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GLYCOGENOSYS TYPE II AND DANON DISEASE: MOLECULAR STUDY AND MUSCLE PATHOLOGY

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1. Riassunto dell'attività svolta

Le malattie da accumulo lisosomale, di cui sono note almeno 40 forme, sono causate da un difetto nel funzionamento delle proteine lisosomali, con conseguente accumulo a livello intra-lisosomale di metaboliti non degradati. Nonostante anni di studi sulle basi genetiche e molecolari di queste malattie, si sa ancora poco sugli eventi che portano da questo accumulo intra-lisosomale alla patologia.
La malattia di Danon e la glicogenosi di tipo II rappresentano due delle forme più conosciute di questo gruppo di malattie, classificate anche come "miopatie autofagiche vacuolari", in quanto i vacuoli autofagici sono la caratteristica morfologica distintiva della patologia.
Scopo di questo studio è stato quello di analizzare a livello molecolare, biochimico e della patologia muscolare due gruppi di pazienti affetti dalla malattia di Danon e da glicogenosi di tipo II, in modo da acquisire nuove informazioni utili a tracciare possibili correlazioni genotipo-fenotipo e a chiarire i meccanismi patologici alla base di queste patologie.

La Glicogenosi di tipo II (GSDII) è una malattia autosomica recessiva (OMIM # 232300) causata da un deficit dell'enzima mitocondriale α-glucosidasi o maltasi acida (EC 3.2.1.20/3), che catalizza l'idrolisi dei legami glicogeno α-1,4 e α-1,6. Tale deficit enzimatico porta all'accumulo a livello lisosomale di glicogeno, che genera diversi fenotipi clinici: la forma severa infantile o malattia di Pompe, in cui la deposizione massiccia di glicogeno causa la rapida progressione e l'esito fatale della malattia per arresto cardiaco nella prima infanzia, e le forme giovanile e adulta (forme ad esordio tardivo), che di solito si manifestano con una miopatia a progressione lenta associata ad insufficienza respiratoria ed assenza di sintomi cardiaci.
La maggior parte dei pazienti con la forma infantile di GSDII non presentano alcuna attività enzimatica a livello dei tessuti, ma alcuni conservano un'attività residua, che sottolinea l'eterogeneità biochimica anche in questa forma clinicamente omogenea della malattia.
Al contrario, nelle forme ad esordio tardivo l'attività enzimatica è ridotta in modo variabile o raramente assente, indicando una relazione tra i livelli di attività enzimatica e la severità del decorso clinico. Il gene codificante per l'α-glucosidasi (GAA, OMIM # 606800) mappa sul cromosoma 17q25.2-25.3, è costituito da 20 esoni, di cui il primo non tradotto, e codifica per una proteina di 952 aminoacidi. L'enzima viene sintetizzato sotto forma di

precursore funzionalmente inattivo, che viene trasportato al comparto pre-lisosomale e lisosomale dove è processato, mediante una serie di proteolisi, nella forma intermedia da 95kDa e nelle forme mature e funzionalmente attive da 76 e 70kDa. Finora sono state identificate più di 200 mutazioni nel gene GAA (riportate nel sito: www.pompecenter.nl). La mutazione più comune nelle forme ad esordio tardivo è la c.-32-13T>G (detta tradizionalmente IVS1-13T>G), che è localizzata nell'introne 1 e causa lo "skipping" dell'esone 2. Circa il 70% dei pazienti di origine caucasica con una forma della malattia ad esordio tardivo presentano questa mutazione in eterozigosi. La delezione dell'esone 18 è invece frequentemente osservata in pazienti con esordio infantile.

Scopo della ricerca è stato quello di analizzare le cause che generano l'ampia eterogeneità clinica caratteristica della glicogenosi tipo II. Tale analisi comprende lo studio a livello molecolare, biochimico e di alcuni aspetti della patologia muscolare. Studi precedenti sono stati condotti su modelli cellulari, ma non sul tessuto muscolare che è primariamente coinvolto nei pazienti affetti.

Abbiamo analizzato 23 pazienti con esordio infantile o tardivo della malattia, 7 maschi e 16 femmine, tra cui 4 coppie di fratelli. Tre pazienti presentavano la classica forma ad esordio infantile con cardiomiopatia (fenotipo Pompe), 3 esordio giovanile (tra i 2 e i 7 anni), 16 esordio tra i 18 e i 57 anni, ed un paziente era asintomatico ma con alti livelli di CK all'età di 14 anni. Abbiamo testato l'attività enzimatica mediante un saggio fluorimentrico nei linfociti e nel muscolo e l'espressione proteica mediante immunoblotting. La patologia muscolare è stata studiata mediante analisi morfologica e immunoistochimica nelle biopsie muscolari. Le colorazioni routinarie per la diagnostica neuromuscolare su sezioni criostatiche seriate con ematossilina-eosina, tricromica di Gomori, PAS, Oil Red O, NADH-tetrazolio reduttasi, citocromo ossidasi, succinato deidrogenasi, ATPasi acida e basica e fosfatasi acida ci hanno permesso di analizzare e misurare la variabilità fibrale, il grado di vacuolizzazione (espressa come percentuale sul totale delle fibre), la reattività degli enzimi mitocondriali e il grado di attivazione degli enzimi lisosomiali (fosfatasi acida). L'analisi immunoistochimica mediante un anticorpo "anti Golgi-zone" ci ha permesso inoltre di indagare la proliferazione del complesso di Golgi. Per testare rapidamente la presenza della mutazione comune nella forma ad esordio tardivo (c.-32-13T>G) abbiamo utilizzato un test allele-specifico (ARMS-PCR). Abbiamo poi effettuato uno screening di mutazioni mediante tecnica SSCP sull'intera sequenza codificante del gene, seguita dal sequenziamento dei casi con migrazione elettroforetica anomala.

Tutti i pazienti con esordio infantile presentavano una severa cardiomiopatia che ha portato al decesso nei primi anni di vita; un paziente con esordio giovanile precoce ha sviluppato una debolezza muscolare grave e insufficienza respiratoria e successiva morte all'età di 4 anni. Nella maggior parte dei pazienti con esordio tardivo la principale caratteristica clinica riscontrata è stata la miopatia o, meno frequentemente, dispnea, astenia o perdita di peso. Sei pazienti hanno sviluppato un'insufficienza respiratoria che ha richiesto l'uso del ventilatore e ha causato la morte in 6 pazienti tra i 45 e 73 anni. Le coppie di fratelli inclusi nello studio confermano la variabilità intrafamiliare.

L'attività enzimatica della maltasi acida è risultata assente o minima nei casi ad esordio infantile e variabilmente ridotta nei pazienti con esordio tardivo. Nei casi ad esordio tardivo, non abbiamo osservato una solida correlazione tra l'attività enzimatica residua a livello muscolare e la severità del decorso clinico.

L'elemento istopatologico che caratterizza la malattia è la presenza di vacuolizzazione fibrale ed autofagia. In pazienti con esordio infantile, la vacuolizzazione e l'autofagia sono risultate essere sempre prominenti (interessando il 95-100% delle fibre), con una severa compromissione della struttura fibrale. Al contrario, nei pazienti ad esordio tardivo il grado di vacuolizzazione era estremamente variabile (dall'1 al 90% delle fibre), e appariva talvolta indipendente dall'età d'esordio e dalla durata della malattia. Il fenotipo clinico nei pazienti con esordio infantile era abbastanza omogeneo e severo, nonostante i diversi tipi di mutazioni, suggerendo che queste hanno tutte un effetto deleterio sul funzionamento proteico.

Le correlazioni genotipo-fenotipo indicano, come già noto dalla letteratura, che la maggior parte dei pazienti ad esordio tardivo presentano la mutazione c.–32-13T>G in eterozigosi (un paziente era omozigote), ma il decorso della malattia è spesso difficile da prevedere solo sulla base delle mutazioni.

Un risultato nuovo ed interessante è derivato dall'analisi mediante western blot dell'espressione dell'α-glucosidasi nei pazienti affetti da GSDII: abbiamo infatti dimostrato che il muscolo di questi pazienti esprime prevalentemente forme inattive/immature dell'enzima α-glucosidasi, mentre la forma matura della proteina era assente o presente a livelli molto ridotti. Inoltre, si è visto che l'eventuale quantità residua di forme proteiche mature riscontrate al western blot correlava con i livelli di attività enzimatica riscontrati nel muscolo di questi pazienti.

Il peso molecolare sia delle forme mature che di quelle immature/inattive è risultato essere maggiore nei pazienti rispetto ai muscoli di controllo. Attribuiamo tali differenze ad

un'eccessiva sialilizzazione delle forme proteiche non funzionali, causata probabilmente da un loro trasporto ritardato o da una loro ritenzione nel complesso di Golgi, in cui agiscono le sialil-transferasi. A sostegno di tale ipotesi, abbiamo riscontrato una proliferazione del Golgi nelle fibre muscolari di tutti i pazienti, sia ad esordio infantile che tardivo,causata possibilmente dalla ritenzione delle forme enzimatiche inattive, che non possono venire correttamente veicolate ai lisosomi. Non abbiamo invece osservato un aumento nell'espressione della proteina della membrana lisosomale LAMP-1, in quanto probabilmente solo una piccola percentuale delle forme proteiche mutanti è in grado di raggiungere i lisosomi. Dallo studio morfologico dei diversi comparti cellulari è derivato anche un altro dato interessante: abbiamo infatti osservato un diverso grado di disfunzione della via endocitica e autofagica in pazienti con esordio infantile rispetto a quelli con esordio tardivo. Nel muscolo di questi ultimi le membrane vacuolari esprimevano le proteine sarcolemmali, come la caveolina-3 e la distrofina (tali vacuoli sono noti come vacuoli di tipo 2), mentre ciò non avveniva nella forma infantile della malattia (vacuoli di tipo 4 o "laghi di glicogeno"). Quanto osservato è attribuibile ad una ridotta proliferazione della membrana e ad un ridotto movimento vescicolare nelle fibre muscolari fortemente compromesse dei pazienti ad esordio infantile, mentre nei pazienti ad esordio tardivo è possibile un rimodellamento di membrana, che probabilmente ha un effetto protettivo che previene la rottura della membrana vacuolare a seguito della contrazione muscolare. Tale osservazione è importante perché la patogenesi degli autofagosomi non è ancora stata studiata a fondo: l'autofagia e il rimodellamento di membrana che caratterizzano la forma tardiva della malattia, potrebbero modificare la risposta alla terapia di sostituzione enzimatica (ERT) e compartimentalizzare l'enzima ricombinante.

La Malattia di Danon ha ereditarietà di tipo dominante legato al cromosoma X, è causata da mutazioni nel gene LAMP2 (Lysosomal Associated Membrane Protein 2) e si presenta con cardiomiopatia ipertrofica, miopatia e ritardo mentale. I sintomi cardiaci in genere iniziano nell'adolescenza, e i pazienti muoiono per insufficienza cardiaca nella terza decade di vita. Al contrario, la miopatia scheletrica è di solito lieve, e la debolezza e l'atrofia interessano prevalentemente i muscoli del cingolo scapolare e del collo, anche se possono essere coinvolti anche i muscoli distali. Il ritardo mentale è presente nel 70% dei pazienti maschi (raro nelle femmine) e di solito è modesto. Nelle femmine, la malattia interessa prevalentemente il cuore ed ha un esordio più tardivo rispetto ai maschi. Il gene LAMP2 mappa nella regione cromosomica Xq24. La proteina LAMP-2 avvolge la

superficie interna della membrana lisosomale ed è composta da un dominio intraluminale, un dominio transmembrana ed una piccola coda citoplasmatica contenente un segnale di localizzazione sulla membrana lisosomale. Si pensa che sia coinvolta sia nella fusione dei lisosomi con altre membrane che nella maturazione dei vacuoli autofagici. Inoltre, opera come recettore che permette alle proteine di essere importate e degradate all'interno dei lisosomi nell'autofagia mediata da chaperoni.

Per studiare gli effetti delle mutazioni nel gene LAMP2 sull'espressione proteica in diversi tessuti, abbiamo effettuato uno screening molecolare ed un'analisi del difetto proteico sul tessuto muscolare, cardiaco, sui leucociti e fibroblasti di 9 soggetti maschi non imparentati tra loro, con cardiomiopatia ipertrofica e miopatia vacuolare.

Tre dei 9 soggetti analizzati hanno evidenziato un deficit proteico di LAMP-2. Sebbene la malattia di Danon sia molto rara nella popolazione generale, la sua frequenza è quindi rilevante (33%) tra quei pazienti che presentano sia miopatia vacuolare che cardiomiopatia ipertrofica, suggerendo che il numero di pazienti riportati finora potrebbe essere stato sottostimato. Il difetto di LAMP-2 è risultato essere generalizzato, in quanto riscontrato in tutti i tessuti da noi analizzati: tessuto muscolare scheletrico e cardiaco, leucociti e fibroblasti. Questo risultato indica che l'analisi biochimica mediante immunoblot può essere svolta in modo non invasivo sui leucociti, e potrebbe quindi essere impiegata nello screening dei soggetti maschi con sospetto di malattia di Danon per le seguenti ragioni: 1) poiché il difetto proteico è riscontrabile in diversi tessuti, ci si aspetta un'elevata sensibilità del test; 2) anche la specificità dovrebbe essere elevata, in quanto non sono mai stati riportati deficit secondari di LAMP-2 in altre malattie; 3) è meno costosa e più rapida rispetto ad uno screening di mutazioni. Un'altra conclusione derivante da questo studio è che il riscontro di un deficit di LAMP-2 generalizzato a diversi organi potrebbe spiegare il coinvolgimento clinico multisistemico.

Abbiamo inoltre esteso lo studio alla madre cardiopatica di un affetto: in questo caso sia l'analisi istopatologica muscolare che l'analisi della proteina LAMP2 sono risultate inconcludenti, in quanto il muscolo, i fibroblasti e i leucociti presentavano livelli proteici comparabili al controllo normale. Riteniamo quindi che un certo numero di pazienti di sesso femminile possa sfuggire alla diagnosi, che può in questi casi essere ottenuta solo mediante screening di mutazioni.

Sono state identificate mutazioni nel gene LAMP2 in tutti e 3 i pazienti maschi e nella femmina eterozigote. Ciascun paziente presentava una mutazione diversa e non riportata precedentemente in letteratura: sono tutte mutazioni nulle (nonsenso o frame-shifting) che

ci si aspetta diano origine ad una proteina tronca, con perdita del dominio trans-membrana.

Sebbene il coinvolgimento cardiaco associato ad elevati livelli di CK riscontrati nel muscolo scheletrico rappresenti una caratteristica costante nei pazienti maschi, è riportato un certo grado di variabilità nel coinvolgimento muscolare cardiaco e scheletrico sia inter che intra-familiare. Soprattutto nelle femmine, il coinvolgimento di organi diversi dal cuore (muscolo e cervello) è piuttosto variabile. Per verificare se tale variabilità potesse essere attribuita ad un'inattivazione non casuale del cromosoma X, abbiamo effettuato un'analisi sul tessuto muscolare e sui leucociti della nostra paziente, che ha escluso tale ipotesi.

La caratteristica istopatologica principale della malattia di Danon è la vacuolizzazione fibrale. I risultati ottenuti dallo studio dell'istopatologia muscolare confermano tale dato, evidenziando una vacuolizzazione estesa e un certo grado di degenerazione, ed una correlazione tra il grado di vacuolizzazione ed il coinvolgimento clinico a livello muscolare, suggerendo un rapporto tra l'accumulo di materiale autofagico all'interno della fibra muscolare e la progressione della malattia. L'analisi immunopatologica del muscolo scheletrico ha evidenziato che non vi è proliferazione del complesso del Golgi nei pazienti e che le membrane vacuolari esprimono le proteine sarcolemmali. Abbiamo dunque dimostrato che, a differenza di altre malattie da accumulo lisosomale, come la GSDII, nella malattia di Danon non vi è ritenzione a livello del complesso di Golgi o del reticolo endoplasmatico di proteina mutante, e quindi una mancata dislocazione sulla membrana lisosomale della proteina sembra non verificarsi in questa malattia. Un problema irrisolto è se il difetto di LAMP-2 causi un danno strutturale o funzionale all'interno del lisosoma: i nostri risultati potrebbero accordarsi ad un danno di tipo funzionale, che comporterebbe un aumento dell'accumulo di materiale lisosomale con conseguente rottura della membrana e fuoriuscita delle idrolasi nel sarcoplasma.

2. Summary

Lysosomal storage disorders (LSDs), of which more than 40 forms are known, are caused by the defective activity of lysosomal proteins, which results in the intra-lysosomal accumulation of undegraded metabolites. Despite years of study of the genetic and molecular bases of lysosomal storage disorders, little is known about the events that lead from this intra-lysosomal accumulation to pathology.

Danon disease and Glycogen Storage Disease type II (GSDII) are two of the best known entities of this group of disorders, which are referred also as “Autophagic Vacuolar Myophaties” (AVMs), because autophagic vacuoles are their pathognomonic morphologic hallmark.

The objective of this study was to examine at molecular, biochemical and muscle pathology level two groups of patients affected with Danon disease and GSDII, in order to get new insights that might help in tracing genotype-phenotype correlations and to delineate their pathological mechanisms.

Glycogen storage disease type II (GSDII) is an autosomal recessive disorder (OMIM # 232300) caused by the deficiency of the lysosomal enzyme acid α-glucosidase or acid maltase (EC 3.2.1.20/3), which catalyses the hydrolysis of α-1,4 and α-1,6 links of glycogen. The enzyme deficiency leads to lysosomal accumulation of glycogen that results in different clinical phenotypes: the severe infantile-onset form or Pompe disease, in which the massive deposition of glycogen causes rapid progression and fatal outcome in early infancy from cardiac failure, and the childhood, juvenile or adult-onset forms (late-onset forms), which usually manifest as slowly progressive myopathy associated with respiratory insufficiency and without cardiac symptoms. Most patients with the infantile form of GSDII have no enzyme activity in tissues, but some patients have residual activity, underlying the biochemical and genetic heterogeneity of this clinically homogeneous form of the disease. Conversely, in the late-onset forms the enzyme activity is variably reduced or rarely absent, indicating a relation between the levels of enzyme activity and the severity of clinical course. The gene encoding acid α-glucosidase (GAA, OMIM # 606800) maps to chromosome 17q25.2-q25.3, consists of 20 exons spread over 28 kb of genomic sequence and encodes for a protein of 952 amino acids. The first exon is not translated and it is separated by a large intron from exon 2, where is located the ATG start codon.

The enzyme is synthesized as a catalytically inactive 110 kDa precursor, which is transported to the pre-lysosomal and lysosomal compartment, where it is processed into the 95 kDa intermediate and the fully active forms of 76 and 70 kDa. More than 200 different mutations in the GAA gene have been identified in patients affected with GSDII (www.pompecenter.nl). The most common mutation in late-onset forms is the “leaky splice” c.-32-13T>G (traditionally called IVS1-13T>G), which is located in intron 1 and causes the splicing out of exon 2. About 70% of Caucasian patients with late-onset forms of the disease share this mutation in compound heterozygote state with a different mutant allele. A deletion in exon 18 is frequently observed in infantile patients.

The aim of this study was to examine at molecular, biochemical, and muscle pathology level the striking clinical heterogeneity resulting from acid α-glucosidase deficiency. Previous studies have been conducted in cellular models but not in the primarily affected tissue (muscle) of GSDII patients.

We investigated 23 patients with infantile-onset or late-onset glycogen storage disease type II, 7 male and 16 female, comprising four pairs of siblings. Three patients had the classic infantile-onset form of the disease with cardiomyopathy (Pompe), 3 had a childhood onset (between 2 and 7 years), 16 had onset between 18 and 57 years of age, and 1 was asymptomatic but with high CK level at age 14 years.

We tested acid maltase enzyme activity by a fluorimetric assay in lymphocytes and muscle and protein expression by immunoblotting. Muscle pathology was studied by morphological and immunohistochemical analysis of muscle biopsies. Serial cryostat sections were routinely stained for haematoxylin-eosin (H&E), Gomori trichrome, PAS, Oil Red O, NADH-tetrazolium reductase, cytochrome oxidase, succinate dehydrogenase, acid and alkaline ATP-ases, and acid phosphatase. The general inspection of stained sections was used to analyse and measure the fiber size variability, the degree of fiber vacuolisation (expressed as percentage of total fibers), and the reaction of lysosomal enzymes (acid phosphatase). Additional sections were processed separately for immunohistochemical analysis to further investigate the proliferation of the Golgi complex, using anti Golgi-zone antibody. In order to quickly screen the presence of the common c.-32-13T>G point mutation in all 23 patients and the common deletion of exon 18 in infantile-onset patients, we adopted an allele-specific test using published primers. Then we performed a mutation screening by SSCP on the entire coding sequence of the GAA gene, followed by sequencing of the samples with abnormal migrating patterns.

All the infantile-onset patients had severe cardiomyopathy that lead to premature death; one patient with childhood onset developed severe muscle weakness and respiratory insufficiency, which has lead to death at age 4 years. In most late-onset patients the main clinical feature was myopathy or, less frequently, dyspnoea, asthenia or weight loss. Six patients developed respiratory insufficiency that required the use of nasal ventilator and caused the death in 6 patients between 45 and 73 years. Furthermore, we confirmed that the age at onset and the disease course was quite different in patients with identical genotype (e.g., siblings), suggesting a role of genetic background and modifying genes or exogenous factors (nutritional, physical exercise, lifestyle) on GAA gene expression. The enzyme activity was absent or minimal in infantile-onset cases and variably reduced in late-onset patients. We observed a poor correlation between the residual enzyme activity in muscle and the severity of the clinical course. The histopathologic hallmark is muscle fiber vacuolization and autophagy. In infantile-onset patients, fiber vacuolization and autophagy were always prominent (involving 95 to 100% fibers) and severely compromised muscle fiber structure. Conversely, the degree of vacuolization was extremely variable in late-onset patients (ranging from 1 to 90% fibers), and appeared sometimes to be independent of age at onset or disease duration. The clinical phenotype in early-onset patients was rather homogeneous and severe, despite the variability of mutation types, suggesting they have a deleterious effect on protein function.

Genotype-phenotype correlation (seven novel mutations were found) showed, as reported by the literature, that most late-onset patients had the heterozygous c.-32-13T>G leaky splicing mutation (one patient was homozygous), but the course of the disease was often difficult to predict on the basis of the mutations alone. One important and novel result from our study came from the Western blot analysis of the different maturative forms of acid α –glucosidase protein in the muscle from patients with GSDII. We have demonstrated that the muscle from patients with GSDII has a predominant expression of inactive forms of acid α-glucosidase protein and severely reduced or absent levels of the mature forms. Furthermore, the residual amount of the mature forms of the protein on blotting correlated with the level of enzyme activity in muscle.

We first report a different molecular weight of the mature and the intermediate forms of the protein between patients and controls that we attribute to an excessive sialylation of mutant proteins. This is likely caused by a delayed transport and longer transit of the inactive proteins in the Golgi where the sialyltransferases are localized. Supporting this hypothesis, we observed that, in both infantile and late-onset patients, there is an

enhanced proliferation of the Golgi apparatus. On the other hand, we did not find any increased expression of LAMP-1 in patients with GSDII, possibly due to the fact that only a minor proportion of mutant enzyme protein is able to reach the lysosomes. Another interesting data rises from the morphologic analysis of the different cellular organelles. Interestingly, we observed a differential degree of dysfunction of endocytic and autophagic pathways in patients with infantile and late-onset GSDII. In late-onset acid maltase deficient muscle, vacuolar membranes expressed sarcolemmal proteins, such as caveolin-3 and dystrophin (previously classified as type 2 vacuoles) and not in the infantile form of the disease (type 4 vacuoles, lakes of glycogen). These features are possibly due to reduced membrane proliferation and vesicular movement in the overcrowded muscle fibers of Pompe disease, and to the membrane remodelling occurring only in patients with late-onset GSDII, which would be a protective mechanism to prevent membrane rupture during fiber contraction.

This observation is important because the pathogenesis of the autophagosomes has not yet been fully investigated. Autophagy and membrane remodelling, which is peculiar to late onset disease, might modify a clear response to enzyme replacement therapy and, also, compartmentalize the delivery of the recombinant enzyme.

