2015

International Ataxia Research Conference

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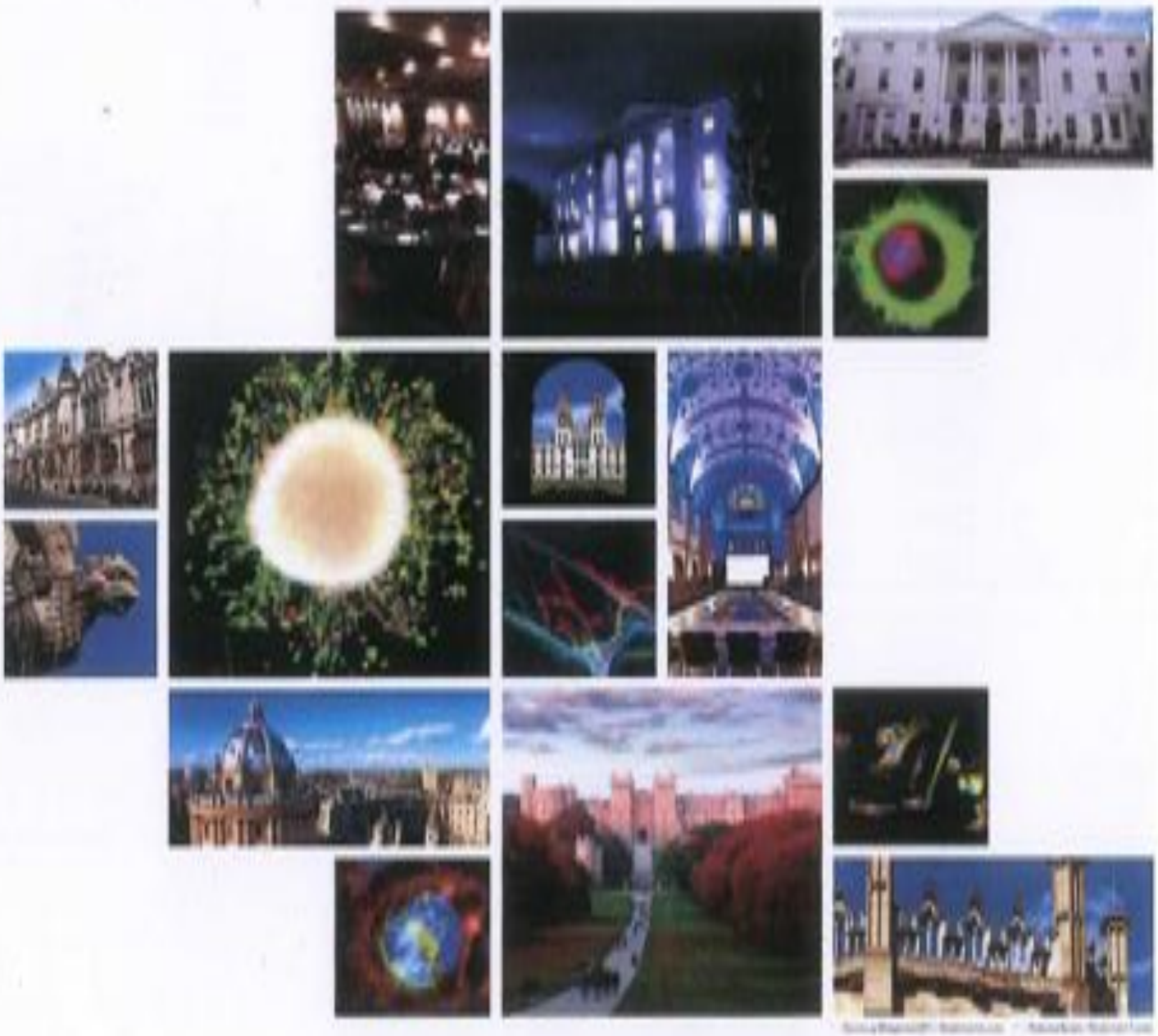

Programme & Abstracts

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Table of content

Welcome

We wish you a very warm welcome to the International Ataxia Research Conference 2015 in Windsor. This conference is hosted and organised by four ataxia organisations from four different countries, which we feel is evidence of the international collaboration platform created to advance treatments for these diseases. The partners, Ataxia UK, Ataxia Ireland, GoFAR (Italy) and FARA (US) have been assisted by a Scientific Steering Committee which has put together a robust programme covering diverse and relevant topics in ataxia research.

The conference covers all hereditary and sporadic ataxias. Sessions have deliberately not been themed by the specific types of ataxias but rather across the spectrum of basic, translational and clinical research relevant to advancing us from diagnosis to treatment as we believe there is significant benefit to be gained from understanding advances in the broader field and applying lessons learned. The sessions have been organised to promote sharing based on topic areas or focus/stage of research; genetic research and diagnosis; molecular mechanisms and cellular pathways; animal and cell models; drug discovery and therapeutic approaches; and clinical research and trials The organisers and Steering Committee were especially encouraged by the number of abstract submissions across all themes, most particularly in drug discovery and therapeutic development. We see this as an important milestone for our scientific and patient communities. We are also very pleased to have an interview with a representative of the European Medicines Agency who will be giving advice and information on the regulation of the development of treatments.

People with ataxia are at the heart of everything we do and to highlight this we have invited three people with ataxia to give presentations from their different perspectives. We are also delighted that many patient group representatives are attending the conference and that the annual meeting of euro-Ataxia, (the federation of ataxia charities in Europe) will also be meeting here in parallel to the conference - giving a perfect opportunity for patients, patient groups and researchers to meet, and learn from one another.

We believe this may be the largest ataxia research conference to date, demonstrated in the number of oral and poster presentations, delegates and sponsors! We are grateful to our Committee and session chairs for the time and effort in reviewing abstracts and preparing our program. We are pleased that so many invited speakers and academic researchers are joining us from around the world and that the conference has attracted a significant number of representatives from pharmaceutical companies – this all highlights the relevant science and collaboration fuelling fast growing interest in ataxia research.

An event this large would not be possible without the generous support of our many sponsors to whom we are extremely grateful. Fourteen pharmaceutical companies have kindly provided sponsorship and four ataxia patient groups have partnered with the four organising charities and given their financial support to the conference.

Over the next few days we will take steps together to bring us closer towards the development of much needed treatments for the ataxias. We hope you learn from the research reported and ideas shared; network with other participants; and leave with new colleagues in your research circle, and new inspiration and urgency for your work.

Sue Millman	Barbara Flynn	Mina Ruggeri	Jen Farmer
Ataxia UK	Ataxia Ireland	GoFAR (Italy)	FARA (US)

Genetic and molecular mechanisms of ataxias

Invited Speaker: Sanjay Bidichandani (University of Oklahoma, US)

Epigenetic promoter silencing in Friedreich ataxia

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The expanded GAA triplet-repeat mutation in Friedreich ataxia causes transcriptional deficiency via epigenetic silencing of the FXN promoter. The ensuing defect of transcriptional initiation, which correlates with the length of the GAA triplet-repeat mutation, is the major cause of transcriptional deficiency in Friedreich ataxia. Epigenetic promoter silencing in Friedreich ataxia is mediated by altered nucleosomal positioning, which obliterates the normal nucleosomal depleted region at the FXN transcriptional start site. A class I HDAC inhibitor, 109/RG2833, currently being developed as a rational therapy for Friedreich ataxia, increased FXN promoter accessibility upon assaying individual chromatin fibers via NOMe-Seq analysis. Metabolic labeling of nascent transcripts revealed that 109/RG2833 significantly improved FXN promoter function in patient-derived cells. Epigenetic promoter silencing in Friedreich ataxia is therefore reversible, and these data implicate class I HDACs in repeat-mediated epigenetic promoter silencing.

This research was made possible by a grant from the NIH to SIB (R01 NS072418)

The impact of compound heterozygous mutations in *FXN* on clinical outcome in Friedreich ataxia: *insights from frataxin structure and function*

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Friedreich ataxia (FRDA) is an autosomal recessive neurodegenerative disease characterised by incoordination and hypertrophic cardiomyopathy. The majority of individuals with FRDA have homozygous GAA trinucleotide repeat expansions in the first intron of FXN with reduced expression of the mitochondrial protein frataxin. The remaining affected individuals are compound heterozygous for a GAA expansion and a point or insertion and/or deletion mutation within FXN. This study examines disease-causing mutations and their impact on frataxin structure and function, and clinical outcome in FRDA. This includes the potential to bind iron and interact with iron sulfur cluster assembly proteins like IscU and Isc8. Clinical information from 111 compound heterozygous individuals with FRDA (81 published and 30 previously unpublished cases) was collated and compared to clinical data from 131 individuals homozygous for GAA expansions. Molecular modelling, in silico protein stability analyses using different prediction algorithms, and systematic review of published experimental data, were used to estimate the impact of each mutation on frataxin structure, function and stability. Each mutation was categorised into one of four groups: three compound heterozygous mutation groups (i) null – no frataxin produced, (ii) moderate/strong impact on protein stability/function and, (iii) minimal impact on protein stability/function; and (iv) homozygous GAA expansions. The difference in age of disease onset between the four categories was examined using regression analysis taking into account GAA expansion size(s). The likelihood of developing cardiomyopathy and diabetes between the mutation categories, taking the expansion into account, was also examined. Individuals with null mutations had a significantly earlier age of onset when compared to homozygous GAA expansions, after accounting for expansion size.

