# The 2nd MDA/AFM International Myotonic Dystrophy Consortium Conference

**Research Triangle Park, NC**
**April 21-23, 1999**

MDA

A F M
Association Française contre les Myopathies

**The Denver Fund For Health & Medical Research**

***GlaxoWellcome***

# The 2nd MDA/AFM International Myotonic Dystrophy Consortium Conference (IDMC-2)

## April 21-23, 1999
## Sheraton Imperial, Research Triangle, NC


**Scientific Organization Committee**
Geneviève Gourdon, PhD [1]
Martine Devillers, PhD [2]
Claudine Junien, PhD [1]
Charles A. Thornton, MD [3]
Allen D. Roses, MD [4]
Tetsuo Ashizawa, MD [5]

**Conference Coordinators**
Reecie Dolan [4]
Lynne Schachte[4]
Karen Gillard [5]

(1) INSERM UR 383, Hôpital Necker - Enfants Malades, Paris, France
(2) Département Scientifique, AFM, Paris, France
(3) Department of Neurology, University of Rochester, Rochester, NY, USA
(4) Glaxo Wellcome, Inc., Research Triangle Park, NC, USA
(5) Department of Neurology, Baylor College of Medicine, Houston, TX, USA



**Acknowledgment**
IDMC-2 is supported by grants from the Muscular Dystrophy Association (MDA), Association Française contre les Myopathies (AFM), the Denver Fund for Health & Medical Research, and Glaxo Wellcome, Inc.


**Cover**
A field of RNA molecules. The RNA was transcribed from a plasmid containing the DMPK gene with 90 CUG's flanked by 150 nt of non-CUG RNA on each end. The resulting molecule folds into a perfect duplex rod about the repeating CUGs, and the non repeat segments generate a ball like bush of unstructured RNA at one end of the rod. This electronmicrograph was provided by Drs. Susan Michalowski and Jack Griffith.

# Program

**April 21,1999**

| | | |
|---|---|---|
| **7:30am-8:30am** | **Registration / Continental Breakfast** | |
| **8:30am-8:40am** | **Welcome** | **T. Ashizawa**<br>**C. Junien**<br>**M. Wagner (Glaxo Wellcome)** |

**I. Invited Speakers' Presentations**

| | | |
|---|---|---|
| **8:40am - 11:30am** | **Invited Speakers: Molecular Pathogenesis** | |
| **8:40am - 9:05am** | **Ion channels and myotonia** | **Frank Lehman Horn** |
| **9:05am - 9:30am** | **Muscle potassium channels and hyperexcitability** | **John Adelman** |
| **9:30am - 9:40am** | **Discussion** | |
| **9:40am - 10:00am** | **Coffee break** | |
| **10:00am B 10:25am** | **Biogenesis of the DMPK transcript** | **Robert Singer** |
| **10:25am - 10:30am** | **Discussion** | |
| **10:30am B 10:55am** | **Spermatogenesis and genome instability** | **Mitch Eddy** |
| **10:55am - 11:20am** | **Skeletal muscle development and Six genes** | **Pascal Maire** |
| **11:20am - 11:30am** | **Discussion** | |
| **11:30am - 1:00pm** | **Lunch** | |

**II. Investigator Presentations:**

**(All presentations are 8-minute platform presentations, and several presentations are discussed together as shown. The presenters of abstracts are shown with the respective abstract numbers in parentheses.)**

**1:00pm -3:00pm** **CTG Repeat Instability (Chairs: Gourdon/Wells)**

**(1) Martorrell**
**(2) Gould**
**(3) Abbruzzese**
**(4) Gourdon**
**Discussion (10 minutes)**
**(5) Ansved**
**(6) Siciliano**
**(7) Ashizawa**

**(8) Spring**
**Discussion (10 minutes)**
**(9) Moore**
**(10) Wells**
**(11) Sinden**
**Discussion (12 minutes)**

**3:00pm - 3:20pm** **Coffee Break**

**3:20pm - 5:40pm** **Disease Mechanisms - DMPK DNA/RNA (Chairs: Singer/Timchenko)**

**(12) Timchenko**
**(13) Lia-Baldini**
**(14) Philips**
**(15) Swanson**
**(16) Gennarelli**
**Discussion (10 minutes)**
**(17) Singer**
**(18) Furuya**
**(19) Mahadevan**
**(20) Furling**
**Discussion (10 minutes)**
**(21) Michalowski**
**(22) Thornton**
**(23) Wieringa**
**(24) Seznec**
**Discussion (16 minutes)**

**6:30pm - 8:00pm** **Reception (heavy hors d'oeuvres and open bar)**

---

**April 22, 1999**

**7:00am - 8:00am** **Continental Breakfast**

**8:00am - 10:10am** **Disease Mechanisms - DMPK protein (Chairs: Housman/Wieringa)**

**(25) Carey**
**(26) Shimizu**
**(27) Wieringa**
**(28) Waring**
**Discussion (10 minutes)**
**(29) Kobayashi**
**(30) Morris**
**(31) Salvatori**
**Discussion (10 minutes)**
**(32) Mounsey**
**(33) Luciano**
**(34) Shimizu**

**(35) Balasubramanyam**
**(36) Perryman**
**Discussion (14 minutes)**

**10:10am - 10:30am** **Coffee Break**

**10:30am - 12:05pm** **Disease Mechanisms - DMAHP (Chairs: Johnson/Thornton)**

**(37) Johnson**
**(38) Harris**
**(39) Sato**
**(40) Winchester**
**(41) Klesert**
**Discussion (10 minutes)**
**(42) Korade-Mirnics**
**(43) Eriksson**
**(44) Gonzalez**
**(45) Tapscott**
**Discussion (13 minutes)**

**12:05pm - 1:30pm** **Lunch**

**1:30pm - 1:50pm** **Disease Mechanisms - 59 (Chairs: Johnson/Thornton)**
**(46) Westerlaken**
**(47) Hamshere**
**Discussion (4 minutes)**

**1:50pm - 3:40pm** **DM Phenotype (Chairs: Eymard/Mathieu)**

**(48) Kinoshita**
**(49)Antonini**
**(50) Eymard**
**(51) Butler-Browne**
**(52) Barbet**
**(53) Jäger**
**Discussion (10 minutes)**
**(54) Johansson**
**(55) Miller**
**(56) Kvist**
**(57) Yamagata**
**(58) Monckton**
**Discussion (12 minutes)**

**3:40pm - 4:00pm** **Coffee Break**

**4:00pm - 4:40pm** **Related Disorders (Chairs: Krahe/Ranum)**

**(59) Mitchell**
**(60) Krahe**
**(61) Day**

**(62) Ranum**
**Discussion (8 minutes)**

**4:40pm - 5:00pm** **Diagnostic Criteria and Nomenclature of DM/PDM/DM2/PROMM**
**Dscussion of a proposal**

---

**April 23, 1999**

**7:00am - 8:00am** **Continental Breakfast**

**8:00am - 8:30am** **Guidelines for DM DNA Testing**
**Baiget/Ashizawa**

**8:30am - 9:00am** **Clinical Rating Scale for DM (Chairs: Ashizawa/Junien)**

**(63) David Hilton-Jones**
**(64) Mathieu**
**Discussion (14 minutes)**

**9:00am - 10:00am** **Treatment Strategies for DM (Chairs: Ashizawa/Junien)**

**(65) Hamshere**
**(66) Furutama**
**(67) Furling**
**(68) Furling**
**(69) Ohsawa**
**(70) Ansved**
**Discussion (12 minutes)**

**10:00am - 10:20am** **Coffee Break**

**10:20am - 10:35am** **Highlight and Summary**
**Wieringa**
**10:35am - 12:00noon** **Discussion - All topics**

**12:00noon** **Adjourn**

## ABSTRACTS

## CTG Repeat Instability

**1.**

**Title:** Intergenerational dynamics of the CTG repeat in myotonic dystrophy: role of age dependent somatic mosaicism and male germline instability

**Authors**: Martorell L [1], Monckton D G[2], Gamez J [3], Baiget M[1]
**Institutions:** [1]Servei de Genètica, Hospital de Sant Pau. Barcelona, Spain. [2]Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow. Glasgow. UK. [3]Servei de Neurología, Hospital de la Vall d=Hebrón. Barcelona, Spain.

The genetic basis of myotonic dystrophy (DM) is the expansion of an unstable CTG repeat located in the 3=UTR of the DM protein kinase gene on chromosome 19. Once into the expanded disease causing range the repeat becomes highly unstable and is biased toward expansion in both somatic and germline tissues. Intergenerational differences usually also reveal an increase in allele size, an observation that is concordant with the high levels of clinical anticipation, that is increasing severity and decreasing age of onset in successive generations, characteristic of DM. In this study we have analyzed a large number of intergenerational transmissions in the Spanish DM population. Our data conform largely with previous studies that reveal clear relationships with parental sex and allele size. We have observed and increased frequency of apparent contractions during male germline transmission, most notable from fathers with > 500 CTG repeats from whom 75% of transmissions appear to result in repeat contractions. However, the overall frequency of such cases was significantly higher ($p<0.001$) in our population than previously reported: 21% of transmissions in our study versus 10% of transmissions in a previous larger study. As in previous studies, the proportion of transmissions that appear to result in contractions is far greater than would be predicted by the high levels of clinical anticipation. As previously suggested this phenomenon may be accounted for by the masking of true germline differences by age dependent somatic expansion in the parent. In order to provide a greather understanding of germline transmission, we have obtained semen samples from six DM males and compared the allele distributions present in the male germline with their blood alleles and those of their offspring by detailed SP-PCR analysis. In all of the cases we have examined the data are consistent with the transmission of alleles that are still present in the fathers sperm, suggesting that the main factor affecting intergenerational differences are germline in origin. However, as indicated by several of these cases it would appear quite possible for germline expansions to be masked by somatic expansion in the father and present as apparent intergenerational contractions. Measuring the intergenerational dynamics of the DM CTG repeat is clearly confounded by the confusing interplay between age dependent somatic mosaicism and germline instability. Thus, without a greater understanding of the dynamics of the somatic expansion pathway or the detailed analysis of individual cases using the SP-PCR procedure, caution should be used in interpreting intergenerational transmissions measured by comparing average allele lengths in the blood DNA of parent and offspring.

**2.**

**Title:** Male germline transmission of normal DM alleles

**Authors:** Gould F K, Monckton D G
**Institutions:** Division of Molecular Genetics, Institute of Biomedical and Life Sciences, Anderson College, The University of Glasgow, 56 Dumbarton Rd, Glasgow G11 6NU, UK.

Myotonic dystrophy is associated with the expansion of a CTG repeat in the 3= UTR of DMPK on chromosome 19q13.3. The repeat is polymorphic in the general population with alleles in the range 5 to 37 repeats. Disease length alleles are >50 repeats and may contain as many as 1,000 or more repeats. Many studies have demonstrated rapid expansions of disease alleles within families and also within somatic and germline tissues of patients. The dramatic anticipation observed in DM families rapidly leads to the generation of effectively lethal alleles. Thus, it might be predicted that the incidence of the disease may decline as the disease length alleles expand

themselves out of existence. However, the incidence of DM appears to have remained constant in recent history suggesting that some mechanism exists generating new mutant alleles. Repeat length distribution and haplotype data have previously been used to infer that new mutations may arise from high end normal alleles >19 repeats. To determine if disease alleles could be derived from a pool of high end normal alleles within the population, we have used the small pool-PCR (SP-PCR) technique to analyse DNA derived from sperm samples from individuals in the general population. Over 340,000 sperm have been screened for large length change mutations. This analysis has revealed that small normal alleles (<19 repeats) are incredibly stable, but that large normal alleles are detectably unstable. Nonetheless, large length change mutations (>+/-5) are relatively rare with only three expansions into the premutation range (40-50 repeats) and only one expansion into the disease range (>50 repeats) having been observed and all from alleles >28 repeats. Single molecule analysis has also been used to quantify more frequent but smaller length change mutations. Once again small alleles are revealed to be very stable, but large normal alleles quite unstable. An increasing mutation rate correlates with increasing allele length, from <10% for alleles of 24 repeats up to ~40% for a 34 repeat length allele. It has also been previously suggested that large normal alleles may be selected for as evidenced by apparent segregation distortion. Our results reveal no evidence for meiotic drive either between alleles <19 or >19 repeats or between lower and higher alleles in heterozygotes. These data indicate that the most likely source of new mutations at the DM locus is from the gradual increase in length of large normal alleles.

**3.**

**Title:** Study of a DM family with two patients having both a pre-mutated and a full mutated allele

**Authors:** Abbruzzese** C., Liguori* M., Tessarolo* D., Costanzi-Porrini* S. and Giacanelli*

**Institutions:** **Chair of Medical Genetics AUniversity of Rome@, *Departement of Neurosciences Service of Neuromuscular Pathology ASan Camillo@ Hospital Rome

We describe a DM family with a male (28 years) and a female (37 years) affected sibling both bearing a pre-mutated allele and a full mutated allele. Molecular analysis showed in both parents the presence of a pre-mutated allele respectively of 43 CTGs in the mother and 50 CTGs in the father. Sequence analysis and XL-PCR analysis showed a genotype of (CTG)43/(CTG)180 in the affected son and (CTG)41/(CTG)500 in the affected daughter. Clinical investigation in the two affected siblings showed a classical DM pattern including myotonic facies, dysphagia, distal and proximal weakness, distal hypotrophy, myotonia, lens opacity at different stages and myotonic discharge at EMG examination. The father, having a border-line allele with 50 CTGs, showed mild DM clinical signs such as DM facies, distal weakness, distal hypotrophy, myotonia and mature cataracts. No clinical signs, as expected, were present in the mother. The presence of the 43 CTGs allele in the son is suggestive of a maternal origin and thereafter the expanded allele is of paternal derivation. The status of the daughter is more puzzling since one of the parental pre-mutated alleles underwent to contraction, and the other one to expansion. A study on the origin of the expanded and the contracted alleles is in progress.

**4.**

**Title:** Transgenic mice carrying the human DM region: a model for CTG repeat intergenerational and somatic instability

**Authors:** Gourdon G[1], Lia A-S[1], Seznec H[1], Hofmann-Radvanyi H[2], Radvanyi F[3], Duros C[1], Fouquet C[1], Junien C[1]

**Institutions:** 1 INSERM U383 Hôpital Necker Enfants Malades, Paris, France; 2 Hôpital Ambroise Paré, Boulogne, France; 3 Institut Curie, Paris, France.

A CTG expansion located in the 3= untranslated region of the DM protein kinase gene (DMPK) has been shown to be the molecular basis of myotonic dystrophy (DM) but the mechanisms involved in the pathophysiology are not fully determined. Dramatic instability with very large intergenerational increases and high levels of somatic mosaicism is observed in patients. Moreover, there is a correlation between repeat size, at least in leukocytes, and

the clinical severity and age of onset. The trinucleotide repeat instability mechanisms involved in DM and in other human genetics diseases remain unresolved.

In order to study the CTG repeat instability mechanisms we generated 3 types of transgenic mice containing 45 kb of the human genomic DM region with the N9, DMPK and DMAHP genes and either 20, 55 or 320 CTG repeat.

The analysis of the CTG repeat in the 3 models ( DM20, DM55 or DM320 ) and over more than 5 generations showed that the CTG repeat intergenerational instability, in our mice, is very similar to the CTG repeat instability observed in DM patients. As in patients, we observed a bias towards expansion, a threshold between 20 and 55 CTG and we observed that the CTG instability is size-, sex-, and age-dependent. We also analyzed by PCR and SP-PCR different tissues of these mice. We observed somatic instability only in the DM55 and DM320 mice. This somatic instability, as in DM patient is size- and age-dependent with a bias towards expansion and no correlation with replication and transcription.

Although we have not obtained yet large increases of the CTG repeat (over few hundred CTG) these results suggest that large genomic DNA and human chromatin environment may be required to recreate the characteristic and the dynamic of the CTG repeat. Furthermore, the different somatic mosaicisms between tissues cannot be explained only by replication rates or by transcription level of the region. Different efficiencies in DNA repair mechanisms during replication and/or in non dividing cells are very likely to be involved.

**5.**

**Title:** Variation of *DMPK* CTG repeat size in satellite cells

**Authors:** Ansved T [1], Anvret M [1], Grandell U [2], Seijersen T [3]

**Institutions:** [1]Department of Clinical Neuroscience, [2]Department of Molecular Medicine, [3]Department of Woman and Child Health and Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

In symptomatic patients with myotonic dystrophy (DM), the expanded CTG repeat in the 3′ untranslated region of the *DMPK* gene is larger in skeletal muscle than in peripheral blood. This indicates tissue-specific somatic events potentiating the transmission of a CTG repeat expansion through the germline. In order to analyze the heterogeneity of the CTG repeat expansion in normal skeletal muscle cells, we have compared repeat lengths between individual single-cell derived satellite cell clones derived from the same muscle, and between different passages of individual satellite cell clones. The size range was between 5 and 38 repeats, which is similar to the sizes previously reported in blood and whole muscle tissue of normal controls. Expansions , contractions, or loss of particular repeat sizes were noted in successive passages of individual satellite cell clones. Variations in repeat size were more frequent in the lower size range. However, in one case, an expansion with novel occurrence of 36-38 CTG repeats was detected. The significance of the variation of the CTG repeat size in individual normal satellite cell clones, and the variation with time in culture, is discussed.

**6.**

**Title:** DM (CTG) repeat instability in tumor tissue

**Authors:** Coolbaugh-Murphy MI[1], Malecki A[1], Evans S[1], Frazier ML[2], Siciliano MJ[1].

**Institutions:** Depts. of [1]Molecular Genetics, [2]Gastrointestinal Oncology and Digestive Disease; Univ.Texas M.D. Anderson Cancer Center; Houston, Texas, U.S.A.;

Microsatellite instability (MSI), indicative of DNA repair defects predisposing to cancer, is not generally detected by methods that allow mutation frequency determination at individual microsatellite loci. Current methods (PCR of thousands of genomes of tumor and normal DNA) have revealed that instability is an early event in certain with DNA mismatch repair defects. Similar analyses have further shown that there is a high level of variability with regard to MSI levels and known MMR gene defects. In order to quantitate MSI frequencies at different loci, we are examining an approach combining the sensitive, quantitative power of small-pool PCR (SP-PCR) with high-throughput fluorescent multiplex PCR analysis. To this end, we are studying different types of microsatellite loci

(e.g. mono-, di-, tri-, tetra-, and pentanucleotide repeats) in tumor and normal material. Since the DM CTG repeat has been shown to be uncommonly stable in individuals not having DM, we have worked out our procedures using that locus and looked for instability in a tumor system known to have high levels of MSI -- hereditary non-poliposis colon cancer (HNPCC). We have also studied a tumor system in which MSI has not yet been observed B soft tissue sarcoma present in the inherited Li-Fraumeni Syndrome (LFS).

Results will be presented indicating that DM is unstable in these tumor systems (15% to 3% instability), instability can also be detected in pre-malignant tissue (up to 3 %), and muliplex analysis using six other microsatellite loci with varying repeat length enables a comparative quantitative analysis of mutation frequencies at these loci in tumor systems. The procedures open a path for studying somatic mosaicism in human tissues and the relationship of such to disease predisposition

**7.**

**Title:** An expansion bias in the instability of the myotonic dystrophy CTG repeat in patient-derived limphoblastoid cell lines

**Authors:** Khajavi M, Ashizawa T

**Institutions:** Department of Neurology, Baylor College of Medicine and VAMC Houston, Texas, USA

In patients with myotonic dystrophy (DM), the expanded CTG repeat in the myotonic dystrophy protein kinase (*DMPK*) gene shows age- and tissue-dependent somatic instability with a bias toward further expansion. We investigated the CTG repeat instability in cell lines derived from DM patients. Our initial observations showed that there are two types of mutations: a frequent Astep-wise@ gain or loss of small number of repeat units, and a Agross@ change of repeat size that occurs infrequently. The two types of mutations appeared to be influenced by the size of the CTG repeat; longer repeats showed more frequent mutations with larger repeat size changes. To investigate the expansion dynamics involved in somatic instability of the CTG repeat, we established lymphoblastoid cell lines from 9 DM patients with various repeat sizes. Each cell line was single-cell cloned using limiting dilutions. Each clone was allowed to grow until the total cell number reaching 30 million, and then 10 million cells were used in each passage. DNA was extracted at each passage and analyzed by the small pool PCR technique using primers flanking the *DMPK* CTG repeat.

In all cloned DM cell lines, the alleles containing expanded CTG repeats showed the frequent Astep-wise@ mutations that resulted in a gradual increase of the modal allele size. The rare Agross@ mutations were also found with a bias toward contraction. However, in some cases, the Agross@ mutations resulted in substantial expansions of the DM alleles. Unexpectedly, one of the larger alleles has become the dominant allele with the gradual disappearance of the progenitor allele during further passages in 3 of the 9 cell lines.

We conclude that the expansion bias of the instability is detected not only in the Astep-wise@ mutations but also in the Agross@ mutations. Furthermore, our data raise a possibility that the cells with a larger allele may have a growth advantage in culture.

**8.**

**Title:** Specific loss of the Myotonic Dystrophy $(CTG)_n$ repeat in somatic cell hybrids containing human chromosome 19.

**Authors:** Spring[1] NV, Monckton[2] DG, Krahe R[3], Siciliano[1] MJ.

**Institutions:** [1]Department of Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, Texas; [2]Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK; [3]Division of Cancer Genetics, Ohio State University, Columbus, OH.