Danon disease is an X-linked dominant disorder due to mutations in the lysosome-associated membrane protein-2 (LAMP2) gene, presenting with hypertrophic cardiomyopathy, skeletal myopathy and mental retardation. Cardiac symptoms usually begin during adolescence, and patients die of heart failure in their third decade. By contrast, skeletal myopathy is usually mild, weakness and atrophy predominantly affect shoulder girdle and neck muscles, but distal muscles may also be involved. Mental retardation is present in 70% of male patients (rare in females) and it is usually mild. In female patients, the disease predominantly involves the cardiac muscle and it has a later onset than in males. LAMP2 gene maps to chromosome region Xq24. LAMP-2 coats the inner surface of the lysosomal membrane and consists of a large intra-luminal head, a transmembrane domain, and a small cytoplasmic tail containing a lysosomal membrane-targeting signal. LAMP-2 is supposed to be involved both in the fusion of lysosomes and with other membranes and in the maturation of autophagic vacuoles, and act as a receptor for proteins to be imported and degraded within lysosomes in chaperone-mediated autophagy.

To investigate the effects of LAMP2 gene mutations on protein expression in different tissues, we screened LAMP2 gene mutations and LAMP-2 protein deficiency in the skeletal muscle of 9 unrelated patients with hypertrophic cardiomyopathy and vacuolar myopathy. We identified 3 novel families (including one affected mother) with unreported LAMP2 gene null mutations and LAMP-2 protein deficiency in skeletal and myocardial muscle, leukocytes and fibroblasts. Although Danon disease is considered very rare in the general population, we showed that its frequency is relevant (33%) among patients presenting with both vacuolar myopathy and hypertrophic cardiomyopathy, suggesting that the number of patients reported so far worldwide could be underestimated.

LAMP-2 protein deficiency was detectable in various tissues, indicating that the biochemical diagnosis can be obtained on leukocytes, instead of on the more invasive skeletal and myocardial biopsies. Clinicians should consider leukocyte immunoblot analysis as a diagnostic screening option when suspecting Danon disease in males for these reasons: 1) it is expected to have high sensitivity because LAMP-2 protein deficiency was found in different tissues of the large majority of mutant patients; 2) it should have high specificity because there are no reports of LAMP-2 protein deficiency in other disorders; and 3) it is much less expensive and time consuming than mutation screening. Another conclusion is that the detection of LAMP-2 deficiency in a variety of cells/tissues supports the clinical evidence that Danon disease is a multisystemic disorder.

We also extended our analysis to the affected cardiopatic mother of a patient: in this case, muscle histopathological and biochemical LAMP-2 protein testing was inconclusive, because muscle, fibroblasts and leukocytes showed LAMP-2 protein levels comparable to controls. Therefore, female patients with Danon disease might escape the diagnosis unless mutation identification is obtained:

We identified mutations in the LAMP2 gene in all 3 male patients and in the heterozygote female. All mutations were unreported and of null type (nonsense and frameshifting), which are expected to generate a truncated protein, with loss of trans-membrane domain.

Although the cardiac involvement associated with a high CK level of skeletal muscle origin is a constant feature in male patients, a different degree of skeletal and cardiac muscle involvement can be observed between inter- and intrafamilial patients. Especially in females, the possible involvement of different organs other than heart (muscle and brain) is variable. Such unpredictability can hardly be attributed to skewed X chromosome inactivation, because we showed that X chromosome inactivation occurred at random in different tissues in our female patient.

The most prominent histopathological feature of Danon disease is the vacuolization of muscle fibers. We confirm that the extent of these changes is related to the degree of clinical muscle involvement, suggesting that the accumulation of autophagic material within muscle fibers correlates with disease progression. Immunopathological analysis of skeletal muscle showed no proliferation of either Golgi apparatus or early endosomes in patients, and expression of sarcolemmal-specific proteins in the vacuolar membrane. As in other lysosomal disorders, such as GSDII, one could speculate that mutant LAMP-2 protein could not be correctly targeted to the lysosomal membrane and could be retained in the Golgi complex or endoplasmic reticulum, but we showed that this is not the case.

An unsolved issue is whether the LAMP-2 protein deficiency might cause structural or functional lysosomal impairment. Our results seem to fit this second hypothesis, where abnormal LAMP-2 function might cause increased lysosomal storage, which, in turn, could trigger the rupture of membrane with the consequent release of acidic hydrolases into the sarcoplasm.

3. Introduction

3.1. The lysosome

Discovered by Christian de Duve over 50 years ago[1], the lysosome is a cytoplasmic cellular organelle that has risen to prominence because of its critical role in cellular function and tissue homeostasis as well as its involvement in numerous human diseases.[2,3] Present in all nucleated eukaryotic cells, the lysosome is delimited by a single-layer lipid membrane and has an acidic internal pH ($\sim$ 5) that is maintained by an ATP-dependent proton pump. The primary cellular function of the lysosome is the degradation and recycling of macromolecules obtained by endocytosis, autophagy and other cellular trafficking pathways. Several classes of macromolecules are hydrolyzed including proteins, polysaccharides, lipids and nucleic acids and this is achieved by the concerted action of numerous soluble catabolic enzymes within the lumen of the lysosome, collectively termed acid hydrolases. Acid hydrolases have evolved to function in the low pH of this organelle and possess a wide variety of enzymatic properties. Over 60 of these enzymes and soluble accessory proteins have been described to date.

In addition to the soluble luminal proteins, many integral and peripheral membrane proteins are associated with the lysosome and have a variety of functions including catalysis, transmembrane transport of substrates and digestion products, establishment of pH gradients, vesicular transport and maintenance of lysosomal structural integrity.[3]

3.2. Lysosomal Storage Disorders (LSD)

The lysosomal system is of considerable biomedical importance as alterations in lysosomes and lysosomal proteins are associated with numerous human diseases.[3,4] The concept of “lysosomal storage disorder” was introduced by Hers in 1965 to explain how genetically determined absence of the lysosomal enzyme α-glucosidase could lead to the fatal disorder called Pompe disease. The undegraded substrate would gradually accumulate within lysosomes, causing progressive increase in the dimension and number of these organelles; the cellular pathology would lead the malfunction of the affected organs. This concept quickly led to the discovery of a number of additional lysosomal storage disorders, the majority of which are caused by the deficiency of a single lysosomal enzyme.

Recent studies including proteomic analysis have identified new soluble lysosomal proteins, including hydrolases, and integral membrane proteins. This has led to estimate that there are at least 50-60 soluble hydrolases and at least 7 integral membrane proteins in lysosomes. In principle, mutations in the genes that encode any of these proteins could cause a LSD. To date, over 40 LSDs that involve soluble hydrolases are known and a number of diseases have been identified that involve the integral membrane proteins.

3.3. Autophagy and muscle disease

Autophagy is a cellular process that mediates the degradation of intracellular components in lysosomes, thus contributing to maintenance of cellular homeostasis, intracellular clearance of damaged structures and adaptation to environmental challenges.[5] Defective autophagy has been linked to common human diseases. Three different autophagic pathways—macroautophagy, microautophagy and CMA (Chaperone-Mediated Autophagy)—have been described in mammalian cells on the basis of their mechanisms for delivery of cargo to lysosomes.[5,6] Whereas in macro- and microautophagy complete regions of the cytosol are sequestered and delivered to lysosomes all at once, in CMA individual proteins cross the lysosomal membrane one by one for their degradation.[7,8] The substrates of CMA are a subset of cytosolic proteins with a motif recognized by the hsc70 chaperone.[9] The chaperone-substrate complex binds to the CMA receptor, the lysosomal-associated membrane protein-2A (LAMP-2A).

Although it has been established that autophagy is regulated by various factors, recent works have demonstrated that autophagy could also occur as a non-regulated spontaneous process for renewal of the molecules and organelles.[10] Skeletal muscles and neuronal tissues are the primary organs where autophagy is physiologically enhanced.[11] In several neuromuscular disorders, the accumulation of autophagic vacuoles is seen in skeletal myofibers.[12-17] Based on this pathological finding, these diseases are called autophagic vacuolar myopathies (AVM). Danon disease and glycogen storage disease type II (GSDII) belong to this group of disorders.

Similar to neurodegenerative diseases, the pathogenesis of myodegenerative diseases may involve either the failure of autophagosomes to fuse with lysosomes or the aggregation of misfolded proteins that exceed the autophagic clearance capacity of the myocyte. The concept that failure of lysosomes to fuse with autophagosomes might contribute to myopathy is further supported by evidence that pharmacological inhibition of

this fusion step (e.g., with chloroquine or hydroxychloroquine) causes severe vacuolar myopathies in rats and humans.[18] Danon disease, is associated with extensive accumulation of autophagosomes in the muscles of LAMP-2-deficient mice and patients. Pompe disease, which is caused by the deficiency of glycogen-degrading lysosomal enzyme acid α-glucosidase (GAA), is often resistant to GAA enzyme replacement in skeletal muscle but not in heart.[19] Endocytic trafficking of recombinant GAA is abnormal in skeletal muscle; it accumulates primarily in autophagosomes and fails to reach the lysosomal compartment, providing an example of how the ”traffic jam” provoked by defective lysosomal function may be pathogenic. Muscle diseases in which autophagy may promote the clearance of disease-causing proteins include sporadic inclusion body myositis, limb girdle muscular dystrophy type 2B, and Miyoshi myopathy.

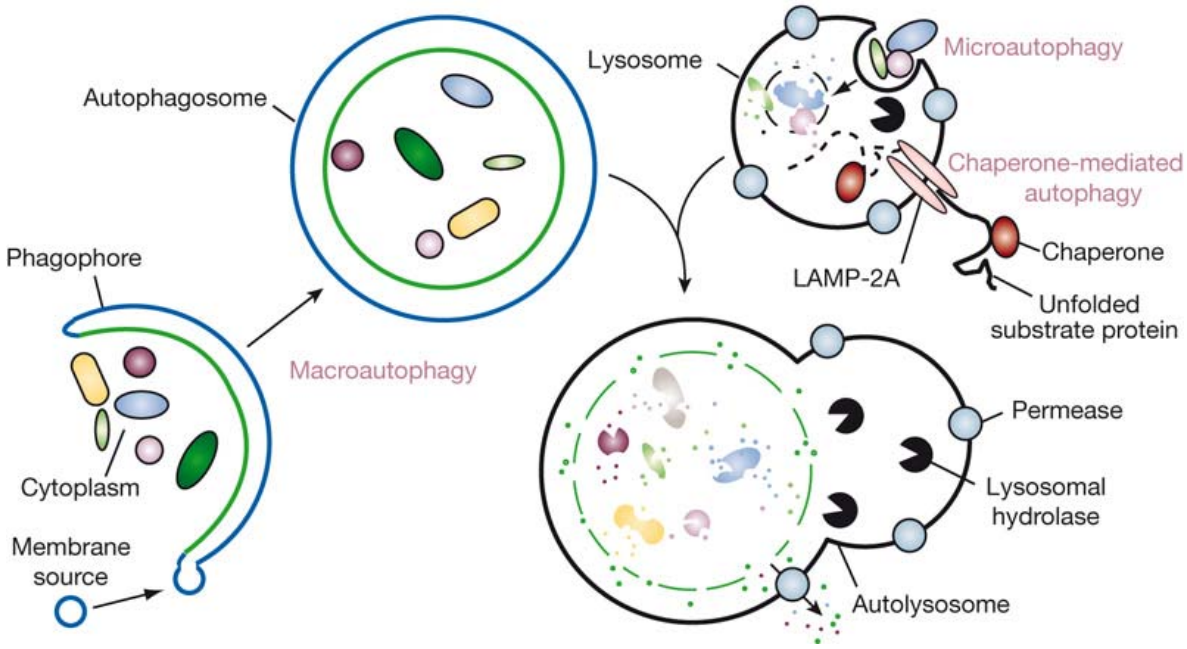

Figure 1. ***Different types of autophagy.*** *Microautophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. The function of this process in higher eukaryotes is not known, whereas microautophagy-like processes in fungi are involved in selective organelle degradation. In the case of macroautophagy, the cargoes are sequestered within a unique double-membrane cytosolic vesicle, an autophagosome. Sequestration can be either nonspecific, involving the engulfment of bulk cytoplasm, or selective, targeting specific cargoes such as organelles or invasive microbes. The autophagosome is formed by expansion of the phagophore, but the origin of the membrane is unknown. Fusion of the autophagosome with an endosome (not shown) or a lysosome provides hydrolases. Lysis of the autophagosome inner membrane and breakdown of the contents occurs in the autolysosome, and the resulting macromolecules are released back into the cytosol through membrane permeases. CMA involves direct translocation of unfolded substrate proteins across the lysosome membrane through the action of a cytosolic and lysosomal chaperone hsc70, and the integral membrane receptor LAMP-2A (lysosome-associated membrane protein type 2A). From Mizushima N. et al., Nature 2008; 451: 1069-1075.*

3.4. Autophagy in the pathogenesis of Pompe disease

GSDII is a systemic disorder, but the major clinical manifestations are seen in cardiac and skeletal muscle. The loss of cell integrity and muscle destruction have been attributed to progressive enlargement and eventual rupturing of glycogen-filled lysosomes.[20,21] Recent studies in patients with Pompe disease and in a knockout (KO) mouse model of the disease suggest that this view of the pathogenesis is simplistic and perhaps inadequate.[19,22-24] The skeletal muscle of KO mice shows a profound failure of productive autophagy. Massive accumulation in the core of muscle fibers of undegraded autophagic material, rather than the buildup of lysosomal glycogen, appears to cause the progressive damage. Furthermore, autophagy-related pathology, observed in type II–rich muscles but not in type I–rich muscles in KO mice, is associated with resistance to enzyme replacement therapy.[19,25]

Autophagy, a major pathway for delivery of proteins and organelles to lysosomes, has been implicated in several other lysosomal storage disorders, suggesting a common mechanism in the pathogenesis of these heterogeneous disorders.

3.5. Autophagy in the pathogenesis of Danon disease

In Danon disease, mere lysosomal dysfunction cannot provide an adequate explanation by which patients develop muscle weakness. Rather, the increase in autophagic vacuoles within the myofibers could be more responsible for the disruption of myofibrillar structures, and ultimately lead to myofiber breakdown and loss of function. It has been demonstrated that LAMP-2 is required for the conversion of early autophagic vacuoles to vacuoles, indicating its involvement in the fusion of autophagic vacuoles with endosomes and lysosomes. LAMP-2-deficient mice exhibit elevated mortality after 20 days of age, and show accumulation of autophagic vacuoles in liver, kidney, pancreas, and cardiac and skeletal muscles. Evidence showing the failure in the normal progression of autophagic process in the absence of LAMP-2 have been presented by using cultured hepatocytes.

Skeletal muscles from the patients with Danon disease show scattered small basophilic granules in myofibers, in addition to mild to moderate variation in fiber size without necrotic or regenerating process.[26] Lysosomal acid phosphatase activity is enriched in these granules, showing accumulation of lysosomal organelles in myofibers. Autophagy-related proteins are also accumulated together with lysosomal proteins. Interestingly, Danon disease, as well as the X-linked myopathy with excessive autophagy (XMEA), has a

peculiar pathological characteristic: sarcolemmal proteins (such as dystrophin and its associated proteins, the extracellular matrix proteins, acetylcholine esterase) are recruited into large vacuolar structures surrounding those lysosomal granules, forming autophagic vacuoles with sarcolemmal features (AVSFs).

Glycogen Storage Disease type II

Glycogen storage disease type II (OMIM # 232300) is an inherited metabolic myopathy. Synonyms for the disease are Pompe disease, acid maltase deficiency and glycogenosis type II. It is an autosomal recessive genetic disorder caused by a deficiency or dysfunction of acid alpha-glucosidase (GAA, EC 3.2.1.20/3), a lysosomal enzyme which catalyses the hydrolysis of α-1,4 and α-1,6 links of glycogen. This enzymatic defect results in lysosomal glycogen accumulation in multiple tissues, with cardiac and skeletal muscle tissues most seriously affected. GSDII has an estimated frequency of one in 40000 in African-American, one in 50000 in Chinese, one in 40000 in Dutch, and one in 146000 in Australian populations.[4,27-31] This disease has been untreatable, but approval in 2006 of enzyme replacement therapy with recombinant human acid α-glucosidase has shown the potential to substantially alter its prognosis.

In the fatal infantile-onset form, the disease presents rapidly with hypotonia, generalized muscle weakness, and hypertrophic cardiomyopathy. Death usually occurs within one year of birth due to cardio-respiratory failure.[4,32]

The late-onset form, which was discovered more than 30 years after the infantile-onset form, is more clinically heterogeneous, with greater variation in age of symptom onset, clinical presentation, and disease progression.[4] Late-onset patients may have residual GAA activity less than 40% of normal when measured in skin fibroblasts.[33] Generally characterized by slowly progressive proximal muscle weakness and respiratory insufficiency, this form can present anytime from childhood until adulthood. It is distinguished from the infantile-onset form by the absence of severe cardiac involvement. While life expectancy can vary, death generally occurs due to respiratory failure.[4]

3.6. History

Dutch pathologist J.C. Pompe first described a 7-month-old infant who died suddenly from the disease in 1932.[34] After observing idiopathic hypertrophy of the heart and the

accumulation of glycogen in all types of tissues, he labeled the disorder "cardiomegalia glycogenica diffusa." Two other reports of infants with similar manifestations soon followed, calling the disorder Pompe disease. Nobel laureate G.T. Cori, who discovered the course of catalytic metabolism of glycogen, classified the disorder as glycogen storage disease type II (GSD-II) in 1954 to reflect the impaired glycogen metabolism of affected patients.[35]

Based on Cori's research and the discovery of a new organelle, the lysosome, Hers and colleagues in 1963 deduced the metabolic basis of Pompe disease by linking the deposition of glycogen to an inherited absence or shortage of lysosomal enzymes.[36] As a result, Pompe disease was the first to be classified as a lysosomal storage disease (LSD). This breakthrough led to the ability to diagnose the disease and enabled the search for the chromosomal location of the genetic mutation. In 1970, Engel published one of the early reports of a late-onset form of the disease, describing four adults with syndromes mimicking that of muscular dystrophy or other myopathies.[37] Nine years later, the gene responsible for the disorder was localized to chromosome 17 and designated GAA on the human gene map.[4]

3.7.Clinical features

GSDII presents as a spectrum of features in which symptoms can manifest at any age (figure 13).7 At the severe end of the spectrum is a subgroup of patients with a clearly defined course. This classic infantile form firstly described by Pompe usually presents in patients within the first months of life. The median age of onset ranges from 1.6 to 2.0 months.[32,38,39] Presenting symptoms are feeding difficulties, failure to thrive, respiratory infections, hypotonia, and very few movements.[40] The heart is characteristically affected. For example, cardiac ultra sound shows a hypertrophic cardiomyopathy with thickening of the ventricular walls and septum, which could lead to outfl ow tract obstruction and cardiac failure.[41] The electro cardiogram (ECG) shows high voltages, repolar isation disturbances, and frequently a short PR interval.[42,43] Motor development is delayed and major motor milestones such as rolling over, sitting, or standing are usually not achieved.

Hearing deficit might be present and has been attributed to pathological changes in the middle ear, inner ear, and auditory nervous system.[44-46]

The mean age of death in studies of large groups of patients was 6.0–8.7 months.[32,38,39] Patients with classic infantile Pompe’s disease rarely survive beyond 1 year of age.

In patients with less progressive forms of Pompe's disease (late-onset forms) onset of symptoms can range from infancy to late adulthood, and a clear distinction of subtypes cannot be made.[4,27,45,47-49]

A questionnaire study in an international cohort of 255 children and adults with Pompe's disease showed age range of first complaints from birth to 62 years.[51] Disease severity, as measured by wheelchair and ventilator use, was more related to disease duration than to age.

Symptoms of children and adults with a non-classic presentation are predominantly related to skeletal muscle dysfunction, resulting in both mobility and respiratory problems. The heart is sporadically affected.[4,50,52-54] Mobility and respiratory problems might progress at a different pace. Patients who are still ambulant might need ventilation at night and those with normal pulmonary function might become dependent on a wheelchair.

Pompe's disease in adults is regarded as a slowly progressive disorder. Many adults show symptoms during childhood.[48,49,51]

3.8. Enzymatic and molecular diagnosis

All patients have a deficiency of the lysosomal enzyme acid α-glucosidase, and can be diagnosed on the basis of this feature.[36] However, the sensitivity and specificity of the enzymatic procedure depends on the choice of tissue specimen, type of substrate, and assay conditions. An artificial substrate (4-methylumbelliferyl-α-D-glucopyranoside) provides an assay that is sufficiently specific and sensitive to detect as little as 1–2% of residual enzyme activity. This test can distinguish infants with the classic infantile form of the disease from children and adults with residual activity (figure 2).[49,55-58] Infants with classic Pompe's disease have less than 1% residual activity; children and adults have residual activity, but usually no more than 30% of average normal activity. Only the assays in fibroblasts and skeletal muscle have proved sufficiently sensitive to correlate the clinical phenotype with the degree of enzyme deficiency (figure 2).[56-58]

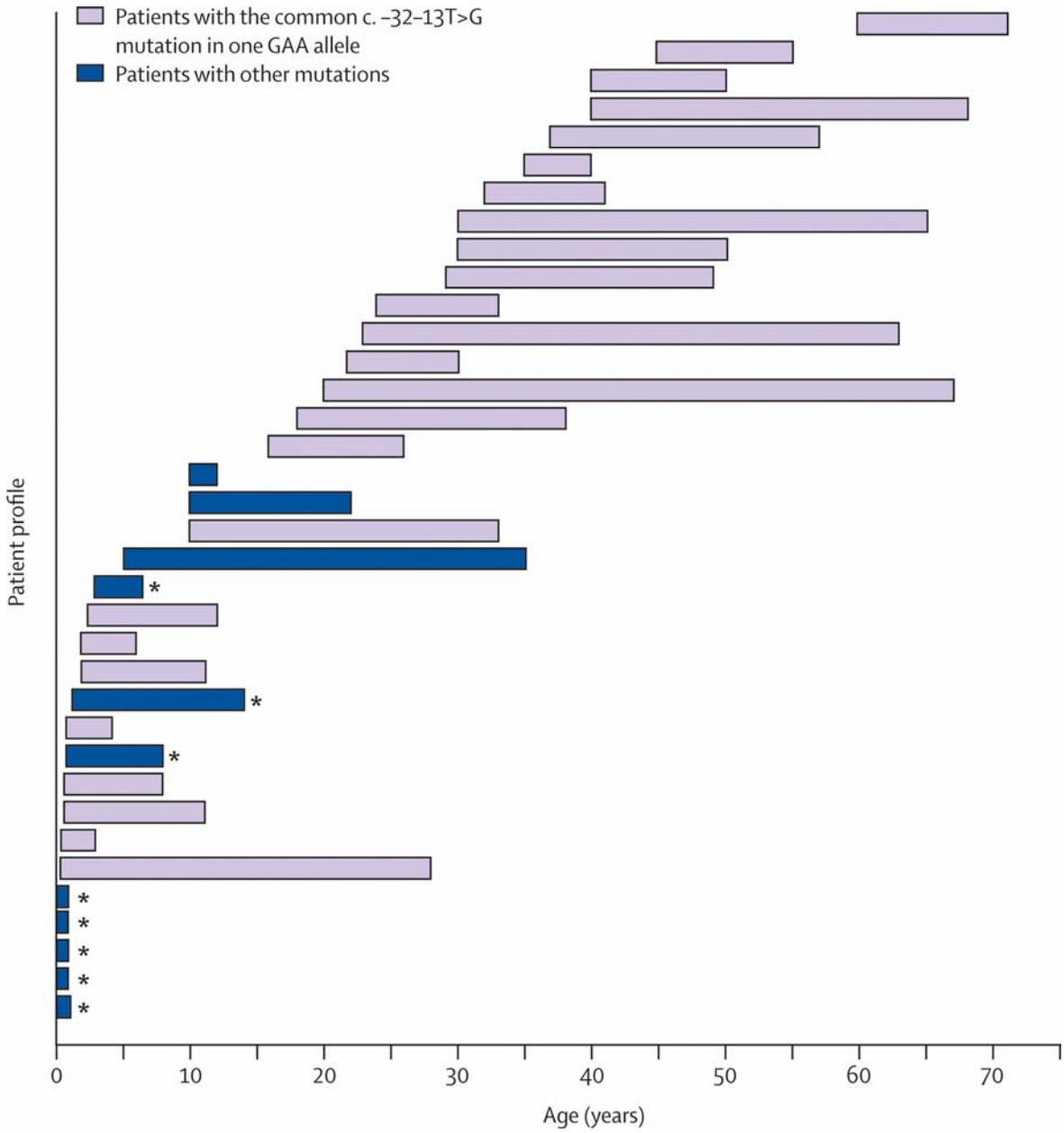

Figure 2. ***Age at onset of symptoms and the current age of a cohort of 36 patients with Pompe's disease***. *Each horizontal bar represents the disease duration of an individual patient. *Patients have died. From: Van der Ploeg A.T. and Reuser A.J.J.; Lancet. 2008; 372: 1342-53*.

