FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD]
2012 International Research Consortium & Research Planning Meeting

Sponsored by:

Boston Biomedical Research Institute
Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center for FSHD Research

FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD]
2012 International Research Consortium & Research Planning Meeting

Tuesday, November 6, 2012
8:00 a.m. – 4:00 p.m.
[Registration and breakfast begins 7:20 a.m.-]

San Francisco Marriott Marquis
Club Room
55 Fourth Street
San Francisco, California 94103 USA

Co-Chairs:	Rabi Tawil, M.D. University of Rochester School of Medicine, Rochester, New York
	Silvère van der Maarel, Ph.D. Leiden University Medical Center, Leiden, the Netherlands
Organizers:	Daniel Paul Perez FSH Society, Inc. Rabi Tawil, M.D. Silvère van der Maarel, Ph.D.

Sponsored By:

Association Française Contre les Myopathies (AFM)
FSH Society
FSHD Global Research Foundation
Muscular Dystrophy Association United States (MDAUSA)
NIH Eunice Kennedy Shriver NICHD Boston Biomedical Research Institute
Senator Paul D. Wellstone MDCRC
Sanofi-Genzyme

PREFACE

November 6, 2012
San Francisco, California

Dear Colleagues,

Welcome to the facioscapulohumeral muscular dystrophy FSHD International Research Consortium 2012. Thank you for coming. Thank you for participating. Thank you for sharing and collaborating. The goal of this meeting is to create synergy to facilitate more progress on FSHD. It is essential for the entire community to work together at every level to communicate clearly on programs, developments and needs. We hope this meeting helps to this end.

In the interest of time, please emphasize new data and ideas. As you present, please try to address which of the four major priority areas identified last year (see below) your work has impact on. Please keep platform presentations to 20 minutes including 5-10 minutes for questions and answers. Poster presenters will have two to three minutes to highlight their work by PowerPoint presentation. Platform speakers please consider bringing a poster as well.

Initially we will revisit last year's four areas that we previously identified as a group as priorities for FSHD. Across the day we will discuss what we have achieved, where we need to focus, and what are the lacunae? By the end of the day we should be able to identify what and if any of these should change or be modified, and if new areas should be considered.

This meeting is organized by the FSH Society and sponsored by Association Française Contre les Myopathies (AFM), the FSH Society, the FSHD Global Research Foundation, the Muscular Dystrophy Association (MDAUSA), the U.S. DHHS NIH Eunice Kennedy Shriver NICHD Sen. Paul D. Wellstone BBRI FSHD Muscular Dystrophy Cooperative Research Center, and Sanofi-Genzyme. It is truly a pleasure to come together to accelerate solutions for FSHD. Thank you for your extraordinary efforts and hard work on behalf of patients and their families!

Sincerely,

Dr. Rabi Tawil, M.D.
University of Rochester School of Medicine, Rochester, New York

Silvère van der Maarel, Ph.D.
Leiden University Medical Center, Leiden, the Netherlands

Daniel Paul Perez
FSH Society, Inc., Watertown, Massachusetts

Tuesday, November 6, 2012

Registration & Breakfast	7:30a.m.-8:00	
Welcome	8:00-8:05	
Review of 2011	8:05-8:30	**Review of 2011 Priorities** as Stated by FSHD Research Moderator: **Louis M. Kunkel, Ph.D.**
Platform Session 1	8:30-9:30	**Clinical, Physiological and Diagnostic Studies** (3x20 minutes)
Flex Time for Discussion	9:30-9:45	
Platform Session 2	9:45-10:45	**Disease Genes and Genetic Studies** (3x20 minutes)
Flex Time for Discussion	10:45-11:00	
Poster Introductions	11:00-11:30	**Poster Introductions** (3 minutes each)
Free assembly session	11:30-12:00	[collect and have **Lunch**]
Platform Session 3	12:00-1:00	**Molecular Studies** (3x20 minutes)
Flex Time for Discussion	1:00-1:15	
Platform Session 4	1:15-2:15	**Therapy** (3x20 minutes)
Flex Time for Discussion	2:15-2:30	
Free Assembly Session	2:30-3:00	
Moderated Discussion	3:00-4:00	**Define 2012/2013 research priorities**, future directions, etc. Moderators: **Alexandra Belayew, Ph.D., Yi-Wen Chen, Ph.D., Rabi Tawil, M.D., and Silvere van der Maarel, Ph.D.**
Adjourn	4:00p.m.	

The FSH Society, Inc. (Facioscapulohumeral Muscular Dystrophy) is an independent, non-profit 501(c)(3) and tax-exempt U.S. corporation organized to address issues and needs specifically related to facioscapulohumeral muscular dystrophy (FSHD). Contributions and financial donations are acknowledged for tax purposes. All inquiries should be addressed to: FSH Society, Inc., Daniel Paul Perez, phones: (617) 658-7811 and (781) 275-7781, fax: (781) 275-7789, e-mail: daniel.perez@fshsociety.org, website: http://www.fshsociety.org

NOTES ON TALKS AND POSTERS

	First Author	Presenting Author	Topic	Session
Platform Session 1				
Chair: **Rabi Tawil, M.D.**				
8:30-8:50 a.m.	Janssen	Padberg	MRI	Clinical and DX Studies
8:50-9:10 a.m.	Lassche	Lassche	Sarcomere	Clinical and DX Studies
9:10-9:30 a.m.	Lunt	Lunt	Clinical Proforma	Clinical and DX Studies
Platform Session 2				
Chair: **Silvere van der Maarel, Ph.D.**				
9:45-10:05 a.m.	Lemmers	Lemmers	FSHD2/FSHD1B	Disease Genes
10:05-10:25 a.m.	Ricci	Tupler	Prognosis	Disease Genes
10:25-10:45 a.m.	Sacconi	Sacconi	FSHD2/FSHD1B	Disease Genes
Poster Introductions				
Chair: **Daniel G. Miller, M.D., Ph.D.**				
11:00-11:03 a.m.	Boyar	Strom/Boyar	Diagnostics	Poster
11:03-11:06 a.m.	Domire	Harper/Wallace	DUX4	Poster
11:06-11:09 a.m.	Gundesli	Gundesli	Inheritance	Poster
11:09-11:12 a.m.	Janssen	Padberg	MRI	Poster
11:12-11:15 a.m.	De Morree	De Morree	mRNA Processing	Poster
11:15-11:18 a.m.	Pandey	Pandey/YW Chen	Autophagy	Poster
11:18-11:21 a.m.	Rahimov	Kunkel/Rahimov	Biomarkers	Poster
11:21-11:24 a.m.	Scionti	Tupler/Scionti	Population	Poster
11:24-11:27 a.m.	Tassin	Belayew/Tassin	DUX4/DUX4c	Poster
11:27-11:30 a.m.	Whittington	Lunt	Diagnostics	Poster
Platform Session 3				
Chairs: **Louis Kunkel, Ph.D.**				
12:00-12:20 p.m.	Harafuji	YW Chen	miR411	Molecular Studies
12:20-12:40 p.m.	Roche	Roche	Fetal FSHD	Molecular Studies
12:40-1:00 p.m.	Wallace	Wallace	DUX4 Promoters	Molecular Studies
Platform Session 4				
Chairs: **Scott Q. Harper, Ph.D.**				
1:15-1:35 p.m.	Block	Block	DUX4	Therapy
1:35-1:55 p.m.	Bosnakovski	Bosnakovski	DUX4	Therapy
1:55-2:15 p.m.	Ansseau	Belayew/Ansseau	AONs	Therapy
Posters **[**	Boyar	Strom/Boyar		Poster
	Domire	Harper/Wallace		Poster
	Gundesli	Gundesli		Poster
	Janssen	Padberg/Lassche		Poster
	Kazakov	Kazakov		Poster
	De Morree	De Morree		Poster
	Pandey	Pandey/YW Chen		Poster
	Rahimov	Kunkel/Rahimov		Poster
	Scionti	Tupler/		Poster
	Tassin	Belayew/Tassin		Poster
	Whittington	Lunt		Poster]

Abstract numbers that are followed by a "[P]" in abstracts that follow denote poster presentation

THE TOP FOUR AREAS IDENTIFIED AS A PRIORITY FOR FSHD BY THE WORLD WIDE SCIENTIFIC COMMUNITY IN NOVEMBER 2011

The international FSHD clinical and research community recently came together at the DHHS NIH NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone MD CRC for FSHD. Almost 95 scientists working on FSHD globally met at the 2011 FSH Society FSHD International Research Consortium, held November 7-8, 2011.

