FARA and NIH collaborated to fund, organize and co-host the second international Friedreich's ataxia scientific research conference that was scheduled to take place from February 14 to 16, 2003, at NIH in Bethesda, Maryland, outside Washington, D.C. About 100 scientists from 12 countries around the world came together to compare findings, share insights and chart the course ahead in the search for treatments and a cure for Friedreich's ataxia (FRDA). Also participating in the conference were representatives of pharmaceutical companies interested in helping develop drug therapies for FRDA, and other patient advocacy groups with which FARA is collaborating to advance FRDA research. The overall objectives of the conference were to integrate the most up-to-date information from the various research disciplines relevant to FRDA, to identify promising new avenues for research, to foster collaborations among researchers in the field, to encourage new investigators to enter the field, and to coordinate approaches to clinical studies and clinical trials.

On what was supposed to be the final day of the conference, Sunday, February 16, the Blizzard of '03 struck the Washington area. The two feet of snow were a blessing in disguise. All the scientists were able to make their presentations on schedule before they were transported to their hotel, where they were stranded for two additional days with little to do but continue their discussions. A great deal was accomplished across what became a five-day conference for most participants.

PROGRAM SCHEDULE

Friday, February 14, 2003

12:00 - 2:00 PM Registration

1:45 - 2:00 PM Opening Remarks

2:00 - 4:00 PM **Session I - The Friedreich's Ataxia Disease Gene**
- oGenotype/phenotype correlations
- oGAA repeat structure and transcription
- oGAA repeat instability
- oPoint mutations
- oModifiers

4:00 - 4:15 PM Roundtable discussion

4:15 - 4:30 PM Break

4:30 - 6:00 PM **Session II - The Friedreich's Ataxia Protein**
- oBiogenesis, structure, and transport
- oMultimerization and iron interactions
- oYFH1p function
- oEffects of point mutations

6:00 - 6:15 PM Roundtable discussion

6:15 - 6:30 PM Transportation to Reception

6:30 - 7:00 PM Opening Reception

7:00 - 9:00 PM Dinner

Saturday, February 15, 2003

7:30 - 8:30 AM Registration and Breakfast

8:30 - 10:00 AM **Session IIIA - Friedreich's Ataxia Cells - Yeast Studies**
- oOxidant damage
- oIntracellular iron distribution
- oMitochondrial dysfunction

10:00 - 10:15 AM Roundtable discussion

10:15 - 10:30 AM Break

10:30- 12:15 PM **Session IIIB - Friedreich's Ataxia Cells - Mammalian Studies**
- oOxidant damage
- oIntracellular iron distribution
- oMitochondrial dysfunction

12:15 - 12:30 PM Roundtable discussion

12:30 - 2:00 PM Lunch

2:00 - 4:00 PM **Session IV - Friedreich's Ataxia Models**
- oLower organisms
- oMouse models
- oCell culture

4:00 - 4:15 PM Roundtable discussion

4:15 - 4:30 PM Break

4:30 - 7:00 PM **Session V - Clinical Studies/Outcome Measures**
- oAtaxia scales
- oCardiac ultrasound
- oSpectroscopic studies

7:00 - 7:15 PM Roundtable discussion

7:15 - 7:30 PM Transportation to Dinner

7:30 - 9:30 PM Dinner

Sunday, February 16, 2003

7:15 - 8:15 AM Registration and Breakfast

8:15 - 10:30 AM **Session VIA - Therapy - Drug Development**
- oDrug screening and development

10:30 - 10:45 AM Roundtable discussion

10:45 - 11:00 AM Break

11:00 - 12:45 PM **Session VIB - Therapy - Clinical Trials**
- oCompleted clinical trials
- oClinical trials in progress
- oPlanned clinical trials

12:45 - 1:00 PM Roundtable discussion

1:00 - 1:15 PM Closing Remarks

1:15 - 2:45 PM Lunch

SESSION CHAIRS

Session I – The Friedreich's Ataxia Disease Gene, Robert D. Wells, Ph.D.

Session II – The Friedreich's Ataxia Protein, Grazia Isaya, Ph.D.

Session III – Friedreich's Ataxia Cells, Massimo Pandolfo, M.D.

Session IV – Friedreich's Ataxia Models, Helene Puccio, Ph.D.

Session V – Clinical Studies/Outcome Measures, Bronya Keats, Ph.D.

Session VI – Therapy, Robert B. Wilson, M.D., Ph.D.

Session I - The Friedreich's Ataxia Disease Gene

oGenotype/Phenotype correlations
oGAA repeat structure and transcription
oGAA repeat instability
oPoint mutations
oModifiers

Sticky DNA: Structure, Replication, Transcription, and Recombination

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Sticky DNA is a long GAA•GAA•TTC triplex formed between a pair of FRDA GAA•TTC tracts located at distant sites in one DNA molecule. We have investigated the formation of sticky DNA from plasmid monomers and dimers; sticky DNA is formed only when two tracts of sufficiently long $(GAA•TTC)_n$ (n = 59–270) are present in a single plasmid DNA and are in the direct repeat orientation. If the inserts are in the indirect (inverted) repeat orientation, no sticky DNA was observed. Furthermore, kinetic studies support the intramolecular nature of sticky DNA formation. Electron microscopy investigations also provide strong data for sticky DNA as a single long triplex between a pair of GAA•TTC tracts which are distantly located in one DNA molecule.

Other types of polypurine•polypyrimidine sequences were also studied for their capacity to form sticky DNA in *E. coli* plasmid systems in order to determine the potential occurrence of this conformation throughout biological systems. A mirror repeat, dinucleotide tract of $(GA•TC)_{37}$, which is ubiquitous in eukaryotes, formed sticky DNA, as did $(GGA•TCC)_n$ inserts (where n = 126, 159, and 222 bp). As shown previously, the control sequence $(GAA•TTC)_{150}$ (450 bp) readily adopted the X-shaped sticky structure; however, this conformation has never been found for the nonpathogenic $(GAAGGA•TCCTTC)_{65}$ of the same approximate length (390 bp). Interestingly, tracts of $(GAA•TTC)_n$ (where n = 176 or 80) readily formed sticky DNA with $(GAAGGA•TCCTTC)_{65}$ cloned into the same plasmid when the pair of inserts was in the direct, but not in the indirect (inverted), orientation. The stabilities of the triple base (Watson-Crick and Hoogsteen) interactions in the DNA/DNA associated triplex region of the sticky conformations account for these observations. The $(GAAGGA•TCCTTC)_{65}$ tract was also found in intron 1 of the FRDA gene. Thus, the presence of these two repeating R•Y tracts may enable the formation of sticky DNA in patients.

Sticky DNA effectively inhibits transcription *in vitro* using the T7 or SP6 RNA polymerases. Furthermore, both transcription and replication are effectively inhibited by this novel DNA conformation in expanded GAA•TTC triplet repeats in transiently transfected monkey COS 7 cells *in vivo*. In addition, sticky DNA reduces the frequency of intramolecular recombination *in vivo* (see abstract by Dr. M. Napierala).

In summary, these results give new insights into our understanding of the capacity of the novel sticky DNA conformation to inhibit biological processes and thereby reduce the level of frataxin protein as related to the etiology of Friedreich's ataxia. (Supported by the NIH, FARA, and the Robert A. Welch Foundation.)

Recent References:

Sticky DNA, a Long GAA•GAA•TTC Triplex That is Formed Intramolecularly, in the Sequence of Intron 1 of the Frataxin Gene. Alexandre A. Vetcher, Marek Napierala, Ravi R. Iyer, Paul D. Chastain, Jack D. Griffith, and Robert D. Wells. *J. Biol. Chem.* 277, 39217-39227 (2002).

Sticky DNA: Effect of Poly Purine•Pyrimidine Sequence. Alexandre A. Vetcher, Marek Napierala, and Robert D. Wells. *J. Biol. Chem.* 277, 39228-39234 (2002).

Sticky DNA Formation in vivo Reduces the Intramolecular Recombination at GAA·TTC Sequences from Intron 1 of the Friedreich's Ataxia Gene

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Homologous recombination enables the expansions and deletions of triplet repeat sequences. Our prior investigations also revealed that long GAA·TTC tracts from intron 1 of the Friedreich's ataxia gene adopted very stable triplex structures (sticky DNA). Sticky DNA formation depends on negative supercoiling and the presence of divalent metal ions. This R·R·Y triplex structure between two tracts of GAA·TTC in one DNA molecule was shown to inhibit transcription of the FRDA gene in vitro and in monkey cells. Herein, we used two recently developed genetic recombination assays to analyze the recombinational properties of GAA·TTC sequences in E. coli. Intramolecular as well as intermolecular recombination determinations showed that the frequency of recombination between GAA·TTC tracts is up to 15 times higher when compared to non-repeating control sequences. These results, together with our previous studies on the myotonic dystrophy (DM) CTG·CAG sequences, suggest that the recombinational hot spot characteristics may be a common feature of all TRS sequences associated with human hereditary neurological diseases. Moreover, biochemical analyses of the recombination products demonstrated that both intramolecular and intermolecular recombination events led to the high instability (expansions and deletions) of the GAA·TTC repeats. Unexpectedly, we found that increasing the length of the homologous sequences, from 33 to 176 GAA·TTC repeats, decreases the intramolecular recombination frequency between TRS tracts. This result is directly contrasting with extensive data from similar studies on the DM CTG·CAG sequence. A correlation was also found between the propensity to adopt the sticky DNA conformation by GAA·TTC tracts and the inhibition of intramolecular recombination. The use of novobiocin (a DNA gyrase inhibitor known to decrease the negative superhelical density of DNA inside living cells and to destabilize the sticky DNA conformation) diminished this effect, thereby restoring the positive correlation between the length of the GAA·TTC tracts and the frequency of intramolecular recombination. Our previous work on sticky DNA formation revealed the capacity of a long tract of GAA·TTC to pair with the intron 1 $(GAAGGA{\cdot}TCCTTC)_{65}$ tract to adopt this unorthodox conformation. Interestingly, intramolecular recombination occurs between these two tracts to generate complex (GAA)n(GAAGGA)m motifs. Thus, we conclude that the influence of sticky DNA on intramolecular recombination proves its existence and function in living cells and therefore strengthens the likelihood of its role in the etiology of FRDA. (Supported by NIH, FARA, and the Welch Foundation).

A persistent RNA*DNA hybrid in the Friedreich ataxia triplet repeat is formed by transcription in vivo

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Expanded GAA*TTC repeats in the first FRDA intron cause Friedreich ataxia by reducing frataxin expression. The degree of reduction correlates with GAA*TTC tract length, but the mechanism of reduction remains controversial. Here we show that transcription of GAA*TTC templates leads to extensive RNA*DNA hybrid formation both in vitro and in live bacteria. The RNA*DNA hybrid can span the length of the GAA*TTC tract, but does not appear to extend beyond it. RNA polymerase arrest during in vitro transcription is tightly coupled to the presence of a hybrid. Transcription in the presence of RNase H1, which specifically degrades the RNA in an RNA*DNA hybrid, produces transcripts that are cleaved within the GAA repeat. RNA*DNA hybrid formation appears to be an intrinsic property of transcription through long GAA*TTC tracts. Hybrid formation in FRDA repeats may contribute to the frataxin transcript deficit by multiple mechanisms.

GAA repeats may induce changes in chromatin configuration *in vivo*, which may underlie the down-regulation of frataxin gene expression in Friedreich's ataxia.

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Friedreich's ataxia (FRDA) is the commonest of the hereditary ataxias. It is caused by the homozygous expansion of the GAA trinucleotide repeat found within the first intron of the frataxin gene, causing marked down regulation of its transcription and expression [1]. The molecular basis for this down regulation *in vivo* is unclear.

DNA is packaged as chromatin within the nucleus. Previously thought of as an inert structural interaction, chromatin packaging of DNA has recently proven to have an essential role in gene regulation. Found in association with highly repetitive DNA at the centromere and associated with gene silencing, is 'closed' chromatin (heterochromatin). 'Open' chromatin (euchromatin) is located at more permissive regions for gene expression such as the chromosomal long arm. Euchromatin is thought to allow access of transcription factors to DNA permitting active gene expression. Placing genes in close proximity to heterochromatin (e.g. centromeres) results in the characteristic phenomenon of position effect variegation (PEV), where a proportion of cells that would otherwise express become silenced. Therefore the hypothesis was addressed that if a pathologically expanded GAA trinucleotide repeat induced 'closed' chromatin formation, then expression from a nearby gene would be expected to variegate regardless of chromosomal integration site. To this end a human CD2 1.3 (hCD2 1.3) reporter transgene linked to a GAA repeat was used to make transgenic mice. The hCD2 1.3 transgene has been previously shown to be sensitive to centromeric heterochromatin in transgenic mice, with expression of hCD2 usually variegating when the transgene was integrated at the centromere but not in the euchromatic chromosomal long arm.[2].

To avoid confounding effects of impaired transcription through the repeat as occurs *in vitro* [3-6], a $(GAA)_{200}$ repeat expansion was linked to the 3' untranscribed region of the hCD2 1.3 transgene. Twelve hCD2 1.3(GAA) transgenic mouse lines were generated with a range of transgene copy numbers. The expression pattern of hCD2 on T lymphocytes from thymus and lymph node was established by flow cytometry. In all transgenic mouse lines hCD2 expression variegated and notably in six the transgene had integrated on the chromosomal long arm, as shown by fluorescence *in situ* hybridisation (FISH). Variegation of hCD2 expression in these chromosomal long arm lines suggested that the GAA repeat had converted the usual euchromatin environment to heterochromatin. DNase I hypersensitive site analysis established that the chromatin configuration of the transgene promoter was converted to 'closed' chromatin in non-expressing T lymphocytes from hCD2 1.3(GAA) mice. The chromatin state of the hCD2 1.3 promoter has been previously shown to correlate with expression status [7].

This work provides a novel *in vivo* mechanism for gene repression caused by expanded GAA trinucleotide repeats; the expanded repeat acts to condense chromatin of nearby gene regulatory elements resulting in variegation of gene expression. Down-regulation of frataxin gene expression in FRDA may be due to a mechanism resembling PEV. The molecular basis for chromatin formation and regulation is becoming increasingly well characterised and many factors involved have been identified. For example, inhibition of histone deacetylases 'opens' chromatin, converting genes from a silent to an actively transcribing state. Perhaps manipulations such as this will provide a means to treat FRDA, one of several neurodegenerative disorders for which there is currently no effective therapy.

References

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4. Ohshima, K., et al., *Inhibitory effects of expanded GAA.TTC triplet repeats from intron I of the Friedreich ataxia gene on transcription and replication in vivo*. J Biol Chem, 1998. **273**(23): p. 14588-95.
5. Sakamoto, N., et al., *Sticky DNA, a self associated complex formed at long GAA*TTC repeats in intron 1 of the frataxin gene, inhibits transcription*. J Biol Chem, 2001. **276**(29): p. 27171-7.
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7. Festenstein, R., et al., *Heterochromatin protein 1 modifies mammalian PEV in a dose- and chromosomal-context-dependent manner*. Nat Genet, 1999. **23**(4): p. 457-61.

