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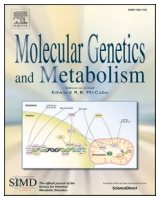

Beyond sialylation: Exploring the multifaceted role of GNE in GNE myopathy

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ABSTRACT

Defects in sialic acid metabolism disrupt the sialylation of glycoproteins and glycolipids, contributing to a spectrum of diseases, including GNE myopathy (GNEM). This rare disorder is caused by mutations in the *GNE* gene that encodes for a bifunctional enzyme required for sialic acid biosynthesis, resulting in progressive muscle atrophy and weakness. There is no approved treatment for GNEM, and the number of affected individuals is underestimated. Although hyposialylation is considered the hallmark of GNEM, evidence showed lack of consistent correlation with GNEM severity and unveiled additional roles of GNE that contribute to the onset and/or progression of GNEM. Recent findings indicate that these mechanisms extend beyond glycosylation, encompassing cytoskeletal dynamics, oxidative stress, and muscle regeneration pathways. Understanding how *GNE* mutations result in a cascade of cellular and molecular dysregulations is crucial for developing targeted therapies aimed at improving the quality of life of patients.

This review comprehensively examines GNEM's pathophysiology, clinical presentation, and therapeutic strategies, highlighting key findings on non-canonical GNE functions that account to GNEM clinical outcomes and emerging therapeutic targets. We propose future research directions to explore alternative target pathways that can ultimately support clinical development.

1. GNE myopathy: facts and numbers

GNE myopathy (GNEM) is a rare neuromuscular disorder characterized by progressive muscle atrophy and weakness, primarily affecting young adults. This condition follows an autosomal recessive inheritance pattern and is predicted to affect 1 to 9 individuals per million worldwide (Orphanet; https://www.orpha.net). Yet, recent research indicates that this prevalence is significantly underestimated due to underdiagnosis and misdiagnosis, and bias introduced by founder allele frequencies. The actual prevalence of GNEM is estimated to be 11–88 cases per million [1].

This myopathy was initially reported in Israel as hereditary inclusion body myopathy (HIBM) and in Japan as distal myopathy with rimmed vacuoles (DMRV). Other designations such as Nonaka myopathy, inclusion body myopathy 2 and quadriceps-sparing myopathy have emerged since then. In 2001, mutations in the *GNE* gene were identified as the causative factor and established that all these myopathies were the same pathological condition and unified into GNEM. This disease is classified as a Congenital Disorder of Glycosylation (CDG), a group of rare genetic disorders with protein and lipid glycosylation affected.

The *GNE* gene encodes for a bifunctional protein, highly conserved in mammals, with UDP-*N*-acetylglucosamine 2-epimerase (UDP-GlcNAc 2-epimerase)/*N*-acetylmannosamine kinase (ManNAc kinase) activity that initiates and regulates the synthesis of sialic acids. Sialic acids are

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terminal monosaccharides of most glycans found on proteins and cell surfaces. The process of sialylation is one of the most important glycosylation modifications, essential for various cellular functions, such as adhesion, signaling, migration, pathogenic recognition, infection, and immune modulation [2,3]. It has been proposed that hyposialylation represents the main pathophysiological hallmark of GNEM.

GNE is constitutively expressed in most tissues and, although the expression is particularly low in the muscle tissue, patients with GNEM have skeletal muscle weakness as their main symptom. Therefore, the pathomechanism that leads to the muscle phenotype in GNEM patients due to *GNE* mutations remains unknown. The complexity of GNEM clinical spectrum and its unique onset often culminates in a diagnostic odyssey for patients. In addition, the biochemical diagnosis in any CDG is difficult since the standard CDG biomarkers are often normal [4].

This review seeks to discuss the latest findings in GNEM, shedding light on the recent clinical features and mechanisms involved in its pathophysiology, while summarizing the current research efforts towards drug target development.

2. Clinical presentation of GNEM

GNEM typically manifests in early adulthood, initially affecting distal muscles of the lower extremities. As disease progresses, it gradually involves the proximal muscles and those of the upper extremities, which may lead to complete skeletal muscle loss. On average, it takes around 12 years from the onset of the disease until patients reach a non-ambulatory status, ascribed to patients who cannot walk a short distance within the house, relying on a wheelchair [5].

The first symptoms of GNEM often include anterior tibialis weakness, gait disturbance, foot drop and inability to lift toes [6–8]. Most GNEM patients retain good quadriceps strength for several decades after disease onset, while only a minority (5 %) experience early quadriceps weakness [9]. This preservation of the quadriceps muscle strength in relation to other muscles is used in differential diagnosis [10]. Interestingly, quadriceps muscles present lower levels of free and total sialic acid than distal muscles, which may explain the sparing of the quadriceps in GNEM patients [11]. Nevertheless, as the disease advances, the quadriceps eventually become affected, with the rectus femoris typically being the first muscle involved and the vastus lateralis the last to show involvement [12].

Research has not clearly established an association between *GNE* mutations and cardiac muscle [13,14]. Yet, some GNEM patients exhibit heart problems, as first-degree atrioventricular block, ST-T abnormalities, and signs of myocardial infarction at a higher frequency than the general population [13].

Unlike cardiac changes, thrombocytopenia has been increasingly associated with GNEM. The GNE enzyme is critical for cell surface sialylation in hematopoietic cells and sialic acid shortage decreases platelet life span and is correlated with increased titters of platelet-associated immunoglobulins G (PA-IgG), a biomarker of immune-mediated thrombocytopenia [15]. Several GNEM patients have a history of idiopathic thrombocytopenia, sometimes in infancy before experiencing muscular manifestations [13], suggesting abnormalities in platelet surface antigens. Other patients have PA-IgGs positive without thrombocytopenia, indicating that sialylation impairment induces PA-IgGs without or before inducing thrombocytopenia [14,16–19].