Comparison of the GAA expansion homozygous group to the minimal impact and moderately or strongly destabilising groups, found no significant differences in age of onset. Interestingly, individuals with homozygous GAA expansions had a greater likelihood of developing cardiomyopathy than individuals in all three compound heterozygous mutation categories. In contrast, those with null mutations were more likely to have diabetes mellitus than those with homozygous GAA expansions. Importantly, in compound heterozygous individuals we find that, in addition to frataxin expression from an expanded allele, expression of a mutant frataxin with partial function is of greater benefit than no frataxin expression. This study provides a foundation for further analyses of the different mutations in frataxin to better understand the link between structure and function, and clinical outcome in FRDA.

R-loop function in pathology of Friedreich ataxia and implications for other expansion disorders

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Around forty human diseases are associated with expansion of small nucleotide sequences. Depending on their location, these expansions can either lead to expression of toxic proteins or transcriptional repression/gene silencing of the host gene. In Friedreich ataxia (FRDA), caused by an expanded (GAA)n repeat sequence in intron 1 of the frataxin (FXN) gene, transcriptional repression has been proposed to be associated with the formation of unusual DNA sequences and repressive chromatin over expanded gene. In our lab we established a DNA immuno-precipitation method (DIP), which allowed us to detect RNA/DNA hybrids (R-loops) in human cells (Skourti-Stathaki et al, 2011). Recently, using DIP we showed that R-loops are formed on expanded FXN alleles and trigger the formation of repressive chromatin in cells from FRDA patients (Groh et al, 2014). We further investigated the molecular mechanism of R-loop-mediated heterochromatin formation in FRDA. In particular, we studied the contribution of RNAi machinery to this process. Our results demonstrate that methyl-transferase G9a, which deposits H9K9me2 mark, is specifically enriched over expanded GAA repeats. Interestingly, we observed the recruitment of RNAi machinery at the promoter region of the FXN gene, which was reduced in FRDA cells. This suggests that R-loops are likely to trigger heterochromatin formation in RNAi-independent manner. We will present the molecular details of this process and its contribution to the pathology of other expansion disorders.

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Expanded GAA repeats induce transcriptional silencing restricted to the FXN locus and decrease the elongation rate through the FXN gene

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Friedreich's ataxia (FRDA) is a severe neurodegenerative disease caused by transcriptional repression, which is induced by expanded GAA repeats located in intron 1 of the FXN gene. FRDA patients homozygous for GAA expansion have 5 – 30 % of frataxin mRNA and protein when compared with healthy individuals. It has been demonstrated, using various model systems along with patients' autopsy samples, that expanded GAA repeats induce epigenetic silencing of the FXN gene. Posttranslational histone modifications that typify heterochromatin are enriched in the vicinity of the repeats, while active chromatin marks in this region are underrepresented in FRDA samples when compared to controls. Thus far, silencing triggers as well as the exact molecular mechanism of FXN silencing remain unknown.

Spreading of heterochromatin and transcriptional silencing has been observed in the proximity of the highly repetitive regions of the genome such us centromeres. However, the extent of silencing induced by expanded GAAs beyond the direct vicinity of intron 1 has not been determined. Results indicating both silencing of the FXN promoter associated with a transcription initiation defect as well as studies demonstrating transcription elongation dysfunction have been reported. Moreover, recent phenotypic observations conducted mostly in FRDA patient-derived fibroblast and lymphoblast cell lines indicate the possibility of a long-range cis silencing mechaniem, spanning a larger region of the FXN locus. The extent of GAA repeat-induced silencing is of particular importance considering two important therapeutic strategies for FRDA: reversal of FXN silencing and gene replacement therapy. In the case of extensive transcriptional silencing of a larger region of chromosome 9, restoring frataxin expression using gene therapy strategies would alleviate only the part of the disease phenotype arising solely from frataxin deficiency. On the other hand, strategies based on epigenetic reactivation of the FXN locus should result in complete reversal of FRDA phenotype. However, if the effect of GAA expansion is localized to the FXN gene, both therapeutic avenues should be equally efficacious.

We present the results of a comprehensive analysis of the transcription status and epigenetic environment of the FXN locus in a large set of 17 primary fibroblast cell lines derived from FRDA patients and 18 lines obtained from unaffected controls. Using next-generation RNA sequencing, we demonstrated a remarkable variability in FXN expression within both FRDA as well as control samples. Additionally, we showed that the epigenetic silencing effect induced by the expanded GAA repeats is confined to the FXN locus and does not affect expression of upstream or downstream neighboring genes. Finally, analysis of FXN pre-mRNA expression between FRDA and control samples revealed a pronounced transcription elongation defect at the expanded GAA region.

Expanded GAA Repeats Impair Frataxin Gene Expression and Promote Repositioning to the Nuclear Periphery at Single-Cell Level

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In Friedreich's Ataxia (FRDA), abnormal GAA trinucleotide–repeat expansions in intron 1 of the frataxin gene (FXN) cause epigenetic changes and reduce FXN mRNA levels in averaged cell samples though a poorly understood mechanism. Dissecting the silencing mechanism in FRDA in situ, through the analysis of FXN nuclear localisation and expression at single-cell level, is crucial to improve our understanding of the disease.

Here, we have developed a human cell model to analyse the link between FXN nuclear localisation and expression in single cells. FXN-MS2-Luc and FXN-GAA-MS2-Luc stable human clones carry a site-specific integration of a single copy of the whole FXN locus with either 6 or ~310 GAA repeats in intron 1, respectively. To fluorescently label the transgenic FXN mRNA, we inserted MS2 protein–binding sites into exon 2 by homologous recombination. The ~310 GAA repeat expansion in the FXN-GAA-MS2-Luc cell line recapitulates the characteristic FXN gene repression in FRDA.

FXN transgene localisation in the FXN-MS2-Luc and FXN-GAA-MS2-Luc lines was determined by DNA FISH. FXN was positioned at the nuclear periphery (NP) in ~44% of the FXN-GAA-MS2-Luc cells compared to ~10% of FXN-MS2-Luc cells. Restoring histone acetylation repositioned FXN-GAA-MS2-Luc away from the NP. To further understand FXN repression, we analysed the transcriptional output of individual transgenic FXN alleles by RNA FISH. FXN-GAA-MS2-Luc cells contained ~5 ± 2 mRNAs per cell and FXN-MS2-Luc cells contained ~9 ± 4 mRNA per cell, therefore ~310 GAA repeats reduce the number of mature mRNA molecules by 44% at single-cell level.

localisation was next analysed in its native genomic environment in carrier, healthy and FRDA patient cells. In carrier cells, the expanded FXN allele localised preferentially closer to and contacted more frequently with the NP than the normal allele. In FRDA versus healthy cells, the GAA expansion increased the probability of an allele to be found at the NP and consequently its probability of being silenced. Moreover, when expanded GAA-FXN alleles did express, it was at significantly reduced levels both in the nucleoplasm and especially at the periphery. We provide evidence that the combined effect of expansion with peripheral relocation results in a catastrophic reduction in expanded FXN transcriptional output, demonstrating a clear link between expanded FXN positioning at the NP and GAA-mediated transcriptional repression.

Collectively, these results suggest repressive epigenetic modifications at the expanded GAA-FXN locus may lead to NP relocation, where further repression may occur.

Lentivirus mediated FXN gene delivery restores genome stability and DNA damage repair potential in human and mouse FRDA fibroblasts

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Friedreich ataxia (FRDA) is a progressive neurodegenerative disease with primary sites of pathology in the large sensory neurons of the dorsal root ganglia (DRG) and dentate nucleus of the cerebellum. FRDA is also often accompanied by severe cardiomyopathy and diabetes mellitus. FRDA is caused by loss of frataxin (FXN) expression, which is due to GAA repeat expansion in intron 1 of the FXN gene. Frataxin is a mitochondrial protein important to iron-sulphur (Fe-S) cluster biogenesis and the electron transport chain (ETC). As a consequence of impaired mitochondrial energy metabolism, FRDA cells show increased levels of and sensitivity to oxidative stress, which is known to be associated with genome instability. In this study, we investigated DNA damage/repair in relation to FXN expression via immunostaining of gH2AX a nuclear protein that is recruited to DNA double strand breaks (DSBs). We found FRDA patient and YG8sR FRDA mouse model fibroblasts to have inherently elevated DSBs (1.8 and 0.9 foci/nucleus) compared to normal fibroblasts (0.6 and 0.2 foci/nucleus, in each case $p<0.001$). By delivering the FXN gene to these cells using a lentivirus vector (LV) at a copy number of ~1/cell, FXN mRNA and protein levels reached 270- and 202-fold, respectively to that of normal fibroblasts, without observable cytotoxicity. This resulted in a reduction in DSB foci to 0.7 and 0.43 (in each case $p<0.001$) in human and YG8sR fibroblasts, respectively and an increase in cell survival to that found for normal fibroblasts. We next irradiated the FRDA fibroblasts (2Gy) and measured their DSB repair profiles. Both human and mouse FRDA fibroblasts were unable to repair damaged DNA. However, repair returned to normal levels following LV FXN gene transfer. Our data suggest frataxin may be important for genome stability and cell survival. We are currently investigating whether lack of DNA damage repair in FRDA to be a factor that influences neurodegeneration.