Expansion of GAA triplet-repeats in the human genome: Unique origin of the GAA triplet-repeat sequence responsible for Friedreich ataxia at the center of an *Alu* element

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Friedreich ataxia is caused by large expansions of a GAA triplet-repeat (GAA-TR) sequence located at the center of an *Alu* element in intron 1 of the *FRDA* gene. Most chromosomes have 8-11 triplets (small normal), however linkage data indicate that "large-normal" alleles (12-29 triplets) serve as a reservoir for disease-causing alleles (>100 triplets), which arise from hyperexpansion of intermediate-sized, premutation alleles (30-60 triplets). Since Friedreich ataxia is the only disease caused by expansion of a GAA-TR, we decided to carry out a comprehensive search for other potential premutation / mutation GAA-TR loci in the human genome. Using a search algorithm to identify all uninterrupted GAA repeats in the human genome we found that GAA-TR sequences have undergone significant expansion. Of the 988 sequences with 8+ GAA triplet-repeats in the human genome, we identified 291 with 20+ triplets, and 30 potential premutation alleles with 30-62 uninterrupted triplets. The GAA repeat motif is especially prone to expand when compared with triplets of similar sequence; a comprehensive search revealed that TAA and CAA triplet-repeats have not expanded beyond 20 triplets, and GGA triplet-repeats have not expanded beyond 30 triplets.

Interestingly, most long GAA-TR sequences map within the 3' poly-A tracts of *Alu* elements and have undergone further expansion during *Alu* evolution. Sixty-three percent of all (GAA)8+ sequences are located in the immediate vicinity of intermediate and old *Alu* elements, 94% of which map within their 3' poly-A tracts. The central *Alu* location of the GAA-TR at the *FRDA* locus is highly unusual; we identified only two other examples of pure (GAA)8+ sequences in the human genome located at the center of an *Alu* element. One of these sequences [(GAA)8 at 8q13] was not polymorphic, but the other sequence [(GAA)9 at 10q24] has undergone a transition to "large-normal" alleles resulting in a similar bimodal distribution of normal alleles at the *FRDA* locus. We also identified a small expansion at this locus indicating that GAA-TR expansions are not limited to the *FRDA* locus. Our data show that GAA-TR sequences have co-evolved with *Alu* elements, undergoing significant expansion during primate genomic evolution with the generation of potential premutation alleles at multiple loci, and locus-specific factors are likely to influence GAA-TR instability.

Molecular and clinical studies in compound heterozygous FRDA Italian patients

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Friedreich ataxia (FRDA) is associated with a GAA-trinucleotide-repeat expansion in the first intron of the FRDA gene (9q13-21). The FRDA gene encodes a 210-amino acid protein named frataxin. In normal subjects, the polymorphic repeat varies in size from 6 to 36 units, whereas FRDA patients carry expanded alleles with 90-1700 repeats. More than 95% of patients are homozygous for the expansion on both alleles. The remaining patients have been shown to be compound heterozygous for a micromutation on one allele and the GAA expansion on the other. In patients, the reduction of both frataxin mRNA and protein was found to be proportional to the size of the smaller GAA repeat allele. We have studied 236 families with recessive ataxia. We have identified 210 families presenting with classical FRDA phenotype and GAA expansion (720±180 GAA units) on both alleles; 4 families presenting with late-onset FRDA associated with reduced number of GAA repeats on both alleles (290±50 GAA units); 11 families in which classical FRDA patients were compound heterozygous for a micromutation on one allele and the GAA expansion on the other allele; and 11 families presenting ataxia with vitamin E deficiency (AVED). Mutational screening of the frataxin gene allowed the identification of disease-causing mutations in all the heterozygous families. Two missense mutations previously described in patients of Italian origin were identified in 4 families: I154F (1 family) and W173G (3 families). In the other 7 families, we identified 5 novel frameshift/nonsense mutations which are predicted to result in a prematurely truncated protein: Y118X (1 family); a 13-nt microdeletion at the exon 3-intron 3 boundary (ex3/IVS3del13) which eliminates the last 4 nt of exon 3 and the splice donor site of intron 3 (1 family), and 3 different 1-nt deletions in exon 1, which are all predicted to cause frameshift and premature truncation of the protein at amino acid position 76: 100delG (1 family), 104delC (1 family) and 157delC (3 families). Western-blot analysis of lymphoblastoid cell lines derived from the compound heterozygous patients and their healthy relatives carrying either the expansion or the microdeletions allowed us to correlate protein expression with the clinical phenotype and the specific molecular lesion. Our results are consistent with the notion that the GAA expansion is a less severe mutation and indicate that (i) among the missense mutations, the W173G is associated with very low residual protein level and a more severe clinical phenotype, and (ii) in patients compound heterozygous for a nonsense mutation and a GAA expansion, disease severity and protein level reduction correlate with the size of the GAA expansion.

The influence of genetic background on mitochondrial disease: European haplogroup J may promote the expression of a new Leber's Hereditary Optic Neuropathy mtDNA mutation

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Leber's Hereditary Optic Neuropathy (LHON) is a form of blindness caused by mitochondrial DNA (mtDNA) mutations. Three mutations, 3460A, 11778A, and 14484C, account for roughly 90% of LHON worldwide and are designated "primary" mutations. Primary mutations strongly predispose carriers to LHON, are not found in controls, are all in Complex I genes, and do not co-occur with each other. Previously, we demonstrated that the 11778A and 14484C mutations occurred more frequently than expected in association with European mtDNA haplogroup J (found in 9% of European-derived mtDNAs), suggesting a deleterious and synergistic interaction among mtDNA mutations increased the probability of disease expression. We have recently discovered a new primary LHON mtDNA mutation, 10663C, which alters the ND4L subunit of Complex I. This missense mutation was homoplasmic in 3 Caucasian LHON families, all of which belonged to haplogroup J. These 3 families were the only haplogroup J-associated LHON families (out of 17 total) that did not harbor a known, primary LHON mutation. Comprehensive phylogenetic analysis of haplogroup J using complete mtDNA sequences from patients and controls demonstrated that the 10663C variant has arisen 3 independent times on this background. This mutation was not present in over 200 non-haplogroup J European controls, 74 haplogroup J patient and control mtDNAs, or 36 putative LHON patients without primary mutations. A partial Complex I defect was found in 10663C-containing lymphoblast and cybrid mitochondria. Thus, the 10663C mutation has occurred three independent times, each time on haplogroup J and only in LHON patients without a known LHON mutation. This makes the 10663C mutation unique among all pathogenic mtDNA mutations in that it appears to require the genetic background provided by haplogroup J for expression. These results provide further evidence for the predisposing role of haplogroup J in mitochondrial disease and for the paradigm of "mild" mtDNA mutations interacting in an additive way to precipitate disease expression.

Friedreich's ataxia screening in the population originating from the Paphos district of Cyprus

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A cluster of Friedreich's ataxia (FRDA) patients has been identified in two neighbouring villages of the Paphos district of Cyprus and has been reported by Dean, Chamberalin and Middleton (1988). The frequency of carriers within the two villages was estimated to be 1 in 5 to 1 in 6. These and additional patients originating from the Paphos district of Cyprus have been studied at the molecular genetic level. Patients that were ascertained at a later stage originate from villages distant from the initially reported neighbouring villages. Haplotype analysis revealed a common disease haplotype in agreement with the hypothesis of a founder effect and all Cypriot patients had a homozygous expansion of the GAA trinucleotide repeat in the first intron of the frataxin gene. In order to better estimate the frequency of FRDA mutation carriers in the population of the Paphos district, we initiated a screening program based on volunteer participation. The program was carried out for 18 months and it included preparation of a leaflet with the relevant facts about FRDA and its high prevalence in the region and many field trips for organized talks at city/village centres, blood collection and genetic counseling sessions to carriers at hospitals. One thousand and fifty individuals, above the age of 18 years old, originating from the Paphos district of Cyprus participated in the program after signed consent. Analysis of the GAA triplet repeat revealed that ninety-eight of the individuals were heterozygous carriers of the expansion accounting for 9.33% of

the sample. Thus, the estimated frequency of FRDA mutation carriers in the greater region of the Paphos district of Cyprus is 1 in 11 to 1 in 10 individuals. This project has been supported by UNOPS.

Session II - The Friedreich's Ataxia Protein

oBiogenesis, structure, and transport
oMultimerization and iron interactions
oYFH1p function
oEffects of point mutations

Yeast frataxin chaperones and stores iron

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Two distinct iron oxidation reactions take place during iron-dependent stepwise assembly of yeast frataxin (mYfh1p). A ferroxidase reaction with a stoichiometry of 2 Fe(II)/O2 is detected at Fe(II)/mYfh1p ratios *0.5; a slower autoxidation reaction with a stoichiometry of 4 Fe(II)/O2 becomes predominant at Fe(II)/mYfh1p ratios >0.5 (Park et al 2002). Under these conditions, mYfh1p assembles into an oligomeric species, α3, that can accumulate ~0.5-5 atoms of iron per subunit (Adamec et al 2000; Park et al 2002). At high Fe(II)/mYfh1p ratios, ferroxidase activity is predominant during the initial assembly step (α*α3) but is rapidly overcome by a slower autoxidation reaction which is influenced by the ionic environment and in turn affects the rate at which α3 assembles into higher order oligomers (α3 to α6 to α12 to α24 to α48). This process is observed at ionic strength and pH values close to those present in mitochondria. During the autoxidation phase, Fe(II) is loosely bound to mYfh1p and can be readily donated to the chelator a,a'- bypiridine or the mitochondrial enzyme ferrochelatase to synthesize heme. In contrast, the oxidized Fe(III) is stably associated with the assembled protein. At physiological salt concentrations, the Fe(II) autoxidation reaction is slowed down resulting in increased Fe(II) bioavailability. The binding and oxidation of Fe(II) by mYfh1p may provide a mechanism to promote iron utilization and prevent accumulation of excess iron in an insoluble form. Supported by the NIH/NIA AG15709

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Assembly and iron binding properties of human frataxin

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When expressed in E. coli, the mature form of human frataxin assembles into a stable homopolymer that binds ~10 atoms of iron per subunit. The iron-loaded homopolymer is detected on non-denaturing gels by either protein or iron staining demonstrating a stable association between frataxin and iron. As analyzed by gel filtration and electron microscopy, the homopolymer consists of globular particles of ~1 MDa and ordered rod-shaped polymers of these particles that accumulate small electron-dense cores (Cavadini et al 2002). Near and far UV circular dichroism (CD) spectroscopy of purified monomer and polymer revealed structural differences between these

two forms. The polymer has an increase in the beta-sheet content as compared to the monomer suggesting that the monomer undergoes a conformational change upon assembly. This change is reversible; when the polymer is disassembled with increasing concentrations of SDS, the far UV CD spectrum of the polymer converts to the spectrum characteristic of the monomer. Two distinct iron oxidation reactions take place during iron-loading. A ferroxidase reaction with a stoichiometry of 2 Fe(II)/O2 is detected at Fe(II)/frataxin ratios *0.5; a slower autoxidation reaction with a stoichiometry of 4 Fe(II)/O2 becomes predominant at Fe(II)/frataxin ratios >0.5. When the polymer is incubated with an excess of ferrous iron, a rapid ferroxidation phase is followed by a slower autoxidation phase during which ferrous iron is available to the chelator a,a'-bypiridine or ferrochelatase allowing production of heme under aerobic conditions in the presence of protoporphyrin IX. Thus, similar to yeast frataxin, human frataxin has the ability to store iron in bioavailable form. Supported by the NIH/NIA AG15709 and by the Muscular Dystrophy Association.
Cavadini, P., O'Neill, H. A., Benada, O., Isaya, G. 2002. Assembly and iron binding properties of human frataxin, the protein deficient in Friedreich ataxia. Hum Mol Genet 33:217-227

Do Frataxin and Ferritin have Different Iron Cores?

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X-ray absorption spectroscopy (XAS) at the iron K-edge is a novel method for examining iron in FRDA. XAS can be used both to characterize metals bound to purified proteins by analysis of the extended X-ray absorption fine structure (EXAFS) and to speciate simple iron mixtures by analysis of the near edge (XANES). We present the XAS analysis of frataxin iron cores assembled in vitro. The yeast frataxin core resembles but is less ordered than that of ferritin, having fewer corner-sharing Fe-Fe interactions at 3.43 Å and slightly fewer face-sharing Fe-Fe interactions. The presence of multiple scattering edge-sharing Fe-Fe interactions in ferritin cores that were not resolved in frataxin cores is further evidence that the frataxin core is composed of small crystallites. The EXAFS analysis supports a model for frataxin core assembly in which small ferrihydrite crystallites, bound to negatively charged sites, aggregate as the polymer assembles.

Iron-binding properties of frataxin and its homologues

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Friedreich's ataxia (FRDA), an autosomal recessive cardio and neurodegenerative disease, is caused by low expression of frataxin, a small mitochondrial protein. At the biochemical level, lack of frataxin causes disregulation of iron homeostasis, oxidative damage and alteration of iron-sulfur cluster proteins concentration in mitochondria. Frataxins are found in organisms ranging from bacteria to humans. The high homology of the amino acid sequences and the conserved three-dimentional structures strongly suggest that the main function of frataxin must be conserved throughout evolution. However, the role of frataxin in mitochondria remains unclear, although it has been suggested to act as a ferritin-like iron scavenger (1). We have undertaken a comparative study using human (hfra), yeast (yfh1) and bacterial frataxins (CyaY) selected as representatives of

different evolutionary steps. We characterised the fold of the proteins and their thermodynamic stability showing that they preserve their fold but have largely different stabilities, which is strongly influenced by the presence of cations. The ability of the proteins to interact directly or indirectly with iron and to form large aggregates under different conditions were also investigated. CyaY was used to perform mutagenesis studies to identify the surface involved in iron interaction and aggregation, and to investigate if such surface was conserved among the three proteins. Our work leads us to a more complex and complete picture of the binding and aggregative properties of frataxins.

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Is human frataxin a storage protein for mitochondrial iron?