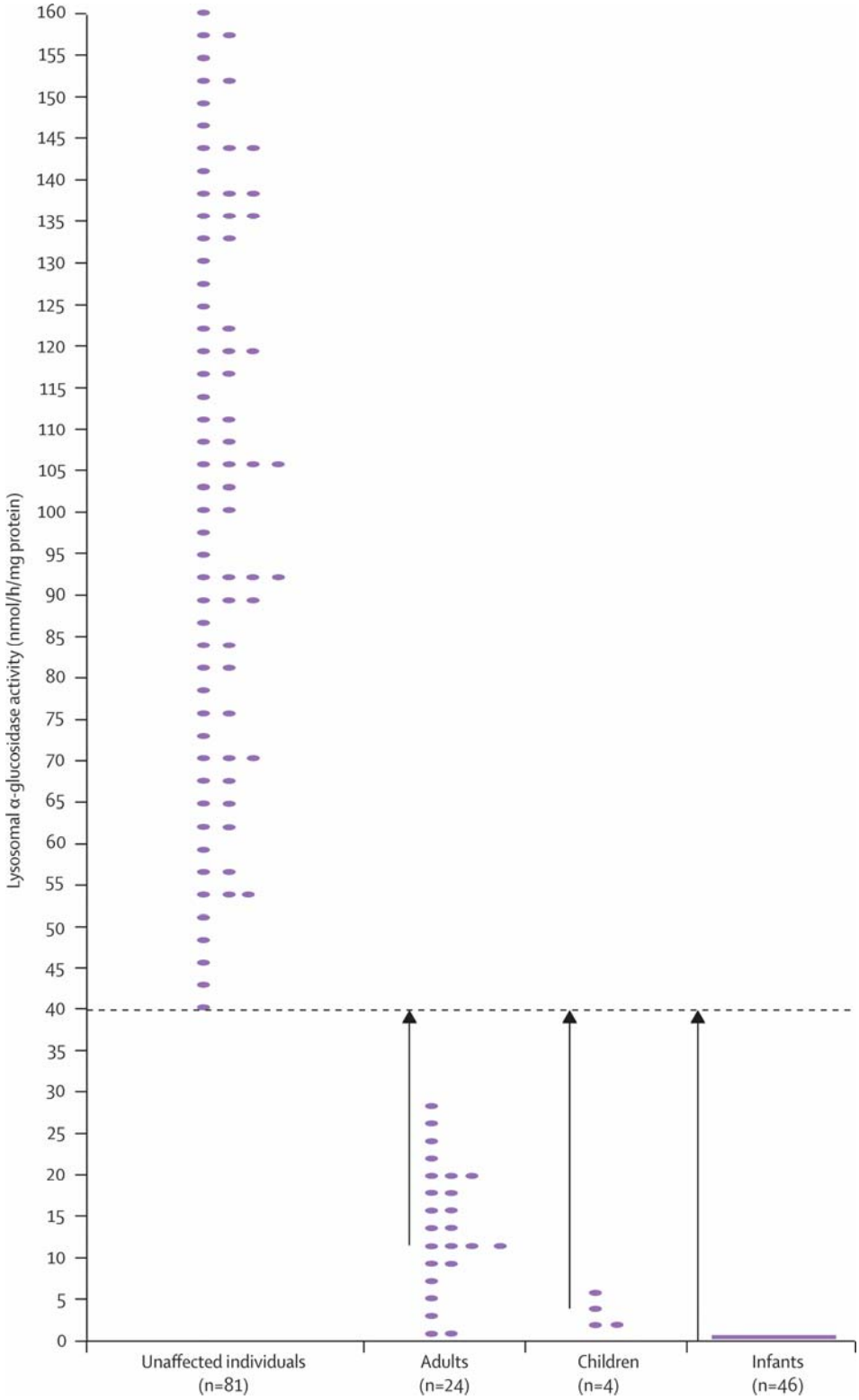

Figure 3. ***Ranges of acid a-glucosidase activity in fibroblasts of healthy individuals and patients of different ages.*** *Lengths of arrows show how much activity needs to be increased to reach lower limit of normal.* From: Van der Ploeg A.T. and Reuser A.J.J., Lancet. 2008; 372: 1342-53.

3.9. The GAA gene and mutations

The gene encoding acid α-glucosidase (GAA, OMIM # 606800) is mapped to human chromosome 17q25.2-25.3 and consists of 20 exons spread over 28 kb of genomic sequence. The first exon is not translated and is separated by a large intron from exon 2, where the ATG start codon is located. The locus is very heterogeneous. The latest update of the Pompe disease mutation database (http://www.pompecenter.nl) brings the number of published variations to 289, the number of non-pathogenic mutations to 67 and the number of proven pathogenic mutations to 197.[59] Most of the non-pathogenic sequence variations are frequent, some are rare, and others seem restricted to isolated ethnic groups. Based on a mixture of criteria including the phenotype of the patient, or the effect of the mutation on enzyme synthesis and function, the pathogenic sequence variations were classified as severe (the majority), intermediate or mild.[59]

Mutations that introduce mRNA instability, such as nonsense mutations, are more commonly seen in the infantile-onset form of Pompe disease as they result in nearly complete absence of GAA enzyme activity. Missense and splicing mutations may result in either complete or partial absence of GAA enzyme activity and therefore may be seen in both infantile- onset and late-onset Pompe disease.[60]

The most common mutation in late-onset forms is the –32-13T>G mutation (traditionally called IVS1-13T>G) is located in intron 1 and causes the splicing out of exon 2. About 70% of Caucasian patients with late-onset forms of the disease share this mutation in the compound heterozygote state with a different mutant allele. The mutation leads to a leaky splice site resulting in greatly diminished, but not absent, GAA enzyme activity and is not associated with the infantile-onset form. A deletion in exon 18 is frequently observed in infantile patients. While these mutations are common in western countries, other single mutations recur in specific populations, possibly because of a founder effect.[60] Considerable molecular heterogeneity is not surprising, considering the complex enzyme processing and the different phenotype expression of the disease. Identification of gene mutations in GSDII patients is fundamental in order to provide genetic counseling to patients' families and to offer heterozygote and prenatal diagnoses. Furthermore, the molecular characterization of such patients is crucial when selecting patients for therapeutic trials.

3.10. Genotype-phenotype correlations

Many studies have investigated genotype-phenotype correlations in GSDII.[48,61-66] The type of mutation is usually a good predictor of clinical phenotype, but in a number of cases this rule seems to fail. In-vitro expression studies of *GAA* gene mutations, focused on the transcriptional effects of mutations on both protein and phenotype,[63,67] were shown to be useful in discriminating disease-causing mutations and for evaluating their effect on enzyme function, whereas they seem less powerful in drawing genotype-phenotype correlations.

GAA enzyme activity may correlate with age of onset and rate of progression as a "rough" general rule:

- It is assumed that a combination of two mutated alleles that encode essentially no enzyme activity results in infantile-onset Pompe disease.
- Various combinations of other alleles resulting in some residual enzyme activity likely cause disease but the age of onset and progression are most likely directly proportional to the residual GAA enzyme activity.

Although a number of mutations seen in homozygosity may suggest a genotype-phenotype correlation, the existence of a number of case reports of both infantile and late-onset Pompe disease in the same family suggests strong caution in extrapolation of these observances.[68]

The clinical heterogeneity primarily relates to the occurrence of different mutations in the acid α-glucosidase gene (GAA; MIM# 606800) that lead to different degrees of acid α-glucosidase (EC; 3.2.1.20/3) deficiency, and different rates of lysosomal glycogen accumulation. Secondary genetic and non-genetic factors are thought to modulate the disease phenotype, but have not been identified yet.[62,63,68-70] These factors will modify the pathogenic effect of GAA genotypes that are associated with some amount of residual activity, but have predictably little effect in case of complete acid α-glucosidase deficiency. Figure 3 depicts the correlation between residual acid α-glucosidase activity and phenotype, and the effect of modulating factors. Model depicting signs of GSDII emerge when the α-glucosidase activity drops below 30% of average normal activity. Enzyme and gene therapy aim to increase the acid α-glucosidase activity so as to exceed the critical threshold. Nutrition and exercise might reduce the critical threshold.[69,70]

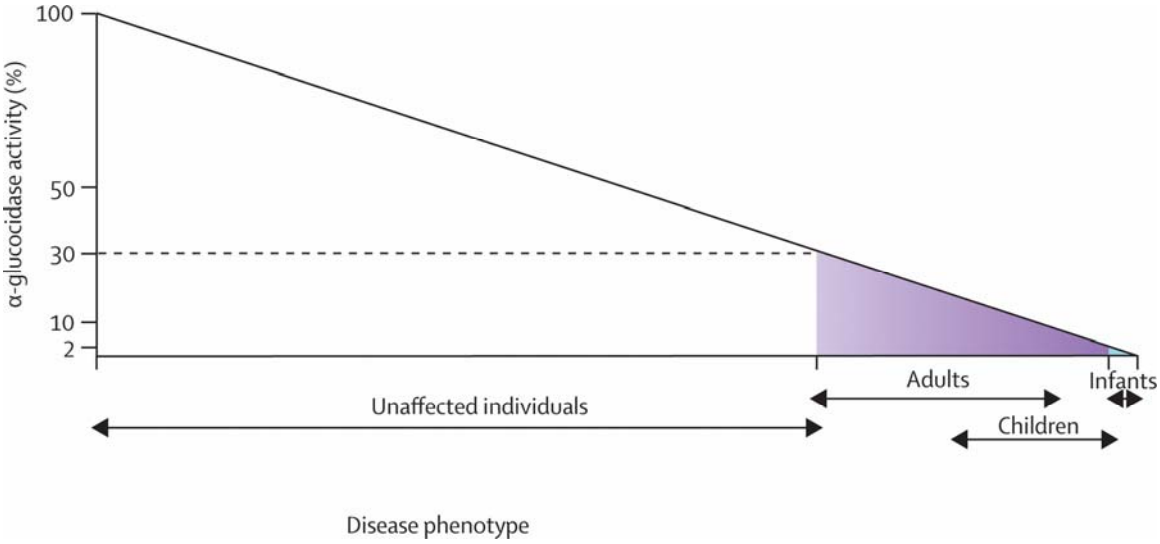

Figure 4. Model depicting that signs of Pompe's disease emerge when the α-glucosidase activity drops *below 30% of average normal activity*. *White to purple zone: disease phenotype aggravates with increasing degree of enzyme deficiency, which is mainly dictated by the nature and combination of the mutations in the two GAA alleles. Within this zone, as yet to be identified, secondary factors modulate the clinical course such that patients with the same degree of enzyme deficiency might manifest symptoms at different ages and with different degrees of severity.* From: Van der Ploeg A.T. and Reuser A.J.J., Lancet. 2008; 372: 1342-53.

3.11. Lysosomal Acid α-Glucosidase (GAA)

The acid α-glucosidase gene (GAA) encodes a precursor protein of 952 amino acids (110 kDa) catalytically inactive, which has a signal peptide at the N-terminus for guiding the nascent protein to the endoplasmic reticulum (ER), where 7 N-linked glycosylations and 2 phosphorylations occur.[71,72] The glycosylated 110 kDa precursor acquires mannose-6-phosphate (M6P) residues in a post ER compartment, binds to the M6P receptor on the Golgi and is principally targeted to the lysosomes; a small amount of precursor protein (about 10%) is secreted into the extracellular environment. Further processing of the precursor protein, which occurs during transport to the Golgi and within the lysosomes, leads to a stepwise proteolysis at both the C- and the N-terminus of the protein, resulting in the generation of a 95 kDa intermediate (formed in the late endosome/lysosome), which is proteolysed first between amino acids 816 and 881, resulting in a 76 kDa form, and then at amino acid 204, to give the 70 kDa mature form. This transport of the protein to lysosomes is a prerequisite for the generation of functional enzyme.

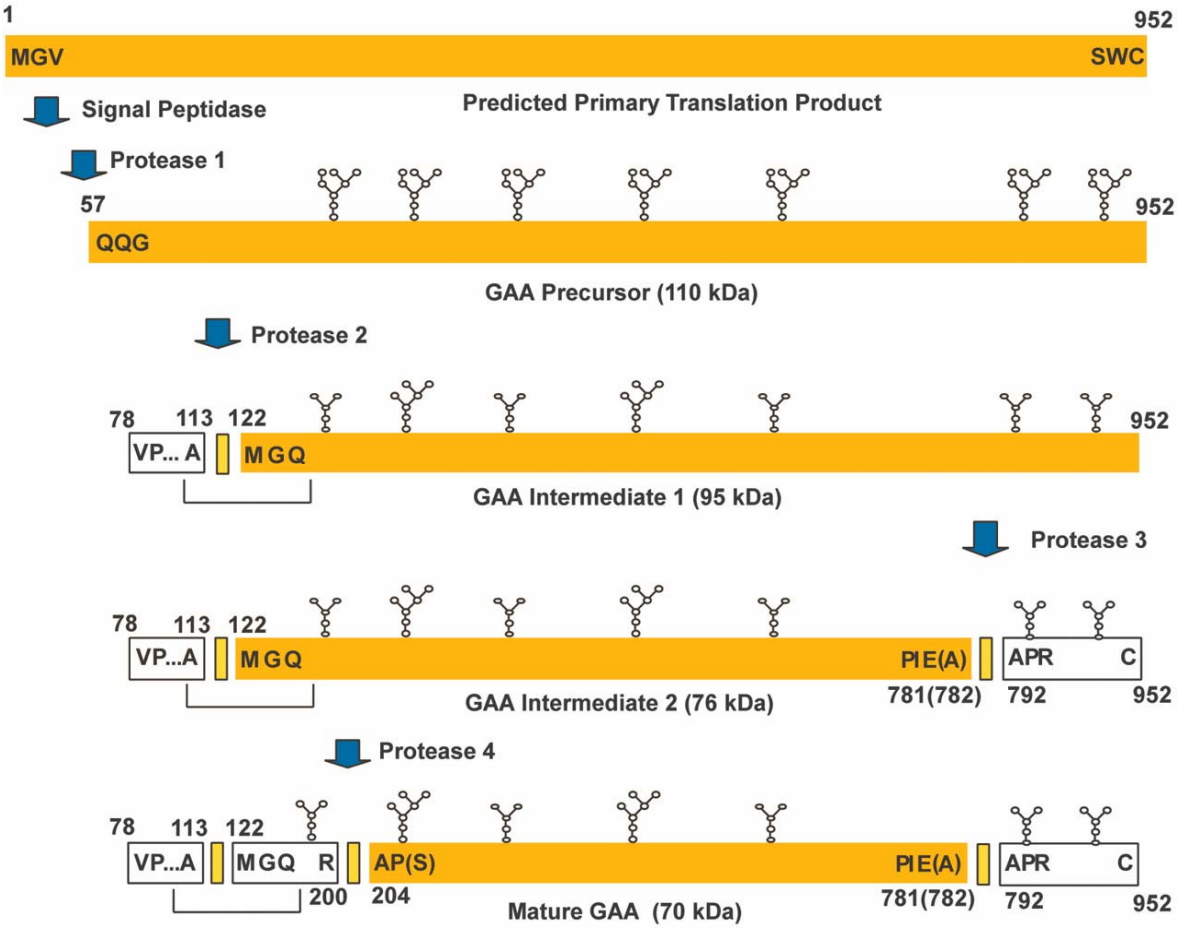

Figure 5. ***Model for the maturation of GAA.*** *From: Moreland RJ et al, J Biol Chem 2005; 280:6780–6791.*

3.12. Pathophysiology

Accumulation of lysosomal glycogen starts when the acid α-glucosidase activity decreases below critical.[4] This threshold amount seems to vary depending on the organ. In knockout mouse models of Pompe's disease with complete enzyme deficiency, storage was seen in almost every tissue and cell type - ie, liver, heart, and skeletal muscle, smooth-muscle cells of the gastrointestinal tract, bladder, blood-vessel walls, kidney, spleen, endothelial cells, and Schwann cells and in the perineurium of peripheral nerves.[72,73] A similar widespread distribution of pathological changes in the tissues were previously noted in infants after post-mortem examination.[4,75] Pathological changes in skeletal muscle, on the other hand, are prominent throughout the entire clinical spectrum.[50,52,53] The different response of heart and skeletal muscle to the same enzyme deficiency can be attributed to

the abundant glycogen in skeletal muscle fibres rather than in cardiomyocytes. Although most glycogen is turned over in the cytoplasm, in theory more cytoplasmic glycogen leads to more lysosomal storage through autophagy.[23,76] The process by which skeletal muscle function is eventually lost is also intriguing. Additional insight is essential to improve understanding of the regenerative capacity of muscle in Pompe's disease and increase the options for treatment. Many investigators have described the pathophysiology of skeletal muscle. They noted an order of events by comparing pathological changes in muscle in various stages of disease in knockout mice and patients.[19,21,22,73-75,77-82] Initially, small vacuoles (being glycogen loaded lysosomes) staining positive with periodic acid Schiff reagent, are seen in the fibres (figure 4). These vacuoles can occur isolated or in linear arrays and are neither necessarily present in all fibres nor present along the entire length of the fibres. When the disease advances, the lysosomes expand, become many, and fuse to form larger structures that interfere with the architecture of the fibre. Additionally, regions develop with lipofuscin deposits, cellular debris surrounded by membranes (identified as autophagosomes) and freely dispersed cytoplasmic glycogen.[19,22,23,75,77,80] With respect to loss of muscle function, both atrophy and reduced performance per unit of muscle mass have a role. In works addressing pathological changes in muscles of a knockout mouse model of Pompe's disease, both mobility of storage vesicles and communication between autophagosomes, endosomes, and lysosomes decreased with disease progression.[19,22,23] Moreover, the endosomal and lysosomal acidification process stopped functioning normally when the autophagosomal and endosomal pathways became clogged with indigestible materials. The autophagic build-up was mainly seen in the fast-twitch type-2 fibres and much less in the slow-twitch type-1 fibres.[22] These findings have led to a new view of the cascade of pathological events in type-2 muscle fibres. The authors suggested that the failure to digest glycogen results in local starvation, which induces autophagy while the autophagic pathway is blocked by the lysosomal dysfunction.

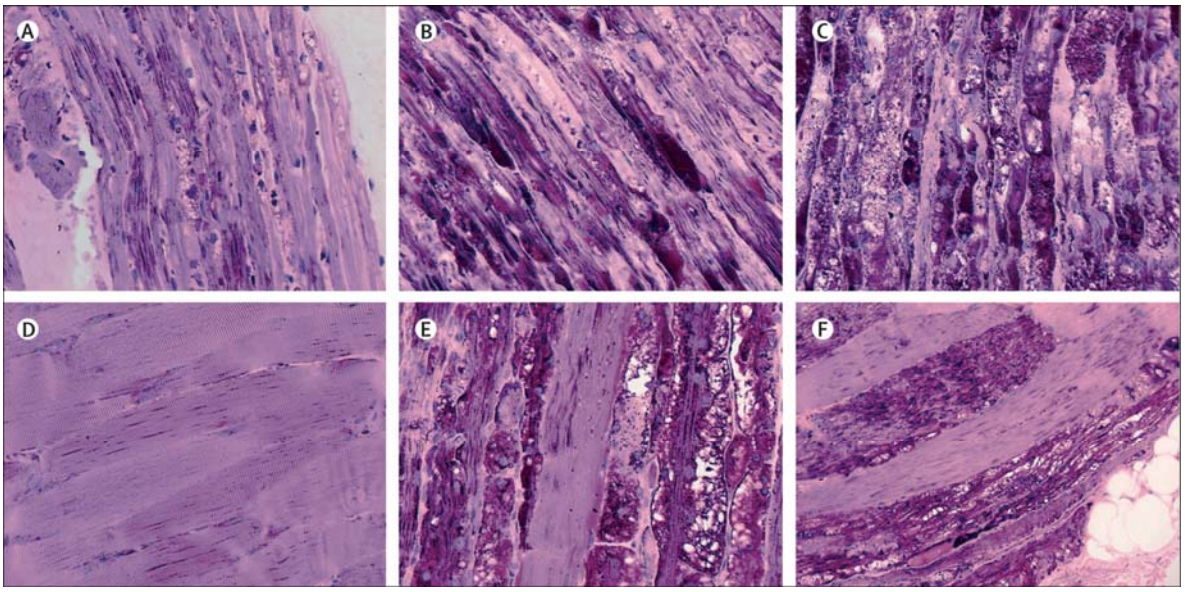

Figure 6. ***Histological changes in progressive muscle damage in infantile (A-C) and adolescent and adult (D-F) disease(A) 2·5-month-old infant.*** *Most muscle fibres containing longitudinal arrays of small PAS-positive inclusions (glycogen), whereas muscle cell morphology is well preserved. Some fibres have areas with larger unstained spaces and loss of cross striation. (B) A more severely affected patient of about the same age. PAS-positive lysosomes are more numerous and larger than in (A). Most muscle fibres now contain lakes of glycogen. Morphology of only very few fibres is well preserved. (C) 8-month-old infant. Massive destruction of muscle tissue by the deposition of glycogen. In the most affected fibres, contractile filaments have been replaced by empty spaces. (D) Changes are mild with normal morphology in muscle fibres, and few and small PAS-positive lysosomes. (E and F) More severely affected adults show variable pathological changes. Some fibres have maintained their structure; others are severely damaged (E) and might be replaced in the long term by connective and adipose tissue, as in (F). From: Van der Ploeg A.T. and Reuser A.J.J.; Lancet. 2008; 372: 1342-53.*

3.13. Animal models for Pompe disease

The mouse models of Pompe disease have been developed by targeted disruption of the murine GAA gene by Bijvoet et al.[73,74] In homozygous knock-out mice, glycogen-containing lysosomes are detected soon after birth in liver, heart, and skeletal muscle cells. By 13 weeks of age, large focal deposits of glycogen and lysosomal vacuolar structures were observed. Electron micrography showed lysosomal glycogen storage. Furthermore, the heart is typically enlarged and the electrocardiogram is abnormal. Recently, Raben's group has demonstrated the importance of autophagy in skeletal muscles, thus shedding some light in the pathogenesis of Pompe disease mouse model.[22] The cellular pathology in this disease affects the pathways involved in endocytic and autophagic processes. They have reported the dramatic expansion of endocytic vesicles, decrease in mobility of late endocytic vesicles, and increase in luminal pH in a subset of late endosomes/lysosomes in

GAA knock-out myoblasts. Using isolated single fibers from these mice, they demonstrated that type 2 fibers contain large regions of autophagic buildup spanning the entire length of the fibers. In addition, they found out that type 2 fibers were resistant to ERT, and this phenomenon is probably influenced by the low amount of proteins involved in endocytosis and trafficking of lysosomal enzymes combined with increased autophagy in these fibers.

3.14. Prenatal screening

Prenatal diagnosis is available for GSDII in cases where it may be warranted, such as subsequent pregnancies in families with an affected child or when a parent presents with the late-onset form. In fact, Pompe disease was one of the first genetic disorders for which researchers attempted diagnosis prior to birth using amniocentesis, with the first published reports appearing in the late 1960s.[4] Today, prenatal diagnosis can be made with either amniocentesis or, more commonly, direct enzyme analysis of uncultured chorionic villi cells, primarily using 4-methylumbelliferyl-a-D-glucoside (4MUG) as substrate.

The direct enzyme analysis of uncultured chorionic villi cells offers additional benefits as it allows for early diagnosis (12th week of pregnancy) and potentially as quick as a one day turnaround for results. In some cases, DNA analysis may also be used as a supportive method to confirm a prenatal diagnosis of Pompe disease when the particular defect involved is known. In addition, it can enable definitive carrier detection in the patient's family.

A recent study has explored the use of plasma and dried blood spots to test for Pompe disease in newborns. It remains to be seen how reliable or accepted this diagnostic technique will be in common practice, however.

3.15. Clinical management

In the absence of an approved treatment for Pompe disease, supportive therapy is used to manage symptoms and minimize complications whenever possible. While these multidisciplinary approaches cannot generally alter the disease course, they may impact quality of life. Physicians play an important role in coordinating the care for Pompe patients and should be consulted whenever adjunctive care is implemented.

As a result of the severe weakening of the diaphragm and other respiratory muscles, respiratory therapy may become a critical component of disease management. Many

individuals with Pompe disease eventually require mechanical ventilation to reduce or eliminate the work of breathing. Other techniques involve the use of an incentive spirometer and intermittent positive pressure breathing (IPPB) to expand the lungs. Patients requiring 24-hour ventilatory support for prolonged periods may be considered for a tracheostomy.[83]

Dietary therapy is sometimes attempted in Pompe disease as case studies have shown that some patients will demonstrate clinical improvement in conjunction with a high-protein, low-carbohydrate diet or, alternatively, a diet rich in amino acids. In addition, patients who are extremely weak--especially infants--may require tube feeding in order to maintain proper nutrition and prevent aspiration.[84,85]

Patients who begin to lose mobility due to weakened muscles may also benefit from physical therapy. A customized exercise and/or physical therapy program may help to preserve range of motion and strength, while the use of assistive devices such as orthotics, canes, or walkers may help with ambulation. In advanced cases, a wheelchair may be indicated. To devise a full spectrum of supportive therapy, consultation with a respiratory therapist, physical therapy.