To study the genetic basis of $(CTG)_n$ repeat instability in the *DMPK* gene in myotonic dystrophy (DM) patients, somatic cell hybrids were constructed between the lymphocytes of DM patients and a variety of Chinese hamster ovary (CHO) cell DNA repair gene deficient mutants. By using small pool PCR(SP-PCR), the instability of the $(CTG)_n$ can be quantitated for both the frequency and sizes of length change mutations. A hybrid containing

the expanded repeat so introduced into an *XRCC1* -deficient CHO background had been shown to have a mutation frequency of 73%, with the majority of mutations being small variations in repeat number. Large repeat deletions were less frequently recovered (1.8%). Here, SP-PCR analysis of 3 hybrids and 17 hybrid subclones revealed 2 subclones showing a marked increase in large repeat deletions, with no intermediate deletions present. Strikingly, in the remaining 15 subclones the human DM $(CTG)_n$ repeat region was completely missing. This result was shown to be due to a specific loss of the repeat region and not due to segregation of the human chromosome 19 in the hybrids. This was demonstrated by the presence of a series of markers scanning chromosome 19 in all the subclones. The extent of the deletion is being mapped by further scanning with high density markers flanking the repeat region. Since the large deletions appear to be occurring in a subsequent hybrid cell culture generation, analysis of the concordant loss of a human genetic element from the hybrids might reveal the location of a gene responsible for maintaining the unstable repeat.

**9.**

**Title:** Triplet repeats form secondary structures that escape DNA repair in yeast

**Authors:** Moore H[1], Greenwell P W[1], Liu C P[2], Arnheim N[2], Petes T D[1]

**Institutions:** [1]Department of Biology, Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599-3280 [2]Department of Biological Sciences, Hedco Molecular Biology Laboratories, Molecular Biology Program, University of Southern California, Los Angeles, CA 90089-1340

Several human neurodegenerative diseases result from expansion of CTG/CAG or CGG/CCG triplet repeats. The finding that single-stranded CNG repeats form hairpin-like secondary structures *in vitro* has led to the hypothesis that DNA secondary structure formation is an important component of the expansion mechanism. We show that single-stranded DNA loops containing 10 CTG/CAG or CGG/CCG repeats are inefficiently repaired during meiotic recombination in Saccharomyces cerevisiae. Comparisons of the repair of DNA loops with palindromic and nonpalindromic sequences suggest that this inefficient repair reflects the ability of these sequences to form hairpin structures in *vivo*.

**10.**

Title: Genetic Instabilities in CTGCCAG Repeats Occur by Recombination

**Authors:** Wells RD

**Institutions:** Institute of Biosciences and Technology, Texas A&M University, 2121 W. Holcombe Blvd., Houston, Texas

The expansion of myotonic dystrophy repeat sequence CTGCCAG is believed from prior studies to be due to DNA replication. We have recently discovered that the expansion of $CTGCCAG_n$ in vivo also occurs by homologous recombination as shown by biochemical and genetic studies. A two plasmid recombination system was established in E. coli with derivatives of pUC19 and pACYC184, which harbor the ampicillin and tetracycline resistance genes, respectively. The derivatives contained various triplet repeat inserts [(CTGCCAG), (CGGCCCG), (GAACTTC), (GTCCGAC), (GTGCCAC)] of different lengths, orientations and extents of interruptions. The availability of the two drug resistance genes and of several unique restriction sites on the plasmids enabled rigorous genetic and biochemical analyses, respectively. The requirements for recombination at the TRS include: repeat lengths >30; the presence of CTGCCAG on both plasmids; and recA and recBC. DNA sequence analyses directly demonstrated the crossing-over and expansion of the homologous CTGCCAG regions. Furthermore, inversion products with the sequence type $[(CTG)_{13}(CAG)_{67}]C[(CTG)_{67}(CAG)_{13}]$ were isolated as apparent results of Aillegitimate@ recombination events on intrahelical pseudoknots. Thus, if these reactions occur in humans, unequal crossing-over or gene conversion may also contribute (along with DNA replication) to the expansions responsible for anticipation associated with myotonic dystrophy.

**11.**

**Title:** Genetic Instabilities of $(CTG)_n\$(CAG)_n$ Triplet Repeats: Slipped Strand Structures and Rates of Short and Large Deletions of in *Escherichia coli*

**Authors:** Sinden R R[1], Hashem V I[1], Potaman V N[1], Oussatcheva E A[1], Pearson C E [2], Shlyakhtenko L S[3], Lyubchenko Y L[3]

**Institutions:** [1]Institute of Biosciences and Technology, Center for Genome Research, Texas A&M Univeristy Houston, TX 77030 USA; [2] Hospital for Sick Children, Department of Genetics, Toronto, Ontario, Canada M5G 1X8; [3]Department of Microbiology, Arizona State University, Tempe, AZ 85287-2701 USA

Slipped strand DNA, formed by the out-of-register pairing of the two complementary strands, can exist within triplet repeats. Slipped strand structures and a particular type of slipped strand DNA, the heteroduplex structure formed between two complementary repeating sequences of unequal length, have been implicated in the development and progression of the mutation responsible for DM (repeat expansion). Slippage of a nascent strand on a template or folding a template strand into a hairpin during replication may result in the formation of a heteroduplex which after processing by the cellular replication or repair machinery leads to a change in the length of repeating sequence. Biochemical, electron microscopic, and chemical probing approaches have not yet revealed the molecular nature of presumed slipped strand structures. We have begun an analysis of model slipped strand structures using biochemical approaches and atomic force microscopy. Slipped strand structures may involve an interaction of the CTG and CAG hairpin arms forming a folded slipped strand structure.

We developed a genetic assay to measure the mutation rates of small deletions and complete deletions of $(CAG)_n\$(CTG)_n$ repeats in either orientation in the *Eco*RI site of the chloramphenicol acetyltransferase gene of pBR325. In-frame triplet repeats, where n> 25, or out-of-frame repeat tracts generate a chloramphenicol sensitive phenotype. In the out-of-frame constructs, which measures complete deletion of the repeat and one copy of the *Eco*RI site, mutation rates increased from $8.70H10^{-9}$ to $9.90H10^{-8}$ with increasing size (n = 13-76) when $(CTG)_n$ comprised the lagging strand. When $(CAG)_n$ comprised the lagging stand, mutation rates decreased from $2.50H10^{-8}$ to $7.40H10^{-9}$ with increasing size. This is consistent with preferential formation of CTG hairpins in the lagging strand during DNA replication. The mutation rate of in-frame constructs decreased with increasing repeat length (n = 26-79) in either orientation. Rates ranged from $4.35H10^{-2}$ to $9.07H10^{-5}$ (CAG, lagging strand) and $5.92H10^{-3}$ to $1.59H10^{-6}$ (CTG, lagging strand). This demonstrates that small deletions occur at a very high rate compared with large deletions. Analysis of stabilities in several genetic backgrounds affecting DNA replication and repair showed that *mut*S is very important for preventing short deletions, while having little effect on the rate of complete deletions.

## Disease Mechanisms - DMPK DNA/RNA

**12.**

**Title:** CTG expansion in Myotonic Dystrophy acts at the RNA level

**Authors:** Timchenko LT, Lu X, Timchenko NA

**Institutions:** Departments of Medicine, Cardiology Section, and Pathology, Baylor College of Medicine, Houston, TX, USA

Myotonic Dystrophy (DM) is an autosomal dominant neuromuscular disorder characterized by manifestations in many tissues. This disease is associated with expansion of CTG triplet repeats in the 3= untranslated (UTR) of myotonin-protein kinase (DMPK) gene. We have hypothesized that DMPK mutation acts at the RNA level. According to our hypothesis, expansion of RNA CUG repeats affects processing of other mRNAs by sequestering specific RNA-binding proteins. As a result of such sequestration, processing of different mRNAs might be altered resulting in tissue-specific features of DM. We identified a CUG triplet repeat RNA-binding protein, CUGBP1, which specifically interacts with RNA CUG repeats. We found that CUGBP1 expression is induced by CUG repeats in cultured cells and in DM patients. Altered expression of CUGBP1 in patients with DM is accompanied by disruption of processing of RNAs regulated by CUGBP1. In nuclei, CUGBP1 regulates splicing of CUG-containing mRNAs. DMPK and cardiac Troponin T mRNAs showed altered splicing pattern in the hearts from DM patients. In cytoplasm, CUGBP1 is involved in the regulation of translation. Single mRNA coding for CCAAT/Enhancer Binding Protein β, C/EBPβ produces several isoforms that are generated by alternative translation from different AUG codons. CUGBP1 binds to the 5= region of C/EBPβ mRNA and regulates translation of C/EBPβ_isoforms in a cell-free system and in tissue culture. Another member of the CUG-binding proteins family, ETR-3, shows 75% of homology to CUGBP1. In contrast to CUGBP1, ETR-3 mRNA is highly abundant in heart, suggesting that it might be important for the regulation of processing of cardiac specific mRNAs. Other proteins, such as EDEN-BP, show striking homology to CUGBP1 and might also be affected by CUG repeats. Our data suggest that there is a family of RNA-binding proteins that specifically interact with CUG repeats. One of these proteins, CUGBP1, is affected by expanded CUG repeats. We suggest that expansion of CUG repeats in DM affects the function of CUG-binding proteins leading to tissue-specific symptoms.

**13.**

**Title:** Sequestration of RNA CUG triplet repeat binding proteins by mutant DMPK transcripts

**Authors:** Lia-Baldini As, Timchenko LT

**Institutions:** Department of Medicine, Cardiology Section, Baylor College of Medicine, Houston, TX, USA

**WIP**

The RNA CUG triplet repeat binding protein, CUGBP1, specifically interacts with CUG repeat sequences in vitro, and possibly plays a role in mRNA processing and/or transport. Indeed, CUGBP1 has been found to bind to the human cardiac Troponin T (cTnT) pre-messenger RNA and to regulate its alternative splicing. In cells from patients with Myotonic Dystrophy (DM), the expression of CUGBP1 is altered and splicing of cTnT is changed. Interestingly, nuclear retention of mutated myotonin-protein kinase (DMPK) transcripts containing large CUG repeat sequence has recently been described by several groups, showing that DMPK mRNA processing is also altered. We suggest that CUG repeat expansion in mutated DMPK transcripts leads to increase of binding sites for CUGBP1 in vivo and alters the function of CUGBP1 in DM disease. We currently use In Situ Hybridization and ImmunoStaining in order to determine if DMPK CUG repeats colocalize with CUGBP1. Muscle cells at different stages of differentiation from normal control, DM, and CDM individuals are used for these experiments. Studies on colocalization of CUGBP1 and mutant DMPK transcripts should clarify whether CUGBP1 protein is sequestered by expanded CUG repeats in DM disease.

**14.**

**Title:** The role of aberrant RNA processing in myotonic dystrophy pathogenesis

**Authors:** Philips AV, Cooper TA

**Institutions:** Department of Pathology, Baylor College of Medicine, Houston, TX 77030

**WIP**

Nuclear accumulation of RNA transcripts from the expanded DMPK allele is likely to make a significant contribution to DM pathogenesis. One model of DM pathogenesis suggests that these RNAs create a gain-of-function by the inappropriate binding of proteins that regulate processing/transport of transcripts from a subset of genes. A proposed mediator of the *trans*-dominant effect is CUG-BP(hNab50), a conserved and ubiquitously expressed hnRNP protein that binds RNA containing CUG repeats. Consistent with a role for CUG-BP in DM pathogenesis, a hypophosphorylated isoform of CUG-BP accumulates in the nucleus of DM cells (Roberts et al., Proc. Natl. Acad. Sci. 94, 13221). We have demonstrated that CUG-BP is a positive-acting splicing factor that promotes inclusion of the cardiac troponin T (cTNT) alternative exon 5 by binding to a conserved intronic element [*Science* (1998) 280, p.737]. This element is necessary and sufficient to promote muscle specific exon inclusion of a heterologous alternative exon [Mol. Cell. Biol. (1998) 18, 4519] and CUG repeats are a component of the CUG-BP binding site within this element. In DM cardiac muscle, the fraction of cTNT mRNAs that include exon 5 is significantly increased, consistent with nuclear accumulation of a positive splicing regulator. The *trans*-dominant effect on cTNT splicing was reproduced in normal muscle cells by cotransfection of cTNT minigenes with DMPK minigenes containing >1400 CTG repeats. We have recently identified several members of a CUG-BP protein family that show different tissue-specific expression patterns and we are currently investigating their role in DM pathogenesis. We have also identified additional genes that express aberrantly spliced pre-mRNAs in DM patients. The pre-mRNAs from these genes are likely targets for regulation by the CUG-BP protein family. Progress on a transgenic mouse model for myotonic dystrophy will also be discussed.

**15.**

**Title:** Involvement of Triplet Repeat Expansion RNA-Binding Proteins in Myotonic Dystrophy

**Authors:** Swanson MS[1], Urbinati CR[1], Miller JW[1], Teng-umnuay P[1], Michalowski S[2], Griffith J[2], Thornton C[3]

**Institutions:** [1]Department of Molecular Genetics and Microbiology, Centers for Gene Therapy and Mammalian Genetics, University of Florida College of Medicine, Gainesville, FL 32610-0266; [2]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [3]Neuromuscular Unit, Box 673, Department of Neurology, University of Rochester, School of Medicine and Dentistry, 601 Elm Ave, Rochester, NY 14620

The RNA dominant mutation model for DM pathogenesis proposes that the DMPK 3'-UTR expansion results in a gain-of-function at the RNA level. We have investigated the molecular mechanisms involved in RNA dominance, and the possibility that $(CUG)_n$ expansion alters the binding properties of triplet repeat RNA-binding proteins (TRR-BPs). Two types of TRR-BPs have been isolated and characterized. The CUG-binding protein (CUG-BP/hNab50) is an hnRNP that preferentially recognizes clustered CUG repeats. Several RNA-binding characteristics of CUG-BP are altered in DM cells, and this protein has been reported to function as an intronic splicing enhancer in muscle and neural cells. CUG-BP may also be required for DMPK 3'-end processing. We have examined the RNA-binding properties and subcellular distribution of CUG-BP in more detail, and visualized the structures of $(CUG)_n$ RNAs by electron microscopy (EM). Although CUG-BP binds preferentially to DMPK RNAs that contain CUG repeats, binding is not proportional to repeat number. Moreover, CUG-BP is a predominantly ssRNA-binding protein that does not recognize the stable dsRNA hairpins formed by large $(CUG)_n$ RNAs. Although DMPK mutant allele transcripts have been reported to accumulate in nuclear foci, the subcellular distribution of CUG-BP in normal and DM cells is similar. These observations suggest that CUG-BP is an important pre-mRNA processing factor for a subset of transcripts that contain CUG RNA regulatory elements, including DMPK. Although this processing activity is affected in DM cells, CUG-BP is not sequestered by the expansion mutation in vivo. In contrast, we have identified the triplet repeat expansion (EXP) RNA-binding

proteins that bind avidly to dsRNA hairpins. The EXP proteins in HeLa cell nuclear extracts do not recognize DMPK RNAs with fewer than 20 CUG repeats, but binding to RNAs with $20 repeats is proportional to CUG repeat length. Moreover, the EXP proteins bind directly to $(CUG)_n$ hairpin structures suggesting an inducible binding model for disease pathogenesis. Possible functions of the EXP proteins in post-transcriptional gene regulation will be discussed.

**16.**

**Title:** Altered distribution of the slow skeletal troponin T (TNNT1) mRNA isoforms in DM muscle

**Authors:** Gennarelli M [1], Pavoni M [1], Angelini C [2], Menegazzo E [2], Dallapiccola B [1],Novelli G [1].

**Institutions:** [1]Chair of Human and Medical Genetics, Department of Biopathology and Diagnostic Imaging, Tor Vergata University of Rome and C.S.S. B Mendel Institute, Rome, Italy; [2]Institute of Neurology, Padua University, Padua, Italy.

The pathogenesis of the myotonic dystrophy disease seems to be related to a gain-of function of the expanded allele at DNA and RNA level, by causing haploinsufficiency of a neighboring genes (e.g. DMAHP) and altering other types of RNAs processing. It has been shown that altered expression of specific RNA CUG-binding protein induced by the expanded repeats result in abnormal translation of C/EBPβ transcription factor and in anomalous RNA splicing of the cardiac troponin T (cTNT) (Timchenko LT, Am J Hum Genet 64:360-364,1999).

To verify if this effect is extended to other genes, we analyzed the mRNA processing of a different troponin, TNNT1 (slow skeletal troponin T) selectively expressed in the muscle. Total RNA extracted from 23 DM muscle biopsies obtained either from different muscles of patients affected by adult type or congenital form of the disease (CDM) and 15 unaffected individuals, was amplified by RT-PCR using a set of primers able in detecting muscle TNNT1 isoforms (TNNT-FL and TNNT-1A). The analysis of skeletal TNNT1 mRNA isoforms distribution using a semi-quantitative RT-PCR method shows a significant difference in the TNNT-FL/TNNT-1A ratio in DM muscle biopsies compared to controls ($P$=0.002 Mann-Whitney test) . The relative ratio varies in different DM muscle biopsies and seem to related to muscle involvement scored by MDRS (Muscle Disability Rating Scale). To investigate the nature of this effect we establish the exon-intron boundary sequence in searching for CUG repeats. No evidence for CUG stretches was found in the TNNT1 gene structure. This result suggests that the anomalous distribution of TNNT1 mRNA isoforms observed in DM muscle, it is independent from the activity of CUG binding proteins and provide evidence that other unidentified molecular mechanisms able to alter the mRNA processing are operative in DM cells.

(Work supported by Italian Telethon (N. 1061) and Italian Ministry of Health.)

**17.**

**Title:**Expansion of a CUG trinucleotide repeat in the 3' UTR of DMPK transcripts results in nuclear retention of transcripts.

**Authors:** Davis BM, Singer RH*, Housman DE

**Institutions:** Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139. *Department of Anatomy and Structural Biology and Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461

Expansion of a CTG trinucleotide repeat in the 3' untranslated region (UTR) of myotonic dystrophy protein kinase *(DMPK)* induces the dominantly inherited neuromuscular disorder myotonic dystrophy (DM). Transcripts containing the expanded trinucleotide are abundant in differentiated cultured myoblasts, and they are spliced and polyadenylated normally. However, mutant transcripts never reach the cytoplasm in these non-mitotic cells; instead, they form stable clusters that are tightly linked to the nuclear matrix, which can prevent effective biochemical purification of these transcripts. In DM patients, reduced DMPK protein levels, consequent to nuclear retention of mutant transcripts, are probably a cause of disease development. Formation of nuclear foci is a novel mechanism for preventing transcript export and effecting a loss of gene function. Additionally, the foci may

interfere with normal nuclear processes and hence provide a gain-of-function defect in the normal nuclear functioning.

**18.**
**Title:** Effect on gene expression of the expanded CTG and CAG repeat in 3'-untranslated region of reporter gene in mammalian culture cells and Xenopus oocytes.
**Authors:** Furuya H[1], Imai N[2], Shiokawa K[2], Kira J[1]
**Institutions:** [1]Department of Neurology, Neurological Institute, Faculty of Medicine, Kyushu University 60, Fukuoka 812-8582, Japan, [2]Laboratory of Molecular Embryology, Department of Biological Science, Graduate School of Science, University of Tokyo 7-3-1, Tokyo 113-33, Japan

Although the expansion of CTG triplet repeat is known to be responsible for myotonic dystrophy (DM), the mechanism by which the expansion induces the disease has not yet been elucidated. We isolated CTG and CAG repeats from the genomic DNA of a DM patient, and tested the effect on gene expression of these repeats (85-135) after inserting them into the 3'-untranslated region (3'-UTR) of galactosylceramidase (GALC) and luciferase genes. Using African green monkey cells (COS-I cells), rat myoblast cells, two different human cultured cells (glioma cells and fibroblast cells), and in addition, Xenopus oocytes, we obtained evidence that while CAG repeat expansion induces a slight decrease in the reporter gene expression, CTG repeat expansion consistently causes a marked decrease in the gene expression. These results are compatible with the low expression of DM protein kinase (DMPK) in patients with DM, and suggest that the low expression of expanded CTG repeat-containing genes is a universal phenomenon. We also found that the inhibition of the expression to be stronger in oocytes, the cells which do not divide than in the growing cultured cells. These results thus suggest that such inhibition is probably due to some type of interference with the nuclear cytoplasmic transport of mRNA.

**19.**
**Title:** A Cell Culture Model of the Myotonic Dystrophy (DM) Mutation
**Authors:** Amack JD, Mahadevan MS
**Institutions:** University of Wisconsin-Madison, Laboratory of Genetics
**WIP**

The DM mutation was identified as an expansion of a CTG trinucleotide repeat in the 3= untranslated region (3=UTR) of the DMPK gene. The mechanism by which this mutation causes disease is unknown. Studies using patient material have provided conflicting data on gene expression. Therefore, we undertook the development of a cell culture model in order to study the effects of the DM mutation in a controlled situation. Using C2C12 mouse myoblasts and transfection of reporter genes, we have found that the expression of the mutant DMPK 3=UTR mRNA is sufficient to cause significant effects on gene expression and myoblast differentiation. The effects on gene expression were measured using various DMPK 3=UTR fragments with $CTG_{(5\ to\ >200)}$ fused as 3=UTRs of heterologous reporter genes (E.Coli β-galactosidase (β-gal) and chloramphenicol acetyl transferase (CAT)). Our results show that the presence of the mutant DMPK 3=UTR has a *cis* effect on gene expression resulting in significant decreases in translation of the reporter gene. This is correlated with the number of CUG repeats in the mRNA and is associated with nuclear entrapment of the mRNA, as detected by RNA-FISH and northern blotting. We have also shown that DMPK 3=UTRs with 57 repeats or greater can form nuclear foci of trapped mRNA and that the CUG repeats alone are sufficient for this effect.

