Published in final edited form as:
Mol Genet Metab. 2007 ; 92(0): 23–35. doi:10.1016/j.ymgme.2007.05.009.

Advancements in the pathophysiology of Friedreich ataxia and new prospects for treatments

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Abstract

On November 9-12, 2006, the Friedreich's Ataxia Research Alliance (FARA) and the National Institutes of Health (NIH) hosted the Third International Friedreich's Ataxia (FRDA) Scientific Conference at the NIH in Bethesda, Maryland, highlighting the exciting research leading now to a variety of clinical trials that show promise of effective treatments for this devastating disorder. Nearly 150 leading FRDA scientists from around the world discussed their new insights and findings. The presence of six pharmaceutical and biotechnology companies underscored the importance of the public-private partnership that has grown in the past years. Some of these companies are already involved in advancing promising drug compounds into clinical trials, while others are eager to help take newer discoveries through drug development and into subsequent clinical trials. National Institute of Neurological Disorders and Stroke (NINDS) Director Dr. Story Landis noted in her opening remarks for the conference that there was a "palpable sense of energy,

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excitement, and enthusiasm" over the scientific progress made since the FRDA gene was discovered over 10 years ago.

Introduction

Friedreich's Ataxia (FRDA), the most common autosomal recessive ataxia (affecting one individual in 50,000), is a neurodegenerative disease characterized by degeneration of the large sensory neurons and spinocerebellar tracts, cardiomyopathy, and increased incidence of diabetes. FRDA is caused by severely reduced levels of frataxin as a result of a large GAA triplet-repeat expansion within the first intron of the frataxin gene [1]. Frataxin is a ubiquitously expressed mitochondrial protein, highly conserved throughout evolution. Neither the precise physiologic function of frataxin nor the pathophysiology of the disease has been unraveled completely, although much progress has been made.

The goal of the meeting was to examine and discuss recent and as yet unpublished molecular, biochemical and clinical work. The major areas covered were: the FRDA disease gene, the frataxin protein, cellular and mitochondrial pathophysiology of FRDA, iron metabolism, cellular and animal models of FRDA, clinical studies/outcome measures and therapy. Whereas a tremendous amount of exciting new science was presented, the audience was elated to learn of the dramatic advances since the 1st International Conference on Friedreich's Ataxia in 1999 and even since the 2nd in 2003. Many new investigators have been attracted to the field and the quality of science continues to improve. The founders and principal supporters of the Friedreich's Ataxia Research Alliance were congratulated and thanked for their continued and important involvement.

The Friedreich's Ataxia Disease Gene

This first session of the symposium covered broad aspects of the DNA biochemistry, cell biology, and genetics of the long GAA•TTC repeats involved in the etiology of FRDA.

Individual talks focused on DNA biochemistry, transcription inhibition, and therapeutic strategies for reversing the transcription inhibition as well as genomics of the *FRDA* sequence. DNA replication studies and gene-therapy strategies were also discussed. In addition, data on somatic instabilities of the GAA•TTC repeats in the expression of *FRDA* were presented.

The session Chair, Dr. Robert D. Wells (Texas A & M University Health Science Center), discussed studies of sticky DNA, which is formed at long GAA•TTC repeats, the capacity of sticky DNA to inhibit transcription, and the reversal of this inhibition by polyamides. Next, Dr. Albino Bacolla (Texas A & M University Health Science Center) and co-workers reviewed genomic studies on the distribution of polypurine•polypyrimidine sequences (especially GAA•TTC) in the human genome [2-4]. Dr. Maria Krasilnikova and Dr. Sergei Mirkin (Pennsylvania State University) presented ingenious studies on the capacity of sticky DNA to inhibit DNA replication in COS-1 cells.

Substantial progress has been realized with two experimental strategies for developing therapeutics to treat FRDA patients. Four laboratories (Dervan *et al*. at California Institute of Technology, Gottesfeld *et al*. at Scripps Research Institute, Napierala *et al*. at the Institute of Biosciences and Technology as well as Pandolfo *et al*. at Universite Libre de Bruxelles) have explored the capacity of small synthetic ligands (polyamides) to alleviate transcriptional inhibition associated with long GAA•TTC repeats in *FRDA*. While this line of investigation seems very promising, an additional strategy using histone deacetylase inhibitors has also been explored to reverse gene silencing in *FRDA*. Both the Pandolfo and

Gottesfeld laboratories reported promising studies in this area, as did Dr. Richard Festenstein (Imperial College London, United Kingdom). Dr. Festenstein had previously shown that transgenes can be silenced *in vivo* by the insertion of GAA•TTC repeats, as found in frataxin alleles, and that the repeats induce heterochromatin formation through position effect variegation [5]. One of the mediataors of position effect variegation is heterochromatin protein 1, which binds to histone 3 via its tri-methylated lysine 9 residue, thereby inducing heterochromatin formation [6]. Dr Gottesfeld presented data suggesting that similar mechanisms of gene silencing (including hypoacetylation of histones 3 and 4 and methylation of histone 3 at lysine 9, which are characteristic of repressed heterochromatin) were observed at expanded *FRDA* alleles in primary lymphocytes derived from patients. Furthermore, treatment of these FRDA cells with a particular class of HDAC inhibitors reversed the *FRDA* gene silencing suggesting that these compounds could be used for treatment of FRDA [7].

Dr. Karen Usdin (National Institutes of Health) and Dr. Ed Grabczyk (Louisiana State University Health Sciences Center) investigated transcriptional effects with emphasis on epigenetic modifications. Dr. Sanjay Bidichandani (University of Oklahoma Health Sciences Center) and colleagues reported the potential role of somatic instability of the triplet repeat in phenotypic expression in FRDA. Dr. Richard Wade-Martins (University of Oxford, United Kingdom) discussed a gene-therapy approach using the entire 135 kbp human *FRDA* locus to complement the *FRDA* deficiency in human cells [8].

The Friedreich's ataxia protein

Frataxin is a small mitochondrial protein whose exact function has been the focus of intense studies for the last decade since the identification of the FRDA gene [1]. Several functions have been proposed for frataxin including a role in the biogenesis of heme and iron-sulfur clusters (ISCs) [9-18], mitochondrial iron homeostasis [19,20], mitochondrial iron storage [18,21-25], and maintenance of antioxidant defenses [26-28]. Talks presented during this session provided recent updates on the aforementioned functions of frataxin and also introduced potential novel functions and cellular location.