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Studies in both yeast and mammals have suggested that frataxin may play a critical role in mitochondrial iron homeostasis and free radical toxicity. It has been reported that purified recombinant mature yeast frataxin homologue Yfh1p (MW of approx. 14 kDa) may assemble in vitro in a macromolecular spherical complex of approx. 60 subunits and approx. 1.1 MDa, following the aerobic addition of iron (Adamec et al., 2000). These higher order multimers would sequester up to 69 iron atoms per subunit maintaning it in a soluble, available, and non-toxic form. A similar role and a similar structure have been proposed for the human counterpart, which would have a binding capability of 10 iron atoms per subunit (Cavadini et al., 2002). These observations prompted the hypothesis that frataxin may play a major role in the handling and storage of iron within mitochondria (Gakh et al., 2002). However, structural studies on the human protein neither confirmed the high-molecular weight supramolecular structure nor disclosed iron binding capability (Adinolfi et al., 2002).
We have investigated the presence in vivo of frataxin macromolecular complexes by using two-dimension blue-native electrophoresis (2D-BNE), a technique designed to separate proteins in their native conformation in the first nondenaturing dimension (1st-D) and then resolve their subunit structure under denaturing conditions in the 2nd-D. Several cell lines (human HeLa cells, murine motor neurons, and human normal and patient-derived lymphoblasts) were analysed. Cells were permeabilized with digitonin to obtain mitochondria-enriched subcellular fractions. Proteins were extracted using different salt concentrations under conditions that preserve native conformation. Several mitochondrial proteins of known structure were used as controls and resolved as expected in their native conformation (1st-D) and subunit composition (2nd-D). Both frataxin and control proteins were revealed by immunoblotting. Following 1stD-BNE, only a strong signal at low molecular weight (<50 kDa) was detected, with no evidence of high-MW species. This observation was confirmed in the 2nd-D, where only the 16-kDa monomer could be detected. Culturing cells under iron overload conditions did not apppear to promote the formation of high-MW complexes. Analysis of frataxin and the other control proteins from rat heart, a tissue severely effected in FRDA, confirmed the data observed in cultured cells. Interestingly, the only experimental condition associated with detection of high-MW frataxin was when the human protein was overexpressed in simian COS1 cells. Under both normal iron and iron overload conditions, the most abundant form of frataxin was still a low-MW species, but a weak signal corresponding to a high-MW product could be observed, suggesting that some frataxin aggregation may occur under non-physiological condition at high protein concentration levels. We have also investigated the in vivo iron binding

capability of human frataxin. HeLa cells were metabolically labeled with 55Fe overnight and proteins were electrophoresed under non-denaturing conditions (lstD-BNE). Autoradiography showed a single 55Fe-containing band >500 kDa, the intensity of which was not affected by immunoprecipitation experiments with saturating amounts of anti-human frataxin Ab. By contrast, immunoprecipitation with anti-cytosolic ferritin Ab resulted in a marked decrease of the radioactive band. These results were confirmed by quantitative analysis, which showed that immunoprecipitation with anti-human frataxin antibody decreased the 55Fe cpm/mg of protein in the mitochondrial preparations by only approx. 10%, a decrease that could be ascribed to nonspecific binding. By contrast, immunoprecipitation with anti-ferritin antibodies resulted in a significant reduction (approx. 60%) of 55Fe cpm/mg of protein. In conclusion, biochemical investigations performed by us on both normal and pathological cell lines could obtain evidence neither for the presence of high-MW human frataxin complexes nor for its ability to bind iron and, therefore, do not lend support to the hypothesis that frataxin may act as a ferritin-like iron storage protein in the mitochondria, a role that could more likely be played by a recently identified mitochondrial ferritin (Levi et al., 2001). (Supported by a Telethon-Italia grant to FT)

Functional interactions between yeast frataxin and Isu1p/Isu2p proteins

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Five yfh1 mutants (A077T, P094A, G107D, P125S and L162F) thermosensitive on high-iron media have been isolated in Saccharomyces cerevisiae by in vitro random mutagenesis. All have mild phenotypes with only slightly decreased aconitase activity and slightly increased mitochondrial iron content, the most severe mutations being P094A and P125S. The yfh1G107D mutant was selected to carry out a synthetic lethal screen on iron media by in vivo EMS mutagenesis. This screen identified a double mutation (G60D-M141V) in ISU1, a gene encoding a member of the mitochondrial Fe-S cluster biosynthesis machinery. In the yeast S. cerevisiae ISU1 has a very close mitochondrial homologue, ISU2. An ISU2 overexpressing vector complements the iron synthetic lethal phenotype of the yfh1G107D isu1G60D-M141V mutant. In a wild-type YFH1 background the isu1 G60D-M141V mutation is mild, as is yfh1 G107D; however, when these yfh1 and isu1 mutations are combined, the strain grows slowly, becomes highly sensitive to iron, and loses aconitase activity. Moreover, the isu1G60D-M141V mutation introduced in a deleted yfh1 strain is lethal, indicating that in the absence of frataxin Isu2p cannot substitute for Isu1p. (Similarly, a strain deleted for both YFH1 and ISU1 is not viable, while a strain deleted for YFH1 and ISU2 is viable.) These synergistic effects show that YFH1 and ISU1 interact genetically and are acting in the same metabolic pathway. Since they cannot rescue one each other by overexpression. Yfh1p and Isu1p should play distinct functions in Fe-S cluster synthesis. One specifically striking difference between an ISU1 deleted (or mutant) strain and the same strain containing also the yfh1G107D mutation is the drastically decreased levels of Isu2p protein in the isu1 yfh1 mutant strain. An interpretation of our data is that wild-type yeast frataxin can interact physically with Isu1p, or with Isu2p in the absence of Isu1p. However, in a yfh1G107D mutant which has an unstable frataxin, Isu2p is destabilized by its incapacity, or decreased capacity, to interact with the G107D mutant frataxin. The hypothesis of a physical interaction between Isu1p (or Isu2p) is still strengthened by the observation that the phenotypes produced by the yfh1 ts mutations introduced in an isu1 deleted strain (or in the G60D-M141V mutant) do not correlate with the severity of the phenotypes observed in a wild-type ISU1 strain.

Session IIIA - Friedreich's Ataxia Cells – Yeast Studies

oOxidant damage
oIntracellular iron distribution
oMitochondrial dysfunction

Biogenesis of cellular iron-sulfur proteins and its implication for Friedreich's ataxia

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Iron-sulfur (Fe/S) clusters are important cofactors of numerous proteins involved in electron transfer, metabolic and regulatory processes. In eukaryotic cells, known Fe/S proteins are localised within mitochondria, the nucleus and the cytosol. Only recently, the molecular basis of the biogenesis of these proteins in a living cell has started to become elucidated (reviewed by Lill & Kispal, 2000; Mühlenhoff & Lill, 2000). Mitochondria perform an essential function in the biosynthesis of cellular Fe/S proteins. The organelles harbour a complex "iron-sulfur cluster (ISC) assembly machinery" consisting of some ten proteins. The ISC proteins (including a cysteine desulfurase, transient Fe/S cluster binding proteins (Isu), a ferredoxin, and an Hsp70 chaperone) are highly conserved from bacteria to man. Their function is crucial for maturation of both mitochondrial and extra-mitochondrial Fe/S proteins. Mitochondria also perform a role in the maturation of cytosolic Fe/S proteins, even though their precise function is still unclear. According to a current working model (Lill & Kispal, 2000), an Fe/S cluster or a derivative thereof is assembled in the mitochondrial matrix and exported by the "ISC export machinery" consisting of the ABC transporter Atm1p, Erv1p in the intermembrane space and glutathione.

By directly measuring the *de novo* maturation of several Fe/S proteins in the model organism yeast, we identified Yfh1p, the yeast homologue of human frataxin, as a novel component of the ISC assembly machinery. Frataxin appears to be directly required for cellular Fe/S protein maturation (Mühlenhoff et al., 2002). Making use of the transient association of an Fe/S cluster to the Isu proteins, we narrowed down the site of requirement for Yfh1p in Fe/S protein maturation by *in vivo* staging experiments. Functional Yfh1p is needed for binding of an Fe/S cluster to the Isu proteins suggesting an early requirement in the process. Current experiments attempt to further pinpoint the site of action of Yfh1p.

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Iron use for heme synthesis is under control of the yeast frataxin homologue (Yfh1)

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Yfh1p (yeast frataxin homolog) has been associated with complex and pleiotropic phenotypes related to iron homeostasis. A link to Fe-S cluster formation has been shown, and here we show a role of Yfh1p in heme synthesis. The Δyfh1 strain had very low cytochrome content. The level of ferrochelatase (Hem15p) was very low as a result of repression of the transcript. However, the low amount of Hem15p was not the cause of heme deficiency in Δyfh1 cells. Ferrochelatase, a mitochondrial protein, able to mediate insertion of iron or zinc into the porphrin precursor, made primarily the zinc protoporphyrin product. Zinc protoporphyrin instead of heme accumulated during growth of Δyfh1 mutant cells, and furthermore, preferential formation of zinc protoporphyrin was observed in real time. The method for these studies involved direct presentation of porphyrin to mitochondria and to ferrochelatase of permeabilized cells with intact architecture, thereby specifically testing the iron delivery portion of the heme biosynthetic pathway. The studies showed that cells lacking Yfh1p were defective in iron use by ferrochelatase. A high frequency of suppressor mutations was observed, and the phenotype of such mutants was characterized by restoration of heme synthesis in the absence of Yfh1p. Suppressor strains exhibited normal cytochrome content and normal respiration, but remained defective in Fe-S proteins and accumulated iron into mitochondria. Our results suggest that Yfh1p mediates iron use by ferrochelatase and that the role of Yfh1p in heme synthesis can be bypassed by suppressor mutations.

Inhibition of Fe-S cluster biosynthesis decreases mitochondrial iron-export: Evidence that Yfh1p affects Fe-S cluster synthesis

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Decreased expression of Yfh1p in the budding yeast, Saccharomyces cerevisiae, and the orthologous human gene frataxin, results in respiratory deficiency and mitochondrial iron accumulation. The absence of Yfh1p decreases mitochondrial iron export. We demonstrate that decreased expression of Nfs1p, the yeast cysteine desulfurase that plays a central role in Fe-S cluster synthesis, also results in mitochondrial iron accumulation due to decreased export of mitochondrial iron. In the absence of Yfh1p there is decreased activity of Fe-S containing enzymes (aconitase, succinate dehydrogenase), while the activity of a non Fe-S containing enzyme (malate dehydrogenase) is unaffected. There was abundant aconitase protein even though the activity of aconitase was decreased in both aerobic and anaerobic conditions. These results demonstrate a direct role of Yfh1p in the formation of Fe-S clusters and indicate that mitochondrial iron export requires Fe-S cluster biosynthesis. We further demonstrate that while introduction of Nfs1p into Nfs1p deficient mitochondria (or introduction of Yfh1p into Yfh1p deficient mitochondria) results in iron mitochondrial iron export; accumulated mitochondrial iron cannot be mobilized by cytosolic iron deprivation. These results have implications in the use of iron chelators to treat Friedreich's ataxia.
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Genetic analysis of iron-citrate toxicity in yeast: Implications for mammalian iron homeostasis

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Deletion of the yeast homologue of frataxin, YFH1, results in mitochondrial iron accumulation and respiratory deficiency (petite formation). We used a genetic screen to identify mutants that modify iron associated defects in respiratory activity in Δyfh1 cells. A deletion in CIT2, a peroxisomal citrate synthase, in Δyfh1 cells decreased the rate of petite formation, as a consequence of decreased cellular citrate levels. Conversely, overexpression of CIT2 under conditions of iron excess, increased the rate of respiratory loss. Citrate toxicity in Δyfh1 cells was dependent on iron but was independent of mitochondrial respiration. Citrate toxicity was not restricted to iron laden mitochondria but also occurred when iron accumulated in cytosol due to impaired vacuolar iron storage. These results suggest that high levels of citrate may promote iron-mediated tissue damage and may explain why cardiac tissue is a target organ in Friedreich's ataxia.
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Progressive mitochondrial dysfunction with loss of frataxin

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The frataxin protein sequesters iron within the mitochondria and reduced levels of frataxin lead to the human disease Friedreich's ataxia (FRDA). To examine the role of iron sequestration and consequences of chronically lower amounts of this protein, we developed a unique, highly regulatable promoter system for expressing this protein in the yeast *Saccharomyces cerevisiae*. While complete deficiency in frataxin leads to genetically defective mitochondria (petites) in all strains examined, we were able to follow changes in several mitochondrial features at varying levels of frataxin in order to mimic features of FRDA. The changes included amounts of iron, extent of damage to both mitochondrial protein and DNA, and mitochondrial morphology. We found that at early stages of frataxin loss, considerable damage may arise in the mitochondria and that the complete deficiency grossly affects mitochondrial function. We suggest that the regulatable frataxin system provides a useful model for understanding consequences of frataxin deficiency and FRDA, enables the study of progressive mitochondrial damage and possibly repair, and is useful for addressing mitochondrial dysfunction associated with other diseases.

Evidence that the expression of human mitochondrial ferritin in yeast rescues respiratory activity in Yfh1 yeast deficient strains.

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Ferritin is a molecule uniquely designed to store large amounts of iron in a non-toxic form. It is mainly cytosolic and composed by two subunit types: the H with ferroxidase activity and the L that assists for more efficient iron incorporation. We have recently found a new form of ferritin which is expressed as a precursor protein with a long 60 amino acid N terminal extension predicted for mitochondrial targeting (Levi et al, 2001). We showed that the new ferritin (MtF) is specifically targeted to mitochondria and processed to form a fully functional ferritin. Expression in HeLa cells

showed that MtF is as active as the cytosolic ferritin in taking up iron and that it reduces cytosolic iron availability (Corsi et al, 2002). This indicates that MtF can act as an efficient mitochondria iron storage protein and that it may protect the organelle from toxicity due to local iron excess. This hypothesis is supported by the finding of large amounts of MtF in the erythroblasts of subjects with sideroblastic anemias (Cazzola et al, 2002), probably the best model of cells with iron-loaded mitochondria, which are not killed by iron. We have produced recombinant human and mouse MtFs and specific antibodies for both. Immunohistochemistry analyses showed that the protein is highly expressed in spermatocytes and spermatozoa, in the interstitial cells of testis, in the islets of Langherans and in the embryo. Lower levels, so far detectable only by RT-PCR, are possibly expressed also in brain and heart. We concluded that MtF has tissue specific expression and its upregulation in sideroblasts (caused by unidentified stimuli) probably protects these cells from the damaging effect of mitochondrial iron overload. Mitochondria iron deregulation is thought to be one of the major causes of pathogenicity in Friedreich's ataxia and Frataxin was found to be able to bind iron and form large aggregates. So far we have no direct evidence that MtF is abnormally expressed in FRDA, but we thought that MtF induction may protect mitochondria from the damaging effect of iron excess, as probably occurs in sideroblastic anemia. To test this hypothesis, we cloned human MtF in a vector for expression in yeast. Analysis of the transfected cells showed that the protein is efficiently expressed, it is properly processed into the mature protein, which assembles in a ferritin localized in the mitochondria. It functionality was demonstrated by the incorporation of radioactive iron inside the yeasts. Next we expressed MtF in a yeast strain deficient of Yfh1, the homolog of human frataxin. We found that this partially restored cell capacity to grow on non-fermentable media and increased cell resistance to H_2O_2. Work is in progress to test a larger number of *yfh1D* strains to confirm the results. The finding indicates that MtF partially complement frataxin deficiency by restoring respiratory activity and increasing resistance to oxidative damage, probably by reducing local iron toxicity. The regulation of iron metabolism in yeast is largely different from that in mammalian cells; for example, they store excess iron in the vacuoles in place of the ferritin. Therefore yeast may not be an adequate model for ferritin functionality, and we plan to develop human cellular models with frataxin deficiency. The newly discovered small interfering RNA technique (siRNA) offers the tool for an efficient transient and constitutive repression of the gene. Preliminary results indicate that the transfection of HeLa cells with short double strand RNAs complementary to a specific region of frataxin mRNA induce a reduction of frataxin accumulation of about 90%. The cloning of the sequence into vectors for stable expression of the functional siRNA is now in progress.

Session IIIB - Friedreich's Ataxia Cells – Mammalian Studies

oOxidant damage
oIntracellular iron distribution
oMitochondrial dysfunction

Iron-sulfur cluster biogenesis in mitochondrial and cytosolic compartments of mammalian cells

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Much evidence indicates that iron-sulfur cluster biogenesis is compromised in the mitochondria of Friedreich ataxia cells. Activities of the iron-sulfur enzymes aconitase and succinate dehydrogenase activities drop when frataxin activity is absent, and mitochondrial iron overload subsequently develops. Deletions of several enzymes known to be involved in iron-sulfur cluster assembly also lead to mitochondrial iron overload. Recent studies suggest that frataxin may directly participate in iron-sulfur cluster assembly. To better understand this process, we have studied mammalian IscS, the sulfur donor, IscU, a presumed iron-sulfur cluster scaffold protein, and NFU, another candidate scaffold protein. In all three cases, we have found that single genes encode isoforms that are

targeted either to mitochondria or to the cytosolic/nuclear compartment. In each case, much less of the enzyme is expressed in cytosol than in mitochondria. Nevertheless, coprecipitation experiments indicate that independent iron-sulfur cluster complexes assemble in mitochondria and cytosol. We suggest that a mitochondrial iron-sulfur protein is important in sensing mitochondrial iron status, and that failure to correctly assemble a cluster causes this unrecognized regulatory protein to enhance mitochondrial iron uptake and/or decrease mitochondrial iron export. Since iron-sulfur clusters are unstable in oxygen, better methods are needed to identify possible iron-sulfur cluster proteins that might play such a role in mitochondria.

