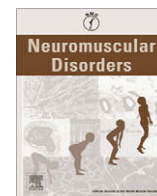




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Workshop report

166th ENMC International Workshop on Collagen type VI-related Myopathies, 22–24 May 2009, Naarden, The Netherlands

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1. Introduction

Twenty-four clinicians, basic scientists and representatives of a patient advocacy organisation and pharmaceutical companies from 8 countries (Australia, England, France, Germany, Italy, Switzerland, Turkey, USA) gathered in Naarden, The Netherlands, to discuss the group of neuromuscular disorders caused by the deficiency of collagen type VI (ColVI), collectively termed ColVI-related myopathies.

This meeting focused on three main areas: (1) the great heterogeneity observed at the genetic and clinical levels and the resulting complexity of the molecular diagnosis; (2) the pathophysiological mechanisms linking mutations in the *COL6A* genes to muscle pathology; (3) the current strategies for therapeutic interventions which have led to the design of upcoming clinical trials. Two sessions were also dedicated to existing and future databases, and to outcome measures.

2. Genetics and clinical phenotypes

2.1. Clinical phenotypes

The clinical spectrum of classical ColVI-related myopathies was presented and discussed by various participants, including Anne Lampe (Edinburgh, UK), Kate Bushby (Newcastle upon Tyne, UK), Enrico Bertini (Rome, Italy), Carsten Bönnemann (Philadelphia, USA), Luciano Merlini (Ferrara, Italy), Francesco Muntoni (London, UK), Susana Quijano-Roy (Garches, France), Beril Talim (Ankara, Turkey).

Anne Lampe (Edinburgh, UK) reviewed the main clinical features of Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM), the most commonly recognized forms of ColVI-related myopathies, as well as the variety of genetic defects involved. Although UCMD and BM were originally described as completely separate disorders, they share common clinical features and are both caused by mutations in the genes encoding ColVI, with over 30 papers published to date confirming this. BM was

classically described as a dominantly inherited relatively mild disorder characterised by proximal muscle weakness and distal joint contractures and UCMD as a recessively inherited congenital muscular dystrophy causing severe muscle weakness associated with proximal joint contractures and distal hyperlaxity. However, it is important to bear in mind that a congenital presentation or lack of family history does not preclude a diagnosis of BM and that contractures in BM can be highly dynamic in the first few years of life. Over 45 different dominantly acting mutations have now been reported in more than 65 BM families, more than 35 mutations in 30 patients with autosomal recessive (AR) UCMD and more than 38 dominantly acting mutations in 58 patients with autosomal dominant (AD) UCMD, so that AD UCMD now appears to be at least as common as AR UCMD. Most mutations causing AR UCMD are nonsense mutations and there are no obvious mutation hotspots, while most dominantly acting mutations in UCMD patients are (de novo) splice changes/deletions causing in-frame deletions that maintain the Gly-X-Y amino acid motif and spare the cysteine in the N-terminal region of the triple helical (TH) domain. For patients with a BM phenotype, the most common mutational mechanisms are glycine substitutions in the N-terminal region of the TH domain that interrupt the Gly-X-Y amino acid motif. Of note, identical mutations have been identified in patients who were described to have a BM or UCMD phenotype.

Luciano Merlini and Francesca Gualandi (both from Ferrara, Italy) presented two patients with a BM phenotype (mild axial and limb-girdle weakness, preserved ambulation at age 27 and 48, fingers contractures) and autosomal recessive inheritance [1]. Both patients carry a truncating *COL6A2* mutation on one allele and missense changes in the partnering allele within the C2 domain of the $\alpha 2(\text{VI})$ chain. They showed decreased amounts of ColVI in the basal lamina of muscle fibres and in dermal fibroblast cultures, and altered behaviour of ColVI tetramers. Biochemical studies supported the pathogenic effect of identified amino acid substitutions, which involve strictly conserved residues. Recognizing that similarly to UCMD, BM may also be caused by recessive mutations has relevant implications both for genetic counselling and molecular characterisation, as well as for genotype-phenotype correlations. Other participants acknowledged that they also identified AR BM patients (Bushby, and Bönnemann [2]).

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Enrico Bertini (Rome, Italy) reported descriptive statistics on clinical aspects of his series of 14 patients with UCMD who received molecular genetic confirmation and have been followed up for at least 3 years. Two patients had never been able to walk (14%), while 5 (36%) patients lost walking ability by the age of 9 years (range 5–9 years), and 7 (50%) patients were still walking at the time of the last examination although they were all under the age of 10 years. Immunohistochemical studies in this series showed that ColVI was absent or abnormally reduced in all patients. Most of the patients with UCMD lose ability to walk by the end of the first decade, emphasizing the importance of this parameter as a clinical endpoint that can be easily detected. Among their series of patients, only one died a few days after exacerbating respiratory distress with the chest X-ray showing bronchopneumonia, diaphragmatic paresis and a severely reduced chest volume. Autopsy showed severe right ventricle hypertrophy of the heart consistent of a *cor pulmonale*, generalized severe reduction of muscle fibres that were mostly replaced by fibrotic tissue in the diaphragm, and by fat and fibrosis in the other muscle groups.

In addition, Bertini reported on a long lasting follow-up of a published family [3] with two siblings presenting a very mild phenotype due to a missense mutation in the *COL6A3* gene (c.G6421A causing a p.G2056R substitution in the triple helix domain). Interestingly, both sibs developed a chronic osteochondritis that is still persistent but attenuated at the age of 25 and 30 years. Thus, chronic chondritis may be an unusual manifestation of BM, particularly in patients with relatively preserved muscle strength.