Several studies have reported the role of frataxin as an iron chaperone in the synthesis of both heme and ISCs [9-18,29]. Frataxin interacts physically both *in vitro* and *in vivo* with the mitochondrial iron scaffold protein IscU2 (or its yeast homologue Isu1p) [11,30,31] and this interaction allows frataxin to deliver its bound iron to IscU in the initial steps of [2Fe-2S] synthesis [12]. Similarly, direct interaction between frataxin and ferrochelatase, the enzyme that catalyzes the last step in heme synthesis, was confirmed *in vitro* using an aerobic heme synthesis assay [18,22], plasmon surface resonance [17], isothermal titration calorimetry [13] and nuclear magnetic resonance (NMR) [32]. The binding of frataxin to either ferrochelatase or the ISC proteins could only be observed in the presence of iron, suggesting that these interactions were a function of frataxin iron delivery [13]. Residues involved in the binding of iron by frataxin and frataxin interactions with other proteins were hypothesized to localize to a conserved acidic patch on the protein, which consists of α-helix 1 and β strand 1 [32-35]. Dr. Timothy Stemmler (Wayne State University) presented additional data from mutational analysis of carboxylate residues in the conserved acidic patch of yeast frataxin. Results obtained by nuclear magnetic resonance spectroscopy (NMR) with several mutant proteins (D50, E52, H44, E42, D35, H32/D35 and D35/E42/H44/D50/E52) suggested that these residues are important for frataxin iron binding and protein-protein interactions with ferrochelatase and ISU proteins. Additional clues on the interaction of frataxin with its binding partners were provided by Dr. Leonid Sazanov (Medical Research Council, United Kingdom), who presented the identification of a novel subunit, Nqo15, of respiratory complex I from *Thermus thermophilus* [36], which has a fold

similar to that of frataxin. Nqo 15 is permanently bound to the adjacent subunits in complex I and provides a reasonable model by which frataxin could interact with ferrochelatase and Isu proteins [36]. Indeed, Nqo15 interacts with complex I through its β sheet, the same surface through which frataxin is hypothesized to interact with its partner proteins. By analogy with frataxin, Nqo15 could function in the storage of iron and in the regeneration of ISCs of respiratory complex I. On the other hand, the presence in respiratory complex I of a protein structurally similar to frataxin supports the hypothesis that frataxin might have an important role in mitochondrial energy production [37]. In agreement with a possible link between frataxin and the mitochondrial electron transport chain, Dr. Francesc Palau's group (Instituto de Biomedicina, Spain) identified physical and functional interactions between yeast frataxin and subunits of complex II, namely succinate dehydrogenase subunits Sdh1p and Sdh2p. Similar interactions were also observed between the human proteins, frataxin and succinate dehydrogenase subunits SDHA and SDHB, as well as the corresponding *C. elegans* homologues [38,39]. In addition to interactions with the succinate dehydrogenase complex, interactions were also identified between frataxin and the electrons transfer flavoproteins ETFα and ETFβ. The fact that these interactions are conserved across species supports a probably important function of frataxin as a component of the electron transport chain. The authors suggested that frataxin could regulate entry of electrons into the electron transport chain via complex II or affect the stability and/or assembly of the succinate dehydrogenase complex.

The multi-functional nature of frataxin could be attributed to its ability to exist in both monomeric or assembled forms [22]. Earlier studies by Dr. Grazia Isaya's group (Mayo Clinic College of Medicine) showed that while both monomeric and oligomeric frataxin can bind and deliver ferrous iron, assembled frataxin also has the ability to sequester and detoxify excess iron [22]. Recent structural studies from Dr. Salam Al-Karadaghi's laboratory (Lund University, Sweden) suggest that trimer is the primary functional unit of yeast frataxin and also serves as the building block of larger frataxin oligomers [24]. Dr. Al-Karadaghi presented a possible mechanism to explain the multiple roles played by frataxin in iron binding and delivery or detoxification. The frataxin trimer would mainly be involved in the initial binding and delivery of iron to other proteins, while higher order oligomers have structural features suitable for iron detoxification and storage [24].

Dr. James Cowan (Ohio State University) presented evidence that a truncated ~14 kDa form of human frataxin (frataxin minus its 22 N-terminal residues) exhibits significant differences in iron binding and interactions with other partner proteins relative to the larger 17 kDa form [40], an important finding since several studies of frataxin have been performed with the shorter version. The 17 kDa form of human frataxin corresponds to the protein present inside mitochondria immediately after cleavage of the mitochondrial targeting sequence from the frataxin precursor by the mitochondrial processing peptidase (MPP) [41]. Further cleavage of the 17 kDa form of human frataxin to a product of 14 kDa has been found to occur both in mitochondria [41] as well as during expression of human frataxin in *E. coli* [42,43]. Dr. Cowan presented data suggesting that *in vitro* the 17 kDa form of frataxin has an iron-mediated self-cleavage function, which results in the 14 kDa form [40]. In addition, Dr. Ngolela Babady from Dr. Isaya's laboratory, presented the interesting finding that a well known mitochondrial enzyme, dihydrolipoamide dehydrogenase (DLD), has a "moonlighting" proteolytic activity that converts the mature 17 kDa form of frataxin to its shorter 14 kDa form [44]. Because this cleavage makes frataxin unable to assemble and detoxify iron *in vitro*[30], the authors suggested that the 14 kDa form of human frataxin might be the result of regulatory processing. Indeed, the primary function of DLD is to serve as the E3 component of the three α keto-acid dehydrogenase complexes, which is essential for energy metabolism across eukaryotes. However, solution conditions or point mutations that destabilize the DLD homodimer enable the enzyme to function as a protease via a

catalytic dyad (S456-E431) normally buried at the homodimer interface. Mutations in the DLD homodimer interface have been linked to an atypical form of DLD deficiency characterized by hypertrophic cardiomyopathy [45,46], one of the manifestations of FRDA. These findings should lead to future studies to assess if the moonlighting proteolytic activity of DLD contributes to the phenotypic variability of FRDA, and whether it could represent a target for therapies of the disease.