Respiratory function is generally not significantly affected in GNEM patients. However, in the first report monitoring program, 10 % of the patients had asthma and difficulty in breathing [5]. Additionally, sleep apnea syndrome is diagnosed more frequently in GNEM patients than in the general population [14]. Neuromuscular disorders have typically a higher risk of sleep-disordered breathing, namely pharyngeal weakness, and dysphagia, which are generally not associated with GNEM. Despite this, the association between GNEM and sleep apnea syndrome underscores the importance of monitoring the sleep of patients [13].

In blood tests, patients often have mildly to moderately elevated levels of creatine kinase (CK), crucial for muscle energy production. Increased CK levels in the blood are usually associated with skeletal and cardiac muscle damage. Although elevated levels of CK in the blood are not observed in all GNEM patients, when present, they can be another indicator for GNEM diagnosis [5,20,21].

Cognition in GNEM patients remains intact with no significant impairments, which contrasts with many other conditions with altered sialylation within CDG [14].

3. From initial symptoms to a confirmed diagnosis of GNEM

The initial symptoms of GNEM are generally bilateral foot drop and distal muscle weakness. As GNEM diagnosis still relies on clinical observations, patients who do not present the classic symptomatology may remain misdiagnosed. The proper GNEM diagnosis demands for a combination of methods, including muscle imaging, muscle histology, and genetic testing.

Muscle imaging, particularly magnetic resonance imaging (MRI), is a widely used non-invasive tool that enables the identification of patterns of muscle atrophy and fat infiltration in GNEM. Usually, GNEM muscles with major deterioration are biceps femoris and ankle dorsiflexors, including tibialis anterior and the extensor digitorum longus, while quadriceps muscles remained relatively spared [12,22,23]. The study of the pattern of muscle atrophy in GNEM patients allows hinting different patterns of affectation at distinct stages of the disease progression [12], and considered as an outcome measure in clinical trials [24,25].

Fiber size variation and atrophic muscle fibers with rimmed vacuoles that correspond to autophagosomes are histopathological findings usually present in muscle biopsies of GNEM patients. Although rimmed vacuoles strongly suggest a GNEM diagnosis, they may be absent in unaffected muscles, such as the quadricep muscles, or be also observed in other muscle disorders. Amyloid β (Aβ) aggregates are also observed in atrophic muscle fibers of GNEM patients [26]. This may be related to decreased activity of neprilysin, a sialylated endopeptidase involved in Aβ clearance, found to be hyposialylated and with reduced activity in GNEM muscles [27]. Additionally, GNEM muscle biopsies usually have no inflammatory cells, but there are reports of muscle inflammation [28–30] and a case with granuloma formation [31].

The final GNEM diagnosis is made by genetic testing identifying pathogenic mutations in both alleles of *GNE* gene through DNA sequencing. The incorporation of *GNE* sequencing into panels that analyse neuromuscular disorders has accelerated GNEM diagnosis.

Following diagnosis, it is important to assess and monitor muscle strength and function for effective disease management. Established methods, such as six-minute walk test (6MWT) and Quantitative Muscle Strength Assessment, evaluate the distance walked and peak muscle strength, respectively [25], while Manual Muscle Testing (MMT) and Gross Motor Function Measure (GMFM) offer better long-term evaluation parameters for GNEM progression [32]. To fulfil the need of a GNEM specific instrument to measure the functional impairment, the GNEM functional activity scale (GNEM-FAS) was developed [33], which assesses the capacity and independence of the patient in mobility, upper extremity use, and self-care. GNEM-FAS data correlates well with the physical assessments, supporting its use for clinical observations during clinical trials [10,34]. Patient reported outcomes, such as the inclusion body myositis function rating scale (IBMFRS) [25] that evaluates 10 functional tasks (including swallowing, handwriting, dressing, sit to stand and walking) have been introduced, to include patient perspective in fully understanding the impact of the disease.

4. Genotype-phenotype correlation

The human *GNE* gene, located on chromosome 9p13.3, has 13 exons and multiple mRNA isoforms. The hGNE1 isoform (ORF length 2169 bp; 722 aa, 79.275 kDa, GenBank NM_005476) is ubiquitously expressed, whereas other isoforms (hGNE 2 – hGNE 8) are differentially expressed.