Increased IRP1 Activity Associated with Decreased Activities of Iron Sulfur Cluster Respiratory Complexes in Friedreich Ataxia

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In low-iron conditions, the cytosolic iron-regulatory protein IRP1 binds to iron-responsive elements (IREs) in mRNAs encoding iron-regulated proteins. In high-iron conditions, IRP1 incorporates an iron-sulfur cluster (ISC), which interferes with IRE binding and thereby prevents intracellular iron accumulation. Here we demonstrate an incomplete shift of IRP1 to its ISC form in Friedreich ataxia (FRDA) fibroblasts, associated with decreased activities of ISC respiratory complexes. Our data demonstrate an impaired adaptive response to iron accumulation in FRDA cells.

Frataxin, iron-sulfur cluster and heme synthesis

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Data from different investigators indicate that frataxin may be involved in the process of iron-sulfur cluster (ISC) synthesis in mitochondria. Iron is used by mitochondria for ISC and heme synthesis. Δyfh1 yeast does not have any obvious heme synthesis or transport abnormality, and the same appears to be the case for other ISC synthesis mutants that accumulate iron in mitochondria. In addition, individuals with Friedreich ataxia are not more anemic than the general population. Increased erythrocytic protoporphyrin IX has been reported in these patients, but human ferrochelatase is an ISP, so this finding, if confirmed, may reflect ISP deficiency rather than impaired heme synthesis. One is then tempted to conclude that mitochondrial iron trafficking for heme and Fe-S cluster synthesis occurs through separate pathways. When cells become programmed for extremely high levels of heme synthesis, as is the case for erythroblasts, it is conceivable that the Fe-S pathway may be downregulated. Des Richardson et al. recently observed a decrease in frataxin when heme synthesis was stimulated in cultured cells. We analyzed whether any modification in frataxin level accompanies the induction of erythropoiesis in wild type and frataxin-overexpressing mice. For this purpose, mice were exposed to phenyl hydrazine (PHZ, three injections of 60 mg/Kg body weight), which induces hemolysis followed by compensatory erythropoiesis. We could demonstrate a marked decrease in frataxin in the spleen of these animals that coincided with the maximum induction of erythropoiesis, as shown by CD71 flow cytometry. We are currently analyzing the level of expression in the spleen of these mice of a recognized ISC synthesis enzyme, the Nfs1 cysteine desulfurase. Our findings suggest that the ISC and heme synthesis machineries are counter-regulated. A different ratio between these two activities of mitochondrial iron metabolism in different cell types may be part of the reason for specific cell vulnerability in Friedreich ataxia. We are also conducting experiments with purified components to determine the step of ISC synthesis affected by frataxin. The first hypothesis that we are testing is

that frataxin may help the very first steps of the process, possibly by preventing the formation of toxic iron-cysteine complexes.

Erythroid differentiation and protoporphyrin IX down-regulate frataxin expression: Characterisation of frataxin expression compared to molecules involved in iron metabolism and hemoglobinisation

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Friedreich's ataxia (FA) is due to decreased frataxin expression that results in mitochondrial iron (Fe)-overload. However, the role of frataxin in mammalian Fe metabolism remains unclear. In this investigation we examined the function of frataxin in Fe metabolism by implementing a well-characterised model of erythroid differentiation, namely, Friend cells induced using DMSO. We have characterised the changes in frataxin expression compared to molecules that play key roles in Fe metabolism (the transferrin receptor (TfR1) and the Fe transporter Nramp2) and hemoglobinization (β-globin).

DMSO-induction of hemoglobinization resulted in a marked decrease in frataxin gene (*Frda*) expression and protein levels. To a lesser extent, *Nramp2* mRNA levels were also decreased upon erythroid differentiation, while *TfR* and *β-globin* mRNA levels increased. Intracellular Fe depletion using desferrioxamine (DFO) or pyridoxal isonicotinoyl hydrazone (PIH) which chelate cytoplasmic or cytoplasmic and mitochondrial Fe pools respectively, had no effect on frataxin expression. Furthermore, cytoplasmic or mitochondrial Fe-loading of induced Friend cells with ferric ammonium citrate, or the heme synthesis inhibitor, succinylacetone, respectively, also had no effect on frataxin expression. While frataxin has been suggested by others to be a mitochondrial ferritin, the lack of effect of intracellular Fe levels on frataxin expression was not consistent with an Fe-storage role.

Significantly, protoporphyrin IX (PIX) but not heme down-regulated frataxin protein levels, suggesting a regulatory role of frataxin in Fe and/or heme metabolism. This was observed in Friend cells and also a variety of cell lines, including mouse LMTK- fibroblasts and human SK-N-MC neuroepithelioma cells. The effect of PIX was rapid and reversible occurring within a 1 h exposure to this agent. This could suggest that an increase in mitochondrial PIX levels could act as a signal to coordinate Fe uptake and haem synthesis within the mitochondrion. That is, as PIX levels increase, this could result in a decrease in frataxin expression that would allow Fe uptake by the mitochondrion and incorporation of Fe into PIX to form heme.

Since decreased frataxin expression leads to mitochondrial Fe-loading in FA, our data suggest that reduced frataxin expression during erythroid differentiation results in mitochondrial Fe sequestration for heme biosynthesis.

Alterations in Sulfur Amino Acid metabolism in Friedreich's ataxia cells

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Deficiencies in the mitochondrial protein frataxin cause the neuro- and cardio-degenerative disease Friedreich's ataxia. Five hypotheses for frataxin's physiological function in human cells have been proposed, including as a mitochondrial iron transporter, iron-sulfur cluster assembler, iron-storage protein, antioxidant and stimulator of oxidative phosphorylation. We have carried out microarray analysis of gene expression in frataxin-deficient and frataxin-transfected human cells in three cell types. The most consistent cell-autonomous effects of frataxin-deficiency are on genes involved in Sulfur Amino Acid (SAA) homeostasis, which are downregulated, in concordance with an involvement of frataxin in biogenesis or maintenance of Iron Sulfur Clusters (ISCs), and probing specific transcripts and proteins involved in ISC synthesis indicates a specific deficiency in these molecules in FRDA cells. Biochemical analysis of Amino acid level indicates a specific alteration in SAA concentrations in this disease. The second largest number of genes altered was in the apoptotic category, predominantly an up-regulation of genes involved in the Fas/TNF pathway, suggesting an apoptotic mechanism for the cell degeneration in FRDA. Furthermore an FRDA-specific cellular deficiency is rescued by small molecules related to these microarray-based hypotheses. Thus these results support only one of the current hypotheses for frataxin's function in human cells, and suggest that there are multiple steps in the FRDA pathophysiologic mechanism, which could each be relevant for pharmacotherapy.

Microarray analysis of frataxin deficient mouse brain identifies compensatory changes in gene expression and supports the role of oxidative stress

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Friedreich's ataxia is caused by a large GAA repeat expansion within the first intron of the frataxin gene, which diminishes frataxin transcription. Frataxin is not evenly distributed in the nervous system, and its deficiency appears to have the largest effect on regions where its expression is normally highest. Frataxin deficiency leads to iron accumulation within mitochondria, increasing free radical production by fenton chemistry and altering oxidative metabolism. The direct pathway(s) by which this results in specific neuronal cell death is not known. To begin to identify the early alterations caused by frataxin deficiency and the cellular compensatory response to this stress, we analyzed gene expression in frataxin deficient mice.
The frataxin deficient model was generated by the crossing of $(GAA)_{230}$ repeat knockin mice with frataxin heterozygous knockout mice (Miranda et al., 2002). These mice have been examined at ages up to 6 months and have no observable neurological phenotype or neuropathology that has been identified (Miranda et al., 2002). A Custom 10K-element cDNA microarray was probed with cDNA from 3 brain regions dissected from 4 frataxin knockout/knockin mice, which express 25 to 36% of wild-type frataxin levels, and controls. Several analyses were performed including comparison of gene expression in males and females, and identification of genes that were altered in common to cervical spinal cord and brain stem. Each of these analyses identified different, but overlapping sets of genes, which suggests that there is a significant neuroprotective compensatory response to the 70% reduction in frataxin, despite the absence of an overt neurological phenotype. Genes with significant alterations included those within functional categories of iron homeostasis regulation and the oxidative stress response, in addition to signal transduction, metabolic, and the neuronal developmental pathways. Regional differences were observed, but these need to be confirmed using more animals and independent methods, such as RT-PCR.
The absence of neurodegeneration in these mice has allowed us to identify the specific cellular response to frataxin deficiency, rather than detecting changes due to cell loss or inflammation, as is

the unfortunate case in most microarray studies of neurodegenerative processes. In this context, the differentially expressed genes likely reflect the true cellular dysregulation and compensation for frataxin loss, as the cell struggles for continued survival. These putative compensatory pathways become novel therapeutic targets.

Stress kinase pathway in Friedreich's ataxia cells

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Friedreich ataxia (FRDA), the most common form of recessive hereditary ataxia, is caused by the severe reduction in mRNA and protein levels of the mitochondrial protein frataxin, encoded by X25 gene. The cell-damaging oxidative stress plays an important role in the pathogenesis of this disease. We demonstrated that the mitogen activated protein kinase kinase 4 (MKK4) mRNA is down regulated by the human frataxin overexpression. MKK4 is a component of the c-Jun N-terminal kinase (JNK) subgroup of the mitogen activated kinases, which is involved in the oxidative stress response. The analysis of the JNK stress kinases pathway, performed in frataxin overexpressing cells, showed a reduced JNK phosphorylation. The analysis of fibroblasts from Friedreich ataxia patients showed an up-regulation of phospho-JNK and phospho-c-Jun, associated to an increase of the caspase-9 activation. Taken together, those data indicated the presence, in FRDA cells, of a "hyperactive" stress-signalling pathway. In order to explore the final effectors of the JNK pathway, we are completing the analysis of the activation of AP-1 DNA binding complex. The AP-1 complex, a dimer consisting of members of c-fos and c-jun families, regulates the transcription of stress response involved genes, as antioxidants. Stress-induced up-regulation of several antioxidant genes has been described in frataxin deficient cells. We are evaluating the AP-1 binding activity in FRDA versus control fibroblasts by electrophoretic mobility shift assays (EMSA). We are also investigating compositional changes in the members of the AP-1 complex, examined by super-shift assay and western blot analysis. We are also studying by real time PCR the mRNA levels of AP-1 regulated transcripts.

Session IV - Friedreich's Ataxia Models

oLower organisms
oMouse models
oCell culture

Development of mouse cellular models and therapeutic trials for FRDA

Hervé Seznec, Delphine Simon, Laurence Reutenauer, Nadège Lagarde, Michel Koenig and Hélène Puccio

Deciphering the molecular mechanisms involved in FRDA and finding therapeutics for this disease would be facilitated by the availability of cellular models. To date, only two cellular models are available. The absence of spontaneous phenotype and the genetic heterogeneity in cell lines derived from FRDA patients make them unsuitable for large-scale drug screening. We have therefore decided to develop mouse cellular models deleted for frataxin, using the Cre-lox homologous recombination system, derived from mouse models that have been generated in the lab. These mouse models reproduce the pathophysiology of the disease and have demonstrated that the Fe-S deficit precedes iron accumulation, and are good tools for deciphering the molecular mechanisms and finding therapeutics for FRDA.
Using our different mouse models, our first strategy was to generate inducible immortalized fibroblasts cell lines in which the deletion of frataxin was obtained by adding tamoxifen to the media. Even under "anaerobic" conditions (supplemented with uridine and sodium pyruvate), no clone with homozygous deletion for frataxin was isolated. Indeed, after tamoxifen treatment, a certain percentage of floating (dead) cells were observed. Enrichment of these floating cells by pre-plating enabled us to verify by PCR that these cells were homozygous for the frataxin deletion, confirming the lethality of frataxin deficiency at the cellular level. These results seem at first in contradiction with the 10 weeks survival of cardiomyocytes and the complete absence of skeletal muscle phenotype in our conditional cardiac knockout where we showed complete frataxin deletion in both skeletal and cardiac muscle. In fact, our results indicate that the absence of frataxin is more deleterious in dividing cells than in post-mitotic cells, even rich in mitochondria, a surprising result. However, this is in concordance with the very early embryonic lethality that we demonstrated in complete frataxin knockout mice.
We are now trying to establish primary cultures of frataxin-deficient myoblasts (derived from our conditional cardiac models) and fibroblasts derived from new inducible mouse models in order to study the lethality phenotype. Our results show that primary myoblasts can be established and maintained for at least 3 weeks, and can be differentiated into contracting myotubes. Preliminary results show that the recombinase responsible for the deletion is expressed at the myoblast stage. We are currently determining at the cellular level whether frataxin is deleted in these primary cell cultures.
In parallel, we are developing an "in vitro therapeutic trials" using the inducible immortalized fibroblasts cell lines in order to test different molecules and their ability to rescue the lethal phenotype observed in our cell system.

Generation of a neuronal specific mouse model for Friedreich Ataxia

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Friedreich ataxia, the most common form of inherited ataxia, is a decade-long disabling disease. We have found that Friedreich ataxia is caused by partial deficiency of frataxin, a mitochondrial protein

most likely involved in iron-sulfur protein biogenesis and/or iron homeostasis, due to an intronic expansion in the defective gene. In order to develop a mouse model that recreates only the neurological features of the human disease, we have used the tamoxifen inducible Cre-ERT system under the control of a neuron-specific prion promoter in order to have a spatio-temporally controlled deletion of the mouse frataxin gene flanked by loxP sequences. We have obtained two different lines which exhibit a progressive neurological phenotype with slow evolution. The mice were tested by accelerating rotarod measurements and by dynamometric grip-tests which revealed a general locomotor deficit without defect of muscle strength beginning at around 10 weeks. This locomotion defect progresses, and by 30 weeks of age, the mutant animals perform half the time than control littermates. We have further analyzed this locomotion defect by footprint analysis which clearly demonstrates that the mutant animals present a progressive ataxia until loss of spontaneous ambulation at around 1 year of age. EMG studies show normal sensory nerve conduction on the caudal nerve (almost exclusively composed of small diameter sensory neurons) and normal motor evoked potential. In contrast, there is a significant decrease in the sensorimotor reflexes after sciatic nerve stimulation ($p<0.01$) indicating that the large myelinated proprioceptive sensory neurons are functionally defective causing sensory and spinocerebellar ataxia, a distinctive pathological characteristic of FRDA. Preliminary histological data show both spinal cord and dorsal root ganglia pathology, with no sensory axonal defect. These mutant mice represent therefore an excellent model of the human disease and will be tested with anti-oxidant compounds, currently the best pharmacological candidates against the partial frataxin deficiency and ensuing mitochondrial defects. Locomotion testing represents an appropriate end-point for such trials

An inducible frataxin mouse model

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Current mouse models for Friedreich ataxia (FA) include conditional knock-out animals whose frataxin gene is completely inactivated in specific tissues and a specific time of development. While these models have demonstrated to be excellent for numerous purposes, including the study of biochemical abnormalities in FA and the testing of new treatments, they do not faithfully reproduce the human disease, characterized by frataxin deficiency with some residual protein in all cells and throughout development. An attempt to mimick the human disease by inserting a GAA triplet repeat expansion in the first intron of the mouse gene has not succeeded in generating symptomatic animals, probably because of the small size of the expanded repeat (only 230 triplets).
We are now generating a new mouse model that will eventaully contain a single, inducible copy of the frataxin gene. We started by creating two constructs containing containing the human frataxin coding sequence preceded by portions of the mouse frataxin promoter sufficient to drive reporter gene expression in cell culture (Pandolfo, unpublished data). A 1.7 Kb fragment driving reporter gene expression 7X, and a 1.1 Kb fragment driving reporter gene expression 1.5X were used. The Tet operator was inserted between the promoter and the frataxin coding gene. These constructs were then inserted by homologous recombination into a single site in the the genome of mouse ES14TG2a cells. These cells are Hprt $^-$ (exons 1 et 2 are absent), exons 1 and 2 of Hprt and the following intron are included in the construct to promote homologous recombination and the reconstitution of a functional Hprt gene, for which cells are the selected. We obtained clones of ES cells for both constructs that are now being used to generate chimeric mice. As mice trasmitting the construct through the germline will be obtained, the next step will be to cross them with mice expressing the Tet-On repressor. The resulting animals will carry an inducible frataxin gene in tissues that depend on the Tet-On repressor promoter utilized. These animals will then be crossed with frataxin KO animals. If there will be enough leaky transcription from the transgene to allow survival through development, the resulting animals will at the same time be a model for FA and will be amenable to “cure” by inducing frataxin expression at any desired time. If such model will not

be successful (eg too low leaky trasncription to allow embryonic growth), the mice will be crossed with conditional KO animals to study the effect of re-expressing frataxin after variable periods of complete deficiency.

