The Journal of Rare DISORDERS

# International Partnership: Patient Advocacy and Engagement

Julie Greenfield[1], Mina Ruggieri[2], Barbara Flynn[3], Jennifer Farmer[4], Jane Larkindale[4]

[1]AtaxiaUK, [2]GoFar, [3]Ataxia Ireland, [4]Friedriech's Ataxia Research Alliance

The Friedreich's Ataxia Research Alliance (FARA), Ataxia UK, Ataxia Ireland, and GoFAR (Italy) partnered to organize and host the largest and most comprehensive International Ataxia Research Conference held to date on March 25-28, 2015 in Windsor, England. The conference highlighted important research advances for hereditary and sporadic ataxias, including Friedreich's ataxia, spinocerebellar ataxias, ataxia with oculomotor apraxia and episodic ataxia. More than 350 international delegates from academic institutions, from the biopharma industry and from medical, healthcare and advocacy organizations attended and presented the latest research findings from basic, translational and clinical investigations.

People with ataxia are at the heart of everything we do, and four people with ataxia shared their personal experiences with the research community. Giving the research community an opportunity to learn from patient stories related to diagnosis, living with the disease, participation in research and personal experiences is not only motivational and inspiring but truly expands and deepens understanding of the disease. These new insights and shared experiences can also spur new hypotheses or avenues for research, as well as inform researchers about the aspects of disease that patients identify as most important to them.

The ataxias are all rare diseases.  Working on each disease individually in individual laboratories or clinics results in slow progress, but by bringing together researchers from around the world from multiple disciplines we can learn from each other and accelerate research.  When the global community works together to look at the same problems in different ways new ideas are generated, new concepts are linked and new pathways may be identified that can help move therapies forward in unexpected ways.  The International Ataxia Research Conference was a venue where this could happen, and many new partnerships and collaborations were forged.

There are no treatments available commercially for the vast majority of the ataxias.  At the Conference, eight different therapeutics in clinical development were presented.  This represents the enormous advances in the field in the past years.  Furthermore, there was discussion of a range of pathways that may be involved in one or more ataxia, and ways that these could be leveraged for potential therapeutics.  There was vigorous discussion of the value of each of these pathways and their therapeutic potential, as well as next steps to prove or disprove their value, and offers of collaboration to complete that work. This suggests that work following the meeting will result in even more progress over the next few years.

Patient advocacy organizations can make meaningful contributions to advancing research, patient engagement and growing the research community by organizing and hosting research conferences. These conferences are the playing field where we assemble and engage all stakeholders; academic investigators, physicians and health care providers, biotech and pharma industry representatives, government and regulatory representatives, and patients, to promote timely dissemination of information, debate and discussion of new research findings and hypotheses, learn from experiences observed in the clinic or living with the disease and network with colleagues to build partnerships and collaborations; all of which advances progress toward treatments.  Our advocacy organizations will continue to work together as we believe we are more effective working in partnership rather than isolation and hope that the next international meeting will be even bigger and better – with more new therapies closer to a reality.

# INTRODUCTION

Hereditary ataxias are progressive, neurodegenerative disorders, associated with degeneration and dysfunction of the cerebellum and/or sensory pathways. Friedreich's ataxia (FRDA) is the most common hereditary ataxia, with an estimated prevalence of approximately 1 in 40000 individuals in Caucasian populations [1]. No treatment to prevent or slow the progression of hereditary ataxias has yet been found, with only symptomatic treatments and palliative care currently available for patients. However, research in this field is robust, and as we develop further understanding of the diseases, new therapeutic concepts are being developed.

The International Ataxia Research Conference 2015 covered all aspects of the ataxias from understanding the causes of diseases through to clinical trials testing potential ways to treat them. Sessions covered the discovery of new genes and new diagnostic techniques, new developments in understanding the mechanisms and pathways of the diseases, new models to study the diseases and developments in drug development, as well as the development of tools to support future drug development. Drug development is moving rapidly in this field, with 8 projects discussed that are in the clinic, mostly for Friedreich's Ataxia or SCA3. Many additional projects are still at a preclinical stage, offering significant hope for patients. This report summarizes some of the conference sessions, focusing on oral presentations. Significant additional work was presented in poster form.

1. Parkinson, M.H., et al., Clinical features of Friedreich's ataxia: classical and atypical phenotypes. *J Neurochem*, 2013. 126 Suppl 1: p. 103-17.

# NEW GENES AND DEVELOPMENTS IN THE DIAGNOSIS OF ATAXIAS

Matthis Synofzik[1,*], Andrea H. Németh[2]

[1] Hertie Institute for Clinical Brain Research, Tübingen; Germany;

[2] Nuffield Department of Clinical Neurosciences, University of Oxford, UK.

* To whom correspondence should be addressed: Dr. Matthis Synofzik, Center for Neurology and Hertie-Institute for Clinical Brain Research, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany; phone: +49 70712982060, e-mail: matthis.synofzik@uni-tuebingen.de

MS is currently funded by the Robert Bosch Stiftung, Germany, and previously by AtaxiaUK. AHN is currently funded by Action Medical Research and The Henry Smith Charity, and previously by AtaxiaUK.

Unraveling the diagnosis and molecular disease cause in patients with so far unexplained ataxias has been revolutionized by the introduction of next-generation sequencing (NGS) methods like targeted capture of large sets of genes ("gene panels"), whole-exome sequencing (WES) and, more recently, whole-genome sequencing (WGS). As demonstrated by the 10 presentations in the opening session of the ARC International Research Conference (given by Andrea Nemeth/Oxford, Michel Koenig/Montpellier, Marie Coutelier/Paris, Marios Hadjivassiliou/Sheffield, Rebekah Jobling/Toronto, Stefania Magri/Milano; Rebecca Schüle/Miami and Tübingen; Matthis Synofzik/Tübingen, Dineke Verbeek/Groningen, Margit Burmeister/Michigan), these NGS methods have led to a rapidly increasing number of newly identified ataxia genes, to new insights into associated molecular pathways and common mechanisms linked to the pathogenesis of degenerative ataxias, and to cost-effective comprehensive diagnostic approaches which now allow to provide a large number of so far unexplained ataxia patients with a molecular diagnosis.

*Novel ataxia genes*

NGS enabled the identification of identification of several novel genes in a short time-frame. Within only the last 3 years the following ataxia genes were identified:

1. recessive mutations in ***WWOX*** leading to a broad range of severe infantile onset neurodevelopmental syndromes, reaching from early lethal microcephaly syndrome with epilepsy growth retardation and retinal degeneration in patients with truncating *WWOX* mutations[1] via epileptic encephalopathies[2] to more benign ataxia-epilepsy syndromes[3] in patients with missense *WWOX* mutations in hypomorphic alleles[2] (Michel Koenig and team).
2. recessive missense mutations in ***SLC9A1***, a protein encoding for NHE1 (Na+/H+ exchanger family member 1) and involved in pH-regulation at the inner ear, leading to an ataxia-deafness syndrome (Lichtenstein-Knorr syndrome)[4] (Michel Koenig and team)
3. recessive mutations in ***PMPCA***, encoding the alpha subunit of mitochondrial processing peptidase (a-MPP), leading to a non-progressive cerebellar ataxia syndrome[5]. Interestingly, a-MPP is the primary enzyme responsible for the maturation of most nuclear-encoded proteins, including the maturation process of frataxin, which is depleted in Friedreich ataxia (Rebekah Jobling and team)[5].
4. recessive loss-of-function mutations in the BiP co-chaperone ***DNJAC3,*** leading to combined cerebellar and peripheral early-onset ataxia with hearing loss and diabetes mellitus (ACPHD; OMIM #616192) as part of a widespread neurodegenerative process (Matthis Synofzik and team)[6].

<u>*Novel phenotypic spectra and frequency estimates of ataxia genes*</u>

These NGS methods also led to novel insights into the relative frequencies and new phenotypic spectra of known disease genes.

a. ***SPG7*** mutations are a common cause not only of recessive hereditary spastic paraplegias (HSPs) and spastic ataxias[7,8], but also of pure ataxias and of undiagnosed ataxias in general[8,9].

b. Similarly, the complicated HSP phenotype (SPG49) caused by recessive ***PNPLA6*** mutations is only *one* end of a broad spectrum of neurodegenerative diseases caused by PNPLA6. Matthis Synofzik and colleagues have now shown that, along this continuum, PNPLA6 is also a frequent cause of the so-called Boucher-Neuhäuser Syndrome (early onset ataxia plus hypogonadism and chorioretinitis) and Gordon Holmes Syndrome (early onset ataxia plus hypogonadism), which might or might not be accompanied by spastic paraplegia[10-12]. Even more recently, it was shown that on the other end of the spectrum, *PNPLA6* can also cause syndromes comprising of trichomegaly, congenital hypopituitarism and retinal degeneration with choroidal atrophy (Oliver -McFarlane syndrome)[13] and even pure ophthalmological phenotypes like Leber congenital amaurosis, photoreceptor degeneration and various other forms of childhood blindness[12,14].

c. While single heterozygous OPA1 variants have long been acknowledged as common cause of dominant optic atrophy, Matthis Synofzik and colleagues now showed that the combined mutational load of *two* **biallelic *OPA1*** alleles explains the phenotype of complex optic atrophy-plus phenotypes including severe ataxia, sometimes also dubbed "Behr syndrome" (OMIM %210000)[15]. As one of these two *OPA1* alleles might be an *OPA1* modifier variant, which does not lead to disease itself, even in a homozygous state (e.g. the p.I382M variant [15]), and as several *OPA1* variants have a reduced penetrance, the parental generation of these subjects can be unaffected, despite the fact that the index subjects carry two variants of an, in principle, dominant disease. The pedigree of optic atrophy-plus subjects can thus present as a seemingly "recessive pedigree", as exemplified by the family presented by Matthis Synofzik[15]. The etiology of *OPA1* disease is even more complicated by the fact that deep-intronic mutations – which are not detected by Sanger, panel or WES – are a recurrent finding in both pure optic atrophy syndromes and complex optic atrophy plus syndromes[15].

d. Non progressive early-onset ataxia phenotypes, frequently (mis-)named "ataxic cerebral palsy", are usually not caused by injury at birth, as often assumed. Nemeth and colleagues have shown that the ataxic subtype of cerebral palsy can be caused by *de novo* point mutations ***KCNC3***, ***ITPR1***, and ***SPTBN2****[16]*.

e. Brown-Vialetto-Van-Laere syndrome type 2 (BVVL2), is known to be caused by recessive mutations in the riboflavin transporter gene ***SLC52A2***, and is characterized by early childhood onset, deafness, bulbar dysfunction, severe diffuse muscle weakness resulting in respiratory insufficiency (+/- optic atrophy). Michel Koenig now showed, by example of an Israeli-Palestinian family, that it can also cause a spinocerebellar ataxia syndrome with blindness and deafness, which had been introduced earlier as SCABD = SCAR3 (OMIM # 271250)[17]. Identifying ataxia patients due to *LC52A2* mutations will be important in the future, given that *SLC52A2*-related syndromes result from a defect in riboflavin metabolism, with some patients benefiting from high-dose riboflavin supplementation[18], as also indicated for the case presented by Michel Koenig.

f. Recessive loss-of-function mutations in ***GRID2***, encoding the glutamate receptor channel delta-2 subunit, which is largely selectively expressed in cerebellar Purkinje cells, have already been identified as a cause of recessive ataxia[19,20]. Findings from Marie Coutelier and colleagues[21] now indicate that *single heterozygous missense* mutations in *GRID2* might also lead to cerebellar ataxia, with an inheritance pattern that is consistent with semidominant transmission and leading to a putative gain of function mechanism, as supported by findings from *Lurcher mice* models. The severity of the ataxia thereby ranges from very mild adult onset to congenital onset ataxia, depending on the genotype (heterozygous vs. homozygous *GRID2* mutation) and the position of the mutation within the *GRID2* gene domains[21].

*Novel insights into mechanisms of hereditary ataxias*

Given this "explosion" of novel, but all very rare, genes the challenge in ataxia research is now to find common denominators between ataxia genes, which might then allow us to target shared pathways or mechanisms susceptible to treatment interventions. Indeed, the growing list of recessive ataxia genes points to some common themes, as pointed out by Michel Koenig and Andrea Nemeth. Ataxia can result from mutations in genes encoding proteins involved in the following types of pathways [22-25], including:

- mitochondrial
- peroxisomal
- lysosomal
- ion channel
- DNA repair
- ciliopathy
- cytoskeleton
- phospholipid metabolism

However, each theme unites only a minor fraction of novel ataxia genes, indicating that the variety of distinct pathways underlying ataxia is large. This fact indicates that ataxia and the corresponding degeneration of the cerebellum/ spinocerebellar tracts is not the result of disturbances in specific pathways, but rather the common downstream result of an unspecific sensitivity of these neurons to even mild metabolic insults which can result from very different pathway disturbances[22]. Thus, rather than looking for alterations in *common metabolic pathways* shared between some ataxias, Michel Koenig proposed as an alternative perspective to look for shared *genetic mechanisms*. He suggested three shared genetic mechanisms leading to ataxia:

i) partial loss of function (in particular in genes of metabolic ataxias, but also e.g. *FRDA, SYNE1, POLG, C10orf2/Twinkle, WWOX, SLC9A1*)

ii) mutations in specific members of redundant gene families (*ATM, APTX, SETX, ADCK3, ABHD12, ANO10*)

iii) mutations in detoxifying pathways (*TTPA* and *MTTP* in vitamin E deficiency; chaperones in ARSACS and Marinesco Sjögren)

He emphasized that partial loss of function in manifold genes can lead to ataxia, given the assumption that prominent spinocerebellar ataxia occurs only when pathways are mildly affected, whereas compete or near-to-complete loss of function would lead to much more severe lethal and/or encephalopathic syndromes. He illustrated this hypothesis by examples of *WWOX*, *PEX6* and *PEX10*.