The discussion on the different forms of human frataxin was further stimulated by Dr. Roberto Testi (University of Rome, Italy) who raised the question of what is the actual size of the mature form of the protein. A construct expressing mature frataxin, as previously characterized after *in vitro* processing [41], migrated more slowly than the mature protein derived from *in vivo* processing in human cells transfected with wild-type frataxin. Direct N-terminal sequencing of the *in vivo* generated product and complementation experiments in frataxin-deficient cells suggested that the mature functional form of human frataxin is a 130 amino acids protein (i.e. the 14 kDa form discussed by Drs. Cowan and Babady) [47]. This interesting finding is in contrast with several earlier studies which analyzed the processing of the precursor form of frataxin into its mature form as detected in isolated human mitochondria, human tissues, and patient-derived cell lines [30,41,48,49]. Together, the results presented by Drs. Cowan, Babady and Testi underscored the importance of further investigating the processing of human frataxin, its functional implications, and how these aspects might be influenced in the context of different experimental settings.

The session ended with Dr. Testi challenging the notion that frataxin only localizes to mitochondria. Dr. Testi's group unexpectedly found an extramitochondrial localization for frataxin in different cell lines, including lymphoblasts, HeLa and HEK-293 cells [50]. Studies of this pool of extramitochondrial frataxin (EF) suggested that localization of frataxin to mitochondria was unnecessary for frataxin's role in protecting against oxidative stress and apoptosis. A transiently expressed mature EF effectively protected frataxin-deficient cells against ceramide- and staurosprine-induced ROS production. The authors suggested that the extramitochondrial localization of frataxin might be accomplished through an export mechanism, which remains to be defined.

Iron Metabolism

The session on iron metabolism in FRDA focused on the observation that mitochondrial iron overload develops in the heart and central nervous system of FRDA patients. The reason why the mitochondria of frataxin-deficient patients develop iron overload is unknown. However, the mitochondrial iron overload occurs not only with loss of frataxin, which compromises ISC assembly, but also when other recognized iron-sulfur cluster assembly proteins are dysfunctional. For instance, in yeast, Dr. Andrew Dancis (University of Pennsylvania) showed that mutations of the cysteine desulfurase responsible for providing sulfur to ISCs cause mitochondrial iron overload, and reported similar observations for other ISC assembly proteins [51].

Dr. Tracey Rouault (National Institutes of Health) reported that ISC assembly takes place not only in mammalian mitochondria, but also in mammalian cytosol. Dr. Rouault and colleagues have shown that the mechanism for cytosolic ISC assembly involves targeting of small fractions of ISC assembly enzymes and scaffolds to the cytosol [52]. Examples include ISCS, the cysteine desulfurase, in which a second downstream initiation AUG encodes a form of the enzyme that lacks a mitochondrial targeting signal, and ISCU, a scaffold protein in which alternative splicing leads to formation of two isoforms, one of which is mitochondrial, whereas the other is cytosolic. Similarly, alternative splicing of another scaffold protein, NFU, leads to formation of both mitochondrial and cytosolic forms.

To further study the role of ISC assembly in mammalian cellular iron homeostasis, Tong and Rouault used RNA interference technologies to interfere with ISC biogenesis in all compartments of the cell, or in the cytosolic compartment only. Using levels of cytosolic iron regulatory protein 2 (IRP2) as a measure of cytosolic iron status, they discovered that interfering with mitochondrial ISC assembly leads to cytosolic iron depletion, whereas interfering with cytosolic ISC assembly does not affect cytosolic iron status [53,54]. They speculated that a mitochondrial iron-sulfur protein regulates mitochondrial iron homeostasis, and that this mitochondrial regulatory protein signals the cell that mitochondria are iron deficient when it lacks an iron-sulfur cluster. Cells respond by increasing mitochondrial iron content, most likely by increasing mitochondrial iron uptake and decreasing mitochondrial iron export, perhaps by changing transcription of genes involved in mitochondrial iron import and export.

Dr. Arnulf Koeppen and his colleagues (Stratton VA Medical Center) presented studies that support the hypothesis that iron dysmetabolism is the underlying mechanism by which frataxin deficiency affects the heart and the central nervous system in FRDA. They found normal levels of total iron and ferritin in heart tissue of patients with FRDA, establishing that the heart in FRDA does not have global iron overload. Instead, electron microscopic studies revealed that iron excess was restricted mainly to mitochondria in heart muscle fibers, and iron deposits appeared to co-localize with ferritin and mitochondrial ferritin. Dr. Koeppen also showed that there is atrophy of the dentate nucleus of the cerebellum in FRDA. Similar to the heart, total dentate iron content was normal, but ferritin was decreased in the oligodendroglia, possibly because the neurons with which oligodendrocytes were associated died, and neurons and oligodendrocytes were replaced by ferritin-rich microglia and astrocytes. The authors found that the iron exporter, ferroportin, was present in clusters of terminals surrounding degenerating nerve cells in the dentate nucleus. These clusters, termed “grumose” degeneration, are believed to derive from axons of Purkinje cells. The authors concluded that there is partial Purkinje cell dysfunction in FRDA, even though Purkinje cells appear normal in tissue samples, because ferroportin expression in Purkinje cells is abnormal in FRDA patients [55].

Dr. Dancis discussed his use of the yeast model system to define the role of frataxin in ISC assembly. He and his colleagues purified mitochondria from yeast that lacked frataxin and assessed assembly of ISCs on two intramitochondrial ISC proteins, ferredoxin (Yah 1) and aconitase. They demonstrated that import of frataxin into mitochondria that lacked frataxin restored ISC assembly in the two target proteins. In yeast mitochondria that lacked both frataxin and the two mitochondrial iron importers, MRS3 and MRS4, addition of frataxin failed to restore ISC assembly, but addition of iron to media overcame the block to ISC assembly. Thus, their results supported the model that MRS3/4 import iron into mitochondria, whereas frataxin makes iron within mitochondria available for use in ISC assembly [56].