3.16. Treatment with Enzyme Replacement Therapy (ERT)

Since there is currently no treatment to cure or slow the progression of Pompe disease, most patients receive symptomatic treatment. Current investigations are primarily focused on two approaches: enzyme replacement therapy (ERT) and gene therapy. Bone marrow transplantation has also been explored.

Enzyme replacement therapy (ERT), using recombinant human GAA, is now available in clinical practice in the US, Canada, Europe, Middle East, Latin America, and Asia Pacific.[86] As currently Pompe disease is the only hereditary muscle disease for which ERT is available, clinicians and pathologists should always consider the possibility of Pompe disease, especially in patients with late-onset form, even though it may be difficult to make a diagnosis solely based upon clinicopathological features. ERT seems to be highly effective especially in infantile cases. Preliminary studies showed that ERT can also benefit some patients with late-onset form to some extent; however, the effect in adult cases remains to be established. The efficacy of ERT seems to be better when it is given early in the course of symptom development and before irreversible muscular damage has occurred. This notion is especially relevant for patients with severe pathological changes,

where increased cytoplasmic glycogen released from lysosomes is probably inaccessible to the membrane receptor-dependent targeting mechanism.[87]

Danon Disease

Danon disease (OMIM #300257) is a rare X-linked dominant disorder predominantly affecting striated muscle. The disorder was originally reported as ‘‘lysosomal glycogen storage disease with normal acid maltase” because pathologic features apparently resemble those of acid maltase deficiency.[88] However, Danon disease is not a glycogen storage disease as the disease is caused by the primary deficiency of a lysosomal membrane protein, lysosome-associated membrane protein-2 (LAMP-2), instead of a glycolytic enzyme.[89] The typical clinical picture is characterized by a triad of hypertrophic cardiomyopathy, myopathy, and mental retardation.[88] Myopathy is usually mild and is evident in most male patients (90%), whereas it is seen only in one third of female patients.

3.17. History

In 1981 Danon et al. reported two unrelated 16-year-old boys with mental retardation,[88] proximal muscle weakness, fatal hypertrophic cardiomyopathy and cardiomegaly, and, in one case, hepatomegaly. Histochemical and electron microscopy of muscle biopsies showed lysosomal glycogen storage disease, with normal glycogen content and acid maltase activity. In 2000 Nishino et al. reported 10 unrelated patients who had primary deficiency of the lysosomal-membrane-protein-2 (LAMP-2), resulting in a vacuolar myopathy with hypertrophic cardiomyopathy.[89]

The identification of the protein and the gene responsible for Danon disease has allowed confirming or excluding this diagnosis in a number of patients previously described. On the contrary, a severe and rapidly fatal infantile form of lysosomal glycogenosis with normal acid maltase, which in the pre-molecular era seemed to correspond to a phenotype of Danon disease,[90-92] was later excluded to be due to LAMP-2 defect. Indeed, besides Danon disease, autophagic vacuolar myopathies include several additional diseases:[93] the infantile autophagic vacuolar myopathy (AVM),[94] the X-linked myopathy with excessive autophagy (XMEA),[95] the autophagic vacuolar myopathy with late-onset and multiorgan involvement,[12] the X-linked congenital autophagic vacuolar myopathy,[16] and the

autophagic vacuolar myopathy with late-onset and cardiomyopathy.[96] XMEA (chromosome region Xq28, OMIM # 310440) is clinically characterized by mild and slowly progressive proximal myopathy without cardiomyopathy.

Except for Danon disease, in the other forms the causative genes are not known, but they are expected to encode proteins related to lysosomal function because the pathological features are quite similar to those in Danon disease. Indeed, they all share a unique pathologic feature: vacuolar membranes with sarcolemmal features. Unlike Danon disease, XMEA and AVM display the presence of complement membrane attack complex on the surface of injured muscle fibers,[97] suggesting a similar pathogenetic mechanism.

3.18. Clinical features of the disease

In male patients the triad of hypertrophic cardiomyopathy, skeletal myopathy, and mental retardation typically characterizes the disease. The onset of the disease is always manifesting at cardiac level; it occurs in males at age variable from early childhood to the second decade (in average in adolescence) and in adulthood in females.[98] The disease has a rapid progression toward end-stage heart failure; the course of the disease is invariable lethal in male patients, and the death occurred for congestive heart failure or sudden death before the age of 30 years in most cases. In female patients the course is more benign and death occurs for cardiac failure in the fourth or fifth decade.

1. Cardiac involvement. It is present in all patients of both sexes, and shows typical hypertrophic and concentric features. Since the early stages of the disease, all patients present abnormal electrocardiography, with ventricular pre-excitation pattern, showing short PR intervals, short delta waves, high precordial voltage.[99] Heart blocks and Wolff-Parkinson-White syndrome were present in 35% of cases. Chest X-ray frequently shows cardiomegaly.[100] Some patients present at onset the cardiac symptoms typically seen in hypertrophic cardiomyopathy, including chest pain, palpitations, easy fatigability, syncope and cardiac arrest.[99,100] Echocardiography shows concentric left ventricular hypertrophy with thickness of the septal and posterior walls. Patients usually develop severe and progressive heart failure associated with end-stage systolic left ventricular dysfunction. Atrial tachicardia or fibrillation and life-threatening ventricular arrhythmias also characterize the disease; these latter are frequent cause of syncope and sudden death, and indicate the implantation of a pacemaker or a cardioverter-defibrillator. Heart transplantation has been successfully conducted in some patients of both genders.[100-104]

2. Skeletal muscle involvement. The skeletal myopathy is present in about 90% of male patients but it is more rarely observed in females.[98] Muscle weakness and atrophy are often mild, affecting predominantly the shoulder girdle muscles, the trunk and the neck; distal muscle may also be involved. All except one patient remained ambulatory[103] and about 10% male patients had fatigability without fixed muscle weakness. One 20-year-old asymptomatic male patient with high CK levels has been reported to suffer from Danon disease.[105]

Serum creatine kinase (CK) level is always elevated in male patients (from 4 to 35 fold the normal values) and mildly elevated or normal in females. EMG is myopathic in all patients, with signs of myotonia in 30% of cases.

3. Mental retardation. Mental retardation of mild degree was found to be present in 70% of male patients, and only in 6% of female patients.[98] Central neurological symptoms, such as seizures and EEG abnormalities have been reported, are uncommon.[88,98] Brain MRI may show mild cortical cerebral atrophy.[106,102,104] These abnormalities have been related to the microvasculature involvement, but an abnormal cerebral glucose metabolism may underlie mental retardation in Danon disease.[107]

4. Liver involvement. Hepatomegaly was present in 35% of cases, and many patients had hepatopathy with high liver enzymes levels since childhood.[100]

5. Ocular involvement. Ophtalmic findings have not been considered part of the classic phenotype of the disease and have received only limited attention

Ocular involvement with chorio-capillary atrophy,[102,108] pigmentary degeneration, lens changes, maculopathy, and myopia,[100,107,109-111] were reported in several patients also of female gender.[110]

6. Involvement of other organs or systems. Foot deformities were reported in 38% of male patients.[98] Axonal polyneuropathy mimicking Charcot-Marie-Tooth disease was sometimes suggestive of a neuropathic disease rather than a primary muscle disorder.[109] Abnormal platelet function has occasionally been reported.[112]

3.19. Treatment of the disease

There is no specific treatment for Danon disease. The therapeutic aim for patients is to prevent progressive heart failure and to reduce the risk of arrhythmias and sudden death. Management with inotrope negative of chronotrope negative agents, used for hypertrophic cardiomyopathy, should be used with caution in Danon disease because of he frequent

evolution towards systolic left ventricular dysfunction and atrioventricular block.[108] As the disease progresses, the only remaining therapeutic option is cardiac transplantation. Heart transplantation appears the most effective treatment for this otherwise lethal cardiomyopathy and can considerably prolong the life expectancy of the transplanted patients.[102,104] Since mental reatardation and skeletal myopathy are not progressive, the prognosis after transplantation is good. The genetic diagnosis of Danon disease is crucial for the proper treatment of these patients.

3.20. Skeletal muscle pathology

Skeletal muscle shows variable degree of non-specific myopathic changes such as increased fiber size variability, increased central nuclei and variably increased PAS positive staining.

The pathological hallmark of the disease is the presence of cytoplasmic vacuoles containing autophagic material and glycogen (Figure 7). Myofibrillar ATP-ase reaction indicated that both type 1 and type 2 fibers are affected by vacuolization.[103] Intracytoplasmic vacuoles contain lysosomal enzymes, as documented by positive histochemical stain for acid phosphatase and esterase.

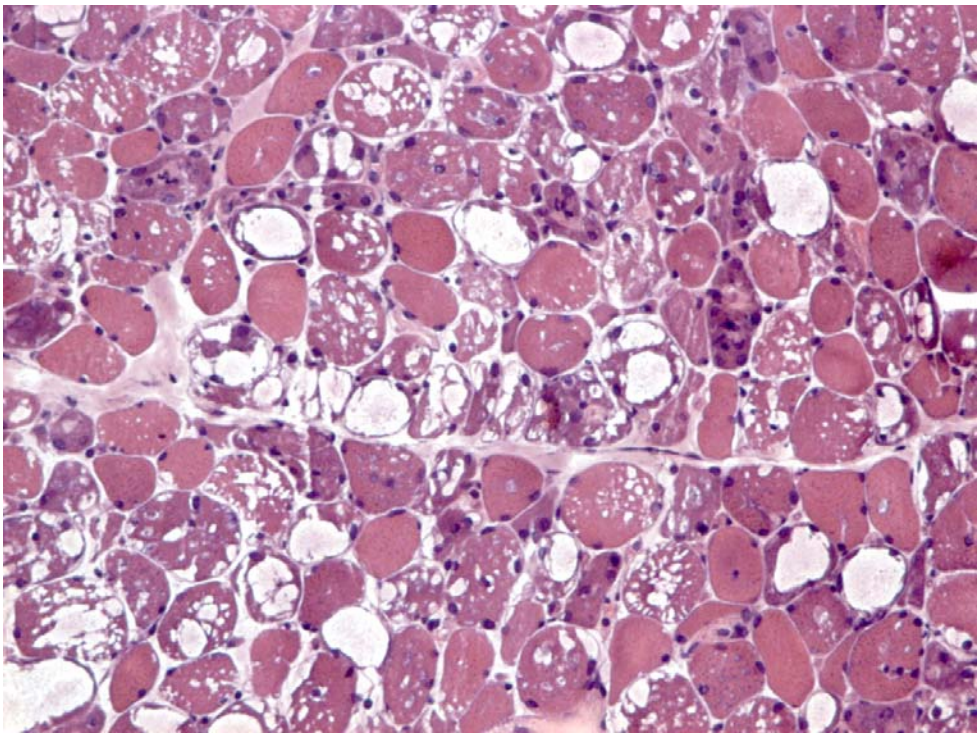

Figure 7. ***Routine hematoxylin-eosin stain of skeletal muscle biopsy section from a patient with Danon disease***. Note diffuse fibre atrophy, and extensive muscle fibre vacuolisation and degeneration. The majority of fibers contain vacuoles that are sometimes large enough to replace all the cytoplasm; few fibers appear relatively spared by vacuolisation. Microscope magnification x 200. From: Fanin et al., *Genetic Inheritance Patterns, Nova Science Publishers, New York 2008.*

On electron microscopy the vacuoles are filled with free or membrane-bound glycogen particles and cytoplasmic debris.[89,102-104,113,114]

The vacuolar membrane occasionally merges with indentations of the sarcoplasmic membrane and stains with antibodies to sarcolemmal proteins[100,115,116] such as dystrophin, laminin, caveolin-3 (Figure 8).

Furthermore, the limiting membrane of endosomal/lysosomal vacuoles are lined by basal lamina, which is a peculiar characteristic of sarcolemmal, indicating that the vacuoles in this disorder share features of both lysosomes and plasma membrane. These vacuolar features are in common with the other autophagic myopathies.

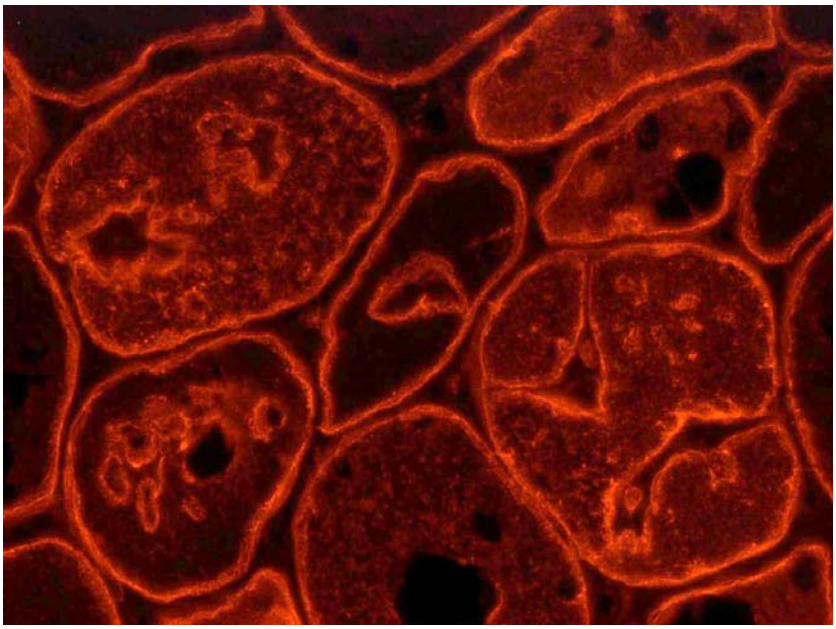

Figure 8. ***Cryostat section of skeletal muscle from a patient with Danon disease, immunolabelled with antibodies against caveolin-3.*** *Caveolin-3 immunolabelling was positive in the plasma membrane of muscle fibers and also in the membrane of the lysosomes that delineate most vacuoles. The cellular membranes that make up both the plasmalemma and the lysosomes are often in continuity. Microscope magnification x 400. From: Fanin et al., Genetic Inheritance Patterns, Nova Science Publishers, New York 2008.*

Conversely, a distinctive histopathological feature of Danon disease is the lack of the complement C5b-9 membrane attack complex on the surface of injured muscle fibers (which is observed for instance in XMEA), and the absence of immuno reactivity to lysosomal membrane protein-2 (LAMP-2) which is primarily defective in this disorder. In some patients with Danon disease, the C5b-9 membrane attack complex was present in some of the vacuoles but not at the surface of the muscle fibers or in the sarcoplasm.[105,113]
The number of vacuoles seems to increase with age[98,114] and their number and extent varies greatly between patients,[113] suggesting that the absence of vacuoles might contribute to diagnostic difficulty. The diagnosis of Danon disease might be delayed if it is not considered also in patients with isolated cardiomyopathy.[103]
In female patients with Danon disease the muscle biopsy has been rarely investigated and showed no remarkable abnormalities,[100,117] or slight focal vacuolization in muscle fibers.[101]

3.21. Cardiac muscle pathology

Cardiac tissue both from endocardial biopsy and explanted heart has been analysed in patients with Danon disease. Common pathological features are hypertrophic

cardimyocytes with enlarged and picnotic nuclei, vacuolated cytoplasm and myofibrillar disarray.[99,100,102,113] Necrotic cardiomyocytes and collection of invading macrophages can be observed, and increased acid phosphatase staining indicates active degeneration of cardiomyocyes.[100]

3.22. LAMP2 gene and transcripts

The human LAMP2 gene (MIM # 309060) maps to chromosome region Xq24[118] and encodes for alternatively splices transcripts, called LAMP2a, LAMP2b and LAMP2c, which have identical luminal domains but different transmembrane and cytoplasmic domains. The LAMP2 isoforms show different subcellular localization and tissue distribution patterns. The mRNA encoding LAMP2b has a tissue-specific expression: high levels were found only in striated muscle and brain. Its absence seems to be the causative defect in Danon disease.[89] LAMP2a is ubiquitously expressed, with high levels in placenta, lung, and liver,[119] and, unlike the other isoforms, it seems to function in chaperone-mediated autophagy.[120] LAMP2a isoform was also shown to facilitate MHC class II presentation of cytoplasmic antigens, probably through chaperone-mediated autophagy. It is not known whether the human LAMP2c isoforms has specific functions.

The LAMP2 gene has an open reading frame consisting of 1233 nucleotides and encodes 410 aminoacids. The gene has 9 exons: exons 1 to 8 and part of exon 9 encode the luminal domain. The rest of exon 9 encodes the transmembrane and cytoplasmic tail (Figure 9). The exon 9 undergoes alternative splicing, which produces the isoforms LAMP2a, LAMP2b, LAMP2c.

Mutations in the LAMP2 gene may affect both LAMP2a and LAMP2b isoforms (the most common pattern leading to the typical disease manifestation) or the LAMP2b isoforms only (no mental retardation in males).

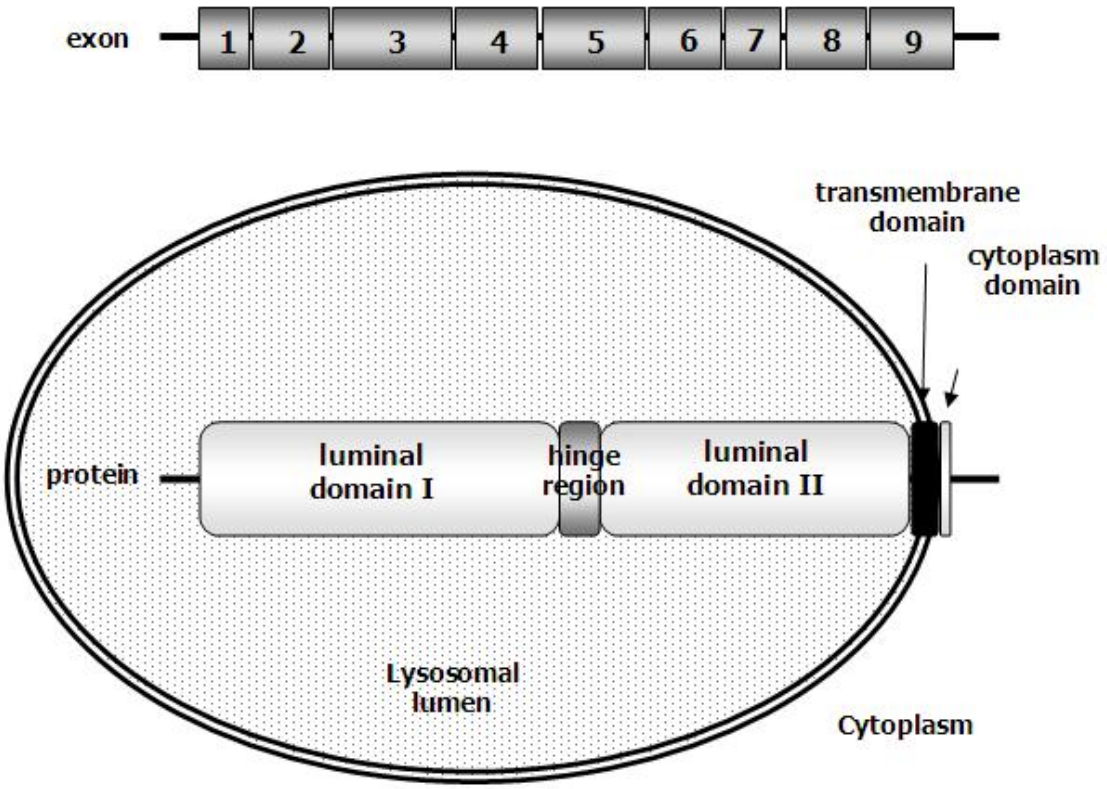

Figure 9. Schematic representation of LAMP2 gene, whose coding region is subdivided into 9 exons, and LAMP-2 protein structure with the corresponding domains: a large luminal domain divided into 3 subdomains (I, hinge region, II), a small lysosomal transmembrane domain, and a small cytoplasmic domain. Exon 9 of the gene encodes for both the transmembrane and the cytoplasmic domains of the protein. From: Fanin et al., *Genetic Inheritance Patterns, Nova Science Publishers, New York 2008.*

3.23. Lysosomal associated membrane proteins (LAMP) and LAMP-2

Two of the most abundant lysosomal components are the lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2, respectively).[121] LAMP-1 and LAMP-2 exhibit considerable sequence homology and have similar domain structure and biochemical properties. LAMPs are transmembrane proteins with a large, heavily glycosylated luminal domain and a short cytosolic tail.[121] The conserved cytosolic tails of LAMP-1 and LAMP-2 are 11 residues long and contain necessary information for their intracellular targeting after biosynthesis. Despite their 37% aminoacid sequence homology, LAMP-1 and LAMP-2 are distinct proteins which most likely diverged relatively early in the evolution as evidenced by their localization on different chromosomes. LAMP-1 is ubiquitously expressed, while LAMP-2 expression is developmentally regulated. The two glycoproteins are similar in

several ways but are distinguishable as different molecules by their molecular and antigenic properties. The molecular mass of the polypeptide backbone of human LAMP-1 and LAMP-2 is 40-45 kDa, however, after glycosylation the mass of the glycoproteins is apparently about 120 kDa (LAMP-1 has an apparent molecular weight of 105-115 kDa and LAMP-2 of 100-110 kDa). The glycosylation constitutes about 60% of the total mass of these proteins, making them the most densely glycosylated proteins known.[121] LAMPs form a continuous carbohydrate lining on the inner leaflet, generating a glycocalyx. Therefore, LAMPs were believed to function in the maintenance of the structural integrity of the lysosomal membrane by protecting it from the hostile luminal environment. However, recent observations are inconsistent with this notion. Depletion of N-glycans with endoglycosidase H caused rapid degradation of LAMP-1 and LAMP-2, yet no changes in lysosomal integrity were noted.[122] This suggested that LAMPs must have alternative functions. Accordingly, LAMP-2 was proposed to serve as a receptor for chaperone-mediated autophagy of cytosolic proteins[120] and was implicated in MHC class II presentation of cytoplasmic antigens.[124] Generation of LAMP-1 and LAMP-2 single- and doubleknockout (DKO) mice revealed additional details of LAMP function. LAMP-1-deficient mice have a near-normal phenotype, with no significant alterations in their lysosomal properties. However, they display elevated levels of LAMP-2, which is thought to compensate for the loss of LAMP-1.[125] By contrast, LAMP-2- deficient mice exhibit elevated postnatal mortality, and the surviving mice are small.[126] Several tissues show accumulation of autophagic vacuoles.[126,127] Moreover, hepatocytes from LAMP-2-deficient mice display elevated secretion of lysosomal enzymes, improper cathepsin D processing and abnormal retention of mannose-6-phosphate receptors in autophagic vacuoles.[127] Thus, LAMP-2 may be involved in autophagy and lysosome biogenesis. Consistent with the notion that LAMP-1 and LAMP-2 may have redundant function, deletion of both LAMP-1 and LAMP-2 causes embryonic lethality.[128] As in LAMP-2- deficient mice, fibroblasts isolated from DKO embryos accumulate abnormally high amounts of autophagic vacuoles and were found to accumulate non-esterified cholesterol in endosomes/lysosomes.

3.24. X-chromosome inactivation in female patients

While in male patients the cardiac involvement associated with a high CK level of skeletal muscle origin is a constant feature, in females the possible involvement of different organs other than heart (muscle and brain) is variable. One explanation of such unpredictability

could be the skewed X-chromosome inactivation occurring in female cells and tissues. However, a random X-inactivation pattern was found in blood and muscle from one female patient with Danon disease suggesting that this possibility is unlikely.[100]

On the other hand, since a preferential X-chromosome inactivation of wild-type allele in female patients could not explain why the disease is clinically manifest in all female heterozygotes (at least in the heart), the dominant inheritance in Danon disease is likely to be due to haploinsufficiency.