Defects in muscle maturation are commonly seen in congenital DM. In order to study the effects of the DMPK 3=UTR mRNA on muscle differentiation, we established a stable C2C12 cell line in which there is a single copy integration of our transgene expressing β-gal with a DM $3{=}UTR_{(CTG>200)}$. These cells show a significant defect in myoblast fusion and differentiation when grown in differentiation media. By using a Cre-Lox system, we have corrected this defect by selectively silencing the expression of this transcript. In addition, in the course of screening this cell line, we identified several subclones that exhibited normal myoblast fusion. Subsequent analysis

has shown that these clones underwent a spontaneous deletion event which deleted most of the 3=UTR sequences after the CUG repeats and also decreased the number of repeats to 5. These clones express a transgene with a DMPK 3=UTR containing the entire sequence from the stop codon to the repeats (the Aupstream element@). Thus, this clone provides strong genetic evidence that the expression of the upstream element does not cause myoblast fusion defect. These experiments demonstrate a *trans* effect of the DMPK3=UTR mRNA on myoblast fusion and provide support for the hypothesis that RNA mediated processes contribute to DM pathogenesis.

**20.**

**Title:** Myotonic dystrophy protein kinase (DMPK) expression in primary skeletal muscle cell cultures derived from normal and DM patients

**Authors:** Furling D, Puymirat J

**Institutions:** Laboratory of Human Genetics, Department of Medicine, Laval University Medical Research Center, CHUQ, 2705 Blvd Laurier, Ste-Foy, Quebec, Canada.

Expression of DMPK was investigated in primary human skeletal cell cultures derived from fetal myotonic dystrophy (DM) and normal muscles. We showed that the expansion (about 750 CTG repeats) was retained in vitro as determined by Southern blot analysis. Distribution of the triplet repeats (CTG) of DMPK transcripts were analyzed by in situ hybridization using a fluorochrome-labeled PNA probe. We found that mutated DMPK transcripts were detected as foci in the nucleus of DM cells. No signal was found in cytoplasm of DM cells nor in normal cells. Discrete foci were observed in the nuclei of DM myoblasts and the number of foci as well as their intensity increased during myoblast differentiation. Northern blot analysis confirmed increment of DMPK transcripts in both DM and normal cells during myoblast differentiation. The levels of mutated transcripts were similar to the levels of the normal transcripts in DM myoblasts. The levels of total (normal and mutated) DMPK mRNAs in DM cells were comparable to those found in normal myoblasts. Expression of DMPK protein correlate with these results. Western blot analysis (using polyclonal and monoclonal anti-DMPK antibodies) showed that DMPK levels were reduced by about 50% in DM cells compared with normal cells. Since fusion of cultured DM myoblasts seems to be not affected, we investigated myogenic differentiation in DM and normal myoblasts using specific markers of differentiation.

**21.**

**Title:** Visualization of double stranded RNAs from the Myotonic Dystrophy Protein Kinase gene and their interaction with human Nab 50 protein

**Authors:** Michalowski S, Urbinati C, Swanson M, Griffith J

**Institutions:** Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill NC 27599 and Department of Biochemistry, University of Florida at Gainsville, Gainsville Florida

Myotonic Dystrophy (DM) is associated with an expansion of a block of repeating CTG nucleotide triplets in the 3' untranslated region of the dystrophic myotonia protein kinase gene (DMPK). In this study RNAs were generated containing 90 or 130 repeats of CUG or CAG triplets flanked by short non repeat RNA or with a segment of the DMPK sequence. Electron microscopic examination of these RNAs revealed perfect double stranded RNA segments whose lengths, as measured against a fully double stranded RNA, were that expected for duplex RNA with a 2.6 A per base pair rise. The non-repeat segments formed a bush-like tail at one end. Incubation of these RNAs with human Nab50 protein resulted in structures consisting of the duplex RNA stems with the protein bound at the junction between the duplex and unstructured RNA segments and binding required both elements.

**22.**

**Title:** Potential mechanism for RNA gain-of-function: expanded CUG repeats in mutant DMPK transcripts interact with proteins that mediate the double-stranded (ds) RNA response

**Authors:** Thornton C , Tian B, White R, McClain C, Xia T, Turner D, Mathews M

**Institutions:** Departments of Neurology and Chemistry, University of Rochester, New York, and the Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, Newark

RNA folding algorithms predict that CUG repeats, like single strand DNA CTG repeats, can form stable hairpins. The stem of the putative hairpin is an imperfect duplex in which G$C and C$G base pairs are separated by a periodic U$U mismatch. The most stable structure predicted for any CUG repeat RNA is the hairpin with a single loop and the longest stem, but multi-stem/loop structures are also possible since their predicted free energies are only slightly less favorable. To test these predictions, a panel of triplet repeat RNAs was synthesized by in vitro transcription. Thermal melting experiments confirm that CUG repeats form a very stable secondary structure with a $T_m$ of 75°C under ~ physiologic ionic conditions. Melting temperatures of CUG transcripts were independent of RNA concentration or polymer length. Nuclease mapping experiments show that expanded CUG repeats preferentially adopt a simple, single-loop hairpin with a long stem, rather than a complex multi-loop structure. To determine if CUG repeats are recognized as a duplex structure, protein binding experiments were conducted with PKR, the dsRNA-activated protein kinase. CUG repeats with 15 or more repeats bind to the dsRNA binding domain of PKR, and expanded CUG repeats can activate PKR in vitro. These observations raise the possibility that mutant DMPK transcripts can interact with proteins that mediate the dsRNA response, a cellular stress response that is activated during viral infections. A model system is being developed to study the potential toxicity of CUG repeats in myogenic cells. Progress toward this goal will be discussed.

**23.**

**Title:** Functional and Structural Behaviour of the Myotonic Dystrophy Locus in Mouse Models.

**Authors:** Groenen P, van den Broek W, Coerwinkel-Driessen M, *Wieringa B.

**Institutions:** Dept. of Cell Biology, Medical Faculty University of Nijmegen, The Netherlands

Myotonic dystrophy (DM) is caused by expansion of a (CTG)n triplet repeat situated in the 3=noncoding exon of the DMPK gene and the promoter-enhancer region of the DMAHP gene, in an evolutionary conserved and gene-dense chromosome area. To gain insight in the biological significance of genes from the Myotonic Dystrophy (DM) region and the consequences of (CTG)n expansion we have used conventional transgenesis and Cre-loxP mediated mutagenesis in ES cells to generate different mouse models.

Animals which overexpress the hDMPK protein from tandemly integrated transgenes were used to compare the alternative splicing behaviour of the endogenous (mouse) and human DMPK transcripts.The orthologous pre-mRNAs behave almost identical with regard to distribution-frequency and cell type-dependency of splice site selection. Together, three major alternative splicing events account for the production of four different 68-76 kDa DMPK isoforms in heart, skeletal muscle, smooth muscle and brain.

Animals carrying a "humanized" DMPK gene-end with short (CTG)11 or long (CTG)85 repeat segments in lieu of the cognate endogenous 3'-terminus are not overtly abnormal and express chimaeric mouse-human RNA transcripts of the expected size. However, the DMPK transcripts in these animals show a high frequency of anomalous splicing events, resulting in deletion of exon 13 sequences in most tissues. Until now, age-dependent somatic (but no meiotic) instability has exclusively been found in the DNA from the stomach of animals carrying the (CTG)85 repeat, and is more pronounced when placed in a mixed-inbred genetic background. Further data regarding the genotypic and phenotypic typing of heterozygous and homozygous mutants will be presented.

**24.**

**Title:** Analysis of the CTG repeat amplification influence in transgenic mice

**Authors:** Seznec H[1], Agbulut O[2], Fouquet C[1], Duros C[1], Hagege A[3], Butler-Browne G[2], Junien C[1], G Gourdon G[1].

**Institutions:** 1 INSERM U383 Hopital Necker Enfants Malades, Paris, France; 2 CNRS Faculté de médecine Pitie-Salpetriere, Paris, France; 3 Hopital Boucicault, Paris, France.

Myotonic Dystrophy is caused by the amplification of a CTG repeat located in the 3=UTR of the DMPK gene but the mechanisms involved in the pathophysiology of this affection are not fully understood. We have generated 3 types of transgenic mice containing 45 kb of the human genomic DM region with the 59, DMPK and DMAHP genes and either 20, 55 or 320 CTG repeat, in order to study: i) the implication of the CTG repeat expansion on tissue-specific expression of the 3 genes ii) if the expression of a 3=UTR carrying an expanded CTG could lead to a DM phenotype.

To define precisely which gene is affected by the CTG expansion and what is the size-threshold for the CTG repeat to affect one particular gene, we analysed the transgenes expression (DMPK, 59, DMAHP) in the three models. We used Ribonuclease Protection Assay (RPA) on total RNA (RNAzol extraction) from various tissues of adult mice (transgenic and control) and for each lines carrying 20 CTG, 55 CTG or 320 CTG. The tissue-specific expression=s level of the 3 transgenes are precisely quantify by comparaison with an internal control and the endogenous genes.

Various phenotype analyses have been performed on the mice carrying at least 320 CTG (DM328): histological studies in muscle, eyes examination, echocardiography and conduction analysis in heart, behaviour tests and insuline response to oral glucose.

The results concerning the transgenes expression and the phenotype analyses will be presented and discussed.

## Disease Mechanisms - DMPK Protein

**25.**

Title: An *in vitro* system to identify the intracellular targets of dmpk in adult skeletal muscle

**Authors:** Carey N, Okoli G, Dunn MJ, Gosling M, Johnson KJ*, Watt DJ
**Institutions:** Imperial College School of Medicine, London, UK; *University of Glasgow, Glasgow, UK
**WIP**

To date there has been very little published on the intracellular targets of DMPK, and this lack of information will hinder any attempts to intervene pharmacologically in this disorder. Studies using purified proteins suffer from the drawbacks of limiting the analysis to specific candidates, and failing to control for the complex interactons which occur in intact cells. To combat these difficulties, we overexpressed the murine DMPK cDNA in the mouse C2C12 muscle cell line, using the pcDNA3 mammalian expression vector. Cultures overexpressing DMPK were radiolabelled with $^{32}P$, and protein lysates analysed by two-dimensional gel electrophoresis. The patterns of phosphorylated proteins were compared with those from control cells transfected with the pcDNA3 vector. Our data show that overexpression of DMPK does not lead to promiscuous phosphorylation changes. A 19 kDa protein (pI of 6.2) was 40% more intensely phosphorylated in the cells overexpressing DMPK than the control cultures. Interestingly, a 25 kDa protein (pI of 4.4) was phosphorylated in the control cultures, but was completely unphosphorylated in the cultures overexpressing DMPK, suggesting activation of a specific phosphorylase pathway. We are attempting to identify and characterise these proteins by direct micro-chemical analysis (N-terminal microsequencing, peptide mass fingerpinting by MALDI-MS and amino acid compositional analysis) of the proteins isolated by micro-preparative 2-DE. When the proteins have been identified we will investigate the direct and indirect pathways by which DMPK influences their phosphorylation status.

Both clinical and experimental evidence suggest that ion channel activity is abnormal in DM, and that DMPK expression may modulate ion channel activity. To test this hypothesis in a more physiologically relevant setting than the more commonly used Xenopus co-transfection system, we are using this same over-expression system to determine if levels of DMPK in muscle cells alter ion channel activity.

**26.**

**Title**: DMPK as a molecular integrator: convergent of interactions with multiple signaling molecules

**Authors: Shimizu M**[1], Wang W[2], Epstein HF[2].
**Institutions:** [1]Department of Developmental Biology, National Institute for Basic Biology, Okazaki, 444-8585, JAPAN. [2]Department of Neurology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, 77030, USA.

Myotonic Dystrophy Protein Kinase (DMPK) is a Ser/Thr protein kinase, which has been confirmed by characterizing bacterially expressed recombinant DMPK as enzymatically active. DMPK represents a new group of proteins called "the DMPK family". We are interested in the molecular interactions of DMPK in several model systems, including the biochemical interactions of DMPK with the p21 small GTPases Rac1 and RhoA which are associated with the actin cytoskeleton-membrane systems and Raf-1which is associated with tyrosine-kinase: Ras:MAP kinase pathways. Since DMPK shares strong similarity of amino acid sequence with a group of Rho-interacting kinases in both its kinase domain and alpha-helical coiled-coil zipper region, a Rho GTPase is also likely to be one of its regulators. Results from two different lines of experimental approaches indicate that DMPK may physically interact with Rac and Rho. Because Rac1 activates DMPK kinase activity in a GTP-sensitive manner, the interaction may physiologically relevant. Raf-1 kinase produces significantly greater phosphorylation of DMPK than DMPK, suggesting that DMPK may be its biological substrate. Control experiments with kinase mutant K100A revealed the direct phosphorylation of K100A by Raf-1.

M.S. is a Research Fellow of the Japan Society for the Promotion of Science. This research was supported by grants from the National Institutes of Health, the National Aeronautics and Space Agency (through the National Space

Biomedical Research Institute), the Muscular Dystrophy Association, and the National Science Foundation to H.F.E.

**27.**

**Title:** DMPK: Component of a supramolecular signalling web?

**Authors:** Groenen P, de Mey JGR+, van den Broek W, Wormskamp N, *Wieringa B.
**Institutions:** Dept. of Cell Biology, Medical Faculty University Nijmegen and +Dept. Pharmacology, University Maastricht, The Netherlands.

**WIP**

DMPK is a serine/threonine-type of protein kinase and a member of the subgroup of Rho-kinases to which also p160-ROCK, dWTS, CRIK and CRIK-SK proteins belong. To learn more about the cellular environment of DMPK we have performed a yeast two-hybrid screening to search for associating partner proteins in a rat heart cDNA library. Among the positive interactors were the myosin binding subunit of smooth muscle phosphatase MBS-PP1M and the myosin light chain MLC2A. This result suggest that DMPK may indeed be involved in signalling pathways that affect MCL phosphorylation and Ca-sensitization of (smooth) muscle contractility. To follow up on these results we have compared agonist/antagonist induced contraction-relaxation behaviour of mesenterial arteries, aorta en arteria femolaris from wild type and DMPK knockout mice. Although the maximal contraction force generated by the smooth muscle in these arteries (in N/m; response to 45 mM $K^+$ plus 30 nM U46619 or 10 ?M phenylephine) was consistently smaller in mutant animals than in wild types the differences were not yet significant. Also the forces generated during decremental relaxation evoked by acetylcholine were not significantly different between the two types of muscle. Possible complicating factors related to the mixed inbred nature of the animal models, and the different nature of the smooth muscle linings in the three types of atery tested, will be discussed.
(* Presenting author).

**28.**

**Title:** Myotonic dystrophy protein kinase structure and gene function.

**Authors:** Waring JD, Storbeck CJ, Korneluk RG
**Institutions:** Solange Gauthier Karsh Laboratory, Children=s Hospital of Eastern Ontario, Ottawa, Ontario, CANADA.

We have continued investigation of the structure and function of the myotonic dystrophy protein kinase (DMPK). Previous results have suggested that DMPK associates in a multimolecular complex and that this is specified by the C-terminus. We have verified this phenomenon for DMPK expressed in mammalian cells using non-reducing polyacrylamide gel electrophoresis. Deletion of both exons 14 and 15 was necessary to prevent multimerization; therefore, the exon 15 hydrophobic domain does not appear to play a role. Inhibition of artefactual cystine bond formation upon lysis prevented the appearance of the non-reduced complex, but mutation of the cysteine residues found in exons 14 and 15 did not likewise completely inhibit the complex. Therefore DMPK appeared to self-associate prior to lysis of the cells. This was verified by chemical cross-linking, which indicated the presence of a dimer species. Surprisingly, this was mapped to exon one and not the C-terminus. Thus DMPK may initiate self-association within exon one, which is then followed by inclusion into a larger complex. We also found that full-length DMPK was resistant to extraction by standard lysis procedures. Again, this property was mapped to exons 14 and 15; their removal releases DMPK to the soluble fraction. This suggests that DMPK binds to the cytoskeleton in agreement with its similarity to rho-binding kinases. This behavior is also likely closely related to large complex assembly. We are currently investigating the effects of disrupting the cytoskeleton. The function of the exon 15 hydrophobic domain remains unknown. We were not able to detect the association of DMPK with cytoplasmic membranes as suggested by the prediction of this sequence as a transmembrane helix motif.

We have also continued studies of the effects of the DMPK 3= untranslated region (UTR) on C2C12 murine myoblast cells. Previous results have demonstrated that 3= UTR expression inhibits terminal differentiation. We found that inclusion of a mutant length repeat (100 CTG) in the 3= UTR was detrimental to the growth of derived stable cells lines. Further investigation demonstrated that these cells have increased sensitivity to staurosporine, an apoptotic trigger. We are currently investigating whether the mutant length repeat is capable of upregulating the expression of proapoptotic factors.

**29.**

**Title:** Localization of Myotonic Dystrophy Protein Kinase and Its Pathophysiological Role in Myotonic Dystrophy (DM)

**Authors:** Kobayashi T[a], Shimokawa M[a], Yamamoto M[a], Kameda N[a], Mizusawa H[a], Ueda H[b], Ohno S[b], Ishiura S[c*]

**Institutions:** [a] Department of Neurology, Tokyo Medical and Dental University School of Medicine, Tokyo113-8519, [b]Department of Anatomy, Yamanashi Medical University, Yamanashi 409-38, Japan, [c]Institute of Molecular and Cellular Biosciences, the University of Tokyo, Tokyo 113-0032, Japan.

It is well known that the mutational expansion of a repetitive trinucleotide sequences (CTG)n located in 3= noncoding region of the DMPK gene is responsible for increasing disease severity and the earlier onset of disease from generation to generation called genetic anticipation. However the molecular pathophysiological mechanisms of DM muscle degeneration and myotonia have still not been clear.

We previously reported that immunoelectron microscopically DMPK was localized in the terminal cisternae of sarcoplasmic reticulum (SR) of skeletal muscle. Now we also examined the subcellular localization of DMPK in human cardiac muscles with confocal laser-scannning microscopy and electron microscopy. Confocal images of the DMPK showed striated banding patterns similar to those of skeletal muscle and, in addition, it was strongly observed around the intercalated disc. Immunoelectron microscopically DMPK was mainly expressed in both corbular and junctional SR, but not in network SR.

To clarify the pathophysiological role of DMPK in DM skeletal muscle, we performed Western blot analysis and immunohistochemical studies on DM skeletal muscles. Western blot analysis of DMPK showed that DMPK protein in DM skeletal muscles dramatically decreased, compared with the controls. Confocal images of double labeling of DMPK/slow or fast myosin heavy chains and DMPK/ SERCA II ATPase clearly showed that DMPK was exclusively localized in slow (type I) muscle fibers with various intensity in the control and DM muscles. DMPK-positive muscle fibers showed typical DM pathological changes such as type I atrophy, central nuclei, nuclear chains, and sarcoplasmic masses. In degenerative DMPK-positive muscle fibers, cross-striated bands disappeared, and irregular granular DMPK-positive materials appeared in sarcoplasm. By immunoelectron microscopy, DMPK was localized in the terminal cisternae of SR in DM skeletal muscle as well as controls. Swollen DMPK-positive SRs were detected between well-preserved myofibrils. In severely damaged regions, degenerated intramembraneous structures with DMPK and accumulation of mitochondria were observed between disorganized myofibrils. The pathophysiological basis of degeneration of DM skeletal muscle is possibly related to the disregulation of intracellular calcium in the terminal cisternae of SR. We will also show the pathological changes of DMPK in the skeletal muscles of congenital DM.

**30.**

**Title:** Localization of myotonic dystrophy protein kinase in human and rabbit tissues using a new panel of monoclonal antibodies

**Authors:** Pham YCN, Nguyen thi Man, Le Thanh Lam, Morris GE[*]

**Institutions:** MRIC Biochemistry Group, N. E. Wales Institute, Mold Road, WREXHAM, UK, LL11 2AW

We have used a new panel of monoclonal antibodies (mAbs) to determine the pattern of expression of DMPK protein in a number of human and rabbit tissues.

Antisera raised against the catalytic and coil domains of DMPK recognized a major 55kD protein and a minor 72-80kD doublet on Western blots of human skeletal muscle. However, ten mAbs, 5 against the catalytic domain and 5 against the coil region, recognized only the 72-80kD doublet, suggesting that these are authentic DMPK. The 72kD protein was present in all tissues tested, though it was more abundant in the central nervous system. The 80kD component was variably expressed, mainly in skeletal and cardiac muscles. This doublet appears to correspond to two proteins identified in the literature by using antisera against the C-terminal domain. The 72kD protein was absent in a DMPK knockout mouse and was greatly increased in a transgenic mouse over-expressing human DMPK, confirming its identity as authentic DMPK.

Two mAbs against the catalytic domain recognized only the more abundant 55kD protein on Western blots and this protein was found only in skeletal muscle. It was even absent from cardiac muscle. It appears to be different, therefore, from a 52-55kD proteins in heart, brain and fibroblasts previously described in the literature.

Nine out of ten mAbs located DMPK to intercalated discs in human heart, an affected tissue in myotonic dystrophy. However, co-localization of DMPK with acetylcholine receptors at neuromuscular junctions (NMJs) was not observed with any of the mAbs. In the brain, DMPK is found in many neuronal cell types, including both granular and pyramidal neurons, but its abundance varies considerably. Subcellular fractionation and sedimentation analysis suggest that a major proportion of the DMPK in skeletal muscle and brain is cytosolic. A cytoplasmic protein kinase might still play a role in muscle relaxation and myotonia through indirect effects on ion channel proteins.