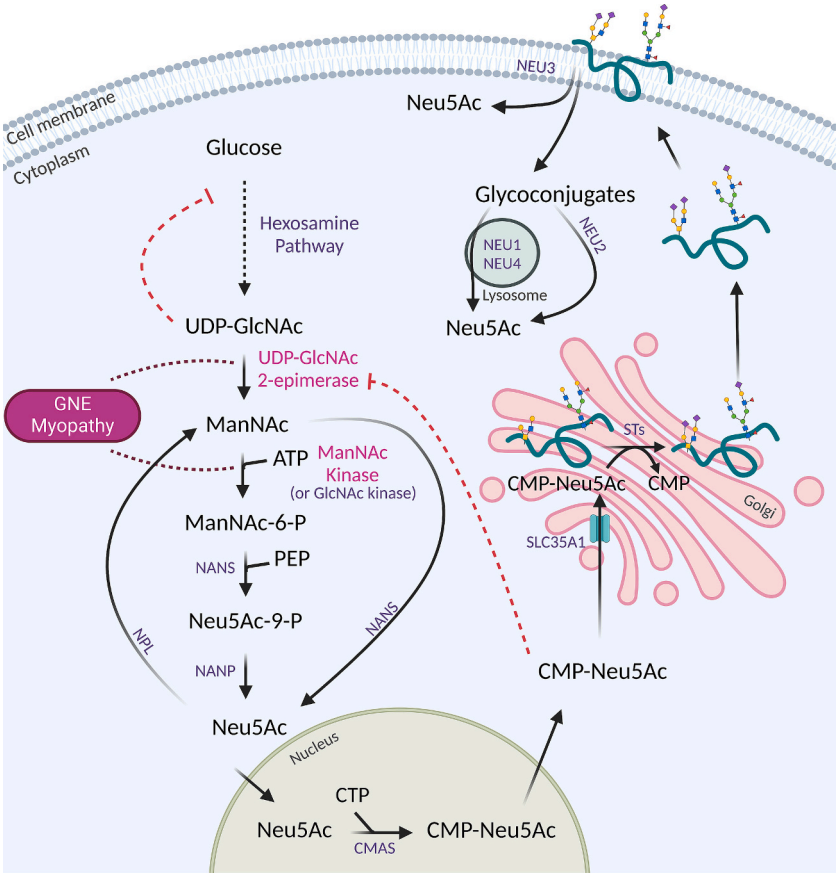

Fig. 1. Disruption of sialic acid metabolism and impact on sialylation in GNE myopathy. Adenosine triphosphate, ATP; cytidine monophosphate *N*-acetylneuraminic acid synthetase, CMAS; cytidine monophosphate, CMP; cytidine-5-monophosphate-*N*-acetylneuraminic acid, CMP-Neu5Ac; cytidine triphosphate, CTP; *N*-acetylmannosamine, ManNAc; *N*-acetylmannosamine-6-phosphate, ManNAc-6-P; *N*-acetylneuraminate pyruvate lyase, NPL; *N*-acetylneuraminic acid phosphatase, NANP; *N*-acetylneuraminic acid synthase, NANS; *N*-acetylneuraminic acid, Neu5Ac; *N*-acetylneuraminic acid-9-phosphate, Neu5Ac-9-P; Neuraminidase, NEU; phosphoenolpyruvate, PEP; sialyltransferases, STs; UDP-*N*-acetylglucosamine, UDP-GlcNAc. The red dotted lines denote the feedback inhibition reactions. Created with BioRender.com.

hGNE2 (ORF length 2262 bp; 753 aa, 83.066 kDa, GenBank NM_001128227) is currently the longest splice isoform described and, therefore, used for mutation annotation. Besides, it is suggested to be the main expressed variant in different tissues, including skeletal muscles [35].

GNEM is inherited in a recessive manner and over 200 *GNE* mutations have been identified, including predominantly missense mutations, along with insertions, deletions, nonsense, intronic, and splice site mutations [1]. Remarkably, no GNEM patients have been identified with two null mutations, suggesting that absent GNE activity is incompatible with life and GNE is essential for embryonic development [36]. While some ethnic founder mutations have been identified in Middle Eastern (p.M743T), Japanese (p.C44S, p.D207V, and p.V603L), Roma Bulgarian (p.I618T) and Indian/Asian populations (pA.662 V and p.V727M), most are sporadically found within single families.

Some asymptomatic carriers with confirmed causative *GNE* mutations have been reported [37,38], suggesting an incomplete penetrance or the influence of environmental or other genetic factors in GNEM. Moreover, there have been reports [39,40] of GNEM patients with apparent mutations solely in one allele, in which the second mutation consists in a complex genomic alteration as copy-number variations (CNVs) or deletions that lead to Alu-mediated recombination [39,41]. Notably, *GNE* is an Alu-rich gene [39], which contributes to genetic diversity and disease through insertional mutagenesis and as non-allelic homologous recombination. In light of these discoveries, genomic rearrangement analysis should also be considered in the patient diagnosis.

A reliable correlation between genotype-phenotype in GNEM remains challenging, partially due to the lack of statistical significance studies in such rare conditions. Most of the current knowledge comes from small cohorts, with a large variability of symptoms and disease features across *GNE* mutations. Even though patients with the variant p.D207V have predisposition to milder phenotype [20], while the p.L539S variant leads on average to an earlier onset [42]. Additionally, phenotypic differences between homozygous and compound heterozygous carriers were also reported, with p.V603L homozygous patients experiencing more severe phenotype than heterozygous ones [42]. Furthermore, patients who have mutations in both enzymatic domains of GNE tend to experience earlier disease onset and become non-ambulant, when compared to those who have both mutations within the same enzymatic domain [5,43].

Understanding how specific mutations in the *GNE* gene correlate with enzyme activity and disease prognostic is essential. Studies with purified GNE mutant proteins showed that each mutation affects GNE activity differently and mutations in one enzymatic domain also affects the activity of the other domain. Interestingly, the p.I618T and p.V727M kinase mutants showed no change in the kinase activity but drastic effect on epimerase function [44]. Despite these genotype-phenotype correlations, studies with patients homozygous for p.M743T show high phenotype variability [45], even between families, suggesting that the type of mutation only partially contributes to disease severity [5,42].

Another point to consider in a condition such as GNEM, is that certain mutations may lead to distinct muscle patterns. A recent study established a relationship between a specific pattern of muscle atrophy

