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Workshop report

251st ENMC international workshop: Polyglucosan storage myopathies 13–15 December 2019, Hoofddorp, the Netherlands

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1. Introduction

The organizers of the 251st ENMC workshop welcomed 22 participants including a patient representative and three industry representatives from European countries, Israel and the United States of America to the first workshop on polyglucosan storage myopathies, which are a group of glycogen storage diseases with aggregation of polyglucans that resemble glycogen, but are less branched. Polyglucosan is an amylopectin-like polysaccharide associated with defective glycogen metabolism and, unlike normal glycogen, it is to some extent resistant to α-amylase digestion. It has a characteristic, partly filamentous appearance under the electron microscope. Polyglucosan may aggregate into dense inclusions known as polyglucosan bodies. Its accumulation can be found in various tissues and to some degree in normal aging, but it is also the hallmark of some diseases associated with defects in glycogen metabolism. These diseases frequently involve both skeletal and cardiac muscle tissue, causing myopathy with muscle weakness and wasting and cardiomyopathy with arrhythmia, conduction block, and cardiac failure. Although the diseases have the muscle polyglucosan storage in common, some of them also affect other tissues such as the brain, which in some cases cause the main symptoms.

The main aim of the ENMC workshop was to create a multidisciplinary discussion forum among clinical and basic researchers working on polyglucosan storage-related issues. The specific aims of the workshop were:

- Update on the current concepts regarding glycogenin and glycogen synthesis.
- Comprehensive overview of diseases with polyglucosan accumulation.
- Discussion on ongoing and future research on pathophysiology and treatment of myopathies with polyglucosan accumulation.

The attending experts discussed recent discoveries of new and established disease entities, their clinical and genetic background, pathologic findings, and pathophysiological mechanism. This was followed by discussions about pharmacological or gene treatment options derived from current knowledge of disease mechanisms. Several animal models were described and the promising results from preclinical treatment studies in these animal models with the aim to reduce the amount of polyglucosan storage to cure the diseases or prevent progression. After a brief introduction and welcome from Alexandra Breukel, the managing director of ENMC, the workshop started with an overview of the clinical background.

2. Overview of polyglucosan storage disorders

Pascal Laforêt and Edoardo Malfatti presented an overview of muscle glycogen storage disorders (GSD) and polyglucosan storage myopathies. Most frequent GSDs are *Pompe disease* (GSD II), debranching enzyme deficiency or *Cori-Forbes disease* (GSD III), and myophosphorylase deficiency or

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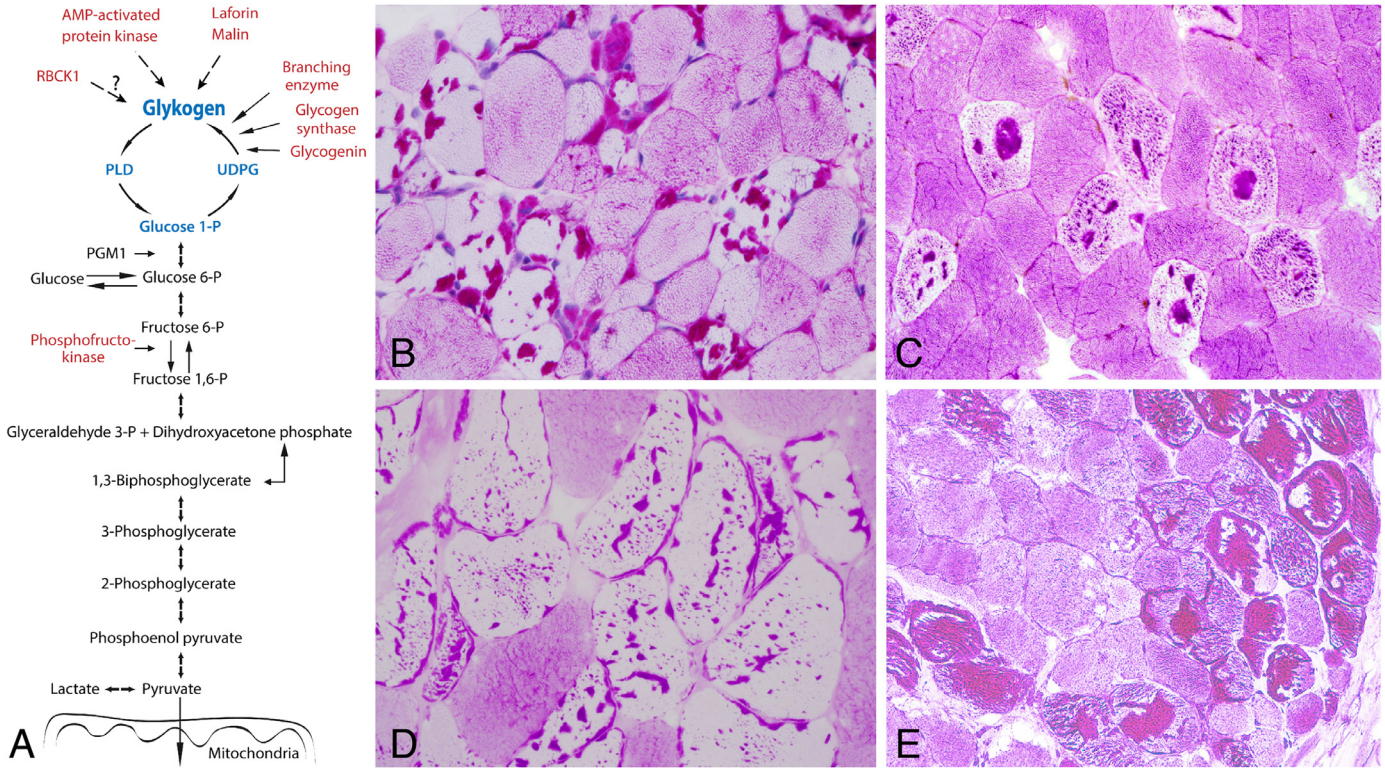

Fig. 1. Glycogen metabolism and polyglucosan storage disorders. (A) Simplified flowchart of glycogen metabolism. The most important enzymes involved in polyglucosan storage myopathies are indicated in red color. (B) Muscle biopsy from a patient with branching enzyme deficiency (PAS). (C) Muscle biopsy from a patient with glycogenin-1 deficiency (PAS). (D) Muscle biopsy from a patient with RBCK1 deficiency (PAS). (E) Muscle biopsy from a horse with autosomal dominant equine polysaccharide storage myopathy type 1 due a mutation in the gene encoding glycogen synthase (PAS).