3.25. Haploinsufficiency

It is still not known how LAMP2 gene mutations produce a dominant effect. A model involving a dominant-negative effect of mutation is incompatible with X-linked inheritance where female patients express about 50% of protein levels. Although the absence of mutant protein in hemizygote male patients indicates a loss-of-function, the phenotype in heterozygote females could only originate from haploinsufficiency, where the normal protein product of the wild-type allele does not reach the threshold level necessary for normal function.[114]

3.26. Frequency of the disease

To date, less than 30 families worldwide have been reported with genetically confirmed Danon disease. Therefore, the frequency of the disease in the general population is very low but it is probably underestimated for the reason that in some cases the early fatal cardiomyopathy might have not been sufficiently investigated and because other signs or symptoms suggestive of Danon disease (e.g. skeletal myopathy and mental retardation) were mild or absent. Furthermore, it is possible that some families might have escaped diagnosis given that the X-linked dominant pattern of inheritance may mimic that of the more frequent autosomal dominant traits, and it is sometimes recognized with difficulty (especially in patients with isolated cardiomyopathy).

Although Danon disease is very rare in the general population, its frequency has been estimated to be 4% among patients with an unclassified form of hypertrophic cardiomyopathy [Charron et al. 2004], and relevant (33%) among patients presenting with both vacuolar myopathy in muscle biopsy and hypertrophic cardiomyopathy.[100]

3.27. Genetic counseling

To distinguish hypertrophic cardiomyopathy from Danon disease has important consequences for prognosis, therapy, mode of inheritance and genetic counseling. The information and genetic counseling given to patients and relatives is different for hypertrophic cardiomyopathy and Danon disease, especially as regards the risk of transmission. Germline mosaicism has been reported in one family with Danon disease (two affected children with LAMP2 gene mutations were born from an healthy non-mutated mother).[129] Furthermore, mothers of patients may be clinically healthy because of de-novo mutations and some patients might present as sporadic.[98,99,108] These possibilities must be taken into account when investigating on the first generation of affected individuals and when offering genetic counseling. Prenatal diagnosis can be offered in genetically defined families, where a lethal disease is segregating.

3.28. Nomenclature

The original report by Danon et al. in 1981 called the disease “lysosomal glycogen storage disease with normal acid maltase” because pathologic features superficially resemble those of acid maltase deficiency. However, the current nomenclature of “glycogen storage disease type IIB” (GSDIIB) seems inaccurate because Danon disease is not a glycogen storage disease but a primary lysosomal storage disease. LAMP-2 deficiency or Danon disease appears more appropriate. Danon disease has OMIM # 300257.

4. Aims

The objective of this study was to examine at molecular, biochemical and muscle pathology level two groups of patients affected with two different forms of Lysosomal Storage Disorders (LSDs): Glycogen Storage Disease type II (GSDII) and Danon Disease.

Glycogen Storage Disease Type II

We studied 23 patients with infantile-onset or late-onset glycogen storage disease type II to analyse the causes of the striking clinical heterogeneity that result from acid α-glucosidase deficiency. Previous studies have been conducted on cellular models but not on muscle, the mainly affeceted tissue of GSDII patients. We performed a screening for enzyme activity, protein expression, GAA gene mutations, in order to get some new insights to trace genotype-phenotype correlations. Identification of gene mutations in patients with GSDII is crucial in order to provide genetic counselling to patients' families and to offer heterozygote and prenatal diagnoses. Furthermore, the molecular characterization of such patients is crucial when selecting patients for therapeutic trials (i.e. Enzyme Replacement Therapy). We analysed also muscle pathology, including immunolabeling for Golgi and sarcolemmal proteins, to better understand the pathophysiology of the disease.

Danon Disease

To identify patients with Danon Disease, we screened LAMP2 gene mutations and LAMP-2 protein deficiency in the skeletal muscle of 9 unrelated subjects with vacuolar myopathy and hypertrophic cardiomyopathy. We performed an immunopathological study on skeletal muscle from LAMP2 mutant patients to correlate the extent of pathological changes in different cellular compartments with the severity of skeletal myopathy and cardiomyopathy. Furthermore, we analyzed the expression of LAMP-2 protein not only on striated muscle, but also on fibroblasts and leukocytes, in order to explain the multisystem involvement observed at clinical level, and provide an easier and less invasive diagnostic tool in Danon Disease.

5. Materials and methods

Glycogen Storage Disease Type II

5.1. Selection criteria of patients

We selected 23 patients with molecular diagnosis of GSDII, attending or referred to the Neuromuscular Center, University of Padova. Diagnosis was obtained following clinical examination, muscle biopsy histopathology, measurement of acid maltase activity in muscle or lymphocytes, and/or identification of mutations in the GAA gene. In two patients the diagnosis was obtained by clinical examination and genetic testing (enzyme activity was not available).

5.2. Morphological and immunohistochemical study of muscle biopsies

Open muscle biopsies were done as part of diagnostic procedure, after written consent from patients or their parents. Muscle specimens were frozen in isopentane, chilled in liquid nitrogen and then stored at –80°C until processed.

A set of 13 serial cryostat sections was obtained for each biopsy. The first ten sections (10 μm thick) were routinely stained for haematoxylin-eosin (H&E), Gomori trichrome, PAS, Oil Red O, NADH-tetrazolium reductase, cytochrome oxidase, succinate dehydrogenase, acid and alkaline ATP-ases, and acid phosphatase. The general inspection of stained sections was used to analyze and measure the fiber size variability, the degree of fiber vacuolization (expressed as percentage of total fibers), and the reaction of lysosomal enzymes (acid phosphatase).

The additional three sections (six μm thick) were collected in polylysinated slides and processed separately for immunohistochemical analysis to further investigate for proliferation of the Golgi complex, anti Golgi-zone antibody (1271, Chemicon, Temecula, CA) diluted 1:100 in phosphate buffer saline (PBS) was used. The expression of membrane-related proteins in the vacuolar membrane was also investigated using anti caveolin-3 (Transduction Lab., Lexington, KY) and anti dystrophin (DYS2, Novocastra Lab. Newcastle upon Tyne, UK) antibodies diluted 1:100 in PBS. Primary antibodies were incubated in a humid chamber at room temperature for one hour. Specific labeling was developed using secondary anti-mouse cyanine-3 conjugated immunoglobulines (Caltag, Burlingame, UK) diluted 1:100. The same optical fields were identified in serial sections,

which were analyzed and photographed with a Zeiss Axioskop epifluorescence microscope, equipped with CoolSnap Photometrics digital camera and an imaging system analysis (Roper Scientific).

5.3. Fluorimetric assay of acid α-glucosidase activity in lymphocytes and muscle

The diagnosis of acid maltase deficiency was confirmed by determination of its enzyme activity using a fluorimetric technique with α-methyl-umbelliferone-α-glucoside or maltose. Briefly, for lymphocytes extraction, 20 ml of blood were diluted in an equal volume of PBS, then further diluted (7+3 ml) in Lymphoprep and centrifuged for 20 minutes at 2000 rpm. The lymphocytes obtained were resuspended in 0.9% NaCl, centrifuged at 2500 rpm for 10 minutes, washed twice in PBS and stored at –20°C. Before use, the pellet was re-suspended in 2 ml water and sonicated in ice 3 times for 5 seconds at 100 W.
About 40 mg of fresh or frozen muscle was homogenized in 5% water using a glass Potter. Muscle homogenate was used, after incubation in 0.1N NaOH, to determine the amount of non-collagen protein using the Bradford method. The reaction incubation medium consisted of 100 µl of 2mM of α-methyl-umbelliferone-α-glycoside, 100 µl of 0.4M acetate buffer at pH=4 (acid maltase) or at pH=6.5 (neutral maltase) in a final volume of 500 µl. The reaction was started by adding 50 µl of muscle or lymphocyte homogenate and then stopped, after 1 hour, with 300 µl of 50 mM BaOH2, 300 µl of 5 mM ZnSO4. After centrifugation at 3000 rpm for 10 minutes, 400 µl of supernatant were added to 1.6 ml of 1M glycine buffer. Activity of α-glucosidase was measured using a fluorimeter primary filter at 313-366 nm and a secondary filter at 470 nm. A standard α-methyl-umbelliferone curve was used to determine the concentration.

5.4. Muscle immunoblot analysis of acid α-glucosidase and LAMP-1

Skeletal muscle biopsy sections were dissolved in Laemmli buffer, boiled for 3 minutes and centrifuged. Muscle proteins contained in the supernatant were loaded in 8% polyacrylamide gels, resolved by overnight SDS-PAGE electrophoresis, and blotted to nitrocellulose membrane for three hours. Post-transfer gels were stained with Coomassie Blue, whereas blots were air-dried, blocked for one hour with non-fat milk in Tris-buffered saline with Tween-20 (TTBS) and incubated overnight with a rabbit polyclonal antibody raised against recombinant α-glucosidase protein (rh-GAA, gift from Dr. D.Bali, Duke

University, Durham, NC), and with a mouse monoclonal antibody against lysosomal-associated-membrane-protein-1 (LAMP-1; H4A3, Developmental Studies Hybridoma Bank, Iowa City, IO) diluted 1:200 in TTBS. Immunoreactive bands were visualized using anti-mouse or anti-rabbit peroxidase conjugated immunoglobulines (Amersham, UK) diluted 1:1000 in TTBS for one hour and developed using the chemio-luminescence system (ECL, Amersham). Visualization of specific bands was obtained by exposure of blots to photographic films. The amount of each protein band was normalized to the amount of tissue loaded, as determined by the skeletal myosin bands in the post-transfer Coomassie blue-stained gels. The molecular weight of the immunoreactive bands was determined using a broad range, prestained, molecular weight marker (Bio-Rad Lab., Hercules, CA) as reference.

5.5. PCR amplification of genomic DNA sequence

Genomic DNA was extracted from either peripheral blood samples or muscle tissue using the GenElute Mammalian Genomic DNA kit (Sigma, St. Louis, MO). The entire coding sequence of the GAA gene was amplified in 18 amplicons, using published intronic primers 16, except for one primer that was redesigned (sequence available on request). PCR reactions were performed under standard conditions.

5.6. Screening of GAA gene mutations and sequencing of genomic DNA

In order to quickly screen for the presence of the common -32-13T>G (IVS1) point mutation in all 23 patients, we adopted an allele-specific test (ARMS-PCR, Amplification Refractory Mutation System) using published primers.[65] The deletion of exon 18, which recurs in Pompe patients, was investigated in infantile-onset patients by PCR amplifications, using standard conditions and published primers.[130]

The analysis of the entire coding sequence of the GAA gene was conducted by SSCP (Single Strand Conformational Polymorphism) analysis. PCR products were mixed with denaturing loading buffer (formamide 95%, 10mM NaOH, traces of bromophenol blue), denatured by heating at 95°C for 5 minutes, and immediately placed on ice. Gel electrophoresis was conducted at 4°C or at room temperature and at different voltage/current/time conditions, depending on the composition and length of PCR fragments, using 10% polyacrylamide gels in Tris-borate-EDTA buffer. The gels were

silver stained using standard procedures. The DNA sequences containing nucleotide changes and distinguishable from aberrant migration bands, were directly sequenced.
PCR products were directly sequenced using the Big Dye dideoxy-terminator cycle sequencing kit and the 377 ABI-PRISM automated sequencer. Sequence analysis was obtained using both ClustalW software and the human GAA sequence as reference.

Danon Disease

5.7. Selection criteria of patients

Our skeletal muscle biopsy tissue bank, which contains more than 6,000 specimens, was surveyed for patients affected with an unidentified form of vacuolar myopathy associated with hypertrophic cardiomyopathy. A total of 9 skeletal muscle biopsies matched our selection criteria and were selected for the screening of both LAMP-2 protein deficiency (by immunohistochemical and western blot analysis) and *LAMP2* gene mutations. Three patients, in whom *LAMP2* gene mutations were identified, were the objects of the present study; the remaining 6 patients are currently undergoing the search for an alternative molecular diagnosis.

5.8. Case reports

Patient 1 (Figure 10, Family 1, II-2). This 20-year-old man of Balkan origin complained of easy fatigability, anorexia and abdominal pain. He progressively developed diffuse muscle hypotrophy, and showed Wolff-Parkinson-White (WPW) syndrome and heart failure. He had several syncopal episodes. Elevated creatine kinase (CK) level was found. At 22 years he was hospitalised to assess his eligibility to cardiac transplantation. Chest X-ray showed cardiomegaly and pleural effusion. An echocardiography showed “end-stage“ hypertrophic cardiomyopathy, i.e. with severe reduction of left ventricular ejection fraction (LVEF=20%), and cardiogenic ascites. A pacemaker was implanted to control the severe bradi-arrhythmias in presence of atrial fibrillation. On neurological exam he had cachectic appearance, marked atrophy of trunk and paraspinal muscles, marked weakness in upper girdle muscles, and diminished deep tendon reflexes. EMG was myogenic. CK was 283 U/L (n.v. 0-190). A quadriceps femoris muscle biopsy showed severe vacuolar myopathy with storage of glycogen and filamentous material. The physical exam showed tender and

enlarged liver and ascites. Ultrasounds revealed hepatosplenomegaly and small kidneys. Laboratory tests (bilirubin, transaminases, PT, PTT, INR, albumin, creatinine clearance, microalbuminuria) resulted abnormal. Viral hepatitis was excluded by serological tests. He had mild mental retardation and in childhood attended special schools. Spirometry showed severe restrictive pulmonary insufficiency. He died at 22 years of age (one month after hospitalisation) of heart failure.

Patient 2 (Figure 10, Family 2, III-1). At age 9 years this boy showed scleral jaundice, abnormal hepatic laboratory tests, and chronic hepatitis at liver biopsy with normal serology. At age 18 an ECG revealed WPW syndrome. Since age 22, he noticed exertion dyspnoea and difficulty in climbing stairs, and complained several syncopal episodes; hypertrophic cardiomyopathy was diagnosed. At age 24 an implantable-cardioverter defibrillator (ICD) was implanted, and one year later the cardiac transplant was considered. Echocardiography showed “end-stage” hypertrophic cardiomyopathy. Cardiac SPECT and PET showed reduced caption.

Endomyocardial biopsy, which was obtained during heart catheterisation, suggested a form of hypertrophic cardiomyopathy with glycogen storage disease. On neurological examination, he had mild waddling gait with hyperlordosis, winging scapulae, diffuse muscle hypotrophy, diminished tendon reflexes, Gower’s sign, arachnodactyly, and weak facial muscles. CK was 558 U/L. A quadriceps femoris muscle biopsy showed vacuolar myopathy with storage of PAS positive material. On physical exam he had cachectic appearance and hepatomegaly, and on abdominal ultrasound he had also a mild reduction of kidney size. Laboratory tests (bilirubin, transaminases, creatinine clearance, proteinuria) resulted abnormal. No mental retardation was present. Spirometry showed severe restrictive pulmonary insufficiency. The patient died at age 29 years of heart failure while waiting for cardiac transplant.

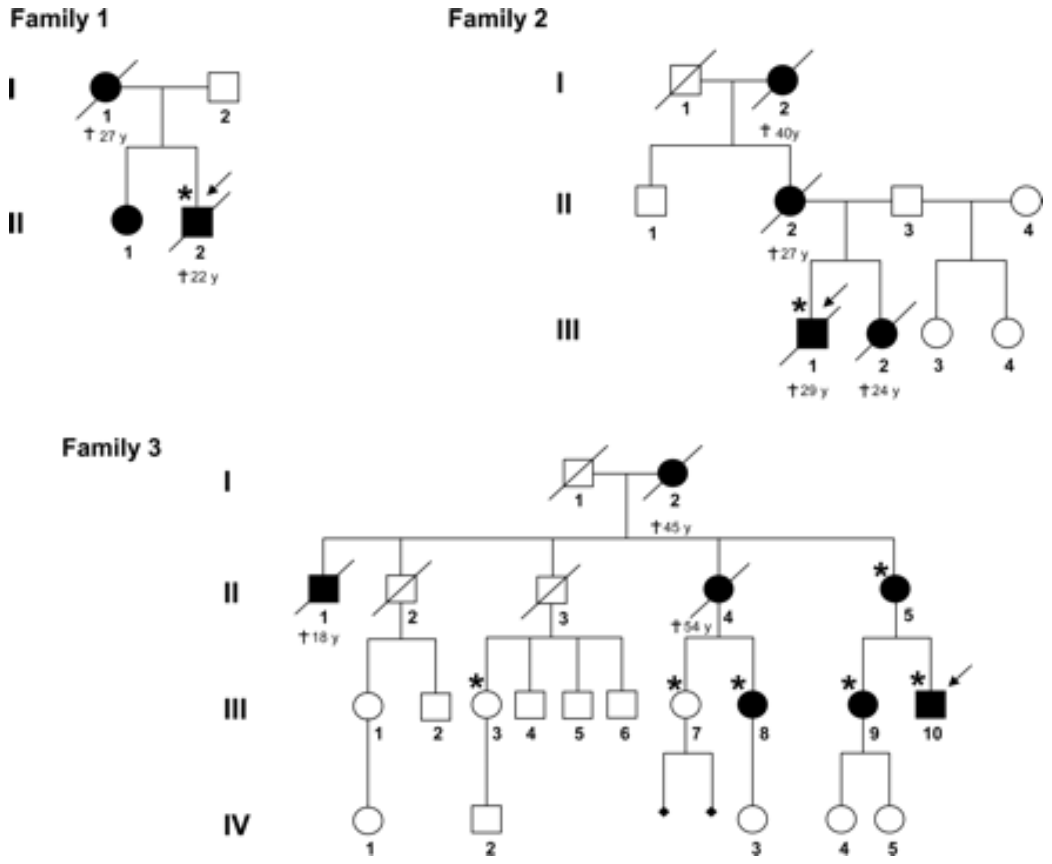

Figure 10. Family pedigree of the index patients involved in this study (arrows). Filled circles and squares indicate affected female and male patients, respectively. The diagonal slash indicates patients who have died (age at death is reported). The asterisks indicate subjects in whom molecular analysis was performed (DNA samples available). From Fanin et al., *Am J Pathol. 2006; 168(4):1309-20.*

Patient 3. (Figure 10, Family 3, III-10). At age 12 years he had sub-clinical jaundice but viral hepatitis was excluded by serology. At 18 years he noticed easy fatigability after mild effort. At 19 years ECG revealed bradycardia and WPW syndrome. At age 22 he had very low aerobic resistance by Tread-Mill test. At age 27 chest X-ray showed mild cardiomegaly. An ophthalmologic exam revealed severe myopia with pigmented epithelial retinal dystrophy. An abdominal echography showed hepatomegaly and chronic pathology, but hepatic biopsy was normal. He was hospitalised at age 28 to investigate muscle weakness and high CK (1094 U/L). Skeletal muscle biopsy showed a vacuolar myopathy with accumulation of PAS positive material. Muscle CT scan revealed moderate proximal and distal atrophy of lower limbs. Echocardiography showed mild hypertrophy of intraventricular septum. Mild restrictive ventilator dysfunction was present on spirometry. At age 30 he complained of mild difficulty in climbing stairs and lifting weights. On physical

examination he had mild waddling gait with hyperlordosis, Gower's sign, distal leg muscle hypotrophy, mild weakness of proximal and distal girdles, neck flexor and facial muscles. A non-obstructive hypertrophic cardiomyopathy with moderate septum hypertrophy (21mm) was diagnosed by a further echocardiography. On neuropsychological evaluation he had delayed psychomotor development, poor school achievements, IQ (WAIS-R) was 77. EEG revealed diffuse slow dys-arrhythmia.

Patient 4. (Figure 10, Family 3, II-5). This woman is the mother of patient 3. She suffered since age 26 of palpitations and easy fatigability; at age 29 an ECG showed WPW syndrome with paroxysmal atrial flutter, which have been later cardioverted first electrically, then pharmacologically. Few years later, an echocardiography revealed hypertrophic cardiomyopathy. At 38 years, aspecific hepatitis was diagnosed on the basis of echographic hepatosplenomegaly and hypertransaminasemia with negative serology. At age 47, she developed a moderate bilateral neurosensorial hypoacusia and progressive heart failure with effort dyspnoea, orthopnea, worsening asthenia, and several syncopal episodes. Aerobic capacity was markedly reduced. An echocardiography showed "end-stage" hypertrophic cardiomyopathy with severe reduction of LVEF (33%), and diffuse hypokinesia. Three years later after colecistectomy, she had an episode of acute heart failure complicated by ventricular fibrillation and an ICD was implanted. At age 52, she underwent cardiac transplantation. Since then, she did not have cardiac complaints but still reported muscular weakness and myalgia. At age 54, on neurological exam she had mild proximal and flexor muscle weakness, facial hypomimia, distal upper and lower limbs atrophy. Severe myopia and weakly reactive pupils were recorded. Liver was tender to palpation. CK level was normal. On neuropsychological exam her cognitive records were normal (IQ= 82 by WAIS-R scale). No EEG abnormalities were present. A mild reduction of cortical kidney thickness and some cystic lesions were demonstrated by ultrasounds. Quadriceps femoris muscle biopsy showed mild myopathic changes without fibre vacuolisation.

5.9. Cardiological evaluation

A cardiac evaluation was obtained from clinical history and non-invasive methods (ECG, Holter monitoring, M-mode, 2D and Doppler echocardiography). The presence of ventricular pre-excitation was diagnosed on short PR interval, delta waves or both, and left ventricular hypertrophy on voltage.[131] Hypertrophic cardiomyopathy was diagnosed on

echocardiography showing unexplained left ventricular hypertrophy (maximal wall thickness ≥ 15 mm). Two patients underwent invasive cardiac techniques (heart catheterisation and endomyocardial biopsy) and one patient underwent heart transplantation.

5.10. Fibroblast cell culture

Fragments of skin biopsy were collected sterile at the time of biopsy, placed in 10% DMSO in rich medium [70% M199 medium (Seromed, Berlin, Germany) + 30% foetal calf serum (FCS) (Euroclone, Torquay Devon, UK)], gradually frozen, and then stored in liquid nitrogen. To start the cell culture, specimens were thawed quickly, and incubated in a freshly prepared mixture of 80% rich medium + 20% human plasma that was filtered directly on the disk. When fibroblast proliferation was visible, the specimens were trimmed and placed on gelatinised dishes. Fibroblasts were cultured in DMEM nutrient medium (Seromed) supplemented with 10% FCS, L-glutamine and penicillin-streptomycin in 75 mm2 flasks at confluence, collected and directly dissolved in electrophoresis loading buffer.

5.11. Histopathology and immunohistochemistry

Sections of skeletal muscle biopsies from patients and controls were routinely stained to evaluate overall muscle morphology or used for immunohistochemistry. The same reaction were also performed in myocardial biopsy from patient 2, whereas histoenzymatic and immunohistochemical reactions in the explanted heart from patient 4 were prevented by its embedding in paraffin. We used a panel of antibodies to study the following subcellular compartment or cell component: lysosomal membrane using LAMP-2 antibody (H4B4 luminal domain, Developmental Studies Hybridoma Bank, Iowa City); plasma membrane by caveolin-3 (Transduction Lab., Lexington, KY) and dystrophin (DYS2, Novocastra, Newcastle upon Type, UK); nuclear membrane by lamin A/C (LAM-A/C, Novocastra) and emerin (Novocastra); basal lamina by laminin A (1924, Chemicon, Temecula, CA); proliferation of Golgi apparatus by anti-Golgi (1271, Chemicon); early endosome membrane by rab5 (Transduction); cytoskeleton by desmin (1698, Chemicon); muscle regeneration by fetal myosin (MHCn, Novocastra); inflammatory features by macrophages (EBM11, Dako, Carpinteria, CA) and MHC class I molecules (W6/32, Dako). Sections were incubated for 1 hour with primary antibodies (diluted 1:100, except LAMP-2 diluted

1:20). After washes in PBS, sections were incubated for 30 minutes with anti-mouse cy-3 conjugated Ig (1:100) (Caltag, Burlingame, UK), and examined by fluorescence microscopy.

5.12. Western blot analysis

Except for the endomyocardial biopsy sample, which was insufficient for this analysis, LAMP-2 western blotting was conducted in a panel of tissues and cell types from mutant patients and control. Skeletal muscle biopsies, leukocytes and skin fibroblasts were dissolved in the electrophoresis-loading buffer used, and processed as previously reported,[132] using the same anti LAMP-2 antibody used for immunohistochemistry (diluted 1:300). LAMP-1 antibody (H4A3, Developmental Studies Hybridoma Bank, Iowa City) was used to evaluate a potential cross-reaction with LAMP-2 protein (for both proteins the immunogen was the native protein). LAMP-2 and LAMP-1 antibody (diluted 1:200) were used in duplicate in adjacent lanes of the same gel. The protein quantity of each sample was normalised to the amount of tissue loaded, as determined by the skeletal myosin or the actin bands in the post-transfer Coomassie blue-stained gels. The amount of protein in patients was determined by densitometry (ImageJ software v.1.34) and expressed as percentage of control.