The summary and recommendations of the group state that given the recent developments in our definition of FSHD and the potential that within one to two (1-2) years, evidence-based intervention strategies, therapeutics, and trials being planned and conducted. Our immediate priorities should be to confirm the DUX4 hypothesis, if valid then understand normal DUX4 function, and finally, understanding the naturally occurring variability should allow us to manipulate the disease in our favor. We need to be prepared for this new era in the science of FSHD, by accelerating efforts in the following four areas:

1. Genetics / epigenetics

It is now broadly accepted that the disregulation of the expression of D4Z4 / DUX4 plays a major role in FSHD1 and FSHD2. Additional FSHD (modifier) loci are likely to exist.

FSHD molecular networks. The relaxation of the chromatin structure on permissive chromosome 4 haplotypes leads to activation of downstream molecular networks. Importantly, the upstream processes – triggering of activation – are equally important.
Detailed studies on these processes are crucial for insight in the molecular mechanisms of FSHD pathogenesis and may contribute to explaining the large intra- and interfamily clinical variability. Importantly such work may lead to intervention (possibly also prevention) targets.

Additional FSHD genes. FSHD2 is characterized by hypomethylation of D4Z4 on chromosome 4 as well as chromosome 10. This also leads to bursts of DUX4 expression. Identification of the responsible factor (gene) and molecular mechanisms is of utmost importance. This work will be facilitated by the recruitment of additional families. Also other genes need to be considered that may give rise to FSHD-like phenotypes. These include, but are not limited to, CAPN3 and the FAT1 gene that was recently suggested to be involved in FSHD.

2. Clinical trial readiness

It is now broadly accepted that disregulation of the expression of D4Z4 / DUX4 is at the heart of FSHD1 and FSHD2. This finding opens perspectives for intervention along different avenues.

Clinical Trial Readiness. Intervention trials are envisaged within the next several years. The FSHD field needs to be prepared for this crucial step. To design and coordinate this important translational process, it was envisaged to install an international task force Clinical Trial Readiness (FSHD-CTR), with Dr Rabi Tawil as leader. The FSHD-CTR needs to be a

multidisciplinary group, including members with expertise, not only in FSHD but also, in trial design and execution, statistics, (non-invasive) biomarkers etc. Important issues are:

Natural history
Homogeneous clinical criteria
Biobanks, biomarkers, etc.
Reliable outcome measures
Patient registries

Biomarkers. Sensitive biomarkers are needed to monitor intervention: they might also improve diagnosis. Important to consider biomarkers established from easily accessible sources like blood. Non-invasive methods like imaging needs further attention. Quantitative muscle function methods are instrumental as are patient-reported indicators.

3. Model systems

There are a plethora of cellular and models, based on different pathogenic (candidate gene) hypotheses. Moreover, the phenotypes are very diverse and often difficult to compare with the human FSHD phenotype.

FSHD Model Data Base. The importance of a systematic database was recognized. This data base should contain detailed information on the molecular characteristics of the model (design and phenotype). Particular emphasis should be paid to the muscle pathology. Non-muscle phenotypes – described also in FSHD patients deserves attention.

Human pathology and bio-banking. Importantly, this data base should also contain well documented muscle pathology data of patients – astonishingly difficult to find in the literature.

Human cellular resources continuously deserve attention. Recent progress in ES-cell technology, including iPS lines, allows for inter-group distribution and dedicated molecular (epi)genetic studies.

4. Sharing

Timely sharing of information and resources remains a critical contributor to the progress in the field. There are several initiatives that create large repositories of data and resources, e.g. NIH Wellstone MD CRC FSHD and R.T. Fields Center. Their websites should be used for sharing of information (e.g. protocols, guide to FSHD muscle pathology (images), model systems, contact information), reagents, and resources.

1.

Evaluation of new antisense oligomers targeting the *DUX4* mRNA as a therapeutic strategy for FSHD

Ansseau E.*[1], Vanderplanck C.*[1], Wallace LM.[2], Tassin A.[1], Domire JS.[2], Guckes SM.[2], Yip C.[1], Laoudj-Chenivesse D.[3], Coppée F.[1], Wilton S.[4], Harper SQ.[2] and Belayew A.[1]

*Contributed equally to this study
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[4]Molecular Genetic Therapy Group, University of Western Australia, Nedlands, 6009 Western Australia, Australia.

There is now compelling evidence that expression of DUX4 is implicated in the pathogenesis of FSHD. The expertise of Prof. Wilton's group in antisense oligomer (AO) design and induced exon skipping in Duchenne muscular dystrophy (DMD) has led to a collaboration with our group to knockdown DUX4 expression in an *in vitro* model of FSHD [1]. Several AOs designed to target exons 2 and 3 of *pLAM* for exclusion from the *DUX4* mRNA, synthesized as 2'-O-methyl on a phosphorothioate backbone, were able to suppress DUX4 protein expression in primary human myoblast cultures. The effect was specific, although some of the AOs were more efficient than others, in that they suppressed DUX4 expression at lower transfection concentrations and, more importantly the AOs did not alter expression of the homologous DUX4c protein. While all the AOs suppressed DUX4 expression, the ones targeted to exon 3 appeared to be more effective.

Empirical AO optimization can influence dystrophin exon skipping efficiency significantly [2] and select AO combinations can greatly increase specificity of exon skipping in DMD [3,4]. In a similar strategy we have tested AO cocktails and also 2 additional AOs targeting other features of the *DUX4* mRNA that could affect its splicing or polyadenylation. As expected, the AO cocktails and new AOs suppressed endogenous DUX4 mRNA and protein expression as well as FSHD markers in FSHD primary myotubes. Moreover, the DUX4 inhibition prevented development of the atrophic myotube phenotype.
The 3 most effective AO sequences were prepared as "vivo-morpholino" to test them in a mouse model. In collaboration with Prof. Scott Harper's group, we performed a preliminary experiment to test those AOs in an alternative FSHD model in which recombinant AAV (adeno-associated virus) vectors expressing DUX4 were injected in the *tibialis anterior* muscle of mice [5]. After co-injection with one of these AOs we observed a decrease in the amount of *DUX4* mRNA.

These experiments will be repeated to confirm a putative efficiency of these AOs *in vivo*.

References:

[1]Vanderplanck C, Ansseau E, Charron S, Stricwant N, Tassin A, et al. PLoS One. 2011;6(10):e26820.
[2]Harding PL, Fall AM, Honeyman K, Fletcher S, Wilton SD. Mol Ther 2006; 15: 157-66.
[3]Mitrpant C, Fletcher S, Iversen PL, Wilton SD. J Gene Med 2009; 11: 46-56.
[4]Adams AM, Harding PL, Iversen PL, Coleman C, Fletcher S, Wilton SD. BMC Mol Biol 2007; 8: 57.
[5]Wallace LM, Garwick SE, Mei W, Belayew A, Coppee F, Ladner KJ, et al. Ann Neurol 2011; 69: 540-52.

2.

