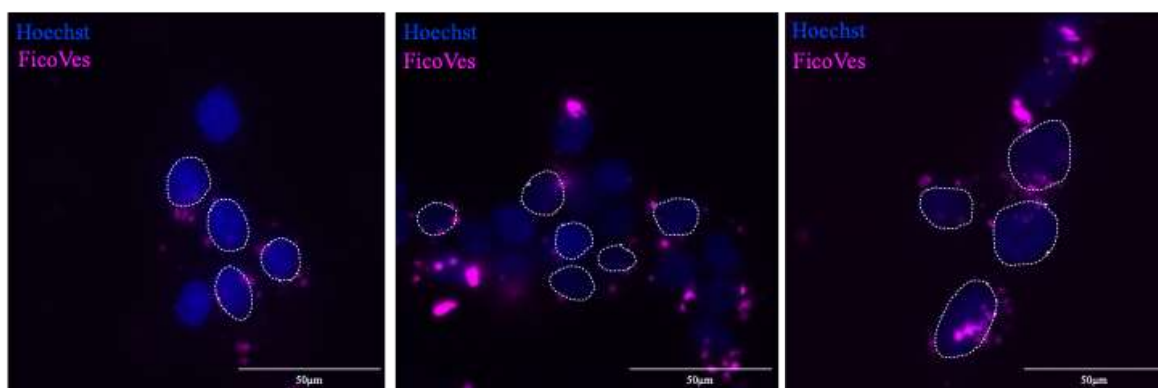


Fig. 23 Panel showing the uptake of ATTO-labeled FicoVes after treatment at three different concentrations (from left 5 µg/mL, 25 µg/mL and 50 µg/mL) for 24h.

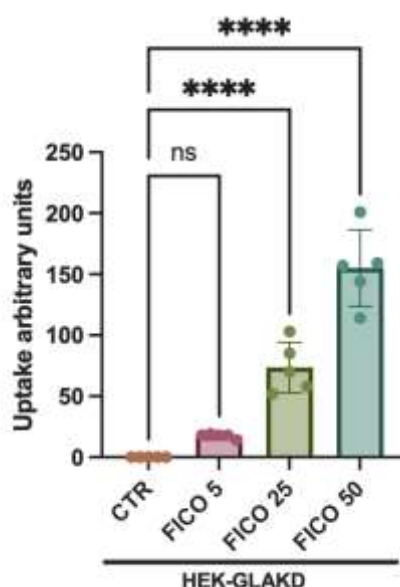


Fig. 24 Histogram showing the uptake levels of ATTO-labeled vesicles, measured by fluorescence analysis using the GloMax® microplate reader (Promega). Fluorescence was detected with an excitation wavelength of 627 nm and emission range of 660–720 nm. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (**) $p < 0.005$.

5. ABSENCE OF MORPHOLOGICAL ALTERATIONS IN HEK-GLAKD CELLS AFTER TREATMENTS

In this study, it was sought to evaluate the morphological aspects following the treatments applied to HEK-GLAKD cell line. Morphological analysis provides a general overview of the ‘cell system’ and allows the assessment of treatment-induced alterations. By maintaining identical microscopic magnifications across all observations, it was documented the

experiments through the acquisition of images of both treated and untreated cellular monolayers.

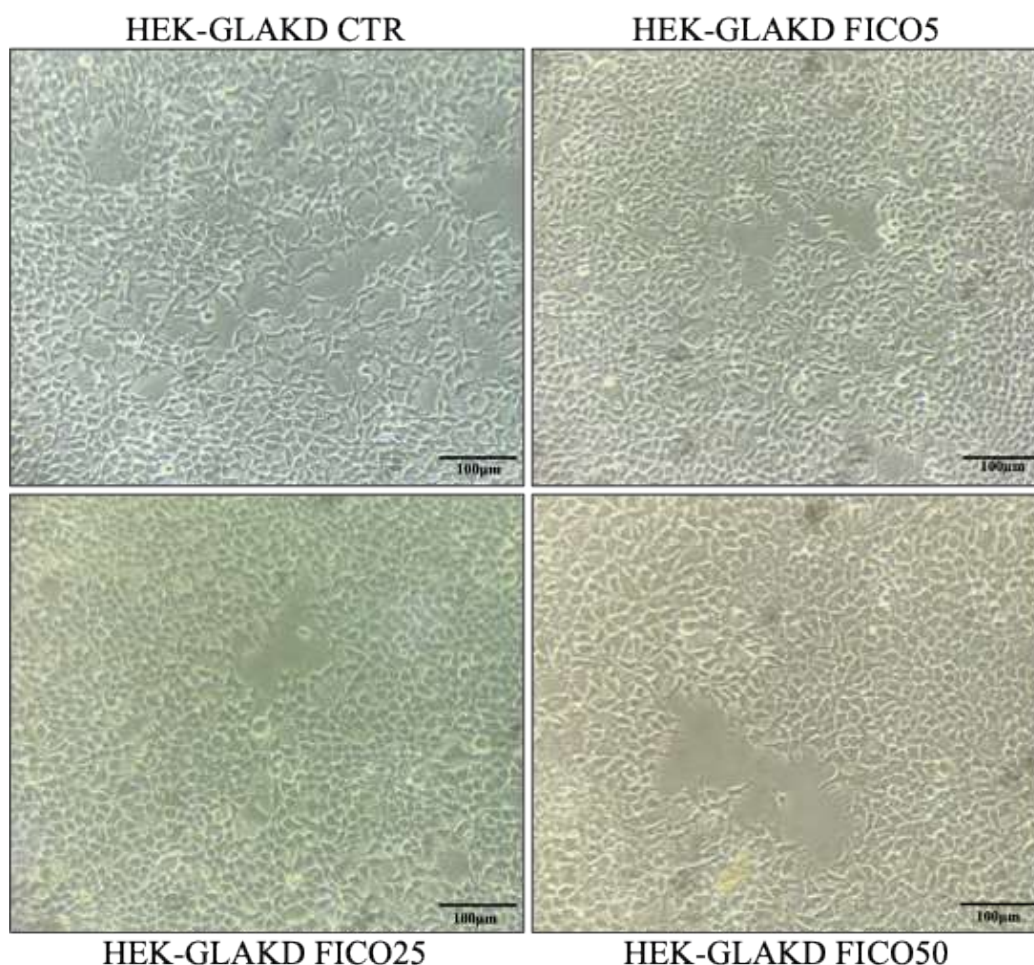


Fig. 25 Panel of images illustrating cell morphology following 24 h of treatment.

As shown in the panel (Fig. 25), none of the three treatments induced significant morphological changes in the examined cells.