McArdle disease (GSD V). In GSD III and GSDV there is mainly a cytoplasmic accumulation of glycogen, whereas glycogen storage is located mainly in the lysosome in Pompe disease. In other rarer glycogenosis, the abnormal glycogen accumulation appears as polyglucosans (Fig. 1).

Andersen disease (GSD IV) or branching enzyme deficiency is an autosomal recessive disorder associated with mutation in the *GBE1* gene. GSD IV has a wide range of clinical presentations and age of onset [1]. Patients can manifest the classic hepatic form, isolated myopathy, cardiomyopathy, arthrogryposis, or a variable combination of clinical features. Polyglucosan bodies are found in different organs including brain, liver, and skeletal muscle (Fig. 1B) [2]. Adult polyglucosan body disease (APBD) is a chronic neurologic disease with spasticity, leukodystrophy, peripheral neuropathy and neurogenic bladder caused by recessive *GBE1* mutations [3]. It is pathologically characterized by a massive accumulation of polyglucosan in central and peripheral neuronal processes and astrocytes [4]. Polyglucosan bodies can also be found in skeletal muscle, although the presence of a myopathy in not formally demonstrated in APBD patients [5].

Glycogenin-1 associated, autosomal recessive disorders include GSD XV, manifesting as a pure cardiomyopathy with myocardial polyglucosan bodies and non-functional glycogenin-1 (*GYG1*), and the Polyglucosan body myopathy 2, (PGBM2), presenting with a pure skeletal myopathy with variable distribution of muscular weakness. Muscle biopsies from PGBM2 patients show storage of glycogen and polyglucosan and depletion of glycogenin-1 protein (Fig. 1C and 2A)) [6].

RBCK1 (HOIL-1) deficiency can manifest as an autosomal recessive lethal immunodeficiency with autoinflammation and polyglucosan storage in muscle, heart and liver [7] or Polyglucosan body myopathy 1 (PGBM1) with severe dilated cardiomyopathy and polyglucosan bodies in the skeletal muscle and the heart [8] (Fig. 1D and 2B). Another constituent of the linear ubiquitination chain assembly complex (LUBAC) complex, HOIP, has also been associated with immunodeficiency and a sublinical amylopectinosis [9].

Phosphofructokinase deficiency (GSD VII) is a disorder of glycolysis associated with recessive mutations in the *PFKM* gene. Five different forms have been described: exertional myopathy and hemolysis, isolated myopathy, isolated hemolysis, partial deficiency of red cell phosphofructokinase, and a fatal myopathy of infancy [10]. Polyglucosan bodies have been observed in skeletal muscle of rare cases presenting the myopathic form [11].

Lafora disease is a central nervous system disorder manifesting with increasingly intractable epileptic seizure, myoclonus, dysarthria, ataxia, emotional disturbances and dementia [12]. Lafora bodies are polyglucosan bodies, which

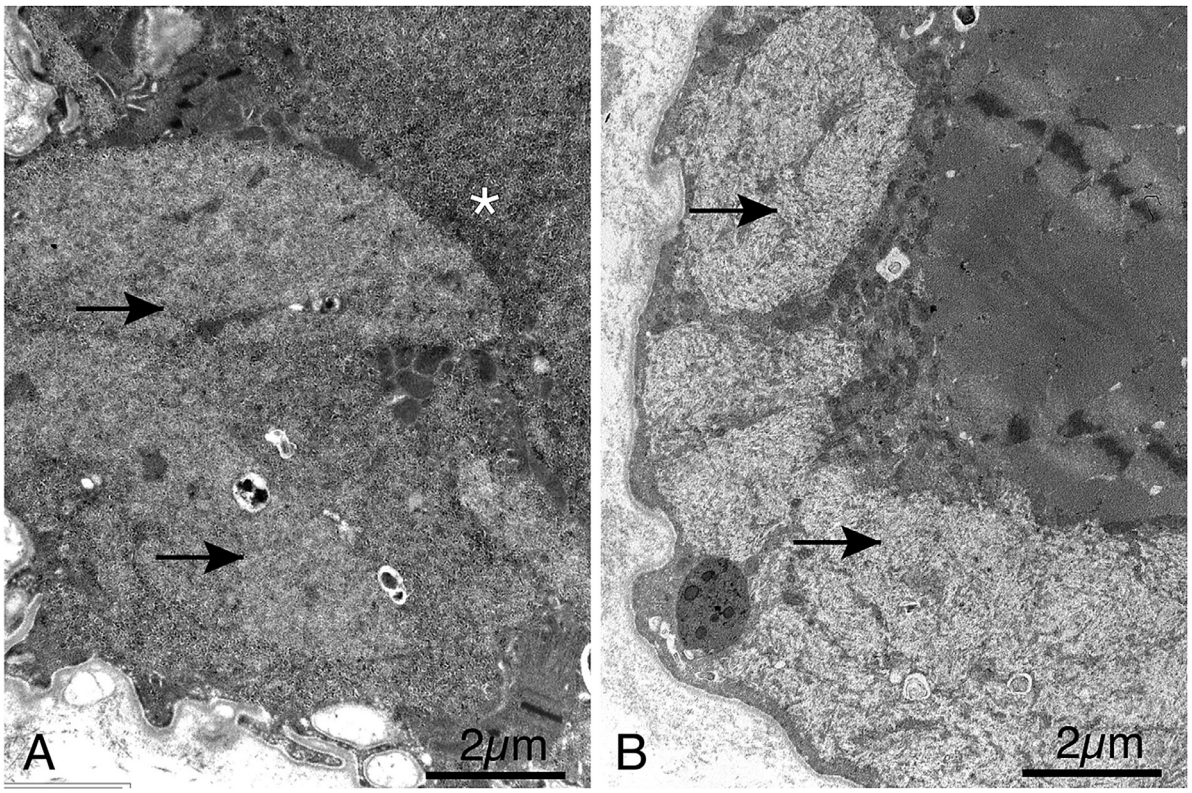

Fig. 2. Electron micrographs of polyglucosan in muscle. (A) Glycogenin-1 deficiency showing fibrillar storage material (arrows) together with granular glycogen particles (asterisk). (B) RBCK1 deficiency showing fibrillar storage material (arrows) and absence of normal glycogen.

