The 220th ENMC workshop: Dystroglycan and the Dystroglycanopathies held on the 27-29 May 2016, Naarden, The Netherlands.

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1. Introduction

The aims of this workshop were to bring together researchers working on the clinical and basic aspects of the post-translational modification of dystroglycan, whether it was in relation to the pathophysiology of patients, animal models of dystroglycanopathies, or cellular systems addressing the effects of post-translational modification on dystroglycan function. By combining clinical and basic scientists, and a range of diseases / models / cellular systems, especially with an emphasis on neuroscience, we aimed to provide a platform for the sharing of ideas, reagents, animal models, to stimulate novel hypotheses and open new avenues of research into these diseases for which there are as yet no forms of therapy. With these objectives in mind seventeen researchers and clinicians, one patient with limb girdle muscular dystrophy and his partner and one patient representative met in Naarden 27-29 May 2016. Participants were from 7 countries (6 UK, 3 USA, 2 Germany, 2 Netherlands, 2 Sweden, 1 France, 1 Mexico).

2. Background

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Dystroglycan is a highly glycosylated extracellular matrix (ECM) receptor that is critical for the structure and function of skeletal muscle and the central nervous system. The post-translational modification of α-dystroglycan (α-DG) is essential for its ability to function as a receptor for laminin G (LG) domain-containing ligands, such as laminin, agrin, neurexins, perlecan, pikachurin, and Slit. The dystroglycanopathies describe a group of diseases caused by the loss or reduced binding of α-DG to its extracellular ligands. This can be caused by mutations in the gene encoding to dystroglycan itself (*Dag1*), known as primary dystroglycanopathies, or the genes encoding for proteins/enzymes that assist with the glycosylation of α-DG, so called secondary dystroglycanopathies). Defects in at least 17 genes fall into this latter category. Recent studies have elucidated the function of many of these enzymes and have positioned them in the O-mannosylation glycosylation pathway of α-DG. Several advances have been made since the last workshop and the overall aim of the present gathering was to discuss these within the context of the disease process and potential strategies for treatment.

3. The spectrum of CMD/LGMD2 (from patient to clinic)

3.1 A patient's perspective.

Neil Bevan provided a moving perspective of his life as his disease progressed, which included his time at college where he met his future wife Lyndsey, their wedding and their hopes and aspirations for their future life together with their eclectic collection of animals. His contribution was particularly valued by those participants who never come into contact with patients; the overall feeling being that it gave focus to their work and

would enable them to share this with students. We were all very grateful that Neil chose to share so much with us.

3.2 Update on diagnosis of the dystroglycanopathies.

Natalie Seta (Paris, France) discussed the genes involved in α-DG glycosylation which encode for 13 glycosyltransferases (POMT1, POMT2, POMGNT1, FKRP, FKTN, B3GALNT2, B4GAT1 (=B3GNT1), LARGE, POMGNT2, TMEM5, DPM3, 2, 1), 2 kinases (Dol kinase, Protein-O-mannosyl kinase) and 2 enzymes involved in nucleotide sugar production (ISPD, GMPPB). She explained that in France diagnosis is implemented on the basis of clinical and biological features associated with congenital muscle dystrophy, limb girdle muscular dystrophy, lissencephaly, and elevated serum CK; a muscle biopsy is used to confirm muscular dystrophy. Using antibody IIH6 (which identifies the glycosylated epitope of α-DG), abnormalities can be shown either in muscle biopsy or using cultured skin fibroblasts. Currently next generation sequencing allows for the testing of all 18 genes in one operation. When no mutation in these genes is revealed, whole exome sequencing is performed, in order to identify new related genes. Professor Seta reported that in the last 10 years more than 250 patients were diagnosed in her laboratory: 125 fetal cases, 88 children and 39 adults. These diagnosis led to 5-10 prenatal diagnosis a year. *POMT1, POMT2, POMGNT1, TMEM5, ISPD* mutations were present in 2/3 of the fetal cases; *POMT1, POMT2, POMGNT1, FKRP* mutations in 3/4 of the children; *FKRP* was the most common mutated gene in adults. The molecular diagnosis efficiency?? was 85% in the fetal cases which was much better than that for children (36%), and adults (22%). Finally, she explained that no strong genotype-phenotype correlation was evident, with

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POMT1, POMT2, POMGNT1, FKRP, FKTN, ISPD mutations being implicated across the entire clinical spectrum from the most severe to the mildest presentation.