Beta-Cell-Specific Disruption of the Frataxin Gene in Mice Causes Superoxide-Mediated Dysregulation of Beta-Cell Proliferation Leading to Progressively Deteriorating Diabetes Mellitus

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Friedreich Ataxia is an inherited degenerative disease caused by reduced expression of frataxin, leading to impaired glucose metabolism in approximatively one third of affected individuals. Employing the cre/lox system, we have removed exon 4 of the murine frataxin gene in pancreatic beta-cells using tissue specific expression of Cre-recombinase under control of the Ins2 promoter. Mice were born apparently healthy, but developed impaired glucose tolerance starting at 6 weeks of age (16.3 mM vs. 8.4 mM 120 min after i.p. injection of glucose, $P<0.001$). With increasing age, animals showed increased postprandial glucose levels (12 weeks, $P=0.026$; 24 weeks, $P=0.011$) and finally –at high age– increased fasting glucose levels (36 weeks, $P<0.001$). Knockout mice in parallel exhibited decreased insulin levels after i.p. glucose load (61% reduction at 30 min, 54% reduction, both $P<0.05$, at 12 weeks) deteriorating with increasing age. Computerized morphometry of pancreatic sections revealed severely reduced insulin positive area with increasing age (P=n.s. at 6 weeks, $P<0.001$ at 36 weeks), which were associated with elevated levels of superoxide production in knock-out animals only (81 nM vs. not detectable). Finally, immunohistochemical methods revealed increased apoptosis (activated caspase 3, 3.2 vs. 1.3 cells per $1/100^{th}$ mm^2 islet area, $P<0.001$) and decreased beta-cell proliferation (Ki-67 and BrdU, both $P<0.001$).
Together, disruption of frataxin in pancreatic beta-cells causes increased levels of free radicals, leading to induction of apoptosis and inhibition of proliferation, cumulating in decreased islet mass. These sequelae explain the development of diabetes mellitus following frataxin deficiency in both mice and men. Furthermore, since free radical formation is induced by both hyperglycemia and FFA oxidation, these observation may –at least in part– explain the chronic deterioration of beta-cell function commonly observed in various subtypes of diabetes.

Human FRDA YAC transgenic mice containing GAA repeat expansions

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Establishing an accurate GAA repeat expansion animal model of Friedreich's ataxia (FRDA) is considered to be essential for investigating potential therapeutic strategies for this disorder. In our initial studies towards this goal, we have demonstrated functional rescue of frataxin knockout mice with wild-type human genomic YAC and BAC FRDA transgenes. Three YAC transgenes and two BAC trangenes, ranging from 370kb to 140 kb, all produced successful rescue of frataxin knockout mice. More recently, we have generated human genomic YAC FRDA transgenic mice that also contain human GAA repeat expansion mutations. In particular, we have established a 200 GAA repeat-containing transgenic line that shows intergenerational instability, including both increases and decreases in GAA repeat size. Initial molecular studies of this line detected high levels of human frataxin expression and we are currently performing histological and neurobehavioural analyses on these mice to determine a possible phenotype. We also intend to perform biochemical analysis and more detailed frataxin mRNA and protein expression analysis on tissues from different sized GAA repeat FRDA transgenic mice. A second line that contains two combined human FRDA YAC transgenes of approximately 100 and 200 GAA repeats is also under preliminary investigation. These transgenic mice may be of considerable use for future investigations of in vivo GAA mutation correction therapies and therapies aimed at alleviating GAA blockage of frataxin transcription, as well as other FRDA gene and drug therapies.

Towards humanized mouse models of Friedreich ataxia

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Over 95% of patients with Friedreich ataxia have at least one allelle with an expansion of the (GAA)n repeat in intron 1 of the *FRDA* locus. The development of a transgenic mouse model carrying a GAA expansion in the human *FRDA* locus should therefore be invaluable for the development of novel therapies for Friedreich ataxia.

We have initially characterised a number of genomic DNA fragments in PAC/BAC clones carrying the human *FRDA* locus. One of these clones, BAC265, containing exons 1-5b of the *FRDA* locus and flanking sequences in a 188 kb genomic DNA fragment, was sequenced in the course of the Human Genome Project. We have used the genomic insert in BAC265 to establish three independent transgenic mouse lines carrying the intact BAC genomic fragment. Fluorescent *in situ* hybridisation (FISH) showed that each line had a single site of integration of the transgene. A competitive PCR assay was used to estimate the transgene copy number in all the lines. RT-PCR analysis of the lines containing the intact genomic fragment demonstrated that human *FRDA* mRNA is expressed in all three lines. While there is some variation in the human/mouse mRNA ratio between the three lines, all lines show similar differences in the expression of the human transgene in relation to the endogenous locus in different tissues. The human/mouse mRNA ratio is lowest in the blood, while heart and liver show similar levels of expression. In contrast, the human transgene is expressed at much higher levels than the endogenous locus in the brain of all three lines. These studies indicate that the human *FRDA* locus in BAC265 may contain a number of regulatory elements that confer tissue specificity in gene expression and that may be differentially recognised by the mouse transcriptional machinery in different mouse tissues.

Homozygous *Frda* knockout mice, which normally die *in utero*, are rescued by the presence of the 188 kb human transgene, in hemizygous or homozygous form in at least two of the three transgenic lines. Animals carrying the human transgene on a mouse knockout background develop without any obvious signs of abnormality and have normal fertility. To date, this is the smallest human

genomic fragment shown to complement the embryonic lethal phenotype of the *Frda* knockout mutation. The availability of the complete sequence of the genomic insert in BAC265 should greatly facilitate the targeted modification of the *FRDA* gene by the introduction of the GAA trinucleotide expansion, or known point mutations, for the generation of accurate humanised mouse models of Friedreich ataxia.

To facilitate studies of frataxin gene expression *in vivo*, another two transgenic lines have been established with a genomic reporter construct consisting of an in-frame fusion of the gene encoding enhanced green fluorescent protein (EGFP) at the end of exon 5a of the *FRDA* gene. Both lines exhibit whole-organ green fluorescence confirming the expression of the fusion protein in these mice. Differential levels of *FRDA-EGFP* expression in different tissues in these mice can be analysed by flow cytometry after enzymatic dissociation, while subcellular patterns of EGFP expression can be examined by cryosectioning of various tissues. These *FRDA-EGFP* genomic reporter mice should greatly facilitate the examination of spatial and temporal aspects of *FRDA* gene expression, and the evaluation of pharmacological agents on *FRDA* expression in the context of a whole animal model.

Development of genomic reporter assays for the upregulation of expression of the *FRDA* gene for the therapy of Friedreich ataxia.

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A number of studies indicate that the expansion of the (GAA)n repeat in intron 1 of the *FRDA* locus results in reduced synthesis of frataxin by partial inhibition of transcription. A possible approach to the therapy of Friedreich ataxia may therefore involve targeted pharmacological upregulation of frataxin expression. The variation in severity of the disease with the length of the GAA expansion further indicates that a moderate increase in frataxin expression may have a significant impact on the development and progresssion of the disease.

In order to facilitate studies on the regulation of frataxin expression and to develop targeted pharmacological approaches, we have developed a sensitive genomic reporter assay for frataxin expression from the intact *FRDA* locus. A fully sequenced bacterial artificial chromosome (BAC) clone containing exons 1-5b of the human *FRDA* gene and surrounding sequences (present on a 188 kb genomic fragment) was first shown to complement fully the embryonic lethality in homozygous knockout mice. Using the *GET Recombination* system, two in-frame fusions between the *FRDA* and *EGFP* (Enhanced Green Fluorescent Protein) genes were then constructed in this fragment. One fusion is within exon 2 of the *FRDA* gene. The other is at the end of exon 5a, producing the entire frataxin protein fused to EGFP. Both constructs were shown to drive the expression of EGFP from the regulatory elements of the *FRDA* locus, with the frataxin-EGFP fusion proteins targeted to the mitochondria. Targeted deletion of conserved non-coding sequences on these *FRDA-EGFP* fusion constructs should identify the regulatory elements determining the tissue and developmental specificity of *FRDA* gene expression and facilitate the development of a rational approach for upregulation of frataxin expression.

The exon 5a *FRDA-EGFP* fusion construct was used to establish stable cell lines of BHK21 (hamster) and HeLa, 293 and neuroblastoma cells (human). Stable cell lines were exposed to a number of test compounds and EGFP fluorescence was measured by flow cytometry. Hemin and butyric acid induced 30% and 19% increases in *FRDA* expression, respectively. The iron chelators deferiprone and desferioxamine both elicited an almost two-fold increase in *FRDA* expression, albeit at concentrations not optimal for cell growth. These cell lines provide a robust assay to

facilitate high-throughput screening for pharmacological compounds that can modulate the expression of the *FRDA* gene in a clinically relevant manner.

As described in the accompanying abstract, the exon 5a *FRDA-EGFP* fusion construct was also used to create transgenic mice expressing the fusion protein in various tissues. These mice provide an invaluable *in vivo* genomic reporter assay for the further preclinical evaluation of pharmacological agents identified in high throughput screening. These cellular and transgenic genomic reporter assays should allow the development of novel pharmacological therapies for Friedreich ataxia that are based on the restoration of frataxin expression.

A Caenorhabditis elegans model of Friedreich's ataxia

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Studies on yeast, mouse and human cell cultures have led to substantial advances in understanding the biochemistry of frataxin and the cell pathophysiology caused by frataxin deficiency. These biological model systems will continue to be extremely important in the study of frataxin and Friedreich's ataxia. Nevertheless we believe that there is a strong case for a multicellular animal model, which will allow the kind of genetic and transgenic studies that are difficult in mouse and the same time address function in whole tissues especially the nervous system. Moreover, such a model may also underpin the use of rapid large scale screening of candidate drugs.

In order to identify functions for frataxin in C. elegans we have addressed three main points: i) cloning and genomic analysis of the frataxin orthologue gene, ii) expression analysis, and iii) generation of mutant worms. As a first step we confirmed by multiple alignment analysis that the F59G1.7 gene, now called frh-1, is the orthologue of the frataxin gene. The gene has three exons and encodes a protein of 136 amino acids. frh-1 is located very closed to two flanking genes, F59G1.1 and F59G1.5. This data suggested that frh-1 may be part of an operon. To investigate this we have characterized the genomic region around frh-1 and the trans-splicing pattern of every gene. We conclude that frh-1 is located within an operon, CEOP2232, that includes eight genes and spans approximately 30 kb.

To determine the pattern of frh-1 expression we generated a number of constructs that include: a variable 5' genomic fragments of variable size, the whole or partial frh-1 gene and the gfp reported gene. Similar studies have also been performed for the other genes belonging to the frataxin operon. Hermaphrodites expressed frh-1 in some neurons of the head and body, the gut, the pharynx, and muscles. The expression pattern is variable depending on the genomic fragment included in the construct, suggesting that several promoters or regulator sequences are regulating the expression of frh-1. By immunostaining analysis with a polyclonal antibody against human frataxin (gift of Dr. F. Taroni) we have also observed expression during embryonic development.

We have also performed genetic analyses by generating transient knock-down mutants of frh-1 using RNA interference (RNAi). To knock down frataxin in worms we used three methods: feeding on E. coli producing dsRNA, injection of dsRNA, and by making stable transgenic worms that express dsRNA for the frataxin gene. Worms of the F1 generation show a specific pleiotropic phenotype: egg laying defective (egl), thin and short worms, slow and arrhythmic pharynx pumping, and a decrease in the rhythm of defecation.

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Session V - Clinical Studies/Outcome Measures

oAtaxia scales
oCardiac ultrasound
oSpectroscopic studies

Friedreich's ataxia: Spinal cord atrophy or hypoplasia?

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The number of intronic guanine-adenine-adenine (GAA) trinucleotide repeats strongly affects the clinical phenotype of Friedreich's ataxia (FA). Long repeats cause early onset and great disease severity; short repeats delayed onset and modest ataxia. Severe hypertrophic cardiomyopathy is characteristic for early onset cases. Invariably, sections of the myocardium of severely affected FA patients show perinuclear accumulation of iron. In patients with late onset or long survival, heart disease is absent and iron stains are negative. In contrast, it is uncertain how the length of the GAA expansion affects the rather uniform neuropathological phenotype. The spinal canal in FA is narrower than normal, and this observation suggests that the spinal cord never reaches its normal size. In control persons, imaging techniques can accurately determine the anteroposterior and transverse diameters of the spinal canal. In many patients with FA, scoliosis interferes with such measurements though several investigators have reported narrowing of the *cervical* spinal canal. In seven autopsy cases of FA with known lengths of their GAA repeats, measurements were made of the cross-sectional area of the thoracic spinal cord at comparable levels. Age of onset ranged from 7-32 years, age of death from 28-87 years. The correlation between the length of the short allele and age of onset conformed to published data. In three neurologically normal persons, the cross-sectional area of the thoracic spinal cord was 34.3±3 mm^2. In the FA cases, the average spinal cord area was 20.7±4.6 mm^2 (range 14.2-26.3). Plots of the lengths of the short and long alleles versus the spinal cord area showed only a modest correlation. Greater GAA expansions caused smaller spinal cords. Two long-term survivors (42 and 55 years, respectively) had severe reductions of their spinal cord areas (14.2 and 22 mm^2). Neither patient had iron deposits in the myocardium. An additional patient with ataxia for over 30 years had classic spinal cord lesions of FA but a normal cross-sectional area (35.7 mm^2). She had not been diagnosed during life, and polymerase chain reaction on deoxyribonucleic acid extracted from autopsy tissue showed one expanded allele (300 GAA repeats) and one normal-length allele (7 GAA). A point mutation is likely. The described findings may allow the interpretation that the spinal cord does not undergo *atrophy* as currently defined. Instead, even short GAA expansions appear to have an early deleterious effect on spinal cord development, and the pathogenesis is more likely *hypoplasia*. The embryonal lethality of homozygous frataxin deficiency in knockout mice also supports this interpretation.
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Iron in the dentate nucleus in Friedreich's ataxia

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Frataxin deficiency causes the accumulation of iron in the hearts of patients with Friedreich's ataxia (FA). Experiments *in vivo* and *in vitro* support the concept that the iron excess occurs in mitochondria. For many years, neuropathologists have emphasized the characteristic

neuropathological phenotype of the spinal cord and the ganglia of the dorsal root. There is no known iron accumulation in these ganglia or in the spinal cord proper. The most common *cerebral* lesion in FA is atrophy of the dentate nucleus (DN). The normal DN contains abundant iron and offers a vexing opportunity to study brain iron in FA. Magnetic resonance (MR) imaging suggests that FA causes iron accumulates in the DN. However, at autopsy, this cerebellar nucleus is often reduced in size; its efferent fibers are grossly atrophic; and macrostaining for ferric iron with potassium ferrocyanide in hydrochloric acid (Perls's solution) shows greatly reduced reaction product. Sections confirm neuronal loss and gliosis. When vibratome sections of normal control specimens are reacted by immunocytochemistry for ferritin (as an indicator of local iron), the iron-carrying protein is found mostly in microglia and oligodendroglia. Oligodendroglia often abut against the cell membrane of DN neurons. In FA, ferritin immunoreactivity in microglia is more prominent, and juxtaneuronal oligodendroglia are lost in step with nerve cell atrophy. Scattered astrocytes become ferritin-reactive. Western blots of extracts from dentate tissue show modest reduction in the amounts of ferritin. The observations allow the conclusion that FA changes the distribution of ferritin-containing cells. It is likely that signal reduction in T2-weighted MR images reflects the physical state of iron in the atrophic dentate nucleus during life rather than true accumulation of the metal. It may be suggested that ferritin aggregation in reactive microglial cells accounts for the low signal.
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Oxidative stress in patients with Friedreich's ataxia

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Increased generation of reactive oxygen species may underlie the pathophysiology of Friedreich's ataxia. We measured concentrations of 8-hydroxy-2'-deoxyguanosine (8OH2'dG), a marker of oxidative DNA damage, and carboxymethyllysine (CML), a marker for the formation of advanced Glycation Endproducts (AGEs), in urine and of dihydroxybenzoic acid (DHBA), a marker of hydroxyl radical attack, in plasma of 33 FRDA patients. We found a 2.6-fold increase in normalized urinary 8OH2'dG but no change in plasma DHBA and urinary CML concentrations as compared with controls. Oral treatment with 5 mg/kg/day of the antioxidant idebenone for 8 weeks significantly decreased urinary 8OH2'dG concentrations, indicating that 8OH2'dG may be useful in monitoring therapeutic interventions in FRDA patients.