5.13. DNA and RNA analysis

5.13.1. PCR amplification of genomic DNA sequence.

Genomic DNA was extracted from blood leukocytes or muscle biopsy, using the GenElute Mammalian Genomic DNA kit (Sigma, St. Louis, MO). The entire coding sequence of the *LAMP2* gene was amplified in 10 amplicons, using primer sequences designed using the human *LAMP2* sequence as reference (GenBank accession #AC002476.1). PCR reactions were performed under standard conditions. [132,133]

5.13.2. DNA sequencing.

PCR products were purified by enzyme reaction (ExoSap-I, Amersham, UK), quantified on agarose gel and directly sequenced using the Big Dye dideoxy-terminator cycle sequencing kit and the 377 ABI-PRISM automated sequencer, at the CRIBI Biotechnology Centre, University of Padova.

5.13.3. PCR amplification with allele-specific primers (ARMS-PCR).

To confirm 2 of the 3 novel mutations found by direct DNA sequencing (W98X and 796-797insC) we used appropriate ARMS-PCR tests by allele-specific primers designed *ad-hoc*. PCR amplifications were performed under standard conditions using a touchdown PCR protocol.[132,133] An ARMS-PCR test was not required to confirm the third mutation found (22 bp deletion), as the reduced size of the amplicon was easily detectable by 2% agarose gel electrophoresis.

5.13.4. RNA isolation and semi-quantitative RT-PCR.

Total RNA was isolated from muscle biopsies using the SV total RNA isolation system (Promega Corp., Madison WI) according to the manufacturer's protocol, including DNAse treatment. To estimate the amount of *LAMP2* RNA in skeletal muscle, total RNA isolated from control and patients' muscle biopsies was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, San Diego, CA) and oligo-dT primer in a 20 µl reaction volume, according to the manufacturer's recommendations. The full-length *LAMP2 gene* cDNA was amplified using specific primers (forward 5'-TGGTGTTGCAGCTGTTGTTG-3'; reverse 5'-CGTAAGCAATCACTATAACGATAAT CAA-3') designed on the human *LAMP2* mRNA sequence (GenBank # NM_013995) by Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The amplification product was expected to be 1418 bp in size. A fragment of 838 bp of the beta-actin cDNA was used as an internal control for normalization. A primary solution of 150 µl was prepared and distributed in 10 aliquots of 15 µl, each corresponding to different points of the amplification curve measured during the exponential phase of the PCR for the two genes (from 25 to 39 cycles).[134] The solution was as follows: 8 µl of template cDNA, 200 µM dNTPs, 0.2 µM of each primer for beta-actin and 0.4 µM of each primer for *LAMP2* (to favour amplification), 1x Taq Platinum buffer, 1.5 mM $MgCl_2$ and 1U Taq Platinum (Invitrogen). PCR parameters were: 94°C for 2 min; 39 cycles of 94°C for 30 s, 55°C for LAMP2 gene and 61°C for beta-actin gene for 30 s, and 72°C for 1 min; with a final elongation step of 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis. The level of each transcript expression was determined by densitometry (using ImageJ software v.1.34n) and expressed as percentage of control.

5.13.5. X-chromosome inactivation.

The X-chromosome inactivation pattern was determined by PCR analysis of polymorphic CAG repeats in the first exon of the *AR* gene.[135] DNA, extracted from blood leukocytes and

muscle biopsy of heterozygote female patient, was digested using methylation-sensitive restriction enzymes (*Hpa*II, *Cfo*I). The PCR primers used for the AR locus were previously described.[136] After digestion, DNA amplification occurred only in presence of methylated restriction sites (inactive allele). PCR products were separated on an ABI 377 automated sequencer and analysed by GeneScan software (Applied Biosystems). Because the smaller allele amplifies more efficiently, a correction factor was generated using the undigested samples so that both alleles were represented equally in the calculations.[136] All samples were analysed in triplicate and the average values were used in calculating the degree of X inactivation. The X inactivation pattern was classified as skewed when 90% or more of the cells preferentially used one X-chromosome.

6. Results

Glycogen Storage Disease Type II

6.1. Patients

Our study involved 23 patients, 7 male and 16 female (sex ratio 1:2.3), and four pairs of siblings (table 1). Three patients had the classic infantile-onset form of the disease with cardiomyopathy (Pompe), 3 had a childhood onset (between 2 and 7 years), 16 had onset between 18 and 57 years of age (mean 36.2 years), and 1 was asymptomatic but with high CK level at age 14 years.

All the infantile-onset patients had severe cardiomyopathy that led to premature death before the age of 4 years; one patient with childhood onset developed severe muscle weakness and respiratory insufficiency, which led to death at age 4 years.

In most late-onset patients the main clinical feature was myopathy or, less frequently, dyspnea, asthenia, weight loss. Six patients developed respiratory insufficiency that required the use of ventilator and caused death in six patients between 45 and 73 years (table 1).

The pairs of siblings included in this study illustrate the interfamilial phenotype variability in this disease: the age at onset was rather different between siblings; furthermore, in one pair of sisters, one had severe respiratory insufficiency whereas the other had limb-girdle myopathy and a slight decrease of forced vital capacity.

One late-onset patient, who was found to be homozygous for the -32-13T>G leaky spicing mutation, developed severe proximal weakness and, when age 23, was unable to rise from a chair and only able to climb stairs with aid.

6.2. Acid [alpha]-glucosidase enzyme activity in muscle and lymphocytes.

The acid maltase activity in muscle from our GSDII patients ranged from 0 to 32% of controls (table 2). The highest levels of enzyme activity were found both in two patients with childhood-onset form of the disease and in a pair of siblings, who only had myalgia and proximal weakness. Conversely, in two adult-onset GSDII patients (including an asymptomatic patient with a high CK level) acid maltase was absent. We therefore

observed a poor correlation between the residual enzyme activity in muscle and the severity of the clinical course.

The level of acid maltase activity in lymphocytes was on average higher than in muscle, although, in most cases, it was reduced below 50% of controls. It is possible that the assay in lymphocytes is less sensitive than in muscle, the latter remaining the gold standard for biochemical diagnosis. The levels of neutral [alpha]-glucosidase activity in muscle and lymphocytes were variable.

Table 1 Clinical and muscle pathology data

Patient no.	Sex	Phenotype	Age at onset	Age at biopsy	Disease duration, y	Age at death	Main clinical features	Ventilator use (age, y)	Vacuolated muscle fibers (%)
1	M	Infantile-onset	1 y, 3 mo	1 y, 3 mo	—	2 y	Cardiomyopathy	No	100
2	M	Infantile-onset	15 d	4 mo	—	1 y	Cardiomyopathy	No	95
3	F	Infantile-onset	2 y, 6 mo	3 y, 6 mo	1	4 y	Cardiomyopathy	No	95
4	F	Childhood-onset	2 y	3 y, 8 mo	—	4 y, 4 mo	Myopathy	Yes (3)	70
5	F	Late-onset	19 y	NA	19	—	Weight loss, myopathy	Yes (35)	NA
6*	F	Childhood-onset	7 y	7 y	—	—	Myopathy	No	20
7*	F	Childhood-onset	2 y	NA	—	—	Myopathy	No	NA
8	M	Late-onset	18 y	52 y	34	62 y	Dyspnea	Yes (30)	10
9	M	Late-onset	20 y	48 y	28	—	Myopathy, dyspnea	No	1
10	F	Late-onset	53 y	53 y	—	63 y	Dyspnea	Yes (52)	10
11†	F	Late-onset	55 y	69 y	14	73 y	Myopathy, dyspnea	No	2
12†	F	Late-onset	44 y	54 y	10	68 y	Myopathy	No	80
13‡	F	Late-onset	40 y	56 y	16	—	Myopathy, dyspnea	No	1
14‡	F	Late-onset	57 y	NA	—	67 y	Dyspnea, weakness	Yes (57)	NA
15	F	Late-onset	34 y	54 y	20	—	Dyspnea	Yes (50)	20
16	F	Late-onset	41 y	42 y	1	—	Myopathy	No	3
17	F	Late-onset	42 y	47 y	5	—	Asthenia, myopathy	No	30
18§	M	Late-onset	27 y	28 y	1	—	Myopathy	No	10
19§	F	Late-onset	32 y	32 y	—	—	Myopathy	No	70
20	M	Late-onset	20 y	25 y	5	—	Myopathy	No	90
21	F	Late-onset	28 y	38 y	10	45 y	Myopathy	No	20
22	M	Late-onset	14 y	14 y	—	—	High CK	No	80
23	F	Late-onset	50 y	59 y	9	—	Weight loss, myopathy	Yes (59)	5

*, †, ‡, § = pairs of affected siblings.
NA = not available.

From: Nascimbeni AC et al., Neurology 2008; 70(8):61

Table 2 α-Glucosidase enzyme activity

Patient no.	Phenotype	Muscle			Lymphocytes		
		Acid glucosidase activity (pH = 4.0)*	% of control	Neutral glucosidase activity (pH = 6.5)*	Acid glucosidase activity (pH = 4.0)*	% of control	Neutral glucosidase activity (pH = 6.5)*
1	Infantile-onset	0	0	27.5	26.7	10	566.5
2	Infantile-onset	4.2	6	189.5	5.9	2	609.7
3	Infantile-onset	0	0	166.7	—		
4	Childhood-onset	21.8	32	110.3	—		
5	Late-onset	—			26.0	8	906.0
6	Childhood-onset	19.2	28	89.6	—		
7	Childhood-onset	—			10.0	3	795.4
8	Late-onset	2.0†	6	125.5†	—		
9	Late-onset	1.4	1	48.8	50.0	16	664.0
10	Late-onset	0.3†	6	—	32.0	10	454.0
11	Late-onset	8.4	12	113.5	—		
12	Late-onset	—			—		
13	Late-onset	5.0	5	76.0	191.8	60	1036.0
14	Late-onset	—			186.3	58	1000.0
15	Late-onset	1.8	7	—	—		
16	Late-onset	1.3†	12	5.26†	—		
17	Late-onset	0.3†	3	—	—		
18	Late-onset	11.7	15	308.7	—		
19	Late-onset	13.3	17	—	—		
20	Late-onset	NA			—		
21	Late-onset	NA			—		
22	Late-onset	0	0	240.0	—		
23	Late-onset	0	0	—	—		
*Controls (pmol/min/mg prot.)		34–138		53.4–274.2	200–613		522–1109
†Controls (nmol/min/gww)		5.2–23.5		7.9–29.0			

NA = data not available for this study.

From: Nascimbeni AC et al., Neurology 2008; 70(8):617-26.

6.3. Muscle morphology and immunohistochemistry.

The histopathologic hallmark is muscle fiber vacuolization and autophagy. In our patients, the vacuoles were of various sizes and shape, showed PAS-positivity and strong reaction for lysosomal acid phosphatase (figure 11). In infantile-onset patients, fiber vacuolization and autophagy were always prominent (involving 95 to 100% fibers) and severely compromised muscle fiber structure. Conversely, the degree of vacuolization was extremely variable in late-onset patients (ranging from 1 to 90% fibers), and appeared sometimes to be independent of age at onset or disease duration (table 1, figure 11): some patients with >10 years of disease duration had myopathic changes but almost absent vacuolization (Patients 9, 11, 13), whereas one asymptomatic patient had 80% vacuolated fibers. Furthermore, we observed a striking variability in the degree of fiber vacuolization between different muscles from the same patient. We observed a predominant vacuolization of type 1 fibers, though it varied in different muscles of patients; autophagy, as shown by acid phosphatase reaction (figure 11), involved both vacuolated and non-vacuolated fibers.

Immunostained sections revealed that in late-onset GSDII the vacuoles colocalized with Golgi-zone labeling (figure 11) and were delimited by membranes that reacted for the sarcolemmal proteins caveolin-3 and dystrophin, indicating an extensive fiber remodeling. On the contrary, in infantile-onset patients the Golgi proliferation was clearly evident but vacuoles did not show membranes reacting for sarcolemmal proteins (figure 11).

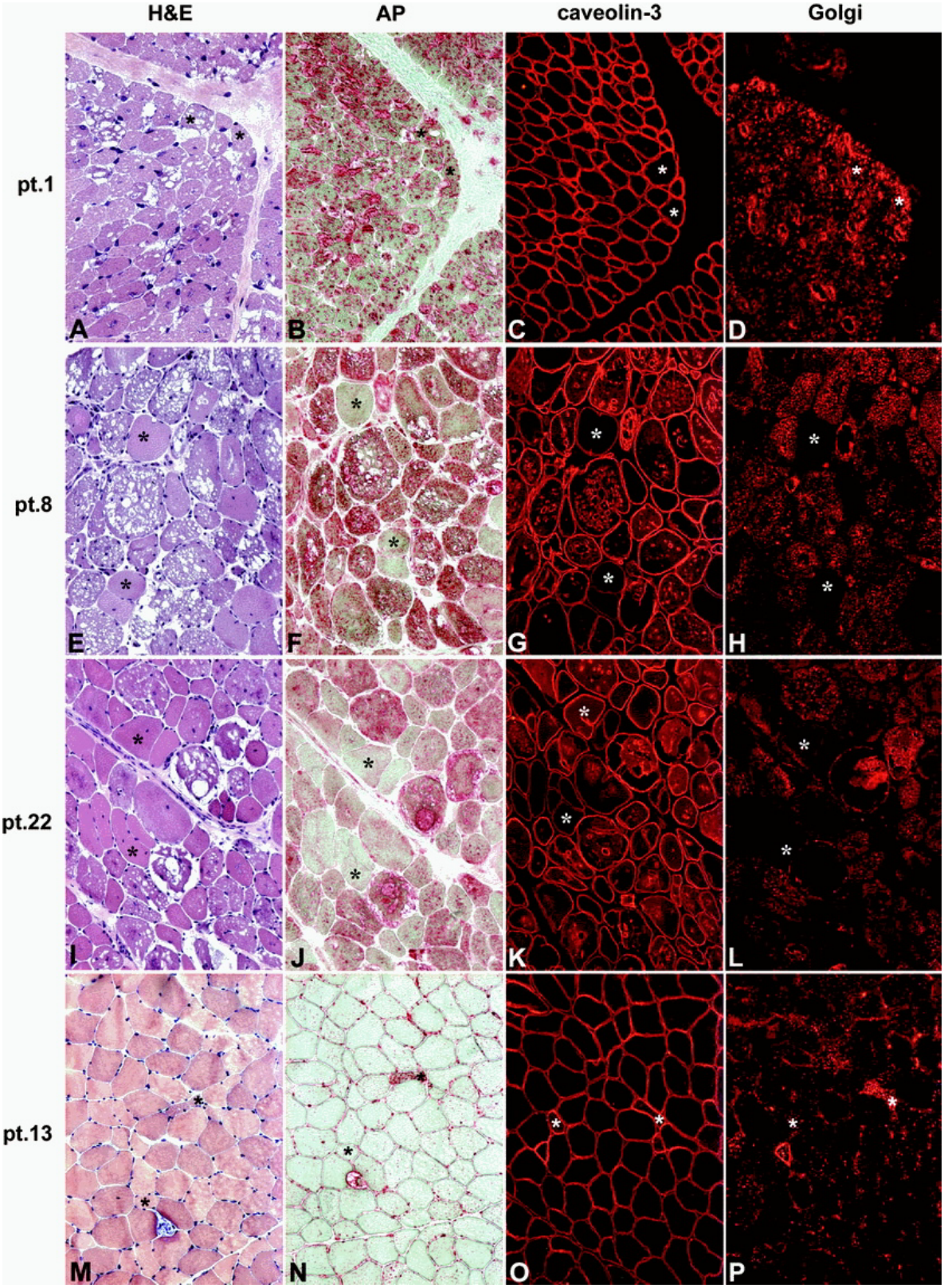

Figure 11. ***Serial sections of muscle biopsy from an infantile-onset GSDII patient (Patient 1) and three late-onset cases (Patients 8, 22, and 13) routinely stained for H-E and acid phosphatase (AP), and immunolabeled with antibodies against caveolin-3 and Golgi zone****. Different degrees of skeletal muscle*

fiber involvement can be observed: the infantile-onset patient had extensive fiber vacuolization (A) and positive AP activity (B), whereas late-onset patients showed variable degrees of vacuolization (E, I) and many fibers were apparently normal. One late-onset patient (no. 13) showed mild myopathic changes but rare fiber vacuolization (M, N). In all muscle biopsies analyzed, there was increased Golgi labeling (D, H, L, P), which was detectable in fibers with cytoplasmic vacuoles but absent in fibers with apparently normal features (asterisks indicate the same fibers in different sections from the same patient). Cytoplasmic type 2 vacuoles were delimited by membranes that reacted for the sarcolemmal protein caveolin-3 in late-onset GSDII patients (G, K), but not in type 4 vacuoles in the infantile-onset patient (C). Magnification ×200 (panels A–D) and ×100 (panels E–P). From: Nascimbeni AC et al., Neurology 2008; 70(8):617-26.

6.4. Muscle immunoblot analysis of acid [alpha]-glucosidase and LAMP-1.

Immunoblot analysis of acid [alpha]-glucosidase using anti rh-GAA antibody in the skeletal muscle of our GSDII patients showed variably increased amounts of the inactive forms (110 kDa and 95 kDa) and very low or absent amounts of the mature forms (76 and 70 kDa) (figure 12). The molecular weight of the mature and the intermediate forms appeared higher in patients' samples than in the control muscle, suggesting impaired processing of the mutant proteins.

LAMP-1 immunoblot analysis showed a similar quantity of the protein (110 kDa) in muscle from infantile-onset and late-onset GSDII patients when compared to control (figure 13). In two late-onset patients (20, 21) we observed an additional band at slightly lower molecular weight, possibly due to unknown cross-reacting material.

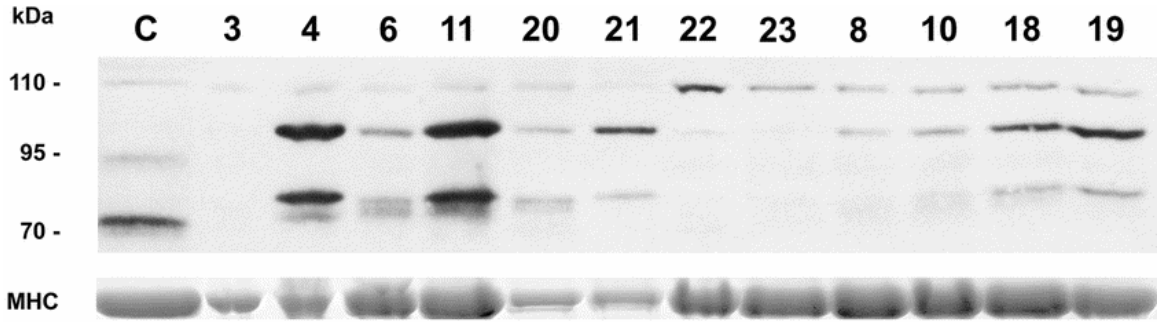

Figure 12. ***Acid [alpha]-glucosidase Western blotting using anti rh-GAA antibody.*** *Muscle samples from both infantile and late-onset GSDII patients (3, 4, 6, 8, 10, 11, 18, 19, 20, 21, 22, 23) show variably increased expression of the nonfunctional forms (110 kDa and 95 kDa) and very low or absent expression of the mature forms (76 kDa and 70 kDa). Furthermore, the molecular weight of the mature and the intermediate forms appeared higher in patients' muscle than in the control muscle (C), suggesting an impaired processing of the mutant proteins. Myosin heavy chain (MHC) amount in the post-transfer Coomassie blue stained gel was used to normalize the protein quantity. From: Nascimbeni AC et al., Neurology 2008; 70(8):617-26.*

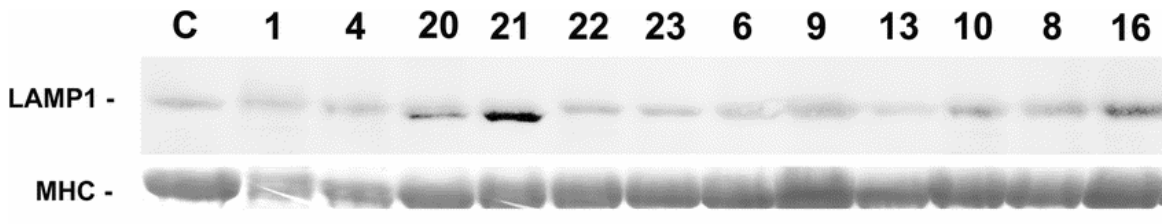

Figure 13. ***LAMP-1 Western blotting*** *showing a similar quantity of the protein in muscle from both infantile and late-onset GSDII patients (1, 4, 6, 8, 9,10, 13, 16, 20, 21, 22, 23) and control (C). Myosin heavy chain (MHC) amount in the post-transfer Coomassie blue stained gel was used to normalize the protein quantity. From: Nascimbeni AC et al., Neurology 2008; 70(8):617-26.*

6.5. Mutation screening in the GAA gene.

We first adopted the analysis of the most common mutations (the -32-13T>G leaky splicing mutation and the deletion of exon 18 in infantile-onset patients) using the allele-specific test, and then the SSCP screening of mutations throughout the gene. We identified a total of 20 different GAA gene mutations, widespread along the gene (table 3): 11 of null type (one splice site mutation, one leaky splice mutation, two nonsense, seven deletion or duplication causing frame shifting), two in-frame deletions, and seven of missense type. Seven of the mutations found are new, according to the Pompe mutation database (table 3). In 22 of the 23 patients both mutant alleles were identified (2 homozygous, 20 compound heterozygous) and in two cases only one mutant allele was found, indicating a very high detection rate (98%).

The most frequent mutant allele found in late-onset patients was the -32-13T>G leaky spicing mutation, which accounted for 42% of all mutant alleles: it was present in the heterozygote state in 18/20 of the late-onset patients, in the homozygote state in 1/20 in the late-onset patients, but it was absent in two late onset patients. The deletion in exon 18, which has been reported to recur in Pompe patients, was present only in 1/3 of our infantile-onset cases.

6.6. Genotype-phenotype correlations and effect of mutations on protein level and activity.

Of the three patients with infantile onset (table 3), Case 2 was a compound heterozygote for one null mutation and one deleterious deletion, and had very low level or residual enzyme activity. Case 3, with one missense and one null mutant allele, had absent

enzyme activity and absent mature forms of the protein. The clinical phenotype in this subgroup of patients was rather homogeneous and severe, despite the variability of mutation types, suggesting they have a deleterious effect on protein function.

In one childhood-onset patient (no. 4), the relatively high level of residual enzyme activity in muscle was due to the effect of a novel missense mutation, as the other was a null mutant allele.

In late-onset GSDII patients, who were mostly compound heterozygote for the IVS1 leaky splicing mutation, age at onset and rate of disease progression was variable, even between pairs of siblings. Indeed, one pair (nos. 18, 19) had a null mutation and onset of symptoms in the third decade; the other two pairs (nos. 11, 12 and 13, 14) had different missense mutations, onset after 40 years, and considerable difference between the members in both age at onset and clinical presentation. One patient (no. 20), who was homozygous for the IVS1 mutation, a rather uncommon genotype that is expected to allow the synthesis of a certain amount of residual enzyme activity, has developed severe proximal muscle weakness, but at age 33 still had no respiratory insufficiency.

We observed a relationship between the enzyme activity in muscle and the amount of the mature forms of the protein on Western blotting (figure 12): patients with the highest (>10%) residual enzyme activity (nos. 4, 6, 11, 18, 19) had detectable amounts of 70 and 76 kDa mature forms of the protein; conversely, patients with absent enzyme activity in muscle (nos. 1, 3, 22, 23) showed only the inactive forms of the protein (110 kDa and 95 kDa). Only in Case 1, homozygous for a missense mutation, we found mature but inactive forms of the protein, suggesting a loss of enzyme function rather than abnormal protein processing and expression.