3.3 The Myo-Seq project

Volker Straub discussed the MYO-SEQ project which is based at Newcastle University, UK where they apply whole exome sequencing to a large cohort of patients with unexplained limb-girdle weakness. Professor Straub stated that more than 500K exomes and 50K genomes of normal healthy individuals. He discussed the Care and Trial Site Registry (CTSR) which includes 320 registered sites worldwide. Blood and consent are taken in each centre and then DNA is shipped to Newcastle. Only known genes are evaluated i.e. those associated with neuromuscular disorders. Variants have been identified in 58 of 169 candidate genes which is less than 1%. However, it is difficult to determine what mutations are disease causing as for example everyone has titin variants – essentially polymorphisms. The most common mutations were in calpain, Ryr1 and dysferlin, POMT2 mutations were more common than anticipated. The MYO-SEQ project uses a database called PhenoTips. Phenotypic data from 1,100 patient records is contained in myoseq. The average age at recruitment is 39.1 yrs for males and 54 for females. Likely disease causing variants have been identified in 214 patients which equates to 42%. This does however, vary between centres as it depends on the experience of the clinicians. The aim now is to collect additional clinical data for deep phenotyping, undertake trio sequencing and include an additional 1000 samples.

Volker Straub also discussed some interesting clinical findings, namely those relating to the POMT2 clinical spectrum which included one patient who was still ambulant at

52. He also mentioned that sometimes a deficiency in either dystroglycan or laminin was not that evident. Further discussions on MRI data seemed to suggest that the tibialis anterior was the best preserved muscle in all the dystroglycanopathies. Pattern recognition was discussed and the value of MRI for diagnostics and as an outcome measure in clinical trials. Particularly since it is something that can be done on a patient at 5 years without anaesthesia. Since the percentage of fat does not change with age in normal individuals it would be feasible to quantify fat as a pathological readout.

4 The identification of new genes/mutations and biochemical aspects of the glycosylation process.

4.1 Novel mutations at the severe end of the clinical spectrum

Hans van Bokhoven described the work of his group in identifying known and novel mutations at the severe end of the dystroglycanopathy spectrum. They have collected DNA and cell lines from patients diagnosed with Walker–Warburg syndrome (WWS) or the slightly milder Muscle–Eye–Brain (MEB) phenotype. Using exome sequencing the patient data is first analysed for variants in known genes namely DAG1 (primary dystroglycanopathy), POMGnT1, POMGnT2 (GTDC2/C3ORF39), POMT1, POMT2, FCMD, FKRP, LARGE1, TMEM5, B3GNT1 (B4GAT1), B3GALNT2, and POMK (secondary dystroglycanopathy), and those engaged in combined N- and O-glycosylation defects DPM1/2/3, DOLK, ISPD, and GMPPB (tertiary dystroglycanopathy). In the cohort of 100 families, at least 69 are explained by mutations in these genes. This is likely to be an underestimate, as not all genes have been fully covered in all families. In addition, the cohort does contain a low proportion of families with an atypical phenotype. The question arises as to how many other genes are involved in severe-end dystroglycanopathy cases. According to the present data

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there is room for some additional genes, but their identification has to rely on genome-wide sequencing efforts.

All genes known so far have been uncovered by the lassavirus screen presented by van Bokhoven [1]. However, besides SLC35A1 this screen has not revealed any other obvious candidate genes for dystroglycanopathies. Genetic studies have yielded several gene mutations of interest for genotype-phenotype associations. One is a family in which a homozygous DAG1 mutation was identified. This highly consanguineous family has five individuals with the manifestations of Walker-Warburg syndrome (WWS). Mutations in DAG1 are rare and so far only four missense mutations have been reported that were associated with LGMD with or without cognitive impairment and one case with a Muscle-Eye-Brain-Disease-like phenotype. The mutation in the present WWS-like family is a frameshift c.743C>delC, predicting a premature termination codon p.Ala248Glufs*19 affecting the synthesis of both α- and β-dystroglycan. Indeed, Western blot analysis and laminin-overlay analysis confirmed the absence of both dystroglycan subunits in patient myoblasts. This is the first report of a full DAG1 loss-of-function allele in humans ([2]). The associated phenotype is severe and associated with early lethality.

Another remarkable observation is the identification of a B3GALNT2 mutation associated with a mild phenotype. Diagnostic exome sequencing in two brothers of 14 and 8 years of age with an unexplained type of intellectual disability identified a compound heterozygous mutation in the B3GALNT2 gene (Ile276Leufs*; Arg330Cys). The boys presented mild-moderate intellectual disability, speech disorder, no structural brain anomalies and no muscular dystrophy. Interestingly, another family with a comparable phenotype and a homozygous missense mutation in B3GALNT2 was identified by a collaborating group in the UK (Dr. A. Crosby). The phenotypes of

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patients in both families is remarkably mild in comparison to those cases previously reported to be caused by mutations in B3GALNT2. Strikingly, the differential phenotypes cannot be explained by the residual activity of the mutant proteins as a complementation assay with wild type and mutant proteins could not identify a relationship between the severity of the phenotype and the capacity to restore IIH6-positive glycosylation in B3GALNT2-deficient cells.