Myotubes generated from FSHD-muscle biopsies undergo DUX4-dependent apoptosis and enable the identification of chemicals and pathways that reduce DUX4 expression

Gregory J. Block[1,4], Divya Narayanan[1,4], Amanda M. Amell[1,4], Lisa M. Petek[1,4], Kathryn Davidson[2,4], Rabi Tawil[5], Randall Moon[2-4], and Daniel G. Miller[1,4]

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[2]Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington
[3]Howard Hughes Medical Institute, University of Washington School of Medicine, Seattle, Washington
[4]Institute for Stem Cell and Regenerative Medicine, University of Washington School of Medicine, Seattle, Washington
[5]University of Rochester Medical Center, Rochester New York

Cultured myoblasts from FSHD-muscle biopsies express DUX4 in 0.01% of nuclei and grow and differentiate normally, thereby making the identification of pathways that regulate DUX4 and disease progression challenging. Using a novel method of myoblast differentiation, we demonstrate that primary FSHD-myotubes undergo apoptosis initiated by myotube-specific activation of DUX4. Chemical inhibitors of apoptosis prevent myotube death; however, we have also identified key cellular pathways that reduce DUX4 expression and concomitant myotube death. Finally, we demonstrate that genetic pathways thought to alter chromatin structure of D4Z4 also modify DUX4 expression levels and myotube viability, suggesting that these genes may modify disease severity and penetrance in FSHD patients. Therefore, cultured myotubes from FSHD patients are an excellent preclinical platform to identify compounds, signaling pathways, and chromatin modifiers involved in FSHD.

3.

Small molecules with antioxidative properties are prevalent DUX4 inhibitors within HTS library

Darko Bosnakovski[1,2], Si Ho Choi[2], Erik Toso[2] , Michael A. Walters[3], Jessica M. Strasser[3] and Michael Kyba[2]

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[3]Institute for Therapeutics Discovery and Development, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota, 55014

DUX4 has emerged as the key molecular target in Facioscapulohumeral Muscular Dystrophy (FSHD). We have shown that DUX4 induces cell toxicity in skeletal muscle at high levels of expression while at low levels it interferes with differentiation and myogenic gene expression related to the FSHD molecular phenotype. A first step towards developing a targeted therapy for FSHD is to discover chemical compounds that inhibit the activity of the DUX4 protein. We have taken advantage of the conditional toxicity of DUX4-inducible myoblasts to develop a small molecule screening platform for identifying inhibitors of DUX4. The assay was based on rapid toxicity of high level DUX4 expression which leads to myoblast death within 24 hours. High throughput screening (HTS) of more than 200.000 small molecules as a part of UT Southwestern HTS compound library was done identified approximately 700 compounds with significant rescue effect. To identify direct inhibitors, we have conducted serial follow up assays, including secondary screens for interference with the conditional gene expression system, reversion of toxicity in other DUX4-expressing cell types (fibroblast, human ES cells) and protection against other cell death-inducing signals.

We are currently working with a subset of 100 purchased compounds. These were selected based on pharmacological desirability analyses and the presence of multiple related compounds that form a chemical series within the hit set. While the majority of hits are singlets, our analysis identified 46 distinct chemical series. Rescreening these purchased compounds demonstrated Almost all of the purchased compounds demonstrated some degree of protection from DUX4-induced cell death indicating the success of the primary screen. Follow up screens indicated that the majority of these hits were also acting independently of the dox-inducible system. Interestingly, further analyses revealed that a very large subset of the selected compounds confer protection against an oxidative insult (tBHP). The data to date highlight the potential usefulness of antioxidant therapy in FSHD.

4. [P]

DNA combing assay for detection of contraction-dependent facioscapulohumeral muscular dystrophy (FSHD1)

F.Z. Boyar[1], P. Chan[1], V. Sulcova[1], D. Tsao1, R. Owen[1], P. Walrafen[2], C.D. Braastad[3], M. Jocson[3], W. Sun[1], A. Anguiano[1], C.M. Strom[1]

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[3] Athena Diagnostics Inc., Worcester, Massachusetts

Abstract: FSHD, the third most common muscular dystrophy, is an autosomal dominant disorder with a prevalence of 4-10 per 100,000. It is characterized by often asymmetric progressive weakness of facial, scapular and upper arm muscles. FSHD1 is associated with contraction of the D4Z4 region (3.3 kb repeat motif that contains DUX4, double homeobox 4, gene) at the subtelomeric region of 4q35 and activation of the normally epigenetically silenced DUX4. Affected alleles have 1- 10 copies of D4Z4 on the 4qA allele, while unaffected alleles have 11-100 copies. Contractions of the partner 4qB allele or a homologous region on chromosome 10q26 are non–pathogenic. Previously, detection of truncated D4Z4 has been based on Southern blot (SB) analysis. SB analysis can only provide a size approximation, yields ambiguous results in at least 20% of cases and, as we show in this study, can miss affected mosaic individuals.

DNA combing provides an attractive alternative method for the diagnosis of contracted alleles in FSHD. In this technology, individual DNA fibers are visualized and measured, allowing differentiation between A and B alleles and 4q and 10q alleles, accurate sizing of all alleles and identification of mosaic affected individuals. We validated a laboratory developed test based on DNA Combing for the diagnosis of FSHD. The validation series consisted of combed DNA from 2 cell lines and from leukocyte plugs from 35 individuals submitted to Athena Diagnostics for SB. The samples were blinded to laboratory personnel performing the analysis. There was 100% concordance in the 7 samples determined to affected by SB analysis and the 2 cell lines. One of the 28 samples determined to be unaffected by SB analysis was demonstrated to be a mosaic individual with 2 normal sized alleles and a contracted affected allele. Our findings underscore the utility of a DNA combing assay for more accurate measurement of the D4Z1 locus on the pathogenic 4qA allele and diagnosis of FSHD1. This represents the first successful validation of a molecular combing assay in a United States commercial laboratory.

5. [P]

Regulates Expression of the Pro-Apoptotic Gene, p63

Jacqueline Domire, Lindsay Wallace, Susan Guckes, Jian Liu, Scott Q. Harper

Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, 43205 USA

Facioscapulohumeral muscular dystrophy (FSHD) is a dominant disorder that most commonly affects specific muscles of the face, shoulder girdle, and limbs. There is no effective treatment for FSHD, and compared to other muscular dystrophies, translational research has been stagnant. Understanding the molecular mechanisms underlying a disease is necessary for developing targeted therapeutic strategies. For FSHD, translational research was impeded because the pathogenic events required for diseased development were elusive and/or controversial. The landscape has changed recently with the emergence of DUX4 gene expression as an underlying pathogenic event in FSHD. We can now begin developing rational therapies that directly target DUX4 as well as downstream pathways it controls, since the DUX4 transcription factor likely initiates a cascade of gene expression changes that are incompatible with normal muscle homeostasis.

The objective of this study was to characterize prospective DUX4-controlled pathways. We hypothesized that this approach would help further define the pathogenic events involved in FSHD and identify new therapeutic targets. Toward this end, we used microarray data from DUX4-overexpressing muscle to identify potential transcriptionally regulated targets of DUX4. We found that several genes involved in apoptosis pathways were up-regulated, including p53-pathway members. The latter finding was consistent with our previous finding that DUX4-associated myopathy was p53-dependent in mice. Since DUX4 is a transcription factor, we therefore hypothesized that it could directly activate p53-pathway genes. In this study, we focused on one such gene, p63, because it was increased in DUX4-expressing muscles and cells, known to transactivate p53 target genes, and sensitize cells to apoptosis. We found that DUX4 binds to the p63 promoter to regulate its expression, and p63 inhibition mitigates the pro-apoptotic activity of over-expressed DUX4. We showed preliminary data supporting the DUX4-p63 relationship at last year's FSH Society meeting, and will discuss additional data accumulated during the last year on this project, including p63 co-localization with DUX4 in vivo. Together, these results suggest that p63 may be a downstream target of DUX4, and may play an important role in DUX4-mediated cell death. As such, p63 could be a therapeutic target for FSHD, and we are currently investigating p63 expression, and that of other p53 pathway members, in biological material from human FSHD patients.