Evaluation of cardiomyopathy in Friedreich's ataxia by volumetric magnetic resonance tomogrophy

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Friedreich's ataxia is associated with hypertrophic cardiomyopathy in 11% of patients. Cardiomyopathy is thought to be the life-limiting factor in most patients. Abnormal ECGs are found in 92% of patients. Echocardiography depicts hypertrophy of the interventricular septum and dorsal wall in 9% of patients. Evaluation of left ventricular mass by echocardiography has been used to monitor pharmaceutical trials, e.g. with idebenone. However, echocardiographic determination of left ventricular mass shows large interrater variability of about 25%. Aiming at a

more robust measure of left ventricular dimensions and function we performed a volumetric cardiac MRT study.

Twenty patients with genetically confirmed Friedreich's ataxia and a repeat length in the shorter allele ranging from 192 to 808 (mean: 485 +/- 196) GAA repeats were included in this study. Mean age was 35.2 +/- 10.9 (range: 18 – 58 years) and age at onset of symptoms varied from 5 to 36 (mean: 16.1 +/- 7.6) years.
Cardiac MRT with breath holding technique was performed using a 1.5 T-scanner with quantum gradient (Symphony, Siemens, Erlangen, Germany). ECG-triggered cine true-fisp sequences were recorded in the short axis plane with 8mm-slices positioned from the apex to the mitral valve. Boundaries of left ventricular structures were marked manually and left ventricular mass, enddiastolic volume, endsystolic volume as well as ejection fraction were calculated by a dedicated software (ARGUS). Two investigators evaluated data sets independently and blinded for clinical symptoms. Interrater variability (IV) was calculated according to the formula: IV [%] = (Rater1-Rater2) / [(Rater1+Rater2)/2]*100.

LV mass varied between 73.0g and 173.7g in FA (mean: 124.9+/-23.9g). LV mass adjusted for body surface was 44.4g – 93.5g/m2 (mean: 70,3+/-12.6g/m2). Cardiac hypertrophy was most pronounced in young and early onset patients and mild in elder and late onset patients as demonstrated by an inverse correlation of LV mass per body surface with age (r=-0.29) and age at onset (r=-0.26). LV mass was not siginificantly influenced by GAA1 repeat length. Interrater variability for the evaluation of left ventricular mass by volumetric cardiac MRT varied from 1.0% to 11.9% (mean: 4.2 +/- 3.0%) between investigators.

Excellent reproducibility recommends MR tomographic volumetry of the left ventricle as a superior tool for therapeutical trials aiming on cardiac hypertrophy e.g. in Friedreich's ataxia. Low interrater variability allows substantial reduction of sample sizes required to receive significant results.

Validation of Assessment Protocols to determine Clinical Progression in Friedreich's Ataxia

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Using a large cohort of patients with genetically proven Friedreich's ataxia we have evaluated the International Co-operative Ataxia Ratings Scale (ICARS) and a variety of tests designed to evaluate co-ordination, speech and nerve conduction. There is currently little data regarding the natural history of the Friedreich's ataxia and what factors influence disease progression. The evidence that oxidative stress, mitochondrial respiratory chain dysfunction and mitochondrial iron accumulation are involved has prompted the use of antioxidant and energy enhancers as therapeutic strategies in FRDA patients. Central to the evaluation of these therapies is the validation of scales to measure the clinical symptoms and obtain data relating to the natural history of FRDA and the factors affecting disease progression. We have clinically evaluated a heterogeneous group of 77 patients with genetically defined FRDA using a range of assessments including; ICARS; the BRAIN test; peg board; hand and foot co-ordination; speech, echocardiography and neurophysiological assessments. The ICARS scores covered a broad range indicative of the diversity of the patient group. The subdivision of the scores into the 4 component parts revealed a spread of data with no floor or ceiling effects with the exception of the posture and gait score which reached a ceiling at approx 23 years. This suggests that ICARS is a useful tool to assess the clinical symptoms of patients with FRDA. ICARS scores correlated with patient age, disease duration and the smaller GAA repeat size. However, the correlations were more significant when age or disease duration was also related to GAA repeat size. As previously reported the smaller GAA repeat sizes correlated with a later

disease onset, however, it was clear disease progression was faster in the groups with longer GAA repeats. The Brain test, hand and foot co-ordination test, peg board and speech assessments all correlated significantly with the ICARS scores suggesting they are excellent quantitative markers of disease progression. We have identified assessment protocols that are useful in assessing a range of symptoms associated with FRDA. Longitudinal follow up studies will help confirm this cross sectional data to determine the variability of disease progression between patients.

A set of simple assessment tools for disease progression in Friedreich ataxia

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There is a need for simple, sensitive, reliable assessment tools for the follow-up of Friedreich ataxia (FA) patients. The availability of candidate drugs for the disease and the planning of clinical studies renders this need even more acute. In recent years, we developed a simple ataxia scale (AS20) that is easily and rapidly administered in the clinic. This scale uses five modified items from the much more complex ICARS that evaluate upright posture, gait, upper limb coordination, dysarthria and eye movements. The score varies between 0 (normal) and 20 (very severe ataxia). Preliminary data suggest excellent intra- and inter-rater concordance. As will be shown at the meeting, AS20 allowed to demonstrate disease progression in a set of FA patients followed for up to 3 years and seems to correlate well with overall disability.
A simple additional quantitative assessment tool that can be added to AS20 in the clinical setting is an upper limb coordination test that makes use of two counters placed 30 cm apart. Data will be presented about the reproducibility of this test and its correlation over time with other indicators disease progression.
More sophisticated equipment, but not out-of-reach for most centers, is required for a quantitative analysis of upright posture using a platform. Again, our data indicate a good sensitivity to disease progression and a good correlation with other indicators.

Clinical Measures in Friedreich Ataxia

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Objective: Define clinical measures that quantitatively assess progressive neurological dysfunction in Friedreich ataxia (FRDA).

Background: FRDA is a progressive neurodegenerative disease of children and adults. While the genetic basis of FRDA is well understood, clinical heterogeneity of FRDA complicates serial clinical analysis. Patients can present as late as age 50, and clinical features include spasticity as well as ataxia. Traditional scales for assessment of ataxia are based on subjective quantification of the neurological exam, and have been insensitive and poorly reproducible. In this study, we sought to define quantitative neurological markers of FRDA using simple functional measures derived from the multiple sclerosis functional composite.

Methods: 27 patients with genetically confirmed FRDA were examined using the timed 25 foot walk (25FW), the 9 hole peg test (9-HPT), and low contrast Sloan letter charts (LCSLC). 9HPT and 25FW testing were performed and scored as in the multiple sclerosis functional composite. For LCSLC, the combined letter score from the high contrast, 5%, 1.25%, and 0.6% charts was used as the measure. Data were analyzed by correlation of these tests with each other, and with disease features including patient age, triplet repeat length, and symptomatic disease duration. Linear regression analysis was used to account for effects of the multiple factors on the test results.

Results: Scores of each test (25FW, 9HPT, LCSLC) correlated with disease duration. Correlations were best for the 9HPT (r_S=0.74, p<0.0001), followed by the 25 foot walk (r_S=0.71, p=0.0001) and the LCSLC, (r_S=-0.54, p=0.006). In linear regression models, test results were also predicted by the combination of age and triplet repeat length. The predictions were best for the LCSLC (R^2=0.57, P=0.0001), followed by the 9HPT (R^2=0.44, P=0.0024), and the 25FW (R^2=0.50, P=0.0005). However, the distinct measures showed only moderate correlations with each other, suggesting that they capture different dimensions of neurologic dysfunction in patients with FRDA (r_S =-0.71 9HPTvs. LCSLC; r_S =-0.58 25FW vs. LCSLC; r_S =0.79 9HPT vs. 25FW).

Conclusion: These results suggest that a combination of simple functional measures may be useful in serial clinical evaluation of patients FRDA. A composite score derived from such functional measures may provide the best measure for clinical trials in FRDA.

Health-related quality of life in adults with Friedreich Ataxia (FRDA).

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FRDA is a progressive neurodegenerative condition that primarily affects speech, balance and coordination. Both positive and negative changes in health-related quality of life (HRQOL) are important outcomes in treatment/prevention trials. This study characterizes HRQOL in FRDA patients and seeks to validate a measure of HRQOL in FRDA. While no disease-specific HRQOL measure has been developed for FRDA, the symptomatology and clinical profile for FRDA overlaps with that of Multiple Sclerosis, a disease group in which such measures have been validated. The MS Quality of Life Index (MSQLI) has demonstrated high levels of reliability and validity. Several design features of the MSQLI make it a prototype measure for developing disease-specific HRQOL for FRDA, such as inclusion of an established and widely-used generic core (the SF-36), and 9 symptom-specific scales that capture fatigue, pain, sexual function, bladder/bowel function, vision, perceived deficits, mental health, and social support. The SF-36 has proven reliability and validity across a variety of conditions to detect effects of treatment and general population norms are well established.

Methods: The MS Quality of Life Inventory (MSQLI) was administered to 14 adults with genetically-confirmed FRDA. Data was entered into an Excel spreadsheet and checked for errors. The SF-36 compared subject results with norms for the general US population. All scores are transformed to Z scores based on a population mean of 50 with a standard deviation of 10. Subjects' scores on the symptom-specific subscales were also calculated.

Results: The SF-36 has two summary scales: the Physical Components Summary score (PCS) and Mental Components Summary score. In our cohort the mean value for the Physical Component Summary is significantly lower at 33.6 (P<0.0001) than the norms for the general population. The physical functioning subscale score is markedly decreased at 29.1 (P<0.0001). The other subscales measuring general health, pain and role-physical are less affected (41.0-43.7) but still reach statistical significance when compared with population norms. The mean of the Mental Components Summary was 55.4 (P<0.049) compared with the general population.

Conclusion: FRDA has important and lasting effects on health-related quality of life (HRQOL) that may not be entirely captured by quantitative performance measures of neurologic impairment. In our presently small sample size we identify significant effects of quality of life from the SF-36; however we are not yet able to determine symptom-specific subscales most relevant for FRDA. Limitations of physical functioning have an impact on quality of life in patients with FRDA such that this may be useful in patient assessment in therapeutic trials. Most interestingly, quality of life

related to vitality, social functioning, role-emotional and mental heath (all making up the Mental Component Summary of the SF36) is near that of the general population.

Quantification of Movement Abnormalities and Clinical Applications in Friedreich's Ataxia

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Since the discovery of the X25 gene for FA, several reports have documented the considerable phenotypic variability within and among different families. It is clear also that there can be a considerable variation in the age of onset and the rate of progression of the disease process (cf., Michele et al, *Neurol*, 47:1260-1264,1996; Montermini et al, *Ann Neurol*, 41:675-682, 1997). However, since the early clinical descriptions (Geoffroy et al, *Canadian J Neurol Sci*, 3:279-286, 1976; Harding, *Adv Neurol,* 61:1-14, 1993) of the symptoms and movement abnormalities in FA, there have been relatively few reports quantifying eye movement (Furman et al, *Arch Neurol*, 40:343-346, 1983), arm and hand movements, and postural abnormalities (cf., Hallett, *Advances in Neurology*, 87:155-163, 2001) in FA, and no quantitative protocols have been adopted for routine clinical evaluation. The aims of this study are to a) develop a protocol for quantifying the force, accuracy, and timing of a finger pointing task and b) characterize eye-hand coordination during the pointing task based on simultaneous measurements of eye saccades and finger position. Two hypotheses to be tested are: 1) the direction of gaze determines finger touch position in a coordinated eye-hand task wherein the eyes make a saccade to the target and 2) macro square wave jerks (MSWJs) (which have a fixed direction that is independent of eye position during attempted fixation) are not correlated with directional errors in finger touch. The results, in combination with measurements of postural stability, should further our understanding of the pathophysiology of motor control and help to identify the neuronal populations involved as the disease progresses in FA. In addition, the results of quantifying the metrics and timing of eye saccades and finger pointing movements could be used to a) track the progression of the disease process over time and b) evaluate the efficacy of drug treatment.

A dedicated Friedreich ataxia clinic- Overview of the clinic and results of echocardiographic and foot deformity management studies

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In Melbourne, Australia, we have set up a dedicated multidisciplinary Friedreich ataxia (FRDA) clinic. This has been conducted monthly for over two years. The aim has been to maximise quality of life for those with FRDA, prevent complications and provide a basis for clinical research. The FRDA community has embraced the clinic with people coming from interstate and overseas to be seen.

Here the results of two studies from the clinic will be presented; one related to the management of foot deformity and the other to echocardiographic studies.

We have found many patients have had progressive foot deformity related to spasticity that has resulted in impairment of mobility and importantly, the capacity to independently transfer. We have managed this aggressively by splinting, botulinum toxin injection and surgery. We have found that this improves mobility, transfer skills and independence and therefore quality of life. The severity

of foot deformity was categorised as (1) requiring surgery, (2) botulinum toxin or (3) milder or no intervention. The more severe foot deformities correlated with age (surgery v no intervention $p<0.05$; botulinum toxin v no intervention, $p<0.05$; surgery v botulinum toxin, not significant) and years since onset (surgery v no intervention $p<0.05$; botulinum toxin v no intervention, $p<0.05$; surgery v botulinum toxin, not significant) but not with GAA repeat size or age at onset. Three of seven patients who underwent surgery had significant complications however. We conclude therefore that aggressive management of foot deformity is warranted and that active measures to prevent permanent foot deformity should be pursued as surgery is associated with significant potential morbidity in this patient population.