4.2 The biochemistry of the dystroglycanopathies

Dirk Lefeber (Nijmegen, The Netherlands) discussed the tertiary dystroglycanopathies which include those genes/proteins that indirectly modify α-DG glycosylation. Glycosylation of proteins requires sufficient availability of nucleotide-sugars as building blocks. Defects in their levels could also result in deficient O-mannosylation of α-DG. The first defect identified in this category was DPM3 deficiency which was shown in an adult patient with LGMD, this was followed by the discovery of mutations in DPM2 [3] and DPM1 in young patients with neurological symptoms and skeletal muscle involvement, without overt structural brain defects, subsequently GMPPB mutations were identified in patients with LGMD, MEB or LGMD with additional myasthenia symptoms. Interestingly, some defects (DOLK with dilated cardiomyopathy [4] and SRD5A3 with mainly eye and brain pathology) presented in patients with isolated symptoms, indicating the presence of tissue-specific pathways for α-DG glycosylation. In summary, the synthesis of the dolichol-P-mannose building block requires 6 different genes. Additionally, many genes are involved in the synthesis of glucuronic acid, xylose, N-acetyl-glucosamine, N-acetyl-galactosamine and ribitol, most of which are not yet associated with disease.

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Recent studies [5] uncovered a novel pathway in human sugar metabolism, involving synthesis of CDP-ribitol by ISPD (isoprenoid synthase domain containing). Subsequent work revealed that CMP-sialic acid transporter SLC35A1 is also required for dystroglycan O-mannosylation, independent of sialic acid [6]. Since nucleotide-sugars are commonly involved in multiple glycosylation pathways, (ISPD is likely to be the exception), genetic defects in sugar metabolism result in clinical phenotypes that share symptoms with the dystroglycanopathies for example the Congenital Disorders of Glycosylation (CDG). Further in analogy with CDG due to deficient protein N-glycosylation, it can be speculated that defects in Golgi homeostasis will also result in (tissue-specific) abnormal α-DG O-mannosylation. As an example, the subunits of the COG complex, known CDG defects, are known to be important for normal α-dystroglycan O-mannosylation [7].

Finally, Dr. Lefeber presented novel methodology for a highly sensitive and specific monitoring of sugar metabolites in samples of patients and model systems. This will be applied to dissect the tissue-specific mechanisms in sugar metabolism, and to test compounds that are able to restore deficient glycosylation as potential future therapeutic strategy.

4.3 Recent findings regarding the posttranslational processing of dystroglycan.

In the first of a two-part presentation **Kevin Campbell** reviewed the novel post-translational processing that is required for dystroglycan function. Ligand binding by α-DG is reduced when O-glycosylation is perturbed. Recent genetic data shows that mutations in at least 18 genes encoding known and putative glycosyltransferases disrupt the O-glycosylation of α-DG and cause muscular dystrophy. His previous efforts to understand the molecular mechanism underlying dystroglycan's ability to bind the

extracellular matrix had led to the identification of a novel phosphorylated O-mannosyl trisaccharide (N-acetylgalactosamine-β3-N-acetylglucosamine-β4-mannose) on α-DG. In this presentation he showed that three of the newly identified proteins (GTDC2, B3GALNT2, and SGK196) are involved in synthesizing the phosphorylated trisaccharide. Interestingly, SGK196 phosphorylates the 6-position of O-mannose, using ATP as the donor, and the addition of this phosphate residue is a prerequisite for formation of the ligand-binding motif. The SGK196-mediated phosphorylation occurs only when the GalNAc-β3-GlcNAc-β-terminus is linked to the 4-position of O-mannose, indicating that this disaccharide serves as the substrate recognition motif for SGK196.

Kevin Campbell also described LARGE1 as a bifunctional enzyme that has both xylosyltransferase (Xyl-T) and glucuronyltransferase (GlcA-T) activities, and showed that it generates the novel heteropolysaccharide [-GlcA-β1,3-Xyl-α1,3-]n, which has now been termed "matriglycan". He confirmed that this polysaccharide structure is present in native tissues, identifying two exoglycosidases that collectively can hydrolyze glycans on native α-DG: β-glucuronidase (Bgus) from T. maritima, and α-xylosidase (Xylsa) from S. solfataricus. He first tested these enzymes with a chemically defined substrate, the LARGE-synthesized pentasaccharide G5. Alternating treatment of G5 with the two enzymes removed one sugar at a time from the nonreducing end, demonstrating that Bgus and Xylsa cleave β-linked GlcA and α-linked Xyl, respectively, without measurable endoglycosidase activity. Finally, he demonstrated that simultaneous treatment of rabbit skeletal muscle α-DG with Bgus and Xylsa dramatically reduced the apparent molecular mass of α-DG, as assessed by SDS-PAGE, and abolished reactivity with the matriglycan-specific antibody IIH6, whereas treatment of α-DG with either exoglycosidase alone had no such effect. Similar results

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were obtained with mouse skeletal muscle α-DG: wild-type mouse α-DG treated with both Bgus and Xylsa migrated similarly to α-DG from Large$_{myd}$ mice, which have an inactivating mutation in the Large1 gene; this protein also failed to react with matriglycan-specific antibodies IIH6 and VIA41, as well as with laminin. Overall, **Kevin Campbell's** results demonstrate that native α-DG in skeletal muscle of rabbit and mouse is modified with multiple [-GlcA-β1,3-Xyl-α1,3-] repeats, and that this modification is required for α-DG to bind laminin.