6. [P]

Breaking the rule of autosomal dominant inheritance in Facioscapulohumeral muscular dystrophy

Hulya Gundesli[1], Ana Nikolic[2], Rachele Piras[3] , Stefania Murru[3], Isabella Scionti[2], Maurizio Moggio[4], Giuliano Tomelleri[5], Maria Antonietta Maioli[3], Rossella Tupler[1,2]

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Facioscapulohumeral muscular dystrophy (FSHD) is the third most common, progressive and genetically heterogeneous myopathy with estimated prevelance of 1 in 20,000 people. FSHD has been causally related to reduction of tandemly arrayed 3.3 kb repeat units (D4Z4) on chromosome 4q35. The number of D4Z4 repeats varies from 11 to 100 in the general population, whereas less than 11 repeats are present in sporadic and familial FSHD patients.

FSHD is considered an autosomal dominat disorder. However, several cases, which escape this model, have been identified. Determining the mode of inheritance in FSHD families is important to understand how to analyze these patients and could play a critical role in identifying genetic defect/s which has not been discovered yet. For this reason in this study, we have analyzed 101 families both clinically and genetically. Among these, we have identified nine FSHD families suggestive of autosomal recessive inheritance. In one of these families, Family 1, there are two affected sisters present clinical features of severe FSHD. Neither parents nor other distant relatives display myopathic signs. Molecular analysis of the FSHD locus at 4q35 failed to detect alleles with a reduced number of D4Z4 repeats. Haplotype analysis of the family through three generations including parents and children by using genetic markers close to 4q35 region demonstrated that both affected sisters and unaffected brother share the same haplotype. No hypomethylation at the D4Z4 locus was found in two sisters in comparison with other family members. Interestingly, this family is from a small village in inner Sardinia and three sibs died at very young age of beta-thalassemia. Both parents are heterozygotes for the ß‸039 mutation in the beta globin gene. Collectively, these observations suggest that in this family FSHD is most likely caused by an autosomal recessive defect not associated with the 4q-FSHD locus. The other family, Family 2, has two affecteds with several unaffected relatives. Parents of the affecteds have no FSHD which supports the mode of inheritance as autosomal recessive. Molecular genetic analysis has demonstrated that there is no correlation between D4Z4 repeats, 4A161PAS haplotype and disease. In addition, we have identified another autosomal recessive inherited FSHD family (Family 3) with two affecteds in which parents of the two affected-siblings are all healthy. There is no correlation between D4Z4 repeat size and disease phenotype.

At present, no FSHD genetic locus other than 4q35 has been identified. Extended clinical and molecular analysis of FSHD families demonstrate that there are families in which FSHD is inherited as autosomal recessive disease. Studying these families will be invaluable to find novel genetic defect/s and will provide critical information to understand the pathogenesis of FSHD.

7.

miRNA-411 negatively regulates YAF2 and myogenesis

Naoe Harafuji[1], Peter Schneiderat[2] and Maggie C Walter[3], Yi-Wen Chen[1,4]

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[4] Department of Integrative Systems Biology and Department of Pediatrics, George Washington University, Washington DC

Previous studies suggest that defects in myogenesis are involved in the disease mechanisms of facioscapulohumeral muscular dystrophy (FSHD). In this study, we hypothesized that misregulation of microRNAs (miRNAs) in FSHD is involved in the myogenic defects and disease progression. To identify misregulated miRNAs in FSHD myoblasts, miRNA expression profiling was performed using TaqMan microRNA arrays. Eight miRNAs were differentially expressed in FSHD myoblasts during proliferating. The differential expression of miR-411 was validated by individual quantitative RT-PCR assay and additional arrays. In situ hybridization showed cytoplasmic localization of miR-411 in the myoblasts. The mRNA expression changes of the myoblasts were determined by mRNA expression profiling, and 4 potential miRNA-411 targets including YY1 associated factor 2 (YAF2) were identified. Over-expression of miR-411 in murine myoblasts, C2C12 cells, showed a reduction of YAF2 mRNA expression and myogenic factors myoD and myogenin. The findings suggest that the higher expression of miR-411 in FSHD primary myoblasts might be involved in the defects of myogenesis reported in FSHD.

8.

MR as biomarker for disease evaluation in facioscapulohumeral dystrophy

Barbara H. Janssen[1], Rob J.W. Arts[2], Nicoline B.M. Voet[3], Christine I.H.C. Nabuurs[1], Alexander Geurts[3], George W. Padberg[2], Baziel G.M. van Engelen[2] and Arend Heerschap[1]

[1] Department Radiology, Radboud University Nijmegen Medical Centre, the Netherlands
[2] Department Neurology, Radboud University Nijmegen Medical Centre, the Netherlands
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Introduction: Facioscapulohumeral dystrophy (FSHD) is the third most common muscular dystrophy. The disease is typically characterized asymmetry of skeletal muscle involvement. FSHD is associated with contraction of D4Z4 microsatellite repeats on chromosome 4q35, leading to a stabilized distal DUX4 transcript.

The aim of this study was to exploit whether MR imaging and spectroscopy can serve as a non invasive biomarker to asses disease severity, enable therapy evaluation and study disease pathobiology. Methods: 30 genetically proven FSHD patients were examined on a Sienens Trio 3T system. The measurements were performed on the upper leg to study the thigh muscles. Fat infiltration was quantitatively determined with the method earlier described by Kan (2009) . The ratio of the high energy compounds (PCr, ATP and Pi) and the pH of the muscles were studied with 31-phosphourus MRS. The strength of the quadriceps and hamstrings muscles was assessed by a myometry setup. Results and discussion: 353 thigh muscles were investigated, of which 232 twice. Analysis of the fat infiltration fraction showed that muscles of FSHD patients are nearly binary dived over a high and a low fat fraction. The muscle with an intermediate fat infiltration (around 50%) showed the largest increase in fat fraction over time. This combined suggests that once disease onset is triggered in a muscle this inevitably rapidly leads to muscle dysfunction. Fat fraction of the quadriceps and hamstring muscles correlate with the muscle strength. PCr/ATP showed a significant decrease with increasing fat fraction, possibly in an attempt to compensate for overload in development towards a FSHD condition, for instance by adapting to a more oxidative phonotype.

9. [P]

Natural progression of fatty infiltration and beneficial effects of non-pharmaceutical interventions in FSHD detected by quantitative MRI

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Introduction: Facioscapulohumeral dystrophy (FSHD) is the third most common muscular-dystrophy and is typically characterized by asymmetry of skeletal muscle involvement.

The aim of this study was to employ the muscular fat fraction as a biomarker to follow the natural progression of the disease and to assess the effects of two non-pharmaceutical interventions: cognitive behavioral therapy (CBT) and exercise training (AET).

Materials and methods: Twenty-four genetically proven FSHD patients were included. Patients were randomly placed in the control group (n=8), CBT group (n=8) or AET group (n=6) and were examined on a Siemens Trio 3T system. Measurements were performed on the thigh muscles of the patients before and four months after usual care or intervention period. Fat fraction was quantitatively determined with a method earlier described.

Results: The difference in fat fraction between before and after measurement was shown to be dependent on the initial fat fraction. In the controls relative most changes in fat fraction were observed in muscle having an initial intermediate fat fraction. This observed change was also largest in these muscles. Unpaired two-tailed t-test revealed significantly smaller increases in fat fraction over time for the CBT and AET group. However no differences were observed between the intervention groups.

Discussion and conclusions: The difference in fat fraction between before and after control or intervention period was shown to be dependent on the initial fat fraction. In controls the fat fraction increases significantly over time. Remarkably, the increase in fat fraction was more common and larger in the intermediary muscles compared to the normal muscle.

Non pharmaceutical interventions like cognitive behavioral therapy or exercise training appears to be beneficial in FSHD as it was shown to slow down disease progression defined as increasing fatty infiltration.