Table 3 Mutations in the *GAA* gene

Patient no.	Phenotype	Mutant allele 1; mutation effect	Mutant allele 2; mutation effect
1	Infantile-onset	1933G>A, D645N, ex.14; missense	1933G>A, D645N, ex.14; missense
2	Infantile-onset	2481+102_2646+31del, ex.18; in-frame	2237G>A, W746X, ex.16; null
3	Infantile-onset	1396delG, V466fsX11, ex.9, null*	784G>A, E262K; ex.4; missense
4	Childhood-onset	461G>C, R154P, ex.2, missense	2227C>T, Q743X, ex.16; null
5	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	1465G>A, D489N, ex.10; missense
6†	Childhood-onset	IVS1(-32-13T>G), int.1; leaky splicing	1655T>C, L552P, ex.12; missense
7†	Childhood-onset	IVS1(-32-13T>G), int.1; leaky splicing	1655T>C, L552P, ex.12; missense
8	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	2604delG, L868fsX18, ex.18; null*
9	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	25delT, S9fsX33, ex.2; null*
10	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	258dupC, N87fsX9, ex.2; null
11‡	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	1927G>A, G643R, ex.14; missense
12‡	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	1927G>A, G643R, ex.14; missense
13§	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	307T>G, C103G, ex.2; missense
14§	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	307T>G, C103G, ex.2; missense
15	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	2219delTG, V740fsX55, ex.16; null
16	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	546+1G>T, int.2; splicing*
17	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	546+1G>T, int.2; splicing
18¶	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	2066_2070AGCCGdup, A691fsX6, ex.15; null
19¶	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	2066_2070AGCCGdup, A691fsX6, ex.15; null
20	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	IVS1(-32-13T>G), int.1; leaky splicing
21	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	2740dupC;2742dupG, Q914fx30, ex.19; null
22	Late-onset	1655T>C, L552P, ex.12; missense	not identified
23	Late-onset	IVS1(-32-13T>G), int.1; leaky splicing	2530_2541del, A844_L847del, ex.18; in-frame*

*Novel mutations.
†, ‡, §, ¶ = pairs of affected siblings.
From: Nascimbeni AC et al., Neurology 2008; 70(8):617-26.

Danon Disease

6.7. Patients

Of the nine male patients who showed vacuolar myopathy and hypertrophic cardiomyopathy and who were screened for both LAMP-2 protein defect and *LAMP2* gene mutations, three cases were affected by primary Danon disease. The latter showed a multiorgan deficiency of LAMP-2 protein and had null mutations in the *LAMP2* gene (table 4). Although all three patients had a positive family history (figure 10), in only one family (family 3), the mother of a male patient (patient 4) was alive at the time of the survey and was included in this study. The onset of cardiac symptoms occurred in late adolescence (except in the female patient, who had onset in the third decade) with exertional dyspnea and easy fatigability. All patients had WPW syndrome (figure 14; table 5), supraventricular arrhythmias, or atrial fibrillation. In two patients, ICD was implanted for ventricular tachycardia or fibrillation. Echocardiography showed concentric left ventricular hypertrophy in patients 1 and 2 and asymmetric hypertrophy with a moderate left ventricular maximal hypertrophy in patient 3 (figure 14; table 5). Three of the four patients developed severe and progressive heart failure associated with end-stage systolic dysfunction; two male patients died of heart failure at age 22 and 29, and the female was transplanted at 52 years of age.

The severity of the cardiomyopathy did not match the severity of skeletal myopathy; all male patients presented skeletal myopathy of variable severity, which, however, never compromised ambulation. Muscle weakness appeared generalized and involved both proximal and distal limb girdles, trunk, neck, and facial muscles. The duration of muscle disease was not related to age at onset of symptoms. A mild mental retardation was present in two male patients. Hepatic involvement, which was present in all patients, had been clinically evident since childhood in two patients (table 4). A milder involvement of organs other than striated muscles, such as kidney, spleen, and eye, was also observed. The female patient had mild myopathy and no mental retardation.

Table 4. Clinical, histopathological and LAMP-2 molecular data

Pt., sex	Family history	Age and symptoms at muscle onset	Age and muscle involvement at last examination	CK * level (U/L)	Age at hepato-pathy (yrs)	MR	LAMP2 gene nt. change	Amino acid change	Tissues available for LAMP-2 protein test	Skeletal muscle pathology
1, M	+	20, easy fatigability	22, marked trunk and limb atrophy, severe girdle muscle weakness	283	22	+	796-797insC	-	Muscle, fibroblasts	Diffuse atrophy and vacuoles
2, M	+	22, difficulty in climbing stairs	22, diffuse hypotrophy, waddling gait, Gowers' sign	558	9	-	680-701del	-	Muscle, heart	Vacuoles in many fibres
3, M°	+	18, easy fatigability	30, waddling gait, Gowers' sign, moderate neck, facial, distal limb weakness	1094	12	+	294G>A	W98X	Muscle, leukocytes	Vacuoles in many fibres
4, F°	+	26, easy fatigability	54, myalgia, mild muscle weakness and distal atrophy	normal	38	-	294G>A	W98X	Muscle, leukocytes, fibroblasts	No vacuoles

° : relatives (pt. 4 is the mother of pt. 3). * CK: Creatine Kinase (normal values 0-190 U/L). MR: mental retardation. nt: nucleotide.

From Fanin M et al., **Am J Pathol. 2006; 168(4):1309-20.**

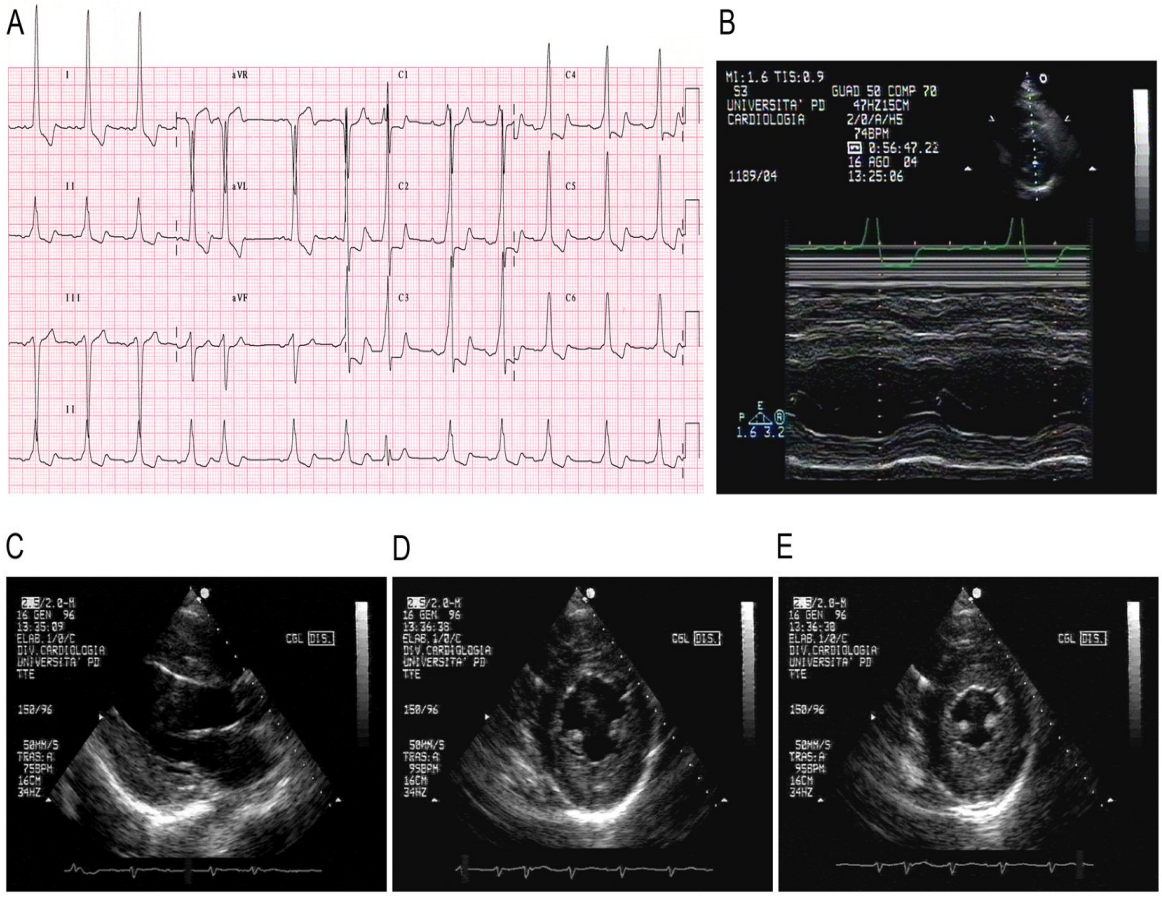

Figure 14. ***A:*** *An electrocardiogram from patient 3 demonstrates delta waves.* ***B:*** *M-mode echocardiogram in the same patient shows moderate asymmetric hypertrophy of the septum. The parasternal, long axis (**C**) and short axis views at the level of papillary muscles (**D**, diastolic frame; and **E**, systolic frame) of an echocardiogram from patient 2 show concentric hypertrophy. From Fanin M et al.,* Am J Pathol. 2006; 168(4):1309-20.

Table 5. Cardiological, electrocardiographic and echocardiographic data

Pt. sex	Age at HCM diagnosis	Symptoms at onset	ECG	Holter ECG	LAD* (mm)	LVEDD* (mm)	LVEF* (%)	Maximal LVWT* (mm)	PWT* (mm)	Pace-maker (years)	Age and cause of death
1, M	22	Syncope	WPW	AF, AVB (pause 5350 ms)	58	63	20	19	18	Yes (22)	22, heart failure
2, M	22	Syncope, NYHAII	WPW	AF, SVT	38	60	46	25	22	ICD (24)	29, heart failure
3, M	28	Fatigability	WPW	SV arrhythmia	39	53	50	21	14	-	-
4, F	36	Fatigability	WPW	AF, NSVT	55	63	33	10	10	ICD (51)	52, heart transplant §

*LVEDD: left ventricular end-diastolic diameter. LVEF: left ventricular ejection fraction (normal values = 53-63%). LVWT : left ventricular wall thickness. PWT : posterior wall thickness. WPW : Wolff-Parkinson-White syndrome. AF: atrial fibrillation. AVB : atrio-ventricular block. SVT : supra-ventricular tachicardia. NSVT : not sustained ventricular tachicardia. ICD: implantable cardioverter defibrillator. LAD: left atrium diameter. * Echo values are referred to last control at our Department. §: transplanted at age 52 and currently alive at age 55. From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

6.8. Skeletal and Cardiac Muscle Histopathology

Skeletal muscle histopathology showed extensive muscle fiber vacuolization and degeneration and focal storage of PAS-positive material. The degree of fiber vacuolization and of overall muscle architectural derangement was variable in the three male patients (table 4; figure 15). Patient 1 had generalized fiber atrophy and degeneration. In addition, the majority of fibers (46%) contained vacuoles that were sometimes large enough to replace all of the cytoplasm. Patients 2 and 3 had multiple, relatively small-sized vacuoles that were seen in a smaller proportion of fibers (24%). Female patient 4 had no fiber vacuolization. The accumulation of PAS-positive material and lysosomal acid phosphatase reaction were evident in small vacuoles of fibers undergoing degeneration. Cardiac muscle histopathology in patients 2 and 4 showed hypertrophic cardiomyocytes with enlarged and picnotic nuclei, empty cytoplasmic vacuoles, and myofibrillar disarray (figure 15). Extensive replacement fibrosis associated with necrotic cardiomyocytes and collections of invading macrophages was observed in both patients. Increased acid phosphatase staining indicated active degeneration of cardiomyocytes.

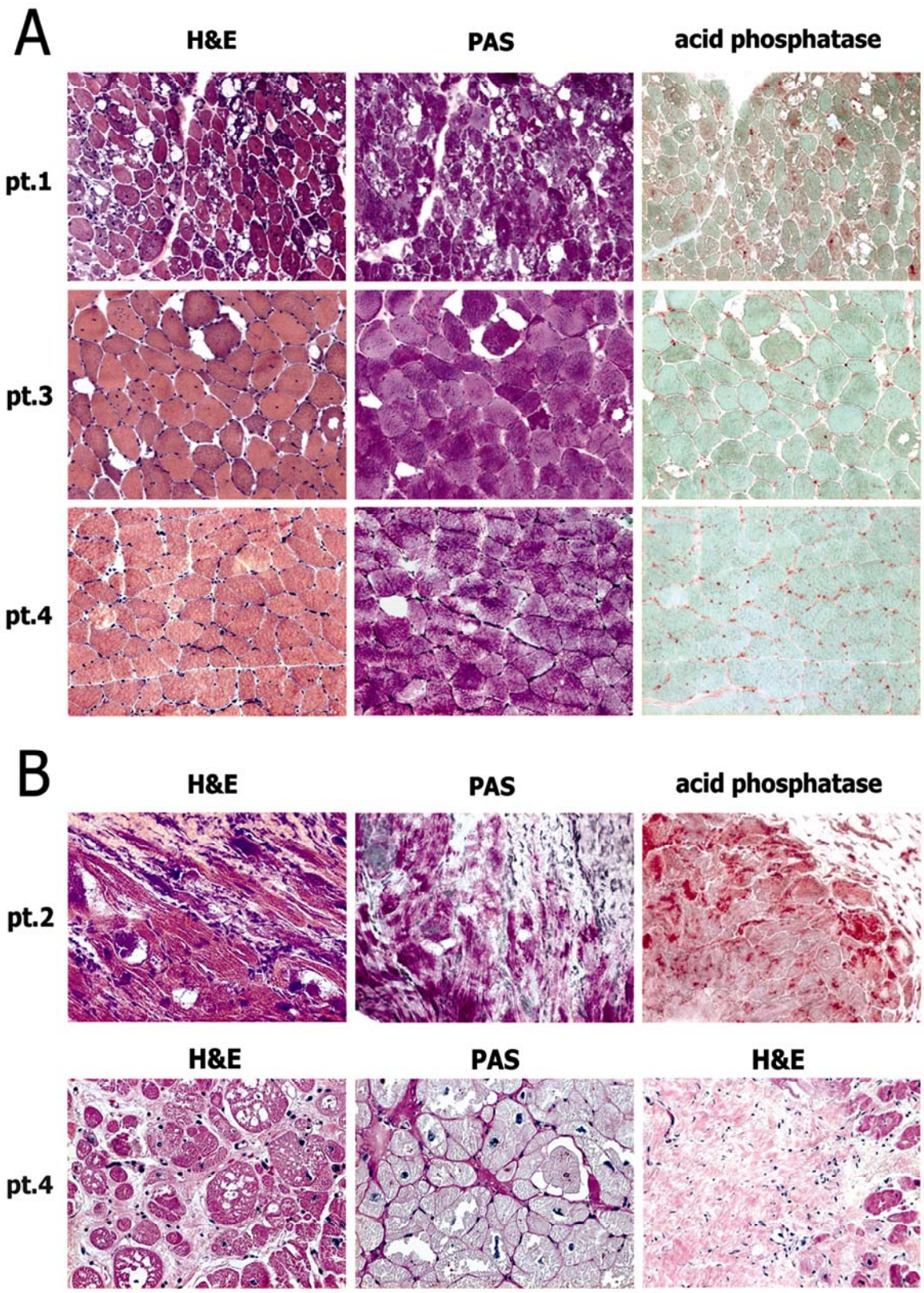

Figure 15. ***Routine histopathological stains of skeletal and cardiac muscle biopsy sections. A:*** *Different degrees of skeletal muscle fibers involvement. Patient 1 had diffuse fiber atrophy, extensive fiber vacuolization and degeneration, accumulation of PAS-positive material, and positive acid phosphatase*

activity in some atrophic fibers; patient 3 showed a milder degree of vacuolization and many fibers with apparently normal features; patient 4 showed mild myopathic changes but no fiber vacuolization or degeneration. ***B:*** *Vacuolization and autophagic degeneration of cardiomyocytes and extensive replacement fibrosis in cardiac muscle from patients 2 and 4. Collections of infiltrating macrophages and focal PAS-positive accumulation were also present. Microscope magnification, x400. From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

6.9. Immunohistochemical studies

Immunofluorescence analysis on both the skeletal and cardiac muscle biopsy from the male patients showed a complete absence of LAMP-2 protein when compared with controls in which there was diffuse intracytoplasmic reaction (figure 16). Conversely, in female patient 4, LAMP-2 immunolabeling was of intensity similar to controls. The labeling of proteins typical of plasma membrane and basal lamina (caveolin-3 [*trans*-membrane], dystrophin [intracellular], and laminin-A [basal lamina]) seemed to be increased in the cytoplasm because of the proliferation of lysosomes and vacuoles with their membranes. Lysosomal and vacuolar membranes were often in continuity with the plasma membrane (caveolin-3 and dystrophin) (figure 16) but not with the nuclear membrane (lamin A/C and emerin). Muscle fiber regeneration and phagocytosis were not active. No proliferation of either Golgi apparatus or early endosomes was observed. Desmin was strongly expressed in the cytoplasm of vacuolated fibers. MHC class I molecules were expressed in the vacuolar membrane.

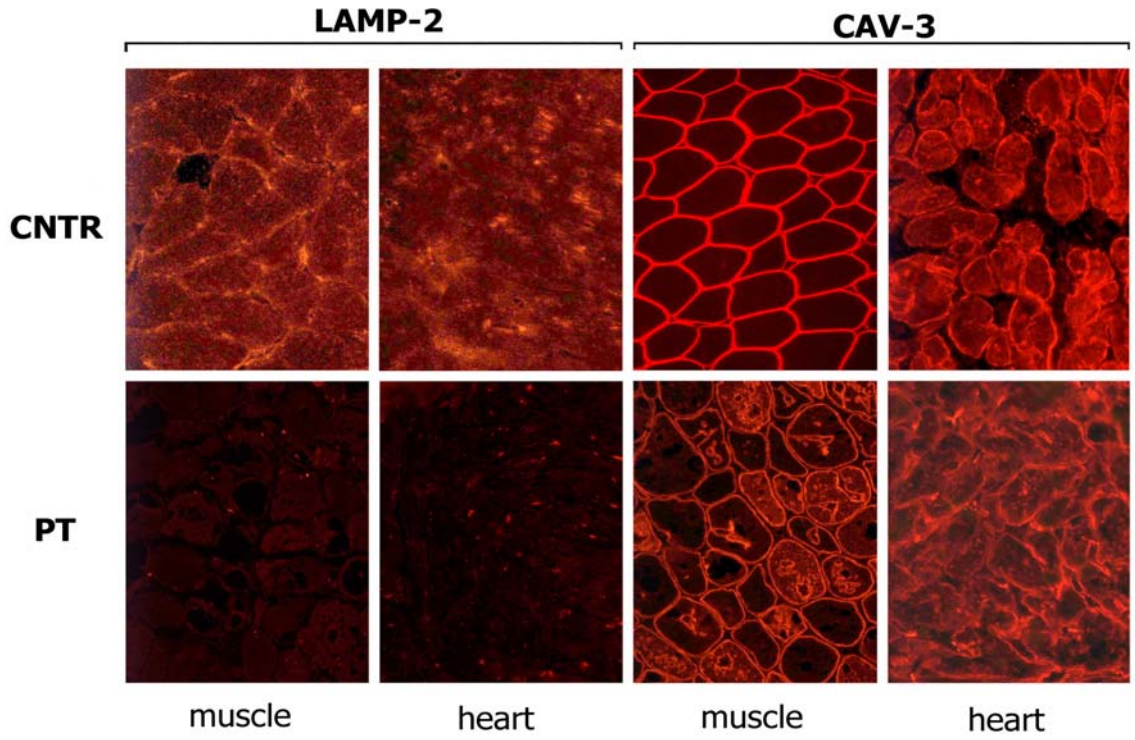

Figure 16. ***Cryostat sections of skeletal and cardiac muscle from patient 2 (PT) and control (CNTR), immunolabeled with antibodies against LAMP-2 and caveolin-3 (CAV-3)****. LAMP-2 reaction was absent in muscle and heart from the patient compared with the diffuse positive reaction observed in control. Caveolin-3 immunolabeling was positive in the plasma membrane of muscle fibers and cardiomyocytes of both patient and control. In the skeletal muscle from Danon disease, caveolin-3 labeling was observed also in the membrane of the lysosomes that delineate most vacuoles. The cellular membranes that make up both the plasmalemma and the lysosomes are often in continuity. Microscope magnification, x400. From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

6.10. Western Blot Analysis

In the skeletal muscle from all male patients, LAMP-2 protein was virtually absent (figure 17). On the contrary, the muscle from the female heterozygote patient showed LAMP-2 protein levels that were not significantly reduced compared with control. Fibroblasts and leukocytes showed absent LAMP-2 protein in male patients and nearly normal amounts of protein in the heterozygote female patient. To check the possibility that residual LAMP-2 immunolabeling in muscle tissue from male patients is attributable to a cross-reaction with the highly homologous LAMP-1 protein, we performed duplicated Western blotting for LAMP-1 and LAMP-2 that showed bands of similar molecular weight (figure 17). A weak

binding cross-reaction between these two proteins or between LAMP-2 and some unknown immunoreactive material cannot be excluded. In control muscle, LAMP-2 was considerably more highly expressed than LAMP-1 (figure 17). LAMP-1 protein levels were similar in control and in patients' muscle. LAMP-1 immunolabeling was similar in the leukocytes of patient and control.

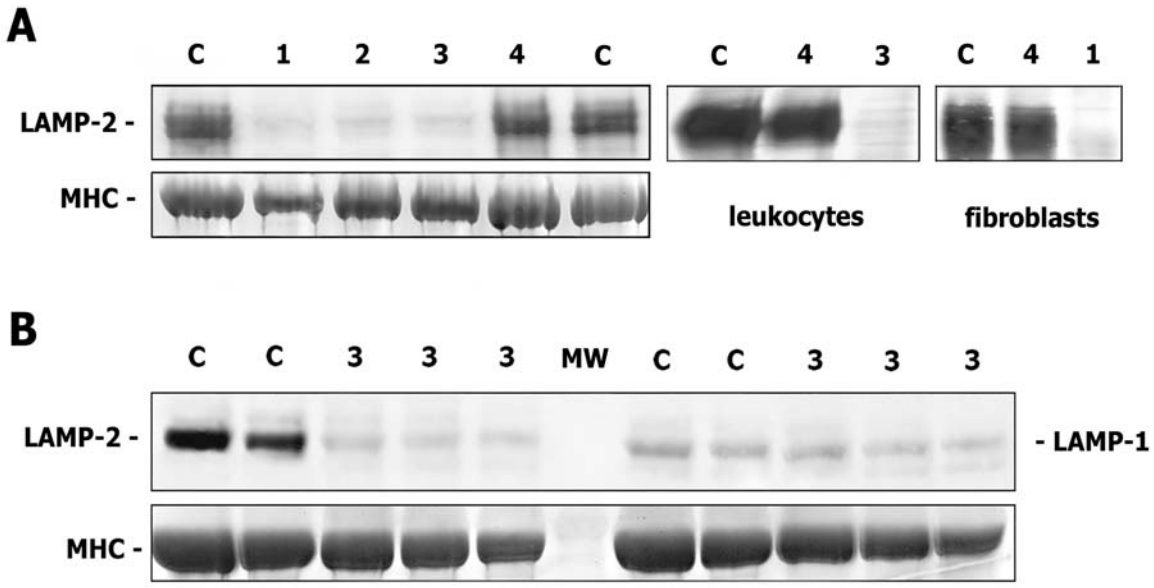

Figure 17. ***LAMP-2 and LAMP-1 Western blotting. A:*** *Skeletal muscles from patients 1 through 4 and control (C) showed that all male patients (1 through 3) have virtually absent LAMP-2 protein, whereas the female heterozygote patient 4 showed nearly normal amounts of protein when compared with control. Leukocytes and fibroblasts from male patients 3 and 1, respectively, showed virtually absent LAMP-2 protein, whereas the corresponding samples from the female patient 4 showed nearly normal amounts of protein when compared with controls.* ***B:*** *Skeletal muscle from control (C) and patient 3 loaded in duplicate and labeled with antibodies against LAMP-1 (right) and LAMP-2 (left). Control and patient samples are shown with different loading quantity of protein, as judged by MHC band in the Coomassie blue-stained gel. From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

6.11. LAMP2 Gene Mutation Analysis

LAMP2 gene mutations were identified in all three male patients who showed LAMP-2 protein deficiency and in the heterozygote female patient 4. Each patient showed a different and previously unreported mutation (table 4; figure 18): a 22-bp deletion in exon 5 (680–701del), causing a frame-shift (fsX8); a C nucleotide insertion in exon 6 (796–797insC), causing a frame-shift (fsX7); and a nucleotide substitution at position 294 in exon 3 (294G>A), causing the change of a tryptophan to a stop codon at position 98 (W98X).

The mutations found by sequence analysis were confirmed by allele-specific tests or by electrophoretic determination of amplicon size (figure 18). All three mutations produce null alleles (nonsense or frame-shift mutations), which are predicted to prematurely truncate protein synthesis and result in the loss of the transmembrane domain (figure 19).