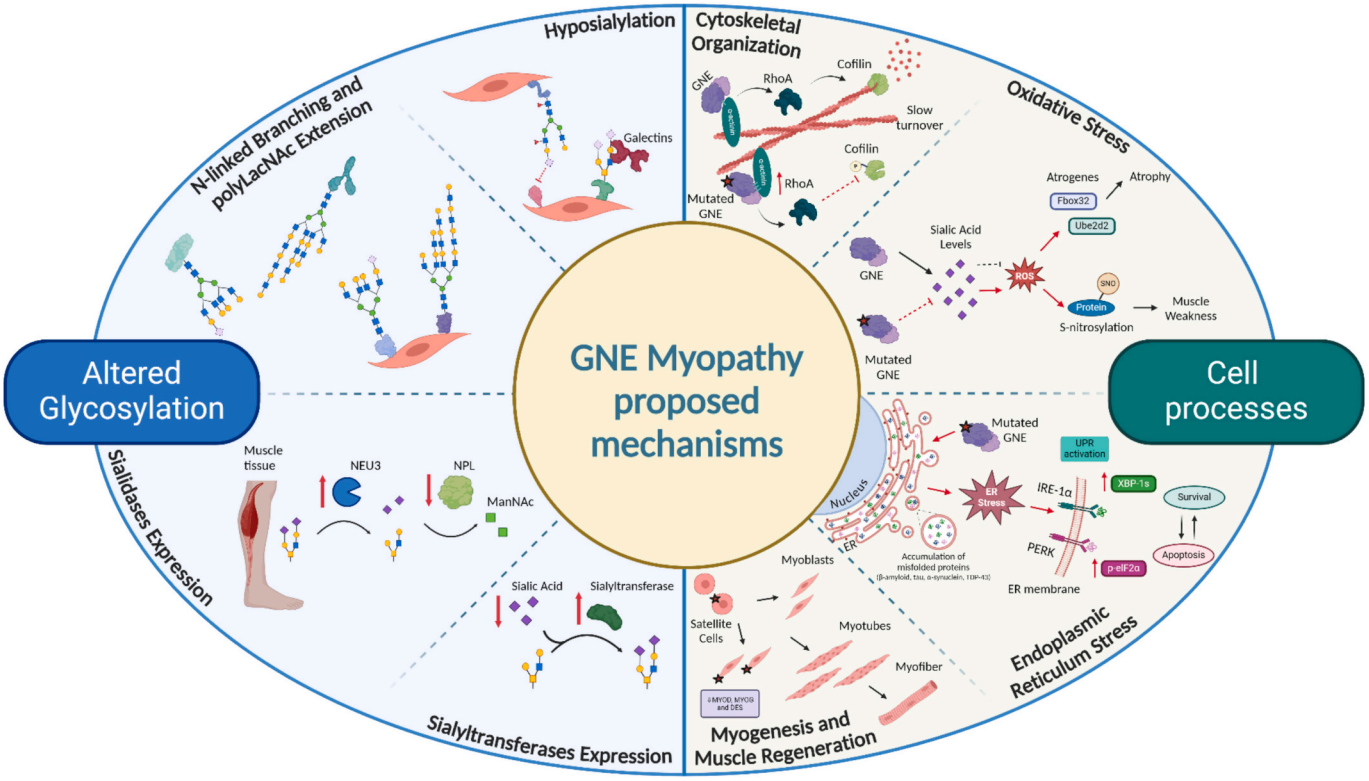

Fig. 2. Overview of the proposed cellular and molecular mechanisms underlying the pathophysiology of GNE myopathy. Created with BioRender.com.

and Iranian typical mutations [22]. Although these associations have not been fully understood, many advances in imaging techniques, such as quantitative MRI, will allow for a better evaluation of muscular atrophy.

In GNEM the proportion of men and women affected is similar, like in most genetic muscle disorders [46]. However, two cohort studies reported that women tend to have an earlier onset of the disease than men [5,20]. Although no direct link has been established, the onset of GNEM occurs during the reproductive age of women which may be linked with an aggravating effect in case of pregnancy due to increased sialic acid requirements and on overall muscle strength [28,47].

4.1. Sialylation in GNEM

GNE is a key enzyme in the biosynthetic pathway of sialic acids (Fig. 1). Sialic acids, a family of derivatives of neuraminic acid, serve as terminal monosaccharides of most glycans found on lipids and proteins at cell surface, where they mediate different biological functions. *N*-acetylneuraminic acid (Neu5Ac) is the most abundant sialic acid in humans, while *N*-glycolylneuraminic acid (Neu5Gc) is the most expressed sialic acid in non-human mammals. Humans cannot convert Neu5Ac to Neu5Gc due to a deletion mutation in the CMAH gene, which encodes for monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH) responsible for this conversion.

The sialic acid biosynthesis is related to the hexosamine biosynthetic pathway (HBP) of the GNE epimerase domain (UDP-GlcNAc 2-epimerase) which converts UDP-GlcNAc into *N*-acetyl-D-mannosamine (ManNAc), and the kinase domain (ManNAc kinase) then phosphorylates ManNAc into ManNAc-6-phosphate (ManNAc-6-P) (Fig. 1). Subsequently, the sialic acid synthase, through condensation with phosphoenolpyruvate (PEP), converts ManNAc-6-P into Neu5Ac-9-P, which is then dephosphorylated to Neu5Ac. ManNAc can also be directly converted into Neu5Ac through the action of the *N*-acetylneuraminate synthase (NANS) enzyme, bypassing the intermediate steps [48]. Additionally, GlcNAc kinase can replace ManNAc kinase in phosphorylating ManNAc to ManNAc-6-P [49]. In the nucleus, Neu5Ac is activated into cytidine-5-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac), which is translocated into the Golgi apparatus by the SLC35A1 transporter, to become substrate of sialyltransferases. This pathway is regulated through two feedback inhibition mechanisms: inhibition of glutamine-fructose-6-phosphate transaminase (GFPT) by its product and by UDP-GlcNAc, and inhibition of UDP-GlcNAc 2-epimerase *via* binding of cytoplasmic CMP-sialic acid to its allosteric site (Fig. 1).

4.1.1. Hyposialylation in GNEM

GNE mutations presumably affect glycan sialylation since they affect the enzymatic activity of GNE. Hyposialylation of cell surface glycans in GNEM patients, including specific skeletal muscle glycans such as α-dystroglycan, neural cell adhesion molecule (NCAM), neprilysin, GM3 ganglioside, and O-linked glycans, have been reported to be hyposialylated in GNEM [11,27,50,51]. In particular, the staining of muscle biopsies from GNEM patients using *Sambucus nigra* agglutinin (SNA) lectin, which predominantly recognizes terminal sialic acid in α(2,6)-linkage in N-glycans [52], showed reduced sialylation of approximately 50 % [53].