Dr. Gloria Ferreira (University of South Florida) discussed ferrochelatase, the enzyme that catalyzes the terminal step of the heme biosynthetic pathway by inserting ferrous iron into protoporphyrin IX. Previous work suggested that frataxin interacts physically with ferrochelatase, perhaps to deliver iron for insertion into the protoporphyrin IX ring. Using resonance Raman (RR) spectroscopy and time-resolved crystallography, her group examined the structural properties of ferrochelatase-bound porphyrins and porphyrin metallation, especially with respect to the porphyrin deformation occurring in the active site. They observed that ferrochelatase distorts its porphyrin substrate, enhancing the reaction rate and also modulating which divalent metal ion is incorporated into the porphyrin ring [57]. They proposed that the physical interaction between frataxin and ferrochelatase

controls the type of metal ion delivered to ferrochelatase and consequently regulates iron and heme metabolism.

Dr. Paolo Arosio (University of Brescia, Italy) reported on experiments performed to analyze X-linked sideroblastic anemia, a disease in which loss of function of a mitochondrial transporter known as ABCB7 causes mitochondrial iron overload in developing red cells. To better understand the role of ABCB7, he and his colleagues used RNA interference techniques to decrease (silence) ABCB7 in HeLa cells, a human cultured cell line. They found that silencing of ABCB7 resulted in a six-fold increase in mitochondrial iron content. Unexpectedly, the excess iron was not incorporated into mitochondrial ferritin, indicating that the excess iron was not available for ferritin loading. In addition, they found that levels of protoporphyrin IX increased, indicating that the excess iron was also poorly available for heme synthesis [58]. They observed that cytosolic aconitase activity was decreased, consistent with the possibility that ABCB7 exports iron sulfur clusters, or that the cytosol is iron depleted as a result of avid iron uptake by mitochondria from the cytosolic iron pool.

Cellular and Mitochondrial Pathophysiology of Friedreich's Ataxia

The mechanisms through which frataxin mutations cause the disease remain unclear, but studies of mitochondrial and cellular dysfunction have shed light on potential pathogenic pathways. To delineate cellular pathways that are altered in Friedreich's ataxia, several groups have studied transcript levels, protein expression, and protein functions in cells, tissues, and whole-organism models with decreased expression of frataxin.

Dr. Gino Cortopassi (University of California, Davis) presented data demonstrating that frataxin interacts with the mitochondrial proteins ISD11 and GRP75; both are components of the ISC biogenesis and insertion machinery in mammalian cells [59]. In addition, he demonstrated that two disease-associated mutations interrupted the ISD11-frataxin interaction, indicating specific alterations of iron metabolism that may contribute to FRDA. Using inducible small interfering RNA (siRNA) knockdown of frataxin in HEK294 cells, Dr. Cortopassi showed that frataxin deficiency initially impaired ISC proteins, followed by oxidative stress, and finally affected the heme pathway [60]. Frataxin knockdown caused defects in adrenodoxin, heme a, and cytochrome c oxidase activity, indicating a connection between ISC function and the heme pathway through the ISC enzyme adrenodoxin, which is critical for synthesis of heme a, a component of cytochrome c oxidase.

Dr. Giovanni Coppola (University of California, Los Angeles) presented microarray studies of frataxin knock-in/knock-out mice [61] revealing tissue-specific effects [62]. Heart tissue showed coordinated down-regulation of contractile-protein genes that could contribute to cardiomyopathy. In contrast, skeletal muscle revealed changes in genes involved in lipid metabolism and fatty acid oxidation, while liver demonstrated downregulation of glycolytic genes including phosphofructokinase and upregulation of lipid metabolism genes; together these changes may contribute to insulin resistance.

Dr. Jordi Tamarit from the laboratory of Dr. Joaquim Ros (Universitat de Lleida, Spain) observed that yeast cells lacking the yeast frataxin homolog (Yfh1p) have increased levels of anti-oxidant proteins including manganese superoxide dismutase (MnSOD), but decreased MnSOD activity, which was attributed to intracellular manganese deficiency possibly induced by accumulation of iron [63]. Proteomic studies of *yfh1*-null yeast revealed increased levels of carbonylated (oxidatively damaged) proteins. Dr. Javier Diaz-Nido (Universidad Autonoma de Madrid, Spain) noted that defects of mitochondrial respiratory chain enzyme complexes and glycogen synthase kinase-3 (GSK-3) activity have been

implicated in the pathogenesis of several neurodegenerative diseases. Using rotenone inhibition of mitochondrial complex I in cultured mammalian neurons (a cellular model of neurodegeneration), Dr. Diaz-Nido demonstrated that inhibition of GSK-3 may be neuroprotective by enhancing anaerobic glycolysis, which may overcome the defect of oxidative phosphorylation. Finally, using siRNA to decrease frataxin expression by ~80% in U2OS osteosarcoma cells, Dr. Christopher Halweg from Dr. Michael Resnick laboratory (National Institutes of Health) observed increased ROS sensitivity, which was attributed to excess iron and hydrogen peroxide. Lymphoblastoid cell lines from FRDA patients revealed increased susceptibility to DNA damage.

Friedreich's ataxia models

A number of model organisms (*S. cerevisiae*, *C. elegans*, *D. melanogaster*, *M. musculus*) have been developed over the past years to understand the function of frataxin and to model the disease. In this session, speakers presented the new advances in the characterization of these models as well as the development of new ones.

In order to identify novel pathways implicated in frataxin function, Dr. Astrid Haugen (National Institutes of Health) presented a comparative genomic study using a *Δyfh1* yeast mutant and a yeast model involving progressive shutdown of *Yfh1*. By mapping the expression data onto the yeast regulatory network of protein/protein and protein/DNA interactions, both frataxin knockout and knockdown strains exhibit severe impairment of the cytochromes, aconitase, mitochondrial protein synthesis, heme, and ISC assembly pathways even at the early stages of frataxin depletion, before any mitochondrial DNA lesions are observed. These candidate genes are being systematically knocked-out in the frataxin knockdown mutant in order to better characterize their genetic interaction with frataxin.

Dr. Thomas Johnson (University of Colorado at Boulder) investigated the inactivation of frataxin in *C. elegans* by RNAi-mediated suppression of the frataxin homolog gene frh-1 [64]. Frataxin-deficient animals have a small body size, reduced fertility and altered responses to oxidative stress. Interestingly, frataxin suppression by RNAi (25% residual frataxin) also significantly extends the lifespan of the worms, and appears to act independently from other major pathways that increase *C. elegans* lifespan. However, these data contradict those of Dr Palau's group, which reported that lowering frataxin in *C. elegans* decreases lifespan [39]. Discussions provided insight on the experimental conditions of RNAi administration and on the rate of frataxin depletion, two parameters which might explain the differences observed. Most particularly, this phenotypic difference might reflect a frataxin-level threshold effect [64].