(This work was supported by a grant from the Muscular Dystrophy Group of Great Britain and Northern Ireland.)

**31.**

**Title:** Dual localization of the myotonic dystrophy protein kinase in rat cardiac muscle

**Authors:** Mussini I, Biral D, Marin O, Furlan S, Salvatori S

**Institutions:** Department of Biomedical Sciences and C.N.R. Unit for Muscle Biology and Physiopathology, University of Padova, and C.R.I.B.I. Biotechnology Center (OM), University of Padova, Italy.

Recently, a number of studies have been devoted to the identification of DM gene product in the muscle. Several localization sites has been reported in both heart and skeletal muscle. In particular, in cardiac muscle, a marked concentration of DMPK has been observed at intercalated discs, the function of which is to connect the myocardial cells to each other, in the meantime allowing the passage of ions and other small solutes.

We used polyclonal antibodies raised against a peptide sequence of the human DMPK to analyze the precise subcellular localization of the protein in tissues relevant to the disease. We focused our attention on cardiac myofibers and further analyzed the DMPK subcellular distribution by confocal laser scanning microscopy and immuno-electron microscopy. Thin fascicles of fibers obtained from rat papillary muscles were incubated with anti-DMPK antibodies and studied by confocal laser scanning microscopy. In the sarcoplasm a transverse pattern of immunofluorescence was visible, regularly spaced and punctate in appearance. The most intense immunostaining however, was found at the intercalated discs, as expected. By appropriate conditions, the immunoreactivity was evidenced in form of discrete patches very similar to those observed after treatment with anti-connexin 43 antibodies. Simultaneous immunostaining of the two proteins demonstrated that they exactly colocalize at intercalated discs.

At higher resolution with immunogold electron microscopy we observed that DMPK is localized at the cytoplasmic surface of junctional and extended junctional sarcoplasmic reticulum, suggesting that DMPK may be involved in the regulation of the excitation-contraction coupling. On the other hand, along the intercalated disc DMPK was found associated with gap junctions, while it was absent in the two other kinds of junctional complexes (fasciae adherentes and desmosomes). Immunogold labelling of gap junction purified fractions showed that DMPK co-localized with connexin 43, the major component of this type of intercellular junctions, suggesting that DMPK may play a regulatory role in the transmission of signals between myocytes.

Such a dual localization, i.e. terminal cisternae of sarcoplasmic reticulum and gap junctions of the intercalated discs, strongly suggest that DMPK may play a relevant role in signaling transduction, namely regulation of both excitation-contraction coupling at the junctional SR level and intercellular communication at the gap

junction level. This work might be of help in addressing the point of the DMPK substrate(s).

**32.**

**Title:** Effects of DMPK deficiency on mouse skeletal muscle Na channels

**Authors:** Mounsey JP, Mistry D, Moorman JR, Reddy S.

**Institutions:** University of Virginia, Charlottesville VA. USA & University of Southern California, Los Angeles. CA. USA

In DM, myotonia results from abnormal Na channel gating. Lehmann-Horn and co-workers[1] showed multiple late Na channel openings in skeletal muscle isolated from patients with myotonic dystrophy. This could lead to persistent, late Na current and myotonia. To test the hypothesis that DMPK deficiency accounts for this abnormality of Na channel gating, we characterized Na currents in skeletal myocytes isolated from wild-type and DMPK-deficient mice. Myocytes were enzymatically dissociated from flexor digitorum brevis, and cell attached patch recordings of macroscopic and single channel Na currents were made at room temperature. The microelectrodes were filled with Tyrode solution (120 mM NaCl) containing (mM) $BaCl_2$ (2), $CdCl_2$ (2), TEA (5), 4AP (5), 9AC (2) to block K and Cl currents.

In comparison to wild-type muscle, DMPK-deficient muscle had:

(1) Reduced peak Na current density (340"45 pA vs. 193"31 pA, mean (SE) 16-18 patches, $p<0.02$).
(2) Reduced threshold for Na channel opening. There was a hyperpolarizing shift of 5.5 mV in the current-voltage relationship ($p<0.05$, 10-12 patches).
(3) Faster current decay.
(4) Reduced maximum possible Na current amplitude at any potential. The relationship between channel availability and potential was shifted by 6 mV in a hyperpolarizing direction ($p<0.05$, 11-13 patches).
(5) An increased number of late Na channel openings with prolonged depolarizations, which would result in a persistent Na current. Late Na current was measured after the macroscopic current had subsided, 10 msec after the start of a depolarizing pulse. At a test potential of 0 mV, the percentage of depolarizations with Na channel opening for >10% of a 100 msec pulse was 3.4% in DMPK deficient muscle compared with 1.0% in wild-type muscle ($p<0.001$).

We conclude that DMPK-deficient mice have significant changes in Na channel gating which could contribute to myotonia in DM. The absence of myotonia in DMPK-deficient would suggest that other mechanisms contribute as well.

[1] Franke C, Hatt H, Iaizzo PA, Lehmann-Horn F. J Physiol . 1990; 425:391-405

**33.**

**Title:** Function Analysis of DMPK

**Authors:** Luciano BS[1], Davis BM[2], Chen E[1], Housman DE[1]

**Institutions:** [1]Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA. [2]Div. Of Geographic Medicine and Infections Diseases, New England Medical Center, Tufts University School of Medicine, Boston, MA.

**WIP**

The expansion of a (CTG)n repeat in the 3=UTR of the Myotonic Dystrophy (DM) gene *DMPK* has multiple consequences, including reduced DMPK protein production, altered behavior of specific trinucleotide binding proteins, and altered expression of neighboring genes. The extent to which each contributes to the pathology of DM is unknown. Analysis of *DMPK* -/- mice strongly suggests that DMPK deficiency has etiologic significance; however, the precise molecular consequences of this deficiency are unclear. To determine the function of DMPK and hence pathways affected by its absence we are using 2-dimensional electrophoresis to detect differences in phosphorylation between proteins from affected tissues of *DMPK* +/+ and -/- mice. Proteins showing altered

mobility are identified via matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Analysis of DMPK=s function is complicated by the existence of multiple splice forms. We have discovered that the variant carboxyl terminal sequences within alternatively spliced forms of DMPK determine the subcellular distribution of these isoforms. These data suggest that each splice form may encounter distinct substrates and be subject to differential regulation.

**34.**

**Title:** Molecular interaction of myotonic dystrophy protein kinase: the structure and function of DMPK family members in *C. elegans*.

**Authors:** Shimizu M[1], Epstein HF[2].

**Institutions:** [1]Department of Developmental Biology, National Institute for Basic Biology, Okazaki, 444-8585, JAPAN. [2]Department of Neurology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, 77030, USA.

WIP: yes but can be published

Myotonic Dystrophy Protein Kinase (DMPK) is a Ser/Thr protein kinase and it represents a new group of proteins called "the DMPK family". We are interested in the molecular interactions of DMPK in several model systems, including the structure and function of DMPK family members in *C. elegans*. Human DMPK has four domains: an amino-terminal leucine-rich repeat (LRR), ser/thr protein kinase, a predicted coiled-coil/zipper region, and a carboxy-terminal putative transmembrane domain (tail). There are two *C. elegans* homologues with respect to the catalytic domain have been identified through sequencing the entire *C. elegans* genome. The closest homologue for DMPK is the predicted product of a locus on chromosome V that we call CeDMPK which has 53.5% identity in its catalytic domain to that of human DMPK. In addition to its DMPK-like ser/thr kinase domain, CeDMPK contains four other functional domains: a large coiled-coil region, a cysteine-rich zinc-finger domain (CRD), a pleckstrin-homology motif (PH), and a Cdc42/Rac binding domain (CRIB). To study the temporal and spatial expression pattern of CeDMPK in *C. elegans*, a transgenic line was established by injecting the gfp expression vector which contains a 1.9 Kb upstream of the start codon through a part of exon 3 of CeDMPK genomic DNA fused in frame to the GFP protein. We found GFP expression at different cell types through the developmental stages including at pharyngeal muscles at all developmental stages from late embryos to adults, in body-wall muscles at L2-L3, and the vulval precursor descendants (hypodermal cells) at L3-L4. Since the expression pattern is very similar to *egl-19* (alpha-1 subunit of an L-type voltage-activated calcium channel) except at vulva, this result maybe consistent with a possible involvement of the the human DMPK in the calcium homeostasis. Potential knockout phenotypes obtained by RNA-mediated inhibition include protruding vulva, abnormal morphology at pharynx, production of a few progeny, and Lumpy-Dumpy phenotype which suggest the possible roll of CeDMPK in morphological development of the worm. Isolation of CeDMPK null mutant is underway to apply the powerful genetic study of *C. elegans* to clarify the regulators of DMPK in the cell signaling cascade.

M.S. is a Research Fellow of the Japan Society for the Promotion of Science. This research was supported by grants from the National Institutes of Health, the National Aeronautics and Space Agency (through the National Space Biomedical Research Institute), the Muscular Dystrophy Association, and the National Science Foundation to H.F.E.

**35.**

**Title:** Myotonic dystrophy protein kinase (DMPK) regulates myogenic genes via serum response factor

**Authors:** Iyer D, Belaguli N, Wei L, Fluck M, Rowan B, Zhou W, Booth F, Weigel N, Schwartz RJ, Balasubramanyam A

**Institutions:** Departments of Medicine and Cell Biology, Baylor College of Medicine, Houston, TX, USA, and Department of Integrative Biology, University of Texas Health Sciences Center, Houston, TX, USA.

**WIP**

The transformation of replicating myoblasts into terminally differentiated myotubes is a critical process in muscle development. The key events are reiterated during muscle repair and hypertrophy after development. Abnormalities in this process are thought to underlie several defects in the contractility and metabolism of skeletal, cardiac and smooth muscle, but the intracellular signals that regulate it are poorly understood. There is evidence that DMPK may be a regulator of myogenic differentiation (Bush et al, *JBC* 271:548-552, 1996).

We have found that DMPK enhances the transactivation of genes critical to both muscle proliferation (e.g., c-fos) and terminal muscle differentiation (e.g., α-skeletal actin). Both these sets of genes are known to be regulated by serum response factor (SRF), a key myogenic transcription factor. DMPK requires SRF for its transcriptional effects. It phosphorylates SRF both in vitro and in vivo on unique sites, one of which is in the critical DNA-binding domain of the transcription factor. This covalent modification enhances the binding of SRF to its cognate promoter element, the serum response element. DMPK also interacts physically with SRF, and this interaction may determine some of its functional effects.

The transcriptional effects of DMPK mimic those of the small GTPase RhoA on myogenesis, as we have previously demonstrated (Wei L. et al, *JBC* 273:30287-30294, 1998). Since DMPK is homologous to known effectors of RhoA (the Rho kinases), our observations suggest the presence of a novel myogenic signaling cascade involving RhoA, kinases of the DMPK/Rho kinase family, and regulatory phosphorylation of SRF.

**36.**

**Title:** Myotonic dystrophy protein kinase isoform generation and enzymatic activity

**Authors:** Bush EW, Helmke S, Perryman MB

**Institutions:** University of Colorado Health Sciences Center

**WIP**

The myotonic dystrophy disease locus encodes a multidomain protein kinase of unknown function, myotonic dystrophy protein kinase (DMPK). DMPK was the first identified member of a novel class of serine/threonine protein kinases with sequence similarities to both protein kinases A and C. Using recombinant DMPK we have shown that DMPK does not phosphorylate PKA substrates but does phosphorylate a subset of PKC substrates. DMPK was not inhibited by a PKA inhibitor but was strongly inhibited by a pseudosubstrate PKC inhibitor peptide. Neither PKA nor PKC activators had any effect on DMPK activity. DMPK was unable to phoshorylate myosin light chain, a Rho-kinase substrate. The basal catalytic activity of DMPK is thus distinct from PKA, PKC, and the more closely related Rho-kinases and indicates that DMPK possesses unique catalytic properties.

In addition to the catalytic domain, DMPK contains leucine zipper, coiled-coil and membrane association domains. Using domain-deleted constructs we examined the effects of loss of the coiled-coil and membrane association domains on DMPK oligomerization and activity. DMPK lacking the membrane association and coiled-coil domains is monomeric and has three-fold less enzymatic activity than a construct lacking only the membrane association domain. The membrane association domain deleted DMPK behaves as a single oligomeric complex, most likely a dimer. These observations suggest that coiled-coil-mediated oligomerization correlates with enhanced catalytic activity. Interestingly, full length DMPK has six-fold less enzymatic activity than the membrane association domain deleted DMPK.

DMPK is expressed as two isoforms in myocardial and skeletal muscle. We have now shown that the higher molecular weight form (88 kDa), designated DMPK-1, is the primary full length translation product and

the lower molecular weight form (80 kDa), designated DMPK-2, is produced by proteolytic cleavage. The proteolytic activity is found in salt washed membranes and is partially blocked by a protease inhibitor cocktail. The proteolytic processing event removes the membrane association domain and converts membrane-associated full length DMPK-1 into soluble DMPK-2. As noted above removal of the membrane association domain also increases the basal enzymatic activity of DMPK. The proteolytic processing of DMPK-1 into DMPK-2 may affect both cellular localization and enzymatic activity and the two isoforms may have different substrates and activators.

## Disease Mechanisms - DMAHP

**37.**

**Title:** Functional analysis of SIX5/DMAHP and its role in DM pathogenesis

**Authors:** Johnson K, Winchester C, Harris S, Hamilton G, Clark B, Ferrier R, Bailey M
**Institutions:** University of Glasgow, Glasgow, UK

Myotonic dystrophy is the commonest neuromuscular disease affecting adults and is inherited in an autosomal dominant fashion. The underlying mutation is a CTG triplet repeat expansion, although the path from genotype to phenotype is unresolved. At least three separate mechanisms are currently under intensive investigation, including (i) a gain of function mediated at the mRNA level through a CUG repeat and (ii) haploinsufficiency of a transcription factor, SIX5 (previously called DMAHP) and (iii) a direct role of DMPK. All of these mechanisms may be acting simultaneously in DM tissues to bring about the characteristic phenotype. We have focussed on the functional analysis of SIX5. We have determined its developmental and adult patterns of expression, which indicate potential pathological involvement of specific cranial nerves in the distribution of the disease. We have implicated haploinsufficiency for this protein in cataract formation. Several regulatory targets of this transcription factor have been suggested and these are strongly supportive of a key role for this mechanism in major pathological features of DM. The genes which may be regulatory targets of SIX5 include myogenin and ion channels.

**38.**

**Title:** Expression and Functional Analysis of SIX5

**Authors:** Harris SE, Johnson KJ
**Institutions:** Division of Molecular Genetics, IBLS, University of Glasgow, UKTopic

**WIP**

*SIX5* (*DMAHP*) is a member of the *SIX* subfamily of homeobox genes of which 6 mammalian and 3 *Drosophila* genes have been identified. All members of this family encode a homeodomain that is highly diverged from other known homeodomains and a second homologous domain, the SIX domain, which lies immediately upstream of the homeodomain.

A bacterial expression system was used to overexpress GST-fusion proteins containing the SIX domain, the homeodomain and both domains together. These SIX5-fusion proteins were used in gel retardation assays with the known DNA binding site of the related protein SIX4 (the $Na^+K^+$ ATPase $\alpha 1$ subunit regulatory element - ARE) to investigate the DNA binding properties of the 2 conserved domains. The 2 fusion proteins containing the homeodomain bound to the ARE, but the SIX domain alone showed no specific DNA binding activity. However, both domains were required for high affinity specific DNA binding. It was hypothesised that SIX5 regulates *DMPK*. Therefore, the 3 fusion proteins were tested for their ability to bind to 2 putative binding sites in the promoter of *DMPK*. However, they showed no binding activity with either of these sites.

A whole genome PCR based screen was used to identify genomic DNA sequences to which SIX5 binds, as an initial stage in the identification of genes which it regulates. Five DNA sequences were identified and are currently being analysed.

**39.**

**Title:** Synergy of Six and Eya in gene transcription

**Authors:** Sato S, Ohto H, Kamada S, Ozaki H, Kawakami K
**Institutions:** Department of Biology, Jichi Medical School, Tochigi, 329-0498 Japan

**WIP**

Six5, the murine equivalent of SIX5/DMAHP, was originally identified in our effort to isolate genes

homologous to Six4, which encodes a transcription factor that binds and transactivates the Na,K-ATPase alpha 1 subunit gene promoter in vitro (Kawakami et al., Nucleic Acids Res. 24, 303-310, 1996; Kawakami et al., FEBS Lett. 393, 259-263, 1996). Since SIX5 is expressed in several tissues affected by DM and the transcription of SIX5 was shown to be repressed by the causative CTG-repeat expansion, it has been proposed that SIX5 is involved in some aspects of DM pathogenesis. To understand how the CTP-repeat expansion exerts its effect through SIX5, it is essential to elucidate the mechanism of SIX5 transcription repression, and to identify the target genes and their transcription regulation not only by SIX5 but also by other SIX family genes. Recently, we reported the functional analysis of the mouse Six5 promoter (Murakami et al., Hum. Mol. Genet., 7, 2103-2112, 1998). In the present study, we analyzed the function of the murine Six proteins including Six5 as transcription factors.

Considering the synergy of so and eya genes in Drosophila compound eye formation, and the overlapping expression patterns of the murine homologues, Six and Eya genes, we hypothesized that Six and Eya proteins cooperate to regulate their target genes. Co-transfection assays were performed using various combinations of Six and Eya proteins to assess their effects on a synthetic promoter, the TK promoter fused to Six binding sites, and on a potential natural target, myogenin promoter. Clear synergistic activation of these promoters were observed by a certain combination of Six and Eya. To investigate the molecular basis for the cooperation, we first examined the intracellular distribution of Six and Eya proteins in transfected COS7 cells. Co-expression of Six2, Six4 or Six5 induced the nuclear translocation of Eya1, Eya2 and Eya3, which are otherwise distributed in the cytoplasm. Surprisingly, co-expression of Six3 did not result in the nuclear localization of Eya proteins. Six- and homeodomains, two evolutionarily conserved domains, were necessary and sufficient for the nuclear translocation of Eya proteins. In contrast, Eya domain alone was not enough for the translocation, and the presence of an additional 62 amino acid portion of Eya was required. Six and Eya proteins were co-immunoprecipitated from the nuclear extract of co-transfected cells. In addition, specific interaction was observed in yeast two-hybrid assays. These results suggest that transcription activation of certain target genes by Six proteins requires cooperative interaction with Eya proteins: complex formation through direct interaction and nuclear translocation of Eya proteins.

**40.**

**Title:** Expression of *DMPK* and *SIX5* (*DMAHP*) in the human eye

**Authors:** Winchester CL[1], Ferrier RK[2], Bailey MES[1], Cree L[1], Clark BJ[3], Johnson KJ[1]
**Institutions:** [1]Division of Molecular Genetics, IBLS, University of Glasgow, UK
[2] University Department of Pathology, Western Infirmary, Glasgow, UK
[3] Department of Pathology, Institute of Ophthalmology, University College London, London, UK
**WIP**

Distinctive ocular abnormalities occur in myotonic dystrophy (DM), which include cataracts, retinal degeneration, corneal lesions, ocular hypotonia, ptosis, blepharitis, extraocular weakness and myotonia. Therefore, we have analysed the expression of *DMPK* and *SIX5* (*DMAHP*) in normal adult and fetal eyes at the RNA level by RT-PCR and *in situ* hybridisation and the DMPK protein by western blotting and immunohistochemistry. We report the normal expression patterns for these genes in a number of eye tissues and show that the expression of *SIX5* is consistent with the sites of pathological change in DM, whereas the overlapping *DMPK* expression pattern is restricted. We propose that dysfunction of SIX5 rather than DMPK is likely to contribute to the principal ophthalmic feature of DM, the cataract. We are currently analysing eye material from DM patients to qualify this hypothesis.

*SIX5* is a member of the *SIX* homeobox gene family, related to the *Drosophila* eye development gene *sine oculis*. We present a preliminary expression analysis of other human SIX genes in the eye.

**41.**

**Title:** CTG-repeat expansion suppresses DMAHP expression: a possible role in myotonic dystrophy?

**Authors:** Klesert TR, Tapscott SJ
**Institutions:** Fred Hutchinson Cancer Ctr, Seattle, WA

One hypothesis that may explain the variable and pleiotropic nature of myotonic dystrophy is the involvement of loci in addition to DMPK. Any role for adjacent genes as mediators of aspects of the DM phenotype, however, remains undetermined. Given its homologies, reported patterns of expression, proximity to the CTG repeats, and putative role as a regulator of cellular ion homeostasis, DMAHP represents an intriguing candidate. Previously, our lab has shown that CTG-repeat expansion correlates with allele-specific changes in both chromatin structure and gene expression at the DMAHP locus that are consistent with gene silencing in cis. Specifically, repeat expansion is associated with loss of DNase-I hypersensitivity at an upstream DMAHP transcriptional enhancer, a 70-90% reduction in steady-state DMAHP transcripts from the expanded allele, and an overall 2-4 fold reduction in total steady-state DMAHP mRNA levels. To better understand what role, if any, these changes play in the natural history of myotonic dystrophy, we are currently generating mice that have been genetically altered at the six5 (murine orthologue of DMAHP) locus to determine the in vivo consequences of loss of the hypersensitive site and loss of DMAHP expression.