Cellular and molecular models of Friedreich's ataxia

Invited Speaker: Michelle Lufino (University of Oxford, UK)

Cell and animal FXN genomic reporter models of Friedreich's ataxia

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Currently, there is no treatment available for Friedreich's ataxia and the silencing mechanism induced by GAA expansions still needs further elucidations. The generation of cell and animal models which closely recapitulate the characteristic molecular features of FRDA is of great importance as it can facilitate the identification of promising therapies and improve our understanding of the FRDA pathogenesis. We have previously reported the generation of a FXN-GAA-Luc reporter cell model which carries a ~310 GAA repeats expansion within the context of the whole FXN genomic DNA locus, thus providing physiologically-relevant FXN expression. Here, we present an update on this model and the generation and use of two further reporter models, namely FXN-GAA-MS2 cells and FXN-GAA-Luc mouse model.

We have recently described the use of FXN-GAA-Luc cells to identify a novel FXN-increasing molecule, named C5, which is able to increase FXN expression in FRDA patient primary cells. Since C5 is characterized by a relatively high EC50, we have carried out structure-activity relationship (SAR) studies in order to identify compounds with improved frataxin up-regulating properties. We synthesized a series of derivatives of C5 and we tested their effect on frataxin protein levels using the FXN-GAA-Luc cell model. We report the identification of a new small molecule with an ~18-fold reduction in EC50 compared to C5, reducing the active concentration to the low uM range and representing a major improvement over to its parent molecule.

FXN-GAA-Luc cells provide an optimal readout for frataxin protein levels, however they do not represent a suitable tool to investigate the transcriptional kinetics of the FXN gene. To achieve this and to assess the effect of the GAA expansion of FXN transcription in live cells and at single-cell resolution, we modified the FXN-GAA-Luc cell model in order to utilize the MS2-based RNA imaging system. By performing Fluorescence Recovery After Photobleaching (FRAP), we show that the presence of a ~310 GAA repeat expansion greatly slows FXN transcriptional kinetics. Moreover, we demonstrate for the first time at single-cell resolution that expanded GAA repeats reduce FXN transcriptional output by inhibiting preferentially FXN transcription initiation.

Finally, we have recently developed a novel FXN-GAA-Luc mouse model for in vivo live visualization of frataxin protein levels and we show that that bioluminescence generated from the FXN-Luciferase fusion protein can be easily detected in live anesthetized animals. Since the light intensity represents a readout of frataxin protein levels, this model is particularly suitable for in vivo testing of frataxin-increasing therapies, allowing detection of frataxin levels before and after compound administration, therefore providing information on the extent and duration of frataxin up-regulating strategies.

In conclusion we describe a series of genomic-reporter models suitable for different applications.

Inducible and reversible frataxin knock-down mouse model for Friedreich's ataxia

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Friedreich's ataxia (FRDA) is an early-onset neurodegenerative disease that progressively impairs motor function, leading to ataxic gait, cardiac abnormalities, and multiple other co-morbidities, ultimately resulting in early mortality (median age of death, 35 years). It is the most commonly inherited ataxia, and is caused by severely reduced levels of frataxin (Fxn) that usually result from a guanine-adenine-adenine (GAA) trinucleotide repeat expansion within the first intron of the Fxn gene. Animal models are valuable tools for mechanistic analysis and therapeutic development in FRDA. Existing transgenic and heterozygous knockout (homozygous are embryonically lethal) animal models, are either mildly symptomatic or restricted in their ability to recapitulate and evaluate the spatial and temporal aspects of FRDA pathology, as they are engineered to be tissue-specific conditional knockouts. We have developed an inducible mouse model for FRDA that permits reversible frataxin knockdown and detailed studies of the temporal progression or recovery following restoration of frataxin expression. We targeted a single copy shRNA against the Fxn transgene (doxycycline-inducible) under the control of H1 promoter gene into the rosa26 genomic locus. This allowed us to circumvent the lethal effect of organism-wide knockout, while permitting significant frataxin reduction in all tissues. Fxn knockdown was achieved to control the onset and progression of the disease depending on the dose of doxycycline (Dox). We observed ataxia, degeneration of dorsal root ganglia, scoliosis, and iron deposition, parallel to what is observed in FRDA patients. Rescue experiments were carried out by withdrawal of Dox to reverse the acceleration of disease progression even after significant motor dysfunction was observed. By controlling the onset and progression of the disease, and attempt rescue via restoration of frataxin levels, we aim to refine our understanding of the pathogenesis of FRDA and its reversibility at different stages of disability, assess biomarker response to drugs, and to test effective therapeutic agents.

Induced pluripotent stem cell-derived neurons from Friedreich's ataxia patients have a cellular phenotype that can be reversed by frataxin inducers

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Background and aims: We employed induced pluripotent stem cell (iPSC)-derived neurons obtained from FRDA patients and healthy subjects to unveil phenotypic alterations related to frataxin deficiency and investigate if they can be reversed by treatments that upregulate frataxin. In a parallel study, we found that FRDA-neurons show mitochondrial oxidative stress-mediated activation of the intrinsic pathway of apoptosis, which can be prevented by cAMP-inducing drugs, such as GLP-1 agonists used in the treatment of type 2 diabetes. Here, we characterise alterations related to defective iron-sulfur cluster biogenesis and dysregulation of the oxidative stress response and iron handling. Our preliminary findings suggest that these alterations can be reversed by molecules that upregulate frataxin expression.

Materials and Methods: iPSCs were from two FRDA patients and two controls. Neurals cells were differentiated as previously described (Hick et al., 2012). We confirmed the stability of the GAA expansions throughout the differentiation process by PCR. Cellular identity at all stages of differentiation was determined by morphological analysis and by expression analysis of specific markers by immunfluorescence or by RT-PCR. Patch-clamp was used to assess electrophysiological properties. Protein levels were estimated by western blot. Videomicroscopy using specific fluorescent probes was used for iron and oxidative stress analyses.

Results: FRDA and control iPSCs were equally capable of differentiating into a neuronal or astrocytic phenotype. iPSC-derived neurons had a cortical phenotype and generated sodium currents and action potentials. We confirmed a slight delay of maturation of FRDA-neurons. The expression of two iron-sulfur proteins, the NDUFS3 mitochondrial Complex I subunit and mitochondrial aconitase and of two lipoic acid (synthesized by Fe-S proteins) -containing proteins, pyruvate dehydrogenase and alpha-oxoglutarate dehydrogenase was decreased by 25-50% in FRDA- vs. control-neurons. Conversely, the expression of mitochondrial superoxide dismutase was robustly increased in FRDA-neurons. FRDA-neurons showed a ± 5-fold increase in labile iron pool and a ± 2-fold decrease in reduced glutathione. Oxidative stress-mediated cell death after administration of 100 µM H_2O_2 was 5-fold higher in FRDA cells.

Treatment with the HDAC inhibitor 109 increased iron-sulfur protein levels, downregulated SOD2 levels and almost fully protected FRDA-neurons from oxidative stress-mediated cell death. Preliminary results suggest a similar effect of exendin-4.

Conclusions: iPSC-derived neurospheres from FRDA patients differentiate properly into neurons and astrocytes, though their neuronal functional maturation appears to be slightly delayed. FRDA-neurons show lower levels of iron-sulfur proteins, higher LIP and lower GSH levels, and enhanced sensitivity to oxidants compared to control-neurons, indicating deficient iron-sulfur cluster biogenesis, altered iron metabolism, and oxidative stress. Treatment with drugs that upregulate frataxin appears to reverse these phenotypic changes. Our findings suggest that correction of frataxin deficiency may not only stop disease progression, but also lead to clinical improvement by rescuing still surviving, but dysfunctional neurons.

Analysis of mitochondrial dynamics in cultured sensory neurons and in *in vivo* mouse models of Friedreich's ataxia

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Friedreich's ataxia (FA) is a primary mitochondrial disorder which causes degeneration of different neuronal types, with a preference for large sensory neurons, cerebellar neurons, and corticospinal tracts. Very few studies, none of which in mammalian systems, have addressed the involvement of mitochondrial dynamics, such as axonal transport, fusion and fission, in FA neurons. Since work from us and others suggest that alterations of mitochondrial dynamics compromise neuronal function, synaptic activity, and the architecture of the cell, we hypothesize that abnormal dynamics may participate to the specificity of neuronal cell loss in FA.

We performed live imaging fluorescent microscopy in isolated sensory neurons and in the whole, living, mouse to examine mitochondrial morphology, distribution along axons, transport, and fusion and fission events. We have identified relevant pathogenic phenotypes for FA, such as fragmentation of mitochondria in axons and cell bodies as early as 4 days *in vitro* and impaired mitochondria bioenergetics, in sensory neurons of two different FA mouse models: the KIKO and the Sarsero mouse. Moreover, we have successfully imaged mitochondrial transport in the sural and femoral nerves of living FA mouse models after crossing them with the mitoDendra transgenic mouse. These *in vivo* studies allowed us to investigate mitochondria morphology and dynamics in affected and non-affected nerve tissues in longitudinal studies. We are currently investigating the cellular consequences of these mitochondrial abnormalities in neuronal architecture and function.