6. FicoVes CONTRIBUTION ON GENOMIC AND GENIC DNA METHYLATION

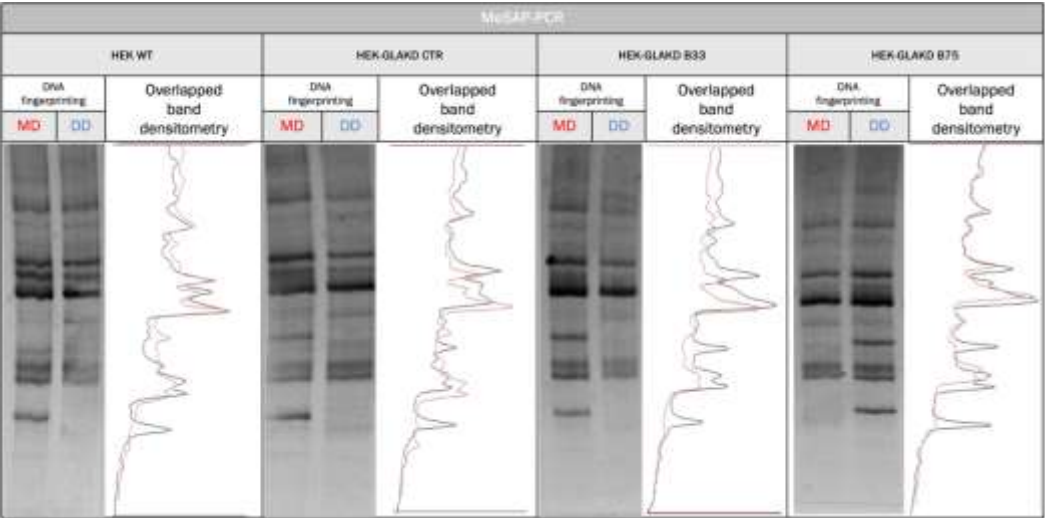
In order to assess whether treatment with FicoVes in HEK-GLAKD cells genomically unstable due to their role as a model of genetic disease could reverse this condition through an epigenetic mechanism, it was performed a genomic DNA methylation analysis using MeSAP-PCR. Furthermore, to evaluate the potential anti-inflammatory activity of FicoVes

in the context of AFD, the effect of the treatment on the methylation status were investigated of the promoters of key inflammation-related genes: IL-6, IL-8, and *IL-10*.

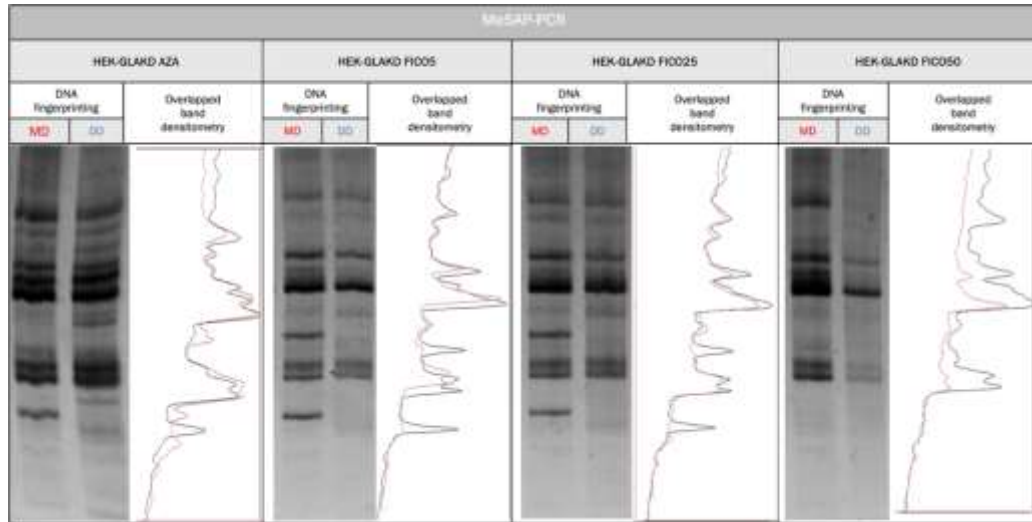
6.1 FicoVes TREATMENT DRIVES GLOBAL DNA METHYLATION TOWARD EPIGENETIC STABILITY

As expected, the results indicate that HEK-GLAKD cells, when compared with HEK293T WT cells, exhibit a global hypomethylation status, as shown in Fig. A and C. Regarding the control treatments, B33 induced a state of hypermethylation, while AZA led to global hypomethylation (Fig. 26 A, C). Treatment with the three different concentrations of FicoVes revealed: a consistent hypermethylating effect at comparable levels for FICO5 and FICO25, whereas FICO50 preserved the global hypomethylated condition of HEK-GLAKD cells (Fig. 26 B, C).

A



B



C

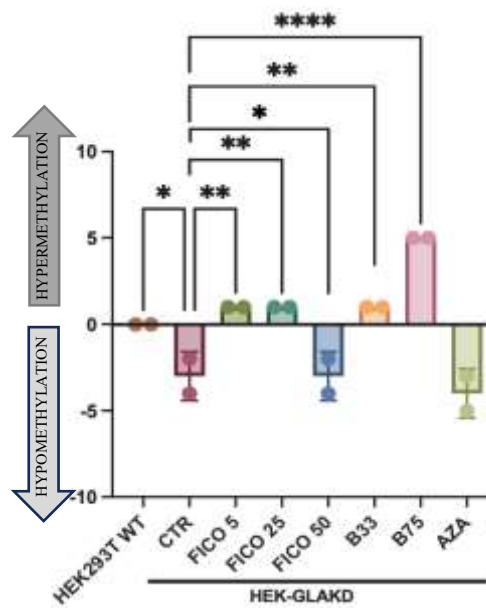


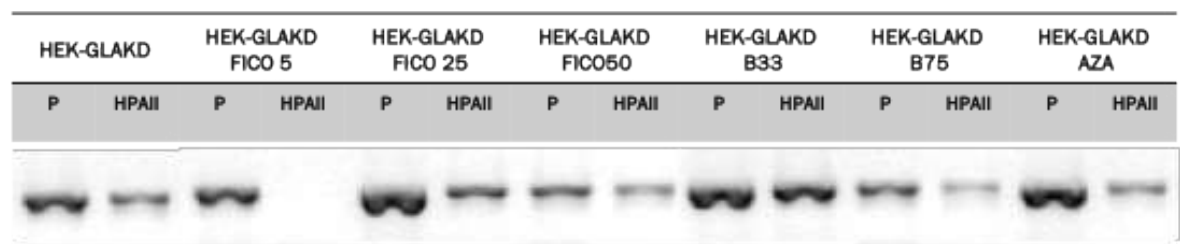
Fig. 26 (A, B) Representative images of DNA fingerprinting and the corresponding densitometric analysis from one replicate of the MeSAP-PCR; (C) Histogram showing the ImageJ-based analysis of the data obtained from MeSAP-PCR. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (*) $p < 0.05$; (**) $p < 0.01$; (****) $p < 0.0001$.

6.2 IL-6 MSRE-PCR

All data were normalized to the HEK-GLAKD control (CTR) (Fig. 27). As expected, B33, used as a positive hypermethylating control, confirmed its effect at sites 1, 3, and 4, while

B75 showed consistent hypermethylating activity at sites 2, 3, 4, and 5. Conversely, AZA, a demethylating control, confirmed its effect at sites 1, 3, and 6. Treatments with FicoVes displayed heterogeneous effects across the different sites: FICO5 induced complete demethylation at site 1 of IL-6, a mild demethylation at site 6, while promoting hypermethylation at the remaining sites. FICO25 caused hypermethylation at site 4 but produced variable degrees of demethylation at the other sites. Finally, FICO50 resulted in hypermethylation at sites 4 and 5, and a modest demethylation (~20%) at the remaining sites.