5. Animal Models of the dystroglycanopathies.

5.1 Insight into the disease process using mouse models.

Sue Brown (London, UK) Mutations in the *FKRP* gene cause a wide range of clinical phenotypes in the human. In this presentation the various mouse models used for investigating the disease process were discussed and summarised. Focusing on the structural brain defects of the mouse with the global reduction in Fkrp, Dr. Brown discussed the early disorganisation evident in the neuroepithelium, and the mislocalisation of the Cajal Retzius cells which appears to correlate with later defects in the pial basement membrane. She also discussed analyses using new monoclonal antibodies to the core dystroglycan protein generated by Glenn Morris' lab which showed some interesting differences with respect to staining of the tongue compared to the LARGEmyd and POMGnT1null mice. Further discussions centred on the correlation between IIH6 and Fkrp expression during embryonic/fetal development in a mouse in which FKRP expression is reported by EGFP. Finally in the FKRP$_{MD}$ mouse in which there is a restoration of Fkrp in the central nervous system (via the expression of Cre recombinase under control of the Sox1 promoter), it was shown that there was increased levels of α-DG glycosylation and laminin α2 expression relative to the global

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knock down (FKRP^{KD}) which influences the separation of secondary myotubes from the parent primary myotube during the second half of gestation.

5.2 The expression of functional α-DG in dystroglycanopathy models.

Qi Lu, (Carolinas Medical Center, Charlotte NC, USA) reported that a small number of fibres expressing functional α-DG at up to normal levels is a general feature of diseased muscles in patients with FKRP mutations. These fibres are often marked with the expression of embryonic myosin heavy chain suggesting that the expression of functional α-DG is related to muscle regeneration. These "revertant" fibers were also observed in mouse models bearing fkrp P448LNeo- mutations and a severe phenotype. This line expresses relatively normal levels of the P448L mutant transcript. Lu's lab confirmed the association of functional α-DG expression with muscle regeneration by notexin and cationic polymers induced degeneration and regeneration. No revertant fibers were detected in either LARGE$_{myd}$ mice or FKRP P448LNeo+ mutant mice which express significantly reduced levels of mutant FKRP transcript. These results suggest that the expression of functional α-DG is dependent on LARGE and FKRP. Nonetheless mutant FKRP carrying the P448L mutation is sufficient for inducing normal levels of functional α-DG in regenerating fibers. **Qi Lu** further showed that muscle fibres of the P448LNeo- mutant mice express near normal levels of functional α-DG in skeletal muscle during the first postnatal week. Importantly, expression, although at much lower levels than in skeletal muscles, is also detected in the cardiac muscle. This postnatal expression remains FKRP and LARGE dependent. These results suggest that modulation of FKRP expression or other factors could be explored in future experimental therapies. Interestingly, enhanced expression of functional α-DG is observed in muscles of the mutant fkrpP448LNeo- mouse models after steroid

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treatment. **Qi Lu**'s studies with gene delivery also showed that overexpression of P448L mutant FKRP is able to rescue the glycosylation defect of α-DG and prevent disease progression in the FKRP mutant mice, strongly suggesting that mutant P448L FKRP retains much of its biological activity with regard to the glycosylation of α-DG. These data provide the rationale for interventions that either enhance the expression of endogenous mutant FKRP or modulate the glycosylation pathways for achieving therapeutic restoration of functional α-DG.

5.3 What can other models tell us about the pathogenesis?

Halyna Shcherbata presented her initial characterization of the Dystrophin-Glycoprotein Complex (DGC) in Drosophila. This complex appears to perform similar functions in the muscle and nervous system of Drosophila as their homologs in humans. Abnormalities in the Drosophila include age-dependent muscle degeneration, reduced mobility, hyperthermic seizures, a shorter life span, myotendinous junction and brain development defects [8-12]. **Shcherbata**'s lab took advantage of the genetic tractability of Drosophila to search for novel DGC-interacting components, as well as factors involved in its signaling and regulation, including miRNAs. This work showed similarities in miRNA profiles under either stress or dystrophic conditions, indicating that mutations in DGC-linked proteins are associated with an entourage of regulatory and homeostatic anomalies [13]. Importantly, the expression of multiple miRNAs, including those which are stress-sensitive, directly depend on the DGC-NOS-HDAC signaling pathway [13, 14]. This suggests that future in depth analysis of DGC interacting proteins and their regulatory miRNAs will promote a better understanding of DGC signaling and regulation and facilitate the development of alternative therapeutic pathways for neuromuscular disorders using miRNAs.