Our data provides a novel readout of FA pathology, which will allow us to evaluate therapeutic approaches targeting mitochondrial function and dynamics, and also to assess the efficacy of therapies that increase frataxin levels by monitoring the downstream consequences on mitochondria, and their impact on neuronal viability.

Impact of frataxin-deficiency on mitochondrial dynamics

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Frataxin is a highly conserved mitochondrial protein that plays a major role in the biosynthesis of iron-sulfur clusters. Effects of frataxin downregulation in human samples and other disease models of Friedreich Ataxia (FA) include diminished activity of several mitochondrial enzymes, impaired ATP production and depolarization of mitochondrial membrane, among others. Remarkably, *Drosophila* models of FA have been able to reproduce all these biochemical features. As expected, frataxin-deficient flies display reduced membrane potential, aconitase activity and ATP levels as well as hypersensitivity to oxidative stress.

Moreover, mitochondria are dynamic organelles that fuse and divide according to the energetic demands of the cell. Therefore, we aimed to analyze whether cells suffering from an energy deprivation due to loss of frataxin would modify their mitochondrial network to compensate this defect. Indeed, alterations in mitochondrial morphology have been reported in FA models but this aspect has not been analysed in detail. Interestingly, we could show that frataxin deficiency affects mitochondrial morphology in glia. Our histological and molecular results also show a strong mitochondrial accumulation in an age and stress-dependent manner. Importantly, using the autophagy marker p62, we can conclude that mitochondrial accumulation in glia is mainly caused by an impaired degradation of damaged mitochondria. In agreement with this hypothesis, we also found that frataxin-deficient flies accumulate mtDNA. We are trying now to decipher the underlying mechanisms. Our results indicate that changes in the expression of *Drosophila mitofusin* (*dMfn*, a gene involved in mitochondrial fusion and degradation) might be a central event. In this sense, a genetic screen, carried out to find putative suppressors or enhancers of FA defects in *Drosophila*, revealed that *dMfn* downregulation is sufficient to counteract some of the frataxin-deficient phenotypes. Moreover, we have found that frataxin overexpression completely alters the mitochondrial network by triggering a strong clustering of mitochondria probably due to its capacity to increase ATP production. Our results link frataxin with the dynamic control of stability, integrity and homeostasis of mitochondria, providing new ideas for the development of potential therapeutic targets.

A new *Drosophila melanogaster* model to identify genetic modifiers of transcriptional repression caused by GAA expansion in *FXN*

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Background/Hypothesis: Friedreich ataxia (FRDA), a neurodegenerative disorder with recessive autosomal inheritance, is caused by pathological GAA expansions within the first intron of the *FXN* gene, which leads to a reduction in the level of the encoded protein frataxin. The most accepted hypothesis to explain the transcriptional repression caused by this triplet expansion is the heterochromatinization of the *FXN* locus. However, the underlying molecular mechanism of this process is not completely understood yet.

Methods: In order to identify potential factors involved in this process, we developed a new *Drosophila melanogaster* model that consists of two strains expressing the reporter gene firefly luciferase preceded by 9 GAA repeats (normal expansion) in one strain, and 300 GAA repeats (pathological expansion) in the other. To achieve this, three genetic constructs (UAS-Renilla; UAS-9GAA-firefly and UAS-300GAA-firefly luciferases) have been developed based in the pACMAN platform in combination with the UAS-GAL4 system to control the expression.

Results: We checked that the 300 GAA repeat expansion represses the firefly luciferase expression, analogously to *FXN* gene repression in FRDA. It was also observed a higher level of chromatin compaction in the luciferase construct of the 300 GAA line compared to the 9 GAA line. Next, we started a genetic screen by crossing both model lines with several *Drosophila* strains carrying alleles of genes involved in posttranslational histone modifications, heterochromatin formation and maintenance, and transcriptional activation or repression. So far, we have identified some potential regulators of the repression mediated by the GAA pathological expansion, as Su(var)3-9, Su(var)2-5 and Su(var)2-1 , which human orthologous are SUV39H and HP1.

Conclusions: We have developed a new model in *D. melanogaster* suitable for high-throughput screening to identify specific genetic modifiers involved in transcriptional silencing of *FXN* and thus potential therapeutic targets for the treatment of FRDA.

Cellular and systemic pathways

DNA Repair Deficit and Neuroinflammation as Potential Contributors to the Physiopathology of Friedreich's Ataxia

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Friedreich's ataxia (FA) is a recessive and predominantly neurodegenerative disorder caused by a decreased level of frataxin protein. To gain some insight into the molecular mechanisms contributing to neurodegeneration in FA we have studied human neural cell models subjected to frataxin knockdown.

We have obtained an inducible neuron-like cell model for frataxin deficiency by stable transduction of the human neuroblastoma SH-SY5Y cell line with a tetracycline-inducible lentiviral vector encoding for a specific shRNA. Enhanced oxidative stress, DNA damage and activation of apoptotic cell death was observed upon frataxin knockdown in this model. Interestingly, frataxin down-regulation was also accompanied by significant changes in the expression of various proteins implicated in DNA repair. These changes were reversible after up-regulation of frataxin gene expression. In view of these data we suggest that increased DNA damage in frataxin-deficient neuronal cells may be due not only to oxidative stress but also to diminished DNA repair systems.

In order to study the contribution of glial cells to the physiopathology of FA, we have analyzed the consequences of frataxin knockdown in cultured human astrocytes, which also results in increased oxidative stress and apoptotic cell death. Interestingly, frataxin silencing in astrocytes is also accompanied by an enhanced expression and secretion of some pro-inflammatory cytokines.

To test for non-cell autonomous interactions we cultured wild-type mouse neurons in the presence of frataxin-deficient astrocyte conditioned medium, which provoked a delay in the maturation of these neurons, a decrease in neurite length and enhanced cell death. These findings indicate a detrimental effect of frataxin silencing, not only for astrocytes but also for neuron-glia interactions, underlining the need to take into account the role of non cell-autonomous processes in the pathogenesis of FA.

Furthermore, our studies performed with cultured olfactory mucosa stem cells, which are obtained from biopsies from FA patients, also indicate a deficiency in DNA repair-related proteins as well as an increased expression and secretion of some pro-inflammatory cytokines. These results support the view that both DNA repair deficit and neuroinflammation may be potential contributors to the physiopathology of FA.

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The role of acetylation in the pathogenesis of Friedreich's ataxia

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Background/Hypothesis: The heart's ability to adaptively use metabolic substrates to drive energy production—known as energetic substrate flexibility—must be maintained, as even subtle variations in efficiency have severe impacts on cellular metabolic health. Lysine acetylation and its regulation by the mitochondrial NAD^+-dependent protein deacetylase sirtuin 3 (SIRT3) are emerging as important regulators of cardiac energy homeostasis. Reduction in SIRT3 activity in the heart results in hyperacetylation of metabolic enzymes, hypertrophy, and markedly reduced ATP levels (>50%)—demonstrating an important role for acetylation in regulating cardio-bioenergetics. In the well-established cardiac mouse model of Friedreich's Ataxia (MCK-FA), mice experience progressive, reversible hyperacetylation of mitochondrial proteins, implicating a role for acetylation and SIRT3 activity in mediating energy homeostasis in FA hearts. Taken together, we predict that hyperacetylation of cardiac metabolic proteins contributes to the impaired, gradual decline in substrate flexibility and ultimate cardiac failure in FA.

Methods: To determine the role of acetylation in the pathogenesis of FA, we aim to modulate acetylation status and SIRT3 activity in MCK-FA mice using three parallel strategies: (1) dietary supplementation with $NAD+$ precursors to boost sirtuin activity, (2) genetic manipulation of SIRT3 to modulate expression and (3) manipulation of metabolic substrate use to control oxidation and subsequent acetylation. We will monitor changes in cardiac function, substrate usage and bioenergetics to examine a role of SIRT3 and acetylation in regulating energy homeostasis in FA.

Results/Conclusions: Prior studies show that NAD^+ and its precursors can reduce cardiac hyperacetylation in mouse models and protect animals against hypertrophy in a SIRT3-dependent manner. First, using NAD^+ precursor nicotinamide mononucleotide (NMN), we boosted NAD^+ levels and reduced mitochondrial acetylation (-1.43 fold) in the MCK-FA heart. Further functional studies are underway to assess changes in SIRT3 activity with NMN supplementation. Second, we are collaborating with R. Mark Payne, MD to generate SIRT3 knockout or overexpression MCK-FA mice to directly test the function of SIRT3 activity on energy metabolism; data will be presented separately. Third, we used transcriptomic, metabolomic, proteomic and other analyses to explore a role for acetylation in contributing to the compromised substrate flexibility of the MCK-FA heart. Our studies in late stage MCK-FA animals have thus far revealed transcriptional downregulation of pathways involved in catabolism of fatty acids, ketones and amino acids. These transcriptional data are further supported by metabolomic analyses. Furthermore, functional assays reveal significantly reduced oxidation of fatty acids and ketones in the MCK-FA heart (-1.64 and -1.67 fold, respectively). We are currently conducting temporal biochemical utilization studies to further understand and ultimately manipulate the metabolism of substrates that may most contribute to the progressive hyperacetylation. Overall, this work allows us to explore altering protein acetylation as a therapeutic strategy to modulate mitochondrial energy homeostasis in FA hearts.