are prevalent in the brain and also in skeletal muscle, liver and heart. Lafora disease is associated with recessive mutations in laforin (*EPM2A*) or malin (*EPM2B*).

AMP-activated protein kinase deficiency, PRKAG2, is an autosomal dominant condition variably manifesting with cardiomyopathy, Wolff-Parkinson syndrome and atrioventricular conduction block. Polyglucosan bodies are found in the cardiac tissue [13].

Equine polysaccharide storage myopathy type 1 (PSSM1) is an autosomal dominant disease caused by a specific (p.R309H) gain of function mutation of the glycogen synthase gene, *GYS1*. Horses show exertional rhabdomyolysis, muscle atrophy, gait abnormalities and paresis with glycogen accumulation and polyglucosan bodies in their skeletal muscle (Fig. 1E) [14].

3. Basic aspects of glycogen metabolism

Monique Piraud presented the current methods allowing diagnosis of liver and muscle glycogenoses. Glycogen is a branched chain polysaccharide that is synthesized from blood glucose through glycogenesis, as a reserve for cell energy. It is mainly stocked in liver as a reserve for the whole organism, and in muscle to support energy for muscular contraction. Energy is liberated through glycogenolysis and glycolysis. Glycogen storage diseases (GSDs) are disorders affecting glycogen metabolism, leading to various symptoms, mainly hypoglycaemia (liver) or exercise intolerance and muscular weakness (muscle).

Liver and muscle metabolism are very similar, but with a few differences. Glucose-6-phosphatase, which allows the release of glucose from the cell into the blood stream, is only present in liver (GSD I). Generally, enzymes are encoded by only one gene, but in some cases, the enzyme activity can be complex (*i.e.* phosphorylase activation system). Many genes/enzymes are specific to liver or muscle (*i.e.* phosphorylase and glycogen synthase) while some others are common (*i.e.* β subunit of phosphorylase kinase).

3.1. Biochemical diagnosis

Very few biochemical studies can be performed in blood cells (phosphorylase kinase, amylo-1,6-glucosidase) for the diagnosis of GSDs. Frozen muscle biopsy is the best sample for studying the glycogen metabolism, but is invasive.

The functionality of glycogenolysis and glycolysis can be screened by incubation of a muscle homogenate with different substrates under anaerobic conditions, leading to lactate production; a blockage at one step of the metabolism without production of lactate indicates a deficiency of the corresponding enzyme, and is a clue for the corresponding GSD diagnosis. Single enzyme activities can also be measured to confirm the suspected deficiency or to explore not screened diseases. Gene sequencing is performed to complement the diagnosis.

3.2. Genetic diagnosis

Massive parallel sequencing allows the simultaneous analysis of numerous genes. All the genes involved in muscle glycogenoses are typically covered in such analyses. The detection of two known or unknown but deleterious variants in a GSD gene can allow the genetic diagnosis of GSD in some cases. Biochemical studies are important to confirm the diagnosis, especially when the genetic findings are not conclusive.

3.3. Glycogenin function

Joan Guinovart discussed the importance of glycogenin and the effects of inactivation of the gene in a mouse model [15]. Glycogenin was traditionally considered essential for glycogen synthesis, as it acts as a primer for the initiation of the polysaccharide chain. Against expectations, glycogenin-deficient mice (Gyg KO) accumulate high amounts of glycogen in striated muscle. Furthermore, this glycogen contains no covalently bound protein, thereby demonstrating that a protein primer is not strictly necessary for the synthesis of the polysaccharide in vivo. Strikingly, in spite of the higher glycogen content, Gyg KO mice show lower resting energy expenditure and less resistance than control animals when subjected to endurance exercise. These observations can be attributed to a switch of oxidative myofibers toward glycolytic metabolism. These results may explain the muscular defects of GSD XV patients, who lack glycogenin-1 and show high glycogen accumulation in muscle.

Furthermore, Gyg KO mice have high perinatal mortality (90%) due to respiratory failure. The lungs of Gyg KO embryos and P0 mice have a lower glycogen content than wild-type counterparts. Embryonic lungs were found to have decreased levels of mature surfactant proteins SP-B and SP-C, together with incomplete processing of precursors. Furthermore, non-surviving pups showed collapsed sacculi, which may be linked to a significantly reduced amount of surfactant proteins. A similar pattern was observed in glycogen synthase-deficient mice, which are devoid of glycogen in the lungs and are also affected by high perinatal mortality due to atelectasis. These results indicate that glycogen availability is a key factor for the burst of surfactant production required to ensure correct lung expansion at the establishment of air breathing [16].