A



B

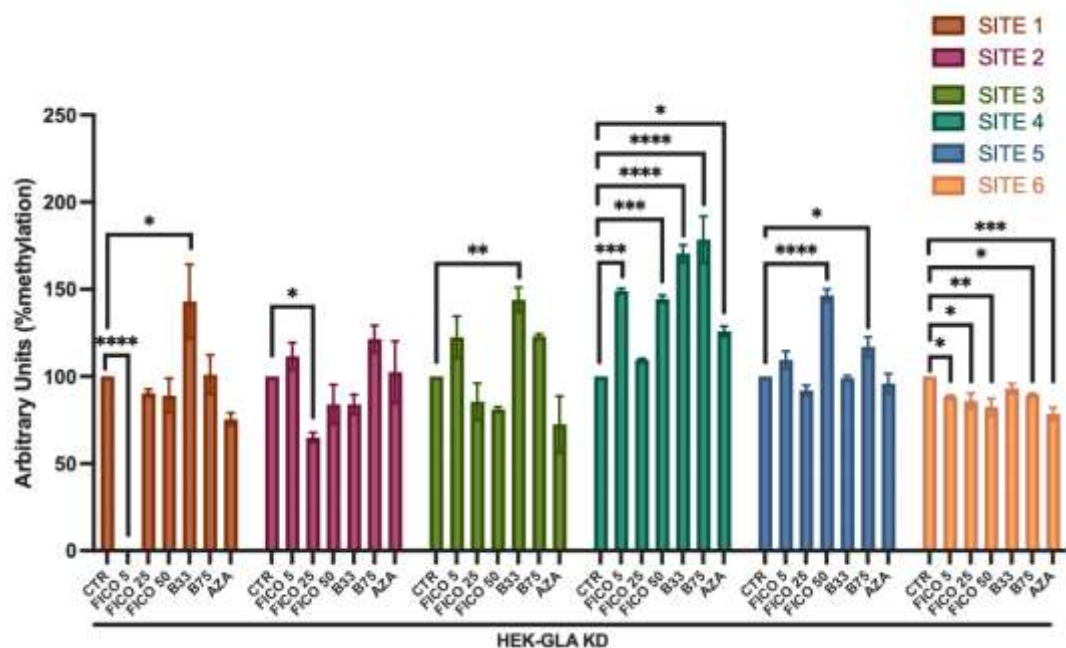
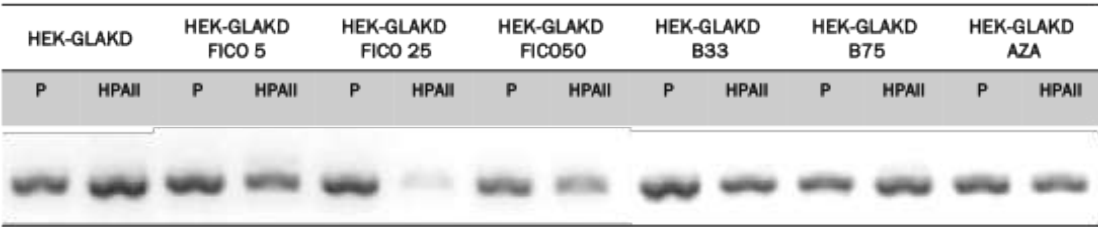


Fig. 27 (A) Representative image from one replicate of the MSRE assay at IL-6 site 1; (B) Histogram summarizing the MSRE results on the methylation status of all analyzable sites within the IL-6 promoter. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at () $p<0.05$, (**) $p<0.02$ (***) $p<0.001$, (****) $p<0.0001$.*

6.3 *IL-8* MSRE-PCR

All data were normalized to the HEK-GLAKD control (CTR) (Fig. 28). The control treatment with B33 induced a slight demethylation at both sites, while B75 caused a mild demethylation at site 2 but produced a 19% hypermethylation at site 1. AZA, instead, yielded comparable results at both sites, with a modest reduction in methylation ($\approx 10\%$). Regarding the FicoVes treatments, FICO5 led to a 21% demethylation at site 1 and a very slight hypermethylation at site 2. FICO25 strongly reduced methylation at site 1 (76%) and to a lesser extent at site 2 (5%). Similarly, FICO50 decreased methylation at both sites, with a 36% reduction at site 1 and a 20% reduction at site 2.

A



B

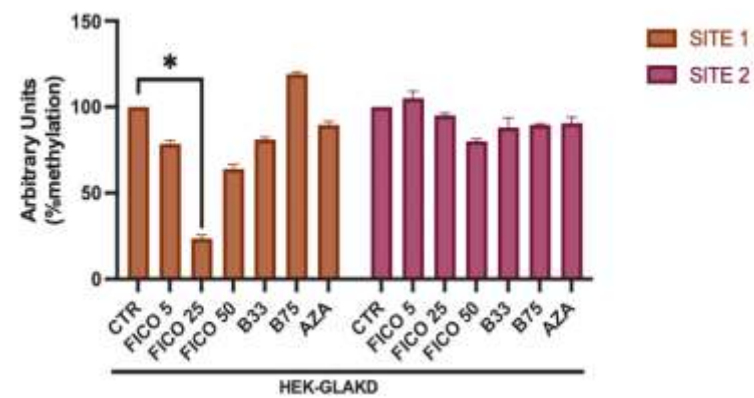


Fig. 28 (A) Representative image from one replicate of the MSRE assay at *IL-8* site 1; (B) Histogram illustrating the MSRE results on the methylation status of the analyzable sites within the *IL-8* promoter. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (*) $p < 0.05$.

6.4 *IL-10* MSRE-PCR

All data were normalized to the HEK-GLAKD control (CTR) (Fig. 29). The analyzed site within the *IL-10* promoter exhibited hypermethylation upon treatment with the controls B33

and B75, while AZA induced a slight demethylation. Regarding the FicoVes treatments, the concentration of 5 µg/mL resulted in an 18% demethylation of the site, whereas FICO25 and FICO50 preserved the methylation status unchanged.