10. [P]

On nosological place of facioscapuloperoneal (or facioscapulolimb, type 2) 4q35-linked muscular dystrophy once again

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We observed the pattern of muscle weakness in 28 patients from fourteen 4q35-linked EcoRI/BlnI DNA fragment size 13-30 kb facioscapuloperoneal muscular dystrophy (FSPMD) families (**Table**). The 13 patients (8 men and 5 women) from these families were re-examined by V.K. after 27-29 years (4 patients), after 36-37 years (n=5), after 43 years (n=1), after 48 years (n=1) and after 49 years (n=2) (**Table**). In the first examination the next phenotypes of muscle weakness were found: facio(scapular) [F(S)] (1 patient), (facio)scapular [(F)S] (n=3), facioscapular (FS) (n=1), (facio)scapuloperoneal [(F)SP] (n=5), (facio)scapuloperoneal-(femoral) [(F)SP(F)] (n=1), scapuloperoneal (SP) (n=1), and facioscapuloperoneal-(humeral) [FSP(H)] (n**=**1) (**Table**). On re-examination (V.K.) these patients after 27-49 years the next muscle phenotypes were found: facio-scapulo-peroneal-femoro (posterior thigh muscles)-gluteo (gluteus maximus) (FSPFG) (1 patient), FSPF(G) (n=1), (F)SPFG (n=1), facio-scapulo-peroneal-femoro (posterior thigh muscles)-gluteo (gluteus maximus)- (humeral; biceps brachii) [FSPFG(H**)**] (n=3), [(F)SPFG(H**)**] (n=1), facio-scapulo-peroneal-humero (biceps brachii) - femoral (posterior thigh muscles)-gluteal (gluteus maximus) (FSPHFG) (n=1), (F)SPHFG (n=1), (facio)scapuloperoneal [(F)SP)] (n=2), facioscapuloperoneal (FSP) (n=1) and facioscapuloperoneal-(femoral) [FSP(F)] (n=1) (**Table**). Thus, in nine patients their phenotypes of muscle weakness were transferred in final ones: in FSPFG or FSPFG(H) in seven patients and in two other patients – in FSPHFG phenotypes in who the biceps brachii muscles were severe affected after involving of tibialis anterior muscles. Three patients (F2, III-10 and VI-8; F8, III-25) after 37, 36 and 27 years, respectively, had the same clinical pure FSP phenotype and in one patient (F8, VI-17) after 36 years the FSP phenotype predominated as well but with slight involvement of posterior thigh muscles (**Table**). However, in all these patients (excluding F8, III-25) on MRI study the severe involvement of some posterior thigh muscles and rectus femoris was found. Thus, in discussed patients the disease began with initial involvement of the face (in minimal/slight degree) and shoulder girdle muscles and some time later of the peroneal group (anterior tibial) muscles. However, later the dystrophic process gradually was extended to the thigh muscles (posterior group, namely; the quadriceps were preserved), pelvic girdle muscles (gluteus maximus, namely; the gluteus medius were preserved) and not always on upper arm muscles (biceps brachii, namely; slightly weakened on the one side in four of 13 patients only; in two other patients these muscles were severe affected). In patients having the final phenotype the abdominal muscles were involved after affection of the peroneal group muscles and the increased lumbar lordosis was due to weakness of abdominal and gluteus maximus muscles but not the erector trunci ones. All patients having the final phenotype of muscle weakness could walk independently and climb the stairs with the aid of a railing excluding two patients (F13, III-8 and F8, II-13) who could walk with aid of a stick on short distances only.

In other 10 symptomatic patients who were re-examined (V.K.) after 3-24 years the similar final FSPFG (n=2), (F)SPFG (n=1) FSPFG(H) (n=1), FSPHFG (n=3), FSPHF(G) (n=1), (F)SP(FGH) (n=1) and FSP(F) (n=1)

phenotypes were found and only 1 patient had pure FSP phenotype after re-examination over 7 years (**Table**).

The term “facioscapulolimb muscular dystrophy, type 2 (FSLD2), descending with a “jump” with initial FSP phenotype (Erb, Landouzy and Dejerine type)” would be more correct. The FSP or (F)SP phenotype constitutes merely a stage in the development of FSLD2. We suppose that classical AD FSPMD (or FSLD2, a descending with a “jump” type with initial FSP phenotype) is different from the classical AD FSHD which was called as a facioscapulolimb muscular dystrophy, type 1 (FSLD1), a gradually descending with initial FSH phenotype (Duchenne de Boulogne type) and may be these diseases are connected with the various 4q35 chromosomal mutations (1-3).

Table

Dynamic of myogenic phenotypes and degree of severity disease in 28 patients from 14 families with different EcoRI/BlnI DFS

№F	DFS Kb	№P in ped.	Age ex.	Ph1	D.S.1	DLWD1	Re ex. yrs	Age reex.	Ph2	D.S.2	DLWD2
1	26/23	IV-42	31	(F)SP	Pr	1	24	55	(F)SP(FGH)	Modd	3
2	27/24	III-7	36h	(F)SP	Modd	2	37	73	FSPFG(H)	Sdd	3
		III-10	36	(F)SP	Sdd	2	37	73	(F)SP	Sdd	2
		VI-8	6	(F)S	Pr	1	36	42	FSP	Modd	2
5	25/22	IV-7	23	(F)SP(F)	Sdd	2	29	52	FSPF(G)	Sdd	3
		V-4	1,5	Norma			22.5	24	(F)S(P)	Pr	1
8	23/20	II-13	60	(F)SP	Sdd	2	28	88	(F)SPFG(H)	Sdd	4
		III-25	28	(F)SP	Pr	1	27	55	(F)SP	Mdd	1
		VI-17	5.5	F(S)	Pr	1	36	41	FSP(F)	Sdd	2
18	27/24	III-3	18h	(F)S	Mdd	1	49	67	FSPFG(H)	Sdd	3
20	16/13	III-2	73	FSP(FG)	Sdd	2	7	80	FSPFG(H)	Sdd	3
		III-10	62	FSPH(FG)	Sdd	2	3	65	FSPHF(G)	Sdd	3
		IV-2	18h	FS	Mdd	1	43	61	FSPFG(H)	Sdd	3
19	20/17	III-4	47h	(F)S	Mdd	1	8	55	(F)SPFG	Sdd	2/3
		IV-2	19h	FSP	Mdd	1	13	32	FSPHFG	Sdd	3/4

16	23/20	III-5	45	FSP	Sdd	2	13	58	FSPFG	Sdd	3
		IV-9	21	FSP	Modd	2	15	36	FSP(F)	Modd	2/3
		IV-10	17	FSPH(FG)	Sdd	2	18	35	FSPHFG	Sdd	3/4
21	17/14	II-6	49	FSP	Mdd	1	11	60	FSPFG	Modd	3
		Ш-5 III-6	25 14	FSPHFG (F)S(P)	Sdd Pr	3 1	14 12	39 26	FSPHFG (F)S(P)	Sdd Pr	4 1
22	23/20	III-1	42	FSP	Modd	2	7	49	FSP	Sdd	2
13a	26/23	III-1	18h	(F)SP	Pr	1	27	45	FSPFG	Sdd	2
9a	16/13	IV-1	25h	(F)S	Pr	1	49	74	(F)SPFG	Sdd	3
13	28/25	III-8	26h	FSP(H)	Sdd	2	37	63	FSPHFG	Sdd	4
15	 24/21 33/30	IV-3 V-10 V-17	20h 10 15	SP S (F)S	Pr Pr Pr	1 1 1	48 24 24	68 34 39	(F)SPHFG (F)S(P) (F)S(P)	Sdd Pr Pr	3/4 1 1

List of the abbreviations used in the Table

№F = number of family; DFS kb = EcoRI/BlnI DNA fragment size (kilobas); №P in ped. = number of patient in pedigree; Age ex. = age of patient in first examination; 18h, 47h and other = age of patients in who the myogenic pattern was take from the case history; Ph1 = patient's phenotype in first examination; D.S.1 = the degree severity of disease in first examination: Pr – presymptomatic, Mdd – mild degree of disease, Modd – moderate degree of disease, Sdd- severe degree of disease (see Acta Myol. 2000; vol. XIX, p.71); DLWD1 = daily-life work disability in first examination (see Acta Myol. 2000; vol. XIX, p. 72); Age reex. = the patient's age in which he was re-examined; Reex. yrs = after what years the patient was re-examined by Dr. V. Kazakov.; Ph2 = patient's phenotype after re-examination; D.S.2 = the degree severity of disease after re-examination; DLWD2 = daily-life work disability after re-examination.