Viability of frataxin-deficient dorsal root ganglia neurons is recovered by calcium chelators and mitochondrial pore inhibitors

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To understand the cellular consequences of frataxin deficiency we use primary cultures of dorsal root ganglia (DRG) neurons as cell model because this tissue is primarily affected in the disease. Reduction of 80% of frataxin levels in these cells was achieved by transduction with lentivirus containing shRNA silencing sequences. These frataxin-deficient cells show neurite degeneration and apoptotic cell death. Phosphorylated neurofilament NF-200, cleavage of caspase 3 and increased levels of Bax and phosphorylated CREB are, among others, markers observed in these cells. A significant increase of free intracellular Ca^{2+} levels and alteration in Ca^{2+}-mediated signaling pathways was also observed; in this context, the activation of calpain was observed by cleavage of one of its substrates, α-fodrin; such cleavage can be avoided by BAPTA, an intracellular calcium chelator. These results suggest that altered calcium homeostasis can play a pivotal role in neurodegeneration caused by frataxin deficiency. These features can be reversed with the addition of a cell-penetrant TAT peptide coupled to the BH4 anti-apoptotic domain of Bcl-xL protein. Additionally, frataxin depletion caused mitochondrial membrane potential decrease. We have recently observed that after frataxin depletion, a marked increase in cyclophilin D, a protein involved in opening the mitochondrial permeability transition pore, occurs. In an attempt to avoid toxic effects caused by low frataxin levels, we treat the cultures with cyclosporin A, a cyclophilin D inhibitor. Preliminary results indicate that survival is recovered to a significant extent. Other compounds are also currently tested in DRG neuron cultures with the aim of decreasing the deleterious impact of frataxin reduction on cell physiology.

As a conclusion, the use of this cell model provide precise clues to understand the physiological events taking place after frataxin depletion and the rationale for new therapies.

Mitochondrial protein hyperacetylation is associated with early diastolic dysfunction in a model of Friedreich's ataxia hypertrophic cardiomyopathy

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Background: We hypothesized that mitochondrial protein hyperacetylation is associated with diastolic dysfunction in Friedreich's Ataxia (FRDA) cardiomyopathy. We had reported that Frataxin (FXN) loss results in decreased activity of the mitochondrial deacetylase, sirtuin 3 (SIRT3), and cardiac mitochondrial protein hyperacetylation. SIRT3 targets enzymes important to energy homeostasis, suggesting hyperacetylation contributes to metabolic derangement in FRDA.

Methods: A conditional mouse model with ablation of FXN in heart and skeletal muscle (FXN MCK-Cre KO, or "FXN KO") was compared to controls at postnatal days 30, 45 and 65. Heart function was measured using echocardiogram and cardiac catheterization. Heart lysate was probed for lysine acetylation. Myocardial histology was performed using Masson's Trichrome and electron microscopy. Respiration of isolated cardiac mitochondria was measured with a Seahorse analyzer.

Results: FXN KO hearts show age-progressive mitochondrial hyperacetylation associated with a slower rate of oxidative phosphorylation ($p < 0.01$). Electron microscopy demonstrates abnormal mitochondrial morphology as early as day 30 in FXN KO mice with loss of cristae content, and disorganized dysmorphic mitochondria by day 65. Histology demonstrates increased myocardial fibrosis. Diastolic dysfunction is evident by day 45 (n=8), with FXN KO mice having LVH ($p<0.01$), and increased mitral E/A ratio vs controls ($p<0.01$), yet no difference in systolic parameters. At day 65 (n=8), diastolic dysfunction in FXN KO is apparent by increased mitral E/A ($p<0.01$), tissue Doppler E/E' ($p<0.01$), IVRT, and Tau (τ) ($p<0.01$), and decreased -dP/dt ($p<0.001$). Systolic failure is evident in FXN KO at day 65 (n=8) with reduced EF, FS, +dP/dt ($p<0.001$), and ESPVR ($p<0.01$). To explore the role of SIRT3, we generated SIRT3-FXN KO mice, with loss of both SIRT3 and FXN cardiac expression ("double KO"). Double KO mice demonstrate a more severe functional cardiac phenotype compared to FXN KO, primarily in systolic parameters (N=2), with decreased EF, FS and SV ($p<0.05$). Left ventricular mass and wall thickness were no different between groups. Interestingly, the double KOs were more susceptible to stress (surgery and echo). The double KO (N=8) developed obesity with higher average body weight compared to FXN KO (24.9g vs 21.4g, $p<0.01$). Heart weight was unchanged, leading to statistically higher heart:body weight ratio in the FXN KO ($p=0.02$).

Conclusions: Mitochondrial protein hyperacetylation is associated with abnormal mitochondrial function and early diastolic dysfunction in a mouse model of FRDA hypertrophic cardiomyopathy. Loss of expression of both SIRT3 and FXN results in a more severe cardiac phenotype and obesity. This may reflect impairment of the normal post translational regulation of metabolic proteins by SIRT3. We are currently investigating the relationship between SIRT3 activity, expression, and acetylation, and its impact on heart function, which will provide important insight into the pathophysiology of FRDA cardiomyopathy.

Drug discovery & emerging therapeutic strategies

Invited Speaker: Joel Gottesfeld (Scripps Research Institute, USA)

Mechanism of action of 2-aminobenzamide HDAC inhibitors in reversing gene silencing in Friedreich's ataxia

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Loss of the essential mitochondrial protein frataxin in Friedreich's ataxia is due to heterochromatin-mediated silencing of the nuclear *FXN* gene. While the mechanism whereby expanded GAA•TTC triplet repeats in the first intron of the *FXN* gene induce heterochromatin has not been fully established, histone posttranslational modifications near the repeats and at the *FXN* promoter are fully consistent with an epigenetic silencing mechanism. Our laboratory has generated patient induced pluripotent stem cell (iPSC) lines, and we find that iPSC-derived neuronal cells recapitulate heterochromatin signatures and *FXN* gene silencing first identified in patient lymphoid cells and fibroblasts. Previous studies identified a class of small molecule histone deacetylase (HDAC) inhibitors that increase *FXN* mRNA levels and frataxin protein in patient cells, mouse models and in the FRDA neuronal cells. We find that only 2-aminobenzamide HDAC inhibitors that target the class I HDAC enzymes (HDACs 1 – 3) are active in restoring *FXN* gene expression. Structural analogs of the active HDAC inhibitors that selectively target either HDAC1 or HDAC3 do not show similar increases in *FXN* mRNA levels. Chromatin signatures indicate that histone H3 lysine 9 is a key residue for gene silencing through methylation and reactivation through acetylation, mediated by the HDAC inhibitor. One member of our library of 2-aminobenzamide HDAC inhibitors has been investigated in a Phase Ib clinical trial in FRDA patients. Drug treatment lead to increases in *FXN* mRNA and histone acetylation at the *FXN* gene in peripheral blood mononuclear cells in treated patients. As in the neuronal cells, increases in histone H3 lysine 9 acetylation paralleled increases in *FXN* mRNA. Interestingly, the concentration of drug required to induce epigenetic changes in neuronal cells is comparable to the exposure in patients required to observe increases in histone acetylation and gene activation. While the 2-aminobenzamides are promising therapeutics for FRDA, further development of this compound class will be necessary to identify molecules for chronic use. We have explored the mechanism of action of this compound class and our efforts to identify improved molecules for future clinical study will be summarized. Additionally, by interrogating microarray data from neuronal cells treated with inhibitors of different specificity, we identify two genes encoding histone macroH2A (*H2AFY2*) and Polycomb group ring finger 2 (*PCGF2*) that were specifically down-regulated by the inhibitors targeting HDACs1 and 3 versus the more selective inhibitors. Both genes are involved in transcriptional repression and we speculate their involvement in *FXN* gene silencing. Our results shed light on the mechanism whereby HDAC inhibitors increase *FXN* mRNA levels in FRDA neuronal cells.

BB-FA (TAT-MTS(cs)-Frataxin) exhibits promising potential as a protein replacement drug candidate for Friedreich's Ataxia

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Bioblast Pharma is a clinical stage company focus on developing multiple drugs for rare diseases. Bioblast's mitochondrial Protein Replacement Therapy (mPRT) platform is based on a novel fusion protein, comprised of a delivery moiety that contains TAT (as membrane carrier) and mitochondrial-transport-signal (MTS) that enables cleavage and anchorage in the mitochondria, fused to a therapeutic replacement protein. MTS origin is either homologous (native to the protein) or heterologous (taken from another mitochondrial protein). This platform is currently in preclinical development for two diseases: Friedreich's Ataxia and Ornithine Transcarbamylase Deficiency.

In vitro studies conducted in collaboration with Prof. Haya Lorberboum-Galski (the Hebrew University Medical School in Hadassah, Jerusalem) demonstrated that our unique fusion protein – TAT-heterologous-MTS-FXN – had better bacterial expression, higher cells penetration in patient's cell lines, more efficient internalization into the mitochondria and consequently better mitochondrial activity, as compared to TAT-homologous-MTS-FXN. Indeed, the recombinant fusion protein (BB-FA) containing the human mitochondrial citrate synthase MTS (TAT-MTS(cs)-FXN fusion protein) internalized successfully into the mitochondria and demonstrated increased Aconitase activity in several patient's cell lines.