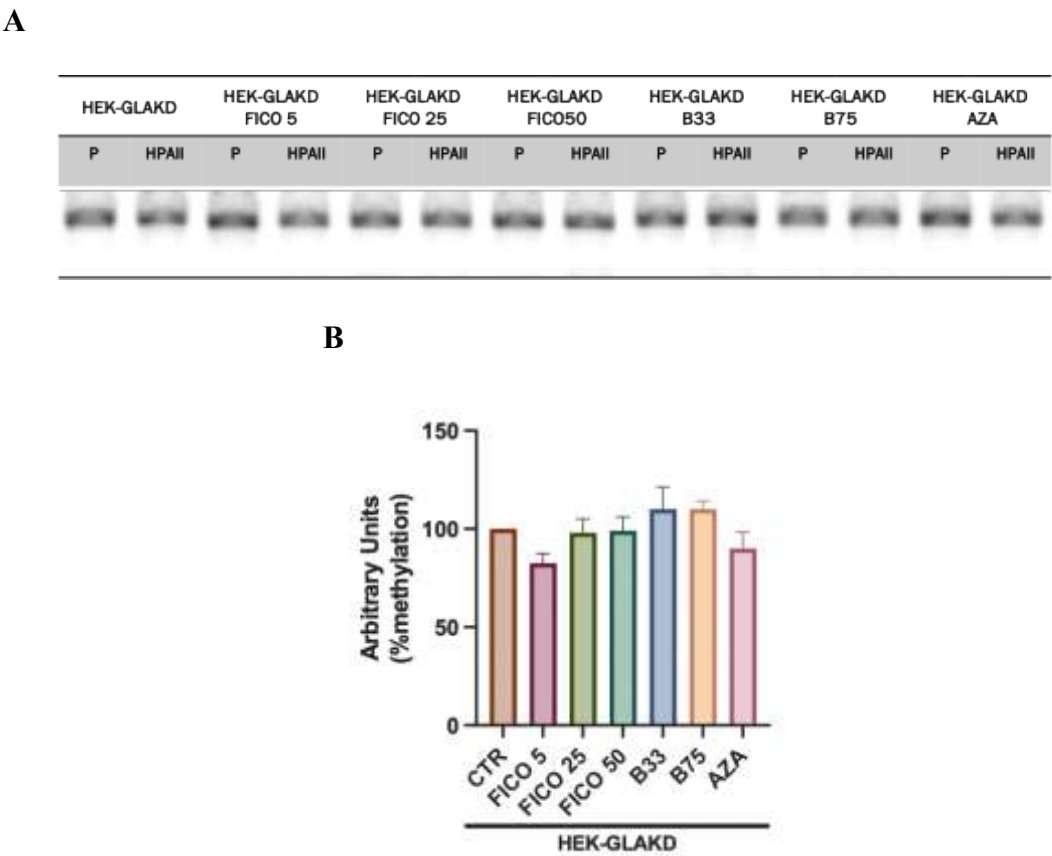


Fig. 29 (A) Representative image from one replicate of the MSRE assay at IL-10 site; (B) Histogram showing the MSRE results for the methylation status of the analyzable site within the IL-10 promoter.

7. FicoVes TREATMENTS SIGNIFICANTLY RESTORE INTERLEUKIN 6-INCREASED PRODUCTION

The gene expression level of the cytokines IL-6 was evaluated following 24hs treatments with FicoVes, BOBCAT339 and 5-Azacitidine and compared to untreated control (HEK-GLAKD CTR). As shown in Fig. 30, all FicoVes treatments significantly reduced IL-6 expression, in a manner comparable to B75. Furthermore, B33 also significantly reduced IL-6 expression, unlike AZA, for which the data are not significant.

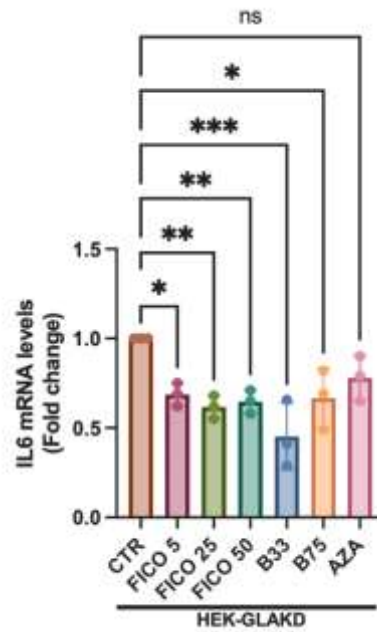


Fig. 30 Expression level of IL-6 gene in HEK-GLAKD cells treated for 24hs with Bobcat 33 μ M (B33), Bobcat 75 μ M (B75), Azacitidine 10 μ M (AZA), FicoVes 5 μ g/mL (FICO5), FicoVes 25 μ g/mL (FICO25), FicoVes 50 μ g/mL (FICO50). Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (*) $p < 0.05$; (**) $p < 0.01$, (***) $p < 0.001$.

8. FicoVes TREATMENTS REDUCE IL-6 SECRETION LEVEL

ELISA analysis of IL-6 levels revealed that treatment of HEK-GLAKD cells with FicoVes led to a significant, though modest, reduction in cytokine secretion compared to untreated cells. This effect was consistent across all three tested concentrations, indicating a homogeneous trend in response to FicoVes exposure. A similar pattern was observed in the control treatments with Bobcat and Azacitidine.

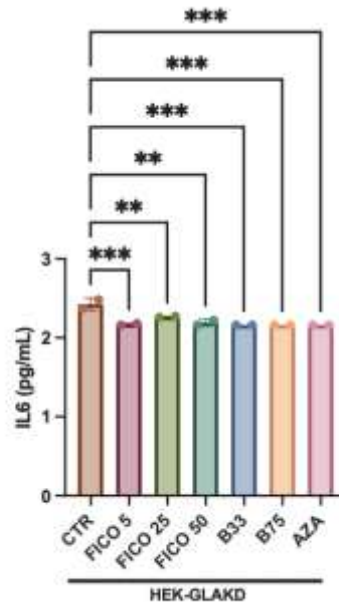


Fig. 31 Histogram showing the IL-6 released levels after cell treatments with Bobcat 33 μ M (B33), Bobcat 75 μ M (B75), Azacitidine 10 μ M (AZA), FicoVes 5 μ g/mL (FICO5), FicoVes 25 μ g/mL (FICO25), FicoVes 50 μ g/mL (FICO50). Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (**) $p < 0.01$, (***) $p < 0.001$.

9. FICO 5 AND FICO 50 SIGNIFICANTLY RESTORE INTERLEUKIN 8-INCREASED PRODUCTION

The gene expression level of the cytokines IL-8 was evaluated following 24hs treatments with FicoVes, BOBCAT339 and 5-Azacitidine and compared to untreated control (HEK-GLAKD CTR). As shown in Fig. 32, FICO5 and FICO50 treatments significantly reduced IL-8 expression, in a manner comparable to B75 and B33. Furthermore, FICO25 increase IL-8 expression, unlike AZA, but the data are not significant.

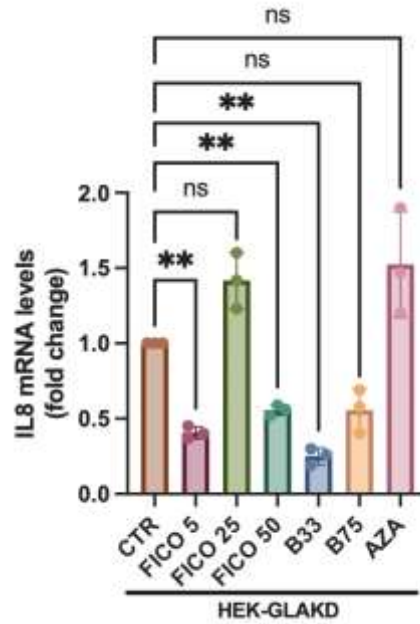


Fig. 32 Expression level of IL-8 gene in HEK-GLAKD cells treated for 24hs with Bobcat 33 μ M (B33), Bobcat 75 μ M (B75), Azacitidine 10 μ M (AZA), FicoVes 5 μ g/mL (FICO5), FicoVes 25 μ g/mL (FICO25), FicoVes 50 μ g/mL (FICO50). Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (**) $p < 0.01$.