In light of the variability in the clinical severity and progression of ColVI-related myopathies, the participants have recognized the need for a revised clinical classification taking into account patients with intermediate presentation. Indeed, to date 19 individuals have been described whose phenotype appears to be intermediate in severity between what was originally described as UCMD and BM. Luciano Merlini pointed out that without a clear-cut definition of the two major phenotypes the same patient may be described as a severe BM or a mild UCMD with evident confusion and uncertainty in the correlation with the genotype. Reviewing the literature it is evident that the age at onset of the symptoms, limbs and fingers contractures, distal laxity and skin changes, are not different enough to separate clearly the two allelic forms [4,5]. The distinguishing features between the two forms are instead the motor and respiratory functions. The patients with UCMD never acquire ambulation or walk for a limited period of time [6]. On the contrary patients with BM are able to walk during adulthood with only some needing aids in the sixth decade of life [7–10]. Respiratory function is invariably and early compromised in UCMD patients who usually require mechanical ventilation at the beginning of the second decade of life, while in BM the respiratory function remains mainly normal with only a few patients requiring assisted ventilation later in life [9,11,12]. Merlini suggested restricting the label of UCMD to the patients who never walked or lose the ability to walk by the age of 12, the BM label to the patients who are able to walk during adulthood and considering as intermediate the patients who lose the ambulation during their teens.

Luciano Merlini reported on the myosclerosis myopathy (MM), recently recognized as a ColVI-disorder allelic to UCMD and BM. In a consanguineous family two siblings presented the characteristic feature of MM: early, diffuse, and progressive contractures resulting in severe limitation of movement of axial, proximal, and distal joints, and slender muscles with firm “woody” consistency [13]. The two patients had a novel homozygous nonsense *COL6A2* mutation (p.Q819X); the mutated messenger RNA escaped nonsense-mediated decay and was translated into a truncated $\alpha 2(VI)$ chain, lacking the C2 domain. The truncated chain associated with the

other two chains to form monomers and dimers while tetramers were almost absent. Secreted collagen VI was quantitatively reduced and structurally abnormal in cultured fibroblasts. Mutated collagen did not correctly localize in the basement membrane of muscle fibres and was absent in the capillary wall. Ultrastructural analysis of muscle showed an unusual combination of basement membrane thickening and duplication, and increased number of pericytes.

Susana Quijano-Roy (Garches, France) reported on two retrospective studies to identify predictive factors of severity in patients with early-onset (hypotonia, weakness or joint or spinal deformities before 2 years of age) ColVI-related myopathies, recruited through the French CMD Network. Indeed, the significant variability of severity in the clinical presentation and course of the myopathy makes it difficult to identify phenotype–genotype correlations and to predict the course of the disease in the everyday clinical setting. The first analysis was performed in a series of 22 patients (4–21 years, mean 14 years; 86% older than 10 years); all were personally examined by S. Quijano-Roy, many with more than 5 years of follow-up with serial forced vital capacity (FVC) measurements in sitting and lying positions at Raymond Poincaré's Hospital (Garches, France). The results obtained were compared to a larger series of 48 patients from different countries, recruited by the French network, (4–34 years of age, mean 16y), but this series lacked of a long-term follow-up data. These analyses showed that motor and respiratory function impairment evolved in parallel: those patients who never walked required mechanical ventilation in the first decade, while ambulatory patients had a variable course, from a progressive and often very severe one, similar to non ambulatory patients, to a less progressive or mild course. Loss of walking happened usually before mechanical ventilation (1–4 years before, although one case with severe scoliosis required ventilation before she lost ambulation). Patients were classified in three “prognosis” groups according to their motor performances and course, as proposed in [14]: (1) patients never walking (*early severe, ES*); (2) Patients who acquired walking and lost this ability or had only indoors ambulation (*moderate-progressive, MP*); (3) patients walking outdoors and not requiring ventilation (*mild, M*). In both studies, the MP group was more represented (~50%). The conclusion of these studies were that proximal joint contractures, spinal or cervical deformities at birth are the early clinical signs most frequently observed in severely affected patients, and therefore appear associated with a worse prognosis. Progressive respiratory restrictive insufficiency with diaphragmatic involvement is a typical and major feature of early-onset patients. Therefore, spirometry studies with special emphasis on FVC in supine position are very important in follow-up for adequate management. The best prognostic factor for severity in ambulatory patients was the FVC% during the first decade of life. Those patients with antecedent of CV over 70% sitting or 60% in supine at the end of the first decade were still ambulant and had not major respiratory insufficiency at last visit. The period of high risk of more rapid progression of complications is the growth period (7–13 years) and has to be closely followed (risk of rapidly progressive scoliosis and respiratory insufficiency). Prospective studies in a larger number of patients and longer follow-up are required to confirm these results, before evaluation of the impact of therapeutic interventions. Other parameters or signs may also need to be studied in prospective studies: motor function scales (MFM), contractures (degree and localization), spinal deformity (degree and level), and surgical tenotomies (localization and number of interventions in the same patient).

Francesco Muntoni (London, UK) reported on the course, complications and long-term outcome in a population of 17 UCMD cases followed in London, with special reference to life changing events, including loss of ambulation and respiratory insufficiency.

In order to have an insight on the long-term prognosis, only individuals who were 15 or older at the last clinical review were included in this study. In this population, the mean age at onset of symptoms was 11 months (SD 13). Twelve patients (70.6%) acquired independent ambulation at a mean age of 1.5 years (SD 0.7). Thirteen patients (76.5%) became constant wheelchair users at a mean age of 12.1 years (SD 4.2). Three patients continued to ambulate with assistance. FVC values were abnormal in all patients from 6 years of age onwards. The mean FVC (% predicted) declined at a mean rate of 3.4% (SD 1.6) yearly. Thirteen patients (76.5%) started non-invasive ventilation at a mean age of 14.9 years (SD 4.4). Two patients died from respiratory insufficiency which had been diagnosed but the family had elected against the initiation of non-invasive ventilation. This study showed that the invariable deterioration of motor ability and respiratory functions of UCMD patients does not correlate with severity at presentation, which is important to acknowledge when counselling new cases [15]. Muntoni also discussed a large collaborative study between London and Philadelphia, inviting other centres to participate as well. Data from more than 140 UCMD and BM cases have already been collected; the scope of this study is to address the spectrum of clinical features in individuals with collagen VI disorders, from the severe end of the UCMD spectrum to mild BM, encompassing individuals with intermediate severity.