In animal studies, following biweekly administrations of 100 and 400µg BB-FA (TAT-MTS(cs)-FXN) for 21 days, BB-FA fusion protein was shown to internalized into the mitochondria of FA mice model (FVB.B6-Tg(FXN)1Sars Fxntm1Mkn/J, aka "Sarsero" model). The internalized protein was processed in mitochondria of animals from day 4 and significantly increased mitochondrial functionality in the hearts of 21-days treated animals. Additional studies are currently performed in the well-characterized conditional mouse model of complete Frataxin deletion in cardiac and skeletal muscles (Mck-Cre-FxnL3/L– mice, aka "Puccio" model) which demonstrates most features of FA cardiomyopathy.

In conclusion, BB-FA (TAT-MTS(cs)-Frataxin), exhibits promising potential as a protein replacement drug candidate for FA. Our findings offer a new platform for protein replacement in the treatment of various genetic mitochondrial metabolic disorders characterized by deficiency of a functional critical protein, diseases which currently have no cure.

The neuroprotective and neuroregenerative properties of bone marrow stem cell mobilising drugs in Friedreich's ataxia

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Despite the large amount of research into pathogenic mechanisms which operate in Friedreich's ataxia, at the present time, therapies show little ability to protect nerves specifically and no capacity to promote neuroregeneration. A large body of experimental evidence, including our own, has indicated that bone marrow-derived stem and progenitor cell populations show therapeutic promise. They represent a cell therapy that is likely to have a real impact in neurological diseases and they act via multiple mechanisms which are particularly apposite to a disease such as Friedreich's ataxia.

We have performed a series of experiments showing that the bone marrow stem cell mobilising drugs (granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF)), display strong neuroprotective properties and activate cell survival pathways in mature neurons and in cells derived from patients with Friedreich's ataxia. Furthermore, the administration of both G-CSF and SCF in the YG8R mouse model of Friedreich's ataxia leads to improvement in the neurological phenotype associated with the disease. Mice treated over a six month period show significant improvements in motor phenotype using an accelerating rotarod, grip strength, string test and open field, when compared to untreated controls. In addition, using bone marrow transplantation in the YG8R mouse model, to establish bone marrow chimeric mice that stably express both enhance green fluroescent protein (EGFP) and 'normal' copies of the frataxin gene, we also show that GCSF/SCF treatment stimulates bone marrow-derived cells carrying the 'normal' frataxin gene to enter the peripheral circulation and subsequently migrate throughout the central nervous system (including the dorsal root ganglia, cerebellum and spinal cord) where they co-express either neuronal or glial cell markers, providing a link to functional motor recovery.

In summary, our studies have provided novel and fundamental insights into the ways in which nerve cells in Friedreich's ataxia can be protected or replaced and their survival prolonged with the administration of stem cell mobilising drugs. We have also shown that the induced migration of bone marrow-derived cells into the CNS could indeed serve as a tool to aid the delivery 'healthy donor' cells and/or frataxin genes to sites of CNS injury. We therefore propose that administration of stem cell mobilising drugs may have the potential to be developed into a simple, non-invasive and effective neuroprotective and regenerative therapy in patients with Friedreich's ataxia.

Stabilization of FXN mRNA Using Oligonucleotides for the Treatment of Friedreich's ataxia

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Friedreich's ataxia (FRDA) is a recessively inherited neuromuscular disorder that arises due to cellular depletion of frataxin (FXN) protein and resulting defects in mitochondrial functions. The protein coding sequence of FXN is normal in the majority of FRDA patients, suggesting that upregulation of endogenous FXN expression could be an effective therapy. The most common molecular cause of this disease is the expansion of GAA/TTC triplet repeats in the first intron of FXN gene. Repeat expansion beyond a certain threshold causes defects which reduce FXN mRNA and protein levels. DNA-DNA and DNA-RNA interactions formed in the long triplet repeat stretches, defects and alterations in splicing patterns and the formation of a heterochromatin-like structure are among the potential causes of repeat-induced FXN silencing. We developed a novel oligotherapeutics-based strategy to upregulate genes by targeting mRNA end regions. This strategy likely involves acting at the post-transcriptional level and is therefore independent of GAA-repeat induced mechanistic changes and defects. We applied this strategy to FXN mRNA and observed significant upregulation in both FRDA cells in vitro and in a FRDA mouse model. This oligonucleotide-based therapeutic approach represents a novel strategy for the treatment of FRDA and other human diseases.

Therapeutic strategies to prevent the ubiquitin/proteasome-dependent degradation of frataxin

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Frataxin levels critically affect onset and progression of Friedreich ataxia. Our therapeutic approaches are therefore aimed at increasing frataxin levels. This can be in principle achieved by increasing the transcription rate or by interfering with its degradation. We previously discovered that a significant amount of frataxin is degraded by the ubiquitin/proteasome system before it reaches mitochondria and we identified the critical ubiquitination site on frataxin. We then described the therapeutic potential of small molecules that increase frataxin levels by docking on the frataxin ubiquitination site, thus preventing frataxin ubiquitination and degradation. We called these compounds ubiquitin competing molecules (UCM). Through an iterative process of computational docking, chemical synthesis and cell-based functional assays, we identified a set of compounds that efficiently promote frataxin accumulation. These compounds directly interact with frataxin and prevent its ubiquitination. Most importantly, these compounds are able to promote frataxin accumulation and aconitase rescue in patients-derived cells, strongly suggesting their therapeutic potential.

In light of these results, another attractive therapeutic strategy to increase frataxin levels would be the inhibition of the enzyme responsible for its ubiquitination. To identify the frataxin-specific E3 ligase we performed a siRNA-based functional screening of an E3 ligase-restricted siRNA library, targeting more than 600 different genes. Knock-down of the frataxin-specific E3 ligase is expected to result in the accumulation of frataxin protein. Through this screening procedure we isolated one gene that consistently promotes frataxin accumulation when its expression is silenced in cells. Importantly silencing of this gene induces frataxin accumulation also in fibroblasts derived from patients. Moreover, the overexpression of the corresponding cDNA, but not its catalytic inactive mutant, promotes frataxin ubiquitination. This gene may actually code for the enzyme responsible for frataxin ubiquitination and may represent a novel therapeutic target for Friedreich ataxia.

Together our data indicate that the strategy aimed at preventing the ubiquitin/proteasome-dependent degradation of frataxin has therapeutic potential for FRDA.

An AAV9 coding for frataxin clearly improved the symptoms and prolonged the life of Friedreich ataxia mouse models

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Friedreich ataxia (FRDA) is a genetic disease due to increased repeats of the GAA trinucleotide in intron 1 of the frataxin gene. This mutation leads to a reduced expression of frataxin. We have produced an AAV9 coding for human frataxin (AAV9-hFXN). This AAV was delivered by intra-peritoneal injection to young conditionally knockout mice in which the frataxin gene had been knocked-out in some tissues during embryogenesis by breeding them with mice expressing the Cre recombinase gene under the MCK or the NSE promoter. In the first part of the study different doses of virus (i.e., 6x1011 v.p. to 6x109 v.p.) were tested from in NSE-cre mice. All doses led to an increase in life spentime of the mice. The higher and the lower dose were also tested in MCK-cre mice. A single administration of the AAV9-hFXN at 6x1011 v.p. more than doubled the life of these MCK-cre mice. In fact the MCK-cre mice treated with the AAV9-hFXN were sacrificed for further molecular investigations at the age of 29 weeks without apparent symptoms. Echography analysis of the heart function clearly indicated that the cardiac systolic function was better preserved in the mice that received 6x1011 v.p. of AAV9-hFXN. The human frataxin protein was detected by ELISA in the heart, brain, muscles, kidney and liver with the higher dose of virus in both mouseice models. Thus gene therapy with an AAV9-hFXN is a potential treatment of FRDA.

This work was supported by grants from Ataxie Canada and from Association Française de l'Ataxie de Friedreich.

Src inhibitors modulate frataxin protein levels

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Defective expression of frataxin is responsible for the inherited, progressive degenerative disease Friedreich's Ataxia (FRDA). There is currently no effective approved treatment for FRDA and patients die prematurely. Defective frataxin expression causes critical metabolic changes, including redox imbalance and ATP deficiency. Since these alterations are known to activate the tyrosine kinase Src, we investigated whether Src might in turn affect frataxin expression. We found that frataxin can be phosphorylated by Src. Phosphorylation occurs primarily on Y118 and promotes frataxin ubiquitination, a signal for degradation. Accordingly, Src inhibitors induce accumulation of frataxin but are ineffective on a non-phosphorylatable frataxin-Y118F mutant. Importantly, all the Src inhibitors tested, some of them already in the clinic, increase frataxin expression in frataxin-deficient cells derived from FRDA patients. Thus, Src inhibitors emerge as a new class of drugs able to promote frataxin accumulation, suggesting their possible use as therapeutics in FRDA.