4. Polyglucosan storage diseases

4.1. Lafora disease

Berge Minassian described clinical aspects and molecular pathogenesis of Lafora disease, which is a devastating neurodegenerative disorder in children and adolescents. The presenting symptom is usually epileptic seizures of various kinds that are increasingly intractable [17]. Myoclonus and occipital seizures are frequent. Dysarthria and ataxia follow, in addition to emotional disturbances and confusion–and later in the course, dementia. The patients usually die within 10 years of onset, of causes related to the neurodegeneration and status epilepticus [18]. The morphological hallmark is Lafora bodies, which are mainly located in astrocytes and the perikarya of the neurons in the brain cortex, basal ganglia, thalamus, cerebellum, and spinal cord. These are round polyglucosan bodies, 10–30 μm in diameter. Lafora bodies can be seen in various other organs and tissues, especially in the liver and skeletal muscle, but they usually do not give rise to signs and symptoms from tissues other than the central nervous system. Two genes have been associated with most cases of Lafora disease, *EPM2A* (laforin) and *EPM2B* (malin). Laforin has a role in glycogen metabolism [19], and together with NHLRC1/malin–an E3 ubiquitin ligase–laforin appears to be involved in the clearance of toxic polyglucosan and protein aggregates through multiple pathways.

Laforin has a carbohydrate-binding domain, of a very specific type (CBM20), which is utilized in the plant kingdom by proteins that interact with starch. In fact, laforin has been shown to preferentially bind longer versus shorter chain glucans. Laforin also has a phosphatase domain with which it dephosphorylates glycogen. In the absence of laforin, glycogen phosphate increases. However, remarkably, the hyperphosphorylation of glycogen does not appear to underlie the transformation of normally branched glycogen to long-branched polyglucosans [20]. This was shown by expressing phosphatase-silent laforin in laforin-lacking mice, which prevented polyglucosan and Lafora body formation, but not correcting the glycogen hyperphosphorylation. Crossing phosphatase deficient mice with malin-lacking mice did not prevent polyglucosan and Lafora body formation, suggesting that the element driving the disease process is absence of malin. For the moment, the following, still speculative, pathogenic concept has emerged. Some glycogen particles likely develop branches that are too long and thus predisposes the molecule at risk of precipitation. The laforin-malin complex detects these through laforin CBM20 and malin acts on proteins involved in branch elongation (perhaps glycogen synthase) to check them and prevent progression of chain lengthening beyond the threshold of solubility. The role of glycogen phosphate remains unclear [21].

Jordi Duran discussed mouse models of Lafora disease. Knockout mouse models of both genes, EPM2A, encoding laforin and EPM2B, encoding malin, have been widely used for the study of the pathophysiology of the disease. Lafora bodies, the glycogen aggregates that accumulate in the brain and that are the hallmark of the disease, were traditionally considered to be found exclusively in neurons. However, the resemblance of some Lafora bodies with the glycogen aggregates that accumulate in normal brains with age (known as *corpora amylacea*) [22] suggest that part of the Lafora bodies could accumulate in astrocytes, since *corpora amylacea* are known to accumulate in this cell type.

The study of the distribution of Lafora bodies in the brain of the malin knockout mouse model [23] with cell type-specific markers demonstrated that, indeed, astrocytes

also accumulate Lafora bodies. In fact, in regions like the hippocampus, the vast majority of the aggregates accumulate in astrocytes and only a few can be found in neurons. In contrast, in regions like the cortex, most Lafora bodies are found in neurons [24]. The study also demonstrated that astrocytic and neuronal Lafora bodies differ in shape and size. The first are smaller and amorphous, while the second are normally bigger and round, located close to the neuronal nucleus. These results identify astrocytes as a key player in the physiopathology of the disease, which has important implications for the design of putative treatments.

4.2. Branching enzyme deficiency

Berge Minassian reported on the clinical presentation of branching enzyme deficiency (*GBE1*), which has a very heterogeneous phenotype with variable tissue involvement possibly due to tissue specific splice variants [25]. The typical presentation is rapidly progressive liver disease with cirrhosis and death in early childhood [26]. However, several neuromuscular forms have been described with perinatal, congenital/infantile, juvenile and adult onset forms. Pure myopathic forms have also been described [27,28]. When muscle is affected there is accumulation of PAS positive material, some of which appears as polyglucosan, which may look filamentous or amorphous in comparison to normal glycogen particles. These polyglucosan bodies are to some extent resistant to α-amylase digestion.

A special form is known as adult polyglucosan body disease (APBD) and manifests as a chronic neurological disease with neurogenic bladder, spastic gate, and peripheral neuropathy. Polyglucosan bodies are widely distributed in the central and peripheral nervous system. Most patients with Ashkenazi Jewish background are homozygous for the c.986A>*C* (p.Y329S) variant [3].

4.3. RBCK1-associated disease

Elaine Murphy presented the clinical manifestations of homozygous or compound heterozygous pathogenic variants in *RBCK1*, which cause a systemic condition characterized by polyglucosan body accumulation. The exact role of RBCK1 in the molecular pathogenesis of underlying polyglucosan formation and/or clearance is still unclear. RBCK1 is one of the components of the linear ubiquitin chain assembly complex (LUBAC), an E3 ligase complex that adds head-to-tail linear polyubiquitin chains to substrate proteins [29,30]. LUBAC regulates activation of the canonical NF-κB pathway, which plays a key role in inflammatory and immune responses. Recessive loss-of-expression and loss-of-function variants in the N-terminal part of RBCK1 were first reported in 3 patients from two unrelated families who presented with a fatal disorder characterized by chronic autoinflammation, recurrent pyogenic infections and skeletal and cardiac myopathies with polyglucosan storage [7]. Functional studies in patients' fibroblasts showed compromised NF-κB activation in response to interleukin-1β (IL-1β). By contrast, patients' monocytes were hyperresponsive to IL-1β. The consequences of RBCK1 and LUBAC deficiencies for IL-1β responses differed between cell types, consistent with the paradox of autoinflammation and immunodeficiency in these patients. Subsequently, 13 patients from 10 unrelated families were reported with homozygous or compound heterozygous variants in the mid- and C-terminal domains of RBCK1 and presenting with childhood or juvenile-onset progressive skeletal and/or cardiac myopathy with polyglucosan storage without immunodeficiency [8,31].