**42.**

Title: Myotonic dystrophy: Tissue-specific effect of somatic CTG expansions on allele-specific *DMAHP/SIX5* expression

**Authors:** [1]Korade-Mirnics Z, [2]Tarleton J, [3]Servidei S, [2]Casey RR, [4]Gennarelli M, [5]Pegoraro E, [5]Angelini C, [6*]Hoffman EP
**Institutions:** [1]Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, PA; [2]Fullerton Genetic Center, Mission St. Joseph Hospital, Asheville, NC; [3]Istituto di Neurologia, Catholic University, Rome, Italy; [4]Department of Biopathology and Diagnostic Imaging, Tor Vergata University of Rome, Italy; [5]Department of Neurological and Psychiatric Sciences, University of Padova, Padova, Italy;
[6]Center for Genetic Medicine, Children=s Research Institute; Washington, DC.

Myotonic dystrophy (DM), the most common inherited muscle disorder, is caused by a CTG expansion in the 3=UTR region of a protein kinase gene (*DMPK*). The complex and variable phenotype is most likely caused by a complex molecular pathogenesis, including deficiency of the DMPK protein, a trans-dominant misregulation of RNA homeostasis, and haploinsufficiency of a neighboring homeobox gene (DM locus-associated homeodomain protein - *DMAHP/SIX5*).

Here, we study the allele-specific transcriptional activity of *DMAHP/SIX5* gene in DM patient tissues. Using a quantitative fluorescent RT-PCR assay, we tested allele-specific accumulation of *DMAHP/SIX5* transcripts in both total and poly($A^+$) pools. In muscle biopsies, we found that transcript reductions of *DMAHP/SIX5* alleles in cis with CTG expansions correlated with the extent of expansion. A patient with ~90 CTG repeats in muscle DNA (normal n<37) showed a 20% reduction of allele-specific transcript levels, while 4 other DM patients with larger expansions showed 80% reductions.

The effects of the CTG expansions on *DMAHP/SIX5* transcription were tissue specific: autopsy tissues from a patient with 1500 repeats showed 80% reductions in muscle and liver. However, RNA from other tissues showed 0-20% reductions (lung, aorta, heart conduction tissue, cerebellum). Our results suggest that the effect of the CTG repeat on the *DMAHP/SIX5* promoter is variable and tissue-specific. Our data is consistent with abnormalities of *DMAHP/SIX5* likely having a more prominent role in disease pathogenesis in muscle, liver, and brain, but may be less important in other tissues.

**43.**

**Title:** Simultaneous Analysis of Expression of the Three Myotonic Dystrophy Locus Genes in Adult Skeletal Muscle Samples - The CTG Expansion Correlates Inversely with DMPK and 59 Expression Levels, but not DMAHP Levels

**Authors:** [1]Eriksson M, [2]Ansved T, [2]Edstrom L, [1]Anvret M, [3]Carey N

**Institutions:** [1]Departments of Clinical Neuroscience and Molecular Medicine, [2]Department of Clinical Neuroscience, Division of Neurology, Karolinska Hospital, Stockholm, 171 76 Sweden [3]Department of Vascular Surgery, Imperial College School of Medicine, Fulham Palace Road, London

The causative mutation in the majority of cases of myotonic dystrophy has been shown to be the expansion of a CTG trinucleotide repeat, but the mechanism(s) by which this repeat leads to the very complex symptomatology in this disorder remains controversial. We have developed a highly sensitive and quantifiable assay, based on competitive RT-PCR, to test the hypothesis that the expansion disrupts the expression of the genes in its immediate vicinity - DMPK, 59 and DMAHP. In order to avoid cell culture-induced artifacts we performed these experiments using adult skeletal muscle biopsy samples, and analysed total cytoplasmic poly-A mRNA levels for each gene simultaneously, as this is more physiologically relevant than allele-specific levels. There was considerable overlap between the expression levels of the three genes in myotonic dystrophy patient samples, and samples from control individuals. However, in the myotonic dystrophy samples we detected a strong inverse correlation between the repeat size and the levels of expression of DMPK and 59. This is the first report of a possible effect of the CTG expansion on gene 59. Our results indicate that whilst a simple dosage model of gene expression in the presence of the mutation is unlikely to be sufficient in itself to explain the complex molecular pathology in this disease, the repeat expansion may be a significant modifier of the expression of these two genes.

**44.**

**Title:** Abnormal regulation of splicing of *DMPK*, *H59*, and *DMAHP* transcripts in myotonic dystrophy

**Authors:** Gonzalez, IL, Frisch, R, Tapscott, SJ, Funanage, VL

**Institutions:** Department of Research, Alfred I. duPont Hospital for Children, Wilmington, DE, USA and Fred Hutchinson Cancer Research Center, Seattle, WA, USA

**WIP**

Myotonic dystrophy (DM), an autosomal dominant disease characterized by progressive muscle weakness and atrophy, myotonia, cataracts, mental retardation, and cardiomyopathy, is associated with expansion of a polymorphic $(CTG)_n$ repeat in the 3' untranslated region of the *DMPK* gene. The repeat expansion results in decreased levels of *DMPK* mRNA and protein, but the mechanism for this decreased expression is unknown. Loss of a nuclease-hypersensitive site in the region of the repeat expansion has been observed in muscle and skin fibroblasts from DM patients, indicating a change in local chromatin structure. We demonstrate through the use of somatic cell hybrids carrying the repeat expansion that splicing of *DMPK* mRNA is altered, similar to what has been observed previously for cardiac troponin T. However, transcription of neighboring genes, *H59* and *DMAHP*, is unaffected by the repeat expansion in the hybrid cell lines. Conversion of the somatic cell hybrids to myogenic cell lines by forced MyoD expression results in decreased levels of *DMAHP* mRNA in terminally differentiated cells. Splicing of *H59* mRNA also appears to be affected by the repeat expansion in terminally differentiated tissues, such as muscle. The pattern of *DMPK*, *H59*, *DMAHP*, and cardiac troponin T mRNA isoform expression in DM tissues is reflective of the isoform pattern found in less differentiated tissues. Thus, DM appears to be a disease characterized by altered RNA splicing and poor tissue maturation. These results may account for the late onset of the disease, explaining, in part, the pathophysiology of the triplet repeat expansion in DM.

**45.**

**Title:** Modeling stochastic gene expression: Implications for haploinsufficiency

**Authors:** Cook DL, Gerber AN, Tapscott SJ
**Institution:** Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Haploinsufficiency refers to a phenotype associated with the inactivation of a single allele in a diploid organism. A common notion of haploinsufficiency is that the phenotype reflects a requirement for greater than 50% of the diploid level of gene product. This assumes relatively stable levels of gene product and precludes gene dosage compensation, i.e., increasing expression from the remaining allele to achieve a steady-state level of product comparable to the diploid state. There is increasing recognition that aspects of the initial activation of gene expression are probabilistic, or stochastic. Therefore, the highly predictable patterns of gene expression in multicellular organisms are achieved by a system that has probabilistic features. We have used a minimal model of stochastic gene expression to illustrate that gene copy number and expression off-rates can be critical variables in achieving predictable outcomes in stochastic systems. We present the hypothesis that some haploinsufficiency diseases result from an increased susceptibility to stochastic delays of gene initiation or interruptions of gene expression, events that are normally buffered by increased gene copy number and that are relatively insensitive to dosage compensation.

## Disease Mechanisms - 59

**46.**

**Title:** Unraveling the biological role of DMR-N9 gene (gene 59), closely upstream of the instable (CTG)n repeat in the myotonic dystrophy (DM) locus.

**Authors:** Westerlaken J*, van Tuijn P, de Rooij D#, de Boer P@, Grootegoed A%, van der Zee I, Wieringa B.
**Institutions:** Dept. of Cell Biology, Medical Faculty University Nijmegen, #Dept. Microscopic Morphology University Utrecht, @Dept. Genetics, Agricultural University, Wageningen, %Dept. Biochemistry EUR, Rotterdam, The Netherlands.

The consequences that (CTG)n repeat expansion in patients with Myotonic Dystrophy may have on the functioning of genes from the DM chromosome area are still not fully understood. We have focussed on the pathobiological significance of the DMR-N9 gene, situated immediately upstream and distal of the DMPK gene. Gene DMR-N9 (also called gene 59 in humans) is composed of 5 exons and encodes products with unknown function. The DMR-N9 protein contains a proline-rich N-terminus and 4 domains with similarity to so-called WD-repeats. WD-repeats are ubiquitous in proteins with propeller-like domains (such as the G-protein β subunit) and found in a very diverse set of proteins (including the F-box proteins). To understand more about the possible role of DMR-N9 we (i) used immunohistological approaches to study its tissue and cell-type distribution and its intracellular localisation, (ii) identified possible interacting proteins by using the yeast two hybrid system, and (iii) made comparisons to DMR-N9 homologs in other organims. DMR-N9 protein is most prominently expressed in brain and testis, where it is found as a 72/74 kDa and 80 kDa protein, respectively. The 80 kDa protein is also present in a wide range of other tissues, where it may originate from the smooth muscle linings.In brain it is widespread expressed, but the highest staining is seen in areas which are rich in synaptic glomeruli. In testis the protein appears in several distinct stages of spermatogenesis, in the sex-vesicle in late pachytene cells and in the sertoli cells. Transfection experiments in COS-7 and N2A-neuroblastoma cells with DMR-N9/EGFP reporter constructs reveal DMR-N9 protein to have predominantly a cytosolic localisation. Preliminary findings indicate that DMR-N9 may associate with HP1-hsgamma, a protein belonging to the HP1-like family of proteins, involved in heterochromatin condensation and chromatin packaging. Finally, from database comparisons we know now that DMR-N9 is highly conserved and orthologous genes can be found in many organisms, including C.Elegans and Aspergillis. Theoretical and experimental data that are of relevance for the role of the protein in these organisms will be briefly addressed. ( * presenting author)

**47.**

**Title:** Triplet repeat expansion in myotonic dystrophy: DMWD transcripts are also affected *in cis*.

**Authors:** Alwazzan M, **Hamshere M,** Brook JD
**Institutions:** Institute of Genetics, University of Nottinham, Nottingham, NG7 2UH, UK

Two main hypostheses have been proposed to explain the molecular basis for myotonic dystrophy. First, there could be a loss of function for the genes that surround the triplet repeat expansion. Second, there could be a gain of function for the expansion derived DMPK RNA that is trapped in the nucleus. In order to explore the first possibility, we have compared the levels of RNA from the expansion and normal alleles for the two genes that flank the repeat (DMWD, previously called 59, and DMAHP). To achieve this, we identified a single base change polymorphism in the 3'UTR of the upstream gene DMWD, which is in linkage disequilibrium with the triplet repeat expansion. Using an allele specific RT-PCR assay we have compared the levels of RNA from each allele, in nuclear and cytoplasmic fractions of DM and control fibroblasts. Data will be presented which indicates that in the cytoplasm there are reduced levels of RNA from the expansion chromosome for both DMWD and DMAHP. However, in the nucleus, although there are similar amounts of both alleles for DMWD, the level for DMAHP more closely reflects the levels seen in thc cytoplasm. This indicates that export of transcripts for DMWD (like those for DMPK) may also be affected *in cis* by expansion of the repeat.

## DM Phenotype

**48.**

**Title:** Genotype-phenotype correlation in myotonic dystrophy

**Authors:** Kinoshita M[1], Ohtake T[2], Komori T[2], Segawa K[3], Osanai R[4], Matsuda A[1], Matsumura O[1], Isoda K[1], Hirose K[5]

**Institutions:** [1]Fourth, [3]Third Department of Internal Medicine, and [4]Department of Otolaryngology Saitama Medical Center, Saitama Medical School, Saitama, [2]Department of Neurology, Tokyo Metropolitan Neurological Hospital, and [5]Department of Neurology, Tokyo Metropolitan Fuchu Medical Center for the Severity Disabled, Tokyo, Japan.

**Objectives:** We investigated whether the degree of $(CTG)_n$ length might be correlated with the extent of endocrinological, cardiological, immunological disorders, and disorders of the central nervous system and muscular involvement in 40 patients with myotonic dystrophy (DM).

**Background:** The characteristic manifestations of DM are well known to be involved multiple organs. The molecular basis of DM is unstable expansion of a $(CTG)_n$ in the gene encoding myotonin protein kinase. The elongation of $(CTG)_n$ length is correlated with increasing disease severity.

**Design/Methods:** We examined the endocrinological disorders with the Ellsworth-Howard (J Neurol 244:613,1997), the thyrotropin releasing hormone (TRH) loading, and the luteinizing hormone releasing hormone (LHRH) loading tests. As one of the cardiac conduction disorders, His-ventricular interval (HIV) using the His bundle electrogram was analyzed. The serum IgG levels and proliferative response of lymphocyte to Con A mitogen were investigated as the immunological disorders. Both intelligence quotient (IQ) and apnea index (AI) was also estimated as the disorders of the central nervous system. Moreover, muscular involvement was evaluated using the muscle disability (MDRS), horizontal saccadic velocity on electronystagmography (ENG) (J Neurol 245:674,1998), myopathy index (MI) of tibialis anterior (TA) and biceps brachii (BB) muscles on electromyography (EMG) and pulmonary function test including % vital capacity (% VC) and peak flow. Southern blot was made of the genomic DNAs extracted from the leukocytes, which were digested with Eco R I, and then was hybridized with a $^{32}P$-labeled cDNA 25 probe (Muscle Nerve: 19,240,1996).

**Results:** The results of the EH test ($p<0.05$), the TRH loading test ($p<0.01$), and the LHRH test ($p<0.05$) were well correlated with the $(CTG)_n$ length. The HVIs ($p<0.01$), the serum IgG levels ($p<0.05$), proliferative response of lymphocyte to Con A mitogen ($p<0.01$), AI ($p<0.01$), and IQ levels ($p<0.001$) also were done with the $(CTG)_n$ length. In addition, the extent of muscular involvement based on the MDRS ($p<0.001$), horizontal saccadic velocity ($p<0.001$), the MIs of TA ($p<0.05$) and BB ($p<0.02$), % VC ($p<0.05$), peak flow ($p<0.01$) was well done with the $(CTG)_n$ length.

**Conclusion:** From these results, it was considered that the $(CTG)_n$ length might be correlated with disease severity in DM.

**49.**

**Title:** The natural history of cardiac involvement in myotonic dystrophy and its relation to the size of CTG trinucleotide repeat. A prospective study.

**Authors:** Antonini G[1], Ceschin V[1], Scioscia F[1], Fiorelli M[1], Gragnani F[1], Clemente A[1], Morino S[1], Amicucci P[4], Gennarelli M[4], Fragola P[3], Mammarella A[2].

**Institutions:** Department of Neurological Sciences[1] and Institute of Medical Therapy[2], University of Rome ALa Sapienza@, Rome- Italy; Department of Internal Medicine[3], School of Cardiovascular Diseases and Department of Biopathology and Diagnostic Imaging[4], University of Rome ATor Vergata@, Rome-Italy.

**WIP**

**Objectives:** To evaluate the frequency and characteristics of cardiac involvement in myotonic dystrophy (MD) and to assess the relationship between cardiac disease and CTG triplet mutation.

**Background:** The MD mutation, identified as an unstable DNA sequence which tends to increase the number of

CTG repeats, produces clinical manifestations of the disease in skeletal muscle, the heart and many organ systems. Sudden death due to complete atrio-ventricular block (AVB) or ventricular arrhythmias is the most dramatic event in MD. The correlation between the size of CTG repeat and the severity of cardiac involvement is debated.
**Methods:** Seventy-five patients with MD, aged 6-63 years (median age 35 years) underwent electrocardiography and 24-h holter monitoring. The diagnosis of MD was established by clinical examination, EMG and DNA analysis. Using the 33rd and the 66th percentile of the observed size of CTG repeats as cut-point, the patients were classified in three groups: E1=26 patients with CTG repeats <300; E2=27 patients with CTG repeats between 300 and 600; E3=22 patients with CTG repeats >600. Sixty-three patients were followed for a period ranging from 12 to 150 months, with periodical ECG and 24-h holter monitoring.
**Results:** The incidence of major conduction changes (atrio-ventricular block, bundle-branch block) was 27% in E1, 22% in E2 and 60% in E3 (p=0.02). The frequency of major arrhythmias as a whole (supraventicular tachycardia, ventricular premature complexes in couplets or with R-on-T phenomenon, atrial fibrillation, and atrial flutter) was 27% in E1, 11% in E2 and 32% in E3. During the follow-up, in a period ranging from 1 year to 10 years, 25/63 patients (40%) developed a "*de novo*" major cardiac complication.
**Conclusions:** Major cardiac conduction disturbances are significantly correlated with CTG repeats in MD. Yearly cardiologic monitoring is warranted in MD patients.

**50.**

**Title:** Cardiopulmonary investigations in Steinert Muscular Dystrophy: Experience of the French Group.

**Authors:** Eymard B[1], Annane D[2], Duboc D[3]
**Institutions:** [1]Institut de Myologie, Salpetrière Hospital, [2]Respiratory Intensive Care Unit, Raymond Poincaré Hospital, Garches, [3]Cardiology Department, Cochin Hospital

Electrophysiologic testing data, clinical status, heart function and DNA mutations size were studied in 83 patients with myotonic dystrophy (DM). Atrioventricular (AV) conduction abnormalities were mainly distal (HV interval 66.3 +- 14ms). AV conduction was generally concordant with surface ECG measurements. However, 11 of 20 patients with normal surface ECG had abnormal sub-hisian conduction. Atrial and ventricular arrhythmias were often inducible. Young age was the most powerful predictor of inducible ventricular tachyarrhythmias. Conversely, the DNA mutation size or the severity of peripheral muscle involvement was not predictive of EP disturbances.

Pulmonary function tests, arterial blood gas, and polysomnography were obtained in 79 consecutive ambulatory patients. 36/79 were hypercapnic (7.7+/-1.2 kPa), 32/79 have decreased forced vital capacity (55+/-20%) and 41/79 have severe sleep apneas (RDI - 33+/-7). The 4 patients with PaCO2>6.1 kPa and normal forced vital capacity (>70% theor), have severe apnea syndrome (RDI=28+/-4). Almost half of patients have diurnal hypersomnia which was not related to sleep disruption or hypercapnia. Correlations between cardiac and respiratory dysfunction are in progress.

**51.**

**Title:** An in vitro model for the analysis of CTG expansion stability, proliferative capacity, telomer length and myogenic differentiation in congenital myotonic dystrophy.

**Authors:** Butler-Browne GS, Coiffier L, Mouly V, Gourdon G*, Barbet JP #.
**Institutions:** CNRS URA 2115,105 bld de l'hôpital, PARIS 75013, France.
*INSERM U383, Hôpital Necker-Enfants Malade, 149 rue de Sevres-75015 Paris, France.
#Département d'Anatomie Pathologique, Hôpital St Vincent de Paul, 74 av. Denfert-Rochereau, 75014,Paris, France

**WIP**

Myoblasts have been isolated from the quadriceps muscles of five fetuses aged between 24 and 34 weeks of development diagnosed as suffering from congenital myotonic dystrophy. These cells have been cultured and

analysed in parallel with myoblasts isolated from the quadriceps muscle of two aged matched normal fetuses. The purity of the cultures has been estimated using a desmin antibody which reacts specifically with myogenic cells.

When differentiated cultures of the same myogenic purity from control and DM fetuses were compared we found that the size and morphology of the myotubes was different. DM myoblasts gave rise to small thin myotubes whereas control myoblasts of the same age gave rise to large branched myotubes containingas many as 200 nuclei.The myosin heavy chain phenotype was also analysed in these fused cultures using antibodies directed against the embryonic, fetal fast and slow isoforms. As we have shown previously the large branched multinucleated myotubes of control cultures expressed all four myosin heavy chains. In cultures isolated from the DM fetuses, embryonic and fetal myosin heavy chains were mainly detected, suggesting that there had been a modification in the myogenic program expressed by the DM myoblasts, resulting in a strong reduction of the adult isoforms as well as the morphological characteristics of the myotubes. A small amount of fast MHC was however observed in the culture with the smallest repeat size. We are now in the process of determining the proliferative capacity of these cells. In addition DNA has been extracted at every 4 or 5 divisions in order to measure the size and stability of the CTG repeat and to calculate the rate of loss of telomeric DNA.

**52.**

**Title:** Immaturity of skeletal muscle in the severe congenital form of myotonic dystrophy

**Authors:** Barbet JP, Agbulut O.*, Junien C#, Gourdon G, Mouly V*, Butler-Browne GS*

**Institutions:** Department of Paediatric Pathology, Hôpital Saint Vincent de Paul, 74 Avenue Denfert-Rochereau, F-75014 France, *CNRS URA 2115, 105 boulevard de l'Hôpital, F-75013 Paris, France, #INSERM U383, Hôpital Necker Enfants Malades, 149 rue de Sèvres, F-75015 Paris, France

**WIP**

The severe congenital form of myotonic dystrophy corresponds to a very particular entity, being always maternally transmitted. This exclusive maternal transmission had previously suggested the role of environmental factors, but instead seems now to be linked to limits of transmission of the genetic defect during meiosis. Indeed, the severe form is only found when there has been an extensive amplification resulting in the presence of more than 2000 base pair repetitions of the trinucleotide CTG in the region 19q13 on chromosome 19.

We have carried out a morphological and biochemical study of the skeletal muscle in a series of fifteen affected foetuses, aged between 12 and 40 weeks of pregnancy. In addition to classical morphological techniques (H&E and enzyme histochemistry), immunocytochemical studies were carried out using antibodies specific for markers of cell proliferation, myoD and myogenin, intermediate filament proteins and different isoforms of the myosin heavy chains. The results were quantified and compared to control foetuses of the same age.