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Dystroglycan (Dg) is subjected to miRNA regulation the mode of which differs depending on the developmental stage and the cell type. During embryonic development, Dg is targeted in an expression-tuning mode, i.e. the miRNA determines the mean of the target gene expression, resulting in an "on-off" expression pattern [12]. The highly evolutionary conserved miR-9a acts as the canalization factor in the dynamic process of muscle attachment establishment and protects tendon precursors from noisy expression of Dg and other critical muscle differentiation genes. Other identified miRNAs that target Dg are the mir-310s, these act in an expression-buffering mode i.e. when a miRNA and its target are co-expressed and the miRNA decreases the variance of the target [14]. This operates via a perceptive-executive mechanism; namely expression of miR-310 is induced by high levels of Dg which can target only a portion of Dg mRNAs with an extended 3'UTR thereby preventing Dg levels from being reduced below a certain threshold. This regulation is of great importance in the developing nervous system to keep the equilibrium of Dg expression. In the CNS, aberrant (too high and too low) Dg levels affect neuronal stem cell division, while higher levels accelerate proliferation and perturb neuron differentiation, causing formation of cobblestone-like structures that outgrow the normal contour of the extracellular matrix defined brain. This phenotype is similar to the cortical abnormalities associated with the dystroglycanopathies in humans, implying that Drosophila Dg mutants can serve as a model for cobblestone lissencephaly [14]. Deregulation of Dg affects the distribution of major cell adhesion proteins, presumably affecting the ECM composition, and consequently resulting in abnormal tissue assembly [15]. Thus, fine-tuning of Dg expression by miRNAs in the nervous tissue is key in the regulation of neuronal development, plasticity, and maintenance.

6 Aspects of dystroglycan cell biology, iPS cells, nuclear dystroglycan and DG proteolysis

6.1 New cellular models for the dystroglycanopathies

Yung-Yao Lin (London UK) presented on CRISPR-mediated genome editing in human induced pluripotent stem cells for modelling FKRP-deficient dystroglycanopathy. Patient-specific induced pluripotent stem cells (iPSC) have been differentiated down a neural lineage cells to investigate pathological mechanisms underlying the CNS involvement. Nevertheless, one major challenge is the lack of an appropriate isogenic control, which minimizes the variability between different genetic backgrounds. Yung-Yao Lin has therefore developed a protocol to carry out targeted gene correction in patient-specific FKRP-iPSC and targeted gene mutation in wildtype iPSC using CRISPR/Cas9-mediated genome editing technology. CRISPR/Cas9 induced homologous recombination was used in combination with the *piggyBac* positive/negative selection cassette, which allows precise modification of the mammalian genome at single base-pair levels without leaving footprints. Targeted gene correction of *FKRP* was shown to restore α-DG functional glycosylation in iPSC-derived neurons, whereas a targeted mutation of *FKRP* disrupts α-DG glycosylation. In the future isogenic pairs of human iPSC-derived cellular models will further elucidate mechanisms underlying the CNS involvement and muscle pathology due to FKRP deficiency. Moreover, these unique cellular models will be exploited to facilitate the discovery of therapeutic targets in FKRP-deficient muscular dystrophies.

6.2 Does dystroglycan have a role at the nuclear envelope?

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Bulmaro Cisneros (CINVESTAV, Mexico) reported that β-dystroglycan has the ability to target to, and conform specific protein assemblies at the plasma membrane and nuclear envelope (NE). However, molecular mechanisms controlling the subcellular fate and abundance of β-DG in each compartment, as well as the physiological consequences of a failure in its intracellular trafficking pathway are largely unknown. Dr. Cisneros Vega presented data showing that β-DG is a nucleocytoplasmic shuttling protein with a functional nuclear export pathway that depends on the recognition of a nuclear export signal located in the transmembrane domain of β-dystroglycan (^{763}ILLIAGIIAM^{772}) by exportin CRM1. He further showed that altered nuclear export of β-dystroglycan is involved in the development of nucleolar stress in Hutchinson-Gilford progeria syndrome cells, thus further demonstrating the importance and biological significance of controlling the physiological overall levels of β-DG as well as the nuclear levels β-dystroglycan.

6.3 The role of dystroglycan in postnatal brain development.

Holly Colognato presented work which showed that dystroglycan is critical for brain development, indeed the dystroglycanopathies can result in profound deficits in brain structure and function. In the developing brain, dystroglycan is found on the basal endfeet of embryonic radial glia [16] and the loss of radial glial attachment to the pial basement membrane is thought to underlie neuronal migration defects in these dystroglycanopathies [17]. In the adult brain, dystroglycan is found on the perivascular endfeet of astrocytes, where it mediates their adhesion to the vascular basal lamina at the blood-brain barrier and regulates Kir4.1 and aquaporin-4 localization. However, there is limited understanding of dystroglycan's function in the developing postnatal brain.