*State-of-the-art ataxia diagnostics: comprehensive NGS panels and exomes*

Exemplified by a consecutive series of 1,288 ataxia patients assessed at the Sheffield Ataxia Centre, Marios Hadjivassiliou reported that about 20% (259/1288) of all patients have a positive familial history, 71% of them with an autosomal-dominant history (183/1288). In 48% of the familial patients and in 30% of the total cohort a genetic diagnosis was found by routine selective genetic sequencing of some ataxia genes, yet with numbers still dating from the pre-NGS era. A genetic cause could also be found in *sporadic* patients, with relative frequencies differing between early versus late onset sporadic ataxia patients: while a genetic diagnosis was found in 46% early-onset cases, a much smaller share of the late-onset cases carried a mutation in an investigated gene.

These numbers are likely to have changed by now, given the advent of NGS procedures to clinical routine diagnostics. NGS panels now covering up to 90-120 ataxia genes and WES covering all coding regions (yet at a variable coverage) can provide a genetic diagnosis in so far unexplained ataxia patients in about 18%[26] by NGS panels and 21%[27]-41%

[28] by WES, respectively. Although the high latter estimate of 41% has to be interpreted with caution, given that it largely exceeds all other findings and that some reportedly pathogenic variants might in fact not be causally pathogenic [29] – exemplifying the problem of over-interpreting NGS findings in ataxia genetics -, these findings nicely demonstrate the power of NGS approaches to diagnose so far unexplained ataxia patients. Similar to these findings, Dineke Verbeek and team identified rare SCA mutations in 7/20 families negative for mutations in the most common SCA genes. In the remaining 13 families, they identified multiple gene candidates (n=39), many of them playing a role in pathways known to involved in cerebellar processing, which are now being functionally validated in the endeavor to identify novel ataxia genes. Stefania Magri and team developed three different ataxia panel approaches (HaloPlex-based gene panel of 127 genes; TrueSeqCustomAmplicon panel of 76 genes; Nextera Rapid Capture Custom Kit of 104 ataxia genes and 100 HSP genes), allowing researchers to identify pathogenic mutations in 25/128 Italian patients (~20%) who were negative for repeat expansion and genes routinely screened by Sanger sequencing. Their NGS panel-based findings included 7 index cases with mutations in the extremely large genes *SYNE1* and *SACS*, where conventional sequencing by Sanger methods is very time consuming and costly. At the same time, the findings from Stefania Magri and Dineke Verbeek also point to a common problem after NGS: They demonstrated that the vast majority of patients have variants of unknown significance in more than one disease genes.

WES also leads to changes in the initial diagnosis in a substantial share of patients (up to 10% of subjects with unexplained pediatric-onset neurodevelopmental disease), by unravelling causative genes known to cause a disease different from the initial diagnosis[30], as highlighted by Andrea Nemeth. It thus leads not only improved diagnosis, but also to changes in the patient management, counselling and therapy[30]. For example, Margit Burmeister presented cases of ataxia due to *POLR3B* mutations where the main phenotypic features had been overlooked on initial referral, but were clinically ascertained after variant detection by NGS.

If WES and NGS panels can provide a diagnosis in 20-40% this implies, in turn, that in 60-80% of ataxia patients no genetic cause can be found by these methods. As outlined by Andrea Nemeth, this might be due to non-genetic causes of the respective ataxias, but also due to digenic/multigenic causes or mutations not detected by WES and NGS, such as copy number variations (CNVs), repeats, deep-intronic mutations (like the *OPA1* mutations exemplified by Matthis Synofzik[15]) or simply mutations in regions with low coverage by these methods.

This share of "missing heritability" in degenerative ataxias might now be substantially reduced by employing whole-genome sequencing (WGS), where costs have come down to $1500-2000 per genome and which can also capture intronic variants and deletions, as described by Andrea Nemeth and Rebecca Schüle. First systematic projects have been started investigating the use of WGS for clinical diagnostics, e.g. the "WGS500 project" launched by the Wellcome Trust Centre for Human Genetics Clinical in collaboration with Illumina, or the "Genomics England" project that aims to produce 100.000 genomes on cancer and rare diseases. First data from the field of intellectual disability indeed indicate that the diagnostic yield by WGS might be up to 50% higher than by WES[31]. Interestingly, however, the vast majority of mutations found by using WGS were found in *coding* regions, in case of intellectual disability mostly de novo SNPs and CNVs[31].

*The future of analyzing NGS results: trans-national collaborative efforts*

NGS approaches like panel sequencing, WES and WGS inevitably require a new framework of thinking. While classical ataxia genetics could be performed in single centers on a regional or national level, these NGS approaches require large -scale transnational collaborative efforts and databases, as convincingly argued by Rebecca Schüle. These efforts are not only needed to manage resources efficiently (the sheer size of *one* genome data-set is 0.1 terabyte, and the analysis and contextual interpretation of variant data for *multiple* genomes is almost impossible to handle for non-bioinformaticians and for genetic centers without sufficient bioinformatic resources). Large-scale collaborative efforts are also needed to produce sustainable results. Given the excess of rare variants in human populations[32], WES commonly produces lists of >250 rare, well conserved heterozygous variants. Rebecca Schüle demonstrated that this can lead to very questionable findings for both dominant and recessive ataxia genes, in particular for large and/or hypervariable genes. For example, a rare conserved variant in *SYNE1* can be found in 7% of all exome data-sets (394

out of 5990 exomes in the database GEM.app[33]). Large-scale collaborative databases here allow to unravel such frequencies and to critically reflect findings on seemingly novel ataxia variants and genes.

Questionable findings might result not only for recessive genes like *SYNE1*, but even more for dominant genes like *TMEM240*[34]. Evidence for pathogenicity of this gene in ataxia was taken from several isolated cases with de novo mutations in TMEM240 and then expanded to additional small families with only limited segregation data and without functional proof of the pathogenicity for several variants. Again, large-scale collaborative exome-databases here allow to broaden the perspective and to critically reflect on such variants. For example, an analysis from the 5990 exomes GEM.app reveals that rare (<0.01%) and well conserved *TMEM240* variants can be found, inter alia, in 1 patient with spastic ataxia, 2 patients with HSP, 1 patient with a mitochondrial phenotype, 2 patients with deafness, 2 patients with epilepsy, 5 patients with ALS, 7 patients with a neuromuscular phenotype, 1 patient with a brain malformation, 4 patients with cardiomyopathy. These findings show that – although some TMEM240 are likely to cause ataxia – other equally rare, well-conserved *TMEM240* variants ubiquitously occur in human disease, and are most likely not linked to ataxia.

In more general and illustrative terms, these findings show that, without access to large-scale multi-phenotype exome databases containing thousands of exomes and without rechecking them to scrutinize target findings, NGS-based gene hunting and variant identification might be mistaken like the blind men in front of the elephant: without having a comprehensive perspective on the different variant frequencies in *various* neurological and non-neurological exomes, one might be inclined to report a novel gene or variant as a novel ataxia gene, HSP gene, mitochondrial gene, deafness gene, ALS gene, or cardiomyopathy gene, without realizing that it might simply present only a little part of the *whole* picture (figure 1).

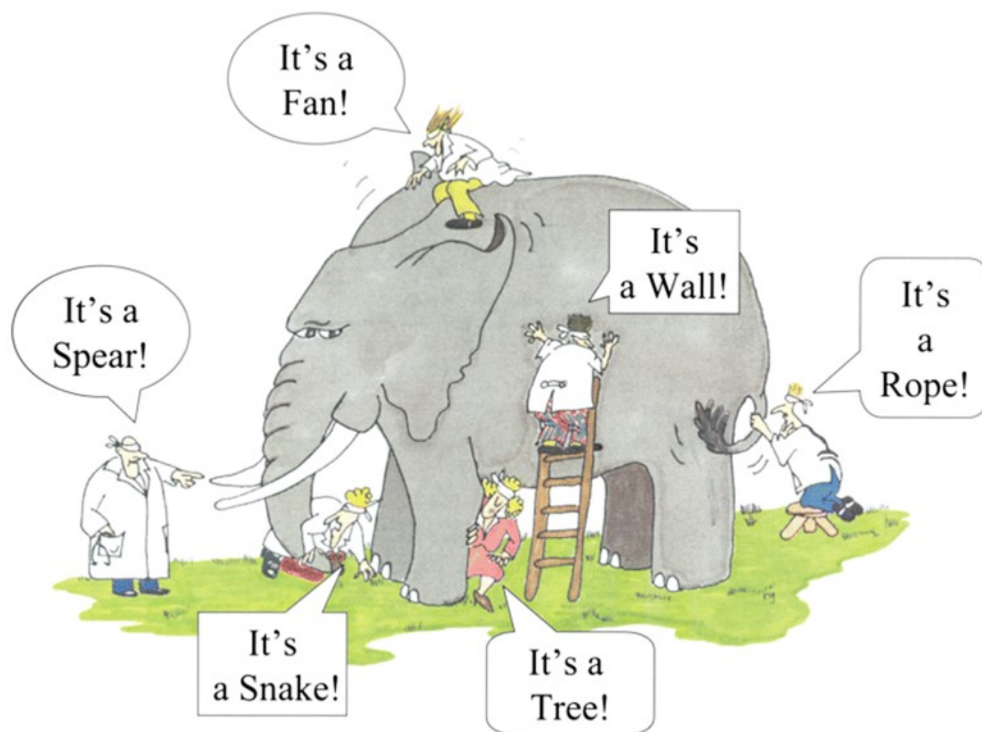


**Figure 1: Blind men and the elephant.**
Analyzing exomes only from a specific disease cohort of interest, without looking at exome-databases as a whole with multiple disease conditions might lead to the identification of a gene which only appears to be a novel gene of the particular condition of interest ("It's an ataxia gene!" "It's an cardiomyopathy gene!"). A broader analysis of this gene also in large exome data-sets of other conditions might have revealed that variants in this gene are in fact an unspecific finding, given that they are ubiquitous in manifold human diseases (figure taken from www.searchenginewatch.com, accessed November 8$^{th}$, 2015).

Such misinterpretations can be avoided by collaborative databases which include large exome sharing platforms, like e.g. GEM.app[33] (now GENESIS) which brings together >600 investigators from 44 countries and >6000 exomes and >1000 genomes from >90 disease phenotypes[35]. Within only 4 years, this collaborative database has allowed to collectively achieve 62 gene identifications or published studies that have expanded phenotype/genotype correlations [35], with large-scale multinational exome studies on *PNPLA6*[10], *STUB1*[36], *OPA1*[15], *DNAJC3*[6] or *KCNA2*[37] as examples from the field of ataxia genetics. This demonstrates that collaborative exome databases also allow to drastically reduce the cycle time to discovery of novel ataxia genes or phenotype/genotype spectra.


1. Abdel-Salam G, Thoenes M, Afifi HH, Korber F, Swan D, Bolz HJ. The supposed tumor suppressor gene WWOX is mutated in an early lethal microcephaly syndrome with epilepsy, growth retardation and retinal degeneration. *Orphanet journal of rare diseases.* 2014;9:12.
2. Mignot C, Lambert L, Pasquier L, et al. WWOX-related encephalopathies: delineation of the phenotypical spectrum and emerging genotype-phenotype correlation. *J Med Genet.* 2015;52:61-70.
3. Mallaret M, Synofzik M, Lee J, et al. The tumour suppressor gene WWOX is mutated in autosomal recessive cerebellar ataxia with epilepsy and mental retardation. *Brain*. 2014;137:411-419.
4. Guissart C, Li X, Leheup B, et al. Mutation of SLC9A1, encoding the major Na(+)/H(+) exchanger, causes ataxia-deafness Lichtenstein-Knorr syndrome. *Human molecular genetics.* 2015;24:463-470.
5. Jobling RK, Assoum M, Gakh O, et al. PMPCA mutations cause abnormal mitochondrial protein processing in patients with non-progressive cerebellar ataxia. *Brain*. 2015;138:1505-1517.
6. Synofzik M, Haack TB, Kopajtich R, et al. Absence of BiP Co-chaperone DNAJC3 Causes Diabetes Mellitus and Multisystemic Neurodegeneration. *American journal of human genetics.* 2014;95:689-697.
7. an Gassen KL, van der Heijden CD, de Bot ST, et al. Genotype-phenotype correlations in spastic paraplegia type 7: a study in a large Dutch cohort. *Brain*. 2012;135:2994-3004.
8. Klebe S, Depienne C, Gerber S, et al. Spastic paraplegia gene 7 in patients with spasticity and/or optic neuropathy. *Brain*. 2012;135:2980-2993.
9. Pfeffer G, Pyle A, Griffin H, et al. SPG7 mutations are a common cause of undiagnosed ataxia. *Neurology*. 2015;84:1174-1176.
10. Synofzik M, Gonzalez MA, Lourenco CM, et al. PNPLA6 mutations cause Boucher-Neuhauser and Gordon Holmes syndromes as part of a broad neurodegenerative spectrum. *Brain.* 2014;137:69-77.
11. Tarnutzer AA, Gerth-Kahlert C, Timmann D, et al. Boucher-Neuhauser syndrome: cerebellar degeneration, chorioretinal dystrophy and hypogonadotropic hypogonadism: two novel cases and a review of 40 cases from the literature. *J Neurol.* 2015;262:194-202.
12. Synofzik M, Hufnagel R, Zuchner S. PNPLA6-Related Disorders. In: Pagon RA, Adam MP, Ardinger HH, et al., eds. GeneReviews(R). Seattle (WA). Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK247161/2014.
13. Hufnagel RB, Arno G, Hein ND, et al. Neuropathy target esterase impairments cause Oliver-McFarlane and Laurence-Moon syndromes. *J Med Genet.* 2015;52:85-94.
14. Kmoch S, Majewski J, Ramamurthy V, et al. Mutations in PNPLA6 are linked to photoreceptor degeneration and various forms of childhood blindness. *Nature communications.* 2015;6:5614.
15. Bonifert T, Karle KN, Tonagel F, et al. Pure and syndromic optic atrophy explained by deep intronic OPA1 mutations and an intralocus modifier. *Brain.* 2014;137:2164-2177.
16. Parolin Schnekenberg R, Perkins EM, Miller JW, et al. De novo point mutations in patients diagnosed with ataxic cerebral palsy. *Brain.* 2015;138:1817-1832.
17. Bomont P, Watanabe M, Gershoni-Barush R, et al. Homozygosity mapping of spinocerebellar ataxia with cerebellar atrophy and peripheral neuropathy to 9q33-34, and with hearing impairment and optic atrophy to 6p21-23. Eur J *Hum Genet*. 2000;8:986-990.
18. Foley AR, Menezes MP, Pandraud A, et al. Treatable childhood neuronopathy caused by mutations in riboflavin transporter RFVT2. *Brain.* 2014;137:44-56.
19. Utine GE, Haliloglu G, Salanci B, et al. A homozygous deletion in GRID2 causes a human phenotype with cerebellar ataxia and atrophy. *J Child Neurol*. 2013;28:926-932.