4.1.2. Sialyltransferase and sialidase expression

The bioavailability and biosynthesis of sialic acid regulates the expression of the cell sialyltransferases and sialidases [54]. Generally, sialyltransferases are upregulated in cells with *GNE* defects, suggesting that the sialic acid availability or the *GNE* itself modulates the expression of sialyltransferases to ensure adequate sialylation [55].

Sialic acid can be cleaved from the glycoconjugates by sialidases (NEU1–4) to generate new free sialic acid that can be reused or catabolized by *N*-acetylneuraminate pyruvate lyase (NPL) into ManNAc and enter the sialic acid pathway (Fig. 1).

A study assessing the tissue specific expression of enzymes of the

sialic acid biosynthesis pathway points to a higher level of hyposialylation in the skeletal muscle, in the case of *GNE* mutations, due to high expression of NEU3 sialidase and low expression of the catabolic enzyme NPL [35].

These studies highlight the need for an integrative understanding of the biosynthetic sialic acid pathways in GNEM.

4.1.3. N-Glycan Branching and PolyLacNAc

Even though hyposialylation is acknowledged to be the main cause of GNEM, the correlation between disease phenotype and reduction in cell sialylation is imperfect [53]. A recent study failed to identify significative changes in the sialylation profile of skeletal muscle glycoconjugates between GNEM patients and healthy controls [56]. Therefore, non-canonical roles for the GNE protein beyond sialic acid biosynthesis have been pointed out.

Besides its key role in sialic acid biosynthesis, GNE activity is predicted to affect the N-linked glycan structures, as *GNE* depletion in cell lines increases N-glycan branching and poly-*N*-acetyl-lactosamine (polyLacNAc) extension [57]. These glycan changes increase binding with galectins [58], in particular galectin-1 [57] implicated in the development, differentiation, repair, and regeneration of the muscle tissue [59,60]. Hence, the muscle impairment in GNEM could result from increased binding of galectin-1; however, no effect on N-linked glycan branching, polyLacNAc extension or galectin binding has been observed in cells with GNEM causing-mutations [57]. Nonetheless, these findings underscore the importance of exploring novel functions of GNE including the glycan recognition by lectins, to enhance our understanding of GNEM pathogenesis.

5. Pathomechanisms implicated in GNEM

Apart from glycosylation, the GNE protein is implicated in various biological processes that may contribute to GNEM pathogenesis (Fig. 2). Recent studies indicate that GNE expression affects cytoskeletal organization, oxidative stress, endoplasmic reticulum (ER) stress, and myogenesis and muscle regeneration.

5.1. Cytoskeletal organization

Cytoskeletal and sarcomere organization has been shown to be altered in the muscle of GNEM patients. There is differential expression of key proteins like actin and its binding proteins, α-actinin-1 and -2, as well as myosin and tubulin [61]. In a GNEM mouse model, proteins implicated in the cytoskeletal network, integrin pathway, focal adhesion and extracellular signal-regulated kinases (ERK) signaling pathway were upregulated [62]. Interestingly, GNE was found to interact with α-actinin-1 and -2 and other microtubule-associated proteins [63–65]. Under normal conditions, GNE interacts with α-actinin-1 and 2 upon integrin activation by recruiting kinases to form the focal adhesion complex, promoting downstream signaling and RhoA activation, leading to phosphorylation and inhibition of an actin-binding protein, the cofilin. However, when GNE is mutated or non-functional, it binds strongly to α-actinins, which consequently leads to an upregulation of RhoA activation and persistent inhibition of cofilin, which prevents actin severing and the generation of actin monomer pool. Therefore, it is proposed that *GNE* mutations lead to slower actin turnover, stress in fiber formation and reduced cell migration [66–68].

5.2. Oxidative stress

Skeletal muscle tissue demands a continuous and adaptable energy supply to fulfil its primary functions, which involves adaptation to metabolic changes and oxygen consumption. In healthy muscles, sialic acid seems to play an important role in suppressing reactive oxygen species (ROS) generated during muscle contraction, by acting as a ROS scavenger or through glycoproteins interactions [69–72]. In GNEM muscles, ROS is upregulated as a consequence of reduced antioxidant capacity, resulting in upregulation of atrogenes (*e.g.*, Fbox32 and Ube2d2), and myofiber atrophy. Higher ROS levels can also increase *S*-nitrosylation on contractile and metabolic proteins, which may induce functional defects on these proteins and lead to skeletal muscle weakness due to impaired contractile network and energy production [73].

5.3. Endoplasmic reticulum stress

Apart from oxidative stress, deregulated ER stress response has also been identified as a source of muscular damage in GNEM. Deposits of β-amyloid, tau protein, α-synuclein, and transactive response binding protein (TDP-43) have been found in the rimmed vacuoles of GNEM patients' muscle [12,21,28,74]. The accumulation of misfolded or unfolded proteins in the ER results in destabilization of ER homeostasis and activates an intracellular signaling cascade, the unfolded protein response (UPR) pathway, to restore a favorable folding environment. In a recent work it was proposed that the ER state and misfolded protein accumulation in *GNE* mutant cells triggers the UPR pathway mediating survival or apoptotic pathways [74] and determining the fate of the cells. Interestingly, in GNEM, the heat shock protein HSP70, involved in protein folding and stress response, is downregulated, which leads to increased apoptosis and protein aggregation [75]. Notably, supplementation with sialic acid failed to restore ER homeostasis, suggesting that ER stress in GNEM is not solely due to the low levels of sialylation [74].