Finally, several participants mentioned patients with phenotypes highly suggestive of UCMD or BM where no mutations can be found in the ColVI-encoding genes, so other genes are likely to be involved and need to be identified.

2.2. Diagnostic regimens

2.2.1. Immunohistochemical approaches

Kate Bushby (Newcastle upon Tyne, UK) presented on the use of a dermal fibroblast-based diagnostic algorithm for BM [16]. Dual immunofluorescent labelling (IF) of ColVI and perlecan in muscle biopsy, a technique used to inform UCMD diagnosis, was shown to be of no additional use to standard single immunolabelling in BM. However, immunofluorescent labelling of ColVI in skin biopsy-derived fibroblast cultures showed abnormalities in over 78% of genetically confirmed BM patients. In addition, in a group of patients with unknown diagnosis studied prospectively, the fibroblast IF technique was highly predictive of the presence of a COL6A mutation, providing a positive predictive value of 75%, a sensitivity and negative predictive value of 100% and a specificity of 63%. From this it was concluded that immunofluorescent labelling of ColVI in fibroblast cultures is a useful addition to current diagnostic services for BM. It can be used to guide molecular genetic testing, the gold standard diagnostic technique for BM, in a cost effective and time saving manner.

Francesco Muntoni presented the strategy followed in the London Centre, funded by the Department of Health NCG (National Commissioning Group), involved in the clinical, pathological and genetic diagnostic and advisory service for congenital muscular dystrophies and congenital myopathies in UK. Regarding the diagnostic muscle and skin biopsy service, patients affected by UCMD are typically diagnosed following a muscle biopsy and the demonstration of abnormal ColVI expression following double immunolabelling studies with another protein which colocalises in the basal lamina (such as perlecan, as described in [17]). In case a muscle biopsy cannot be obtained, fibroblasts studies are performed to look at ColVI expression in fibroblasts permeabilised or not. In BM cases, the diagnosis is established in cases with evocative clinical features (and family history whenever appropriate) after having excluded other conditions with the relevant muscle biopsy and genetic investigations and either ColVI fibroblast analysis or muscle MRI, or both (see below). Muntoni reported that while the sen-

sitivity and specificity of muscle (and fibroblast, whenever done) ColVI expression abnormalities was 100% in UCMD, the specificity of ColVI abnormalities in fibroblasts in BM was lower, with a number of patients with clear abnormalities in skin fibroblasts in whom genetic analysis finally ruled out a primary involvement of the COL6A genes. While not all of these patients had been systematically studied by muscle magnetic resonance imaging (MRI), in several individuals the changes were different from those reported in BM [18], suggesting that the specificity of changes on muscle MRI could be higher than the one of abnormal ColVI processing in fibroblasts, at least in BM. The current diagnostic algorithm therefore gives the preference to the clinical and muscle imaging findings in BM; fibroblast protein and transcription studies are performed subsequently to address the significance of changes identified with the genetic analysis.

Muntoni also reported a pilot project in his unit aimed at assessing the sensitivity and specificity of Fluorescent Analysis Cell Sorting (FACS) in the diagnosis of ColVI-related disorders using fibroblasts. Preliminary data indicate that FACS can be performed much more rapidly compared to the traditional immunoanalysis of cultured fibroblasts (one day compared to 7 days); in addition FACS provides quantitative assessment of protein expression at the cell surface, with clear differences between patients with UCMD compared to patients with BM.

2.2.2. Sequencing approaches

Francesco Muntoni reported that regarding the molecular genetic studies carried out in the London Centre, this is performed typically using direct sequencing of the entire coding region of the three COL6A genes from fibroblast derived mRNA, although more recently genomic sequencing strategies of the three genes had been also pursued. This analysis is performed in the diagnostic laboratories of the unit at Guy's and now, after a period of optimisation and validation, is available to patients referred to the NCG service. The turnaround is expected in 8–12 weeks. A total of 79 cases were studied and reported in the last 18 months (46 cDNA and 33 DNA), of these 44 had clear pathogenic mutations, 15 had no identifiable changes and 20 has unclassified variants which role is currently being established using segregation analysis, whenever possible, or further transcription studies.

Francesca Gualandi's group currently carries out molecular characterisation of ColVI-related myopathies by standard cDNA or genomic sequencing with a mutation detection rates approximating 75–80% in UCMD and 65–75% in BM patients. However, since PCR based techniques obviously miss gross genomic rearrangements as well as Copy Number Variations both in the coding sequence and in intronic regions, they have designed a custom oligonucleotide-based Comparative Genomic Hybridization array (aCGH) (Agilent technology). The coding and regulatory regions of COL6A1, A2, A3, A5 and A6 genes were included in the array as well as 10 additional genes selected on the basis of their functional relationship with ColVI and therefore possible candidate as UCMD/BM genes. The COL6A array was tested in a cohort of 18 UCMD/BM patients negative at sequencing analysis and in three subjects carrying a single COL6A mutation unable to explain the clinical phenotype being either inherited from a healthy parent or shared with unaffected relatives. A 2 kb deletion was identified within intron 1 of COL6A2 gene in a BM patient, occurring in compound heterozygosity with a small deletion in exon 28, detected by routinely sequencing and maternally inherited. RNA studies showed monoallelic transcription of the exon 28-deleted transcript, thus elucidating the functional effect of the intronic deletion that turned out to be paternally inherited. The identified intronic mutation represents the first example of pure intronic, regulatory mutation in COL6A genes.

Kevin Flanigan (Salt Lake City, Utah) presented the experience of the clinical testing program at the University of Utah Genome Center, which is directed by his collaborator Dr. Robert Weiss. Direct sequence analysis of the *COL6A1*, *COL6A2*, and *COL6A3* genes is performed using previously published methods [19]. This clinical testing is available to any physician who orders it, meaning that the clinical phenotype is not verified prior to testing. To date, among 221 non-carrier subjects tested, mutations have been found in 121. Seventy-six of these (63%) have only had a single mutation defined (29 in *COL6A1*, 23 in *COL6A2*, and 24 in *COL6A3*). Four had homozygous mutations (1 each in *COL6A1* and *COL6A2*, and 2 in *COL6A3*). The remaining 41 (34%) had compound heterozygous mutations, with six patients who had more than two potential disease alleles (where the deleterious effect of the variants is not yet established). Including these, the distribution of heterozygous alleles was 23 in *COL6A1*, 43 in *COL6A2*, and 23 in *COL6A3*. This data set has two limitations. First, detailed phenotypic information is not available on these clinically referred patients. This is being addressed by an MDA-funded project to retrospectively gather this data on existing clinical patients, and by the revision of clinical testing protocols to capture this information on all patients. Second, the methodology used does not detect deletions or duplications of one or more exons. The Genome Center is developing an exon copy number assay to restudy all patients in whom only a single disease allele was detected.