RBCK1 deficiency is a very rare condition, with current knowledge based on a few case reports [7,8,31-34]. A small case series from the UK reports 4 additional patients, from 3 kindreds, including 2 sisters with compound heterozygous mid-domain variants in RBCK1, with a phenotype spanning the entire spectrum of the condition, including skeletal and cardiac myopathy, combined immunodeficiencies and auto-inflammation [35].

Combining data from the UK patients and available information from the published literature, the mean age at death in patients who underwent a cardiac transplant for cardiomyopathy ($N=8$) was 25.3 years (28, 33, 15 years), while in those with cardiomyopathy who were not transplanted ($N=6$) the mean age at death was 17.6 years [8,33]. Three of the UK patients survived 13, 16 and 21 years after cardiac transplant.

Muscle MRI findings in a single patient, showed a distinct pattern of selective involvement predominantly affecting quadriceps, sartorius, adductor magnus and, to a lesser extent, hamstring muscles. Marked signal changes in corresponding areas on STIR sequences indicated marked edema suggestive of ongoing inflammation. This pattern showed some similarities, in particular prominent adductor magnus involvement, to the muscle MRI pattern reported in acid maltase deficiency [36].

Anders Oldfors reported on the pathology in RBCK1 deficiency. In most cases there is, in both muscle and heart, an extensive accumulation of polyglucosan that appears highly resistant to alpha-amylase digestion [8]. The polyglucosan is usually present in large and small inclusions in muscle fibers that are depleted of normal glycogen (Fig 1D). Muscle fibers without polyglucosan storage have an apparently normal content and distribution of glycogen. Electron microscopy reveals a monomorphic pattern of fibrillar material in circumscribed deposits that vary in size from less than 0.1 to >10 μm (Fig. 2B). The small aggregates are found between the myofibrils replacing the normal glycogen and the larger globular deposits are frequently found in aggregates replacing the myofibrils separated from each other by a rim of mitochondria.

4.4. PRKAG2 cardiomyopathy

Pascal Laforêt reported that dominant pathogenic mutations in the gene encoding the non-catalytic gamma subunit (PRKAG2) of AMP-activated protein kinase (AMPK) are usually associated with hypertrophic cardiomyopathy,

Wolff-Parkinson-White syndrome, and atrioventricular conduction block. It is essentially a heart-specific glycogenosis, but glycogen storage may also occur in skeletal muscle [37]. Onset is usually in late adolescence, but it can be in early childhood [38]. There is typically glycogen accumulation in the heart, and many patients (but not all) may show some deposits of polyglucosan in the cardiomyocytes [39], but not in skeletal muscle [37].

4.5. Glycogenin-1 deficiency

Pascal Laforêt reported on the clinical findings in glycogenin-1 deficiency. So far, 41 patients have been diagnosed with glycogenin-1 deficiency associated with pure skeletal myopathy (37), combined skeletal and cardiomyopathy (1), or isolated cardiomyopathy (3). Four patients underwent an endomyocardial biopsy showing polyglucosan bodies [40,41] of which, three needed cardiac transplantation. Cardiac abnormalities, consisting of arrhythmia [6], ischemic cardiopathy [42], valvulopathy [43], conduction disorder [6], and pulmonary artery hypertension [6] were reported in 9 patients and were considered unrelated to glycogenin-1 deficiency.

There was no sex prevalence among the GYG1 patients. Age at onset was before 30 years in 25% of cases and after 50 years in 75%. Cases with very late onset have been described [6,44]. Twenty-seven patients showed a slowly progressive myopathy with mainly proximal weakness with variable asymmetry, mimicking a limb girdle muscular dystrophy or Pompe disease [45,46]. Proximo-distal weakness was reported in 8 patients with a scapuloperoneal distribution in some cases [42]. The course was slowly progressive and only a few patients have become wheelchair-dependent. Distal weakness was reported in 6 patients and involved hand and finger muscles in three. Exercise intolerance, effort-induced myalgias and cramps were reported in a few patients [44,47-49]. EMG was myopathic in the majority of cases (28 reported cases) and neurogenic in four. Five patients presented fibrillation and myotonia was reported in one case. Serum CK was elevated (183-1509 UI/L) in nine cases. Central nervous system involvement has been never reported. Of note, two patients had neurosensorial hearing loss [44,46].

Edoardo Malfatti summarized the pathological features encountered in 20 muscle biopsies from 17 patients with myopathic form of glycogenin-1 deficiency, PGBM2. All muscles presented single or multiple PAS positive inclusions with ovoid or rounded shape in both subsarcolemmal and cytoplasmic regions (Fig.1B). The inclusions showed variable resistance to alpha-amylase digestion with bigger inclusions being in general more resistant [50]. With oxidative staining they usually showed an intense peripheral rim. They were intensively reactive for desmin, ubiquitin and P62/SQSTM1 by immunohistochemistry [6]. Electron microscopy confirmed that the inclusions corresponded to polyglucosan bodies with lobulated grape-like structures, separated by mitochondria or darker glycogen granules. Polyglucosan bodies contained partly filamentous material intermingled with round glycogen granules (Fig. 2B). In some cases, autophagic profiles were observed. One case harbored nemaline rods [44]. In conclusions, skeletal muscle in glycogenin-1 deficiency presents a common and recognizable morphological picture. Edoardo Malfatti also reported on a comparison between the muscular morphological pictures found in glycogenin 1-related myopathy and RBCK1-related myopathy with cardiac involvement, showing that there are histopathologic differences among the polyglucosan bodies. In particular, there is a variable alpha-amylase resistance with a major resistance of dotty inclusions in RBCK1-PGBM1 and increased resistance of bigger inclusions in GYG1-PGBM2 [50].

Anders Oldfors summarized the current knowledge about the glygogenin-1 associated cardiomyopathy, which appears to be much rarer than the myopathic form of glycogenin-1 deficiency, and the patients have in general no or only minor skeletal muscle symptoms [40,41]. Only three *GYG1* mutations have been described, including a recurrent p.Asp102His variant. The clinical course is usually an adult-onset cardiomyopathy with arrhythmias and cardiac failure necessitating heart transplantation within a few years after onset of symptoms. Myocardial biopsy reveals storage of abnormal glycogen that ultrastructurally is composed of a mixture on granular glycogen-like and fibrillar material. To a minor extent, it is resistant to alpha-amylase digestion. Mutated glycogenin-1 was expressed in the heart in all studied cases, which may be part of the pathogenesis, since complete lack of glycogenin-1 seems not to be associated with cardiomyopathy.