10. FicoVes TREATMENTS REDUCE IL-8 SECRETION LEVEL

ELISA analysis of IL-8 levels, as well as for IL-6, revealed that treatment of HEK-GLAKD cells with FicoVes led to a significant, reduction in cytokine secretion compared to untreated cells. This effect was consistent across all three tested concentrations, indicating a homogeneous trend in response to FicoVes exposure. Also for this cytokine, a similar pattern was observed in the control treatments with Bobcat and 5-Azacitidine.

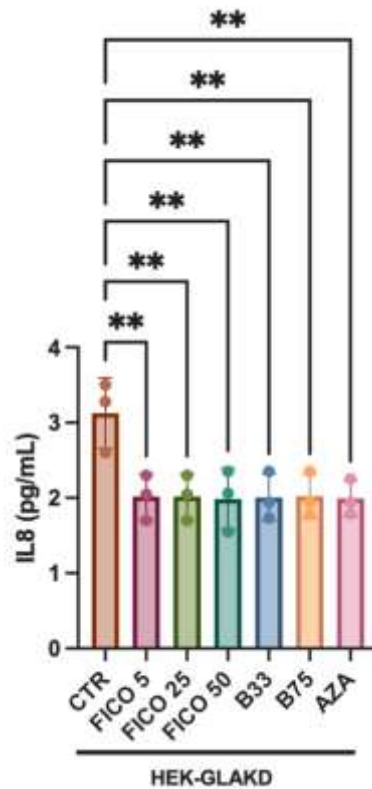


Fig. 33 Histogram showing the IL-8 released levels after cell treatments with Bobcat 33 μ M (B33), Bobcat 75 μ M (B75), Azacitidine 10 μ M (AZA), FicoVes 5 μ g/mL (FICO5), FicoVes 25 μ g/mL (FICO25), FicoVes 50 μ g/mL (FICO50). Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (**) $p < 0.01$.

11. ANALYSIS OF LIPID DROPLETS ACCUMULATION VIA OIL RED-O STAINING

Although Oil Red-O staining is not a standard diagnostic assay for AFD, it is occasionally used in biopsy studies to evaluate the accumulation of lipid vesicles in cells, as GB3 is a glycosphingolipid. This analysis revealed a slight reduction in the presence of intracellular lipid vesicles in cells treated with all three concentrations of FicoVes, with an approximate 30% decrease observed for each treatment.

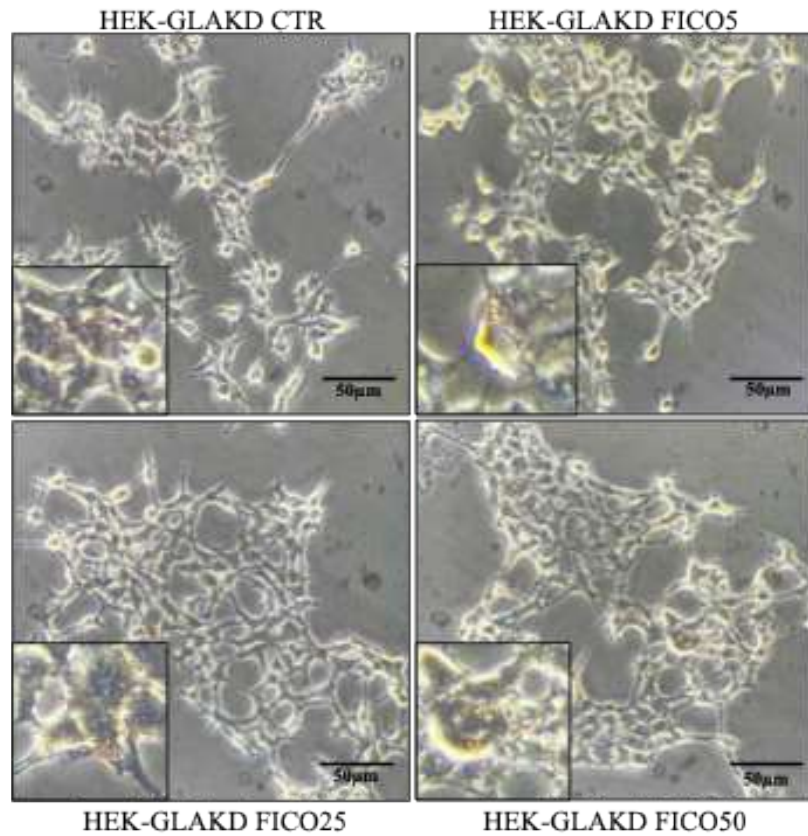
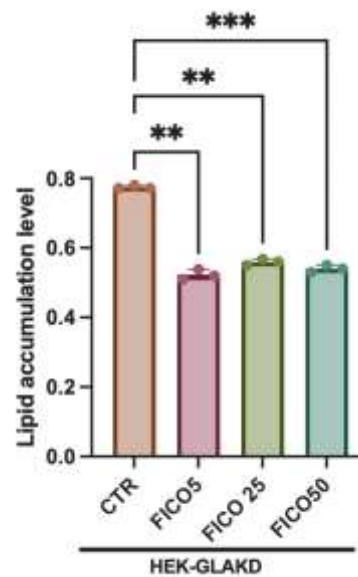
A**B**

Fig. 34 (A) Panel of images illustrating slight lipid accumulation after OIL RED-O staining; (B) Histogram showing the level of lipid accumulation through absorbance analysis measured at 600 nm using a spectrophotometer. Statistical analysis performed with one-way analysis of variance (ANOVA). Differences were considered significant at (**) $p < 0.01$, (***) $p < 0.001$.

DISCUSSION

AFD is a lysosomal storage disorder characterized by a partial or total deficiency of the enzyme α -GAL A that leads to the inability to catabolize glycosphingolipids resulting in accumulation of GB3 in lysosomes. AFD is a X-linked hereditary disease.

DNA methylation is the main epigenetic mechanism that induces the inactivation of X chromosome. Women are therefore a mosaic of two cell types expressing either the maternal or paternal X chromosome (Ørstavik 2009).

DNA methylation is important, also, to establish and maintain cellular identity and genomic stability. The distortion of an epigenetic profile can have a significant impact on cellular functions, which can lead to dysregulation of gene expression and potentially to the development of various diseases called "epigenetic diseases" (Zhang et al. 2020).

Until now research about AFD focused on heterozygous mutations and only partially on the inactivation of the X chromosome which causes symptomatic variability on female patients (Echevarria et al. 2016).

In recent years, the evaluation of DNA methylation as a potential biomarker for predisposition to various diseases has become an increasingly realistic possibility. In this study, the aim was to analyze the DNA methylation patterns of patients affected by AFD and compare them with those of healthy subjects to assess the contribution of DNA methylation in this AFD. Furthermore, by examining the methylation status of the regulatory region of the *GLA* gene, it was hypothesized to propose DNA methylation as a potential diagnostic and prognostic biomarker for AFD.