Secondly, we have been performing annual echocardiographic studies on our cohort (n=26), including measurement of left ventricular (LV) and left atrial (LA) size, LV wall thickness, mass and contraction, as well as a detailed Doppler assessment of LV diastolic function. In comparison to age and sex matched control subjects, FRDA patients had an increase in LV wall thickness, relative wall thickness (septal + posterior wall thickness/LV diastolic diameter), LV mass indexed for body surface area and fractional shortening, but a decrease in LV diastolic diameter ($p<0.05$ for all) and a similar left atrial diameter. There was no correlation between the GAA repeat size and any of the measures of LV hypertrophy. The most common structural abnormality in FRDA was an increase in relative wall thickness (>0.44), which was present in 16/26 (62%). FRDA patients also had evidence of diastolic dysfunction with a 17% reduction in the peak early diastolic transmitral velocity ($p<0.05$) and a 30% reduction in the septal and lateral mitral annulus early diastolic velocities ($p<0.05$). The lateral early diastolic velocity was clearly reduced in 14/26 FRDA patients. This study confirms that diastolic function is significantly impaired in FRDA.

Session VIA – Therapy – Drug Development

oDrug screening and development

Dose-dependent cardioprotective effects of Idebenone in a mouse model for Friedreich ataxia

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Friedreich ataxia (FRDA), a slowly progressive neurodegenerative disorder associated with primary hypertrophic cardiomyopathy, is caused by severely reduced frataxin, a mitochondrial protein presumably involved in Fe-S cluster assembly. We have recently generated mouse models that reproduce important progressive pathophysiological and biochemical features of the human disease, including a cardiac hypertrophy, deficient activities of complex I-III of the respiratory chain and of the aconitases, and mitochondrial iron accumulation. We now report the detailed pathophysiological characterization of the murine cardiac disease due to frataxin deletion in the heart. Through echocardiographic measurements, we show that murine FRDA cardiomyopathy is characterised by an early dilatation with the concomitant development of left ventricular hypertrophy. This dilatation is rapidly followed by reduced systolic function. Our results demonstrate that the Fe-S enzyme deficiency is an early event of the disease occurring prior to cardiac dysfunction, and that mitochondrial iron accumulation is a late event. Furthermore, we show a dose response effect of idebenone, a lipid soluble antioxidant, on the survival of the mutant animals. Idebenone delays the onset of the cardiac disease, without simultaneous correction of the deficiency in Fe-S enzyme activity. Our results thus support the view that frataxin is a necessary albeit non-essential component of the Fe-S cluster biogenesis, and indicate that the antioxidant idebenone can act downstream of the primary Fe-S enzyme deficit. Furthermore, our results demonstrate that idebenone is cardioprotective even in the context of a complete lack of frataxin, which further supports its utilization for the treatment of FRDA.

Targeting Antioxidants to Mitochondria as a Potential Therapy for Friedreich's Ataxia

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Over the past few years we have developed antioxidants that selectively block mitochondrial oxidative damage. Among these molecules are derivatives of the natural antioxidants ubiquinone and Vitamin E. The antioxidant efficacy of these molecules was increased considerably by targeting them to mitochondria, which are the major source of oxidative stress in mammalian cells. This was achieved by covalent attachment of the antioxidant to a lipophilic cation. Due to the large mitochondrial membrane potential, these cations accumulate several hundred fold within mitochondria, protecting them from oxidative damage far more effectively than untargeted antioxidants (1-4).
To see if this approach could be used to prevent mitochondrial oxidative damage in vivo, we next determined whether these compounds could be directed to mitochondria within mice. Non-toxic doses of mitochondrially targeted antioxidants could be fed to mice safely and led to the accumulation of intact antioxidant by mitochondria in the heart, skeletal muscle, liver and brain. As mitochondrial oxidative stress is thought to be central to the etiology of Friedreich's Ataxia,

mitochondria-targeted antioxidants may have potential in treating Friedreich's Ataxia. This idea is now being explored.

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Journal of Biological Chemistry (2001) 276 4588 – 4596

Drug Discovery for Friedreich Ataxia: Cellular models, drug targets and pharmacological options

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We have developed a cellular assay system that discriminates between fibroblasts from FRDA patients and unaffected donors on the basis of their sensitivity to pharmacological inhibition of de novo synthesis of glutathione. We characterized potential drug candidates and found that idebenone, a membrane associated antioxidant that ameliorates cardiomyopathy in FRDA patients, as well as other lipophilic and mitochondrially targeted antioxidants protected FRDA fibroblasts from cell death. Furthermore, with this assay we observed that supplementation with selenium effectively improved the viability of FRDA fibroblasts indicating that bioavailability of selenium for incorporation into detoxification enzymes such as glutathione peroxidase (GPX) may also be limited in FRDA patient cells. Moreover, our results also demonstrate for the first time that small-molecule GPX mimetics hold the potential as a novel treatment strategy for Friedreich Ataxia.

Mito Q: Progress Towards a Clinical Study in Friedreich's Ataxia

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Antipodean is undertaking the development of Mito Q (mitoquinone) the targeted mitochondrial antioxidant discovered and patented by Michael Murphy, MRC Dunn, Human Nutrition Unit, Cambridge, UK and Robin Smith, University of Otago, NZ [1]. Seven institutions in New Zealand

are undertaking the GMP manufacturing, formulation, pharmacokinetic and toxicology studies. A sensitive HPLC/MS assay has been developed. A phase I clinical study is planned for May 2003 and hopefully efficacy studies in Friedreich's Ataxia can be initiated in the second half of 2003. We will present data on the synthesis, analytical technique, bioavailability and CNS penetration of mito Q.

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Brain Permeable Iron Chelators and Radical Scavengers to Attenuate Neuronal Cell Death Resulting from Iron Deposition

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The increase of iron at neurodegenerative sites in Parkinson's (PD), Alzheimer's brains and Friedreich's Ataxia is thought to be a contributing factor to the onset of oxidative stress and pathogenesis of aging brain in these neurodegenerative disorders. Neurochemical studies on animal models of PD and other neurodegenerative diseases confirm these findings. Our recent cDNA microarray gene expression studies on midbrain, substantia nigra of MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treated mice have indicated a cascade of domino events that involves over-expression of genes associated with iron metabolism, oxidative stress, inflammatory processes, glutamate receptors and nitric oxide. It has been demonstrated that age-related decline in neuronal signal transduction is reversed by iron chelators and radical scavengers. However, many of presently available agents (e.g. desferal) do not cross the blood brain barrier (BBB). Recently, we have developed a number of BBB permeable isoquinolic iron chelators (eg. VK-28) and identified several naturally occurring polyphenol iron-chelator-radical scavengers, such as (-)-epigallocatechin-3-gallate (EGCG), from green tea. These compounds inhibit iron-induced lipid peroxidation of brain mitochondrial fraction and prevent 6-hydroxydopamine and MPTP-induced cell damage in neuronal cell lines and in *in vivo* models of Parkinson's disease in rats and mice. In an attempt to elucidate the neuroprotective actions of these compounds and possible gene targets for their action, we have employed customized cDNA microarray and have shown that these compounds have a concentration-dependent action on neuronal cell survival and death-inducing genes. At low concentrations they are protective antioxidants, while at higher concentrations they are pro-oxidant and pro-apoptotic. Our studies clearly suggest a possibility of protecting the neurons at sites in the brain where iron accumulates, via the action of these brain penetrating, iron chelator-radical scavengers such as Vk-28 and EGCG .

Iron Chelators of the 2-Pyridylcarboxaldehyde Isonicotinoyl Hydrazone Class as Potential Agents to Treat Friedreich's Ataxia

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Desferrioxamine (DFO) is the most widely used drug for the treatment of the iron (Fe) overload diseases despite suffering from a range of disadvantages. Among these are a lack of oral activity, poor chelation efficacy, and the inability to effectively permeate membranes including those of the mitochondrion. Hence, DFO cannot be used for the treatment of disorders such as Friedreich's ataxia.

Pyridoxal isonicotinoyl hydrazone (PIH) and deferiprone (L1) have both shown potential as orally active alternatives to DFO. However, the lack of patent protection has stifled the development of PIH, while the use of deferiprone remains controversial. Recently, Fe chelators of the 2-pyridyl-carboxaldehyde isonicotinoyl hydrazone (PCIH) class have shown high chelation efficacy, accessibility to mitochondrial Fe pools and low toxicity *in vitro* (Becker, E. and Richardson, D.R. (1999) *J. Lab. Clin. Med.* 134:510-521; Richardson, D.R. et al. (2001) *Biochim. Biophys. Acta* 1536, 133-140). Hence, these compounds have potential for the treatment of FA.

An important consideration in the characterization of iron chelators for the treatment for FA, is that the ligand does not cause toxicity by promoting Fe-mediated oxidative damage to DNA. With this in mind, the effect of chelators on the redox activity of Fe has been investigated using the ascorbate oxidation, benzoate hydroxylation and DNA strand break assays. Further, we examined interactions of the chelators with DNA using the absorbance hypochromicity, topoisomerase I inhibition, DNA precipitation and DNA alkaline elution assays.

Ascorbate oxidation in the presence of Fe(III) was not enhanced by PCIH or any of its analogues (PCIH, PCTH, PCBH, PCBBH) when compared to the control, while in the presence of EDTA, ascorbate oxidation was enhanced to 918 $\pm$ 14% of the control. The PCIH analogues did not enhance benzoate hydroxylation in the presence of Fe(II) but facilitated low level redox cycling to varying degrees in the presence of Fe(III) (105 $\pm$ 4 to 344 $\pm$ 1% of the control). Plasmid DNA strand breaks increased with the concentration of PCIH analogues in the presence of Fe(II) and H_2O_2. This DNA damage appeared to relate to weak interaction of ligands and Fe complexes with DNA in the DNA-dependent hypochromicity assay. However, these interactions were not sufficient to inhibit topoisomerase I activity or damage DNA in cultured cells. In contrast, the potent intercalating agent Doxorubicin (10 μM) caused complete inhibition of topoisomerase I, decreased the precipitable fraction of DNA to 22 $\pm$ 3% of control and reduced the fraction of DNA remaining on the filter to just 25 $\pm$ 4% of the control. In agreement with a lack of cytotoxicity, these data indicate a weak and physiologically insignificant ability of the PCIH analogues to cause Fe-mediated DNA damage. Our results support the potential of these chelators for the treatment of FA.

Development of Iron Chelators of the PCIH Class for the Treatment of Friedreich's Ataxia: Chelators that Bind Mitochondrial Iron

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Friedreich's ataxia (FA) is a severe neurodegenerative disease that has recently been discovered to be associated with iron (Fe) overload within the mitochondrion. This accumulation of Fe results in oxidative damage that is thought to disrupt mitochondrial function. At present, there is no treatment for this crippling disorder and new treatment regimens are essential. One potential therapeutic

intervention may be the development of a specific Fe chelator that could permeate the mitochondrion to bind Fe. Obviously, the only chelator in widespread clinical use, desferrioxamine (DFO), will not be suitable for the treatment of FA, as it does not readily penetrate the plasma membrane to chelate intramitochondrial Fe pools.

In this study we have implemented the only well characterized model of mitochondrial Fe overload to examine the potential of a new class of Fe chelators known as the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) analogues (Becker, E. and Richardson, D.R. (1999) *J. Lab. Clin. Med.* 134:510-521). The PCIH analogues have been designed to be lipophilic enough to permeate the mitochondrion and to bind Fe strongly. In the current study, we examined the Fe chelation efficacy of the ligands using rabbit reticulocytes treated with the haem synthesis inhibitor succinylacetone (SA) that results in an accumulation of non-haem mitochondrial Fe.

Our experiments demonstrate that several of the PCIH analogues show very high activity at mobilizing ^{59}Fe from the ^{59}Fe-loaded reticulocytes. In fact, one of the most effective analogues examined was 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), which mobilized 21% of cellular ^{59}Fe after 15 min. The amount of ^{59}Fe released by PCIH after 15 min was more than that mobilized by PCBBH, PCAH, PCHH, or FIH after a 240 min incubation, viz. 17%, 15%, 6%, and 4% respectively. Desferrioxamine was the least effective chelator examined, being only slightly more effective than control medium. In all experiments, the ^{59}Fe released was ethanol-soluble, indicating that it is a low molecular weight complex that is not bound to protein. Further studies examining the Fe chelation efficacy and toxicity profile of these agents in animal models has been initiated.

Development of a Gene Therapy Approach for the Treatment of the Peripheral Nervous System Manifestations of Friedreich Ataxia

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The peripheral sensory neuropathy and associated ataxia, caused by progressive loss of dorsal root ganglia (DRG) sensory neurons, is a major component of the morbidity experienced by individuals with Friedreich Ataxia (FRDA). Existing treatment options remain inadequate, and there is a pressing need to explore novel therapeutic approaches. We are investigating the potential of gene therapy targeted to DRG sensory neurons as a strategy for ameliorating these peripheral nervous system (PNS) manifestations of FRDA. In a previous study, we have shown that recombinant adeno-associated virus (rAAV) and lentivirus (rLV) vectors exhibit significant promise as vehicles for the gene delivery to PNS sensory neurons (Fleming *et al.*, Hum. Gene Ther. 12:77-86, 2001). In dissociated mouse DRG cultures, rAAV and rLV vectors, encoding EGFP under the transcriptional control of the cytomegalovirus immediate early (CMV-IE) promoter, achieved efficient transduction at low multiplicities of infection (MOI 1 and 10), and sustained transgene expression throughout a 28-day culture period. Neuron-specific antibody (NeuN) staining, performed 8 to 10 days following vector exposure, demonstrated that 68% (rAAV) and 97% (rLV) of EGFP-positive cells were neurons. Significantly, both vectors also achieved efficient and stable transduction of human DRG sensory neurons in dissociated culture. In an effort to more closely reflect an *in vivo* gene delivery approach, we also investigated direct microinjection of both vectors into intact murine DRG explants. Maximal transduction efficiencies of 20 (rAAV) and 200 (rLV) transducing units per neuronal transductant were observed. More recently, we have constructed rAAV and rLV vectors encoding the human frataxin cDNA gene under the transcriptional control of the CMV-IE promoter. These vectors have demonstrated the capacity to increase frataxin expression (visualised using frataxin-specific antibody staining) in the human cervical carcinoma

cell line (HeLa) and human embryonal kidney cell line (HEK 293), both of which have low endogenous frataxin expression. Co-localisation of frataxin expression with mitochondria has also been implied by dual labeling of cells with MitotrackerÔ, a mitochondrial dye. Since measurable differences between the phenotype of FRDA patient fibroblasts and age matched controls have been reported, we are now attempting to functionally validate these frataxin-encoding vector constructs by phenotype correction. Transduction of FRDA patient fibroblast cell lines, derived from patients exhibiting 540/810, 460/690, 540/710 and 600/1020 GAA repeats, has resulted in increased frataxin expression. These cell lines are currently being analysed to quantitate transgene expression (RT-PCR and Western blot analysis) and transgene function (sensitivity to oxidative stress and measurement of mitochondrial iron levels). Ultimately, we intend to evaluate phenotype correction in DRG sensory neurons *in vitro* and *in vivo* using a mouse model for FRDA. In a collaborative project with Dr Puccio and Dr Koenig, we have imported the conditional neuron-restricted exon 4-deleted mouse model of FRDA into Australia. We intend to investigate survival of DRG sensory neurons in dissociated cultures and assess the efficiency of frataxin gene expression in these cells using rAAV and rLV vector mediated transgene transfer. If, as expected, we achieve efficient transduction and demonstrate evidence of phenotype correction in these cultures, the most efficient vector will be delivered by direct injection to DRG in the mouse model of FRDA.