In the early stages, the skeletal muscles show a delay in the proliferation and fusion of the myoblasts, resulting in a delayed formation of the secondary generation myotubes. This results in an increased cellularity of the muscles which persists even after the fiber formation has normally ceased (about 20 weeks), with many cells expressing markers of cell proliferation and the early myogenic factors. In older foetuses, skeletal muscles are characterized by a general smallness of the fibers, and by a delayed maturation of the second generation slow fibers. This delayed maturation varies between different groups of muscles, with a predominance in the facial and proximal limb muscles whereas the diaphragm can be less severely affected.

This study demonstrates that the CTG triplet repeat results in a defect in the proliferation and fusion of myogenic precursor cells and in an abnormal neuromuscular interaction, responsible for the abnormal growth and metabolic differentiation of the fibers.

Acknowledgements : This work was supported by grants from the Association Française contre les Myopathies, the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and the Fondation pour la Recherche Médicale

**53.**

**Title:** Possible role of apamin-sensitive potassium channels in MD muscle

**Authors:** Jäger H, Grissmer S, Lehmann-Horn F
**Institutions:** Dept. Applied Physiology, University of Ulm, D-89081 Ulm, Germany

Myptonic dystrophy (MD) is characterized by muscle atrophy and myotonia, a repetitive electrical activity of MD muscle. The injection of apamin, a component of the bee venom, into the thenar muscles of MD patients decrease the basal electrical activity (Behrens *et al.*, *Muscle & Nerve* 17:1264-1270, 1994) and these apamin binding sites were identified as $Ca^{2+}$ activated $K^+$ channel (SKCa) with small conductance. In brain, SKCa channels determine the firing pattern by modulation of the slow afterhyperpolarization (sAHP). One hypothesis assumes that the activity of SKCa channels in MD muscle causes a stronger afterhyperpolarization which results in a faster recovery from inactivation of voltage-dependent $Na^+$ channel. This would explain the hyperexitability by the re-opening of $Na^+$ channels. The $Cl^-$ conductance does not influence the production of myotonia in MD (Franke *et al.*, *J. Physiol.* 425:391-405, 1990). Recently, some members of the family of SKCa channels were cloned from rat brain (Köhler *et al.*, *Science* 273:1709-1714, 1996).

We characterized the electrical behavior of *r*SKCa2 a member of this channel family in COS7 cells in order to understand how the presence of this channel could cause a myotonic phenotype. Cells were transfected by the $Ca^{2+}$-phosphate methode and were investigated 1-2 days after transfection. SKCa channels were activated by whole-cell dialysis with a pipette solution (in mM: 140 K-aspartate, 2 $MgCl_2$, 10 HEPES, 8.7 $CaCl_2$, 10 EGTA, pH 7.2) containing 1 FM free $Ca^{2+}$. Ramp currents were elicited by 400 ms voltage ramps from B160 to 40 mV every 20s before and after application of $K^+$ solution containing $Ba^{2+}$ or peptide toxins. $Ba^{2+}$ blocks the *r*SKCa2 channel in a voltage-dependent manner, similar to the $Ba^{2+}$ block of the apamin-sensitive $K^+$ channel in Jurkat T cells (Hanselmann & Grissmer, *J Physiol.* 496:627-637, 1996). Furthermore, we characterized the toxin binding sites of the outer pore of SKCa channels by introducing mutations in this region.

(Supported by grants from the DFG (Gr 848/4-2))

**54.**

**Title:** Abnormal diurnal rhythmicity of adrenal steroid hormones in dystrophia myotonica

**Authors:** Johansson Å, Forsberg H, Krylborg E, Olsson T
**Institutions:** Dept of Medicine and Clinical Chemistry, Umeå University Hospital, Umeå and Myotonic Dystrophy Centre, Dept of Medicine, Boden Hospital, Boden, Sweden

**Introduction.** Dystrophia myotonica (DyM) is the most common inherited muscular dystrophy and there is a high prevalence of the disease in Norrbotten, the northernmost county of Sweden. Endocrine, metabolic and cognitive disturbances are common features of this disease. Abnormal levels of hormones produced by the adrenal cortex, notably cortisol and dehydroepiandrosterone (DHEA) may be of importance for these disturbances. Our aim was to study basal levels of cortisol and adrenal androgens and the diurnal rhythmicity of cortisol and DHEA in DyM patients.

**Methods.** In the first study, 15 men with mild to moderate DyM and 15 healthy men participated. Saliva samples were collected at 08.00, 16.00 and 22.00 hours for analysis of cortisol and blood samples were collected at 08.00 the first morning for analysis of adrenal steroid hormones. In the second study, 18 men with DyM and 18 healthy men were included. Blood samples were drawn at 07.00, 11.00, 16.00 and 22.00 hours for analysis of adrenocorticotropin (ACTH), cortisol, and dehydroepiandrosterone (DHEA).

**Results.** Serum and saliva cortisol diurnal curves were highly abnormal among patients, with flattening of diurnal rhythmicity (12-38% lower cortisol levels in the morning versus controls, $p=$ NS - $< 0.01$ and $p < 0.05$, for serum and saliva respectively; and 130-426% higher cortisol levels during afternoon and evening; $p < 0.05$ - $0.01$ and $p < 0.001$, respectively). Plasma ACTH levels showed a pattern similar to cortisol levels with significantly increased levels at 16.00 hours ($p < 0.\ 1$). Morning serum adrenal androgen (androstenedione,17-alpha-hydroxyprogesterone, dehydroepiandrosterone sulphate and testosterone) levels were significantly decreased in DyM patients (27-53% decrease vs controls; $p < 0.05$-$0.001$). DHEA diurnal curve was clearly flattened with

decreased levels at all time-points (21-61% vs controls) and significantly so at 07.00 and 11.00 hours ($p < 0.001$ and $p < 0.05$, respectively).
**Conclusion.** There are profound abnormalities in the regulation of adrenal steroid hormones and their diurnal rhythmicity in DyM. These disturbances may be of importance for metabolic and cognitive dysfunction in this disease.

**55.**
**Title:** Frontal loby dysfunction in myotonic dystrophy without loss of general intelligence
**Authors:** Miller G, Rettig G, Ashizawa T
**Institutions:** Baylor College of Medicine, Houston, Texas, USA

Although apathy, poor initiative and compliance, have frequently been commented upon by clinicians managing adults with myotonic dystrophy (DM), neuropsychologic studies have not revealed any abnormal function other than that related to global intellectual impairment. We decided to evaluate this further by examining affected individuals who had no loss of general intelligence. We performed a battery of neuropsychologic tests on 9 individuals with adult onset DM, all of whom had completed high school without difficulty. We found that formal tests of IQ, language, visuo-motor perception, memory and learning were all within the normal range. However on tests that specifically evaluated non verbal reasoning and executive function, in 6 of 9 individuals, the results were abnormal. We suggest that the peculiar personality frequently found in adult onset DM is due to poor executive functioning out of proportion to other aspects of neuropsychologic function, and may reflect a frontal lobe syndrome.

**56.**
**Title:** Focal Sertoli Cell degeneration and impaired spermatogenesis are early events in men with Dystrophia Myotonica and related to lengths of CTG repeats.
**Authors:** Kvist U, Plöen L[1,2], Anvret M[3], Ansved T[3]
**Institutions:** 1) Deparment of Woman and Child Health, Karolinska Hospital, Stockholm, 2) Department of Anatomy and Histology, SLU, Uppsala, 3) Department of Clinical Neuroscience, Karolinska Hospital, Sweden.
**WIP**

Men with DM eventually acquire testicular failure of both spermatogenesis and testosterone production. We studied five men, of whom three were brothers, all with genetic predisposition for DM. They were clinically assessed and the lengths of their CTG repeats were determined. To assess testicular functions semen samples and testicular biopsies ( from three men) and hormonal status were evaluated.

There was a clear relation between clinical findings, length of CTG-repeats and reduction in testicular tubular diameters and spermatogenesis. The man with the most advanced DM symptoms and the most severely impaired spermatogenesis had increased levels of pituitary FSH and also impaired testosterone production with elevated level of LH.

Light and electron microscopy revealed tubules with impaired spermatogenesis in all three investigated men. Focal Sertoli cell degeneration was found in all three but most advanced in the patient with the longest repeat. All, but the patient with most advanced DM, had normal semen emission and ejaculation and their samples were judged as normal. In addition to severely impaired spermatogenesis he had no emission from the Wollffian duct system ( epididymis, vas deferens and seminal vesicles).

We suggest that the Sertoli cell is the testicular cell primarily affected in men with DM and that the effects on spermatogenesis are secondary to the Sertoli cell lesions.

**57.**

**Title:** Meiotic drive at the myotonic dystrophy locus in Japan

**Authors:** Yamagata H, Miki T
**Institutions:** Department of Geriatric Medicine, Ehime University School of Medicine, Ehime 791-0295, Japan.

Myotonic dystrophy (DM) is an autosomal dominant neuromuscular disease caused by CTG repeat expansion. It is interesting to ask how DM, in spite of its reduced fitness in affected, can be maintained in the population with a relatively high prevelence. Meiotic drive at the DM locus has been suggested as being responsible for maintaining the frequency of DM chromosomes capable of expansion to the disease state in the human population. Several studies have found evidence of segregating distortion in favor of transmission of the longer CTG allele, but the data upon which sex carries this effect are conflicting.

In order to address this question, we investigated 64 Japanese DM pedigrees and 9 non-DM pedigrees by clinical and molecular analysis. Among 236 sibs investigated we found a significant proportion of affected sibs (57.6 % vs 42.4 %) (p= 0.019). In addition, we observed a striking distortion of segregation with sex. Father had preferentially transmitted the mutant allele to sibs (59 % vs 41 %) (p= 0.041). We then studied meioses in which the parent was heterozygous with one allele of <19 repeats and the other of $19 repeats. In a total of 156 meioses the larger allele, CTG (n_$19), was transmitted in 66 % of cases, rather than the expected 50 % (p < 0.001).

In conclusion our data suggest the existence of meiotic drive at the DM locus in the Japanese population.

**58.**

**Title:** A database of DM alleles, intergenerational transmissions and clinical correlations

**Authors:** Monckton DG, Hogg GF, Armstrong JD
**Institutions:** Division of Molecular Genetics, Institute of Biomedical and Life Sciences, Anderson College, The University of Glasgow, 56 Dumbarton Rd, Glasgow G11 6NU, UK.

Myotonic dystrophy is associated with the expansion of a CTG repeat in the 3=UTR of the DM protein kinase gene. In normal individuals the repeat ranges in length from 5 to 37 repeats, but is expanded beyond this range containing from 50 up to many thousands of repeats in the DM population. There is a clear correlation between repeat length and disease severity with larger alleles giving rise to a more severe form of the disease. Conversely, age of onset decreases with increasing allele size. However, these correlations are by no means absolute and an accurate prediction of the likely age of onset for asymptomatic individuals cannot be made based on their measured repeat length. Once into the expanded range the repeat becomes highly unstable with frequent length changes from one generation to the next. Such frequent length changes account at least in part for the symptomatic variability observed between patients and in particular the fact that such length changes are biased toward further expansions presents a molecular explanation for the anticipation observed in DM families. Once again though, these correlations are not absolute with a significant subset of transmissions apparently giving rise to contractions in repeat length not associated with a decrease in the severity of the symptoms. Over the last few years we have started to gain some insights into why the repeat length correlations are not as predictive as might have been hoped. It is now realised that the repeat is also highly unstable in somatic tissues and once again biased toward further expansion in a process that appears to proceed through the frequent gain of small numbers of repeats at a tissue specific rate throughout the lifetime of the individual. Thus, the measured allele size in an individuals blood DNA is a moving target, whose size therefore is highly dependent on age at sampling. Failure to correct for such an age effect has almost certainly compromised genotype to phenotype correlations for individuals and from one generation to the next. We are attempting to develop methods for correcting for such effects with t hope of providing more accurate prognostic information to individuals and risk assessments to future generations. Such analyses are proceeding via the detailed analyses of relatively small number of patients, but would be greatly facilitated by the accumulation of larger data-sets. Such large data-sets could be only be accumulated by collaboration between many groups using standardised reporting procedures. We propose to establish a suitable database and will discuss the types of data we should like to collect and explore the feasibility of such an approach with the DM research community.

## Related Distorders (DM2, PROMM, PDM, etc.)

**59.**

**Title:** A genome-wide scan for PROMM

**Authors:** Mitchell A[1], Bailey MES[1], Moxley R[2] , Thornton C[2], Johnson K J[1]

**Institutions**: [1]Division of Molecular Genetics, IBLS, University of Glasgow, UK

[2]Neuromuscular disease center, Department of Neurology, University of Rochester, Rochester, New York, USA

**WIP**

A new myotonic disorder, proximal myotonic myopathy (PROMM) has been identified with an almost identical phenotype to DM. We have performed a primary genome scan of two unrelated three and four generation PROMM families. The suitability of both pedigrees for linkage analysis has been assessed using a simulated linkage analysis computer program, SLINK. Sixty three members of the two families have been genotyped with 283 dinucleotide repeat markers spanning the genome, excluding the X chromosome, at an average interval of 10cM. We report the preliminary results of this scan and its implications for the genetic relationship between PROMM and DM.

**60.**

**Title:** Dominant Multi-Systemic Proximal Myotonic Myopathic Syndromes: Clinical and Genetic Heterogeneity in Three Families.

**Authors:** Krahe R[1], Meola G[2], Ptacek L[3], Lee D[1], Udd B[4]

**Institutions:** [1]Div. of Human Cancer Genetics, Ohio State University, Columbus, OH 43210, USA; [2]Dept. of Neurology, Univ. of Milan, San Donato Hospital, Milan, Italy; [3]Dept. of Neurology and Human Genetics, Univ. of Utah, Salt Lake City, UT 84132, USA; [4]Dept. of Neurology, Vasa Central Hospital, 65130 Vasa, Finland.

**WIP**

Myotonic dystrophy (DM and DM2), proximal myotonic myopathy (PROMM), proximal myotonic dystrophy (PDM), and related proximal myopathic syndromes are dominantly inherited, multi-systemic myotonic myopathies characterized by predominantly distal (DM and DM2) or proximal (PROMM and PDM) localization. DM is caused by the expansion of an unstable (CTG)n repeat in *DMPK* in chromosome 19q13.3; linkage of DM2 to a locus in 3q21 has recently been reported. Some PROMM families appear to be linked to the same chromosomal region in 3q21, while other families with PROMM or a related phenotype appear to be unlinked to either locus, as well as the other two myotonia loci (*CLCN1* in 7q35 and *SCN4A* in 17q23-q25). Two PROMM families from Italy and the U.S. with a total of 21 affecteds and a single PDM family from Finland with 15 affecteds were compared clinically. Patients of all three families show predominantly proximal and less pronounced distal muscle weakness of varying degrees, EMG myotonia, cataracts, and male hypogonadism as the core features of the syndrome. The cardiac system appears relatively unaffected. To assess clinical and genetic heterogeneity in these three families with dominantly inherited multi-systemic myotonic myopathy not linked to *DMPK*, *CLCN1*, and *SCN4A*, genetic linkage analysis was performed. The U.S. PROMM and the PDM family showed linkage to the same region in 3q21 by two-point (D3S1269; $Z_{max} = 5.41$, $\theta_{max} = 0.05$) and multi-point GeneHunter linkage analysis (D3S1269; $lod_{max} = 5.36$), while the Italian PROMM family was unlinked. Our data confirm clinical and genetic heterogeneity for the dominantly inherited multi-systemic myotonic myopathies. Importantly, the observed linkage in two of the three families to the same chromosomal region reported for DM2 and PROMM suggests allelic heterogeneity in a major myopathic locus in 3q21

**61.**

**Title:** The genetic locus for myotonic dystrophy type 2 (*DM2*) also underlies many cases of PROMM

**Authors:** Day JW[1], Ricker K[2], Liquori CL[1], Durand AC[1], Ranum LPW[1]

**Institutions:** [1]University of Minnesota Department of Neurology and Institute of Human Genetics, [2]University of Wuerzburg Department of Neurology

We have studied a 5-generation family (MN1) with multisystemic clinical features that are strikingly similar to myotonic dystrophy (DM), including clinical and electrical myotonia, distal muscle weakness, muscle atrophy, cardiac arrhythmias, and cataracts that are iridescent on slit-lamp examination. Like DM, affected MN1 family members have low serum immunoglobulins, diminished response to insulin and testicular failure. Muscle biopsy findings have ranged from normal (in biopsies of unaffected muscles in young subjects) to severely dystrophic. Because of the overwhelming clinical similarity of the disorder in the MN1 family to DM, we refer to it as DM2.

We genetically mapped the DM2 locus to chromosome 3q. Interestingly, most German PROMM families have also been linked to the DM2 site. We have now identified and begun to collect several additional large Minnesota families that are consistent with linkage to the 3q locus. Some of these families have distal weakness, but others have a more classic PROMM phenotype. Since weakness in these families can be either distal or proximal in location, and since the muscle biopsies may or may not show signs of dystrophy, we now recognize that the salient clinical features of these conditions are myotonia, weakness, and a systemic disease akin to DM. Haplotype analyses of the Minnesota and German families do not suggest the presence of a common founder mutation.

RAPID cloning analysis indicates that a pathogenic CTG expansion is unlikely to be involved in the disease process. We are using a positional cloning strategy to identify the gene involved. Understanding the molecular mechanisms common to both DM2/PROMM and DM should help clarify the pathophysiological causes of these multisystemic myotonic disorders.

**62.**

**Title:** An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8)

**Authors:** Ranum LPW, Moseley ML, Schut LJ, Benzow KB, *Bird TD, Day JW, Koob MD

**Institutions:** Departments of Neurology and Genetics Cell Biology & Development and the Institute of Human Genetics, University of Minnesota, Minneapolis, MN USA and *Department of Veteran=s Affairs Medical Center, Seattle WA USA

To date, myotonic dystrophy (DM) has been the only reported disease caused by the expansion of an untranslated CTG repeat. We have now identified a CTG expansion in the 3= untranslated region of a transcript that causes a novel form of spinocerebellar ataxia (SCA8). The CTG expansion was isolated from the DNA of a single affected individual using RAPID cloning. Subsequent PCR analysis identified eight families among 361 ataxia kindreds with this expansion. The largest of these is a seven generation family (lod=6.8 at $\theta$=0.00). Affected individuals from this kindred have repeat tracts similar in size to those found among mildly affected adult-onset DM patients (107 to 127 repeats). Within this family, 20 individuals with shorter repeats (mean=92.4) were not clinically affected. Although the repeat expansion is transmitted from both males and females, all 21 affected members of the large SCA8 family, and 26 of 30 SCA8 patients overall, inherited an expanded repeat from their mother. This maternal penetrance bias is consistent with the high frequencies of elongation in female transmissions (-11 to +600) and contraction in paternal transmissions (-86 to +7). Three very large increases in repeat length (+250, +375, +600), similar in size to those seen in DM, all resulted from maternal transmissions.

Because all of the dominant SCA genes identified to date are caused by CAG expansions translated as polyglutamine tracts, we expected to find evidence supporting this mechanism for SCA8. However, analysis of the genomic sequence flanking the *SCA8* expansion revealed that there is no extended glutamine ORF and there are no splice sites that could generate an open glutamine reading frame in a spliced mRNA transcript. Furthermore, strand specific RT-PCR analysis detected an RNA transcribed in the CTG but not the CAG direction. As expected from the genomic sequence, RACE analysis confirmed that the CTG repeat is present in the 3= terminal exon beginning at the predicted splice-acceptor site. Identified transcripts contain three or four exons, but no ORFs are

present in either variant. Efforts are continuing to conclusively identify the 5= end of the *SCA8* transcript. The size, orientation, and 3= location of the untranslated *SCA8* CTG expansion are similar to the CTG expansion that causes DM. Further clinical and molecular characterization of SCA8 should help us to more fully understand the pathophysiology of both ataxia and DM.

## Clinical Rating Scale for DM

**63.**

**Title:** Experience with a practical clinical rating scale for myotonic dystrophy (MyD).

**Authors:** Hilton-Jones D

**Institutions:** MDG Muscle & Nerve Centre, Radcliffe Infirmary, Oxford, UK, OX2 6HE

Numerous rating scales have been developed for patients with neurological disease, and several specifically for neuromuscular disorders. Many of these are very time consuming to administer and impracticable in a routine clinic setting, although they may be relevant for formal trials. General assessment of muscle function may not reflect the rather specific changes associated with MyD, such as weakness of grip and the severity of myotonia. Measurement of raw muscle strength must be accompanied by assessment of functional ability reflecting normal daily activities. I will describe our experience with a rating scale developed by Rogers and Harper (Cardiff), with some input from ourselves, discuss the advantages and disadvantages of the scale, and seek views on the design of a scale that could be used internationally.

Successful treatment implies halting the progression, or reversal, of the disease process. It is likely to be some time before this becomes available. The rationale behind our assessment programme is two-fold. Firstly, objectively measuring deterioration of function (and thus plotting the natural history of the condition) over a period of time prior to the start of a therapy trial should help in demonstrating the effectiveness, or otherwise, of the treatment. Secondly, a prolonged study prior to the start of a trial should allow any difficulties in applying the rating scale to be ironed-out.

There are three main components to the Clinic Record. Patients are assessed annually.

*History*: Specific symptoms relating to Swallowing, Cardiorespiratory function, and Somnolence are recorded.

*Examination*: A quantifiable record is made of lens status, weakness (10 movements), myotonia, pulse rate and blood pressure, respiratory function and swallowing function. An ECG is performed and conduction status recorded.

*Mobility index*: A 15 point Rivermead Mobility Index is determined.