20. Hills LB, Masri A, Konno K, et al. Deletions in GRID2 lead to a recessive syndrome of cerebellar ataxia and tonic upgaze in humans. *Neurology.* 2013;81:1378-1386.
21. Coutelier M, Burglen L, Mundwiller E, et al. GRID2 mutations span from congenital to mild adult-onset cerebellar ataxia. *Neurology.* 2015;84:1751-1759.
22. Anheim M, Tranchant C, Koenig M. The autosomal recessive cerebellar ataxias. *N Engl J Med.* 2012;366:636-646.
23. Palau F, Espinos C. Autosomal recessive cerebellar ataxias. *Orphanet journal of rare diseases.* 2006;1:47.
24. Fogel BL, Perlman S. Clinical features and molecular genetics of autosomal recessive cerebellar ataxias. Lancet *Neurol.* 2007;6:245-257.
25. Coutelier M, Stevanin G, Brice A. Genetic landscape remodelling in spinocerebellar ataxias: the influence of next-generation sequencing. *J Neurol.* 2015;262:2382-2395.
26. Nemeth AH, Kwasniewska AC, Lise S, et al. Next generation sequencing for molecular diagnosis of neurological disorders using ataxias as a model. *Brain.* 2013;136:3106-3118.
27. Fogel BL, Lee H, Deignan JL, et al. Exome sequencing in the clinical diagnosis of sporadic or familial cerebellar ataxia. *JAMA neurology.* 2014;71:1237-1246.
28. 28. Pyle A, Smertenko T, Bargiela D, et al. Exome sequencing in undiagnosed inherited and sporadic ataxias. *Brain.* 2015;138:276-283.
29. Sandford E, Li JZ, Burmeister M. Evaluation of exome sequencing variation in undiagnosed ataxias. *Brain.* 2015;138:e383.
30. Dixon-Salazar TJ, Silhavy JL, Udpa N, et al. Exome sequencing can improve diagnosis and alter patient management. *Science translational medicine.* 2012;4:138ra178.
31. Gilissen C, Hehir-Kwa JY, Thung DT, et al. Genome sequencing identifies major causes of severe intellectual disability. *Nature.* 2014;511:344-347.
32. Fu W, O'Connor TD, Jun G, et al. Analysis of 6,515 exomes reveals the recent origin of most human protein-coding variants. *Nature.* 2013;493:216-220.
33. Gonzalez MA, Lebrigio RF, Van Booven D, et al. GEnomes Management Application (GEM.app): A New Software Tool for Large-Scale Collaborative Genome Analysis. *Hum Mutat.* 2013;34:842-846.
34. Delplanque J, Devos D, Huin V, et al. TMEM240 mutations cause spinocerebellar ataxia 21 with mental retardation and severe cognitive impairment. *Brain.* 2014;137:2657-2663.
35. Gonzalez M, Falk M, Gai X, Schule R, Zuchner S. Innovative Genomic Collaboration Using the GENESIS (GEM.app) Platform. *Hum Mutat.* 2015.
36. Synofzik M, Schule R, Schulze M, et al. Phenotype and frequency of STUB1 mutations: next-generation screenings in Caucasian ataxia and spastic paraplegia cohorts. *Orphanet journal of rare diseases.* 2014;9:57.
37. Syrbe S, Hedrich UB, Riesch E, et al. De novo loss- or gain-of-function mutations in KCNA2 cause epileptic encephalopathy. *Nat Genet.* 2015;47:393-399.

The Journal of Rare DISORDERS

# GENETIC AND MOLECULAR MECHANISMS OF THE ATAXIAS

Sanjay I. Bidichandani
Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Of the twelve presentations in this session, seven concerned the molecular pathogenesis of Friedreich's ataxia (FRDA), and five described various molecular mechanisms underlying the autosomal dominant spinocerebellar ataxias (SCAs). The presentations covered a wide range of topics, including identification of new gene mutations, molecular consequences of mutations, transcriptome analysis, post-translational modification and abnormal subcellular localization of mutant proteins, and histopathological consequences. Despite the wide range of topics and methodologies, three major themes dominated the discussion: (a) the mechanisms by which the expanded GAA triplet-repeat mutation in FRDA results in *FXN* transcriptional deficiency; (b) the processing and subcellular localization of ataxin-3 as it relates to the pathogenesis of SCA type 3 (SCA3); and (c) the role of DNA damage and the response to damaged DNA in the pathogenesis of inherited ataxias. This review will describe the salient features of all twelve presentations, focusing particularly on the aforementioned three themes. It should be noted that this review will be limited to the information that was presented in this session, and although some contextual background information will be mentioned, due to space constraints it is not possible to reference the vast amount of already published information upon which the presentations were based.

*Molecular Pathogenesis of FRDA*

Almost two decades after the discovery of the expanded GAA triplet-repeat mutation in the *FXN* gene as the most common cause of FRDA [1], we are developing a nuanced understanding of how this mutation ultimately leads to *FXN* transcriptional deficiency. Located in intron 1, the polymorphic GAA triplet-repeat sequence in non-FRDA alleles contains <30 GAA triplets and most individuals with FRDA are homozygous for expanded alleles containing 100 – 1300 GAA triplets. The expanded GAA triplet-repeat results in *FXN* transcriptional deficiency and patients have 5-20% of the normal level of *FXN* transcript. The length of the shorter of the two expanded GAA triplet-repeat alleles determines the severity of *FXN* transcriptional deficiency and the resulting clinical phenotype. Patients who have at least one expanded allele containing <500 GAA triplets tend to have higher *FXN* transcript levels, a later age of onset [2], and slower progression of disease [3].

Deciphering the mechanisms by which the expanded GAA triplet-repeat causes *FXN* transcriptional deficiency was the subject of four of the presentations. Sanjay Bidichandani (University of Oklahoma, USA) presented data in support of epigenetic silencing of the *FXN* promoter in FRDA. The presentation included evidence of spreading of repressive chromatin from the expanded GAA triplet-repeat to the *FXN* promoter, leading to altered nucleosome positioning at the transcription start site as assayed in individual chromatin fibers, and ultimately a severe (5-10 fold) deficiency of transcriptional initiation in living cells[4]. A milder deficiency of promoter function was noted in cell lines from patients carrying one expanded allele with <400 GAA triplets, indicating that promoter silencing is dependent on the length of the repeat, thus further substantiating the etiological relationship between the expanded repeat and epigenetic promoter silencing [5]. The humanized mouse model of FRDA (YG8sR) [6] was also shown to have *FXN* promoter silencing, indicating that this mechanism is not limited to patient-derived cell lines. Treatment with the histone deacetylase inhibitor 109 (RG2833) [7] resulted in significant improvement of both promoter structure and function, indicating that epigenetic promoter silencing in FRDA is reversible. However, the precise mechanism underlying the upstream spread of repressive chromatin to the *FXN* gene promoter remains unknown. Ana Silva (Oxford University, UK) presented elegant data using a single-cell assay, which showed that the expanded *FXN* allele is preferentially repositioned close to the nuclear periphery [8]. The heterologous model system, which is based on the HEK cell line carrying either GAA-6 or GAA-310, allowed spatial localization of the transgene within the nucleus via DNA-FISH, and real-time measurement of transcription via RNA-FISH. Treatment with histone deacetylase inhibitors (106 and nicotinamide) [9,10] caused the transcriptionally silent allele that was localized near the nuclear periphery to be both de-repressed and mobilized away from the nuclear lamina, indicating that the abnormal intra-nuclear positioning of the *FXN* locus in FRDA is reversible. Cells from heterozygous carriers (i.e., with one expanded GAA triplet-repeat allele

and one normal allele) showed that the endogenous expanded *FXN* allele was also transcriptionally silenced and preferentially localized near the nuclear periphery, indicating that the abnormal intra-nuclear localization was not an artifact of the heterologous system. RNA-FISH, using a probe located upstream of the expanded GAA triplet-repeat, revealed significantly reduced nascent RNA production. The deficiency of transcriptional initiation arising specifically from the expansion-bearing *FXN* allele in single cells lends further support to the existence of epigenetic promoter silencing in FRDA. However, it is not yet known if *FXN* transcriptional silencing precedes or follows the physical repositioning of the expanded allele to the nuclear periphery, but Ana Silva (and her colleague at Oxford University, Michele Lufino, who spoke in a later session) speculated that the nuclear periphery may be the site where heterochromatin is established on the expanded allele.

Marek Napierala (University of Alabama, USA), who has amassed an impressive amount of RNA-Seq data (derived from primary fibroblasts of 18 FRDA patients and 17 non-FRDA controls; using both polyadenylated and non-polyadenylated RNA), presented evidence for a reduced rate of transcriptional elongation through intron 1 in FRDA. By assaying almost the entire length (~10 kb) of intron 1, Marek Napierala showed that the rate of transcriptional elongation through the *FXN* gene in FRDA was half of what is seen in non-FRDA controls. Furthermore, he showed that transcriptional deficiency in FRDA is limited to the *FXN* locus, confirming that the expression of neighboring genes remains unaffected by the *cis*-acting silencing effects of the expanded GAA triplet-repeat mutation. Natalia Gromak (Oxford University, UK) presented evidence for R-loop formation *in vivo* in the vicinity of the expanded GAA triplet-repeat mutation in FRDA [11]. Using DNA immunoprecipitation it was shown that the R-loop structure, which is likely formed by the GAA-rich transcript interacting with the GAA-TTC DNA duplex, extends from the expanded GAA triplet-repeat to involve upstream regions of intron 1, although it does not reach the *FXN* promoter. Intriguingly, in a manner reminiscent of RNA-mediated silencing, which is a well-established mediator of heterochromatin formation in *S. pombe* [12], Natalia Gromak found increased occupancy of Argonaut proteins extending towards the *FXN* promoter. These data suggest a dual role for R-loop formation in FRDA; serving to recruit silencing factors that result in epigenetic silencing of the *FXN* promoter, and directly contributing to the reduction in the rate of transcriptional elongation through intron 1.

Whereas the vast majority (>90%) of FRDA patients are homozygous for the expanded GAA triplet-repeat mutation, some patients are compound heterozygous for the expanded GAA triplet-repeat mutation and another (conventional) deleterious intragenic *FXN* mutation [1]. Marguerite Evans-Galea (Murdoch Children's Research Institute, Australia) analyzed a large cohort of compound heterozygous individuals in an attempt to evaluate the phenotypic consequences of deleterious intragenic *FXN* mutations. Clinical information from 111 compound heterozygotes (81 previously published and 30 new patients) was compared with data from 131 homozygous individuals. Deleterious *FXN* mutations were classified into three categories: "null," or based on the predicted relative impact on stability / function of frataxin as "moderate-to-severe" or "mild". Individuals with null mutations showed an earlier age of onset (accounting for the length of the expanded GAA triplet-repeat mutation in the homologous allele), and had a higher risk of developing diabetes compared with homozygous individuals. Interestingly, homozygous individuals had a higher prevalence of cardiomyopathy compared to all three groups of compound heterozygotes, including those carrying null mutations.

Cardiomyopathy (and its sequelae) is the leading cause of mortality in FRDA [13]. Cardiomyopathy in FRDA is typically concentric and shows histopathological features that are consistent with inflammatory necrosis. Arnulf Koeppen (Albany Medical College, USA) presented evidence of cardiac remodeling in FRDA [14]. Using immunohistochemistry and laser scanning confocal immunofluorescence microscopy in 15 FRDA hearts and 12 non-FRDA controls, Arnulf Koeppen discovered a paucity and abnormal morphology of intercalated discs, and abnormal distribution of myocardial gap junctions. Therefore, in addition to the inflammatory pathology, cardiomyopathy in FRDA also includes features of inefficient mechanical coupling and abnormal distribution of ion channels.

*Molecular Pathogenesis of Autosomal Dominant SCAs*

SCA3 (or Machado-Joseph disease) is a neurodegenerative disorder caused by a CAG triplet-repeat expansion in the *MJD1* gene. This leads to an expanded polyglutamine tract in ataxin-3, which forms intra-nuclear aggregates in neurons

of SCA3 patients [15]. Neurotoxicity is thought to stem, at least in part, from the nuclear localization of mutant ataxin-3, which is normally cytoplasmic in neurons [16]. Thorsten Schmidt (and his colleague, Olaf Reiss, from the University of Tübingen, Germany) screened a library of FDA-approved compounds in order to identify potentially therapeutic agents with the ability to reduce nuclear translocation of ataxin-3. They found four such compounds, two of which acted specifically on expanded ataxin-3. These compounds were validated in a *C. elegans* model as an initial step towards their development as therapies for SCA3. In another talk, also focused on SCA3 pathogenesis, Ana Teresa Simões (and her colleague, Luís Pereira de Almeida, from Coimbra University, Portugal) mutated potential calpain cleavage sites in an attempt to determine the portion of ataxin-3 that contributes to neurotoxicity. By mutating three putative calpain sites they identified a 26 kD fragment that, via lentiviral transduction of adult mouse brain, contributed to ataxin-3 nuclear aggregation and neurotoxicity.