Phenotypes: FS = facioscapular; S = scapular; SP =scapuloperoneal; FSP = facioscapuloperoneal; FSPFGH = facio-scapulo-peroneal-femoro (posterior group muscles)-gluteo (gluteus maximus muscle)-humeral (biceps brachii muscle); FSPFG = facio-scapulo-peroneal-femoro (posterior group muscles)-gluteal (gluteus maximus muscle); FSPHFG = facio-scapulo-peroneal-humero (biceps brachii muscle)-femoro (posterior group muscles)-gluteal (gluteus maximus muscle).
(F) = slight atrophy and weakness of upper or lower half of orbicularis oris muscle and slight weakness of orbicularis oculi muscles (orbital part); (S) = slight atrophy lower part of trapezius muscles; (H) = slight weakness (grade 4) of biceps brachii muscle; (F) = slight weakness of posterior thigh muscles; (FG) = slight weakness of posterior thigh and gluteus maximus muscles; (FGH) = slight weakness of

posterior thigh, gluteus maximus and some facial muscles; (P) = slight weakness of tibialis anterior muscles (the patient cannot stand up on his heels/or on one hell). The patient's phenotypes who were re-examined over 27-49 years marked by rich black color.

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11.

Sarcomeric dysfunction in facioscapulohumeral muscular dystrophy

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The expression of sarcomeric proteins is impaired in facioscapulohumeral muscular dystrophy (FSHD), a common hereditary myopathy characterized by muscle weakness. In addition, overexpression of DUX4, the leading FSHD candidate gene, has been shown to activate pathways involved in sarcomeric protein degradation. Here we investigated whether sarcomeric dysfunction contributes to muscle weakness, using demembranated single muscle fibers of FSHD patients and control subjects. The force generating capacity of sarcomeres is significantly impaired in FSHD. Sarcomeric weakness was restricted to type II muscle fibers, in which maximum force generation was only 70% of normal strength. In contrast to active force measurements, a 5- to 12-fold increase in passive force was seen in type I and type II fibers respectively, indicating stiffening of titin molecules. Corroborating these findings, we observed a decrease in myofilament lattice spacing and increase in calcium sensitivity, both physiological consequences of titin stiffening.

Based on these findings, we propose that sarcomeric dysfunction plays a critical role in muscle weakness in FSHD.

12.

Identification of the epigenetic modifier of the D4Z4 epiallele in FSHD2

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Facioscapulohumeral muscular dystrophy (FSHD) is a common form of muscular dystrophy in adults. FSHD is associated with chromatin decondensation of the D4Z4 macrosatellite array on chromosome 4 and ectopic expression of the D4Z4-encoded germ line transcription factor DUX4 gene in skeletal muscle. Two equally common variants of D4Z4 have been identified of which only one is permissive to DUX4 expression. In most patients, DUX4 derepression is caused by contraction of the D4Z4 array (FSHD1) and is inherited as a dominant trait. However, in some patients the D4Z4 decondensation occurs on normal sized arrays (FSHD2) of both chromosomes 4q and 10q and show a more complex inheritance pattern. In both FSHD1 and FSHD2, the chromatin relaxation is associated with a loss of CpG methylation and a loss of repressive histone modifications at D4Z4. Here we show that rare, normally benign variants in a chromatin modifier segregate with genome-wide D4Z4 CpG hypomethylation. FSHD2 patients inherited both variants of this epigenetic modifier and normal-sized D4Z4 arrays permissive for DUX4 expression. In conclusion, we identified an epigenetic modifier of the D4Z4 epiallele that is underlying FSHD2 and possibly other human diseases subject to epigenetic regulation.

13.

Use of a simple Clinical Proforma to identify potential cases of FSHD2 in DNA diagnostic service testing for FSHD1

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The Bristol Genetics Laboratory (BGL) receives over 300 requests for FSHD testing (diagnostic, predictive and prenatal) each year, providing the majority of tests for the UK, and some service provided for Europe and other continents. Most requests (~80%) are for primary diagnostic testing of index cases affected with neuromuscular symptoms using EcoR1/Bln1/Apo1 triple digests where a shortened ≤35kb 4q band is detected with the p13E11 probe in 38% of referred cases.
Further testing is by re-request from the referring clinician, who is required to complete a standard simple Clinical Proforma, which undergoes clinical/laboratory review. These are returned to us for around 30% of the initial negative diagnostic test results (approx 40 proforma returned /year). Exclusion criteria for FSHD such as ptosis, extraocular weakness, marked excess of pelvic over shoulder girdle weakness, or very high serum CK (>1500 IU/l) will result in alternative advice to the referring clinician. However, in clinically compatible cases, including where there is a marked discrepancy between a severe clinical presentation and a borderline-sized residual fragment we recommend reflex testing for proximal deletions using MseI/EcoRI/BlnI digests and the D4Z4 1kb probe finding only 1-3 extended deletion cases per year (estimated ~ 1% of total requests).

Annually approximately 30 test-negative samples remain (10% of total requests) where the proforma suggests that a clinical diagnosis of FSHD remains very plausible. It is amongst this group that we anticipate that a test for FSHD2 will become highly valuable, as well as using a targeted next generation sequencing approach to identify conditions such as calpainopathy and acid maltase (alpha glucosidase) deficiency in the differential diagnosis of FSHD2.

14. [P]

Understanding mRNA Processing

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FSHD develops in individuals who have BOTH a D4Z4 repeat contraction, causing chromatin relaxation, AND a PolyAdenylation Site (PAS) in the pLAM domain distal to the D4Z4 array. This PAS allows for cleavage of the pre-mRNA and subsequent polyadenylation and stabilization of the transcript. In individuals who have D4Z4 contractions but a single base change in the PAS, the cell does not recognize it as a PAS, preventing cleavage and polyadenylation. As a result the DUX4 transcript is unstable, no DUX4 protein is made, and the individuals are protected from getting the disease. Therefore, any intervention that prevents the addition of the poly(A) tail to the DUX4 transcript is a potential therapeutic approach for FSHD. Clearly, it is untenable to interfere with mRNA processing in general because of the toxicity to the cell. Therefore, understanding the mechanisms by which a cell can bypass a specific PAS would suggest a mechanism for selectively blocking the PAS in the DUX4 gene without generally affecting cellular mRNA processing. This would be an effective treatment for patients with FSHD. We hypothesize that sequence elements around the PAS unique to the that gene define the site of mRNA cleavage and polyadenylation and that specific sequences instruct a cell to select or bypass the PAS. The PAX3 gene has three functional PASs in its 3'UTR and encodes a transcript that is alternatively polyadenylated in different populations of quiescent muscle stem cells. Here we describe the characterization of the PAX3 3'UTR and discuss its implications for mRNA processing in general, and we discuss an assay to measure mRNA 3'UTR length.