To evaluate the impact of DNA methylation in AFD, it was performed MeSAP-PCR on DNA from PBMC of 18 female patients characterized by heterozygous mutations causative of AFD, symptomatic and paucisymptomatic and 6 healthy female subjects. The results, showed clearly significant global DNA hypomethylation in AFD patients with respect to healthy subject (Figure 1). In this regard it is important to remember that it has been demonstrated that chronic inflammatory conditions can induce global DNA hypomethylation and, at the same time, this demethylation also affects the promoters of genes involved in inflammation (Naselli et al. 2025). In light of the results presented in this study, it was supposed that the hypomethylation detected in these patients could be both a cause and a consequence of chronic inflammatory state observed in patients with AFD (Kurdi et al. 2024) and this epigenetic alterations may contribute to genomic instability and the variability of clinical manifestations, which in women may not necessarily be strictly linked to X chromosome inactivation.

The analysis of DNA methylation at the *GLA* gene promoter showed that healthy subjects exhibit approximately 50%-70% methylation, with respect to undigested genomic DNA.

This finding is consistent with X chromosome inactivation, which predicts that the inactivated X chromosome is fully methylated at CpG sites. Thus, healthy individuals have at least one methylated copy of the *GLA* gene.

An interesting result was obtained from the methylation status of the *GLA* gene in AFD patients. These patients exhibited approximately 80% DNA methylation, a higher level compared to healthy subjects.

Since peripheral blood is a sentinel tissue, integrating signals from various parts of the body, and methylation is a tissue-specific event, it is possible that a heterogeneous population of cells with different epigenetic profiles coexists in the sample from AFD patients (Meier et al. 2021).

The promoter higher methylation (80%) in AFD patients was explained by the presence, within the analyzed sample, of two distinct cell populations: i) Cells in which both copies of the X chromosome are fully hypermethylated; ii) Cells with partial promoter methylation, where only one X chromosome is methylated due to X chromosome inactivation, while the other remains active as an normal subject. This mixed epigenetic landscape could lead to a dilution effect, influencing the overall methylation measurement.

The presence of *GLA* gene hypermethylation, along with the genetic mutation, can result in double silencing (both genetic and epigenetic) of the *GLA* gene, leading to a more significant reduction in protein expression. It was supposed that this aspect is closely linked to the symptomatology of the disease, potentially resulting in more severe clinical manifestations. Based on our findings, it was asserted for the first time that the methylation status in AFD patients is aberrant. In particular, global DNA hypomethylation, which is commonly associated with genomic instability, inflammation and even cancer (Volpes et al. 2023), could contribute to the cellular dysfunction observed in AFD patients. This highlights the potential role of epigenetic alterations in disease progression and symptoms variability. Biochemical data were then associated on enzymatic activity and protein accumulation with genetic data (type of *GLA* gene mutation) and epigenetic data. The results allow us to speculate that, regardless of the type of mutation (classic or late-onset) affecting AFD patients, all AFD patients share a common feature of global hypomethylation.

The indirect evaluation of *GLA* gene expression can be inferred from lysoGB3 accumulation, as the methylation and subsequent silencing of the *GLA* gene prevent the production of the α -GAL A responsible for GB3 degradation, leading to its inevitable accumulation. Therefore, GB3 accumulation can result either from a *GLA* gene mutation or from its epigenetic silencing. Our results show that 85% of patients with *GLA* gene hypermethylation also exhibit pathological lysoGB3 accumulation value. Conversely, patient with a *GLA* mutation

but without *GLA* hypermethylation have normal lysoGB3 levels, as the gene coding for α -GAL A responsible for GB3 degradation is not silenced. From these results it is possible to suppose that GB3 accumulation can arise from either a *GLA* gene mutation or its epigenetic silencing through hypermethylation.

A limitation of this study is undoubtedly the small number of patients analyzed, partially balanced by the known the rarity of the AFD. The development of a cellular model could facilitate the study of therapeutic targets and contribute to the formulation of new adjuvant therapies to complement existing treatments for disease management.

Immortalized cell lines represent the gold standard for in vitro disease modeling and for the recreation of specific conditions that occur in vivo, both in humans and in animals. Despite significant advances in biomedical research, critical challenges remain in the study of rare, complex and heterogeneous diseases, leading to limitations in both mechanistic understanding and the development of novel therapeutic strategies (Yousef et al. 2024). Two-dimensional (2D) cell cultures have been widely employed over the years in biomedical research to investigate both fundamental aspects, such as cell growth, proliferation, and differentiation, as well as pathogenetic mechanisms underlying cancer and non-cancer diseases, including applications in drug testing (Fusco et al. 2023; Esposito et al. 2019). Engineered cells, such as reporter cell lines, are also commonly used as they are amenable to high-throughput manufacturing and have high reproducibility and lower costs relative to stem cell-based methods. A huge step forward in genome editing, was made with the discovery of CRISPR/Cas9, a natural system used by several prokaryotes for adaptive molecular immunity against bacteriophage infections. Its mechanism was subsequently modified and engineered for use in eukaryotic cells to allow for the cutting or editing of specific DNA sequences in a target cell. Despite the unique advantages of primary, patient-derived cells, these cell models have some important limitations, not only the limited cultivation time, but also the restriction to those cells that can easily be accessed from humans and readily cultivated, confines the spectrum of target cells available for disease modeling (Borisch et al. 2024). The lack of a commercially available in vitro cellular model for the study of AFD slowdown research progress. Therefore, in this work the development of a cell model, of Fabry disease is attempted, specifically, a cell model simulating Fabry disease in a female context in which residual expression of the *GLA* gene occurs, resulting in the characteristic attenuated phenotype. This goal was primarily achieved using HEK293T cells, human embryonic kidney cell line and to a lesser extent with AC16 cells, which are stabilized human adult cardiomyocytes. In this study, it was chosen to use HEK293T and