Extracellular matrix, including the dystroglycan ligand, laminin, has been reported to regulate neural stem cell quiescence in the subventricular zone (SVZ) of the adult brain. However the function of extracellular matrix and its receptors in the developing SVZ remains unknown. **Holly Colognato** discussed a new study in which her lab found that dystroglycan regulates a unique developmental restructuring of extracellular matrix in the early postnatal SVZ. Dystroglycan is furthermore required for ependymal cell differentiation and assembly of niche pinwheel structures, at least in part by suppressing Notch activation in radial glial cells, which leads to the increased expression of *MCI, Myb,* and *FoxJ1*, transcriptional regulators necessary for acquisition of the multiciliated phenotype. Dystroglycan also regulates perinatal radial glial cell proliferation and transition into intermediate gliogenic progenitors, such that either acute or constitutive dystroglycan loss-of-function results in increased oligodendrogenesis. These findings reveal a role for dystroglycan in orchestrating both the assembly and function of the SVZ neural stem cell niche.

7 Understanding the laminin binding, interface/functional consequences how they relate to different tissues/developmental regulation. How can we move forward what models would be useful/what techniques?

7.1 Structural studies of the LARGE glycan.

Erhard Hohenester (London UK) and Kevin Campbell (Iowa, USA) have determined a crystal structure of laminin G-like (LG) domains 4-5 of laminin α2 bound to a LARGE-synthesized oligosaccharide. Their results provide the first atomic-resolution insight into the laminin-α-DG interaction that is compromised in a number of congenital muscular dystrophies. The crystal structure shows that a single glucuronic

acid-β1,3-xylose disaccharide unit of the LARGE glycan straddles a Ca^{2+} ion in the LG4 domain, with oxygen atoms from both sugars replacing Ca^{2+}-bound water molecules. The chelating binding mode explains the high affinity of this protein-carbohydrate interaction, which was determined by using NMR. Specificity for the LARGE glycan is achieved by steric exclusion of C5-substituted sugars from the xylose subsite. The binding mode of the LARGE glycan is predicted to be conserved in all other LG domains, and is consistent with prior mutagenesis results of neurexin domains LG2 and LG6.

7.2 Adhesion complexes in muscle

Mutations in the LAMA2 gene encoding the α-2 chain of laminin-211 cause congenital muscular dystrophy (MDC1A). Laminin-211 chain binds two major cell surface on the muscle cell surface namely dystroglycan, which is part of the dystrophin-glycoprotein complex, and integrin α7β1. Absence of laminin α2 chain does not affect the expression of the dystrophin-glycoprotein complex but leads to a secondary reduction of integrin α7 chain at the sarcolemma. In order to establish the relationship between laminin α2 chain and members of the dystrophin-glycoprotein complex and integrin α7, respectively, **Madeleine Durbeej** and co-workers generated three new mouse models. Mice deficient in either laminin α2 chain and dystrophin (dy3K/mdx) or laminin α2 chain and α-sarcoglycan (dy3K/Sgcb), display extremely severe muscular dystrophy, with massive muscle degeneration, inflammation and fibrosis. Mice devoid of laminin α2 chain and integrin α7, on the other hand, do not display an aggravated phenotype compared to single laminin α2 chain knock-out mice. These data suggest that laminin α2 chain and members of the dystrophin-glycoprotein complex have overlapping but

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non-redundant functions despite being part of the same adhesion complex. In contrast, laminin α2 chain and integrin α7β1 have very similar roles in muscle.

More recently, the **Durbeej** group has performed comparative proteomic analyses of affected muscles from dy3K/dy3K mice in order to obtain new insights into the molecular mechanisms underlying MDC1A. A large number of differentially expressed proteins in diseased compared to normal muscles were identified. A majority of the dysregulated proteins were involved in different metabolic processes and mitochondrial metabolism. These data imply that metabolic alterations could be novel mechanisms that underlie MDC1A and might be targets that should be explored for therapy. Indeed, preliminary data indicate defective metabolism in laminin α2 chain-deficient muscle cells and this new disease driving mechanism can be targeted with good results in mice.

7.3 New insight into LARGE2 and its substrates

LARGE2 is a homolog of LARGE1, and both are bifunctional glycosyltransferases that elaborate a Xyl-GlcA disaccharide repeat on α-DG. This disaccharide repeat forms the functional binding glycan that links α-DG at the cell surface to laminin G domain-containing proteins in the extracellular matrix. **Kevin Campbell** showed that LARGE2 can extend a IIH6 reactive (laminin-binding) glycan on ES cells that are deficient for α-DG or the ability to transfer O-mannose or ribitol 5-phosphate, indicating that functional glycans exist independent of α-DG. He identified glypican-4 and other proteoglycans (i.e. biglycan, syndecans) as endogenous substrates for LARGE2 in vitro and in the kidney, using biochemical and mass spectrometry methods. These findings reveal that LARGE2 has activity toward both heparan sulfate- and chondroitin/dermatan sulfate-containing proteoglycans.

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8. Omics: the search for new mutations, biomarkers and post translational modifications.