The French experience was presented by Pascale Richard (Paris, France) who described the analysis of 150 index patients by a strategy based on immunolabelling of ColVI in cultured fibroblasts, followed by sequencing of the coding sequences. All the variations are subsequently confirmed on genomic DNA from the patients, as well as the relatives whenever available. Seventy-seven patients (50%) carried a disease causing mutation. These patients were classified according to the severity of their phenotype in three groups; the UCMD group (39 patients), the BM group (12 patients) and an intermediate group (26 patients). The distribution between the three genes *COL6A1*, *COL6A2* and *COL6A3* was 39%, 38% and 29%, respectively. The mutations identified in the UCMD group were dominant de novo in 75% of the patients, while the remaining 25% carried recessive mutations. In the BM group, all mutations were dominant and transmitted as familial forms in patients with a family history (92%). In the intermediate group, 63% of the mutations were dominant de novo and 23% recessives. 14% of the patients could not be classified because parents were not available for testing. They showed that genomic mutations are private even if some exon skipping events at the mRNA level are recurrent (*COL6A1*-exon 14, *COL6A2*-exon 5 and *COL6A3*-exon16). In *COL6A1*, all mutations fall within the triple helical domain with a majority of exon skipping and missense substitution affecting one Glycine residue of the [Gly-X-Y] motif. In *COL6A2* and *COL6A3* the spectrum is wider with dominant mutations always affect the TH domain while recessive ones were found in the N- and C-terminal domains. The patients without a molecular defect identified underwent a clinical reassessment and half of them were then excluded as ColVI-related disorders because of a non typical muscle imaging, a cardiac involvement or high CK levels. The other half were confirmed as compatible and warrant further analyses.

Laura Briñas (Paris, France) reported on the quantitative analysis of transcripts of each *COL6A* gene in 48 early-onset patients classified according to the severity of their clinical phenotype as described in [14]. Immunolabelling of cultured fibroblasts from 43 of them displayed an abnormal secretion of the protein. Chain specific transcript quantification was carried out on cDNA obtained from total RNA extracted from fibroblasts by quantitative RT-PCR using specific primers in the C-terminal domain of each chain, and expressed relative to the beta-actin gene (Briñas/Richard et al., under revision). A rather large degree of variability was de-

tected in transcripts levels among control individuals and patients. Nevertheless, most of the patients' transcripts displayed a value below 1 (the normalized value of calibrator which serves as the reference for normal expression level). Moreover, in almost one third of the patients, a striking reduction of one of the chains was observed (values ≤ 0.1). Further sequencing analyses indicated that these chains harboured the pathogenic mutation. Premature termination codons (PTC) causing mutations or in-frame insertions led to transcripts values below 0.1, while missense mutations or in-frame deletions showed values above 0.1. In the case of PTCs, the greatly reduced transcript levels were most likely due to its degradation by nonsense-mediated decay. In an attempt to extract phenotype-genotype correlations, they observed that the five patients carrying homozygous PTCs upstream or within the triple helix domains were the most severe patients, included in the early severe category since they never walked. Their fibroblasts showed no ColVI secretion, and they displayed the lowest transcript levels (≤ 0.1). Eleven out of the 12 patients harbouring splice mutations leading to exon skipping belonged to the moderate-progressive category since they lost ambulation. No secretion or a reduced secretion of ColVI was detected, and the transcripts levels were above 0.1. Finally, the two patients carrying in-frame insertions were in the mild category. They remained ambulant at five and thirteen years of age and they displayed a reduced ColVI secretion and strikingly reduced transcript levels (0.1). For the rest of the patients no strict phenotype-genotype correlation could be established. This study demonstrates that mutations in the *COL6A* genes led to reduced transcript levels and that their quantification is a helpful tool in the diagnosis since it can point to the mutated chain, in particular in the case of PTC-causing mutations or in-frame insertions, which represent almost one third of the patients.

3. Pathophysiology (part 1)

Shireen Lamandé (Parkville, Australia) reviewed the various biological roles of collagen VI, a broadly expressed, perhaps ubiquitous, extracellular matrix (ECM) protein that has long been thought to have both structural and signalling roles. While muscle is the most severely affected tissue in patients with mutations in the ColVI-encoding genes, other tissues are also involved reflecting the wide distribution of ColVI and its various, often poorly understood functions. Collagen VI appears early during wound healing and is one of the first proteins deposited into the extracellular matrix of confluent cultured fibroblasts suggesting that it may be a scaffold for deposition of other matrix proteins. Collagen VI interacts directly with collagen I (Coll) and accelerates the rate of Coll fibril formation *in vitro* [20]. Consistent with this, Coll fibrils are abnormal in the skin of UCMD patients [21], and also in cultured fibroblasts from BM patients. Fibronectin matrix organisation is also disturbed in BM fibroblasts [22]. These data may explain the abnormal scar formation and other skin features in patients with ColVI disorders as well as joint hypermobility and tendon contractures. Collagen I is the major ECM protein in bone, and in cultured osteoblasts, ColVI up-regulates Coll mRNA and protein expression and enhances mineralisation [23]. It is not surprising then that the ColVI null mouse is smaller and has delayed ossification and reduced bone mineral density between 3 and 6 months [24]. Collagen VI is also abundant in cartilage where it is concentrated in the pericellular matrix surrounding chondrocytes. It is highly upregulated during the early stages of osteoarthritis in humans and in experimental mouse models and ColVI knockout mice have increased age related hip arthritis [24]. A role for ColVI in nerves is also emerging. Collagen VI is upregulated in the brains of mice expressing a mutant amyloid precursor protein that causes early-onset familial Alzheimer's disease in humans [25]. This suggested