Thomas Krag presented a study on glycogen metabolism in two patients with mutations in the *GYG1* gene with absence of glycogenin-1 protein expression. Apart from positive stain for glycogen and polyglucosan bodies, electron microscopy clearly demonstrated large pools of glycogen or glycogen-like granules in both patients as well as intermyofibrillar glycogen. The polyglucosan bodies had mostly a heterogeneous content of glycogen, fibrillar molecules and autophagic organelles and stained positive for fibrillar but not sarcomeric proteins. To find out what may initiate glycogen synthesis in the patients, it was tested if glycogenin-2, a liver-specific glycogenin-isoform was expressed and positive bands were found in both patients, suggesting that in glycogenin-1 deficient patients, glycogenin-2 may be expressed as a compensatory mechanism. In addition, adaptations in glucose metabolism and glycogen synthesis was found in both patients, possibly reducing blood glucose metabolism and altering glycogen branching and the energy density of glycogen [51].

Anders Oldfors reported on a recent study on the expression of glycogenin-1 and the liver isoform, glycogenin-2 (GYG2), in normal liver, muscle and heart. The glycogenin-2 was only identified in liver, whereas glycogenin-1 was expressed in all examined tissues. At variance with the results presented by Dr. Krag, no glycogenin-2 could be detected in skeletal or heart muscle biopsies from patients with glycogenin-1 deficiency by applying immunohistochemistry, western blot and mass spectrometry analyses, indicating

that up-regulation of glycogenin-2 does not compensate for glycogenin-1 deficiency [52].

Mads Stemmerik presented a recent case-control study with four Glycogenin-1 deficiency patients investigating their fat- and glucose metabolism during exercise using exercise tests and stable isotope technique. Results showed an impaired lactate production during maximal exercise and handgrip tests and improved exercise tolerance with a glucose infusion during submaximal exercise. Exercise-induced increases in lactate were attenuated to about half normal in patients while palmitate utilization was greater in patients compared to controls [53]. Glycogenin-1 deficiency has previously been thought to be a defect exclusively affecting glycogen build-up and resulting in fixed muscle weakness, but these findings suggest that patients with Glycogenin-1 deficiency not only have abnormal formation of glycogen, but also have impaired muscle glycogenolysis, and as a result of that also have exercise intolerance due to shortage of energy production.

5. Equine polyglucosan storage disease

Richard Piercy discussed the pathogenesis of a common polyglucosan myopathy of horses, known as polysaccharide storage myopathy type 1 (PSSM1). The disorder is caused (unusually for glycogen storage diseases) by a gain of function, dominant, R309H mutation in glycogen synthase 1 (*GYS1*) and the same mutation has been reported in more than 30 breeds of horse from all over the world [54]. Since the disease is so prevalent, homozygotes are occasionally encountered. Affected horses tend to present with one of 2 phenotypes: either with intermittent exertional rhabdomyolysis or with slowly progressive weight loss and lameness or paresis. A significant number of horses that carry the mutation are subclinical. Affected horses store more glycogen and with time, polyglucosan, in skeletal muscles, often in subsarcolemmal, non-membrane bound deposits, or in cytoplasmic inclusions and have a shift in muscle fiber type, having fewer type 2X and more 2A muscle fibres [55]. Horses may have normal or mildly raised plasma creatine kinase (CK) activity which might increase with exercise [55]. The R309H mutation causes constitutive activation of the mutant enzyme – its increased activity occurs despite hyperphosphorylation [56]. Affected horses are managed (often successfully) with alterations in diet and their exercise: in particular, horses do best with regular daily exercise and with a change of diet so that a greater proportion of dietary calories are derived from fat [57]. The mechanisms that lead to the exertional rhabdomyolysis and the muscle weakness are currently unclear, but are the subject of investigation.

Marie-Anne Colle presented results from a study of infrared (IR) microspectral signatures of skeletal muscle from horses with type 1 polysaccharide storage myopathy (PSSM1)). The main histopathological lesions are characterized by intracytoplasmic PAS-positive and amylase-resistant inclusions, consistent with complex polysaccharides. Other fibers contain subsarcolemmal vacuoles that are PAS-positive and amylase-sensitive, consistent with glycogen. The aim of this study was to demonstrate that IR-microspectroscopic analysis could be used to characterize PSSM1. Spectral characterization of inclusions from muscle fibers from horses with and without the disorder were studied and results indicated that spectral microspectroscopy analysis can discriminate glycogen positive fibers. Principal Component Analysis also showed that the distribution of fibers containing abundant PAS positive-amylase resistant material was homogeneous and that inclusions corresponded with complex polysaccharides. Affected fibers can be discriminated from less affected fibers. The work demonstrated that the IR-microspectral carbohydrate signature of PSSM1 horse muscle provides information about histopathological criteria and severity.

6. Biochemical and therapeutic aspects

Christer Thomsen presented results from studies on the protein composition of muscle polyglucosan aggregates in RBCK1 deficiency. By using laser micro-dissection and quantitative mass spectrometry some proteins were found to be accumulated and could be validated by IHC. The major part of accumulated proteins had known functions in metabolic or cellular quality control pathways. Metabolic proteins were involved in various aspects of glycogen metabolism (e.g. glycogenin-1, glycogen synthase, myophosphorylase, laforin) and glycolysis (e.g. 6-phosphofructokinase). Proteins involved in quality control included chaperones (e.g. heat shock protein beta-1, alpha-crystallin B), ubiquitin processing factors (e.g. UCHL1), ubiquitin receptors (e.g. p62/Sequestosome 1, RAD23B) and subunits of the proteasome. The remaining accumulated proteins were functionally diverse; however, some are known components of protein aggregates in muscle and brain pathologies (e.g. desmin, ferritin).