8.1 Biomarkers

Sebahattin Cirak presented clinical data of 48 patients, largely recruited in collaboration with Haluk Topaloglu. The inclusion criteria was based upon clinical phenotype, high CK, brain and eye involvement together with a deficiency of α-DG on muscle biopsy. Forty-eight patients, including 4 affected siblings, from 44 families with α-DGpathy were included in the study, 83% of the cases are solved and 17% remain unsolved. Molecular work-up included whole exome sequencing including Copy Number Variation analysis. The mean age of the patients (27 boys, 21 girls) were approximately 6.4 years (6 months-19 years). Consanguinity was present in 24 families, and 9% (n=4) had an affected sibling. MRI was available in 33 patients (69%), and 28 (84.8%) had abnormal findings. Homozygous or compound heterozygous mutations in α-DGpathy genes were detected in 83% probands. Mutations in POMT2 were the most prevalent (n=9) in our cohort, followed by POMT1 (n=7) and POMGNT1 (n=7), TMEM5 (n=2), FKRP (n=4), FKTN (n=2), GMPPB (n=2), ISPD (n=2), POMK (n=2), B3GNT1 (n=1) and LAMA2 (n=2). Interestingly, two of our cases were patients with a mutation in LAMA2 and one case identified as a double trouble mutation in FKRP and SEPN1 with a congenital muscular dystrophy phenotype.

FKRP mutations were frequently associated with cerebellar cysts although a larger study would be needed to confirm statistical significance. Muscle eye brain patients often displayed spasticity of the lower limbs. Importantly, we reported a patient with LGMD and congenital mirror movements, with a novel homozygous missense mutation

in POMK (exon 5, c.401T>G, p.V134G). A 17-year-old right-handed boy who presented with childhood onset muscle weakness, easy fatigue, clumsiness, and difficulty in running and climbing. Remarkably, physical examination at 19 years revealed mirror movements in the upper limbs indicating a defect in axon guidance. To date, mirror movement have been reported in only one patient with αDGpathy which was due to LARGE mutation [23]. Our etiological yield with exome sequencing has been 83 % so far.

With regard to clinical endpoints/biomarkers **Sebahattin Cirak** discussed a pilot biomarker study undertaken in collaboration with the Wellstone Muscle Center in Iowa. Dr. Mathews provided 22 anonymous LGMD2I serum samples from subjects between 6 and 49 years to analyze potential serum biomarkers that were identified in DMD mouse models and in boys with DMD [19]. In preliminary work, in collaboration with Yethrib Hathout Dr. Cirak used label free mass spectrometry to identify the target proteins. Preliminary Results are shown in Table 1 and validation of these markers in longitudinal studies is in progress.

MMP9 was also discussed since an increase of MMP9 was observed with age in control serum which was not observed in control plasma samples. This suggests that MMP9 serum levels might be age related rather than related to disease progression and is remarkable since MMP9 has been investigated as potential biomarker in other muscular dystrophies. Furthermore, it has been debated that MMP9 is increased with the activation of thrombocytes and the clotting cascade [20] and MMP9 is known to be involved in the proteolysis of β-DG.

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Sebahattin Cirak also reported that he has isolated total RNA from LGMD2I serum samples and quantified 800 known human miRNA with the NanoString technology which allows absolute quantification. A heatmap of 12 miRNAs has been constructed showing a convincing clustering depending on the age of LGMD2I patients. Further biomarkers studies including innovative novel technologies for protein and miRNA quantification from urinary exosomes and plasma is in progress.

8.3 Dystroglycan phosphorylation

Steve Winder (Sheffield, UK) reported that by studying the fate of the dystrophin glycoprotein complex in Duchenne muscular dystrophy (DMD) he has identified tyrosine phosphorylation of dystroglycan, the key transmembrane laminin receptor, as central to the loss of the entire DGC from the sarcolemma. Preventing phosphorylation of dystroglycan in *mdx* mice by mutation of a key tyrosine phosphorylation site ameliorates the dystrophic phenotype ([21]). Studies in mouse myoblasts also demonstrate that pharmacological treatment with proteasome or tyrosine kinase inhibitors can increase levels of non-phosphorylated dystroglycan. Furthermore by inhibiting tyrosine phosphorylation, ubiquitination or proteasomal degradation pharmacologically he demonstrated a reduction in dystroglycan phosphorylation and a rescue of the dystrophic phenotype in *sapje* zebrafish, a fish model of DMD. Through the use of FDA approved cancer therapeutics, he showed a significant improvement in *sapje* zebrafish swimming ability when treated with either proteasome inhibitors or tyrosine kinase inhibitors such as dasatinib ([22]). These studies have now been extended into *mdx* mice and again certain drug regimen demonstrate improvements in muscle pathophysiology including muscle central nucleation, serum creatine kinase

levels, restoration of dystroglycan and sarcoglycan to the sarcolemma and in physical parameters such as wire hanging times.