that ColVI upregulation may be neuroprotective. In neuronal cell cultures, ColVI prevented neurotoxicity of the amyloid protein by sequestering it into large aggregates in the ECM thus preventing the mutant toxic protein interacting directly with the cells. In general, the signalling roles of ColVI are not well understood. There is evidence that ColVI promotes differentiation and proliferation and prevents apoptosis in a number of cultured cell models. Collagen VI binds to integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha10\beta1$ and possibly others [26] as well as NG2 proteoglycan [27], and likely signals through these receptors. Like other ECM proteins, ColVI may also regulate bioavailability of growth factors and cytokines. Collagen VI interacts with interleukin-2 [28], and binds oncostatin M [29], and keratinocyte [30] and platelet derived growth factors [31]; it may well interact with many others.

Carsten Bönnemann (Philadelphia, USA) reported on the cell of synthesis of ColVI in postnatal skeletal muscle. Knowledge about the origin of ColVI in muscle is of practical interest if future treatment approaches requiring cell-specific delivery are to be considered. The production and deposition of ColVI in pure cultures of primary myogenic cells as well as of muscle interstitial fibroblasts from limb muscle of neonatal mice was examined. By Northern blot analysis as well as by real-time PCR analysis there was no appreciable transcription from the *Col6a1*, *Col6a2*, and *Col6a3* genes in differentiated myogenic cultures. Immunofluorescence staining and Western blot analysis showed prominent secretion and matrix deposition of ColVI by interstitial muscle fibroblasts, but no detectable staining in myogenic cells culture. Myoblast conditioned media increased the ColVI transcription of interstitial fibroblasts 1.5–2-fold, whereas no significant change on ColVI expression in myogenic cells was observed when the myogenic cells were co-cultured with interstitial fibroblasts for different time periods. Indirect confirmation of the cell of origin for ColVI in postnatal human muscle was obtained by analysing muscle biopsies from patients with mutations in the *COL6A* genes that lead to intracellular retention of ColVI immunoreactive material, as judged by staining dermal fibroblast cultures from the patients. In the muscle biopsy sections the only cells retaining ColVI immunoreactive material were interstitial cells co-staining for vimentin and the fibroblast marker AB-11133, whereas no retention was seen in the muscle cells [32]. In agreement with these findings, the groups of Giorgio Bressan and Paolo Bonaldo recently characterised an enhancer for the *Col6a1* gene that drives expression in muscle interstitial cells but not in myogenic cells – they determined that this expression is induced by resident myogenic cells during embryonic development, after which the expression in the muscle interstitial cells is autonomous [33]. Thus, in postnatal muscle it is the muscle interstitial fibroblast population and not the myogenic cells that are responsible for deposition of the ColVI matrix.

Raimund Wagener (Cologne, Germany) presented the three recently identified new murine collagen genes, *Col6a4–6*, with extensive homology to the known collagen VI chains [34,35]. Analysis of $\alpha1(VI)$ chain knockout mice and phylogenetic analysis of the new chains clearly show that they belong to the ColVI subfamily and most likely substitute for the $\alpha3$ chain, forming ($\alpha1\alpha2\alpha4$), ($\alpha1\alpha2\alpha5$) or ($\alpha1\alpha2\alpha6$) heterotrimers. In mouse, the new chains are found in or close to basement membranes. In contrast to the ubiquitously distributed ColVI chains $\alpha1–3$, the new chains have a very restricted tissue distribution. Two of the three genes are retained in the human genome, *COL6A5* and *COL6A6*. However, the human *COL6A4* is split into two pieces and both parts have become transcribed non-processed pseudogenes [35,36]. Interestingly, the 5' part of the split *COL6A4* gene has recently been identified as a susceptibility locus for knee osteoarthritis in Japanese and Chinese patients and erroneously named *DVWA* [37]. The authors concluded that *DVWA* codes for a novel protein containing two von

Willebrand factor A (VWA) domains without a signal peptide sequence that intracellularly interacts with tubulin. The *COL6A5* gene, coding for the collagen VI $\alpha5$ chain (referred to as collagen XXIX by the authors) was recently linked to human atopic dermatitis [38].

Patrizia Sabatelli (Bologna, Italy) reported an immunofluorescence and electron microscopy study of the $\alpha5(VI)$ chain in normal and ColVI-deficient skin and skeletal muscle. In normal tissues, $\alpha5(VI)$ chain showed a striking restricted localization: it is expressed at the papillary dermis and related annexes in the skin, and at the myotendinous junctions in skeletal muscle. Immunogold electron microscopy study showed a tight association of $\alpha5(VI)$ chain with fibrillar collagen bundles both in skin and skeletal muscle. To determine whether $\alpha5(VI)$ chain expression and localization may be affected by mutations in *COL6A* genes, six skin and muscle biopsies from genetically characterised UCMD patients, were studied. By immunohistochemistry, all biopsies showed a reduced amount of $\alpha1–3$ chains ranging from moderate to severe. In skin of UCMD patients with mutations in the *COL6A1* and *COL6A2* genes, the $\alpha5(VI)$ chain was markedly reduced, indicating a possible post-transcriptional mechanism affecting intracellular assembly of $\alpha5(VI)$ chain in the presence of mutated $\alpha1$ and $\alpha2(VI)$ chains. In the skin of UCMD patient with *COL6A3* mutation, the $\alpha5(VI)$ chain was apparently normal, suggesting that it may substitute for the $\alpha3(VI)$ chain, forming $\alpha1–\alpha2–\alpha5$ heterotrimers. The restricted localization at junctions in skin and skeletal muscle suggests that $\alpha5$ -containing ColVI may have a specialized function in areas of tissues subjected to tensile stress.