Autosomal dominant SCAs are caused by mutations in over 20 different genes [17], which result in either a pure ataxic phenotype or a complex phenotype that includes ataxia. As part of the UK Brain Expression Consortium, Conceição Bettencourt (University College London, UK) presented a study of whole transcriptome profiles from 788 brain regions of 101 neuropathologically healthy individuals. Expression patterns of 24 different autosomal dominant SCA genes were included in a weighted gene co-expression network analysis (WGCNA) in order to identify possible relationships between the various SCA genes and potentially overlapping pathophysiological pathways. WGCNA revealed two cerebellar co-expression modules that were simultaneously enriched for SCA transcripts and either cerebellar granule cell (GC) or Purkinje cell (PC) markers. The GC module corresponded with SCA genes associated with a complex phenotype and the PC module with those causing pure ataxia. A pilot RNA-Seq study of three SCA3 and four controls revealed that SCA3 is co-expressed with the GC module.

Finally, Eleonora Di Gregorio (University of Torino, Italy) described the discovery of the gene for SCA38, which presents as a slowly progressive pure cerebellar ataxia. By linkage analysis and targeted sequencing they identified missense mutations in the *ELOVL5* gene [18], which encodes an elongase that is involved in polyunsaturated fatty acid synthesis, especially docosahexaenoic acid (DHA). The product of *ELOVL5*, which is normally located in the endoplasmic reticulum (ER), is translocated to the Golgi in SCA38. *ELOVL5* missense mutations cause abnormal activation of the unfolded protein response, supporting a dominant gain-of-function pathogenic mechanism. *ELOVL5* expression is regulated by a DHA-dependent feedback loop, and accordingly DHA-treatment of lymphoblasts from individuals with SCA38 resulted in reduction of *ELOVL5* expression. This observation has potential therapeutic implications for SCA38 however a pathogenic component attributable to loss-of-function in the ER may also contribute to SCA38.

*DNA Damage and Response in Ataxias*

Mutations in various genes involved in DNA repair and the response to DNA damage are known to cause cancer, syndromes of premature aging, and inherited ataxias. Examples of the latter include ataxia telangiectasia, spinocerebellar ataxia with axonal neuropathy 1, and ataxia with oculomotor apraxia types 1 and 2. Steve Jackson (Cambridge University, UK), whose lab studies the cellular response to DNA double strand breaks (DSBs) described the use of genetic screens to identify post-translational modifications of DSB response proteins as potential treatments for genetic diseases. He described the identification of a small molecule that corrects the abnormal nuclear morphology in Hutchinson-Gilford syndrome [19], a form of premature aging. This molecule, acting via an acetyl-transferase protein target (NAT10), reduced DNA damage and cellular senescence, and increased the lifespan of a mouse model. An implication of this success is that such screens may also be fruitful in the identification of potential therapies for inherited ataxias caused by abnormal DNA repair or response to DNA damage.

Michael Themis (and his colleague, Mark Pook, from Brunel University London, UK) found that fibroblasts from people with FRDA and from the YG8sR humanized mouse model [6] have an increased prevalence of DSBs. Both human and murine cells were also deficient in the repair of radiation-induced DSBs. The DSB repair defect was reversed by overexpressing frataxin via lentiviral infection. These data indicate that frataxin deficiency is associated with increased genomic instability, which is reversible in cultured fibroblasts. However, it remains to be determined if this deficiency in DSB repair influences the neurodegeneration and other phenotypes seen in FRDA.

1. Campuzano V, Montermini L, Molto MD, Pianese L, Cossee M, Cavalcanti F, et al. Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science*. 1996;271(5254):1423-7.

2. Durr A, Cossee M, Agid Y, Campuzano V, Mignard C, Penet C, et al. Clinical and genetic abnormalities in patients with Friedreich's ataxia. *N Engl J Med*. 1996;335(16):1169-75.
3. Regner SR, Wilcox NS, Friedman LS, Seyer LA, Schadt KA, Brigatti KW, et al. Friedreich ataxia clinical outcome measures: natural history evaluation in 410 participants. *Journal of Child Neurology*. 2012;27(9):1152-8.
4. Chutake YK, Costello WN, Lam C, Bidichandani SI. Altered Nucleosome Positioning at the Transcription Start Site and Deficient Transcriptional Initiation in Friedreich Ataxia. J Biol Chem. 2014;289(22):15194-202.
5. Chutake YK, Lam C, Costello WN, Anderson M, Bidichandani SI. Epigenetic promoter silencing in Friedreich ataxia is dependent on repeat length. *Ann Neurol*. 2014;76(4):522-8.
6. Anjomani Virmouni S, Ezzatizadeh V, Sandi C, Sandi M, Al-Mahdawi S, Chutake Y, et al. A novel GAA-repeat-expansion-based mouse model of Friedreich's ataxia. *Dis Model Mech*. 2015;8(3):225-35.
7. Soragni E, Miao W, Iudicello M, Jacoby D, De Mercanti S, Clerico M, et al. Epigenetic therapy for Friedreich ataxia. *Ann Neurol*. 2014;76(4):489-508.
8. Silva AM, Brown JM, Buckle VJ, Wade-Martins R, Lufino MM. Expanded GAA repeats impair FXN gene expression and reposition the FXN locus to the nuclear lamina in single cells. *Hum Mol Genet*. 2015;24(12):3457-71.
9. Soragni E, Xu C, Plasterer HL, Jacques V, Rusche JR, Gottesfeld JM. Rationale for the development of 2-aminobenzamide histone deacetylase inhibitors as therapeutics for Friedreich ataxia. *J Child Neurol*. 2012;27 (9):1164-73.
10. Chan PK, Torres R, Yandim C, Law PP, Khadayate S, Mauri M, Grosan C, Chapman-Rothe N, Giunti P, Pook M, Festenstein R. Heterochromatinization induced by GAA-repeat hyperexpansion in Friedreich's ataxia can be reduced upon HDAC inhibition by vitamin B3. *Hum Mol Genet*. 2013;22(13):2662-75.
11. Groh M, Lufino MM, Wade-Martins R, Gromak N. R-loops associated with triplet repeat expansions promote gene silencing in Friedreich ataxia and fragile X syndrome. *PLoS Genet*. 2014;10(5):e1004318.
12. Grewal SI, Elgin SC. Transcription and RNA interference in the formation of heterochromatin. *Nature* 2007;447 (7143):399-406.
13. Tsou AY, Paulsen EK, Lagedrost SJ, Perlman SL, Mathews KD, Wilmot GR, Ravina B, Koeppen AH, Lynch DR. Mortality in Friedreich ataxia. *J Neurol Sci*. 2011;307(1-2):46-9.
14. Koeppen AH, Ramirez RL, Becker AB, Bjork ST, Levi S, Santambrogio P, Parsons PJ, Kruger PC, Yang KX, Feustel PJ, Mazurkiewicz JE. The pathogenesis of cardiomyopathy in Friedreich ataxia. *PLoS One.* 2015;10(3):e0116396.
15. Paulson HL, Perez MK, Trottier Y, Trojanowski JQ, Subramony SH, Das SS, Vig P, Mandel JL, Fischbeck KH, Pittman RN. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron.* 1997;19:333–44.
16. Paulson HL, Das SS, Crino PB, Perez MK, Patel SC, Gotsdiner D, Fischbeck KH, Pittman RN. Machado-Joseph disease gene product is a cytoplasmic protein widely expressed in brain. *Ann. Neurol*. 1997;41:453–62.
17. Bird TD. Hereditary Ataxia Overview. 1998 [updated 2015 Jun 11]. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2015. Available from http://www.ncbi.nlm.nih.gov/books/NBK1138/
18. Di Gregorio E, Borroni B, Giorgio E, Lacerenza D, Ferrero M, Lo Buono N, et al. ELOVL5 mutations cause spinocerebellar ataxia 38. *Am J Hum Genet*. 2014;7;95(2):209-17.
19. Larrieu D, Britton S, Demir M, Rodriguez R, Jackson SP. Chemical inhibition of NAT10 corrects defects of laminopathic cells. *Science.* 2014;344(6183):527-32.

# CELLULAR AND ANIMAL MODELS OF FRIEDREICH'S ATAXIA

Michele M.P. Lufino[1] and Hélène Puccio[2, 3, 4, 5, 6]

[1]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1 3QX, UK

[2]Translational Medecine and Neurogenetics, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), 67400 Illkirch, France

[3]INSERM, U596, 67400 Illkirch, France

[4]CNRS, UMR7104, 67400 Illkirch, France

[5]Université de Strasbourg, 67000 Strasbourg, France

[6]Collège de France, Chaire de génétique humaine, 67400 Illkirch, France

In session 3a of the International Ataxia Research Conference 2015 a series of novel methods and models were described to address poorly understood areas of FRDA, such as the mechanism of *FXN* silencing induced by GAA repeats, the pathological mechanism of neurodegeneration observed in FRDA sensory neurons and the potential contribution of mitochondrial dynamics to the FRDA phenotype.

Hélène Puccio described the development of a novel conditional animal model of FRDA by crossing a mouse expressing Cre-recombinase under the Parvalbumin (Pvalb) promoter with mice carrying a conditional *FXN* allele. This model represents an improvement of the previously developed conditional NSE-Cre and PRP-Cre $ER^T$ models, which have shown limitations such as variability in the efficiency of *FXN* deletion (PrP-Cre $ER^T$), non-FRDA phenotypes such as spongiform neurodegeneration (NSE-Cre) and finally, high disease severity non-representative of FRDA (NSE-Cre). Hélène Puccio demonstrated that Pvalb-Cre KO mice are characterized by markedly reduced *FXN* expression in the DRG and cerebellum and by a progressive ataxia phenotype as demonstrated by motor coordination tests and loss of sensorimotor reflexes. The histological and pathophysiological analysis of the mice is ongoing. This novel model effectively replaces the previously developed conditional models, since it closely recapitulates the sensory neurodegeneration seen in FRDA.

Michele Lufino presented recent advances on the generation and use of genomic-reporter models of FRDA, which carry reporter genes within the context of a GAA-expanded *FXN* genomic DNA locus. Michele Lufino described the use of *FXN -GAA-Luc* cells for a structure-activity relationship (SAR) study around the *FXN* up-regulating compound C5, which led to the identification of derivatives with improved properties. Furthermore, the author presented the development of a *FXN-GAA-Luc* mouse model which provides sensitive detection of FXN-luciferase protein in live mice, thus allowing *in vivo* validation of *FXN*-increasing compounds. Finally, Michele Lufino presented an RNA-FISH study in FRDA lymphoblastoid cells, which demonstrated at single-cell resolution that expanded GAA repeats reduce *FXN* expression predominantly by impairing initiation of *FXN* transcription, in agreement with data presented by Sanjay Bidichandani in session 2 of the International Ataxia Research Conference 2015.

Vijayendran Chandran presented the development of an inducible *FXN* knock-down mouse model of FRDA, where an shRNA targeting the *FXN* gene is expressed following doxycycline administration in drinking water. Vijayendran Chandran showed how the administration of doxycycline effectively resulted in multiple organ *FXN* knock-down, which negatively affected mouse survival and weight. Furthermore, these mice developed an ataxic phenotype with reduced coordination and muscle strength. Interestingly, preliminary results suggest that these FRDA-associated phenotypes can be rescued by removal of doxycycline after 12 weeks of continuous treatment. This model is particularly useful to study the progression of disease and to dissect the reversibility of the FRDA pathogenesis.

Simona Donatello described the generation and molecular characterization of neuronal cells differentiated from control- and FRDA patient-derived induced pluripotent stem cells (iPSCs). Simona Donatello showed that both control and FRDA iPSCs successfully differentiated to neuronal and astrocytic cells, although FRDA iPSCs presented a slight delay in maturation. The author showed that FRDA iPSCs are characterized by reduced expression of frataxin and Fe-S proteins, and by increased oxidative stress. Furthermore, Dr Donatello demonstrated that treatment of differentiated

cells with the HDAC inhibitor 109 and exendin-4 normalized the FRDA-like phenotypes of FRDA iPSCs, thus suggesting the use of FRDA iPSC-derived cells as a platform for functional validation of therapies for FRDA.

Jordi Magrane presented the development of novel methods to analyze the impact of abnormal mitochondrial dynamics on the pathogenesis of FRDA. Jordi Magrane analysed mitochondrial dynamics in cultured FRDA sensory neurons isolated from the GAA-based FRDA mouse models B6.Cg-Tg(FXN)1Sars $Fxn^{tm1Mkn}$ and B6.129-$Fxn^{tm1.1Pand}$ and identified synaptic abnormalities in the FRDA neurons. Furthermore, in order to determine mitochondrial dynamics *in vivo*, Jordi Magrane developed a novel method to visualize mitochondria in live FRDA mice, by crossing the FRDA mice with mitoDendra transgenic mice.

Juan Antonio Navarro Langa developed a model to study mitochondrial dynamics in FRDA by utilizing a drosophila model of FRDA, expressing an shRNA targeting the drosophila frataxin homolog (*dfh*). The author identified an accumulation of mitochondria due to a mitophagy dysfunction, thus preventing mitochondrial degradation. Furthermore, Juan Antonio Navarro Langa showed that knock-down of mitofusin (*mfn*) is capable of rescuing the abnormal mitochondrial accumulation and locomotor performance of the *dfh* RNAi model.

Finally, Jose Vicente Llorens presented a novel drosophila reporter model carrying expanded GAA repeats upstream of a firefly luciferase gene to allow the identification of genetic modifiers of *FXN* silencing induced by GAA expansions. Jose Vicente Llorens generated two constructs, carrying either 9 GAA repeats or 300 GAA repeats, and showed that the presence of 300 GAA repeats reduced firefly expression and caused chromatin condensation in flies. Finally, Jose Vicente Llorens carried out a genetic screen using the 300 GAA-firefly luciferase model and identified SU(VAR)3-9, SU (VAR)2-5 and SU(VAR)2-1 as regulators of the transcriptional repression induced by GAA expansions.