Results are recorded on a computer database. Part of the questionnaire and physical assessment can be performed by suitably trained non-medical personnel. The assessment time for each patient, excluding ECG, is approximately 30 minutes. We will be assessing inter- and intra-observer variability.

**64.**

**Title:** Development of a quantitative motor assessment protocol in myotonic dystrophy

**Authors:** Mathieu J, Richards C

**Institutions:** Neuromuscular Clinic, Complexe Hospitalier de la Sagamie, Chicoutimi, Qc, Canada, Laval University, Quebec, Qc, Canada

**WIP**

**Objective.** To document the reliability of a quantitative motor protocol and to establish baseline data prior to a prospective longitudinal study of the natural history of myotonic dystrophy (DM).

**Design/Methods.** We conducted a cross-sectional study using a protocol of manual muscle testing (MMT), quantitative muscle testing (QMT), and timed functional testing (TFT) on 50 definite DM patients (27 men, 23 women), aged 16 to 67 years. Intra- and interrater reliability of MMT, QMT and TFT scores were analyzed using the intraclass correlation coefficient (ICC). The relationships between MMT, QMT and TFT scores and disease duration were examined using regression analysis. **Preliminary results.** Good to excellent intra/interrater reliability was found for total MMT score, for all QMT measurements and for most TFT results. The muscle weakness was symmetrical and the neck flexors and the distal muscles of upper and lower extremities were weaker than proximal muscles. Using MMT scores, the average strength decline was 1.2% per year of disease duration and was similar for men and women. The strength decline was significantly more rapid for neck flexors and distal muscles than for proximal muscles. QMT scores documented a strength decline per year of disease duration ranging from 1.2-1.6% for hip flexors to 2.0-3.0% for hand grip flexors.

**Preliminary conclusions**. The MMT, QMT and TFT tests have been shown to produce reliable measures of disease state. We observed significant linear relationship between the muscle strenght scores generated by this protocol and the disease duration. The follow-up assessment of a large DM cohort in a longitudinal study will establish whether this quantitative protocol provides sensitive measures of disease progression.

## Treatment Strategies for DM

**65.**

**Title:** Ribozymes: tools to dissect the molecular basis of myotonic dystrophy?

**Authors:** Hamshere MG, Nickerson H, Brook JD
**Institutions:** Institute of Genetics, Universtiy of Nottingham, Nottingham, NG7 2UH, UK

Myotonic dystrophy is associated with expansion of a triplet repeat unit in the 3'UTR of DMPK. However, the underlying molecular basis for the pathophysiology of this disease remains unclear. Two possible models have been proposed. First that the mutation has an effect at the DNA level to alter transcription of all genes which surround the repeat. Second, that there is a gain of function for RNA/protein complexes that may be produced by the failure of expansion derived DMPK transcripts to be exported from the nucleus. It is important to note, however, that these models are not necessarily mutually exclusive; they could have different contributions at different stages of development or in different tissues. In order to address the mechanism raised in the second model (gain of function for RNA) in isolation, we have been developing ribozymes to remove the aberrantly retained DMPK RNA. Two types of ribozyme have been produced: a series based on a hammerhead ribozymes, and a second series based on the P ribozyme of E.Coli. The main difference between these constructs is the potential for the P ribozyme to preferentially target transcripts that contain expanded repeats. These ribozymes have been incorporated into eukaryotic expression vectors, and transfected into DM fibroblasts that are informative for a polymorphism within DMPK. Stable cell lines are currently being established, to study the feasibility for endogeneous expression of these constructs in tissue culture.

**66.**

**Title:** Mechanisms of dehydroepiandrosterone sulfate (DHEA-S) actions to skeletal muscle: possible implication for DHEA-S replacement therapy

**Authors:** Furutama D, Tsuji K, Ohsawa N
**Institutions:** The First Department of Internal Medicine, Osaka Medical College, Takatsuki, Japan

Dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) are the most abundant steroidal products and major circulating steroids in humans. Previous studies reported low serum concentrations of DHEA and DHEA-S in myotonic dystrophy (DM) patients compared with age- and sex-matched healthy controls, and recently a possible improvement of myotonia and muscle weakness following administration of DHEA-S was shown. DHEA-S is synthesized from DHEA by DHEA sulfotransferase (DHEA-ST) mainly in adrenal gland and liver. Surprisingly, DHEA-ST gene localizes to human chromosome 19q13.3, which is adjacent to DMPK gene. These evidences strongly suggest that the DHEA(-S) metabolism is involved in the pathogenesis of DM, however, it is unknown how DHEA(-S) could affect the DM pathophysiology. As the first step, to investigate the mechanism(s) of DHEA(-S) actions, we studied: (1) DHEA(-S) binding sites in skeletal muscle cells by Scatchard analysis, (2) changes of gene expression in skeletal muscle cells by DHEA(-S) using differential display and semi-quantitative RT-PCR, and (3) transcriptional alternation of neighboring genes, including DHEA-ST, by expanded CTG repeats in DMPK gene. We found: (1) DHEA-S, not DHEA, binding sites of Kd value similar to human physiological DHEA-S concentration, (2) changed or unchanged expression of several genes in skeletal muscle, (3) transcriptional suppression of DHEA-ST gene by CTG repeat expansion in human chromosome 19 containing somatic hybrid cells. We believe that further investigation focused on the relationship between DHEA(-S) action and DM pathophysiology would provide new insight into the DM research.

**67.**

**Title:** INSULIN-LIKE GROWTH FACTOR-I OVERCOMES INSULIN DEFECTIVE ACTION IN HUMAN MYOTONIC DYSTROPHY SKELETAL MUSCLE CELLS

**Authors:** Furling D, Marette A, Puymirat J

**Institutions:** Laboratory of Human Genetics, Department of Medicine, Laval University Medical Research Center, CHUQ, 2705 Blvd Laurier, Ste-Foy, Quebec, Canada.

Primary human skeletal muscle cell cultures were established from fetal myotonic dystrophy (DM) muscles. DM myoblasts were allowed to proceed to the stage of myotubes in order to investigate resistance of insulin action described in DM patient muscles, and potential ability of IGF-1 to circumvents this defect. In cultured DM cells, basal glucose uptake remained unchanged but stimulatory effect of insulin on glucose uptake was not observed at 10 nM and was significantly reduced from 24 % at 100 nM and 17 % at 1 FM, when compared with cultures initiated from normal myoblasts. In addition, both basal (-29 %) and insulin-mediated protein synthesis (-15 %) were significantly decreased compared with normal cells. On the other hand, IGF-1 is able to stimulate glucose uptake in DM myotubes to a similar extent as control cells and restore normal protein synthesis level in DM myotubes. These results show that insulin action on both glucose uptake and protein synthesis is impaired in cultured DM muscle cells and that IGF-1 is able to bypass these metabolic impairment. Moreover, insulin receptor mRNA expression and binding were significantly reduced in DM myotubes from 31 % and 14 % respectively as compared to control muscle cells whereas the expression of both GLUT1 and GLUT4 glucose transporters were not affected in these cells. These results suggest that the reduced action of insulin on glucose uptake may be explained by a proximal signaling defect in insulin signaling. Thus, human muscle cell culture provides a relevant cellular model to determine the mechanisms of insulin resistance in myotonic dystrophy. Our results form the rational basis for the use of IGF-1 as an effective treatment of muscle weakness and wasting in DM.

**68.**

**Title:** TRANSPLANTATION OF MYOTONIC DYSTROPHY HUMAN MYOBLASTS IN SCID MICE : A NEW POTENTIAL MUSCULAR MODEL.

**Authors:** Furling D[1], Skuk D[1], Bouchard J-P [1,2],Vilquin J-T[3], Tremblay JP[1], Puymirat J[1]

**Institutions:** [1]Unité de Recherche en Génétique Humaine, Centre Hospitalier de l'Université Laval, Ste-Foy, Québec, Canada; [2]Departement des Sciences Neurologiques, Hôpital de l'Enfant Jésus, Québec, Canada and [3]INSERM U153, Institut de Myologie, Groupe Hospitalier Pitié-Salpétrière, Paris, France.

A possible alternative to develop an in vivo muscular model for DM could be the transplantation of DM myoblasts into mice skeletal muscle. In the present study, we transplanted myoblasts from DM patients in the Tibialis anterior of Severe Combined Immunodeficient (SCID) mice to determine whether this approach could reproduce the muscular characteristics observed in DM. One to four months after the transplantation, a variable number of human muscle fibers recognized by an antibody specific for the human dystrophin were found in transplanted muscles. Southern blot analysis demonstrated the presence of the CTG repeat expansion in the DM regenerated human muscle fibers. Although the characteristic histological features of DM were absent in these fibers, we showed that DM fetal myoblasts produced smaller fibers than normal myoblasts in SCID muscle. Morever, electromyographic recording showed typical myotonic discharges in muscles transplanted with DM myoblasts. The presence of motor end plates in sequential sections, as shown by the acetylcholinesterase reaction, confirmed that human dystrophin fibers were innervated. The specificity of the myotonic runs was demonstrated by its inhibition by apamin, a drug that specifically blocks DM myotonia. We conclude that transplantation of myoblasts from DM patients in SCID mice could provide a potential in vivo model for basic studies on this entity.

**69.**

**Title:** Early phase II study of KB-601 (dehydroepiandrosterone sulfate) in myotonic dystrophy

**Authors:** Ohsawa N, Sugino M, Shinoda K, and KB-601 early phase II study group
**Institutions:** The First Department of Internal Medicine, Osaka Medical College, Takatsuki, Japan

**Background and purpose:** Recently a pilot study of KB-601 (dehydroepiandrosterone sulfate; DHEA-S) in myotonic dystrophy (DM) have suggested that it improved activities of daily living, muscular strength, and myotonia in DM patients (Neurology 51: 586, 1998). As the next step, we performed a early phase II study of KB-601 in patients with DM.

**Patients and Methods:** Thirty-three patients with DM in 16 hospitals were studied. As previously reported (Neurology 51: 586, 1998), the patients underwent daily IV injection of KB-601 (Kanebo Co. Japan; 200 mg/day) for 8 weeks. Their activities of daily living (ADL), muscular strength, and myotonia before and 8 weeks after DHEA-S were evaluated and compared. In addition, to test whether effects or improvements persist after discontinuation of KB-601 administration, we performed the follow-up evaluation for 3 months.

**Results:** Improvement of muscular strength in 8 weeks after 8 weeks administration of KB-601 was noted in 23/32 (manual muscle testing), 21/30 (left grasping power), and 21/31 patients (right grasping power).ADL scores were increased in 21/31 patients. Myotonia grade improved in 10/31 patients. Especially, grip myotonia, which was evaluated by hand-opening time after grip myotonia, improved in 23/31 (left grip) and 26/31 (right grip) patients after KB-601. Improvement of myotonia persist for 3 months in about half of patients. Although several adverse effects, such as abdominal or lumbar pain, headache, and elevation of AST or ALT, appeared in a few patients, the trails was completed without any serious side effects.

**Conclusion:** This result was similar to that of the pilot study by Sugino et.al. They suggest that KB-601 (DHEA-S) is beneficial for patients with DM, and provide a rationale for a controlled study in DM.

**70.**

**Title:** Heavy resistance training in myotonic dystrophy

**Authors:** Ansved T [1], Eriksson S [1], Jenner G [2], Tollbäck A[1], Vargas R[2], Wredenberg A[1], Borg K[1]
**Institutions:** [1]Department of Clinical Neuroscience, and [2]Department of Radiation Physics, Karolinska Institutet, Stockholm, Sweden

It is well established that progressive strength training regimens increase muscle strength in healthy subjects. However, much less is known regarding the effects on diseased muscle. In the present study, nine ambulatory subjects with myotonic dystrophy participated in a supervised twelve-week progressive high-resistance training program. Knee extensor muscles were trained three times a week with free weights, 3x10 repetitions at 80% of one repetition maximum (1RM). One leg was randomly chosen for training and the other served as control. Six patients completed the training program. In the trained leg 1RM increased from 16.4 " 3.4 kg to 21.8 " 2.6 kg (p=0.0002). There was no difference between pre- and post-training concentric or eccentric isokinetic values at 30E/sec in either leg. Muscle biopsy from *m. vastus lateralis* in the trained leg revealed no systematic difference in the degree of histopathological abnormalities before and after training. There was no statistically significant change in the proportion of fibre types, or in muscle fibre cross-sectional area after the training period, although the type I fibres tended (p=0.062) to increase in size. However, the number of subjects was too small to allow any definite conclusions regarding the effects of training on the histopathology. Magnetic resonance imaging revealed no difference in *m. quadriceps* area after training.

In conclusion, patients with myotonic dystrophy can improve their muscle strength without any obvious negative side-effects after a twelve-week high-resistance training program.

**Invited Speakers**

**Title:** Genetics of Spermatogenesis
**Authors:** Eddy EM
**Institutions:** NIEHS, NIH, Research Triangle Park, NC 27709 USA

Spermatogenesis is a developmental process that occurs in closely coordinated mitotic, meiotic and post-meiotic phases. In the mitotic phase, self-renewing stem cells give rise to spermatogonia, which undergo a precise number of divisions that successively produce type A, intermediate, and type B spermatogonia, thereby expanding the number of cells available to give rise to male gametes. The final division produces preleptotene spermatocytes, which undergo the last round of DNA synthesis in spermatogenesis. In the meiotic phase, spermatocytes perform chromosomal synapsis and genetic recombination, and then undergo two reductional divisions in rapid succession. In the post-meiotic phase, the haploid spermatids are remodeled, undergoing acrosome formation, nuclear condensation, flagellar development, and losing the majority of their cytoplasm, to become spermatozoa. Spermatogenesis takes about 35 days in the mouse and 64 days in the human, with each of the three phases requiring roughly the same amount of time. This results in a cell that is highly differentiated in structure and function, but developmentally totipotent. Exceptional genetic processes occur during spermatogenesis. Many unique genes are translated and germ cell-specific transcripts are produced at specific stages of development. Genes expressed in the brain often are expressed in the testis and genes usually associated with oncogenesis and mutagenesis often are expressed in the testis. In addition, DNA breaks and repairs occur during meiotic recombination that result in the exchange of genetic materials between chromosomes derived from different parents. Gene expression is active from the haploid genome during the post-meiotic phase until nuclear condensation. Some mRNAs are subject to translational regulation, being stored in spermatids and translated several days after transcription ends. Changes in genomic imprinting also occur uniquely in germ cells. Expression of certain genes in an offspring depends upon the parent of origin. Although an allele remains imprinted throughout the rounds of DNA replication in the somatic cells of an individual, the imprint is erased during passage through the germ line of the opposite sex. In addition, telomere length, which shortens throughout the life span and is believed to define the replicative capacity of cells, is restored during spermatogenesis. Heritable mutations, including expansion and contraction of trinucleotide repeats, can also arise during spermatogenesis, suggesting that genetic and possibly environmental factors affect genetic stability during this process. The significance of many of these genetic events during spermatogenesis is obvious, but their fundamental basis is obscure.

## Author Index


| | | | |
|---|---|---|---|
| Abbruzzese C | 3 | Durand AC | 61 |
| Adelman J | Guest Speaker | Duros C | 4, 24 |
| Agbulut O | 24, 52 | Eddy EM | Guest speaker |
| Alwazzan M | 47 | Epstein H F | 26, 34 |
| Amack JD | 19 | Eriksson M | 43 |
| Angelini C | 16 | Eriksson S | 70 |
| Ansved T | 5, 43, 56, 70 | Edstrom L | 43 |
| Anvret M | 5, 43, 56 | Evans S | 6 |
| Arnheim N | 9 | Eymard B | 50 |
| Angelini C | 42 | Ferrier R | 37, 40 |
| Annane D | 50 | Fiorelli M | 49 |
| Antonini G | 49 | Fluck M | 35 |
| Amicucci P | 49 | Forsberg H | 54 |
| Armstrong JD | 58 | Fouquet C | 4, 24 |
| Ashizawa T | 7, 55 | Fragola P | 49 |
| Baiget M | 1 | Frazier ML | 6 |
| Bailey M, MES | 37, 40, 59 | Frisch R | 44 |
| Balasubramanyam A | 35 | Funanage VL | 44 |
| Belaguli N | 35 | Furlan S | 31 |
| Barbet JP | 51, 52 | Furling D | 20, 67, 68 |
| Benzow KB | 62 | Furutama D | 66 |
| Biral D | 31 | Furuya H | 18 |
| Bird TD | 62 | Gamez J | 1 |
| Booth F | 35 | Gennarelli M | 16, 42, 49 |
| Borg K | 70 | Gerber AN | 45 |
| Bouchard J-P | 68 | Giacanelli | 3 |
| Brook JD | 47, 65 | Gonzalez IL | 44 |
| Bush EW | 36 | Gosling M | 25 |
| Butler-Browne G | 24, 51, 52 | Grootegoed A | 46 |
| Carey N | 25, 43 | Gould F K | 2 |
| Carey RR | 42 | Gourdon G | 4, 24, 51,52 |
| Ceschin V | 49 | Gragnani F | 49 |
| Chen E | 33 | Grandell U | 5 |
| Clark B | 37, 40 | Greenwell PW | 9 |
| Clemente A | 49 | Griffith J | 15, 21 |
| Coerwinkel-Driessen M | 23 | Grissmer S | 53 |
| Coiffier L | 51 | Groenen P | 23, 27 |
| Cook DL | 45 | Hagege A | 24 |
| Coolbaugh-Murphy MI | 6 | Hamilton G | 37 |
| Cooper TA | 14 | Hamshere M, MG | 47, 65 |
| Costanzi-Porrini S | 3 | Hashem VI | 11 |
| Cree L | 40 | Harris S | 37, 38 |
| Davis BM | 17, 33 | Helmke S | 36 |
| Dallapiccola B | 16 | Hilton-Jones D | 63 |
| Day JW | 61, 62 | Hirose K | 48 |
| de Boer P | 46 | Hoffman EP | 42 |
| de Mey JGR | 27 | Hofmann-Radvanyi H | 4 |
| de Rooij D | 46 | Hogg GF | 58 |
| Dunn MJ | 25 | Housman DE | 17, 33 |
| Duboc D | 50 | Imai N | 18 |

Ishiura S 29
Isoda K 48
Iyer D 35
Jäger H 53
Jenner G 70
Johansson Å 54
Johnson KJ 25, 37, 38, 59
Junien C 4, 24, 52
Kameda N 29
Kamada S 39
Kawakami K 39
Khajavi M 7
Kinoshita M 48
Kira J 18
Klesert TR 41
Kobayashi T 29
Komori T 48
Koob MD 62
Korade-Mirnics Z 42
Korneluk RG 28
Krahe R 8, 60
Krylborg E 54
Kvist U 56
Le TL 30
Lee D 60
Lehmann-Horn F 53, Guest Speaker
Lia A-S, Lia-Baldini AS 4, 13
Liquori CL 61
Liu C P 9
Liguori M 3
Lu X 12
Luciano BS 33
Lyubchenko YL 11
Mahadevan MS 19
Maire P Guest Speaker
Malecki A 6
Mammarella A 49
Marin O 31
Marette A 67
Martorell L 1
Mathews M 22
Mathieu J 64
Matsuda A 48
Matsumura O 48
Menegazzo E 16
Meola G 60
McClain C 22
Michalowski S 15, 21
Miki T 57
Miller G 55
Miller JW 15
Mistry D 32
Mitchell A 59
Mizusawa H 29
Monckton DG 1, 2, 8, 58
Moore H 9
Moorman JR 32
Morino S 49
Morris GE 30
Moseley ML 62
Mouly V 51, 52
Mounsey JP 32
Moxley R 59
Mussini I 31
Nguyen TM 30
Nickerson H 65
Novelli G 16
Ohno S 29
Ohsawa N 66, 69
Ohtake T 48
Ohto H 39
Okoli G 25
Olsson T 54
Osanai R 48
Oussatcheva EA 11
Ozaki H 39
Pavoni M 16
Pearson CE 11
Pegoraro E 42
Perryman MB 36
Petes TD 9
Pham YCN 30
Philips AV 14
Potaman VN 11
Plöen L 56
Ptacek L 60
Puymirat J 20, 67, 68
Radvanyi F 4
Reddy S 32
Ranum LPW 61, 62
Rettig G 55
Richards C 64
Ricker K 61
Rowan B 35
Salvatori S 31
Sato S 39
Schut LJ 62
Schwartz RJ 35
Scioscia F 49
Segawa K 48
Seijersen T 5
Servidei S 42
Seznec H 4, 24
Shimizu M 26, 34

Shimokawa M 29
Shiokawa K 18
Shinoda K 69
Shlyakhtenko LS 11
Siciliano MJ 6, 8
Sinden RR 11
Singer RH 17, Guest Speaker
Skuk D 68
Spring NV 8
Storbeck CJ 28
Sugino M 69
Swanson MS 15, 21
Tapscott SJ 41, 44, 45
Tarleton J 42
Teng-umnuay P 15
Tessarolo D 3
Thornton C 15, 22, 59
Tian B 22
Timchenko LT 12, 13
Timchenko NA 12
Tollbäck A 70
Tremblay JP 68
Tsuji K 66
Turner D 22
Udd B 60
Ueda H 29
Urbinati CR 15, 21
van den Broek W 23, 27
van der Zee I 46
van Tuijn P 46
Vargas R 70
Vilquin J-T 68
Watt DJ 25
Wells RD 10
Wang W 26
Waring JD 28
Wei L 35
Weigel N 35
Westerlaken J 46
White R 22
Wredenberg A 70
Wieringa B 23, 27, 46
Winchester C 37, 40
Wormskamp N 27
Xia T 22
Yamagata H 57
Yamamoto M 29
Zhou W 35