Valérie Allamand (Paris, France) presented her studies on the intracellular retention frequently observed in skin-derived fibroblasts of genetically confirmed UCMD patients. Indeed, out of 149 fibroblasts cultures showing abnormal expression of ColVI (26% with complete absence of secretion, 65% with reduced secretion), 94 (69%) displayed intracellular immunostaining using “pan” antibodies against ColVI [39]. Co-immunolabelling with antibodies against resident proteins of the endoplasmic reticulum (ER) indicated that ColVI was retained in this subcellular compartment. Pre-embedding immunoperoxidase electron microscopy on fibroblasts cultures further confirmed these findings, thereby indicating that the assembly and trafficking of mutant ColVI was altered in these cells. Her group and Carsten Bönnemann’s examined whether the retention of ColVI in the ER triggered the unfolded protein response (UPR) in AR and AD UCMD fibroblasts cultures. By screening ER stress markers, they both demonstrated that this pathway is not involved in these cells.

4. Pathophysiology (part 2)

Paolo Bonaldo (Padova, Italy) presented the ColVI knockout mice, which was generated by his group via targeted inactivation of the *Col6a1* gene [40]. In the absence of the $\alpha1(VI)$ chain, ColVI cannot be assembled and secreted, thus homozygous null (*Col6a1*^{-/-}) have a complete lack of ColVI. *Col6a1*^{-/-} mice display an early-onset myopathic phenotype with histological changes and weakness of diaphragm and other skeletal muscles. A similar, although milder, myopathic phenotype is present in heterozygous (*Col6a1*^{+/-}) mice, which have half the amount of ColVI due to gene haploinsufficiency [40]. The defects displayed by *Col6a1* knockout mice strongly resemble those detected in BM and UCMD patients, and this animal model has provided considerable progress in understanding the pathogenic mechanisms of ColVI myopathies. *Col6a1*^{-/-} muscles are characterised by spontaneous apoptosis and ultrastructural defects of mitochondria and sarcoplasmic reticulum. Lack of ColVI has a major impact on muscle fibres, and triggers the mitochondrial pro-apoptotic pathway through opening of

the permeability transition pore (PTP), a high-conductance channel of the inner mitochondrial membrane playing a role in several forms of cell death and which can be desensitized by cyclosporin A (CsA). Lack of ColVI is the cause of increased PTP opening, and this mitochondrial event sets in motion the executioner mechanism of apoptosis *in vitro* and *in vivo* [41]. By studying skeletal myofibres derived from *Col6a1*^{-/-} mice, it was demonstrated that these defects are reversible and they can be normalized by treatment with CsA or by adhesion onto purified ColVI. Consistently, the mouse model could be cured with CsA, a widely used immunosuppressant that desensitizes the mitochondrial PTP independently of calcineurin inhibition. Short-term treatment of *Col6a1*^{-/-} mice with 10 mg/kg per day CsA resulted in complete rescue of the muscle ultrastructural defects and normalization of apoptosis through PTP inhibition [41].

Paolo Bonaldo and Paolo Bernardi (Padova, Italy) further addressed the role of mitochondrial dysfunction in ColVI-related myopathies both in the mouse model and in tissue cultures established from patients. The main findings are: (i) crossing the *Col6a1*^{-/-} mouse with the *Ppif*^{-/-} mouse lacking cyclophilin (CyP) D, the mitochondrial target of CsA, allowed to rescue the myopathic phenotype of ColVI null mice [42], a finding that provides a striking proof of principle that CyPD (and hence PTP) is a key effector mechanism in this disease model; (ii) cells from muscle biopsies of patients with both UCMD and BM display an abnormal mitochondrial depolarisation upon the addition of oligomycin irrespective of the expression of desmin, which is lost after a few passages in culture (below 0.3% desmin-positive cells at P7); (iii) in one BM patient the response to oligomycin normalized spontaneously after 10 passages in culture, suggesting that time-dependent *in vitro* selection may be a cause of variability, and possibly the basis for the normal phenotype found in BM cultures [43]. This analysis is currently being performed on cultures from different UCMD and BM patients.

On behalf of Debbie Hicks (Newcastle upon Tyne, UK) who could not attend the meeting, Anne Lampe (Edinburgh, UK) presented their *in vitro* studies of mitochondrial dysfunction in UCMD and BM cells [43]. Using the tetramethyl rhodaminemethyl ester (TMRM) assay, mitochondrial depolarisation was observed in only one of the clinically and genetically characterised UCMD fibroblast lines assayed, which could be rescued by CsA. Intriguingly, this fibroblast line is completely null for intra- and extracellular ColVI, a rare pattern only once seen in over 100 ColVI disease fibroblast lines. Furthermore, no mitochondrial depolarisation could be detected in myoblasts of other muscular dystrophies or disorders where it might have been expected, such as the allelic disorder BM, MDC1A, LGMD2A, DMD or Leigh syndrome. However, a rather severe mitochondrial depolarisation was confirmed in two UCMD myoblast lines assayed, and in myoblasts from a LGMD2B patient. Of note, these cell lines were negative for desmin, unlike all of the myoblasts cultures that did not depolarise. In addition, these cell cultures also presented an abnormal morphology and low growth rate. This could be described as a “latent pro-apoptotic phenotype” in keeping with observation of al-Rubeai and Singh that many cell lines will respond to the stressful culture environment by undergoing apoptosis [44]. Furthermore, mitochondrial depolarisation of UCMD muscle cultures could be rescued not only by CsA and ColVI, but also by plating on collagen I and laminin, two unrelated proteins of the ECM, questioning the specificity of the CsA effect and also whether it is an appropriate treatment strategy in ColVI disease. This group concludes that the link between the cellular phenotype as measured by the TMRM assay and development (and in the context of treatment, the progression) of disease needs to be better established, and feel that there is a need for other outcome measures when judging effectiveness of treatment in patients with ColVI-related disorders. These findings also lead to question

whether rather than being causative, apoptosis might in fact be a common pathogenic endpoint in muscle disease.