Biomarkers and functional measures

Invited Speaker: David Lynch (University of Pennsylvania, USA)

Biomarkers in Friedreich ataxia

Friedreich ataxia (FRDA) is an autosomal recessive ataxia with early onset reflecting the deficiency of functional frataxin in cells. As much is understood about the mechanisms of disease in FRDA, many agents are in therapeutic development. This process would be aided by development of biomarkers of disease progression. This presentation will review a series of anatomical and functional biomarkers of frataxin deficiency.

Physiological and anatomical biomarkers are crucial for assessment in FRDA, in particular defining the state of neurological abilities and the structural components of cell loss. Imaging studies have previously used MRI of brain and spinal cord to identify cell loss and other anatomic changes. Modalities like optical coherence tomography can be used to investigate the retina selectively and detailed physiology (SSEP, BAER, LSN-S etc.) maybe useful for identifying abnormal neural properties earlier than clinical evaluation. However these markers do not reveal changes in the pathophysiological process at their earliest point. Identifying biomarkers of pathophysiology is necessary for assessing the effect of molecular therapy.

At the most basic pathophysiological level, cellular frataxin levels provide a unifying biomarker in FRDA. Frataxin deficiency can be measured in a variety of peripheral tissues, and levels correlate with the GAA repeat length on the shorter allele. Most point mutations in FRDA also give rise to lower levels of frataxin protein. Frataxin levels appear to be constant over the course of the disease. However, the degree to which levels in unaffected tissue reflect levels in affected tissues is unclear. In addition, assessment of frataxin is useful mainly for therapies designed to raise frataxin levels, and is without benefit in assessment of downstream pathways.

A second approach is to examine metabolic dysfunction created by frataxin deficiency. Such approaches could concentrate on levels of Krebs cycle intermediates, or other key metabolic species. Using mass spectrometry based approaches; these measurements have the potential to be extraordinarily sensitive. They would be even more useful if they could be combined with anatomical resolution to create novel imaging modalities. Collectively, these finding illustrate the need for a diverse effort in biomarker development in FRDA while being sensitive to the need for efficient use of resources to identify the most useful ones.

Platelet biomarkers of metabolic disturbances in Friedreich's ataxia

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Friedreich's ataxia (FRDA) is a heritable disease characterized by spinocerebellar degeneration and cardiomyopathy, with metabolic abnormalities that are suspected to have a role in disease pathogenesis. Despite this knowledge, the inability to access the highly affected neuronal and cardiac tissues has hampered metabolic evaluation and biomarker development. In this study, we used platelets from patients with FRDA coupled with liquid-chromatography-mass spectrometry methodology to assess their ability to metabolize stable isotope-labeled glucose and palmitate to acyl-coenzyme A (CoA) isotopologues associated with mitochondrial metabolism. Our findings revealed that platelets from FRDA patients (n=10) had diminished relative incorporation of [$^{13}C_6$]-glucose into the Krebs cycle through acetyl-CoA when compared with control subjects (n=10). In addition, the decrease of labeling into acetyl-CoA showed a negative correlation with GAA repeat length (r^2 = 0.39), a known marker of disease severity. This is consistent with studies that have shown diminished pyruvate oxidation in FRDA. In addition to decreased glycolysis, we observed a concomitant increase in the β-oxidation of fatty acids. This was revealed by a shift in metabolism by FRDA platelets toward formation of β-hydroxybutyryl (βHB)-CoA and 3-hydroxy-3-methyl-glutaryl (HMG)-CoA from [$^{13}C_{16}$]-palmitate in FRDA platelets when compared with controls. In contrast to the [$^{13}C_6$]-glucose-derived acetyl-CoA, there was a positive correlation of [$^{13}C_{16}$]-palmitate labeling into βHB-CoA with GAA repeat length (r^2 = 0.51). The β-oxidation of fatty acids results in the removal of contiguous two carbon units from the fatty acyl substrate to generate acetyl-CoA, which can then enter into the Krebs cycle. Consequently, alterations to lipid metabolism might play an important role in cellular homeostasis in times of mitochondrial dysfunction. Taken together these results suggest FRDA platelets exhibit a diminished capacity for oxidative phosphorylation, as decreased [$^{13}C_6$]-glucose labeling into acetyl-CoA has been shown to occur in response to pharmacologic inhibition of mitochondrial complex I. Furthermore, our previous cell culture studies have shown increased β-oxidation of lipids in response to diminished complex I activity, supporting the notion that lipid breakdown plays an important compensatory role in times of mitochondrial dysfunction. Finally, generation of a receiver operator characteristic (ROC) curve combining decreased labeling into acetyl-CoA from [$^{13}C_6$]-glucose together with increased labeling into βHB-CoA from [$^{13}C_{16}$]-palmitate revealed an area under the curve of 0.90. Therefore, our findings demonstrate that platelets can be used as a surrogate tissue for in vivo metabolic studies and lend insight into metabolic defects in heritable mitochondrial and metabolic diseases such as FRDA.

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A longitudinal study of the Friedreich ataxia impact scale

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Background: Quality of life in Friedreich ataxia (FRDA) has been explored using various generic health status measurement tools, most commonly the Short Form Health Survey Version 2 (SF36v2). The tool did not address many specific issues related to disease impact in people with FRDA. The Friedreich Ataxia Impact Scale (FAIS) was developed to examine clinically relevant areas in FRDA. The aims of the current study were to assess the relationship between the FAIS and clinical characteristics of FRDA, as well as to determine the responsiveness of the FAIS to change over one and two years.

Methods: One hundred and four individuals with FRDA aged at least 18 years and homozygous for the GAA expansion in intron 1 of *FXN*, completed the FAIS at baseline. Seventy individuals completed the FAIS again 12 months later and 49 completed the FAIS at 24 months. Clinical parameters and neurologic scales (Friedreich Ataxia Rating Scale (FARS)) were also recorded.

The FAIS comprises 126 items grouped into eight independent subscales, measuring three areas identified as being clinically important to individuals with FRDA: 1) symptoms, 2) physical functioning, and 3) psychological and social impact. Symptoms encompass speech and body movement. The FAIS was designed to be used together with current clinician-administered rating scales to capture the true health impact of FRDA.

Spearman's rank correlation coefficients were utilised to correlate the FAIS subscales with disease parameters; these included age at disease onset, disease duration, GAA1 and GAA2 repeat sizes Friedreich Ataxia Rating Scale (FARS) score. The FAIS subscales were also correlated with the Physical Component Summary (PCS) and the Mental Component Summary (MCS) of Version 2 of the SF-36. Responsiveness was examined by measuring the change in median subscale scores using Wilcoxon signed-rank test between baseline and 12 months, and between baseline and 24 months.

Results: The total FARS score, onset age and disease duration correlated significantly with FAIS subscales measuring symptoms and physical functioning. There were no significant correlations between GAA1 or GAA2 repeat sizes and any of the FAIS subscales. Both summary measures of the SF-36V2 also correlated well with the FAIS subscales. Speech was the only subscale that demonstrated significant change over one and two years.

Conclusions: The FAIS provides valuable insight into the perspective of individuals with FRDA on their health status, and is an important measure of morbidity. It has, however, limited responsiveness to change and its use in intervention studies is questionable.

Abnormal brain function and connectivity in cerebello-cerebral circuits underlying cognitive function in Friedreich ataxia: The IMAGE-FRDA study

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Introduction: Within the brain, the principal consequence of Friedreich ataxia (FRDA) involves the progressive degeneration of the dentate nucleus of the cerebellum. Beyond classically reported motor and sensory symptoms resulting from dentate atrophy, there is increasing acknowledgement that some degree of cognitive impairment also defines the gross phenomenology of the condition. In particular, FRDA has been associated with deficits in working memory, attention, and cognitive control, processes that rely on intact interactions between the cerebellum and prefrontal cerebral cortices. Disruption to cerebello-thalamo-cerebral connectivity may therefore underlie changes to cognitive functioning in FRDA. This study examined the integrity of brain activity and connectivity within cerebello-thalamo-cerebral systems in individuals with FRDA while undergoing a working memory task.

Methods: Twenty-nine individuals homozygous for a GAA expansion in intron 1 of FXN, and 34 matched control participants undertook a functional magnetic resonance imaging (fMRI) protocol. During scanning, participants performed an N-Back working memory task with two levels of cognitive load. Task stimuli consisted of a sequential string of letters, each presented visually for 500ms and separated by 1500ms. The low-load ("0-Back") condition required participants to indicate, via button press, when a pre-instructed letter appeared on screen. The high-load ("2-Back") condition necessitated a button press when the current letter was the same as that presented two letters previously. To isolate brain activations related to higher-order cognitive processing, group differences in the magnitude of the fMRI signal during 2-Back performance was contrasted with the 0-Back condition using gold-standard statistical parametric mapping (SPM) approaches. Cerebello-cerebral functional interactions were inferred based on the covariation of task-related fMRI signals in the cerebellum and the cerebrum.

Results: Behaviourally, there were no significant group differences in reaction time or error rates when 2-Back was contrasted with 0-Back. The imaging data also showed qualitatively similar areas of functional activation across both groups; however, individuals with FRDA showed significantly reduced brain activations in cognitive regions of the cerebellar cortex (i.e., Lobule VI) and associated cerebral cortices, including the anterior insula and lateral prefrontal cortex. Moreover, in individuals with FRDA, the functional connectivity between these regions was significantly reduced, and normal patterns of task-related connectivity dynamics were diminished, as compared to controls. All results are statistically significant at family-wise error corrected $p < 0.05$.