Miguel Weil described his long-term involvement in developing several human cell-based models to find therapeutic solutions for rare diseases. Standard approaches towards drug development are inapplicable to rare diseases, as multi-phase clinical trials cannot be performed to evaluate efficacy due to the low number of patients available. Since a systematic methodology to evaluate the therapeutic efficacy of compounds for rare diseases is lacking, the emergence of image-based high content screening/analysis (HCS/HCA) may help to solve this problem using a personalized patient strategy. HCA allows characterizing phenotypes of cells derived from donors with and without a disease and use it as a multi-parametrical profile or as a personal signature of the analyzed cells. Moreover, these cell or disease phenotype signatures (or biomarkers) could serve for personalization of the drug discovery process even when almost nothing is known about the disease etiology. This personalized strategy involves developing target-based/phenotypic cell-based assays and the use of libraries of small numbers of compounds (including FDA approved drugs). These compounds are highly diverse and representative of large collections making

the personalized approach affordable. Specifically, for cell-based HCA assays we have adopted the use of bone marrow human mesenchymal stem cells (hMSC) or skin fibroblasts that can be maintained and expanded in culture into considerable numbers quite easily. The HCA assay development is designed according to the specific disease based on a measurable disease phenotype [58]. At present, this novel drug screening pipeline strategy has been applied to adult polyglucosan body disease (APBD or GSD type IV) for which a cell-based assay was developed using patient skin fibroblasts for drug high throughput screening in order to identify small molecule inhibitors of PB glycogen accumulation, irrespective of the mechanism of inhibition. These encouraging results from this patient-cell-based drug screening approach are summarized in a recent publication [59], for which the biological effects of one of these novel compounds in the APBD mouse model are described below in Dr. Or Kakhlon's presentation. Potentially this HCA phenotypic screening approach could find beneficial drugs also for other related GSDs, which share abnormal glycogen accumulation in cells.

Or Kakhlon described three therapeutic strategies for treating the prototypical polyglucosan disorder APBD, which has been described above. Axon plugging by the polyglucosan bodies is the presumed key pathogenic factor for the disease. Kakhlon described three pharmacological approaches studied in his lab for ameliorating APBD: Inhibition of glycogen synthase (GYS) activity, activation of GBE1, and polyglucosan reduction.

GYS inhibition reduces the relative levels of the non-branched amylose component in glycogen molecules, thus rendering them relatively more branched and soluble. GYS activity was lowered by the FDA approved flavoring agent guaiacol [60], discovered by screening 1700 FDA approved drugs for their capacity to lower polyglucosan staining in APBD patient cells. Guaiacol also lowered basal and glucose-6-phosphate–stimulated GYS activity and increased inactivating GYS phosphorylation and phosphorylation of the master activator of catabolism, AMP-dependent protein kinase. In vivo, guaiacol increased grip strength and survival in GBE1 knocked-in mice that mimic human APBD. These treatments had no adverse effects except making the mice slightly hyperglycemic, possibly due to the reduced liver glycogen levels. Histopathologically, guaiacol reduced PB in peripheral nerve, liver, and heart, demonstrating the potential therapeutic efficacy of a GYS inhibitor for treating APBD.

Stabilization of mutated GBE1 (GBE1-p.Y329S, the most common homozygous mutation in APBD) was accomplished by the triacylglycerol mimetic, TGM5, a synthetic lipid containing three 2-hydroxy-eicosapentaenoic acid (EPA) moieties. The GBE1 p.Y329S mutation exposed a hydrophobic patch, which reduced GBE1 activity and stability. Through interaction with this hydrophobic patch, TGM5 was able to stabilize GBE1Y329S and increase its activity [61].

Lastly, a new compound (compound A) was tested, which was discovered by high throughput screening of a commercial library (ChemBridge) of ~11,000 compounds designed to cross the BBB [59]. This high throughput screening campaign discovered polyglucosan-lowering compounds in APBD patient cells. The assay was based on polyglucosan level as an endpoint regardless of the mechanism of action. Compound A was found to be both acutely and chronically safe and its predicted absorption, distribution, metabolism, excretion and toxicity profile were impeccable. As required from a potential neurotherapy, compound A reduced polyglucosan levels in the brain and peripheral nerve of APBD modeling mice. The compound also ameliorated animal locomotion and reflexes and significantly increased stride length. The mechanism of action of compound A was tested by the nematic protein organization technique (NPOT, Inoviem, Ltd.) and where it was shown that protein hetero-assemblies uniquely generated around compound A only when it was added to the cell homogenates. Compound A thus illustrates an endpoint-focused approach (reduction of polyglucosans in patient cells) for APBD pharmacotherapy.

Matthew Gentry spoke about recent work his laboratory performed in collaboration with Valerion Therapeutics on pre-clinical Lafora disease mouse models. Lafora disease is a fatal, autosomal recessive glycogen storage disease with patients experiencing severe epilepsy [12,62]. Lafora disease patients and corresponding mouse models develop Lafora bodies (LB) in cells from nearly all tissues. These LBs drive disease progression and multiple modalities are being explored to stop LB synthesis or degrade existing LBs [21,62,63]. Valerion has developed a proprietary cell penetrating antibody fragment (Fab) that is imported into cells via the ENT2 nucleotide salvage transporter [64-66]. The Gentry lab with Valerion developed a novel antibody-enzyme fusion that utilizes the Fab fused to pancreatic α-amylase, named VAL-0417 [67-69]. Gentry showed that VAL-0417 robustly degrades purified LBs in vitro releasing glucose and maltose. He also demonstrated that VAL-0417 degrades LBs in L Lafora disease mouse models after both intramuscular and intravenous injections. Removing LBs in astrocytes and neurons is the key to treating Lafora disease and VAL-0417 likely does not efficiently cross the blood brain barrier. VAL-0417 was continuously provided by intracerebroventricular administration into the brain of Lafora disease mouse models and Gentry showed that VAL-0417 ablated Periodic-acid Schiff positive (PAS+) LBs. He then highlighted a second antibody-enzyme fusion that the team has developed whereby the Fab is fused with α-glucosidase named VAL-1221 and it too ablates PAS+ LBs. Excitingly, VAL-1221 just completed a clinical trial for treatment of Pompe disease patients (NCT02898753). Gentry concluded by presenting metabolomics data from the Lafora disease mouse models that provided novel mechanistic insights into how LBs drive disease progression and recently published data demonstrating nuclear glycogen metabolism [69].