These studies demonstrate the utility of inhibiting dystroglycan tyrosine phosphorylation as a therapeutic strategy for DMD, particularly as several of the compounds that are effective are in existing clinical use. Although this strategy was initially focused on identifying new therapeutic strategies to treat DMD, the fact that the net result of preventing dystroglycan phosphorylation on tyrosine is to stabilize it in the sarcolemma may be of benefit in the dystroglycanopathies. Reduction in alpha dystroglycan binding to laminin in the dystroglycanopathies could perturb outside in signaling from the extracellular matrix and lead to aberrant intracellular signaling that could also affect dystroglycan stability. Blocking this signaling pathway by inhibiting tyrosine phosphorylation may serve to stabilise alpha- and beta-dystroglycan at the sarcolemma and preserve the function of α-DG albeit with a reduced laminin binding capacity as exemplified in the dystroglycanopathies.

9. A parents perspective on caring and potential treatment strategies.

Monika Liljedahl (mother of a boy with Limb Girdle Muscular Dystrophy and representative for Cure CMD) described how her son and his brother were IVF babies of parents 40 years of age. The pregnancy was uncomplicated and they were born at 8 month by C section. A developmental delay was noted already within the first 6 weeks of birth with difficulties holding up the head. Her affected son could not sit up and keep up with his brother. At 7 months weak reflexes were noticed. At 14 months a blood test showed a CPK of 5000, a muscle biopsy taken at 18 months showed muscular dystrophy, but no more specific diagnosis could be given. He could walk at 2.5 years, and carried on until 10 years of age. However, he has never been able to skip or run,

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and has only raised himself up from the floor a few times. He is very healthy apart from his muscle weakness, and is intellectually normal. Sequencing for the FKRP gene at 3 years of age showed that he is a compound heterozygote for two mutations in the FKRP gene. As a parent Monika set up a screen using skin fibroblasts to identify compounds that might up-regulate glycosylated α-DG and increase laminin binding. About 100, 000 compounds have so far been screened, these are mostly marketed drugs and natural compounds. The best hits have been HDAC inhibitors. The boy does various physical therapies that are helpful and the family strives for a normal life by engaging the boy in many spare time activities such as skiing, sailing and hiking. He is very intellectual, has many friends and enjoys computer games. He has a service dog that always stays at his side and which he finds very beneficial.

10. Conclusions.

The aims of this workshop were to bring together researchers working on the clinical and basic science aspects of dystroglycan glycosylation, whether in relation to the pathophysiology of patients, animal models of dystroglycanopathies, or cellular systems addressing the effects of these modification on dystroglycan function. A number of clinicans and scientists with an interest in different aspects of the dystroglycanopathies reviewed a broad range of topics, from patient registries and clinical diagnostic approaches to the functional role of dystroglycan in muscle and brain, as well as potential new therapeutic approaches to treat this group of diseases. We also benefited from presentations from a patient and a parent providing us with insight into what it is like to live with LGMD2I.

DNA sequencing technology has transformed the diagnosis of the dystroglycanopathies and this information is being collected in registries in order to facilitate the

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implementation of clinical trials if and when viable therapeutic approaches become available. With a doubling since the previous workshop ([23]) in the number of identified genes directly responsible for the full function of dystroglycan, much discussion centred around how these genes contribute to the way that dystroglycan performs its role in muscle and brain, as well as other tissues and cells where dystroglycan is also present. Presentations also covered potential treatments for the dystroglycanopathies which have so far been tested in mice with similar disease symptoms. This ranged from simple small molecule inhibitor drugs to alleviate symptoms, to more complicated gene replacement therapies using viruses to get new genes into muscle.

The following key conclusions were reached.

1.   An agreement from those present to form a consortium to address future major scientific funding programmes that may be announced by National or International agencies, such as the EU Horizon 2020 project

2.   A consensus for the naming of dystroglycanopathies which should be classified as Primary Dystroglycanopathies (affecting the dystroglycan gene directly) the Secondary Dystroglycanopathies (affecting genes that directly modify dystroglycan) and Tertiary Dystroglycanopathies (mutations in genes that indirectly affect dystroglycan function). This recommendation will be taken forward to a forthcoming ENMC Workshop that will specifically address naming and nomenclature of LGMD which also encompasses several dystroglycanopathies.

3.   To share important research reagents and tools such as antibodies, cells, tissues and mice through existing biobanks, and to encourage the sharing of therapeutic viruses.

4.   Agreement to apply for funding to hold another meeting in 4 years time.

Participants

- **Neil and Lyndsey Bevan (Patient and Carer Representative) (UK)**
- **Sue Brown (UK)**
- **Hans van Bokhoven (Netherlands)**
- **Kevin Campbell (USA)**
- **Sebahattin Cirak (Germany)**
- **Bulmaro Cisneros (Mexico)**
- **Holly Colognato (USA)**
- **Erhard Hohenester (UK)**
- **Dirk Lefeber (Netherlands)**
- **Monika Liljedahl (Sweden)**
- **Yung-Yao Lin (UK)**
- **Qi Lu (USA)**
- **Natalie Seta (France)**
- **Dirk J. Lefeber (Nijmegen, The Netherlands)**
- **Erherd Honenester (UK)**
- **Halyna Shcherbata (Germany)**
- **Volker Straub (UK)**

- **Steve Winder (UK)**

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