## List of Attendees

Claudia Abbruzzese
Department of Neurosciences
Service of Neuromuscular Diseases
San Camillo Hospital
Circ. Ne Giacolense 87
00182 Roma, Italy
Tel: +39-6-582-097-40
Fax: +39-6-582-097-40
Email: malico@tin.it

John Adelman
Vollum Institute
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road
Portland, OR 97201
Tel: (503)-494-5450
Fax: (503)-494-4353
Email: adelman@ohsu.edu

Giovanni Antonini
Dipartimento Di Scienze Neurologioie
Univ La Sapienza
Roma 00185, Italy
Tel: +39-6-49914701
Fax: +39-6-4457705
Email: md0914@mclink.it,
ANTONINIMUSC@UNIROMA1.IT

Tor Ansved
Department of Neurology
Karolinska Hospital
S-171 76 Stockholm
Sweden
Tel: +46 851 77 37 02
Fax: +46-851 77 37 57
Email: tora@neuro.ks.se

Tetsuo Ashizawa
Dept of Neurology
Baylor College of Medicine
One Baylor Plaza SM1801
Houston, TX 77030
Tel: 713-798-3953
Fax: 713-798-3128
Email: tetsuoa@bcm.tmc.edu

Montserrat Baiget
Genetics Unit
Hospital de la Santa Creu i Sant Pau
AVDA, Pare Mª Claret, 167
Barcelona 08025
Spain
Tel: +34-93-2919194
Fax: +34-93-2919494
mbaiget@hsp.santpau.es

Ashook Balasubramanyam
Division of Endocrinology
Baylor College of Medicine
One Baylor Plaza, Room 537E
Houston,TX 77030
Tel: 713-798-8654
Fax: 713-798-4585
Email: ashokb@bcm.tmc.edu

Patrick Barbet
Service d'Histologie Embriologie Cytogénétique
Faculté de Médecine Cchin Port Royal
24 rue du Faubourg Saint Jacques
75014 Paris, France
Tel: +33 1 44 41 23 56
Fax: +33 1 40 51 09 46
Email: jp.barbet@tahiti.tethys-software.fr

Gillian Butler-Browne
CNRS Laboratoire Cytosquelette et
Développement
Faculté de Médecine Pitié Salpétrière
105 Boulevard de l'Hopital
75634 Paris cedex 13
Tel: +33 1 40 77 98 38
Fax: +33 1 53 60 08 02
Email: Butlerb@ext.jussieu.fr

Nessa Carey
Department of Vascular Surgery
Imperial College School of Medicine
Charing Cross Campus
St Dunstan's Road
London
W6 8RP
Tel: +44 181 846 7250
Fax: +44 181 846 7330
Email: n.carey@ic.ac.uk

Emy J. Chen
Center for Cancer Research
E-17-543
Massachussets Institute of Technology
Cambridge, MA 02139
USA
Tel: 617-253-3020
Fax: 617-253-5202
Email: emychen@mit.edu

Thomas A. Cooper
Department of Pathology
Baylor College of Medicien
One Baylor Plaza
Houston, TX 77030
Tel: 713-798-3141
Fax: 713-798-5838
Email: tcooper@bcm.tmc.edu

John Day
Dept of Neurology Box 295 Mayo Bldg
Univ of Minnesota Med School
420 Delaware St SE
Minneapolis, MN 55455-0323
Tel: 612-625-6180
Fax: 612-625-7950
Email: johnday@tc.umn.edu

Martine Devillers
AFM Département Scientifique
1 rue de l'Internationale
91000 Evry
Tel: +33 1 44 49 45 00
Fax : +33 1 47 83 32 06
Email: devillers@necker.fr

Edward Eddy
NIH
Lab of Reproductive & Dev Toxicology
111 T.W. Alexander Dr
Research Triangle Park, NC 27709-2233
Tel: 919-541-3015
Fax: 919-541-3800
Email: eddy@niehs.nih.gov

Maria Ericksson
Department of Neurology
Karolinska Hospital
S-171 76 Stockholm
Sweden
Tel: +46 851 77 37 02
Fax: +46 851 77 37 57
Email: Maria.Eriksson@cmm.ki.se

Bruno Eymard
Service de Neurologie 2
Hôpital de la Pitié Salpétrière
47/83 Bld de l'Hôpital
75651 PARIS Cedex 13
Tel:+33 1 42 16 22 41
Fax:+33 1 42 16 37 93

Denis Furling
Laboratory of Human Genetics
Dept Med
Laval Univ Med Ctr, CHUQ
2705 Boul Laurier
Ste-Foy Quebec
G1V 4G2 Canada
Tel: 418-654-2186
Fax: 418-654-2207
Email: denis.fuling@crchul.ulaval.ca

Daisuke Furutama
The First Dept of Internal Med
Osaka Medical College
2-7 Daigaku-cho, Takatsuki-city
Osaka 569-8686 Japan
Tel: +81-726-83-1221 X2672
Fax: +81-726-83-1801
Email: in1003@poh.osaka-med.ac.jp

Hirokazu Furuya
Department of Neurology, Omuta Rosai Hospital,
Yoshino, Omuta 837-0904, Japan
TEL.: +81-944-58-0051
Fax: +81-944-58-0317
E-mail: Furuyahiro@aol.com or
furuya@neuro.med.kyushu-u.ac.jp

Massimo Genarelli
Catt. di Genetica Umana
Universita Tor Vergata
Via Tor Vergata 135, ED nord
Roma 00133
Italia
Tel: +39 06 204;273 15
Fax: +39 06 725 960 80
Email: genarelli@utovrm.it

Iris Gonzalez
Clinical Science
A.I. Dupont Institute of the Nemours Found.
1600 Rockland Rd
Wilmington, Delaware 19899
Tel: 302-651-6775
Fax: 302-651-6881
Email: igonzale@aidi.nemours.org, iris@dol.net

Martin Gosling
Dept of Vascular Surgery
Imperial College School of Med
Charing Cross Hosp, Fulham Palace Rd
London W6 8RP UK
Tel: 0181-846-7317
Fax: 0181-846-7330
Email: m-gosling@ic.ac.uk

Fiona K. Gould
Division of Molecular Genetics, IBLS
University of Glasgow
56 Dumbarton Road
Glasgow G11 6NU
UK
Tel: +44 141 330 6220
Fax: +44 141 330 6871
Email: fgould@molgen.gla.ac.uk

Geneviève Gourdon
Inserm U383
Pavilon Maurice Lamy
Hopital Necker Enfants
149 rue de Sèvres
Paris 75015
France
Tel: +33 1 44 49 45 23
Fax: +33 1 47 83 32 06
Email: gourdon@necker.fr

Jack D. Griffith
LCCC
Uinversity of North Carolina
CB#7295 Mason Farm Rd.
Chapel Hill, NC 27599
USA
Tel: 919-966-2151
Fax:
Email: jdg@med.unc.edu

Marion Hamshere
Division of Genetics
University of Nottingham
Nottingham
NG7 2UH
Tel: +44 115 9194477
Fax: +44 115 9709906
Email: hamshere@nottingham.ac.uk

Sarah E. Harris
Division of Molecular Genetics, IBLS
University of Glasgow
Glasgow G11 6NU
UK
Tel: +44 141 330 6220
Fax: +44 141 330 6871
Email: sharris@molgen.gla.ac.uk

David Hilton-Jones
MDG Muscle and Nerve Centre
Dept of Clinical Neurology
Radcliffe Infirmary
Woodstock Rd
Oxford OX2 6HE
U.K.
Tel: 44-1865-224891
Fax: 44-1865-790493
Email: david.hilton-jones@clneuro.ox.ac.uk

David E. Housman
Center for Cancer Research, E17-541
Massachussets Institute of Technology
Cambridge, MA 02139
USA
Tel: 617-253-3013
Fax: 617-253-5202
Email: dhousman@po7.mit.edu

Heike Jaeger
Albert-Einstein Allee
Dept Applied Physiology
ULM Germany D 89081
Tel: 0731 502 3879
Fax: 49 731 502 3260
Email: heike.jaeger@medizin.uni-ulm.de

Åsa Johansson
Department of Internal Medicine
Umeå University Hospital
S-901 85 Umeå
Sweden
Tel: +46 90 785 12 61
Fax: +46 90 77 81 97
Email: Asa.Johansson@medicin.umu.se

Keith Johnson
Division of Molecular Genetics, IBLS
The University of Glasgow
56 Dumbarton Rd
Glasgow G11 6NU U.K.
Tel: 44-0141-330-5101
Fax: 44-0141-330-6871
Email: K.Johnson@bio.gla.ac.uk

Claudine Junien
Inserm U383
Pavilon Maurice Lamy
Hopital Necker Enfants
149 rue de Sèvres
Paris 75015
France
Tel: +33 1 44 49 45 23
Fax: +33 1 47 83 32 06
Email: junien@necker.fr

Mehrdad Khajavi
Department of Neurology
Baylor College of Medicine
Houston, TX 77030
Tel: 713-794-7820
Fax: 713-798-3128
Email: mkhajavi@bcm.tmc.edu

Masanobu Kinoshita
Fourth Dept of Internal Medicine
Saitama Medical School
1981 Tsujido
Kamoda, Kawagoe, Saitama 359
Japan
Tel:
Fax: +81 492 26 8451
Email:

Todd Klesert
Division of Molecular Medicine
Fred Hutchinson Cancer Research Ctr
1100 Fairview Ave N
Seattle, WA 98109
Tel: 206-667-4286
Fax: 206-667-6524
Email: klesert@u.washington.edu

Takayoshi Kobayashi
Department of Neurology
Tokyo Medical and Dental Univ
1-5-45 Yushima, Bunkyo-ku
Tokyo 113
Japan
Tel: +81-3-5803-5235
Fax: +81-3-5803-0169
Email: m-kobayashi.nuro@med.tmd.ac.jp

Zeljka Korade-Mirnics
Dept of Molecular Genetics & Biochem
Univ of Pittsburgh, School of Med
BST W1211, 3550 Terrace St
Pittsburgh, PA 15261
Tel: 412-648-9024
Fax: 412-624-1401
Email: zeljka@hoffman.mgen.pitt.edu

Ralf Krahe
Human Cancer Gen, Med Res
FAC
Ohio State Univ
420 W. 12th Ave
Columbus, OH 43210
Tel: 614-688-4766
Fax: 614-688-4761
Email: krahe-1@medctr.osu.edu, krahe.2@osu.edu

Ulrik Kvist
Andrology Unit, Reproductive Med Ctr
Woman & Child Health
Karolinska Hospital
Stockholm S-17176 Sweden
Tel: 46 155 288430
Fax: 46 155 288430
Email: ulrik.kvist@kbh.ki.se

Frank Lehmann-Horn
Department of Physiology
University of Ulm
Albert Einstein Allee 11
89069 Ulm
Germany
T : +49 731 502 3250 / +49 731 502 3261
F : +49 731 502 3260
Email : frank.lehmann-horn@medizin.uni-ulm.de

Anne-Sophie Lia-Baldini
Division of Cardiology
Department of Medicine
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
USA
Tel: 713-798-8380
Fax: 713-798-7437
Email: aslia@bcm.tmc.edu

Christina Liquori
Dept of Neurology
University of Minnesota
505 Essex St Se Rm 604 Diel
Minneapolis, MN 55455
Tel: 612 626-3521
Fax: 612 626-2639
Email: liqu0003@tc.umn.edu

Brenda Luciano
Center for Cancer Research
E-17-543
Massachussets Institute of Technology
Cambridge, MA 02139
USA
Tel: 617-253-3020
Fax: 617-253-5202
Email: bluciano@mit.edu

Mani Mahadevan
Medical Gen/Pathology/Lab Med
Univ Wisconsin Madison
445 Henry Mall
Madison, WI 53706
USA
Tel:608-265-8634
Fax: 608 262 2976
Email: msmahade@facstaff.wisc.edu

Pascal Maire
Inserm U129
CHU Cochin Port Royal
24 rue du Faubourg Saint Jacques
75014 Paris
Tel: +33 1 44 41 24 24
Fax: +33 1 44 41 24 21
Email: maire@cochin.inserm.fr

Loreto Martorell
Genetics Unit
Hospital de la Santa Creu i Sant Pau
AVDA. Pare Mª Claret, 167
Barcelona 08025
Spain
Tel: +34-93-2919194
Fax: +34-93-2919494
Email: genm@santpau.es

Jean Mathieu, M.D.
305 St-Vallier
Chicoutimi PQ G7H 5H6
Canada
Tel: 418-549-9572
Fax: 418-549-8631
Email: jmathieu@saglac.qc.ca

Susan Michalowski, Ph.D.
Lineberger Cancer Center
CB#7295
University of North Carolina
Chapel Hill, NC 27599
USA
Tel: 919-966-2151
Fax:
Email: smichalo@med.unc.edu

Geoffrey Miller
Neurology
Texas Children Hospital
MC3-3311
6621 Fannin Stret
Houston, TX 77030
USA
Tel: 713-770-3976
Fax: 713-796-2340
Email: gmiller@bcm.tmc.edu

Andrea M. Mitchell
Division of Molecular Genetics, IBLS
University of Glasgow
56 Dumbarton Road
Glasgow G11 6NU
UK
Tel: +44 141 330 6220/6229
Fax: +44-141-330-4878
E-mail: A.Mitchell@bio.gla.ac.uk

Darren G. Monckton
Division of Molecular Genetics, IBLS
The University of Glasgow
56 Dumbarton Road
Glasgow G11 6NU
UK
Tel: 44 141 330 6213
Fax: +44 141 330 6871
Email: dmonck@molgen.gla.ac.uk

Hutton Moore
UNC Chapel Hill
404 Fordham Hall
Chapel Hill, NC 27599-3280
Tel: 919-962-1445
Fax: 919-962-8472
Email: hutton@med.unc.edu

Randall Moorman
University of Virginia
Health Sciences Center
Dept of Internal Medicine
Cardiovascular Division
P.O. Box 6012, MR4
Charlottesville, VA 22908
USA
Tel: 804-982-3367
Fax: 804-982-3162
Email: rmoorman@virginia.edu

G E Morris
MRIC, Postal Point 19,
North East Wales Institute,
Plas Coch, Mold Road,
WREXHAM, LL11 2AW, U.K.
Tel: +44-1978-293330 (direct) or 293082 (secretary)
Fax: +44-1978-290008
Email: morrisge@newi..ac.uk

J Paul Mounsey
Medicine (Cardiology)
University of Virginia Health Science Center #158
Charlottesville, VA 22908
USA
Tel: 804-982-3367
Fax: 804-982-3162
Email: JM6a@virginia.edu

Nakaaki Ohsawa
Professor and Chairman
First Dept of Internal Medicine
Osaka Medical College
2-7 Daigaku-cho
Takatsuki-shi, Osaka 569
Japan
Tel: + 81 726 83 1221
Fax: + 81 726 85 1655
Email: in1001@poh.osaka-med.ac.jp

Christopher E. Pearson
The Hospital for Sick Children
Research Institute
555 University Avenue
Toronto, Ontario M5G 1X8
Canada
Tel: 416-813-8256
Fax: 416-813-4931
Email: cepearson@genet.sickkids.on.ca

Ana Y. Perez
343 W. Houston #702
San Antonio, TX 78205
USA
Tel: 210-225-3744
Fax: 210-222-8497
Email: ap1529809@pol.net

Ben Perryman
Dept of Cardiology
University of Colorado Health Science Ctr
4200 East 9th Ave, Box B139
Denver, CO 80262
USA
Fax: 303-315-3261
Tel: 303-315-3259
Email: benjamin.perryman@uchsc.edu

Anne V. Philips
Department of Pathology
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
USA
Tel: 713-798-5021
Fax: 713-798-5838
Email: aphilips@bcm.tmc.edu

David Pribnow
Department of Cellular and Molecular Biology
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road
Portland, OR 97201
Tel: 503-494-4358
Fax: 503-494-4353
Email: pribnowd@ohsu.edu

Laura P.W. Ranum
Box 295 FUMC
Department of Neurology
University of Minnesota
420 Delaware St. S.E.
Minneapolis, MN 55455
USA
Tel: 612-624-0901
Fax: 612-626-2639
Email: laura@gene.med.umn.edu

Allen D. Roses
Vice-President and World-Wide Director, Genetics
Glaxo Wellcome Research and Development
Five Moore Drive 5-5616
Research Triangle Park, NC 27709
USA
Tel: 919-483-3771
Fax: 919-315-6013
Email: adr69412@glaxowellcome.com

Sergio Salvatori
Universita degli Studi di Padova
Dipartimento di Scienze Biomediche Sperimentali
Viale C Colombo 3
35121 Padova
Italia
Tel:39 49 8276041
Fax:39 49 8276040
Email: patgen08@civ.bio.unipd.it

Shigeru Sato
Dept of Biology
Jichi Medical School
Minamikawachi, Tochigi
320-0498
Japan
Tel: 81-285-58-7312
Fax: 81-285-44-5476
Email: ssato@jichi.ac.jp

Hervé Seznec
Inserm U383 Pavilon Maurice Lamy
Hopital Necker Enfants
149 rue de Sèvres
Paris 75015
France
Tel: +33 1 44 49 45 23
Fax: +33 1 47 83 32 06
Email: seznec@necker.fr

Miho Shimizu
Section of Morphological Dev
Nat'l Institute of Basic Biology
Myodaiji-machi, Okazaki-shi 444-8585
Japan
Tel: + 81 564 55 7572
Fax: +81 564 71 5871
Email: mshimizu@nibb.ac.jp

Michael Siciliano
Dept Molecular Genetics
MD Anderson Hosp & Tumor Inst
TX Medical Ctr
Houston,TX 77030
USA
Tel: 713-792-2910
Fax: 713-794-4394
Email: mjs@mdacc.tmc.edu

Richard R. Sinden
Center for Genome Research
Instute of Biosciences & Technology
Texas A& M Univ
2121 W. Holcombe Blvd
Houston,TX 77030-3303
USA
Tel: 713-677-7664
Fax: 713-677-7689
Email: Rsinden@ibt.tamu.edu

Robert H. Singer
Department of Anatomy & Structural Biology
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461
USA
Tel: 718-430-8646/8695
Fax: 718-430-8697
Email: rhsinger@aecom.yu.edu

Nia Spring
Dept Molecular Genetics
U.T. @ Houston MDACC
1515 Holcombe St Box 045
Houston,TX 77030
Tel: 713-792-8926
Fax: 713-794-4394
Email: nia_spring@molgen.mdacc.tmc.edu

Maurice S. Swanson
Dept of Molecular Genetics & Microbiology
and Gene Therapy Center
University of Florida College of Medicine
Gainesville, FL 32610-0266
USA
Tel: 352-392-3082
Fax:352-392-5914
Email: swanson@college.med.ufl.edu

Stephen Tapscott
Fred Hutchinson Cancer Ctr
C3 168, 1124 Columbia St
Seattle, WA 98104
USA
Tel: 206-667-4499
Fax: 206-667-6524
Email:stapscot@fhcrc.org

Charles A. Thornton
Department of Neurology
University of Rochester Medical Center
601 Elmwood Avenue, Box 673
Rochester, NY 14642-8673
USA
Tel: 716-275-2542 or 2559 716-275-1200
Fax: 716-256-1423
Email: cthorn@mail.neurology.rochester.edu

Lubov T. Timchenko
Division of Cardiology
Department of Medicine
Baylor College of Medicine
One Baylor Plaza
Houston,TX 77030
USA
Tel: 713-798-6911
Fax: 713-798-3142
Email: lubovt@bcm.tmc.edu

Michael Wagner
Department Head, Human Genetics
Glaxo Wellcome, Inc.
PO Box 13398
Research Triangle Park, NC 27709
Tel: (919) 483-5332
Fax: (919) 483-0315
Email: mw29911@glaxowellcome.com

James Waring
Solange Gauthier Karsh Lab
Children's Hosp of Eastern Ontario
401 Smyth Rd
Ottawa Ontario
K1H 8L1 Canada
Tel: 613-738-3927
Fax: 613-738-4833
Email: jamie@mgcheo.med.uottawa.ca

Robert D. Wells
Center for Genome Research
Albert B. Alkek Instute of Biosciences & Technology
Texas A & M Univ
2121 W. Holcombe Blvd
Houston, TX 77030-3303
Tel: 713-677-7651
Fax: 713-677-7689
Email: rwells@ibt.tamu.edu

Jolanda Westerlaken
Department of Cell Biology
Medical Faculty University Nijmegen
Adelbertusplein 1
Nijmegen 6525EK
The Netherlands
Tel: +31-24-3614329
Fax: +31-24-3540525
e.mail: J.Westerlaken@celbi.kun.nl

Berend Wieringa
Department of Cell Biology
Medical Faculty University Nijmegen
POBox 9101 6500HB Nijmegen
Street Address: Adelbertusplein 1
Nijmegen 6525EK
The Netherlands
Tel: +31-24-3614329/3614287
Fax: +31-24-3540525
e.mail: b.wieringa@celbi.kun.nl

Catherine L. Winchester
Division of Molecular Genetics, IBLS
University of Glasgow
56 Dumbarton Road
Glasgow G11 6NU
UK
Tel: +44 141 330 6220 (office)
+44 141 330 6229 (lab)
Fax: +44 141 330 6871
Email: cwinches@udcf.gla.ac.uk

Hidehisa Yamagata
Dept of Geriatric Medicine
Ehime University School of Medicine
Shigenobu-cho, Onsengun,
Ehime 791-0295 Japan
Tel: +81 89 960 5851
Fax: +81 89 960 5852
Email: hideyama@m.ehime-u.ac.jp