5. Therapeutic approaches

Luciano Merlini (Ferrara, Italy) reported on the results of the first pilot trial of cyclosporin A in ColVI-related myopathies [45]. The rationale for the use of CsA was based on the results obtained in the ColVI null mice [41] and UCMD primary myoblasts cultures [46]. An open short-term (one-month) clinical trial was carried out in 5 patients (3 children with UCMD and two adults) with ColVI myopathies. The primary endpoint was to see if CsA was able to correct the mitochondrial dysfunction. Before treatment, all patients displayed mitochondrial dysfunction and increased frequency of apoptosis, as determined in muscle biopsies. Both of these pathologic signs were largely normalized after 1 month of oral cyclosporin A administration, which also increased muscle regeneration [45]. These findings demonstrate that ColVI-related myopathies can be effectively treated with drugs acting on the pathogenic mechanism downstream of the genetic lesion, and they represent an important proof of principle for the potential therapy of genetic diseases. The parents of the 3 children with UCMD asked to continue the treatment after the completion of the one-month trial. After 2 year of continuous treatment the 3 children showed an increase in limb muscle strength, which raised from 140 to 192 N (34.6%) when measured with a myometer as a composite megascore (sum of bilateral hand-grip, elbow flexion, knee flexion and extension). In addition their motor function, which was deteriorating before treatment, remained stable. Their FVC, which was already severely reduced before treatment, did not improve, and one patient required nocturnal ventilation. The 28-year-old woman reported less fatigability in walking after 3 months of treatment, and had a mild increase in muscle strength, but then decided to stop treatment for familial reasons. The 58-year-old patient had a 31% increase in muscle strength in 3 months of treatment with 5 mg/kg/d of CsA, and then he was found to have an advanced prostate cancer. He underwent external-beam radiation and hormone therapy. As a consequence CsA was reduced to 2 mg/kg/d, and finally to 1 mg/kg/d because of an increase in serum creatinine. His muscle strength went down stabilizing just above the baseline in response to the decrease dosage of CsA. In conclusion, long-term CsA treatment stabilized motor function, increased limb muscle strength, but did not have an evident influence on the downhill course of respiratory compromise in the children with UCMD.

Santhera's development program for omigapil in congenital muscular dystrophy (CMD) was presented by Stefanie Possekel (Basel, Switzerland). Omigapil is an anti-apoptotic compound with neuro-rescuing properties originating from Novartis [47]. Omigapil has shown efficacy in two animal models of CMD. Data obtained in the *dy*^W/*dy*^W mouse model for MDC1A has shown that oral administration of omigapil reduces apoptosis in muscle, improves locomotion, reduces body weight loss, improves muscle histology, ameliorates skeletal deformations and reduces early mortality [48]. The anti-apoptotic effect in muscle was similarly seen in the mouse model of ColVI-deficiency in which it was apparent that the treatment effect improved over time. Functional assessment in this model is ongoing. UCMD was selected as the first indication for clinical development. Santhera is planning a 12 month double blind, randomized trial with 70–80 patients aged 5–15 years, distributed over three active dose arms plus placebo. Proposed clinical endpoints such as forced vital capacity and activities of daily living were presented. A comprehensive series of nonclinical studies has already been performed by Novartis and the nonclinical and technical aspects of drug development are well advanced. However, as

the compound has so far only been tested in an adult patient population, a juvenile toxicology study that is required prior to starting a clinical trial in paediatric patients is currently ongoing supported by funding from AFM.

Urs Ruegg (Geneva, Switzerland), reviewed the pharmacology of cyclosporins and their use in muscular dystrophies. Cyclosporin A [49] was isolated in 1972 as a cyclic undecapeptide from the fungus *Tolypocladium inflatum Gams* at the Sandoz Company (now part of Novartis) in Basel, Switzerland. CsA has revolutionized organ transplantation because of its potent immunosuppressive activity. CsA forms a complex with its ubiquitous intracellular binding protein cyclophilin A and calcineurin (protein phosphatase 2B), thereby inhibiting both, the prolyl-peptidyl isomerase activity of cyclophilin, and the calcineurin phosphatase activity. Apart from CypA, other immunophilins were discovered including the FK506 (tacrolimus) binding proteins FKBP, which are also prolyl-peptidyl isomerases and inhibit calcineurin. The Cyp family also increased in size; the new members were called CypB, C, D, etc., their structures, intracellular locations and presumptive functions were assigned. Of interest in the context of cell death pathway is CypD that inhibits the opening of the mitochondrial permeability transition pore (mPTP). With the aim of selectivity for cyclophilins rather than calcineurin (and other targets), several thousand analogues of CsA were prepared semi-synthetically. Debio 025 (UNIL025; D-MeAla3EtVal4CsA) has a 7000-fold higher inhibitory activity for cyclophilins than for calcineurin and was therefore investigated in a multitude of models where cyclophilins play a role including the mPTP. This interaction proved very useful to suppress the downstream pathological consequences of mutations in the ColVI gene [50]. As the mitochondrial death pathway was also suspected to be responsible for other muscular dystrophies, DEBIO 025 was also investigated in the mouse models of Duchenne muscular dystrophy (DMD) and limb-girdle muscular dystrophy [51,52]. Indeed, this compound inhibited the necrosis, the loss in muscle force and to some extent fibrosis in the *mdx* mouse. On the other hand, the degree of protection appears to be less impressive in these disease models than in the ColVI-related myopathies. They believe that while in the latter disorder the mitochondrial pathway plays a major role in the pathology, in *mdx*/DMD the ubiquitin-proteasome pathway might be more prominent. This hypothesis requires further experiments in order to be validated.