15. [P]

Autophagy defects in FSHD

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Using mRNA profiling and immunoblotting, we detected autophagy defects in a tet-repressible muscle-specific Pitx1 transgenic mouse model, including up-regulation of a master autophagy regulator, Dram, up-regulation of p62 and accumulation of ubiquitinated proteins. The up-regulation of DRAM was also observed in FSHD muscle biopsies but not DMD or controls. We further characterized the autophagy state in immortalized FSHD myoblasts by immunoblotting. Similar to the mouse model, higher expression of DRAM, p62 and ubiquitinated proteins were detected in immortalized FSHD myoblasts. In addition, lower LC3B-II/LC3B-I ratio were observed suggesting suppression of autophagy in the FSHD myoblasts. While the activation of DRAM is expected to activate the downstream autophagy pathways, the autophagy process is somehow disrupted in immortal FSHD myoblasts. The findings suggest involvement of autophagy defects in FSHD.

16. [P]

Transcriptional profiling in facioscapulohumeral muscular dystrophy to identify candidate biomarkers

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FSHD is a progressive neuromuscular disorder caused by contractions of repetitive elements within the macrosatellite D4Z4 on chromosome 4q35. The pathophysiology of FSHD is unknown and as a result, there is currently no effective treatment available for this disease. In order to better understand the pathophysiology of FSHD and develop mRNA-based biomarkers of affected muscles, we compared global analysis of gene expression in two distinct muscles obtained from a large number of FSHD subjects and their unaffected first-degree relatives. Gene expression in two muscle types was analyzed using GeneChip Gene 1.0 ST arrays: biceps, which typically shows an early and severe disease involvement, and deltoid, which is relatively uninvolved. For both muscle types, the expression differences were mild: using relaxed cutoffs for differential expression (fold-change ≥1.2 and nominal *P*-value <0.01), we identified 191 and 110 genes differentially expressed between affected and control samples of biceps and deltoid muscle tissues, respectively, with 29 genes in common. Controlling for a false-discovery rate <0.25 reduced the number of differentially expressed genes in biceps to 188 and in deltoid to 7. Expression levels of 15 genes altered in this study were used as a "molecular signature" in a validation study of an additional 26 subjects, and predicted them as FSHD or control with 90% accuracy based on biceps and 80% accuracy based on deltoids.

17.

Prognostic parameters for familial fascioscapulohumeral muscular dystrophy (FSHD): a large-scale genotype-phenotype analysis from the Italian National Registry for FSHD

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Facioscapulohumeral muscular dystrophy (FSHD) is classified as an autosomal dominant myopathy associated with deletions of integral copies of tandem 3.3 kilobase repeats (D4Z4) combined with 4A(159/161/168) PAS haplotype at 4q35. It has been estimated an almost full penetrance of the disease by the age of 20. However, studies reported a unexpected high percentage of asymptomatic or minimally affected subjects carrying a reduced D4Z4 allele (DRA) at 4q. Recently we have reported that 3% of healthy subjects carried alleles with reduced number (4-8) of D4Z4 repeats on chromosome 4q.

Through the Italian National Registry, 530 subjects carrying DRA (367 relatives and 167 probands) from 176 unrelated FSHD families were clinically and molecularly evaluated. Size of DRA, gender, age, degree of kinship and 4q haplotype were evaluated to establish the role of these variable on severity of the clinical expression. The FSHD scale was used to define muscle impairment, which can be translated into a number (FSHD score).

Overall, 32•2% of relatives did not display any muscle functional impairment, with 47•1% of unaffected individuals among second- through third-degree relatives and 27•5% among first-degree family members. The estimated risk of developing FSHD for relatives carrying DRA with 1-3 repeats was 64•3% at age 20, 80•1 at age 40, and 96•2 at age 60. The risk of FSHD for relatives carrying DRA with 4-8 repeats was approximately 20% at age 20, 43% at age 40, 65% at age 60.

Comparison of the FSHD score given to females versus males with DRA of the same size , displays that male relatives received a mean score significantly higher than females relatives (mean FSHD score: 5.4 in males vs 4.0 in females, p0.003). No 4q haplotype was exclusively associated with the presence of disease.

Our work establishes that DRAs with 4-8 repeats have limited prognostic value. In this class of alleles female gender and degree of kinship are associated with a reduced risk of developing FSHD. No specific haplotype is predictive of disease expression.

Collectively our data suggest that the pathogenesis of FSHD is more complex than previously thought and that the current genetic molecular signature needs to be carefully re-considered as predictor of disease outcome, with crucial consequences for genetic counseling and prenatal diagnosis.

18.

FSHD, a fœtal pathology ?

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Introduction: FSHD is a dystrophic pathology mainly of the adolescent or the young adult. The disease is suspected to be associated to a modulation of the expression of different genes located in the 4q35 chromosomal region. Our objective was to characterize the presence of pathological signs during the fetal life and define the expression pattern of the 4q35 genes.

Methods: Muscular biopsies from fetuses were obtained from medical interruption of pregnancy. The biobank is constituted with biopsies from fetuses carrying a deletion of D4Z4 from parents affected with FSHD. The non-carrier controls are not affected by any identified genetic mutation or muscular pathology. RNA was extracted from biopsies, reverse transcribed and amplified by quantitative PCR using a Roche 480 lightcycler. GUSB were used as the reference gene. Expression analysis and Wilcoxon-Mann-Whitney test were performed by Gnumeric spreadsheet software version 1.10.16.

Results: Three groups of gene have been quantified: (i) genes located on the 4q35.1 and 4q35.2 region, (ii) genes related to muscular physiology and (iii) genes involved in metabolism and signal transduction. Expression was measured for the 3 trimesters of pregnancy in biceps, quadriceps, diaphragm, brain and kidney. In FSHD biopsies, we observed an over-expression of different genes located at the 4q35 region, including ANT1. Furthermore, we observed that the muscular physiology was impacted in fetuses carrying a shortened *D4Z4* array. We did not observe an induction of MMP9 or a modulation of PABPN1, usually associated to dystrophic phenotype. We also observed that calcium related genes were over-expressed. PITX1 expression was down-regulated only in brain and kidney but not in biceps and quadriceps.

Conclusion: We confirmed a modulation of some genes from 4q35 region in FSHD biopsies. This modulation was accompanied with signs of a muscular hypertrophic phenotype without signs of dystrophic process. In addition, over-expression of calcium channel genes was shown. Our results indicate that at the molecular level, a latent dystrophic disease is present in FSHD fetuses.

19.

FSHD2 may act as (epi)genetic modifier for FSHD1

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In recent years, the advances of knowledge in clinical, genetic and epigenetic features of facioscapulohumeral muscular dystrophy (FSHD) allowed the identification of two forms of FSHD, the classical autosomal dominant FSHD type 1, and FSHD type 2 characterized by identical clinical phenotype but associated with a different (epi)genetic defect.

FSHD1 patients carry a contracted D4Z4 allele ≤ 11 repeats on a permissive 4QA chromosome, while FSHD2 patients display normal-sized D4Z4 alleles on chromosomes 4, marked hypomethylation of D4Z4 loci on both chromosomes 4 and 10 and at least one permissive 4QA allele. Myotube cultures of FSHD2 patients also express DUX4 in a variegated pattern of few myonuclei expressing abundant amounts of DUX4 protein.

In the present study we analyzed a cohort of 42 unrelated FSHD1 patients (24 women, 18 men, mean age 58, [IQR 49-67]) followed in Nice neuromuscular disease center in order to establish a genotype/phenotype correlation between the number of repeated units and the severity of clinical phenotype evaluated by Manual Muscle Testing and Brooke and Vignos scale. A good genotype/phenotype correlation was established for all but four FSHD1 patients. These patients display a very severe clinical phenotype (3 out of 4 were wheelchair bound) in association with 9 or 10 repeat units. The complete genotype of chromosome 4 and 10 D4Z4 loci and the methylation studies confirmed the presence of a contracted 4QA allele in all these patients consistent with FSHD1 diagnosis, but also uncovered marked hypomethylation of both chromosomes 4 and 10, consistent with a FSHD2 diagnosis.