In summary, a series of advanced *in vitro* and *in vivo* models and methods were described which in our opinion are suitable to address different unknown areas of the FRDA pathogenesis and to validate therapies for FRDA. While *FXN-GAA-Luc* genomic-reporter cells allow easy identification of *FXN* up-regulating compounds, neuronal cells differentiated from FRDA iPSCs closely recapitulate the molecular phenotype observed in FRDA, thus representing an advanced model for the functional validation of *FXN*-increasing compounds. Single-cell techniques are particularly suitable for the dissection of the GAA-induced *FXN* transcriptional silencing mechanism since they overcome the possible artifacts associated with the analysis of averaged cell samples. The Pvalb-Cre KO mouse model by causing a selective loss of frataxin in sensory neurons recapitulates the progressive nature of FRDA, allowing the dissection of the pathophysiological mechanism of FRDA as it develops in time. The *FXN* knock-down model instead, causes a controllable and reversible systemic loss of *FXN* expression, which seems particularly suitable for addressing the question of the reversibility of FRDA after long term exposure to decreased frataxin levels. The *FXN-GAA-Luc* mouse model allows sensitive *in vivo* detection of frataxin protein, thus representing a highly suitable tool for the analysis of the effect of *FXN* up-regulating compounds on frataxin protein over time. Finally, the analysis of mitochondrial accumulation in the drosophila knock-down model and the characterization of mitochondrial dynamics in live mitoDendra mice will allow the assessment of the potential contribution of mitochondrial dynamics to the neurodegeneration observed in FRDA.

# CELLULAR AND SYSTEMIC PATHWAYS

Giovanni Manfredi

Professor of Neurology, Neurology, Weill Cornell Medical College

*Aberrant cerebellar output in ataxia, a common theme- Kamran Khodakhan (Albert Einstein College of Medicine, USA)*

Cerebellar ataxias are caused by the failure of the information that the cerebellum provides to the motor system. In many forms of ataxias the signal to noise ratio of the information is reduced.

The main computational units of the cerebellum are the Purkinje cells, which fire spontaneously with a very precise clock-like pace making rhythm. This precise pace making allows Purkinje cells to accurately encode synaptic information in their firing rate. Causes underlying defective cerebellar output to the motor system include loss of information, such as loss of synaptic function, defects in computation such as dysfunction of ion channels, synapses, dendrites, calcium signaling, plasticity, and connectivity, increased noise of the neurons, or reduced averaging, such as in loss of Purkinje cells and silent Purkinje cells. To study the consequences of increased noise in the cerebellar output optogenetic techniques were used to incorrectly stimulate the cerebellar nuclei *in vivo*.

Another example of ataxia caused by increased cerebellar output noise is the tottering mouse model of episodic ataxia type-2 (EA2) associated with mutation in the gene encoding P/Q-type voltage-gated calcium channels. This mouse has an ataxic phenotype and episodic attacks of dyskinesia triggered by caffeine, ethanol and stress. During peak motor dysfunction in the tottering mouse, cerebellar output is extremely erratic, and the noise in cerebellar output correlates with the severity of the motor symptoms.

The activity of deep cerebella nuclei (DCN) neurons is altered in several ataxic mice. As an example, a model of ataxia telangiectasia was generated by acute knock down of the ATM gene using AAV-shRNA. In this model, there was a good correlation between severity of motor dysfunction and the noise in the cerebellar output. Importantly, improving the precision of pace making in Purkinje cells can improve motor dysfunction in ataxic mice. For example, chlorzoxazone restores DCN firing and improves motor performance in the waddler mouse, suggesting potential therapeutic avenues in patients with cerebellar dysfunction.

*Why does polyglutamine expansion cause dominantly inherited ataxia? Lesson from SCA3 -Henry Paulson (University of Michigan, USA)*

Polyglutamine diseases cause various forms of neurodegeneration, including Huntington disease and spinobulbar muscular atrophy. Polyglutamine (polyQ) repeat expansion in disparate genes is the most common cause of spinal cerebellar ataxia (SCA). However, how such expansions, which are localized in different domains of proteins with different functions, lead to neuronal death is largely unknown.

Specific protein context is likely central to polyQ diseases. In SCA3, the most common form of SCA, the polyQ expansion affects ataxin 3 (ATXN3), a highly specialized de-ubiquitinase (DUB), associated with numerous cellular pathways, including autophagy, endoplasmic reticulum-associated protein degradation (ERAD), and aggreosome formation. All forms of polyQ disease share the neuropathological presence of intracellular protein aggregates, which are reproduced in cellular and animal genetic models of the diseases. While the normal function of the expanded protein can be partly preserved, such as in the case of ATXN3, the interactions with other proteins may be affected. Mutant ATXN3, for example, binds CHIP, a key quality control ligase, more tightly than normal. This change in binding is associated with decreased CHIP (C-terminus of Hsc70 Interacting Protein) in mouse models of SCA3. Since CHIP is at the intersection of major protein quality control pathways, including chaperones, autophagy, and proteasomal degradation, alterations in CHIP levels can lead to severe pathological consequences. This hypothesis is supported by the observation that genetic ablation of CHIP worsens the disease in the mouse model of SCA3. Furthermore, loss of CHIP causes increased aggregation of expanded ATXN3.

With expanded CAG repeats, splice isoforms production can differ, resulting in proteins with markedly different aggregation propensity. A knock in mouse model was generated to recreate different ATXN3 isoforms in the context of the effects of polyQ expansion. In this model, Increased aggregation propensity of mutant ATXN3 was associated with transcriptional changes, suggesting an unanticipated role for oligodendrocytes.

The multilayered pathogenic pathways identified in SCA3, and possibly other polyQ diseases, indicate different levels of potential therapeutic intervention: reduce disease protein expression (e.g., RNAi, antisense oligonucleotides), enhance clearance of mutant protein and abnormal conformers, block formation of toxic oligomers, find agents that lower steady state levels of ATXN3 by cell-based screens (small molecules).

*Yeast Flavohemoglobin (YHB1) and nitric oxide, new players in frataxin deficient yeast -David Alsina, Joaquim Ros, Jordi Tamarit (Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, Spain)*

Frataxin is highly conserved, from bacteria to humans. Lack of frataxin leads to intracellular iron accumulation, loss of iron-sulfur proteins (e.g., aconitase), and increased oxidative stress. In the experimental model utilized, the endogenous promoter of the yeast frataxin homolog YFH1 was substituted by a tet-off promoter for inducible repression of protein expression. Repression of YFH1 resulted in a series of cellular responses. An early event was the activation of the iron regulon. This led to activation of a number of downstream genes involved in different pathways, including mitochondrial transport and use of iron, iron export from vacuole, and metabolic remodeling. A later event was the activation of CYH2, leading to downregulation of iron-sulphur cluster protein synthesis.

A recently identified early event was the induction of yeast flavohemoglobin 1 (YHB1). The protein is localized both in mitochondria and in the cytosol and is able to interact with frataxin. It has nitric oxide (NO) oxidoreductase activity and its expression can be induced by NO and reactive oxygen species (ROS). In line with a role of NO in regulating YHB1, it was found that NO increases early on after YFH1 repression. Furthermore, genetic ablation of YHB1 prevents iron accumulation in cells lacking YHF1, suggesting that YHB1 is involved in the regulation of the iron regulon. Taken together, the results obtained in the yeast mutants point to the interplay among frataxin, YHB1, NO and iron.

*DNA repair deficit and neuroinflammation as potential contributors to the physiopathology of Friedreich's ataxia - Jara Moreno-Lorente, Frida Loria, Sara Perez-Luz, Daniel Oberdoerfer, Yurika Katsu-Jimènez, Oscar Yang, Javier Diaz-Nido (Centro de Biologia Molecular Severo Ochoa, Universidad Autonoma de Madrid, Spain)*

To investigate the molecular mechanisms contributing to neurodegeneration in Friedreich's ataxia (FA) human neural cells were subjected to frataxin knockdown. Neuroblastoma SH-SY5Y cells were engineered with an inducible frataxin shRNA expressed from a lentiviral construct. Frataxin downregulation resulted in enhanced ROS production and oxidative stress, DNA damage, and apoptosis. The expression of a number of DNA repair proteins was also altered in a reversible manner. Intriguingly, cultured olfactory mucosa stem cells from biopsies of FA patients also displayed a decline of DNA repair related proteins. Together, these findings suggest that DNA repair enzyme deficiency likely play a role in the pathogenesis of FA.

To address the non-cell autonomous role of glial cells in neuronal degeneration in FA, frataxin was silenced in cultured human astrocytes, which was accompanied by enhanced expression and secretion of some pro-inflammatory cytokines. When these cells were co-cultured with wild type neurons, they caused neuronal degeneration, further indicating that astrocytes have a non-cell autonomous role in FA pathogenesis.

*Citalopriam treatment ameliorates motor impairment and suppresses ataxin-3 aggregation in a Machado-Joseph disease mouse model - Sofia Esteves, Sara Duarte-Silva, Anabela Silva Fernandes, Andreia Teixeira-Castro, Patricia Maciel (Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Portugal)*

Machado-Josef disease or SCA3 is caused by the polyQ expansion in the c-terminus portion of the ATXN3 gene product, ataxin-3. Citalopram is a selective serotonin reuptake inhibitor (SSRI) that was identified in an unbiased screen of FDA approved compounds as reducing motor impairment and ataxin-3 aggregation in a C. elegans model of the disease. Chronic Citalopram treatment (8mg/Kg or 13 mg/Kg day) in the CMVMJD135 mouse model improved motor defects and weight loss. It also improved the astrogliosis in the substantia nigra and ataxin-3 inclusions in various brain regions

affected by the disease, without decreasing total ataxin-3 levels. The decrease of aggregates suggests that Citalopram contributes to ameliorating proteostasis in affected cells.

*The role of acetylation in the pathogenesis of Friedreich's Ataxia -Angelical Martin, Gregory Wagner, Kathleen Hershberger, Mark Payne Matthew Hirschey (Duke University & Indiana University, USA)*

The heart regulates it substrate utilization based on metabolic needs. Acetylation of several mitochondrial enzymes involved in energy metabolism is regulated by acetylation and SIRT3 is a mitochondrial deacetylase that modulates this process. Reduction of SIRT3 activity in the heart resulted in hyperacetylation of metabolic enzymes and decreased ATP levels, accompanying cardiac hypertrophy. The conditional heart frataxin KO mouse model (MCK-FA) displayed mitochondrial protein hyperacetylation, suggesting a role for sirtuin 3 (SIRT3) impairment.

The NAD+ precursor nicotinamide mononucleotide (NMN) activated SIRT3 and decreased mitochondrial acetylation in the MCK-FA heart. A multi-omics analysis revealed downregulation of the transcription of pathways involved in fatty acid, ketone bodies, and amino acid metabolism. These results were in agreement with metabolomics data showing that the oxidation of fatty acid is reduced in the MCK-FA heart. Taken together these results suggest that energy metabolism alterations in the FA heart are associated with altered acetylation of substrates involved in the regulation of fatty acid metabolism and beta-oxidation, possibly through SIRT3 dysfunction.

*Viability of frataxin-deficient dorsal root ganglia neurons is recovered by calcium chelators and mitochondrial pore inhibitors -Joaquim Ros, Stefka Mincheva, Marta Llovera, Jordi Tamarit (Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, Spain)*

Lentiviral mediated frataxin silencing was achieved in dorsal root ganglia primary neurons. Frataxin levels were decreased by 80% in these neurons, causing neurite degeneration and apoptotic death. Frataxin deficient neurons displayed increased levels of intracellular calcium, which correlated with increased activation of calpains, suggesting that altered intracellular calcium homeostasis could underlie some aspects of neuronal degeneration in FA. Delivery of a TAT-BH4 anti-apoptotic domain of Bcl-xL prevented the apoptotic response in frataxin deficient neurons.

Since increased calcium could cause mitochondrial damage through activation of the permeability transition pore, a cyclophilin D inhibitor (cyclosporin A) was used to prevent calcium-induced mitochondrial permeabilization and depolarization. Cyclosporin A had a protective effect in neurons depleted of frataxin, suggesting that mitochondrial calcium overload may play a role in the degeneration of FA neurons.

*Mitochondrial protein hyperacetylation is associated with early diastolic dysfunction in a model of Friedreich's ataxia hypertrophic cardiomyopathy - Amanda Stram, Gregory Wagner, Melanie Pride, Steven Messina-Graham, Hal Broxmeyer, Matthew Hirschey, Mark Payne (Duke University & Indiana University, USA)*

Mitochondrial acetylation was evaluated in MCK-FA (conditional KO of frataxin in the heart) mice and controls at 30, 45 - and 60 days of age (spanning from the pre-symptomatic to the terminally advanced diseased stages). MCK-FA hearts showed an age-dependent increase in mitochondrial acetylation and decreased oxidative phosphorylation. The mitochondrial ultrastructure was abnormal with loss of cristae content. These changes were paralleled by increased fibrosis of the heart and decreased diastolic function, as early as 45 days of age, while systolic dysfunction became evident at 65 days of age.

SIRT3 KO mice were crossed with MCK-KO mice to generate a double KO in the heart. The MCK-KO/SIRT KO mice displayed a more severe functional heart phenotype, as compared to the MCK-KO mice, and were more susceptible to stress, although there were no apparent differences in left ventricular thickness. Taken together, these observations suggest a functional link among mitochondrial acetylation, SIRT3 activity, and heart dysfunction in the mouse models of cardiac frataxin deficiency.

The Journal of Rare DISORDERS

# DRUG DISCOVERY AND EMERGING THERAPEUTIC STRATEGIES

Alessandra Rufini[1,2], Robert B. Wilson[3] and Roberto Testi[1,2,*]

[1]Laboratory of Signal Transduction, Department of Biomedicine and Prevention, University of Rome “Tor Vergata”, Via Montpellier 1, 00133, Rome, Italy.

[2]Fratagene Therapeutics Ltd, 22 Northumberland Rd, Dublin, Ireland.

[3]Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, United States of America.

* To whom correspondence should be addressed, at Laboratory of Signal Transduction, University of Rome “Tor Vergata”, Via Montpellier 1, 00133, Rome, Italy, phone: +39 06 7259 6503, e-mail: roberto.testi@uniroma2.it

Over the past decade, major advancements in the development of new therapeutics for hereditary ataxias have been described; however, no effective treatments are currently available. In this report we summarize recent progress in drug discovery and the development of new therapeutic strategies that were presented at the International Ataxia Research Conference 2015.