To identify functions for frataxin in *D. melanogaster*, Dr. Juan Navarro (University of Regensburg, Germany) generated RNAi models silencing the *D. melanogaster* frataxin homolog gene (*Dfh*) by means of the UAS-GAL4 system [65]. Full lethality was achieved when *Dfh* was widely silenced, in agreement with previously reported models [66]. However, Dr. Navarro obtained viable flies with moderate systemic reduction of frataxin levels by RNAi that parallel the situation in FRDA patients. Under these conditions, *Dfh*-RNAi flies showed a shortened life span, reduced climbing activity and dramatic reduction of aconitase activity under hyperoxic conditions. As the activities of succinate dehydrogenase, respiratory complexes I and II, and indirectly III and IV, were normal, the authors suggested that the decay in aconitase activity was the primary event in the development of the pathology, and that frataxin's primary function is to protect aconitase from oxidative-stress-dependant inactivation, as has been suggested previously [67]. Dr. Peter Anderson (University of Guelph, Canada) also generated *D. melanogaster*, models of FRDA by RNAi-mediated suppression of *Dfh*. *Dfh* suppression recapitulated cellular

hallmarks of FRDA including decreased activities of heme- and iron-sulfur-containing enzymes, impaired iron homeostasis, and increased susceptibility to iron toxicity. Ubiquitous suppression of *Dfh* produced large, long-lived larvae and conditional, short-lived adults with impaired motility. Dr Anderson further reported that selective reduction of *Dfh* in the peripheral nervous system permits normal pre-adult development but imposes a marked decrease in adult lifespan [66]. To test the possibility that oxidative damage contributed to the deleterious effect of *Dfh* deficiency in the peripheral nervous system, compound transgenic strains over-expressing CoZnSOD, MnSOD, or cytoplasmic catalase in concert with *Dfh* suppression in the peripheral nervous system were generated. Curiously, over-expression of CoZnSOD or MnSOD in the peripheral nervous system exacerbated phenotypes associated with *Dfh* depletion, while over-expression of catalase ameliorated the symptoms. Such experiments on other FRDA target tissues, such as heart and skeletal muscle, are in progress.

Using a recombinase under the control of the human skeletal actin (HSA) promoter, Dr. Helene Puccio (Universite Louis Pasteur, Strasbourg, France) presented a new conditional mouse model completely deleted for frataxin in skeletal muscle. The mice exhibited a greatly decreased lifespan and developed a mitochondrial myopathy characterized by muscle fibers with central nuclei, numerous ragged red fibers (RRF) and abnormal accumulation of mitochondria. Activities of the ISC enzymes of the respiratory chain as well as aconitases were significantly decreased in the mutant mice. Although skeletal muscle is not primarily affected in FRDA, several reports demonstrated mitochondrial dysfunction in skeletal muscle of FRDA patients. The mitochondrial myopathy observed after complete frataxin skeletal muscle deletion in mice suggested that muscular symptoms may be present in FRDA patients but are masked by the neurological symptoms.

In parallel to the development of mouse models with total frataxin depletion, several groups have generated humanized models of FRDA, which contain the entire human frataxin genomic locus with the presence of a long GAA expansion. Dr. Mark Pook (Brunel University, United Kingdom) previously generated two lines of human genomic YAC *FRDA* transgenic mice that contain unstable GAA repeat expansion ($(GAA)_{190}$ and/or $(GAA)_{90}$) within the appropriate genomic context [68]. Dr. Pook described the first "humanized" GAA-repeat-expansion mouse models obtained by breeding either line of human frataxin YAC transgenic mice with heterozygous frataxin knockout mice [69]. These "humanized" mice expressed only decreased quantities of human-derived frataxin from the YAC transgene and were completely deleted for endogenous murine frataxin. These mice exhibited a progressive neurodegenerative phenotype with locomotor activity deficiency without a reduced lifespan. Histologically, these mice showed many abnormalities with the presence of vacuoles, chromatolysis and lipofuscin in the dorsal root ganglia, and lipofuscin and iron depositions in cardiomyocytes without any signs of cardiac hypertrophy. Furthermore, aconitase activity was decreased in the heart. These mice reproduced the early stages of the previously reported conditional mouse models. In addition, these mice exhibited an increase in oxidative stress in several tissues. Dr. Joseph Sarsero (Murdoch Childrens Research Institute, Australia) presented a similar strategy using a BAC vector. He had previously demonstrated that a BAC vector containing the normal human frataxin locus rescued the *FRDA* knockout mutation in transgenic mice [70]. Introducing a $(GAA)_{500}$ expansion in the first intron of the human frataxin gene present on the BAC, Dr Sarsero generated transgenic mice with the modified BAC, leading to decreased frataxin expression. The modified human transgene rescued the embryonic lethality of homozygous frataxin knockout mice. However, despite the low level of frataxin, these animals did not develop any phenotype by 1 year of age. By recapitulating the molecular basis of FRDA, these "humanized" YAC and BAC mouse models offer important perspectives in the analysis of the progressive pathophysiology of the disease and variability thereof, and will be extremely

useful in the evaluation of potential new therapies specifically targeted at overcoming the molecular effects of the GAA expansion.

To obtain a human neuronal FRDA cellular model, Dr. Diaz-Nido (Universidad Autonoma de Madrid, Spain) presented the use of lentiviruses encoding short hairpin RNAs complementary to the frataxin gene in human SH-SY5Y neuroblastoma cells. This clonal cell line has the advantage of being neuronal in lineage and is able to differentiate into a homogeneous population of neuron-like cells [71]. The lentiviral shRNAs were efficient at down-regulating frataxin gene expression to almost negligible levels, leading to neurite retraction, atrophy and cell death in differentiated neuron-like cells. Non-differentiated neuroblastoma cells were less sensitive to frataxin deficiency, with a small increase in cell death but with a significant proliferation defect. This model of frataxin down-regulation in mature neuronal cells could provide a very useful tool to study the molecular consequences of frataxin depletion in neurons.