5.4. Myogenesis and muscle regeneration

Defective myogenesis has also been implicated as a contributing mechanism to the GNEM pathophysiology. A recent study with GNEM patient-derived induced pluripotent stem cells (iPSCs) identified lower levels of myogenic and muscle repair regulatory factors during the differentiation of muscle precursor cells into myoblasts, when compared to controls [76]. As myogenesis and regenerating myofibers involve common pathways, the discovery of these defects can have significant clinical implications, pointing out muscle regeneration as a potential therapeutic target.

6. Attempts to develop research models that recapitulate GNEM

Research models to study GNEM have evolved over the years. Cell lines with *GNE* human mutations or decreased *GNE* expression have been extensively used. More recently, models using patient-derived iPSCs have also been established [76]. Cell models are extremely important due to the low availability of patient-derived cells.

Despite the usefulness of the *in vitro* models, animal experiments remain essential to understand the mechanisms underlying GNEM. A complete knock-out (KO) mouse model of the *Gne* gene is not viable and displays early embryonic lethality [36]. Up to date, there are three *Gne*-deficient mice models available [62,77,78], yet none fully recapitulate the patients' phenotype, which limits their pre-clinical use. Two transgenic mice that express human *GNE* mutations common among the Japanese population (p.D207V and p.V603L) [77,78] exhibit marked hyposialylation, highlighting the potential role of sialylation in GNEM mechanism. The $Gne^{(-/-)}$ hGNED207V-Tg mouse exhibits late onset muscle atrophy and features resembling the disease. However, these mice also show cardiac and diaphragm pathology, not observed in GNEM patients [77]. Despite the high lethality of the *Gne* null mice, with only 10 % survival [36], so far this is the only mouse model that showed hyposialylation and late onset progressive muscle weakness, and thus useful for testing therapeutic supplements [79] and evaluating pathological changes in GNEM [73].

Another available model is the hypomorphic *Gne* mouse model created through homologous recombination to introduce the Middle Eastern founder mutation p.M743T. Unexpectedly, this mouse model

Table 1
Overview of therapeutic options to target GNE myopathy.

Therapy	Principle	phase of development / Testing	Observations	REFERENCE (S)
Intravenous Immunoglobulin (IVIG)	Sialylation-increasing therapy	Phase I Clinical Trial (NCT00195637)	Failed to increase muscle sialylation No histological changes	[85]
		Phase I Clinical Trial (NCT01359319)		
Extended-release formulation of Neu5Ac (Ace-ER)	Sialylation-increasing therapy	Phase II Clinical Trial (NCT01517880) Phase III Clinical Trial (NCT02377921)	Failed to prove clinical efficacy	[86–89]
		Approved in Japan	Approved after Phase III Clinical Trial (NCT04671472)	
N-Acetylmannosamine (ManNAc)	Delivery of sialic acid intermediate	Phase I Clinical Trial (NCT01634750) Open Label Phase II Clinical Trial (NCT02346461)	Preliminary evidence of clinical efficacy	[90,91]
		Multi-center Phase II Clinical Trial (NCT04231266)	Ongoing study	
6′-sialyllactose (6SL)	Sialylation-increasing therapy	Randomized pilot trial	Distributes to the muscles and can ameliorate muscle weakness	[92,93]
O-tetra-acetylated *N*-acetylmannosamine (Ac_4ManNAc)	Delivery of sialic acid intermediate	Pre-clinical studies	Lipophilic ManNAc analogue	[79]
Ac_3ManNAc-6-phosphoramidates	Delivery of sialic acid intermediate	Pre-clinical studies	Improve the low permeability and plasma stability of ManNAc-6-P	[94,95]
AAVrh74.MCK.GNE Viral Vector	Delivery of human WT *GNE*	Pre-clinical studies	Around 50 % of GNEM patients with pre-existent serum antibodies to AAV8	[96]
Metformin	Activator of the autophagic flux	Pre-clinical studies	Protective role in several neurodegenerative disorders	[97]
BGP-15 ($C_{14}H_{22}N_4O_2$·2HCl)	Activator of HSP70 chaperone	Pre-clinical studies	Reduced protein aggregation	[75]

showed a high mortality rate in the first generation due to severe renal damage, which is not observed in GNEM patients. Surviving $GNE^{M743T/M743T}$ mice did not manifest muscle atrophy or histopathology changes throughout their life span [62]. A comparative study of the two $GNE^{M743T/M743T}$ mice phenotypes, with and without kidney manifestations and control ($Gne^{+/+}$) demonstrated that the altered pathways behind kidney protection are well-defined muscle pathways, unravelling a connection between human GNEM and the $Gne^{M743T/M743T}$ KI model [62].

Gne^{FLAG} mice was recently established through the addition of a Flag epitope tag to the carboxy-terminus of mouse *Gne* gene [80], to facilitate the evaluation of GNE levels across different tissues and over time. This model can then be useful for better understanding GNE functions in different cells and tissues.

Overall, none of the currently available mouse models fully recapitulate the GNEM phenotype observed in humans. This highlights that the role of sialic acid to protein glycosylation and function differs between species. A clear indication of this was the success of the supplementation with the extended-release sialic acid in mice, which then failed in human phase 3 clinical trials. Nowadays, efforts are focused on the development of conditional muscle *Gne* KO mice by crossing $Gne^{FLAG\ loxP}$ mice with mice expressing Cre recombinase in skeletal muscle cells [80]. Another approach relies on the use of CRISPR-Cas9 to delete *Gne* gene in mice skeletal muscle tissue, despite challenges due to cell size and the intrinsic composition of muscle fibers with hundreds of myonuclei. Recent studies successfully established mouse models with the deletion of genes crucial to muscle function [81], through the crossing of Cre-dependent $Rosa26^{Cas9\text{-}EGFP}$ KI mice with mice expressing Cre-recombinase in muscle cells [82]. To delete the gene of interest specific single guide RNAs (sgRNA) are delivered using myotropic adeno-associated viruses (AAV) [81]. However, these mice models only with skeletal muscle affectation haven't yet shown the ability to fully recapitulate the GNEM phenotype.