Targeted Corrective Gene Conversion (TCGC): Application to Point Mutation, Gene Rearrangement and Triplet Disorders

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Point mutation, deletion, insertion and triplet contraction and expansion are all classes of mutation involved in human disease. Targeted Corrective Gene Conversion (TCGC) has an obvious potential for treating human conditions involving nonsense, missense and transcriptional splice junction mutations, provided that therapeutic strategies can be delivered effectively to relevant tissue(s). Non-point mutations affect encoded protein function either by disruption of functionally critical peptide sequence or by disruption of codon reading frame. TCGC has some potential in restoring small deletions and insertions to normal sequence at the chromosomal level, and in the case of frame shift, TCGC may be used to restore codon reading frame by either inserting or deleting a single nucleotide at or just downstream of the deletion interface. We have used a TCGC method, Short Fragment Homologous Replacement (SFHR) to correct the exon 23 C3815T nonsense mutation at the Xp21.1 dystrophin locus (*dys*) of the *mdx* mouse, and to restore the *dys* codon reading frame in lymphoblast culture derived from a boy with Duchenne muscular dystrophy (DMD). In these experiments, correction frequencies of the *mdx* mutation have been improved in myogenic precursors to better than 20% of cells in culture. Correction of the *mdx* mutation has been achieved *in vivo*, although to far lower frequencies (<1% of cells). TCGC clearly has a widespread applicability and in our hands has demonstrated utility for restoration of codon reading frame and point mutations at the chromosomal level. We are currently developing a number of additional TCGC applications, including strategies to "loop out" triplet expansions or to "loop in" triplet contractions. The applicability of triplet TCGC strategies to disorders involving triplet expansion/contraction will be discussed.

Session VIB – Therapy – Clinical Trials

oCompleted clinical trials
oClinical trials in progress
oPlanned clinical trials

Four year administration of Coenzyme Q10 and vitamin E to patients with Friedreich Ataxia. A phosphorus MR spectroscopy and clinical follow up study

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Reduction in frataxin expression in the tissues of Friedreich ataxia (FRDA) patients results in mitochondrial iron overload, increased sensitivity to oxidative stress and profound deficit of oxidative phosphorylation (1-4). We previously showed, using phosphorus MR spectroscopy (31P-MRS), that the bioenergetic deficit in cardiac and skeletal muscle of ten FRDA patients was partially reversed after only three months of antioxidant therapy (Coenzyme Q10, 400 mg/day, and vitamin E, 2100 IU/day) (5). We used 31P-MRS, echocardiography and the International Cooperative Ataxia Rating Scale (ICARS) to assess the effect of long lasting Coenzyme Q10 plus vitamin E administration on tissue bioenergetics, cardiological and neurological abnormalities in the same group of patients.
Coenzyme Q10 and Vitamin E therapy resulted in a sustained improvement in cardiac and skeletal muscle bioenergetics in FRDA patients. After 48 months of therapy the mean cardiac PCr/ATP in FRDA patients had increased by 64%, (p=0.001) and skeletal muscle mitochondrial maximum rate of ATP production, Vmax, had risen by 44% (p=0.02). No worsening in the ICARS score and left ventricle hypertrophy was detected after 48 months of treatment in the patients' group. Cardiac fractional shortening significantly (p=0.02) increased.
Antioxidant therapy results in a sustained improvement in cellular bioenergetics in FRDA, as measured directly and in vivo in cardiac and skeletal muscle. In our FRDA patients partial reversal of energy metabolism deficit was associated with lack of progression of both neurological and echocardiographic signs. This pilot study provides a strong rationale for designing larger randomised trials focusing on the clinical response to such a therapy. Such investigation will confirm whether an early diagnosis of FRDA will serve to initiate antioxidant treatment and prevent progression of this disorder.

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Antioxidant treatment of Patients with Friedreich's ataxia: 4 year follow-up and a prospective double-blind randomized trial

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Objective: To determine any clinical and bioenergetic benefits of long term vitamin E and coenzyme Q10 therapy in patients with Friedreich's ataxia.

Background: Decreased mitochondrial respiratory chain function and increased oxidative stress have been implicated in the pathogenesis of Friedreich's ataxia (FRDA). *In vivo* phosphorus magnetic resonance spectroscopy (31P-MRS) has confirmed the decreased mitochondrial energy metabolism in heart and skeletal muscle in patients with Friedreich's ataxia (FRDA). Consequently

patients with FRDA may benefit from an antioxidant / mitochondrial enhancement therapy. Using MRS we have already demonstrated that after 3 and 6 months antioxidant treatment (Coenzyme Q10, 400 mg/day and Vitamin E, 2100 IU/day) cardiac and skeletal muscle MRS bioenergetic parameters were significantly improved in FRDA patients.

Methods: We have followed 10 patients with genetically defined Friedreich's ataxia for 4 years on the same therapy and determined the effect upon the clinical progression of the disease using ICARS and echocardiography, and on the mitochondrial bioenergetics of the heart and skeletal muscle using 31P-MRS.

Results: We have shown that the improvement in heart MRS continued and patient values became similar to controls. In skeletal muscle, MRS continued to improve to between 50 to 60% of control mean over 4 years of therapy as compared to 30% at baseline. Echocardiography data suggests the fractional shortening at the 48 month time point for the patients as a whole was significantly increased relative to the pre-therapy data. Comparison of the progression of the patients' clinical scores with cross-sectional data suggested the clinical scores for 8 patients were better than predicted while 2 patient's scores declined as expected.

Conclusion: This pilot study demonstrates that antioxidant therapy can improve mitochondrial energy synthesis over a prolonged period of time, which is possibly associated with a slowing of the clinical progression of the disease and a significant improvement in heart function. On the basis of the above results, a prospective double-blind randomized trial was established 1 year ago. Fifty patients with genetically proven FRDA have been randomized to receive 600mg coQ10 + 2100iu vitamin E or 30mg coQ10. Assessment is by validated clinical rating scores, e.g. ICARS and echocardiography.

Double-blind placebo-controlled trial of coenzyme Q10 in Friedreich's ataxia

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Antioxidative therapy with idebenone or its natural counterpart coenzyme Q10 is supposed to be beneficial in Friedreich's ataxia. Especially effects on left ventricular mass and cardiac bioenergetics have been reported. However, controlled studies are missing or could not detect significant effects.
To establish the therapeutical effect we perform a double-blind placebo-controlled trial of 400mg/d coenzyme Q10 for 6 months in adult patients with Friedreich's ataxia. Primary outcome measures include left ventricular mass in echocardiography and cardiac magnetic resonance volumetry as well as the international cooperative ataxia rating scale. Measures of cardiac function like ejection fraction will be calculated from echocardiographic and MR data. Subjective improvement or worsening is analysed by visual analogue scale. Data will be analysed for potential effects in subgroups, e.g. patients with cardiac hypertrophy, early onset patients or patients with cardiac complaints.
So far 25 patients have been randomised for this study. We intend to include 40 patients. Final results are awaited in autumn 2003.
This study will help to clarify the potential of Q10 therapy for Friedreich's ataxia. In case of a positive outcome this placebo-controlled trial can help patients to receive reimbursement of costs for coenzyme Q10 treatment from their health insurances.

Idebenone in children and adults with Friedreich ataxia: the Montreal experience

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In 1999, a small open-label study with idebenone was started in 11 children (ages 9 to 17) and 17 adults with FA. The drug was given at 5 mg/kg/day. With a few exceptions, these patients are still being followed and taking the drug after three years. One young adult patient died for the complications of dilated cardiomyopathy shortly after the beginning of the study.
Patients were evaluated at baseline and every 3 months. To assess tolerability, we monitored clinical side effects and performed liver function tests and complete blood counts at every visit. Cardiac evaluation included EKG and transthoracic echocardiography. Neurological function was evaluated using the UADRS. Additional evaluations were only performed in children and included a number of timed tests to asses balance, gait, limb coordination and speech. Plasma malondialdheyde was monitored in children as a sign of lipid peroxidation.
Idebenone at 5 mg/Kg/day was very well tolerated by children and adults, with minimal side effects reported, ususally gastrointestinal distress at the beginning of treatment. There was no case of increased liver enzymes, anemia or leukopenia.
The most significant finding during the first year of treatment was the observation of an average 10% reduction of cardiac mass in 5 out of 6 children whose cardiac mass was > $100g/m^2$ at the beginning of the study ($P<0.002$). The findings concerning the progression of ataxia were less clear. During the first year, two children showed a clear progression of ataxia and the neurological status was overall stable in nine. In adults, severely affected patients showed no changes, but progression was observed in younger, less advanced patients.
Malondialdheyde levels were significantly higher in FA children compared to age-matched controls and were not changed after one year of treatment.
An update after three years of follow-up will be given at the meeting.

Conclusions: At the dosage of 5 mg/Kg/day, idebenone is well tolerated in children with FA and is effective in reducing cardiac mass in most cases with heart hypertrophy. The drug causes no clear improvement in neurological function, but may slow down the progression of the disease. Further evaluation of the drug in larger, controlled trials is necessary to assess its impact on neurological function.

Long term follow up of patients with Friedreich`s ataxia treated with Idebenone: a French open trial for over 3 years

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Background: Based upon the recent advances, a therapeutic trial for Friedreich's ataxia was assessed in three children receiving Idebenone, an analogous of Coenzyme Q10. The preliminary results showed remarkable improvement of cardiac function, septal and left ventricular hypertrophy. Moreover, biochemical *in vitro* studies revealed a significant reduction of iron-induced injuries with Idebenone (Rustin et al., 1999). Considering these encouraging results, we set up a prospective open therapeutic trial for a comparative study of Idebenone efficiency in children and adult patients. We report the results of cardiac and neurological evaluations in this cohort.
Patients and Methods
48 patients (17 children, 31 adults) aged 8-44 yrs were included in a two-centre prospective open therapeutic trial. All patients received oral Idebenone treatment (5 mg/kg/day) during one year. The

clinical outcome was evaluated on the WFN ataxia rating scale (including global, dysarthric, kinetic, posture and gait scores) and the Stewart-Holmes Test, at inclusion and every three months. Electro-oculographic recordings and cardiologic evaluation of the septal thickness, posterior wall thickness and ventricular mass index (VMI) by two dimensional echocardiography were performed at inclusion and every six months. In addition adults are being evaluated for clinical scores, cardiac parameters and ocular recording after 3-4 years of treatment.
Results
The neurological follow-up study showed worsening of the clinical score (44.3±17.1 before treatment vs 47.7±15.9 at month 12, $p<0.0001$). The progression, however, was more severe in the group of children than in adults, where global, kinetic, posture and gait scores were stable with time. The frequency of square-wave jerks increased under treatment (0.48±0.42, vs 0.8±0.42, $p<0.0001$). Cardiologic evaluations revealed a significant reduction of septal thickness (13.4±3.5 vs 12.5±2.4, $p<0.0001$) and posterior wall thickness (12.5±2.4 vs 11.6±2.4, $p<0.0001$) at 12 months. After a 3 year follow up the observed tendency of increased clinical scores was confirmed.
Conclusion
We confirmed the reduction of cardiac hypertrophy in both children and adult patients, but we could not show any neurological improvement with Idebenone treatment. The significance of the increased frequency of square wave jerks remains to be established.

Effects of idebenone treatment in Friedreich patients: Results of one-year-long randomized placebo-controlled trial

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Friedreich ataxia is an inherited neurodegenerative disorder, characterized by progressive gait and limb ataxia, dysarthria, sensory loss, and hypertrophic cardiomyopathy. The disease is due to mutations in the gene encoding frataxin, a mitochondrial protein whose reduction is associated with impaired iron metabolism and increased oxidative damage. These findings suggested a possible therapeutic effect of anti-oxidant agents. We carried out a one-year, randomized, placebo-controlled trial of idebenone, a free-radical scavenger, in Friedreich patients. A total of 29 patients have been randomized to either idebenone (5 mg/kg/day) or placebo. There were 6 women and 23 men, the mean age of participants was 26 years, and the mean disease duration was 15 years. Echocardiography and neurological examination (Ataxia Rating scale) were performed at baseline and after 6 and 12 months. At the end of follow-up we found an average 5.6% decrease in left ventricular mass in idebenone-treated patients, versus 10.7% increase in those assigned to placebo ($p= 0.01$). Regarding septal thickness, we found 4.6% decrease in idebenone-treated patients, and 5.4% increase in the placebo group ($p=0.004$). No improvement was found in other heart ultrasound measures, and in neurological conditions. Our study indicate that idebenone treatment has no serious side effects and is associated with persisting, though moderate, effects on cardiac hypertrophy.

Safety and tolerability of high dose idebenone in patients with Friedreich's ataxia: determining the maximum tolerated dose

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Insights into the pathogenic mechanism of Friedeich's ataxia (FRDA) suggest that antioxidants may be an effective approach to treatment. In particular, recent clinical studies suggest that treatment with low doses of lipid-soluble antioxidants is associated with reduction in cardiac mass indices. These encouraging findings suggest that antioxidant therapy may protect patients from FRDA-associated cardiomyopathy, although no amelioration of the neurological features has been seen. It is hoped that higher doses of lipid-soluble antioxidants will provide greater cardiac protection and effectively treat neurological features as well.

We are conducting a phase I dose-escalation trial examining the toxicity and tolerability of the antioxidant idebenone in patients with FRDA. Our primary objective is to determine the maximum tolerated dose of idebenone in each of these age groups. Our secondary objective is to document the pharmacokinetics of high dose idebenone in this population. The doses of idebenone examined in this study range from 2.5 mg/kg to 75 mg/kg. At present, 25 adults have completed the study and we have found idebenone to be well tolerated up to 75 mg/kg - the maximum dose allowed under the protocol. Fourteen adolescents and 6 children have completed the study with no evidence of toxicity.

A placebo-controlled crossover trial of L-carnitine and creatine in Friedreich's ataxia

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Friedreich's ataxia is caused by drastically reduced levels of frataxin and impaired mitochondrial energy metabolism associated with oxidative damage to iron-sulfur protein clusters including defects to respiratory chain complexes I-III. Decreased mitochondrial ATP formation can be demonstrated *in vivo* measuring phosphocreatine recovery after exercise with ^{31}P magnetic resonance spectroscopy (^{31}P-MRS). Creatine and L-carnitine are main compounds that can enhance cellular energy transduction. Supplementation with creatine is assumed to synthesize more phosphocreatine, an intrinsic energy reservoir that functions to regenerate ATP in times of high energy demand. High doses of L-carnitine may compensate secondary carnitine deficiency due to defects of oxidative phosphorylation with an increased NADH/NAD(+) ratio that inhibit beta-oxidation.
We studied the administration of creatine (6.75g/d) and L-carnitine (3g/d) in a placebo-controlled three-period crossover trial in 15 adult patients with genetically confirmed Friedreich's ataxia. All patients were still able to walk to prevent immobilization effects on ^{31}P-MRS.
As shown in previous studies, ^{31}P-MRS revealed substantially prolonged times of phosphocreatine recovery after exercise under aerobic as well as ischemic conditions. After 4 months on L-carnitine phosphocreatine recovery in ^{31}P-MRS was significantly improved after ischemic exercise ($p<0.03$, t-test). However, in comparison to placebo and creatine this effect was not significant. Scores in the international cooperative ataxia rating scale and echocardiographic data remained unchanged. Creatine treatment had no effect in Friedreich ataxia patients.
Our data suggest larger trials with L-carnitine and underscore the importance of placebo controls in therapeutical trials in Friedreich's ataxia.

CoQ10 treatment for the ataxic variant of CoQ10 deficiency

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