AC16 cell lines for distinct but complementary reasons. HEK293T cells are highly transfectable cells and possess a tetraploid genetic cariotype with four X chromosomes. This unique feature, when properly engineered, allows them to effectively mimic a female AFD model. On the other hand, AC16 cells were selected because they are immortalized, normal-like female cardiomyocytes that can be maintained in culture without particular difficulties. Moreover, considering that one of the main clinical manifestations of AFD involves the cardiac system, the use of AC16 cells provides a relevant model to investigate the cardiac aspects of the disease. The objective was pursued through the engineering of cell lines using the CAS9-wt protein, kindly provided by the Epigenetic Engineering group at LMU, led by Prof./Dr. Stefan Stricker, during the research period abroad in Munich. The cells were treated as previously described with the CAS9-wt protein conjugated to the appropriate gRNA required for the established engineering process and as shown in Tab. 3, the transfection process was markedly more efficient in HEK293T cells due to their high transfectability compared to AC16 cells. Nevertheless, as illustrated in Fig. 16, the programmed deletion occurred in both cell types. Clonal selection was performed for both engineered cell lines, and initial PCR screening on clones derived from HEK293T cells confirmed that selected clones harbored the intended deletion. Specifically, clones C2OLD, C7OLD, and C1NEW displayed the amplicon corresponding to the targeted deletion (Fig. 18 A, B). Given that HEK293T cells are tetraploid, having 8 copies of the *GLA* gene, it is plausible that the synergistic action of the two gRNAs resulted in a complete deletion in only in a part of the four X chromosomes, generating the observed band. In contrast, the other chromosomes may have been affected by only a single guide, producing minor deletions undetectable by conventional PCR, likely leading to small insertions or deletions via non-homologous end joining (NHEJ). To validate this hypothesis, gene expression analysis was conducted, which surprisingly confirmed the expectation: residual *GLA* expression was approximately 10% in C2OLD, 18% in C7OLD, and 32% in C1NEW (Fig. 19). These findings indicate heterogeneous Cas9 activity across the targeted sequences by the using of 2 sgRNA, ultimately leading to a partial reduction in gene expression. Gene expression analysis by qRT-PCR was also performed on selected AC16 clones (Fig. 20) and the most promising knockdown candidates were C3OLD (57% residual expression) and C2NEW (82% residual expression). However, given that these levels did not meet the desired knockdown efficiency, further studies focused on the HEK293T-derived clones, particularly C2OLD, which was subsequently designated HEK-GLAKD. Protein analysis of α -GAL A confirmed a residual expression of approximately 19%, consistent with the observed gene knockdown. Collectively, these data demonstrate that the experimental strategy employing

wild-type Cas9 and specifically designed guide RNAs constitutes a reliable and reproducible standard for generating a cellular model of AFD. The consistency of both gene and protein expression analyses across multiple passages further supports the robustness and predictive value of this model.

Inflammatory mechanisms play a central role in the pathophysiology of AFD. Lysosomal dysfunction resulting from α -galactosidase A (α -Gal A) deficiency leads to the progressive accumulation of glycosphingolipids, primarily GB3 and its deacylated derivative lyso-GB3, this substrate accumulation activates multiple inflammatory signaling pathways. In the innate immune response, GB3 and lyso-GB3 have been shown to stimulate Toll-like receptor 4 (TLR4) and activate the NF- κ B pathway, thereby promoting the release of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 (Biddecki et al. 2025). Indeed, Fabry disease exhibits a distinct inflammatory profile, as demonstrated by the elevated levels of circulating pro-inflammatory cytokines and by evidence from PBMCs of Fabry patients, which show constitutive secretion of TNF- α and IL-1 β (De Francesco et al. 2013), characteristic cytokine pattern of autoinflammatory disorders (De Jesus et al. 2015). Moreover, increased expression of anti-inflammatory cytokines, including IL-10 and TGF- β 1, has also been reported, particularly in patients with missense mutations undergoing enzyme replacement therapy (ERT) (Laffer et al. 2024). Collectively, these findings reinforce the concept that Fabry disease encompasses both pro- and anti-inflammatory immune responses, suggesting that anti-inflammatory co-adjuvant strategies could represent a promising approach to enhance the clinical efficacy of current ERT-based treatments.

FicoVes were first tested for their cytocompatibility, as it is known that natural compounds derived from different cellular species can sometimes trigger undesired cellular responses, potentially leading to cytotoxic effects, although the original source is considered “safe” (Volpes et al. 2023). The MTT assay performed on HEK-GLAKD cells showed that although 24 h and 48 h treatments with a concentration of 50 μ g/mL resulted in a significant reduction in cell viability, none of the values dropped below 70% viability (Fig. 22). This finding complies with the reference guidelines for cell viability assays (Standard, I. *ANSI/AAMI/ISO 10993-5:2009/(R)2014*), confirming that FicoVes can be considered safe at all tested concentrations and time points. Based on these results, all three FicoVes concentrations were used in subsequent experiments, while treatment duration was limited to 24 hours to ensure that cells completed at least one cell cycle (with a doubling time of approximately 22 hours). To further investigate the mechanism of action of FicoVes, the vesicles were labeled with *ATTO633 NHS ESTER* to assess their uptake after 24 hours of treatment. The analysis (Fig.

24) revealed that, although with dose dependent level of internalization efficiency, the labeled vesicles were localized in the perinuclear and cytoplasmatic regions (Fig. 23). This suggests that the vesicle content is released into the cytoplasm, where it could directly modulate post-transcriptional processes and indirectly affect nuclear epigenetic mechanisms, for example by influencing the methylation and acetylation state of chromatin through the nuclear translocation of regulatory proteins or enzymes. Alternatively, FicoVes may act indirectly within the cytoplasm by activating kinase or phosphatase cascades involved in epigenetic regulation.

Additionally, optical microscopy analysis was used to evaluate whether FicoVes treatments altered cell morphology. The results showed that none of the three concentrations caused morphological changes after 24 hours of treatment (Fig. 25). This data supports the MTT data, confirming the absence of cytotoxic effects and the overall cytocompatibility of FicoVes.

Since the anti-inflammatory activity of FicoVes has already been reported in the literature about other cell lines (Naselli et al. 2024), the next objective of this project was to assess their potential anti-inflammatory effects in AFD context and evaluate if it could be mediated by epigenetic mechanisms, particularly by modulating genomic and gene-specific DNA methylation in HEK-GLAKD cells. It was very interesting to analyze the global DNA methylation status of these cells compared to HEK293T WT cells after the development of a cellular model for AFD. As shown in Fig. 26, the genetic engineering of the HEK-GLAKD cells, leads to a global DNA hypomethylation, indicative of genomic instability (Kulis and Esteller 2010), which is consistent with the inflammatory and unstable genomic profile characteristic of AFD. This result also aligns with the initial global DNA methylation study performed on AFD patient samples (Fig. 9), which displayed global DNA demethylation, thus further validating the relevance of our cellular model.

Regarding the treatment of HEK-GLAKD cells with FicoVes, it was observed that 5 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$ concentrations induced significant global hypermethylation, restoring methylation levels closer to those of HEK293T WT cells. Since this level of hypermethylation is comparable to that induced by B33 (rather than B75), a known methylating agent that acts through TET1 inhibition, it is plausible to suppose that FicoVes exert a similar effect, inducing hypermethylation via TET1 inhibition, thereby contributing to genomic stabilization.

Given the global methylation changes, we further examined local methylation levels of inflammation-related genes (IL-6, IL-8 and IL-10) to assess whether FicoVes could modulate specific anti-inflammatory mechanisms. For IL-6, treatment with the three FicoVes concentrations revealed heterogeneous methylation patterns: FICO5 caused a significant demethylation at site 1, a mild but significant hypermethylation at site 4 and demethylation at site 6, FICO25 led to slight but significant demethylation at sites 2 and 6, FICO50 resulted in strong hypermethylation at sites 4 and 5 and mild demethylation at site 6. It can be stated that FicoVes do not display a linear or constant trend across all sites, they alternate between increases and decreases in methylation levels. This observation suggests that their mechanism of action may involve a direct or indirect interaction with the players of the methylation process, such as DNMTs or TET enzymes.

These results indicate that FicoVes can modulate gene-specific DNA methylation across all tested concentrations. Similar to the observed global methylation effects, FicoVes appear capable of dynamically regulating DNA methylation homeostasis, although further studies are required to clarify the molecular mechanism.