Eija Lundstrom (Lausanne, Switzerland) presented the status of the clinical development of Debio 025 at Debiopharm Group, for treatment of chronic hepatitis C (currently in Phase IIb). The mechanism of action of Debio 025 in HCV seems to principally involve binding to CypA, a key player in virus replication. Debio 025's increased Cyp binding, as well as the absence of calcineurin inhibition (determinant for immunosuppressive activity), were obtained via structural modifications of cyclosporine. To date more than 460 subjects have been exposed to Debio 025. Multiple doses have ranged from 50 to 2400 mg/day from 10 days up to 3 months. The drug is rapidly absorbed orally and eliminated mainly by hepatic metabolism and biliary excretion. The most important safety finding related to Debio 025 in clinical studies has been a dose related, reversible increase in serum bilirubin. This is caused by inhibition of biliary canalicular transporters responsible for the transport of bilirubin. There was no increase of transaminases or other liver enzymes. As mentioned before, Debio 025 is also able, by interfering with CypD, to prevent the inappropriate opening of the mPTP, an inner membrane channel that plays a role in several forms of cell death [53]. Treatment of *Col6a1*^{-/-} mice with Debio 025 led to normalization of latent mitochondrial dysfunction in skeletal muscle fibres, a significant reduction in ultrastructural damages and a decreased incidence of apoptosis in diaphragm muscle fibres, suggesting a correlation between PTP opening and pathogenesis of ColVI dystrophies [50]. A clinical pilot study

with Debio 025 is being planned in patients suffering from ColVI-related myopathies to confirm the results obtained with CsA [45], and to investigate also the efficacy on various clinical endpoints, such as muscle mass, muscle strength, motor ability, functional capacity, joint mobility, pulmonary function and quality of life, as well as the safety profile of Debio 025 in this patient population.

6. Patients registries and locus-specific databases (LSDB)

This session was dedicated to already existing registries for the various genes implicated in ColVI-related myopathies. Kate Bushby gave a short introduction on the network of excellence TREAT-NMD (www.treat-nmd.eu).

Anne Rutkowski (Olathe, USA) presented Cure CMD (www.curecmd.org), a non-profit advocacy group focused on CMD awareness, disease education and identification of drugs to slow disease progression. To pursue clinical trial readiness in the CMD will require a synergistic multifaceted approach targeting the development and implementation of CMD care standards, ongoing support for CMD translational research, establishment of a CMD drug pipeline and launch of both a CMD international patient registry and natural disease progression study. The development of the CMDIR (CMD International Patient registry; www.cmdir.org) started in November 2008 with a meeting at Indiana University (USA) bringing together a TREAT-NMD representative, Indiana University registry experts, paediatric neurologists and geneticists. An online beta test of registry questions followed with participation of 30 CMD affected individuals and families. A subsequent meeting sponsored by TREAT-NMD in April 2009 brought together neurologists from the European neuromuscular community to seek input and drive consensus. Since the occurrence of this workshop, a final version of the CMDIR was circulated and launched in French, German, Spanish and English in August 2009. The CMDIR is a patient self report registry which seeks to link those with a confirmed genetic mutation to the TREAT-NMD global database, direct those with disease confirmation through muscle immunohistochemistry to genetic testing and maintain active contact with those individuals who remain undiagnosed. The CMDIR will register patients with the following genetic mutations who span the CMD to LGMD spectrum: *COL6A* genes, *LAMA2*, *FKRP*, *fukutin*, *LARGE*, *POMT1*, *POMT2*, *POMGnT1*, *SEPN1*, *ITGA9*, *ITGA7* and *LMNA*. Patients with an *FKRP* or *LMNA* mutation will be directed to currently established registries for these two genes. There is recognition that the lines between the CMDs, the LGMDs and the congenital myopathies are blurred. Gene specific databases (LSDBs) are simultaneously being formalized. The CMDIR will drive patient and physician interaction with future LSDBs by directing patients with confirmed genetic mutations to LSDBs. A CMD Standard of Care effort has been launched under the direction of Dr. Thomas Sejersen, Dr. Ching Wang and Dr. Anne Rutkowski, with a working group recruited and a workshop held on Nov 14th–16th in Brussels. Deliverables include the publication of three documents: a CMD diagnostic review and two versions of CMD Care Guidelines (professional and lay friendly).

Carsten Bönemann presented the CHOP CoPS (Children's Hospital of Philadelphia Collagen VI Patient Survey) comprehensive study of the clinical manifestations of ColVI-deficiency in the USA. Beril Talim (Ankara, Turkey) reviewed the Myoccluster form that was drawn up several years ago and included a large number of clinical items. Valérie Allamand presented an update on the status of the locus-specific databases UMD-COL6A1-3 that have been developed over the last year in the Institut de Myologie (Paris, France) in close collaboration with the team of Christophe Bérout (Montpellier, France) [54]. To date, these databases contain all the

mutations reported in the *COL6A* genes and will be made accessible online in the course of 2010.

7. Outcome measures

Eugenio Mercuri (Rome, Italy) commented on the importance of identifying reliable outcomes measures that often constitute bottlenecks in designing clinical trials. So far, most measures assess FVC. Indeed, appropriate outcome measures depend on the clinical trial, the disease and the age of the patients. Working with regulatory authorities and having parents express themselves was felt important.

8. Conclusions and future perspectives

In conclusion, the present workshop raised a number of important points concerning collagen VI-related myopathies. In particular, fruitful discussions led to the recognition that a better characterisation of the clinical phenotype, and a common vocabulary within the community were recognized, since the literature is often confusing. Participants discussed the need to define natural history in collaborative studies. Indeed, these issues are of some urgency since clinical trials are being planned.

9. Participants

Valérie Allamand (Paris, France);
Paolo Bernardi (Padova, Italy);
Enrico Bertini (Rome, Italy);
Paolo Bonaldo (Padova, Italy);
Carsten Bönnemann (Philadelphia, USA);
Laura Briñas (Paris, France);
Kate Bushby (Newcastle Upon Tyne, UK);
Kevin Flanigan (Salt Lake City, USA);
Francesca Gualandi (Ferrara, Italy);
Shireen Lamande (Melbourne, Australia);
Anne Lampe (Edinburgh, UK);
Eija Lundstrom (Lausanne, Switzerland);
Eugenio Mercuri (Rome, Italy);
Luciano Merlini (Ferrara, Italy);
Francesco Muntoni (London, UK);
Stefanie Possek (Basel, Switzerland);
Susana Quijano-Roy (Garches, France);
Pascale Richard (Paris, France);
Urs Ruegg (Geneva, Switzerland);
Anne Rutkowski (Olathe, USA);
Patrizia Sabatelli (Ferrara, Italy);
Tanya Stojkovic (Paris, France);
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