Zebrafish models have been recognized as potentially effective for studying GNEM due to the similarity between zebrafish and the human *GNE* sequence. The initial studies with GNEM zebrafish model involved the injection of morpholino (MO)-modified antisense oligonucleotides to knockdown *gne* in zebrafish embryos. This model showed a variety of phenotypic severity, reduced locomotor activity and disturbed muscle integrity, including myofiber loss [83]. In a more recent study, a hypomorphic zebrafish model expressing human *GNE* mutation M743T developed normally, without disease [84]. In contrast, a zebrafish CRISPR-Cas9 KO for the *gne* gene showed 7–8 days of normal development followed by severe abnormalities with rapid death. The *gne* KO phenotype included impaired organization in myofibers with no motor dysfunction, as well as issues in the swim bladder, reduced response sensory stimuli, abnormal eyes and brain, and reduced heartbeat rate. Attempts to rescue the *gne* KO phenotype though sialic acid was unsuccessful, suggesting that the phenotype and death are not solely linked to a deficiency in sialic acid [84].

7. Revisiting therapeutic options to target GNEM

Currently, there is no worldwide approved therapy for GNEM, and patients rely on palliative medicine, such as physiotherapy, and analgesics to manage disease symptomatology. However, efforts have been made to develop therapeutic strategies based either on the supplementation with sialic acid/sialic acid precursors or in *GNE* gene therapy (Table 1).

Supplementation therapy addresses the hyposialylation underlying GNEM pathophysiology and aims at increasing sialic acid levels in patients and restore muscle strength. Initial trials with a highly sialylated glycoprotein, IgG, administered intravenously (IVIG), improved muscle strength and function, but not the sialylation of the muscle glycoproteins and did not provide evident histological changes (ClinicalTrials.gov: NCT00195637) [85]. Still, the improvements observed after IVIG treatment suggest that providing sialic acid has therapeutic potential.

An extended-release formulation of Neu5Ac (Ace-ER) was later developed and orally administered to GNEM patients in a phase 2 randomized, double-blind, placebo-controlled clinical trial (ClinicalTrials.gov NCT01517880). The results from this trial showed that Ace-ER administration stabilized GNEM patients' muscle strength [86].

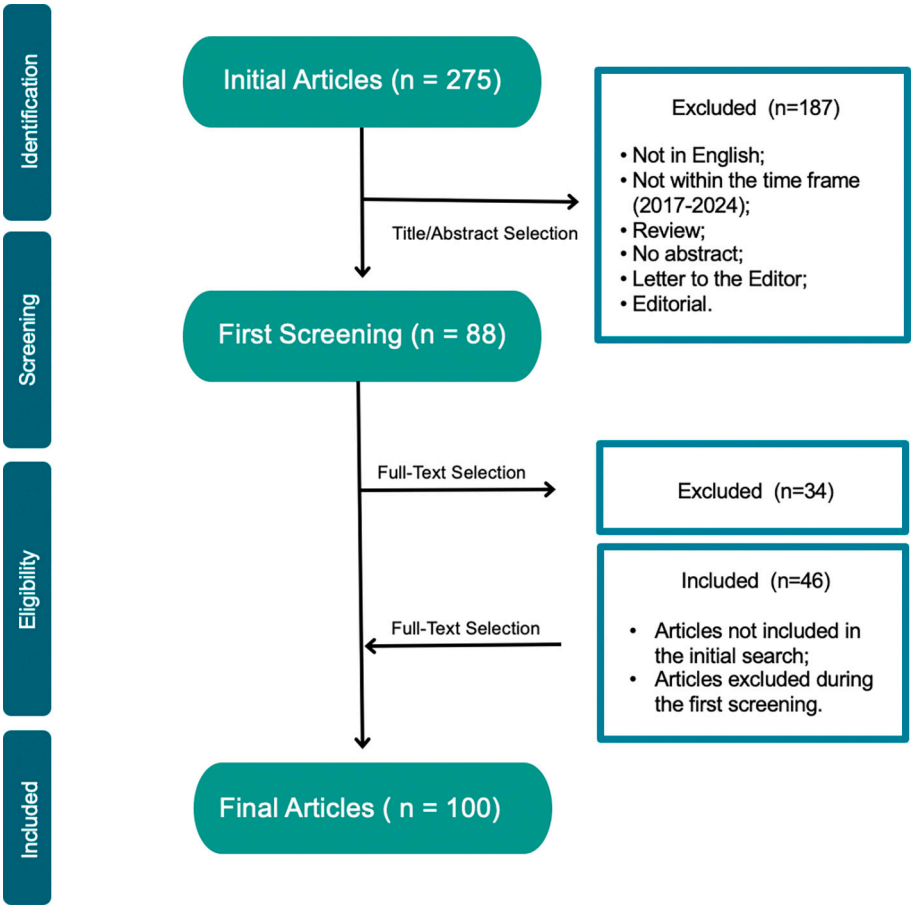

Fig. 3. Flowchart of article selection.

However, subsequent phase 3 trial failed to demonstrate Ace-ER benefits for muscle strength maintenance (ClinicalTrials.gov NCT02377921) [87]. Despite this, Ace-ER was recently approved in Japan after a successful clinical trial conducted in GNEM patients with mild phenotype [88,89]. Differences in the results between the international and the Japanese clinical trials may be attributed to ethnicity and genetic background, or the level of disease progression at baseline [89].

The most recent strategy for sialic acid supplementation is the oral administration of ManNAc. As the only uncharged molecule precursor in the biosynthesis of sialic acid, ManNAc can cross cell membranes more easily. While ManNAc does not overcome the second step catalyzed by GNE enzyme, it bypasses the rate-limiting feedback inhibition step and can be phosphorylated into ManNAc-6-P by other kinases [49]. ManNAc trials showed long-term safety and preliminary efficacy in a phase 1 study (ClinicalTrials.gov NCT01634750) and in a phase 2 trial (ClinicalTrials.gov NCT02346461) [90,91]. A phase 2 randomized, placebo-controlled, double-blind, multi-center study (ClinicalTrials.gov NCT04231266) is ongoing.