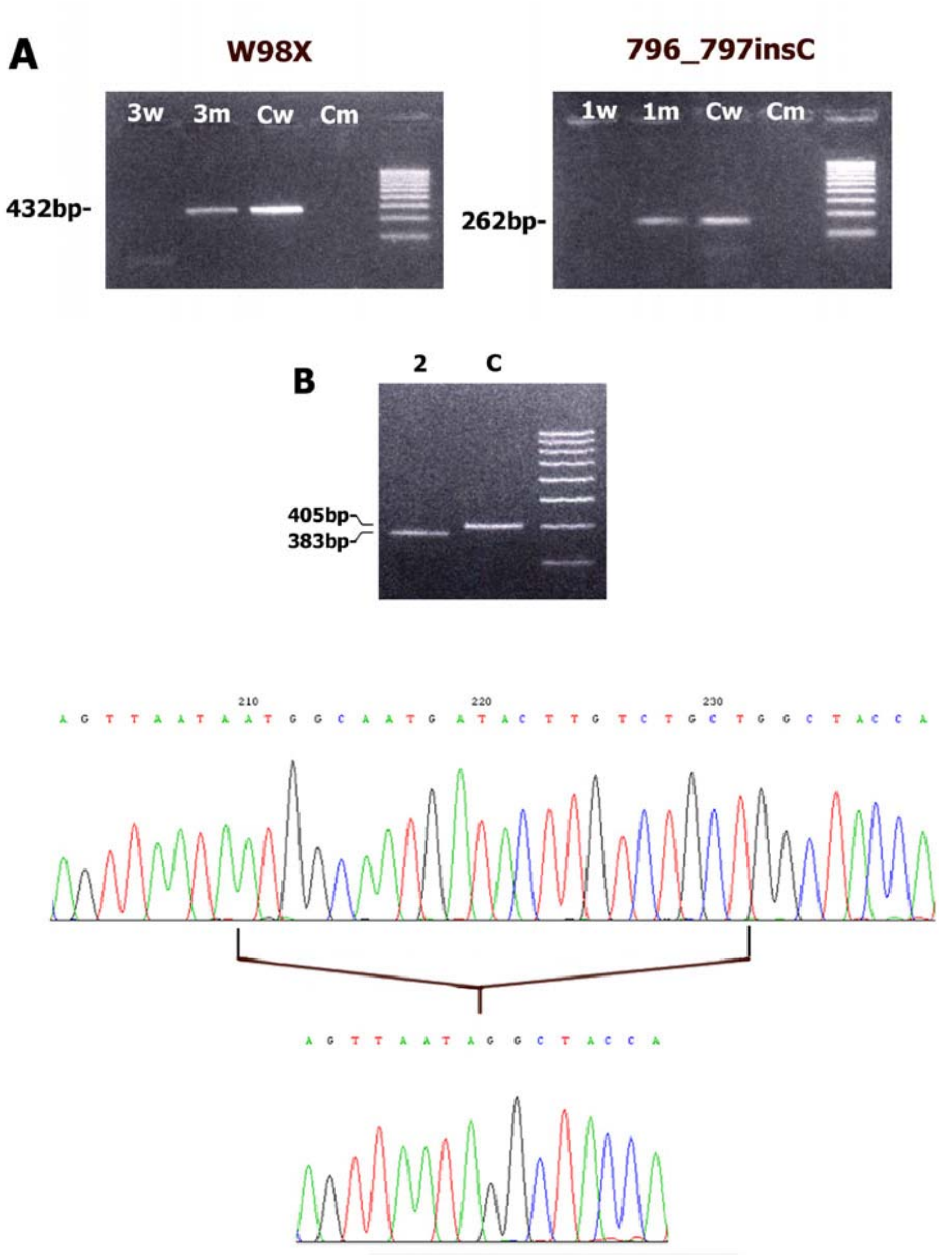

Figure 18. ***LAMP2 gene mutation analysis. A:*** *ARMS-PCR tests for 294G>A, W98X mutation (left) and 796–797insC mutation (right). Patients 3 and 1 and control (C) were tested for both the wild-type (w) and the mutant (m) alleles of the corresponding mutation found. Patients 3 and 1 are hemizygote for W98X and 796–797insC mutations, respectively.* ***B:*** *Left figure shows the agarose gel electrophoresis of the PCR product from exon 5; the amplicon from normal control (C) was 405 bp, whereas the amplicon from patient 2 was 383 bp (indicating a 22-bp deletion). DNA sequence analysis in* patient 2 showed the 22-bp deletion (680–701del). *From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

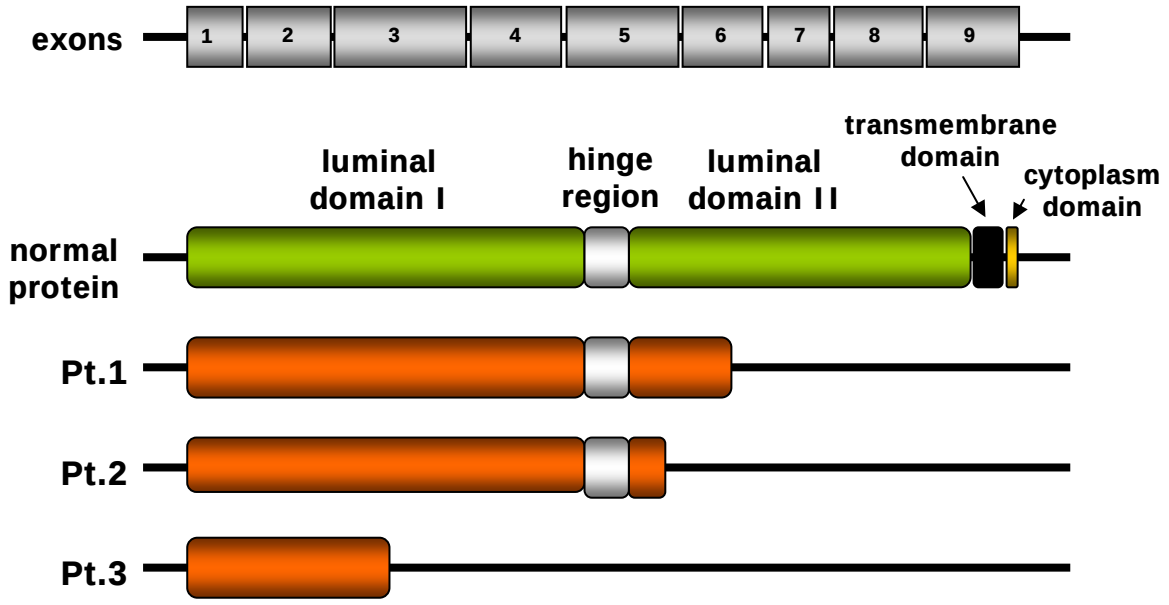

Figure 2. ***Schematic representation of LAMP-2 protein structure*** *with the corresponding domains: a large luminal domain divided into three subdomains (I, hinge region, and II), a small lysosomal transmembrane domain, and a small cytoplasmic domain. The bottom panel shows the truncated proteins caused by null mutations in our three patients in whom the transmembrane domain was lost. From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

6.12. RNA Studies

To study the transcriptional effect of premature truncating codon mutations observed at genomic level, we first analyzed *LAMP2* mRNA in patients' muscle by RT-PCR, using actin transcript as an internal standard for RNA quantity. Because *LAMP2* mRNA was significantly reduced in all patients when compared with control, a semiquantitative RT-PCR was used to estimate the extent of transcript down-regulation. Aliquots were taken at different times during the exponential phase of the PCR. After ß-actin normalization, *LAMP2* mRNA levels were 65, 43, and 21% in patients 1, 2, and 3, respectively, compared with control (figure 20). Considering all male patients in our study, the lowest *LAMP2* mRNA expression was found in patient 3, who has the most proximal premature truncating codon gene mutation (figure 19) and who presented the least severe phenotype.

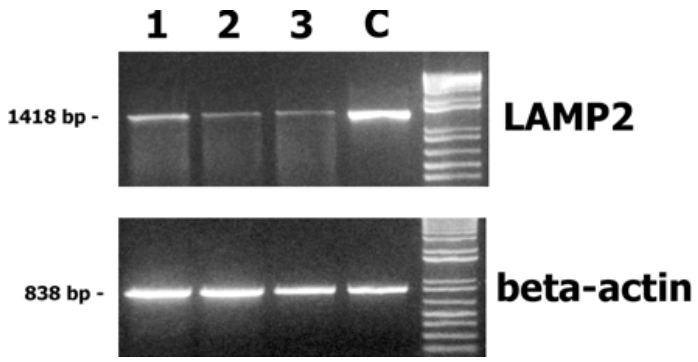

Figure 3. ***RT-PCR analysis of LAMP2 in muscle from patients 1-3 and control.*** *The LAMP2 mRNA is highly down-regulated in patients compared with control (PCR aliquot taken at 35 cycles during the exponential phase of the reaction). ß-Actin transcript was used as an internal standard for RNA quantity and normalization (PCR product at 25 cycles during the exponential phase). After ß-actin normalization, LAMP2 mRNA levels in patients 1, 2, and 3 were 65, 43, and 21% of control, respectively.*

6.13. X-Chromosome Inactivation

X-chromosome inactivation analysis was performed in three independent experiments on both skeletal muscle and leukocytes from the heterozygote female patient (no cardiac tissue was available for the study). After digestion, (CfoI_ HpaII_) PCR product is obtained only from the inactive X-chromosome. After compensation for unequal amplification of the two peaks caused by different product size, the inactivation ratio is calculated for the two CAG repeat alleles of the X-chromosomes. A random pattern of inactivation was found in both tissues analyzed: the mean skewing rate was 66% in skeletal muscle (62, 58, and 78% in the three independent experiments) and 60% in blood leukocytes (62, 48, and 69%) (Figure 21).

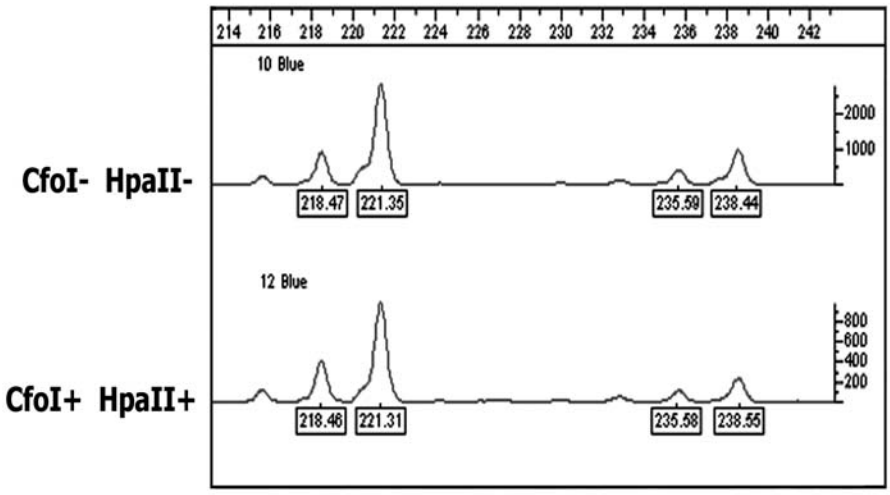

Figure 41. ***X-chromosome inactivation pattern in skeletal muscle from patient 4 using the human androgen receptor (AR) polymorphism (CAG repeat).*** *Undigested (CfoI– HpaII–) and digested (CfoI+ HpaII+) DNA samples are shown in the top and bottom plots, respectively. After enzyme digestion, only the nonmethylated (active) allele disappears. The plots show a quantitative measure of the fluorescent PCR products when analyzed on an automated sequencer and quantified by GeneScan software. The comparison of the areas of the peaks corresponding to the two alleles considered (221 and 238 bp) was similar before and after DNA digestion, indicating a random X-chromosome inactivation pattern. From Fanin M et al., Am J Pathol. 2006; 168(4):1309-20.*

7. Discussion

Glycogen Storage Disease Type II

In our series of patients both the clinical phenotype and the biochemical and molecular features confirmed the diagnosis of GSDII, despite the fact that muscle fiber vacuolization was not always present. Furthermore, the degree of fiber vacuolization was not related to the clinical course and was also variable in different muscles from the same patient,[137,138] suggesting that this morphologic change is not completely reliable when diagnosing and predicting the clinical severity of the disease. It is therefore conceivable that, based on fiber vacuolization alone, some GSDII cases might either have escaped diagnosis (leading to the frequency of the disease being underestimated) or have been subject to delayed diagnosis. Thus, biochemical and molecular analysis should be strongly encouraged when the clinical features suggest disease involvement even though muscle fiber vacuolization is not apparent.

In this study we investigated the cause of the striking clinical heterogeneity that results from acid maltase deficiency. The levels of residual enzyme activity in muscle did not account for the different phenotypes: absent enzyme activity was found both in infantile and in late-onset GSDII. The reason why some late-onset patients have low levels of enzyme activity could be explained by tissue differences in factors affecting the processing and intracellular transport of mutant enzymes.

Genotype-phenotype correlations in our series of patients showed that although the nature of the mutation sometimes matched the phenotype, in most cases, the disease phenotype seemed to be hard to predict on the basis of gene mutations alone. Furthermore, we confirmed that the age at onset and the disease course was quite different in patients with identical genotype (e.g., siblings), suggesting a role of genetic background and modifying genes or exogenous factors (nutritional, physical exercise, lifestyle) on GAA gene expression, which might produce a slight shift in the enzyme biosynthesis rate.

One important and novel result from our study came from the Western blot analysis of the different maturative forms of acid [alpha]-glucosidase protein in the muscle from patients with GSDII. Indeed, previous studies on the effect of mutations on protein processing and expression were all conducted on knock-out mice after ERT,[19] on human cellular models,[61] or on mutant fibroblasts.[63,71,139] We have demonstrated that the muscle from patients with GSDII has a predominant expression of inactive forms of acid [alpha]-glucosidase protein and severely reduced or absent levels of the mature forms. Furthermore, the residual

amount of the mature forms of the protein on blotting correlated with the level of enzyme activity in muscle. We first report a different molecular weight of the mature and the intermediate forms of the protein between patients and controls that we attribute to an excessive sialylation of mutant proteins. This is likely caused by a delayed transport and longer transit of the inactive proteins in the Golgi where the sialyltransferases are localized.[61] The different pattern of expression of the various forms of the protein in patients with GSDII depends mainly on the effect of the mutations, but also on a complex regulation at transcriptional and post-translational level. Indeed, the mutation might affect the synthesis, degradation, or dislocalization of the protein, as well as its enzyme function. The clinical course of the disease, primarily determined by the amount of functional protein,[140,141] might also be influenced by a number of secondary factors.

Another interesting part of our study is the morphologic analysis of the different cellular organelles. We observed that, in both infantile and late-onset patients, there is an enhanced proliferation of the Golgi apparatus. Proliferation of the Golgi was previously described in adult patients with GSDII[37] and it was shown that Golgi derived membranes contribute to the membranes of the autophagic vacuoles. We have demonstrated that patients with GSDII display increased amounts of inactive forms of the protein; these nonfunctional proteins cannot be correctly targeted from Golgi to lysosomes and are likely to be retained in the Golgi complex, causing it to proliferate. On the other hand, we did not find any increased expression of LAMP-1 in patients with GSDII, possibly due to the fact that only a minor proportion of mutant enzyme protein is able to reach the lysosomes. An increased size of the lysosomes was however demonstrated in GSDII,[87] as a result of accumulation of undigested material and the consequent activation of secondary lysosomal enzymes.

Interestingly, we observed a differential degree of dysfunction of endocytic and autophagic pathways in patients with infantile and late-onset GSDII. In late-onset acid maltase deficient muscle, vacuolar membranes expressed sarcolemmal proteins such as caveolin-3 and dystrophin (previously classified as type 2 vacuoles).[142] Conversely, in the infantile form of the disease the lysosomal membrane did not show caveolin-3 expression (type 4 vacuoles, lakes of glycogen). These features are possibly due to reduced membrane proliferation and vesicular movement in the overcrowded muscle fibers of Pompe disease, and to the membrane remodeling occurring only in patients with late-onset GSDII. In adult acid maltase deficient muscle, the expression of sarcolemmal proteins on the vacuolar membranes is likely to strengthen them and would be a protective mechanism to prevent

membrane rupture during fiber contraction and the intracellular release of acid hydrolases that might cause muscle fibers necrosis.

Our results agree with the observation that in young GAA knock-out mice the autophagic vacuoles containing lysosomes have no clear membrane boundaries,[19] suggesting that autophagy might be upregulated to remove damaged lysosomes. A recent study has also shown abundant autophagosome formation and areas of autophagic buildup of a wide range of size in muscle from Pompe patients.[24]

Infantile and late-onset GSDII muscle appears to differ in the expression of sarcolemmal protein in the vacuolar membranes. This observation is important because the pathogenesis of the autophagosomes has not yet been fully investigated. Autophagy and membrane remodeling, which is peculiar to late onset disease, might modify a clear response to enzyme replacement therapy and, also, compartmentalize the delivery of the recombinant enzyme.

Danon Disease

About 30 families with Danon disease have been described in whom LAMP-2 protein deficiency on various cell types and/or *LAMP2* gene mutations have been identified.[89,98,99,102,103,105,108,113,129,143,144] We exploited the availability of a tissue bank with about 6000 skeletal muscle biopsies to select patients who presented vacuolar myopathy associated with hypertrophic cardiomyopathy for subsequent screening of both LAMP-2 protein deficiency and gene mutations. The study of nine patients with vacuolar myopathy and hypertrophic cardiomyopathy led to the identification of three novel families with Danon disease. Although Danon disease is considered very rare in the general population, its frequency is relevant (33%) among patients presenting with both vacuolar myopathy and hypertrophic cardiomyopathy, suggesting that the number of patients reported so far worldwide could be underestimated.

LAMP-2 protein deficiency was demonstrated in the explanted heart of affected patients,[89] but Danon disease could be diagnosed by LAMP-2 immunofluorescence even from the very small tissue samples that are collected during heart catheterization. The search for LAMP-2 deficiency in endomyocardial samples (when available) should be pursued in patients with hypertrophic cardiomyopathy especially when it is associated with skeletal myopathy (even with only high CK) and/or other organ impairment; moreover, a diagnostic skeletal muscle biopsy (less invasive and easier to obtain than myocardial biopsy) should be suggested in potential Danon disease. The identification of LAMP-2 protein deficiency in

such tissue samples is very important because of the consequences of an early molecular diagnosis for both clinical evaluation (therapy and prognosis) and genetic counseling. In two of our male patients, the prognosis was poor for the rapid progression toward heart failure and death. The female patient was transplanted at age 52, and she is still alive.

In all of our male patients, we demonstrated a generalized LAMP-2 protein deficiency, which was detected in striated muscle, in fibroblasts, and in leukocytes. This result should be expected because LAMP-2 is a ubiquitous protein and because null gene mutations were found in our cases; nevertheless, few patients have been reported in which splice site mutations led to LAMP-2 protein synthesis in leukocytes.[99]

Our first conclusion is that the collection of leukocytes is much less invasive than skeletal and myocardial biopsy but it is equally useful for LAMP-2 protein diagnosis in males. *LAMP2* gene mutation analysis ensures complete sensitivity, whereas LAMP-2 immunoblot could fail to identify the patients with a cardiac-predominant phenotype due to partially functional mutant proteins.[99] However, clinicians should consider leukocyte immunoblot analysis as a diagnostic screening option when suspecting Danon disease in males for these reasons: 1) it is expected to have high sensitivity because LAMP-2 protein deficiency was found in different tissues of the large majority of mutant patients;[present study,89,98,102,105,108,113,114,143,144] 2) it should have high specificity because there are no reports of LAMP-2 protein deficiency in other disorders; and 3) it is much less expensive and time consuming than mutation screening.

The second conclusion is that the detection of LAMP-2 deficiency in a variety of cells/tissues supports the clinical evidence that Danon disease is a multisystemic disorder.[98,102] Accurate clinical history collection revealed that hepatic involvement was present in all of our patients (in two cases, it was the first clinical sign), indicating that also in the human disease, as in the *LAMP2* knockout mouse,[126,143] different organs other than striated muscles may suffer from LAMP-2 deficiency.[98,102]

Another important result from our study is that female patients with Danon disease might escape the diagnosis unless mutation identification is obtained: in our heterozygote patient, muscle pathology and LAMP-2 protein analysis was inconclusive, and molecular diagnosis was pursued because of her affected son. The only other female patient so far analyzed showed a reduction of LAMP-2 protein levels in skeletal muscle[114] ; the discrepancy with our results might originate from a different X-chromosome inactivation pattern, a different gene mutation, or other unknown modulating factors. It is thus

conceivable that a number of female patients with Danon disease (especially isolated cases) could remain under-diagnosed.

The most prominent histopathological feature of Danon disease is the vacuolization of muscle fibers. We confirm that the extent of these changes is related to the degree of clinical muscle involvement,[114] suggesting that the accumulation of autophagic material within muscle fibers correlates with disease progression. We observed that the vacuolar membrane occasionally merged with the sarcolemma and was delineated by the basal lamina. The expression of sarcolemmal-specific proteins in the vacuolar membrane could be related to their function as a mechanical reinforcement.[142] As in other disorders, one could speculate that mutant LAMP-2 protein could not be correctly targeted to the lysosomal membrane and could be retained in the Golgi complex or endoplasmic reticulum, but we showed that this is not the case.

The mechanism leading from *LAMP2* mutations to clinical phenotype is an intriguing aspect in the study of Danon disease that requires further studies. One could speculate that, depending on different types of mutations, either mutant LAMP-2 proteins could be degraded or their synthesis could be abolished by the nonsense-mediated RNA decay mechanism in the nucleus.[145-147] Although the demonstration that nonsense-mediated RNA decay mechanism is at play in this disease is beyond the purpose of our study, an eventual synthesis of low levels of prematurely truncated proteins in our cases would be followed by cytoplasmic protein degradation because of the lack of the transmembrane domain.

Although the clinical triad of hypertrophic cardiomyopathy, skeletal myopathy, and mental retardation typically characterizes the disease, a different degree of skeletal and cardiac muscle involvement can be observed between inter- and intrafamilial patients. Although the cardiac involvement associated with a high CK level of skeletal muscle origin is a constant feature in male patients, in females, the possible involvement of different organs other than heart (muscle and brain) is variable. Such unpredictability can hardly be attributed to skewed X-chromosome inactivation, because we showed that X-chromosome inactivation occurred at random in different tissues in our female patient. An alternative hypothesis is that brain and striated muscles have high-energy requirements, high protein turnover, and low regenerative capacity and are therefore likely to be more prone to damage. However, because LAMP-2 protein function is not fully understood, the reason why specific tissues are more vulnerable in Danon disease remains unclear.

It is not known how *LAMP2* gene mutations produce a dominant effect. A model involving a dominant-negative effect of mutation is incompatible with X-linked inheritance where

female patients express about 50% of protein level. Although the absence of mutant protein in hemizygote male patients indicates a loss-of-function, the phenotype in heterozygote females could originate either from skewed inactivation of X-chromosomes or from haploinsufficiency. Because preferential X-chromosome inactivation of wild-type allele could not explain why the disease is clinically manifest in all female heterozygotes (at least in the heart), the dominant inheritance is likely to be due to haploinsufficiency, where the normal protein product of the wild-type allele does not reach the threshold level necessary for normal function.

The pathogenetic mechanism underlying the disease is still unclear. An unsolved issue is whether the LAMP-2 protein deficiency might cause structural or functional lysosomal impairment. One hypothesis is that the lysosomal membrane could be structurally normal but that abnormal LAMP-2 function might cause increased lysosomal storage, which, in turn, could trigger the rupture of membrane with the consequent release of acidic hydrolases into the sarcoplasm.

8. Publications

1. Fanin M, Nascimbeni AC, Fulizio L, Spinazzi M, Melacini P, Angelini C.
Generalized lysosome-associated membrane protein-2 defect explains multisystem clinical involvement and allows leukocyte diagnostic screening in danon disease.
Am J Pathol. 2006 Apr;168(4):1309-20.

2. Fanin M, Nascimbeni AC, Angelini C.
Muscle protein analysis in the detection of heterozygotes for recessive limb girdle muscular dystrophy type 2B and 2E.
Neuromuscul Disord 2006 Nov;16(11):792-9.

3. Pizzanelli C, Mancuso M, Galli R, Choub A, Fanin M, Nascimbeni AC, Siciliano G, Murri L.
Epilepsy and limb girdle muscular dystrophy type 2A: double trouble, serendipitous finding or new phenotype?
Neurol Sci. 2006 Jun;27(2):134-6.

4. Fanin M, Nascimbeni AC, Angelini C.
Screening of calpain-3 autolytic activity in LGMD muscle: a functional map of CAPN3 gene mutations.
J Med Genet. 2007 Jan;44(1):38-43.

5. Angelini C, Nascimbeni AC.
Late-onset GSDII with novel GAA gene mutation.
Clin Genet. 2007 Apr;71(4):374-5.

6. Fanin M, Nardetto L, Nascimbeni AC, Tasca E, Spinazzi M, Padoan R, Angelini C.
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