In the last few years, histone deacetylase inhibitors (HDACi) have been proposed as potential therapeutics for FRDA based on their ability to reverse epigenetic modifications responsible for *FXN* silencing. As reviewed by Elisabetta Soragni, of The Scripps Research Institute, San Diego, California, the 2-aminobenzamide class of HDAC inhibitors can increase *FXN* mRNA levels in lymphoblasts derived from FRDA patients. This effect is associated with a marked increase in the acetylation of H3K14, H4K5 and H4K12 in the region located upstream of the GAA repeats in *FXN*. Improved versions of these compounds were generated and efficacy of these versions, designated 106, 136, and 109 was demonstrated in KIKI [1,2] and YG8R transgenic mouse models [3], in which treatment reversed hallmarks of chromatin repression and increased frataxin expression in the heart and nervous system. This was accompanied by a reduction of neuronal degeneration in the dorsal root ganglia and an increase in brain aconitase activity. Compound 109 also upregulates *FXN* mRNA and protein in FRDA-iPSC-derived neuronal cells, reaching the level of an unaffected carrier sibling [4]. This correlates with an increase in histone acetylation near the frataxin locus. Importantly, only compounds that simultaneously inhibit all class I HDACs, such as 106 and 109, activate *FXN* expression. Kinetic measurements indicate that a slow-on/slow-off mechanism of inhibition, and prolonged residence on the target enzyme, are required for effective gene reactivation [5]. Recently, HDACi 109 (RG2833) was evaluated in a phase I clinical study involving 20 FRDA patients. The drug was well tolerated with no overt adverse effects; however, benzimidazole derivatives were found in patient serum, raising concerns about possible metabolite-induced side effects. Administration of RG2833 to patients inhibited total HDAC activity and increased *FXN* mRNA in blood cells. ChiP assays revealed an increase in H3K9 acetylation in the region upstream of the GAA repeats in *FXN*. The drug concentration required to exert positive effects in cells in vitro is comparable to the dose required in patients. A new generation of compounds has now been generated to circumvent metabolite-induced toxicity and to increase brain penetration. One of these compounds, Click -1, was as effective as HDACi 109 in inducing *FXN* mRNA.

To gain further insight into the mechanism of action of this class of inhibitors, the effect of siRNA-mediated suppression of either HDAC1 or HDAC3 was evaluated in FRDA iPSC-derived neuronal cells. Knock-down of either enzyme increased *FXN* mRNA, further supporting the involvement of class I HDACs in the repression of *FXN* expression. A proteomic approach based on the use of an affinity-profiling probe, and aimed at identifying specific HDACs, initially revealed a direct interaction between HDACi 106 and HDAC3 [6]; more recently, an unbiased chemoproteomic study identified more than a thousand proteins interacting with HDACi 106 [7]. Localization of class I HDACs at the GAA repeats in *FXN* could not be proven by ChiP experiments, suggesting the possibility that other mechanisms leading to *FXN* reactivation may be involved. Microarray analyses identified 88 genes selectively modulated by HDACi 109, but not by the related and ineffective HDACi 233 or 966. Among these genes, histone H2A family member Y2 (H2AFY2) and Polycomb group ring finger 2 (PCGF2), were reproducibly down-regulated by HDACi 109. These genes could be involved in chromatin remodeling and regulation of gene expression, indirectly leading to *FXN* upregulation [8].

Another interesting approach to treat FRDA is based on protein replacement [9]. Dalia Megiddo, of Bioblast Pharma, Tel Aviv, Israel, reported the use of a fusion protein, comprising a TAT moiety, which functions as a trans-membrane carrier, and a heterologous mitochondrial transport signal (MTS), fused to frataxin. Surprisingly, the heterologous MTS from human mitochondrial citrate synthase targeted frataxin to mitochondria more efficiently than the homologous MTS. One possible explanation is that only one-step proteolytic processing is required to remove the citrate synthase MTS, while two-step proteolytic processing is normally required to generate mature frataxin from its precursor. Indeed, when administered to patient-derived cell lines, the recombinant fusion protein, TAT-MTS(cs)-FXN, called BB-FA, was successfully delivered into mitochondria and regularly processed. Moreover, the increase in aconitase activity and ATP levels in these cells suggests that recombinant frataxin can functionally substitute for the endogenous protein. Similar results were obtained in the humanized FRDA transgenic murine model (FVB;B6-Tg(FXN)1Sars $Fxn^{tm1Mkn}$/J) [10]. The recombinant protein was detected in different murine tissues and localized to mitochondria. A significant improvement in mitochondrial function in the heart and brain was observed in these mice after 21 days of treatment. Additional studies were conducted in the FRDA mouse model harboring a complete *FXN* deletion in cardiac and skeletal muscles (Mck-Cre-$Fxn^{L3/L-}$ mice) [7]. Frataxin was successfully delivered and was shown to restore mitochondrial complex II activity and to decrease interventricular septum (IVS) thickness to near normal. Recombinant frataxin was also associated with increased weight gain in the mice, and increased survival.

Clinically approved, bone-marrow-stem-cell-mobilizing cytokines have neuroprotective and neuroregenerative properties [12] and have been proposed as therapeutics for FRDA [13, 14]. Encouraging data were reported by Kevin Kemp, of the University of Bristol, UK. Granulocyte-colony-stimulating factor (G-CSF) and stem-cell factor (SCF) were evaluated in the FRDA transgenic mouse model YG8R. Accelerated-rotarod and grip-strength tests showed improved motor function after simultaneous administration of the two cytokines for 6 months, and electrophysiological recordings revealed improved peripheral nerve conduction. Moreover, increased expression of frataxin protein was observed in the cerebellum and spinal cord of treated mice, and correlated with increased expression of Nrf2, Pgc1-?, SOD1 and SOD2, all of which contribute to improved anti-oxidant defences. The therapeutic potential of bone-marrow-derived cell integration into the central nervous system was also explored in the YG8R mouse model. The mice were given myeloablative bone-marrow transplants from enhanced-green-fluorescent-protein- (EGFP-) transgenic wild-type donor mice, generating chimeric mice with EGFP-expressing bone marrow. Remarkably, bone-marrow-derived, EGFP-expressing cells were detected in the central nervous system of these mice, in the dentate nucleus, Purkinje cells, and medulla, indicating the possibility of delivering healthy donor cells to sites critical for the disease. Moreover, improvement in rotarod function and beam testing was observed in these mice, supporting the therapeutic potential of bone-marrow transplantation.

Non-coding RNAs are emerging as a powerful and innovative strategy to modulate gene expression [15]. Fatih Ozsolak, of RaNA Therapeutics, proposed an oligonucleotide-based approach to upregulate *FXN* expression using an oligo RNA targeting the 3' end region of *FXN* mRNA. By protecting the RNA end from exoribonuclease-mediated degradation, the oligo increased steady-state RNA levels and enhanced protein translation. Alternatively, RNA oligos could be synthesized that interact with both 5' and 3' mRNA regions at the same time, thus inducing a pseudo-circularization of the RNA, which has been shown to enhance translation. Acting at a post-transcriptional level, these approaches are not affected by the presence of the GAA repeat expansion. Upregulation of *FXN* mRNA and protein levels was achieved in fibroblasts derived from patients and healthy donors, independent of GAA repeat status, by treatment with both end-targeting and pseudo-circularization-inducing oligos. Subcutaneous administration of end-targeting oligos in murine models results in broad tissue distribution, without requirement for any delivery system, though intrathecal injection will be required to deliver RNA oligos to the CNS. Simultaneous administration of 5' and 3' end-targeting oligos in a transgenic mouse model of FRDA increased *FXN* mRNA levels in the liver, encouraging further evaluation of this therapeutic strategy.

Due to the presence of the intronic GAA-repeat expansions, *FXN* is transcribed inefficiently in patients; however, the small amount of protein that is produced is functional, since, in the vast majority of disease alleles, the coding sequences are intact. This is the rationale for therapeutics that aim to increase levels of endogenous frataxin. Starting from the idea that both rate of synthesis and rate of degradation of any given protein contribute to determine

intracellular steady-state levels, we (A.R. and R.T.) developed a therapeutic strategy based on the possibility of increasing frataxin levels by preventing degradation. We previously reported that frataxin degradation is regulated by the ubiquitin/proteasome system and that a significant fraction of frataxin precursor is degraded by the proteasome prior to mitochondrial import. Preventing degradation of frataxin precursor increases availability for mitochondrial import and maturation, thereby increasing mature frataxin. A single site on frataxin, K147, is responsible for ubiquitination and critically controls degradation. Accordingly, the substitution of K147 with an arginine generates a more stable form of frataxin. Small-molecule compounds directly targeting the pocket surrounding K147 were identified by computational screening and in vitro validation [16]. These molecules, named ubiquitin-competing molecules (UCMs), can prevent frataxin ubiquitination and degradation and increase frataxin levels. As we reported at this meeting, however, these molecules are ineffective on the "ubiquitin-resistant" frataxin mutant K147R, indicating that their mechanism of action is K147-dependent. Using fluorescence spectroscopy, we showed that these molecules interact with recombinant human frataxin. Moreover, these compounds promote frataxin accumulation in different lymphoblastoid cell lines derived from patients. Importantly, the frataxin that accumulates upon treatment with UCMs is functional, as demonstrated by increased aconitase activity in treated cells derived from patients [17].

An additional player in the regulation of frataxin degradation is the non-receptor tyrosine kinase Src. Florence Malisan, of the University of Rome "Tor Vergata", Italy, reported that frataxin can be phosphorylated directly by Src and, using mass-spectrometry analysis, identified Y118 as the primary Src phosphorylation site on frataxin. Cross-talk between phosphorylation and ubiquitination has been described for several proteins and indeed frataxin phosphorylation was observed to promote ubiquitination, thereby enhancing degradation. Metabolic alterations in frataxin-deficient cells, such as decreased ATP content and increased oxidative stress, might be responsible for Src activation, further contributing to frataxin down-regulation. Indeed, Src inhibitors increase frataxin levels in cells expressing wild type frataxin but not in cells expressing the non-phosphorylatable Y118F frataxin mutant, suggesting that they act by preventing phosphorylation on Y118. Moreover, frataxin accumulation was observed in cells derived from patients upon treatment with Src inhibitors. Of therapeutic relevance, some of the Src inhibitors tested are clinically approved drugs, which might lead to faster development as therapeutics for FRDA [18].

Data in support of gene therapy as an approach to the treatment of FRDA were presented by Jacques P. Tremblay, of Laval University, Quebec, Canada. Conditional knock-out mice in which murine *FXN* is deleted by a Cre recombinase under the control of the muscle-creatine-kinase (MCK) or neuron-specific enolase (NSE) promoter were used as models to assess the effects of an AAV9 vector [19] expressing human *FXN* (AAV9-hFxn). Different doses of AAV9-hFxn were delivered by single intra-peritoneal injection into NSE-Cre mice and significant increases in growth rate and life span were observed. Improved motor activity and decreased heart hypertrophy were also observed in treated NSE-Cre mice; however, no significant improvement in heart function was observed. The human *FXN* transgene and mRNA displayed a broad tissue distribution and frataxin protein was detected by dipstick analysis in muscle, liver, heart, kidney and brain when high vector concentrations were administered. Preliminary evaluation of AAV9-hFxn administration to MCK -Cre mice showed no significant differences in growth rate; however, cardiac hypertrophy was decreased and an improvement in heart function was reported. Lifespan of treated animals was extended more than three-fold compared to untreated mice. As in the NSE-Cre mice, a broad tissue distribution of *FXN* transgene, mRNA and protein after a single administration of AAV9-hFxn was reported in MCK-Cre mice. These data strongly support further evaluation of an AAV9-based gene therapy approach as a potential treatment for FRDA [20].

Spinocerebellar ataxia type 28 (SCA28) is an autosomal dominant, progressive neurodegenerative disorder. Several mutations in the gene encoding the AFG3L2 protein have been associated with the disease. AFG3L2 is part of the m-AAA mitochondrial protease complex involved in mitochondrial protein quality control, and therefore in maintaining mitochondrial morphology and functionality [21]. Haploinsufficient, Afg3l2$^{+/-}$ mice represent the murine model for SCA28, showing progressive motor deficits and dark-cell degeneration of Purkinje cells, associated with mitochondrial damage. Mitochondria from Afg3l2-deficient cells display impaired ability to buffer evoked calcium peaks, leading to increased cytoplasmic calcium concentrations and cell death, as reported by Giorgio Casari, of San Raffaele Institute, Milan, Italy. This was associated with fragmented mitochondrial networks and decreased ER/mitochondria contacts. Impaired mitochondrial calcium handling was therefore proposed as the pathogenic cause of the disease. In support of

this mechanism, genetically decreased expression of mGluR1, by decreasing calcium influx in Purkinje cells, reverts the phenotype of SCA28 mice. Similarly, pharmacological treatment with the cefalosporin ceftriaxone was shown to induce increased expression of the astrocyte glutamate transporter, EAAT2, and to decrease glutamatergic stimulation. Consistent with the proposed disease mechanism, treatment of Afg3l2$^{+/-}$ mice with ceftriaxone was shown to restore motor function and prevent dark-cell degeneration of Purkinje cells. Importantly, rescue of motor skills was also observed when mice were treated after onset of symptoms. Treatment with ceftriaxone was therefore proposed as a therapeutic approach for SCA28 and other SCAs characterized by impaired calcium homeostasis [22].

Machado-Joseph disease (MJD or Spinocerebellar ataxia 3, SCA3) is caused by a CAG trinucleotide repeat expansion in the gene encoding Ataxin-3. The resulting mutant protein accumulates in intranuclear neuronal inclusions, and induces toxicity and cell death in the cerebellum, brain stem, and striatum. No therapy has been found to prevent or slow disease progression. Proposed therapeutic approaches include gene silencing, autophagy activation, proteolysis inhibition, and neuropeptide Y. Recently, the therapeutic potential of cerebellar neural-stem-cell transplantation has been proposed. Luis Pereira de Almeida, of the University of Coimbra, Portugal, reported that by transducing cerebellar neural stem cells with a lentiviral vector expressing green-fluorescent protein (GFP), it was possible to monitor the diffusion of transplanted neural stem cells in the MJD transgenic mouse model. Cerebellar neural stem cells can differentiate into mature functional neurons, astrocytes, and oligodendrocytes, and can thereby reconstitute damaged tissues. Moreover, neural stem cells secrete neurotrophic factors that stimulate neuroregeneration and exert protective effects. Transplanted neural stem cells can survive up to 8 weeks after cerebellar transplantation. Importantly, transplanted animals displayed significant motor function recovery. Although neural stem cells do not differentiate into Purkinje cells, transplantation resulted in an increase in the number of Purkinje cells and reduction of cellular-layer shrinkage. Moreover, transplanted animals displayed a decrease of ataxin 3 aggregates and decreased hallmarks of neuroinflammation.