Vyas Ramanan spoke about the use of large databases of human genotype/phenotype information to inform drug target selection and safety considerations, specifically focusing on

the potential for inhibiting glycogen synthesis as a treatment for GSDs such as Pompe disease [70], Lafora disease [71], and others [72]. The concept of reducing glycogen synthesis as a method of substrate reduction therapy in various GSDs has been proposed for several years, and preclinical data have demonstrated intriguing efficacy. However, there remain potential concerns about the safety of prolonged glycogen synthesis inhibition in humans, in part because of the potentially severe phenotype seen in humans with complete deficiency of the muscle glycogen synthase isoform GYS1 [73]. To explore the effects of chronic glycogen depletion in human subjects on a larger scale, the Maze team and collaborators used the UK Biobank genetic resource to study the effects of a relatively common frameshift mutation in the gene *PPP1R3A*, which results in decreased glycogen synthesis (and increased clearance) and a reduction of 50–65% in muscle glycogen per allele [74]. The team studied the ~350 K individuals of European ancestry in the UK Biobank, and extracted measures related to exercise capacity (grip strength, exercise tolerance test time) and cardiac health (LVEF, LVEDV, CO) from individuals with 0, 1, or 2 PPP1R3A frameshift alleles. Analysis of this data showed that heterozygous carriers of this variant, and even homozygous variant carriers (who would be predicted to have >70% reductions in muscle glycogen), showed no deficits by these measures of skeletal and cardiac muscle function. Furthermore, the analysis showed no association with blood glucose level or diabetes risk, which have previously been associated with variants in the glycogen metabolic pathway through candidate gene studies. Overall, the analysis suggested a therapeutic window for the inhibition of glycogen synthesis in humans for the treatment of GSDs, and this continues to be an area of active investigation for multiple groups.

Federico Mingozzi discussed the potential of gene therapy as a treatment modality for GSDs. Adeno-associated virus (AAV) vectors are currently the most broadly adopted platform for in vivo gene transfer [75], owing to their excellent safety and efficacy profile demonstrated in numerous clinical trials [76-78]. In the treatment of Pompe diseases, promising preclinical results were previously published [79] showing that it is possible to engineer the liver to produce a secretable form of α-acid glucosidase (GAA) and correct all the manifestations of the disease in mice. Furthermore, by providing a steady state supply of the GAA enzyme into the bloodstream, the approach is also efficacious in reversing the pathology in mice with advanced Pompe disease. Additional data were presented in which the efficacy of liver gene transfer with AAV vectors expressing secretable GAA was compared with the standard of care for the disease, enzyme replacement therapy with recombinant human GAA (rhGAA), showing superiority of gene therapy vs. enzyme replacement therapy. Last, experiments in large animal models also suggest that AAV vector-mediated liver gene transfer successfully delivers the GAA enzyme to distal tissues and also to the central nervous system. The investigational gene therapy consisting of liver gene transfer with a secretable form of GAA is currently being tested in adult Pompe patients in a phase I/II clinical trial (Clinicaltrials.gov ID: NCT04093349).

Developing gene therapies to provide a steady-state supply of enzymes via cross-correction is relatively straightforward. Conversely, in the case of glycogen storage disease type III (GSDIII), the development of a gene therapy approach is challenging as the deficient enzyme, glycogen debranching enzyme, is a cytosolic protein and cross-correction is not feasible. An additional limitation that applies specifically to the development of an AAV-based therapy for GSDIII, is the size of the cDNA encoding for glycogen debranching enzyme, which is large (4.6 Kb) and therefore hard to package into an AAV vector. Using a strategy consisting of a dual AAV vector, in which the glycogen debranching enzyme cDNA is split between two vectors, it was possible to correct the disease phenotype in muscle, with corresponding rescue of function. Partial rescue of hypoglycemia, a hallmark of the disease in children, was also demonstrated. These results were recently published [80].

7. New diseases

Anders Oldfors reported on a novel disease with polyglucosan in the heart caused by inactivation of kelch-like protein 24 (KLHL24) [81]. In skeletal muscle, the disease appears with glycogen and desmin accumulation, forming protein aggregates but no apparent polyglucosan. The muscle fibers show a characteristic and unique cog-wheel appearance, due to the desmin and glycogen accumulation.

Polyglucosan accumulation in skeletal muscle may occur in rare cases of Pompe disease and has been observed in several diseases, usually in single cases where gene panels have not disclosed any of the known genes associated with polyglucosan storage. Attempts to identify the genetic defect in such cases may unravel new polyglucosan storage disorders.

8. Conclusions

The main aim and deliverables of the workshop were met, as the meeting formed a multidisciplinary study group of researchers working on polyglucosan storage diseases. It is apparent from the foregoing text that the other aims of describing current concepts regarding glycogenin and glycogen synthesis, providing an overview of diseases with polyglucosan accumulation and to review and discuss pathophysiology and emerging treatment options for polyglucosan body diseases were also met. These diseases, as an experiment of nature, provide very important clues to glycogen metabolism in man, an example being that glycogen can be formed without its primer, glycogenin. Despite the diseases' rarities, several molecular treatment options are emerging. We still need to learn how such diverse phenotypes arise from apparently single enzyme deficiencies in some of the diseases and to what extent the defects impact on not only carbohydrate metabolism in cells, but also on protein expression and cell signaling.

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