Approaches to Therapeutics

A variety of exciting therapeutic and drug-development strategies for FRDA were presented. Both pharmacologic approaches, using CoQ_{10} analogs, iron-chelators or compounds that increase frataxin expression, as well as newer strategies, such as gene therapy, were discussed.

Dr. Jonathan Plehn (National Institutes of Health) and colleagues used echocardiography to examine the prevalence of left-ventricular (LV) hypertrophy and cardiac functional associations in a cohort of 48 young individuals (ages 9 to 17) with FRDA before they were enrolled in a phase II trial of idebenone. LV wall thickening greater than 1.1 cm was found in 39% of subjects and general LV hypertrophy in 45% of subjects. Strikingly, however, LV concentric remodeling, an early marker of hypertensive cardiomyopathy, was found in 73% of subjects as indicated by sub-threshold relative wall thickness (RWT). LV ejection fraction was mildly reduced (<55%) in only two subjects; the group mean (71%) was well within the normal range (55-75%) and cardiac index was unimpaired. These results suggest that there is a high prevalence of cardiomyopathy in FRDA, as detectable by RWT, and that LV hypertrophy correlates poorly with global systolic dysfunction.

Dr. Pierre Vankan (Santhera Pharmaceuticals) described four phase I studies of a new formulation of idebenone, SNT-MC17, in healthy adult males. SNT-MC17 was well-tolerated in single oral doses of up to 1050 mg, and in repeated daily oral doses up to 750 mg three times per day (2250 mg daily), for up to 14 days. The most frequently observed adverse events were gastrointestinal symptoms. In addition, there was a dose-dependent discoloration of the urine, which may complicate placebo-controlled studies. No clinically relevant effects on vital signs, physical findings, or laboratory parameters were observed. These results suggest that daily doses of SNT-MC17 up to 2250 mg may be appropriate in clinical efficacy trials for FRDA.

Dr. Nicholas Di Prospero (National Institutes of Health) described a six-month, double-blind, placebo-controlled phase II trial of idebenone for FRDA, comprising four arms of 12 patients (ages 9 to 17) each: placebo, ~5 mg/kg/day, ~15 mg/kg/day, and ~45 mg/kg/day. The primary endpoint measure was urinary and plasma 8-hydroxy-2-deoxyguanosine, a marker of oxidative stress. Secondary endpoints included the International Cooperative Ataxia Rating Scale (ICARS), Friedreich's Ataxia Rating Scale (FARS), various functional measures, health related quality of life score (SF-10), and activities of daily living (ADL). Idebenone was well-tolerated at all dose levels, with similar types and frequencies of adverse events among all treatment groups including placebo. However, one subject in the high-dose group developed neutropenia after six months on idebenone, which resolved

following discontinuation of the drug. Although an overall statistical analysis did not reach significance for primary or secondary endpoints, there was an indication of a dose-dependent response with ICARS ($p<0.05$). A pre-determined subset analysis excluding those requiring wheelchair assistance showed a trend toward improvement with ICARS ($p<0.01$) and a dose-related response in ICARS, FARS, and ADL scores ($p<0.05$) by Jonckheere analysis. These results suggest that a larger phase III trial is warranted to test whether high-dose idebenone is safe and efficacious.

Dr. Susan Perlman (University of California at Los Angeles) described the plans of Antipodean Pharmaceuticals for a one-year, multinational, placebo-controlled phase II trial of MitoQ (mitoquinone mesylate) for 130 individuals with FRDA. MitoQ is a mitochondrially targeted antioxidant developed to protect mitochondria from lipid peroxidation by reactive oxygen and nitrogen species and thereby prevent apoptosis. The primary endpoint will be the FARS. Secondary endpoints comprise safety measures, pharmacokinetics, components of the FARS, other indicators of disease severity, quality of life scales, and cardiac measures. The goals are to gather preliminary data on whether MitoQ can slow or reverse the progression of FRDA and to properly design and power a potential phase III efficacy trial.

Dr. William Shrader (Edison Pharmaceuticals) described the development of new compounds of the CoQ10 para-benzoquinone class for the treatment of FRDA. The redox properties of the para-benzoquinone head group, and the lipophilicity of the hydrophobic tail, were systematically altered. The goal was to maximize efficacy, by optimizing redox properties in relation to the respiratory chain defects in FRDA, as well as pharmacokinetics and bioavailability. In cell-culture models of FRDA, efficacy was maximized by altering both lipophilicity and the redox potential of the head group, thereby defining an optimized combination of the two for FRDA. A translational strategy for these compounds, involving profiling of serum-derived metabolic biomarkers and enabling rational selection of the most favorable compounds for further clinical development, was described.

With the goal of improving cellular energy production, Vincent Paupe from Dr. Pierre Rustin's laboratory (INSERM U676, Hôpital Robert Debré, France) presented an overview of the rationale for using pioglitazone to counteract FRDA. Pioglitazone is an agonist of peroxisome proliferator activated receptors gamma (PPARγ), which is known to increase β-oxidation of fatty acids and antioxidant defenses and to decrease inflammatory responses. Neuroprotective effects of pioglitazone have been suggested in an amyotrophic lateral sclerosis mouse model [72]. Already commercialized as an antidiabetic drug, pioglitazone crosses the blood-brain barrier without showing strong side effects. The treatment of FRDA patient fibroblasts by pioglitazone revealed no effect on cellular proliferation or the respiratory chain. Dr Rustin plans to perform a one-year pilot clinical trial on a small FRDA patient cohort to test the hypothesis that it is beneficial for FRDA.

Nadege Carelle from Dr. Puccio's team presented preliminary results of a large-scale drug screen using a frataxin-deficient murine cellular model. The model, based on a ribozyme strategy, exhibits a proliferation defect associated with a quantifiable decrease in ATP production. The chemical library tested consists of a collection of more than one thousand drugs, all off patent, selected for their structural diversity and their broad therapeutic spectrum. Forty-two compounds that increase the ATP production rate of the frataxin-deficient cell line have been identified and dose-response assays are in progress to confirm these hits.