Neuropeptide Y (NPY) has a widely recognized neuroprotective action [23-25]; therefore, its effect on the progression of MJD has been investigated. As reported by Joana Duarte-Neves, of the University of Coimbra, Portugal, NPY mRNA levels were decreased in the cerebella of post-mortem patients and in brain tissues from MJD murine models. AAV-mediated overexpression of NYP in the striatum and cerebellum of MJD mice was shown to decrease striatal lesions and to rescue some of the motor deficits associated with the disease, as assessed by rotarod and beam testing, suggesting that NYP overexpression may represent a viable strategy to treat MJD.

1. Rai, M., et al., HDAC inhibitors correct frataxin deficiency in a Friedreich ataxia mouse model. *PLoS One*. 2008. 3(4): p. e1958.

2. Rai, M., et al., Two new pimelic diphenylamide HDAC inhibitors induce sustained frataxin upregulation in cells from Friedreich's ataxia patients and in a mouse model. *PLoS One*, 2010. 5(1): p. e8825.

3. Sandi, C., et al., Prolonged treatment with pimelic o-aminobenzamide HDAC inhibitors ameliorates the disease phenotype of a Friedreich ataxia mouse model. *Neurobiol Dis*. 2011. 42(3): p. 496-505.

4. Soragni, E., et al., Epigenetic therapy for Friedreich ataxia. *Ann Neurol*. 2014. 76(4): p. 489-508.

5. Chou, C.J., D. Herman, and J.M. Gottesfeld, Pimelic diphenylamide 106 is a slow, tight-binding inhibitor of class I histone deacetylases. *J Biol Chem*. 2008. 283(51): p. 35402-9.

6. Xu, C., et al., Chemical probes identify a role for histone deacetylase 3 in Friedreich's ataxia gene silencing. *Chem Biol*. 2009. 16(9): p. 980-9.

7. Shan, B., et al., Quantitative proteomic analysis identifies targets and pathways of a 2-aminobenzamide HDAC inhibitor in friedreich's ataxia patient iPSC-derived neural stem cells. *J Proteome Res*. 2014. 13(11): p. 4558-66.

8. Soragni, E., et al., Mechanism of Action of 2-Aminobenzamide HDAC Inhibitors in Reversing Gene Silencing in Friedreich's Ataxia. *Front Neurol*. 2015. 6: p. 44.

9. Vyas, P.M., et al., A TAT-frataxin fusion protein increases lifespan and cardiac function in a conditional Friedreich's ataxia mouse model. *Hum Mol Genet*. 2012. 21(6): p. 1230-47.

10. Sarsero, J.P., et al., Human BAC-mediated rescue of the Friedreich ataxia knockout mutation in transgenic mice. *Mamm Genome*. 2004. 15(5): p. 370-82.

11. Puccio, H., et al., Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S

enzyme deficiency followed by intramitochondrial iron deposits. *Nat Genet*. 2001. 27(2): p. 181-6.
12. Wilkins, A., et al., Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. *Stem Cell Res*. 2009. 3(1): p. 63-70.
13. Kemp, K., et al., Mesenchymal stem cells restore frataxin expression and increase hydrogen peroxide scavenging enzymes in Friedreich ataxia fibroblasts. *PLoS One*. 2011. 6(10): p. e26098.
14. Dey, R., et al., Human mesenchymal stem cells increase anti-oxidant defences in cells derived from patients with Friedreich's ataxia. *Cerebellum*. 2012. 11(4): p. 861-71.
15. Krishnan, J. and R.K. Mishra, Emerging trends of long non-coding RNAs in gene activation. *FEBS J.* 2014. 281(1): p. 34-45.
16. Rufini, A., et al., Preventing the ubiquitin-proteasome-dependent degradation of frataxin, the protein defective in Friedreich's ataxia. *Hum Mol Genet*. 2011. 20(7): p. 1253-61.
17. Rufini, A., et al., Highly specific ubiquitin-competing molecules effectively promote frataxin accumulation and partially rescue the aconitase defect in Friedreich ataxia cells. *Neurobiol Dis*. 2015. 75: p. 91-9.
18. Cherubini, F., et al., Src inhibitors modulate frataxin protein levels. *Hum Mol Genet*. 2015.
19. Manfredsson, F.P., A.C. Rising, and R.J. Mandel, AAV9: a potential blood-brain barrier buster. *Mol Ther*. 2009. 17(3): p. 403-5.
20. Gerard, C., et al., An AAV9 coding for frataxin clearly improved the symptoms and prolonged the life of Friedreich ataxia mouse models. *Mol Ther Methods Clin Dev*. 2014. 1: p. 14044.
21. Di Bella, D., et al., Mutations in the mitochondrial protease gene AFG3L2 cause dominant hereditary ataxia SCA28. *Nat Genet*. 2010. 42(4): p. 313-21.
22. Maltecca, F., et al., Purkinje neuron Ca2+ influx reduction rescues ataxia in SCA28 model. *J Clin Invest*. 2015. 125(1): p. 263-74.
23. Decressac, M., et al., Neuropeptide Y modifies the disease course in the R6/2 transgenic model of Huntington's disease. *Exp Neurol*. 2010. 226(1): p. 24-32.
24. Croce, N., et al., Neuropeptide Y protects rat cortical neurons against beta-amyloid toxicity and re-establishes synthesis and release of nerve growth factor. *ACS Chem Neurosci*. 2012. 3(4): p. 312-8.
25. Decressac, M., et al., Neuroprotection by neuropeptide Y in cell and animal models of Parkinson's disease. *Neurobiol Aging*. 2012. 33(9): p. 2125-37.

# BIOMARKERS AND FUNCTIONAL MEASURES

Yina Dong[1], Dave Lynch[1] and Bernard Ravina[2]

[1] Departments of Neurology and Pediatrics, The Children's Hospital of Philadelphia and Perelman School of Medicine, University of Pennsylvania, US

[2] Voyager Therapeutics

While there are many agents in clinical trials in ataxia, there is a need for biomarkers that provide reliable information on therapeutic activity in relevant tissues. Finding sensitive and specific cellular and molecular biomarkers for Ataxias is crucial for clinical development of novel drugs. Although identification of such biomarkers is challenging, a number of potential biomarkers aimed at different clinical stages were reported in ARC.

In FRDA, decreased peripheral frataxin (FXN) protein levels were identified in a number of cells, including buccal cells, whole blood, PBMCs, platelets and lymphocytes. Peripheral FXN levels also may respond to agents designed to increase FXN level in clinical trials. David Lynch reported using peripheral frataxin levels by lateral flow FXN dipstick immunoassay as a biomarker of systemic frataxin levels in FRDA. The study from his group including 405 patients for buccal cells and 376 patients for blood demonstrates that FXN level in both buccal cell and whole blood correlated inversely with GAA repeat length and directly with age of onset. In addition, FXN level correlated with the severity of disease as measured by Friedreich's Ataxia Rating scale (FARS). The accessibility of buccal cells and whole blood supports the use of peripheral FXN as a biomarker in clinical trials of systemic agents and a complementary diagnostic tool to aid in characterization of atypical patients. In parallel subjects at the University of Pennsylvania, Ian Blair's study demonstrated that platelets may be used as an alternative tissue for *in vivo* metabolic studies. By using liquid-chromatography-mass spectrometry methodology including isotopologue analysis, Ian Blair found that platelets from FRDA patients have diminished relative incorporation of $\{^{13}C_6\}$-glucose into the Krebs cycle through acetyl-CoA and concomitant increase in the β-oxidation of fatty acids. The decreased glucose metabolism negatively correlated with GAA repeat length and increased fatty acid metabolism positively correlated with GAA repeat length.

Ian Blair's findings contrast somewhat with results from Mark Payne, who found that FRDA patients heart have altered glucose and fatty acid metabolism as well. Instead of decreased glucose metabolism and increased fatty acid metabolism seeing in FRDA platelet, Mark Payne reported that FRDA patients' hearts have impaired ability to metabolize fatty acids and greater glucose utilization than controls. This result was also found in the heart of a Frataxin knockout mouse as demonstrated by the decreased palmitate utilization rate and increased glycolytic rate, suggesting a tissue specificity of metabolic changes in FRDA and providing further rationale for the use of metabolic biomarkers in FRDA. Serum fatty acid binding protein 3, which was significantly elevated in the FRDA patients, can also be a candidate for such a biomarker. The differences between the results of Ian Blair's and Mark. Payne's studies could reflect tissue specific metabolism, or be related to disease stage.

Imaging techniques can complement biochemical markers in FRDA. High field $^1$H magnetic resonance spectroscopy ($^1$H-MRS) is a powerful tool for the non-invasive characterization of biochemical alterations in the brain that enables the measurement of neurometabolite profiles in localized brain regions in humans [1,2]. These metabolic profiles reflect neuronal damage and may provide insights into the disease processes at an early stage. Lenglet's group first reported changes in neurometabolites thought to reflect neuronal damage and gliosis in spinal cord of FRDA patients by using $^1$H MRS. Alterations of axonal integrity were also observed in the cervical spinal cord. By using functional magnetic resonance imaging (fMRI) in a working memory task, Georgiou-Karistianis's group found reduced brain activation and altered connectivity in the regions of the cerebellar cortex that supports cognitive function and associated cerebral cortices, including the anterior insula and lateral prefrontal cortex. This provides further evidence that FRDA can be viewed as a disorder of large-scale, spatially distributed cerebral and cerebellar circuitry. A neuroimaging study with advanced diffusion tensor imaging (DTI) and fMRI also demonstrates the ability to discriminate FRDA patients from controls and may prove to be a useful paraclinical disease marker. As reported by Martinuzzi's group, DTI identified clinically relevant structural changes, and fMRI with motor tasks showed significant abnormalities in activation of the

relevant cerebellar structures in FRDA. The combined use of DTI and fMRI may serve as objective and efficient biomarker to monitor disease progression and response to treatment.

Clinical scales and assessments are also crucial measures of disease progression. The value of biomarkers can also be viewed in how they correlate with detailed clinical methodology. To monitor disease progression in FRDA, the Scale for the Assessment and Rating of Ataxia (SARA) and Activities of Daily Living (ADL) were particularly suitable based on Jorg Schulz's cross-sectional analysis of the European Friedreich's Ataxia Consortium for Translational Studies (EFACTS). SARA strongly correlated with clinical and functional measures, while ADL showed small to medium correlations. The strong correlations between spatiotemporal gait characteristics at different walking speeds and a range of clinical and disease characteristics in individuals with FRDA was also reported by Louise Corben, suggesting that gait analysis was a sensitive indicator of disease progression in FRDA as well. Disease impact on the quality of life in people with FRDA was also reported. Martin Delatycki who presented data on the Friedreich's ataxia impact scale (FAIS) which measures clinically important symptoms, physical functioning and psychological and social impact. FAIS subscales relating to symptoms and physical functioning correlated significantly with many clinical characteristics of FRDA including total FARS score, onset age and disease duration, but did not correlate with GAA repeat size. FAIS highlights the importance of ambulation and physical function in the perception of disease impact in people with FRDA and provides valuable insight into the perspective of individuals with FRDA on their health status. However, its limited responsiveness to change over two years makes its use as a primary outcome in intervention studies questionable.

Biomarkers are also important in other ataxias. Novel candidate blood-based transcriptional biomarkers of spinocerebellar ataxia type 3 (SCA3) were reported by Marfalda Raposo who found that genes related with immune system response (like FCGR3B and SELPLG) and G-protein coupled receptor signaling (like P2RY13) were significantly upregulated in SCA3 compared with controls.

Imaging markers can also be used as diagnostic tools. Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is a rare neurodegenerative disorder caused by mutations in the SAC gene. Michael Parkinson reported using retinal nerve fiber layer thickening by ocular coherence tomography (OCT) as sensitive and specific marker to distinguish ARSACS from patients affected with ataxia including FRDA. A cut-off value of 119 um provides sensitivity of 100% and specificity of 99.4% for ARSACS, suggesting OCT could be part of routine pre-genetic screening. This is important as genetic testing for ARSACS is not routinely available.

A number of measurements were also developed to identify ataxia-specific dysfunction in both pre-clinical and clinical stages of the disease and how such dysfunction impacted the quality of life of affected individuals. Zofia Fleszar reported that deficits of balance like body sway and a multivariate analysis of gait differentiated healthy controls not only from early ataxia subjects, but also from pre-clinical ataxia subjects. These movement features correlated with time of estimated disease onset and were sensitive to disease progression before genetically estimated disease onset, suggesting a potential disease progression marker for the preclinical phase.

1. (1)H MR spectroscopy in Friedreich's ataxia and ataxia with oculomotor apraxia type 2. Iltis I, Hutter D, Bushara KO, Clark HB, Gross M, Eberly LE, Gomez CM, Oz G. *Brain Res*. 2010 Oct 28;1358:200-10. Aug 14.

2. In vivo 1H NMR spectroscopy of the human brain at high magnetic fields: metabolite quantification at 4T vs. 7T. Tkác I, Oz G, Adriany G, Uğurbil K, Gruetter R. *Magn Reson Med*. 2009 Oct;62(4):868-79.