We therefore studied the segregation of the contracted 4QA allele and of the hypomethylation of chromosomes 4 and 10 in two of these patient's families. This revealed that FSHD1 and FSHD2 have different genetic basis since the two diseases segregate independently. Subsequent genetic analysis identified mutations in the recently identified FSHD2 gene segregating with the disease in three of our families.

Collectively, our study shows that FSHD1 and FSHD2 share a common pathophysiological pathway since they present an identical clinical phenotype and FSHD2 genetic defect, and that the FSHD2 gene may act as a modifier for disease in FSHD1 families.

20. [P]

Large scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy (FSHD)

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The practice of medical genetics requires a clear, definite evaluation of the significance of mutations/variations of DNA sequences for diagnosis to provide prognostic information and genetic counseling. This is particularly important for a progressive disease with unpredictable onset and a high variability of clinical expression such as FSHD. Establishing the value of mutational events underlying genetic diseases may be complex even when there are simple patterns of inheritance in diseases with a well-characterized pathologic course.

FSHD seems to fall in this complex pattern even though it is currently considered a fully penetrant disease with a wide variability in clinical spectrum, ranging from subjects with very mild muscle weakness to wheelchair bound patients. However the wide use of this test revealed several exceptions to the original model. Through the years the threshold size of D4Z4 alleles has been increased from the original 28 kb (6 repeats) to 35 kb (8 repeats), with FSHD cases carrying D4Z4 alleles of 38-41 kb (9-11 repeats) considered borderline alleles. Additional genotype-phenotype studies led to the identification of subjects carrying D4Z4 reduced alleles with no sign of muscle weakness in FSHD families as well as in normal controls. The present results from our systematic clinical and molecular analysis of FSHD patients from the Italian National Registry for FSHD as well as a large number of healthy controls challenge the current model for FSHD diagnosis.

Remarkably, our data establish as a general rule rather than an exception that detection of a D4Z4 reduced allele is not sufficient to diagnose FSHD. Although the majority of FSHD patients (70%) carry D4Z4 alleles with 4-8 units, this size range is carried by 3% of healthy subjects from the general population. Additionally there is little predictive value of the 4qA161PAS haplotype in the absence of family history since 1.3% of healthy subjects carry this haplotype, which therefore has the frequency of a common polymorphism (Figure 1) rather than a rare mutation. Finally 49 of 253 probands (19%) do not carry D4Z4 alleles with 1-8 repeats and only 50% of the probands carry the 4A161 permissive haplotype.

In summary, our study indicates that a profound re-thinking of the genetic disease mechanism and modes of inheritance of FSHD are now required and entirely new models and approaches are needed. It is hoped that broadening the scope of investigations including next generation deep sequencing in particular in families with asymptomatic and clinically affected members carrying the same FSHD allele may finally lead to an understanding of the molecular pathogenesis of this complex disease. These findings have important clinical implications for genetic counseling of patients and families with FSHD with particular regard to the interpretation of data in prenatal diagnosis.

21.

Investigations on the FSHD disorganized myotube phenotype

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Primary CD56^{+} FSHD myoblasts fuse into myotubes presenting various proportions of an atrophic or a disorganized phenotype. To better define those phenotypes, we optimized a differential isotope protein labeling (ICPL) combined with 2DLC-MS/MS to study their proteome as compared to healthy control myotubes. FSHD atrophic myotubes presented decreased relative abundance of structural and contractile muscle components. This phenotype suggests atrophy-associated proteolysis that likely results from the DUX4-mediated dysregulation cascade. The skeletal muscle myosin isoforms and non-muscle myosin complexes presented a lower or higher relative abundance, respectively, that likely reflects a differentiation defect. In FSHD disorganized myotubes, myosin isoforms were unchanged, while proteins with increased relative abundance were mostly involved in microtubule network organization and myofibrillar remodeling.

Because the predominantly atrophic or disorganized FSHD cultures that we have analyzed are derived from comparable patients in terms of the number of *D4Z4* units, sex and age, we assume that other factors could intervene to explain the emergence of a non-atrophic phenotype, despite the expression of DUX4. The open chromatin at the *D4Z4* repeat array in FSHD facilitates transcription of several genes in the vicinity, among which *DUX4* within the *D4Z4* unit itself and *DUX4c* located 42 kb centromeric. The DUX4c protein, expressed in myoblasts from healthy control individual and induced in FSHD, could bind to DUX4-target promoters through its identical double homeodomain but has a shorter carboxyl terminal domain and does not share DUX4 toxicity. However, its overexpression in myoblasts led to an increased proliferation rate. We have now evaluated the impact of DUX4c overexpression on myotube phenotypes. Control myoblasts overexpressing DUX4c fuse to form disorganized myotubes with clusters of nuclei, associated with the induction of β-catenin and an abnormal troponin T distribution and sarcomere structure. We developed siRNAs targeting the DUX4c mRNA. The addition of these inhibitors to primary disorganized FSHD myoblasts cultures suppressed DUX4c expression and restored a normal myotube phenotype.

In conclusion, these results suggest that besides *DUX4*, *DUX4c* could be an additional target for the development of a therapeutic approach against FSHD.

22.

The DUX4 promoter is expressed in FSHD-affected tissues

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FSHD is most commonly characterized by progressive wasting and weakness of facial and shoulder-girdle muscles, although no consistent pattern of penetrance or severity exists, even within affected families. A hallmark characteristic of FSHD is asymmetrical muscle weakness. There are also non-muscular features including retinal vasculopathy and high frequency hearing loss. Several recent breakthroughs now support a model in which mis-expression of the myotoxic DUX4 gene is a primary pathogenic event underlying FSHD. We hypothesized that there was a direct correlation between DUX4 expression patterns and the involvement of only selected muscles (and ear and retinal pathologies) in FSHD. In short, we proposed that if DUX4 over-expression is indeed an underlying pathogenic event in FSHD, it must be preferentially expressed in FSHD-affected regions. To test this hypothesis, we developed transgenic reporter mice containing a putative DUX4 promoter cloned upstream of GFP. We generated three separate lines ofDUX4 promoter-GFP mice. We found the DUX4 promoter directed GFP expression in the face and limbs of newborn and adult mice, as well as the retina. Essentially all other organs were GFP negative. Strikingly, all lines showed asymmetrical expression and variable penetrance, even within individual litters. We conclude that our mice faithfully recapitulate expected DUX4 expression patterns in regions of FSHD pathology, and further suggest the role of DUX4 as pathogenic insult in FSHD.

23. [P]

FSHD extended deletion analysis using the D4Z4 1kb probe – a case study

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FSHD1 (OMIM 158900) is an AD disease affecting 1 in 20,000 and is the third most common form of muscular dystrophy, characterized by progressive wasting of upper body muscles. FSHD1 is associated with contraction of D4Z4 tandem repeat units in the subtelomere region 4q35.

Bristol Genetics laboratory offers a UKGTN/international specialist diagnostic FSHD service. First line testing using the p13E-11 probe (to confirm the chromosome of origin and size of the D4Z4 contraction), identifies 95% of individuals with FSHD. In 1-3% of the remaining cases a deletion of the D4Z4 repeat can extend proximally to include the p13E-11 probe site, giving a false-negative p13E-11 result. Reflex testing using MseI/EcoRI/BlnI digests and the D4Z4 probe (binds within the repeat array proper) allows the chromosome origin and number of D4Z4 repeats to be determined and is available for clinically typical FSHD patients, upon return of a clinical proforma.

We present an audit of cases tested by D4Z4 analysis, and clinical information and results of extended analysis for a complex three generation FSHD family, with a deletion of the p13E-11 probe region. The index case presented with asymmetric proximal weakness, scapular winging and mild facial weakness; other relatives presented with variable phenotypes. Confirmation of an extended deletion facilitated urgent predictive testing related to pregnancy. This family illustrates the complexity and clinical utility of extended deletion analysis for FSHD.