As the frataxin coding sequence is usually unaltered in FRDA patients, and as there is a correlation between the amount of residual frataxin and the severity of the disease, a

potential strategy for the treatment of FRDA is pharmacologic upregulation of frataxin gene expression to therapeutic levels. Researchers are using different approaches to find such compounds. Using a stable human cell line containing a human frataxin-EGFP fusion construct [73], Dr. Sarsero reported the initial results from a screen of a library containing 2,000 FDA-approved drugs tested for their ability to increase frataxin-EGFP expression. Eighteen compounds were shown to increase frataxin expression rate more than three-fold relative to untreated cells; these compounds are currently being characterized further. Dr. Michael Hebert (University of Mississippi Medical Center) presented an adapted competition dialysis method [74] to identify small molecules that promote the duplex form of the GAA repeat found in the first intron of the FRDA disease gene. Using stable human cell lines containing a portion of intron 1 with an expanded GAA repeat fused to a GFP reporter, 7/126 compounds were found to increase GFP protein expression [75]. One of these compounds, pentamidine, known as a DNA minor groove binder and already approved for the treatment of infections in patients with HIV, increases frataxin protein levels in lymphoblasts from individuals with FRDA. Screening of additional compounds in patient cells is in progress. Drs. Barbara Scheiber-Mojdehkar (Medical University of Vienna, Austria), Sergio Cocozza (Università 'Federico II', Italy) and Franco Taroni (Istituto Nazionale Neurologico 'Carlo Besta', Italy) presented their data on the effect of recombinant human erythropoietin (rhuEPO) on frataxin expression. RhuEPO was shown to increase frataxin protein levels in isolated primary lymphocytes from FRDA patients, and in other non-FRDA cell types such as human cardiac cells and murine neurons [76]. Dr. Cocozza confirmed a modest increase (9.9%) of frataxin protein level after 24 hours rhuEPO (9.9 IU/ml) treatment of primary fibroblasts from FRDA patients, without any effect on mRNA expression. Dr. Taroni presented preliminary data on primary rat sensory neurons demonstrating an increase in frataxin staining by immunohistochemistry after rhuEPO treatment. As a proof of concept study, Dr. Scheiber-Mojdehkar presented data from an 8-week open trial of 5,000 IU rhuEPO 3 times per week for 12 adult patients with FRDA. A stable upregulation of frataxin protein level was observed in 70% of the patients in whole blood as well as lymphocytes, accompanied by a significant reduction of 8-hydroxy-2-deoxyguanosine in urine. Furthermore, an improvement in the SARA (Scale for the Assessment and Rating of Ataxia) scores was noted. Dr. Taroni outlined an ongoing trial for 16 patients with FRDA comparing the combination of idebenone and rhuEPO against idebenone alone.

Iron chelation is another candidate route for treatment of FRDA. Dr. Des Richardson (University of Sydney, Australia) used the cardiac conditional frataxin knockout mouse model [77] to test the efficacy of a mitochondrially permeable iron chelator, pyridoxal isonicotinoyl hydrazone (PIH) (150 mg/kg/day) [78] in combination with desferrioxamine (DFO) by intraperitoneal injection. Strikingly, both a decrease in total body weight loss and prevention of the development of cardiac hypertrophy (as measured by the heart/body weight ratio) were observed. Furthermore, the treatment of the mice led to an absence of myocardial iron deposits and cardiac fibrosis, without any alteration in hematologic indices. Studies of survival and cardiac function, as well as ISC enzyme activities, are in progress. Dr. Ioav Cabantchik (Hebrew University of Jerusalem, Israel) pointed to the necessity of removing regional mitochondrial labile iron in FRDA patients with minimal interference on systemic iron status. To slow the progression of neurological damage, Dr. Cabantchik, in close collaboration with Dr. Arnold Munnich (Hopital Necker-Enfants Malades and Universite Paris V Rene Descartes, France), performed an efficacy-toxicity phase I-II open label trial with the mitochondrial iron chelator deferiprone (L1) [79]. Eleven adolescents with FRDA received 6 months of either 20-30 mg/kg/day p.o. of deferiprone in 2 daily doses, or placebo, in combination with idebenone at 10 mg/kg/day p.o. in 3 daily doses. Brain iron content was monitored by the measurement of proton relaxation rates R2* by MRI. Deferiprone intake led to a progressive and significant decrease in R2* of the *dentate*

nuclei, whereas no effect was observed in the placebo arm. Dr. Cabantchik also reported clinical improvements of neurologic symptoms in the youngest patients, without significant changes in hematologic indices [79].

Finally, Dr. Filip Lim (Universidad Autonoma de Madrid) and Dr. Mark Payne (Indiana University School of Medicine) presented two non-pharmacologic therapeutic strategies: a viral gene-therapy approach and a protein-replacement approach. Dr. Lim has generated localized frataxin knockout mouse models by stereotaxic injection of Cre recombinase expressing HSV-1 amplicon vectors in the olivocerebellar circuit of frataxin conditional transgenic knockout mice. These mice develop a behavioral deficit on the rotarod detectable 4 weeks after injection of the Cre-HSV-1. A second stereotaxic injection of HSV-1 amplicon vectors expressing human frataxin cDNA rescued the rotarod phenotype. Behavioral recovery was detectable as early as 4 weeks after the second injection, with a stable difference up to 12 weeks compared to mice re-injected with lacZ vector. Dr. Payne presented work on novel protein transduction domains (PTD), notably the transactivator of transcription (TAT) peptide from the human immunodeficiency virus, to deliver multiple proteins to mitochondria both in cell culture and *in vivo* [80-82]. TAT is an 11-amino acid, positively charged peptide that is highly efficient at delivering protein cargos across cell membranes and intracellular membranes as well. They have previously demonstrated that fusion proteins containing TAT with a mitochondrial targeting sequence translocated efficiently through the mitochondrial membrane, with appropriate processing and persistence of the fusion protein within mitochondria. To circumvent frataxin deficiency, they have developed a TAT-human frataxin fusion protein targeted to the mitochondria, and have shown that it is correctly processed in human FRDA fibroblasts in culture. This TAT-frataxin fusion protein will be tested for its capacity to partially or fully rescue the phenotype both of FRDA fibroblasts in culture and in knockout conditional mouse models. TAT-frataxin fusion proteins represent a novel therapeutic approach for FRDA.