To complement these findings, the cytokines expression and secretion from FicoVes-treated HEK-GLAKD cells were evaluated using qRT-PCR and ELISA assays. Firstly IL-6 cytokine was evaluated. As shown in Fig. 30 and 31, all three FicoVes treatments led to a significant decrease in IL-6 both expression and secretion. These data suggest that the reduction in IL-6 secretion is probably associated with the local methylation changes, for example the site 4 and site 5 hypermethylation, and with other unknown mechanism. Probably a compensatory mechanism between hyper- and hypomethylation events might occur, ultimately favoring the effects of hypermethylation-driven regulation. Furthermore, considering that the variation in gene expression is highly comparable to that observed following B75 treatment, as well as to the methylation pattern observed across different sites, it is plausible to hypothesize that the mechanism regulating the expression of this cytokine, after FicoVes treatments, involves the inhibition of the TET1 and TET2 enzymes, as already explained by Bellavia et al. 2022.

In order to complete the investigation about the inflammatory profile of AFD and the effect of FicoVes on it, the methylation status of the IL-8 promoter region was analyzed, too. As shown in Figure 28A, concerning site 1, the only significant result was obtained with the FICO25 treatment, which induced demethylation of approximately 75% of the site. This finding confirms that, similarly to what was observed for IL-6, FicoVes are capable of

exerting an epigenetic modulation on the IL-8 promoter as well. FICO5 and FICO50 also induced local demethylation at site 1, although not to a statistically significant result.

Subsequently, IL-8 expression was evaluated to determine whether its regulation was primarily influenced by the epigenetic mechanism of DNA methylation. As shown in Figure 32, demethylation at site 1 induced by FICO25 resulted in an increased expression of IL-8, supporting the hypothesis that DNA hypomethylation directly affects the expression of this cytokine. This interpretation is further reinforced by the observation that the upregulation of IL-8 expression is comparable to that obtained with 5-Azacytidine, a well-known demethylating agent reported to directly modulate IL-6 expression by Boada et al. 2021, and possible modulator of IL-8 as well. Conversely, the decrease in methylation at sites 1 and 2 of IL-8 observed with FICO5 and FICO50 treatments did not correspond to an increase in expression; in fact, IL-8 expression was significantly reduced in these conditions. This suggests that other epigenetic mechanisms, intrinsic to FicoVes or activated by them, could be responsible for the observed effects, such as for IL-6 cytokine.

Furthermore, the secretion of IL-8, evaluated through ELISA assay after treatments with FICO 5, FICO 50, B33 and B75, as shown in Fig. 33, confirms the results obtained from the gene expression analysis. In contrast, the treatments with FICO 25 and 5-Azacytidine diverge from the gene expression data.

It is possible to hypothesize the existence of post-translational mechanisms leading to the degradation of the soluble cytokine, which, although its expression is stimulated by the FICO 25 treatment, is not subsequently secreted at the same level.

Thus, it can be concluded that, although FicoVes at the concentration of 25 µg/mL stimulates IL-8 gene expression, it ultimately reduces its secretion, suggesting a potential health-promoting effect of FicoVes also in this context.

Finally, no significant methylation changes were observed in the IL-10 promoter following treatment with FicoVes, indicating that these vesicles do not exert a direct effect on the methylation of this anti-inflammatory gene.

Furthermore, in order to assess whether treatment with FicoVes could positively influence lipid metabolism, as previously reported by Pahl and Hou (2023) in tissue samples, an Oil Red-O staining assay was performed. The staining revealed positive signals within HEK-GLAKD cells, consistent with lipid accumulation (Fig. 34). Oil Red-O is a nonspecific lipid stain and positive staining is not diagnostic of any specific lipid type or lysosomal storage

disorder however, the presence of abnormal lipid accumulation or distribution may indicate altered lipid metabolism. Based on the data obtained, it can be stated that FicoVes at concentrations of 5 and 25 $\mu\text{g/mL}$ significantly reduce lipid accumulation, thereby suggesting a beneficial effect on lipid metabolism as well.

CONCLUSION

In conclusion, this pilot study highlights the relevance of epigenetic alterations in AFD patients, characterized by global DNA hypomethylation and localized promoter hypermethylation of the *GLA* gene. These findings suggest a potential role of DNA methylation in disease manifestation and progression, possibly influencing gene expression, inflammation and symptom variability. These findings suggest that DNA methylation may serve as a promising therapeutic target in AFD, acting as an adjuvant to established treatments. Targeting methylation and related epigenetic mechanisms could open new avenues for the development of anti-inflammatory and antioxidant therapies, including those based on naturally derived compounds capable of modulating the epigenetic landscape and thereby contributing to disease management. However, further studies are needed to better define the relationship between epigenetic modifications, X chromosome inactivation and the inflammatory state in AFD.

As previously mentioned, one of the most advancing aspects in AFD research concerns the development of stable *in vitro* cellular models, which are essential for accelerating scientific investigation. Part of this work was therefore focused on this objective, through the use of the CRISPR/Cas9 system. The development of this model represents a crucial step in AFD research, driving significant progress in understanding of the disease. In the first instance, it can be used to perform *in vitro* experiments aimed at elucidating the underlying pathogenic mechanisms and, potentially, at testing novel therapeutic approaches to complement existing treatments.

Furthermore, by standardizing the methodology described above identified as optimal for establishing the cellular model through the use of specific gRNAs and the CAS9-WT protein, it will be possible to generate additional disease-specific cellular models.

The AFD cellular model generated through the CRISPR/Cas9 system reproduces the global hypomethylation features previously demonstrated in the first part of this study. Treatment with FICO5 and FICO25 was able to restore the global demethylation state observed in HEK-GLAKD cells, an indicator of inflammation, bringing it back above normal levels. This finding confirms, already at the genomic level, the FicoVes anti-inflammatory activity previously demonstrated by Naselli et al. 2024 in other cell lines. Furthermore, with regard to the investigation about pro-inflammatory cytokines, it is possible to assert that, the obtained results indicate that, as part of a pro-survival response, cells may activate adaptive mechanisms upon FicoVes treatment to reduce excessive IL-6 and IL-8 secretion, which could otherwise be detrimental. Such regulation might also be mediated by specific miRNAs

delivered by FicoVes or miRNAs already into the cells but activated after the treatments with FicoVes, capable of sequestering and degrading IL-6 and IL-8 mRNA, such as Mir26b and Mir124 for IL-6 and Mir200c for IL-8. This hypothesis is supported by the cytoplasmatic localization of the vesicles after internalization and by their miRNA-rich genetic cargo as preliminarily demonstrated (data not shown).

Taken together, these results highlight the promising anti-inflammatory potential of FicoVes treatments in the context of AFD. FicoVes may be considered a effective co-treatment to existing replacement therapies, acting not only as a global epigenetic modulator but also as a regulator of the inflammatory profile in a health-promoting manner. By influencing both the epigenetic landscape and inflammatory pathways, FicoVes have the potential to contribute to an overall improvement in the clinical condition of treated patients, creating opportunities for novel therapeutic strategies that combine conventional treatments with bioactive, naturally derived compounds.

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