However, both Ace-ER and ManNAc supplementations have disadvantages, including the need for high daily dosages (from 3 g to 12 g) and many adverse gastrointestinal events, such as flatulence and diarrhea, which compromise patient compliance.

Another promising supplementation is with sialyllactose, a source of sialic acid extracted from human milk. After administration, sialic acid is released due to the action of intrinsic sialidases and then incorporated into the body. Studies with 6′-sialyllactose (6SL) showed that it effectively distributes to the muscle and can restore its function [92]. A recent randomized pilot trial showed that 6SL is well tolerated by GNEM patients, increases free and bound sialic acid levels and can prevent muscle weakness [93].

In a study conducted with the $Gne^{(-/-)}$ hGNED207V-Tg mouse model, supplementation with a lipophilic ManNAc analogue, O-tetraacetylated *N*-acetylmannosamine (Ac_4ManNAc), effectively restored sialylation and ameliorated GNEM phenotype [79]. This work uncovered the advantage of prodrugs for sialic acid precursors. Additionally, new prodrugs of ManNAc-6-P formulated using ProTide technology [94,95], improved *in vitro* the permeability and plasma stability of ManNAc-6-P. These prodrugs are then promising options to overcome the problems of low absorption and toxicity of the charged molecular precursors of sialic acid.

Alongside the development of these supplementation strategies, gene therapy for GNEM has recently gained greater interest. Gene therapy for GNEM aims to deliver a healthy copy of the *GNE* gene to skeletal muscle tissue to restore the muscle function or reduce atrophy. It was shown that the adeno-associated viruses 8 (AAV8) vector can effectively deliver the WT *GNE* gene to GNEM patients' muscle cells and to the skeletal muscle of healthy and GNEM mouse model [96,98]. Further results showed that the rAAVrh74.MCK.GNE viral vector led to long-term expression of human WT *GNE* in the muscle cells and a mild improvement in the mouse phenotype [96]. A recent work established an assay to demonstrate the potency of AAV gene vectors in replacing sialic acid and to determine the dosage for human *GNE* gene therapy [99]. The assay intends to facilitate the clinical approval of any AAV-based GNEM therapy, by evaluating the bioactivity of various production lots of AAV and their stability over time.

Like other rare diseases, drug repurposing has been explored for GNEM. Metformin, the first-line therapy for type 2 diabetes, activates the autophagic flux and has protective role in neurodegenerative disorders and cardiomyopathy. Metformin was found to increase cell viability and restore the autophagic flux in GNEM patient-derived

fibroblasts [97], highlighting the possibility of using metformin to manage GNEM. BGP-15, a new insulin sensitizer currently under clinical phase II trial for insulin resistance treatment, has already shown beneficial effects for other muscle disorders, such as Duchenne dystrophy. In *GNE* mutant cells, BGP-15 increased the expression of HSP70, reduced the β-amyloid aggregates and apoptosis [75] and improved the GNE epimerase activity [44].

While many of these therapies are promising, the lack of reliable animal models, as discussed before, hinders the development and their approval.

8. Conclusions and future perspectives

Sialylation of glycoproteins and glycolipids on cell surfaces plays key roles during development and regeneration, as well as in the pathogenesis of various diseases, including certain hereditary myopathies. While deficiency of sialic acid production and decreased sialylation of muscle glycoproteins have been widely accepted as the main pathophysiological hallmarks of GNEM, the suggested impact of *GNE* variants on sialylation remains controversial, raising the question: "Can we blame it solely on a sialic acid defect?". Considering the emerging evidence thoroughly summarized in this review it is possible to assume that the knowledge of the mechanisms underlying the molecular-pathological spectrum of GNEM is still limited, but genotype-phenotype correlations, or the unknown relationship between them, imply other GNE functions rather than sialic acid biosynthesis. Uncovering novel functions for GNE may then be the answer towards understanding the molecular-pathological spectrum of GNEM and future drug target development. The rare incidence of disease, limited preclinical models, lack of reliable biomarkers, and slow disease progression are some of the most challenging factors to drug development. In addition, clinical trial designs to assess treatment efficacy require a better understanding of the disease, its natural history, priority symptoms, and GNEM pathophysiology. To address these challenges, several tools need to be refined, including multiomics analyses and/or single cell sequencing. Findable, Accessible, Interoperable and Reusable (FAIR) principles also need to be implemented to optimize the reuse of data in the domain of rare diseases, namely for GNEM. Plus, a patient-centric research that secures the engagement of patients throughout the research cycle to prioritise research needs to be a standard practice.

9. Materials and methods

9.1. Literature search

A literature review was carried out to understand the current findings in GNEM. As a mean of systematizing the initial article screening, an automated python search tool was used to retrieve articles through the Medline database, using PubMed as the search engine. Query terms "Hereditary inclusion body myopathy" AND "GNE", "Distal myopathy with rimmed vacuoles" AND "GNE", "GNE myopathy", and "GNE-CDG" AND "GNE" were used as the code primary input [100]. Selection criteria were chosen to screen the extracted articles focusing on the most relevant literature for this study (Fig. 3).

CRediT authorship contribution statement

Beatriz L. Pereira: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Mariana Barbosa:** Writing – review & editing, Investigation, Conceptualization. **Pedro Granjo:** Writing – review & editing, Formal analysis. **Hanns Lochmüller:** Writing – review & editing. **Paula A. Videira:** Writing – review & editing.

Declaration of competing interest

The authors have nothing to declare.

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Data availability

No data was used for the research described in the article.

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