Phase III Efficacy Measures

As a number of therapeutic approaches to FRDA come to light the importance of accurate sensitive outcome measures becomes critical. Presentations included clinical examination based measures as well as surrogate outcome measures.

The first presentation by Dr. David Lynch (University of Pennsylvania) reported work from a collaborative study involving six sites in the USA. One hundred and fifty five individuals with genetically confirmed FRDA were assessed with the Friedreich Ataxia Rating Scale (FARS) and a series of performance measures: the 9-hole peg test, the timed 25-foot walk, the Sloan low-contrast letter chart, and the PATA test of speech). All measures demonstrated significant change over 12 months, albeit that the changes were modest. The effect size of change was greater after 2 years of measurement. The authors of this study concluded that while both the FARS and the composite measure were valid measures of disease progression, the functional composite was likely to be modestly more sensitive than the FARS at measuring change in FRDA.

A study was presented by Dr. Michael Fahey from Dr. Martin Delatycki's group (Murdoch Childrens Research Institute, Australia), looking at the performance of the FARS compared to the International Cooperative Ataxia Rating Scale (ICARS), the Functional Independence Measure (FIM) and the Modified Barthel Index (MBI). Here 43 subjects were tested 12 months apart. The FARS demonstrated the greatest change as measured by effect size and power calculations based on the number of subjects needed to be recruited for clinical trials.

There was discussion surrounding the greater change in the FARS over 12 months in the Australian compared to the US study. Possible explanations for this include that the US cohort were at a more advanced stage of disease at entry to the study, that the Australian patients were all examined by one examiner, and that antioxidant use is much greater amongst subjects in the United States than in Australia. Further studies are required to resolve these discrepancies.

Dr. Alexandra Durr (Salpetriere Hospital, France) presented data from long-term (median of 5 years) follow-up of individuals with FRDA. At study entry none were on antioxidant therapy, but 88 of 104 undertook treatment with idebenone during the study. The deterioration in ICARS score during the treatment period was greater in those untreated by Idebenone compared to those who were treated. However, the untreated patients had significantly shorter disease duration at inclusion. There was a significant decrease in cardiac hypertrophy as measured by left ventricular mass index in the group treated with Idebenone.

Louise Corben from Dr. Delatycki's group, presented work on assessment of motor processing. The 15 subjects with genetically confirmed FRDA were tested on a tapping board which required responding to change in the distance moved or the directional movement, or a combination of the two. Individuals with FRDA were significantly slower than controls in all of the changes in movement. The highest correlations were between the change in movement time and age of onset, as well as with the FARS score. The authors of this presentation concluded that the results suggest an impact of FRDA on developing cognitive capacity and motor planning.

Dr. Fahey presented data on comprehensive studies of eye movement in FRDA. This was undertaken using scleral search coils and infrared oculography. The team found significant fixation abnormalities, particularly square wave jerks and flutter that were broadly associated with disease progress. Vestibular abnormalities were evident from prolonged saccadic latency and decreased vestibulo-occular reflex gain. The authors concluded that the results implicate abnormalities in the brainstem, cortical and vestibular systems in FRDA.

Dr. Chris Gomez (University of Chicago) presented ^{1}H magnetic resonance spectroscopy (MRS) data. The levels of N-acetyl aspartate and glutathione were significantly lower in subjects with FRDA compared to controls. There was also a significantly decreased glutamate-to-glutamine ratio in the vermis. The authors of this study concluded that MRS can non-invasively quantify neuronal viability and oxidative stress in individuals with FRDA.

Dr. Raffaele Lodi (University of Bologna, Italy), presented his group's data on proton MRS and diffusion-weighted imaging (DWI) in FRDA. They studied 9 subjects with genetically confirmed FRDA and 8 controls. They found that N-acetyl aspartate is deceased in individuals with FRDA and that the average diffusivity map values were increased in the cerebellum in individuals with FRDA. There was a correlation between these values and GAA repeat size and score on the ICARS scale. The authors of this study concluded that the results indicate that proton MRS and DWI can quantify *in vivo* the extent of neurodegeneration in FRDA and provide robust surrogate markers of disease progression that are useful for evaluating therapeutic interventions.

Dr. Bennett Van Houten (National Institutes of Health) presented data from the NIH idebenone trial on mitochondrial DNA damage and gene-expression profiling in lymphoblastoid cells and peripheral lymphocytes. Levels of mitochondrial DNA damage using quantitative PCR were measured before and after idebenone treatment. In addition,

gene-expression profiling using a 22,000 oligonucleotide gene array chip was undertaken. The latter study revealed global transcriptional changes associated with cell death, cardiovascular disease, neurological disease and muscular and skeletal disorders. Further work was to be undertaken to look at the gene-expression profile after idebenone treatment, and the authors hypothesized that the pattern of gene-expression profiles and mitochondrial DNA damage will change with treatment, and that such tests will be useful biomarkers to monitor disease progression and response to therapeutic interventions.

The final presentation was from Dr. Michael Marusich (MitoSciences Incorporated in Oregon). Dr. Marusich presented data related to a dipstick test that the company has developed for measuring frataxin levels. He presented data that showed that decreased frataxin levels in lymphoblastoid cell lines from individuals with FRDA can be detected with the dipstick. The residual frataxin levels measured with the dipstick correlate inversely with the number of GAA repeats in the cells. He presented data indicating that the dipstick can measure frataxin levels in cheek-swab and finger-prick-blood tests. He concluded that the dipsticks can be used as a diagnostic test and for assessing the efficacy of therapies in FRDA.

The presentations in the session on Phase III efficacy measures revealed a number of clinical and surrogate biomarkers that can be used to assess the efficacy of therapeutic agents. This work will be of importance in testing the ever-increasing number of agents that show promise as therapies for FRDA.

Acknowledgments

The authors would like to acknowledge the Friedreich's Ataxia Research Alliance (FARA), the National Institutes of Health Office of Rare Diseases (NIH/ORD) and the National Institute of Neurological Disorders and Stroke (NINDS) for the organization of the conference as well as financial support. The authors would also like to extend special thanks to the organizing committee, Ronald Bartek (FARA President), Jennifer Farmer (Conference Manager), and the many FARA volunteers who made the conference possible.

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