# CLINICAL TRIALS & TRIAL DESIGN

Rosella Abeti[1], Michael H Parkinson[1], Myriam Rai[2], Massimo Pandolfo[2], Paola Giunti[1]

[1] Department of Molecular Neuroscience, UCL, Institute of Neurology, Queen Square WC1N 3BG, London, UK

[2] Department of Neurology, ULB-Hôpital Erasme, Brussels, Belgium

Session 7 reported clinical trials in Friedreich's and other types of ataxia. Presentations included trials at various phases and stages of planning, some due to start within the next year, along with the underlying scientific rationale. The session started with an interview with Pavel Balabanov from the European Medicines Agency (EMA), who provided advice on specific regulatory issues facing academics and companies willing to develop novel treatments for rare neurological diseases such as the ataxias.

An interview conducted by Massimo Pandolfo with Pavel Balabanov from the Human Medicines Evaluation Division of the European Medicines Agency (EMA) gave useful advice on various aspects of drug development, such as preclinical studies normally needed for a drug to reach the market. He also stressed the importance of contacting the EMA for advice at the earliest stage possible as the EMA provides scientific advice in all stages of developing a medicine. Even before completing the pre-clinical studies, if someone has a drug in development, it is advisable to contact the EMA. A report of the interview can be seen here: https://www.ataxia.org.uk/international-research-conference.

The session continued with an invited presentation by Richard Festenstein (Imperial College London) describing important progress in the treatment of Friedreich's ataxia (FRDA). Festenstein first discussed how epigenetic changes around the expanded GAA repeat at the *FXN* locus lead to gene silencing and frataxin deficiency. These changes include increases in the histone marks $H3K9me^3$ and $H3K27me^3$, both associated with heterochromatin formation and reduced DNAse I accessibility. Modifying these epigenetic changes offers the possibility of improving frataxin expression and eventually the clinical outcome [1]. Richard Festenstein used the class III histone deacetylase (HDAC) inhibitor nicotinamide (closely related to vitamin B3) in preclinical studies, showing how this compound increased histone acetylation, decreased DNA compaction and increased DNAse I accessibility at the *FXN* locus. Accordingly, nicotinamide upregulated frataxin in primary peripheral blood mononuclear cells (PBMCs) from FRDA patients.

Based on these promising pre-clinical data, RIchard Festenstein and Paola Giunti (UCL/UCLH,UK) went on to perform an exploratory, proof-of-concept, open-label, dose-escalation study of nicotinamide in adult human FRDA patients with upregulation of frataxin expression as the primary endpoint. They also studied safety and tolerability, clinical outcomes and changes in heterochromatin formation [2]. Nicotinamide was well tolerated with mild, dose-related nausea as the main adverse event, which resolved spontaneously or after dose reduction or antiemetic medication. Administration of single doses of nicotinamide resulted in dose-related increases in frataxin expression over 0 to 8 hours post-dose ($p=0.0004$). Daily dosing at 3.5-6 g resulted in sustained and significant increases in frataxin expression ($p<0.0001$) over 8 weeks with a significant decrease in $H3K9me^3$ and decreased heterochromatin formation at the *FXN* locus. The levels of frataxin obtained were comparable to those seen in asymptomatic carriers of a single *FXN* repeat expansion, who do not develop any clinical manifestation of FRDA. These initial results are very encouraging, but further long-term, randomized controlled trials are necessary to establish if nicotinamide is a valid therapy for FRDA.

Robert Molinari showed preclinical data on the neuroprotective effect of the stabilisation of mitochondrial fats to block their peroxidation. He explained the role of lipid peroxidation in neurodegeneration and how Retrotrope Ltd compounds can prevent this process. These are poly-unsaturated fatty acids (PUFAs) carrying a deuterium instead of a hydrogen at a key site. Several collaborating groups confirmed the beneficial effect of Retrotrope new PUFAs. In particular, RT001 and RT002 significantly protected human FRDA fibroblast from iron toxicity (Wilson Lab, UPENN). A significant increase of lipid peroxidation is one of the major causes of cell death of cerebellar granule cells from the YG8R (Pook) FRDA mouse model [3]. RT001/2 prevented significantly YG8R granule cell death (Giunti Lab UCL, UK). Retrotrope Ltd has now completed pre-clinical toxicity studies and a Phase 1 human trial. The company is ready to move forward to a phase 2 trial for FRDA and planning clinical trials in other neurodegenerative conditions as Parkinson's disease.

Colin Meyer from Reata Pharmaceuticals discussed the rationale to use Nrf2 activators for neuroprotection. These compounds massively increase endogenous antioxidants, reduce pro-inflammatory mediators (such as NF-kB), and ameliorate mitochondrial bioenergetics. RTA 408, the Reata lead compound, increased activity of Complex I of the mitochondrial electron transport chain and of thioredoxine reductase, an important antioxidant enzyme. A phase 2 study on FRDA will be started very soon in sites in North America and Europe. Colin Meyer provided information on the design and outcome measures for this phase 2 trial.

Gino Cortopassi showed interesting data on the reduced level of endogenous antioxidants, such as GSH, in cerebellum and dorsal root ganglia (DRGs) of YG8R mice (Pook FRDA mouse model) and in patients' lymphoblast. Thioredoxine reductase was also defective in these models. The Cortopassi lab linked frataxin deficiency to decreased Nrf2 activation, which, in addition to reducing antioxidant levels, also led to an inflammatory effect, as demonstrated by increased arachidonic acid metabolites and microglial susceptibility to lipopolysaccharide (LPS) activation [4].

Cortopassi utilized the increased sensitivity to diamide (a pro-oxidant) of patient's fibroblasts and of frataxin-deficient DRGs to screen 1600 compounds for protective effect. His group found some EMA/FDA-approved drugs that protect patient's fibroblasts from diamide stress. One of these drugs is dyclonine, which, in addition to protecting FRDA cells from diamide stress, also increased FXN mRNA in mouse cerebellum. Dyclonine engages the Nrf2/ARE antioxidant pathway response and upregulates frataxin by inhibiting the methyltransferase G9AE. Dyclonine also rescued the iron-sulfur cluster defects found in the FRDA models, increasing aconitase and succinate dehydrogenase activities. Moreover, dyclonine improved balance in KIKO mice (a mouse model with 30% of wild-type frataxin levels).

Dyclonine is available in a mouthwash. A pilot trial with 1% dyclonine mouthwash 2x daily was conducted on 8 FRDA patients and 2 control subjects. Buccal cells frataxin showed a significant increase after treatment, which correlated with disease severity [5]. To move to more advanced clinical testing, it will be necessary to develop a dyclonine formulation for systemic administration or to find an analogue with overlapping pharmacological properties.

Eppie Yiu et al., revaluated clinical trial in FRDA with resveratrol, a compound with antioxidant properties. Treatment of FRDA patients with two different doses of resveratrol over a period of 12 weeks resulted in an increase in frataxin mRNA level in patient's lymphocytes. Oxidative markers were lower. The higher dose of resveratrol also improved ataxia in this open-label study.

Saute et al reported on a study on lithium carbonate in SCA3. They presented a detailed analysis of their results with clinical rating scales – the neurological examination score for the assessment of spinocerebellar ataxia (NESSCA) and the scale for the assessment and rating of ataxia (SARA) – as well as with the spinocerebellar ataxia functional index (SCAFI), which was used only in the subgroup of patients with independent gait. They also calculated sample size estimates and other data relevant for planning future trials. NESSCA, SCAFI and CCFS showed evidence of lithium efficacy. The interaction of disease severity and treatment response indicated that early stages patients should be preferentially recruited.

Argov et al, on behalf of BioBlast Pharma, described a proposed randomized, controlled, double-blind phase 3 trial of high dose trehalose in SCA3. Trehalose is a naturally occurring α-linked disaccharide with known protein-stabilizing properties, which is already used in the food and in the pharmaceutical industry as a food stabilizer and as an excipient ingredient in medicinal products. Trehalose has been shown to enhance autophagy and accelerate the clearance of mutant Huntingtin and α-synuclein. Trehalose may therefore stabilize mutant ataxin-3 as well, and allow its clearance by increased autophagy.

In a lentiviral-based mouse model of SCA3, non-significant reductions in brain lesion volumes were seen with trehalose treatment. In a transgenic mouse model producing truncated ataxin-3, latency to fall from a stationary rotarod was significantly improved in animals treated with trehalose compared to controls, although the effect was most pronounced in female animals.

In humans, trehalose is broken down in the intestinal epithelial brush border and so it must be given intravenously. In a human trial in oculopharyngeal muscular dystrophy (OPMD), intravenous trehalose was well-tolerated without adverse

events, and pharmacokinetic data showed that after a single 30g intravenous dose, plasma trehalose levels reached concentrations determined in animal studies as sufficient for intracellular activity. Increases in plasma and urinary glucose were noted.

BioBlast Pharma is now planning a multicenter, randomized, open-label study of 30g iv trehalose given weekly over 52 weeks to SCA3 patients, with an untreated parallel control group. Weekly administration is justified by pharmacokinetic studies showing that plasma concentrations are retained for a number of hours following administration of the drug, and it is posited that the intracellular protein-stabilizing effect long outweighs this; but whether the weekly dosing schedule will be sufficient is not known. The investigators will collect data on safety, tolerability and efficacy. Change in the SARA will be the primary endpoint; the secondary endpoints will include change in the NESSCA, 9-hole peg test, 8 meter timed walk and quality of life scores. An obvious shortcoming of this proposed trial is the lack of a placebo group, but it was felt this would pose problems for recruitment.

Paola Giunti presented work from Bunn et al. who studied the relative contribution of different sensorimotor processing deficits (vestibular, proprioceptive & visual) in determining balance impairment in patients with SCA6, which is known to cause a relatively pure cerebellar form of ataxia [6]. They undertook whole body motion analysis as a measure of balance response after galvanic vestibular stimulation, muscle vibration and exposure to moving visual scenery in 16 patients with SCA6 and 16 healthy controls. The results were compared with the SARA as a measure of disease severity.

They found that visually evoked balance responses were approximately three times greater in the SCA6 patients compared to normal subjects and correlated with disease severity. The vestibular and proprioceptive balance responses were not significantly different from healthy controls. The group speculates that cerebellar degeneration may disturb visual-evoked balance responses through disinhibition of extracerebellar visuomotor centres.

Based on these findings, Bunn et al. designed a randomized pilot clinical trial that was home-based training intervention with optokinetic stimuli on 12 SCA6 patients with pure cerebellar ataxia. Therapy group participants undertook balance exercises in front of optokinetic stimuli during weeks 4-8, while control group participants received no intervention. Test-retest reliability showed strong (intraclass correlation coefficient >0.7) for selected outcome measures evaluating balance at impairment and activity levels. Some measures reveal trends towards improvement for those in the therapy group. They found that this study was feasible and the outcome measures employed were reliable.

Nanri et al found a 54% prevalence of GAD, gliadin, deaminated gliadin and thyroid antibodies amongst a group of 59 Japanese cases with idiopathic cortical cerebellar atrophy and cerebellar ataxia. Of the antibody-positive cases, 57% were said to respond well to immunotherapy, underlining the importance of not missing these potentially treatable causes of ataxia.

Riluzole has several potential modes of action and has known efficacy in preventing progression in amyotrophic lateral sclerosis. Romano et al postulated that its property of opening calcium-activated potassium channels might reduce neuronal excitability and be useful in the treatment of cerebellar ataxia. They performed a multicenter, randomized, double-blind, placebo-controlled trial of 60 patients with hereditary cerebellar ataxia over 12 months. The riluzole arm performed significantly better at both 3 months and 12 months as measured by the SARA, and the proportion of patients stable or improved at these time-points was significantly higher in the riluzole group. There were no major adverse events. It is difficult to interpret the generalizability of studies including mixed groups of patients, studies in individual genetic ataxias over longer time periods are therefore warranted.

This session included presentation of the rationale behind a number of potential therapies being developed for ataxias, mostly for FRDA and SCA3. Several trials are planned, in some cases phase I or pilot studies have already been carried out with encouraging results. However, as Vogel's review showed for dysarthria, there is often little systematic evidence guiding treatment decisions in the cerebellar ataxias, and still no disease-modifying treatments licensed. Rigorous basic science is necessary to guide translation of research findings to the clinic, followed by rigorous, well-structured, properly controlled clinical trials.

1. Heterochromatinization induced by GAA-repeat hyperexpansion in Friedreich's ataxia can be reduced upon HDAC inhibition by vitamin B3. Chan PK, Torres R, Yandim C, Law PP, Khadayate S, Mauri M, Grosan C, Chapman-Rothe N, Giunti P, Pook M, Festenstein R. *Hum Mol Genet*. 2013 Jul 1;22(13):2662-75.

2. Epigenetic and neurological effects and safety of high-dose nicotinamide in patients with Friedreich's ataxia: an exploratory, open-label, dose-escalation study. Libri V, Yandim C, Athanasopoulos S, Loyse N, Natisvili T, Law PP, Chan PK, Mohammad T, Mauri M, Tam KT, Leiper J, Piper S, Ramesh A, Parkinson MH, Huson L, Giunti P, Festenstein R. *Lancet*. 2014 Aug 9;384(9942):504-13

3. Targeting lipid peroxidation and mitochondrial imbalance in Friedreich's ataxia. Abeti R, Uzun E, Renganathan I, Honda T, Pook MA, Giunti P. *Pharmacol Res*. 2015 Sep;99:344-50.

4. Frataxin deficiency leads to defects in expression of antioxidants and Nrf2 expression in dorsal root ganglia of the Friedreich's ataxia YG8R mouse model. Shan Y, Schoenfeld RA, Hayashi G, Napoli E, Akiyama T, Iodi Carstens M, Carstens EE, Pook MA, Cortopassi GA. *Antioxid Redox Signal*. 2013 Nov 1;19(13):1481-93.

5. Dyclonine rescues frataxin deficiency in animal models and buccal cells of patients with Friedreich's ataxia. Sahdeo S, Scott BD, McMackin MZ, Jasoliya M, Brown B, Wulff H, Perlman SL, Pook MA, Cortopassi GA. *Hum Mol Genet*. 2014 Dec 20;23(25):6848-62.

6.Sensorimotor processing for balance in spinocerebellar ataxia type 6. Bunn LM, Marsden JF, Voyce DC, Giunti P, Day BL. *Mov Disord*. 2015 Aug;30(9):1259-66.