Conclusions: These results provide evidence that cerebellar pathology in FRDA directly links with changes in cerebral activation and connectivity during working memory performance. Taken together, this study supports the conceptualization of FRDA as a disorder of large-scale, spatially distributed cerebral and cerebellar circuitry, providing further explanation for the non-motor symptoms associated with this disease.

MRS and diffusion MRI of the spinal cord in Friedreich's ataxia

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Purpose: Although spinal cord atrophy is a hallmark of Friedreich's ataxia (FRDA), there have been very few MR studies of the spinal cord in patients with FRDA[1] and, to our knowledge, none using ^{1}H MRS or DTI, due in part to technical challenges (B_0 shim, motion artifacts). Here, our objective was to characterize neurodegeneration in early stage patients with FRDA using ^{1}H MRS and DTI of the spinal cord.

Methods: We studied 15 patients and 15 age-matched controls. All measurements were performed on a Siemens Trio 3T scanner (Siemens, Erlangen, Germany). ^{1}H MR spectra (TE = 28 ms, TR = 5 s, 256 averages) were acquired using a modified semi-LASER sequence[2] in an 8 × 8 × 30 mm^3 voxel positioned along C4-C5 vertebrae. Spectra were quantified with LCModel using water as an internal reference. Diffusion MRI at the C2-C3 level was acquired using a readout-segmented echo-planar sequence[3] with 1.1x1.1x3.3mm^3 resolution and with correction of geometric and eddy current distortions[4]. All subjects were also assessed by the Friedreich's Ataxia Rating Scale (FARS).

Results: Patients had FARS scores averaging 45±17 (mean ± SD, range 10-81) and age 20±7 years (range 11-32). We observed 33% lower NAA ($p<1e-5$) and 32% higher myo-inositol ($p<0.005$) levels in spinal cord of patients vs controls, reflecting neuronal damage and gliosis. Similarly, fractional anisotropy was lower in the cervical spinal cord of patients (FA = 0.46±0.04 in patients vs. 0.59±0.06 in controls, $p = 0.001$), reflecting alteration of axonal integrity. In spite of the large differences observed between controls and patients, there was no correlation between these parameters and FARS scores on this small group of patients, suggesting that these changes occur very early in the disease process, possibly even before the apparition of clinical symptoms. The number of fibers, however, correlated negatively with FARS, likely reflecting spinal atrophy.

New results to be presented at the ARC 2015 meeting: The second year of this MR study is focused on a) 12-month follow-up of patients scanned in 2013-2014; b) recruitment of additional early stage patients; and 3) assessment of the precision of MR parameters (test-retest). These data are currently being acquired (as of Nov 2014). Therefore the results are not available for this abstract but will be available for presentation at the meeting.

Conclusion: This is, to our knowledge, the first report using 1H MRS or DTI to study spinal cord in patients with FRDA. Such multi-modal MRI/S measurements in the spinal cord may yield further insight into disease mechanisms and provide markers of neurodegeneration in patients at an early stage to assess therapeutic efficacy in clinical trials.

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Potential neuroimaging biomarkers validated in Friedreich's ataxia: DTI and functional magnetic resonance findings

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Background: Friedreich's ataxia (FRDA) is a progressive hereditary neurodegenerative condition caused by an autosomal recessively inherited GAA repeat in the FXN gene. In this study we used clinical measures and advanced tractography combined to functional MRI (fMRI) to explore white matter (WM) connectivity and motor dysfunction in a cohort of FRDA patients.

Methods: Molecularly defined FRDA patients (n=17) were clinically assessed with the specific ataxia scales. Patients and age matched healthy controls underwent a neuroimaging study protocol on a 3T MRI scanner that included advanced neuroimaging DTI and fMRI. After the pre-processing, a nonlinear monoexponential model was used to calculate fractional anisotropy (FA), mean, radial and axial diffusivity (MD, RD, AD) maps. Non-parametric voxel-based permutations were performed on the WM maps regions of interest (ROI), considering age and sex via a general linear model (GLM) with critical threshold 0.05 while correcting for multiple tests. An fMRI sequence was acquired during a simple block design finger-tapping task. After a standard pipeline pre-process, intra- and intergroup GLM analysis were conducted, considering age and sex variables and also $p < 0.001$ threshold.

Results: Our cohort included early onset FRDA patients, mean age at onset 10.65 ± 5.08 (range 4-20 years); F/M: 13/4; mean GAA expansion in the smaller repeat was 651,07 ± 234.39 (n=16) and one patients with a single base pair deletion and 170 GAA repeat. Mean age at assessment was 27.82 ± 10.51years (12-51), mean disease duration was 17.17 ± 8.43 (4-33). The mean age of the control group was 23 ± 4.83 years; F/M= 5/8. From both the voxel-based and ROI-based analysis altered FA and MD parameters were consistently found in the following four Central Nervous System areas: cerebellar WM (superior, median and inferior peduncles), long sensory-motor pathways (corticospinal and lemnisceal systems, cerebral peduncles), major commissural fibres (splenium and tapetum of the corpus callosum), the thalamic and the optic radiations. The fMRI data were analyzed from 13 patients (mean age 30.05 ± 11.76 years) and 8 controls (mean age 24.5 ± 3.85 years). The finger-tapping task demonstrated intragroup activation of the controlateral motor cortex and the ipsilateral cerebellar cortex both in patients and healthy controls. Intergroup analysis demonstrated a consistent and significantly higher cerebellar cortex activation, in controls compared to the FRDA patients, in particular in the lobules V and VI.

Discussion: We show that a comprehensive MRI protocol consistently discriminates FRDA patients from controls. DTI changes in selected areas and BOLD signal in the cerebellar ipsilateral cerebellar cortex in response to a simple motor task show strong intergroup discriminating power and may prove to be useful paraclinical disease markers. A longitudinal study is undergoing to explore the sensitivity of these indicators to disease progression.

Sensitivity of spatiotemporal gait parameters in Friedreich ataxia

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Friedreich ataxia (FRDA) is an autosomal recessive disease with average symptom onset between 10-15 years of age. Initial symptoms are 'clumsiness' and gait ataxia, however mobility progressively declines and people with FRDA typically become non-ambulant 10 to 15 years after disease onset. Loss of ambulation has a significant impact on quality of life in people with FRDA. Thus a more comprehensive understanding of gait dysfunction will provide a better basis for targeting specific therapeutic interventions. The primary aim of this study was to examine the interrelationships between spatiotemporal gait characteristics at different walking speeds and a range of clinical and disease characteristics in individuals with FRDA. Thirteen people with FRDA walked along an 8.3 meter GAITRite® mat six times each at their preferred, fast and slow speeds. Relationships between spatiotemporal gait parameters, variability of spatiotemporal parameters and a range of clinical and disease characteristics were also examined. Significant correlations were found between spatiotemporal gait characteristics at each of the walking speeds and Friedreich Ataxia Rating Scale (FARS) score and disease duration. GAA1 repeat expansion positively correlated with double support percentage of the gait cycle in all speed conditions demonstrating a relationship between the genetic mutation and compensatory strategies for impaired dynamic balance. Age of onset negatively correlated with speed and cadence in the preferred and fast speeds, suggesting that earlier onset of FRDA has an effect on gait maturation. Heel-to-heel base of support positively correlated with the FARS lower limb coordination subscale in the preferred (r=0.602), fast (r=0.589) and slow (r=0.644) speed conditions, whilst the FARS upright stability subscale positively correlated with intra-individual variability of stride length in the preferred (r=0.579) and fast (r=0.660) speed conditions. There were no significant correlations between the FARS peripheral nervous system subscale and any spatiotemporal gait parameter measured. In all speed conditions, including at slow speed, there were correlations between a range of spatiotemporal gait characteristics and the timed 25 foot walk test, a well-established measure of gait mobility. This study reveals several interrelationships between spatiotemporal gait characteristics and a range of genetic and clinical markers of FRDA, suggesting earlier disease onset impairs the ability to compensate for stability challenges in gait. Moreover, these findings indicate that spatiotemporal gait parameters are a sensitive measure of gait decline in individuals with FRDA, and should be considered for inclusion in intervention studies whilst participants are still ambulant.

Fatty acid oxidation is disrupted in the FRDA heart

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Background: Fatty Acid Oxidation (FAO) supplies ~70% of ATP demands in normal hearts, making β-oxidation of fatty acids a key metabolic activity to interrogate in the cardiomyopathy and heart failure of Friedreich's Ataxia (FRDA). We recently reported the novel finding that mitochondrial proteins in the heart from the FRDA animal model become heavily acetylated concurrent with cardiac hypertrophy and heart failure, and this is partially caused by inhibition of the NAD+-dependent SIRT3 deacetylase. We tested the hypothesis that mitochondrial dysfunction in FRDA leads to altered patterns of myocardial metabolic substrate utilization, and that mitochondrial protein expression can serve as a biomarker of disease severity. We predicted that the FRDA heart preferentially utilizes glucose due to mitochondrial dysfunction, thus placing FRDA patients at risk of death or morbidity with stressful events. We used cardiac Positron Emission Tomography (PET) to quantify glucose and fatty acid uptake in FRDA patient and control hearts, and in FRDA KO and control mouse hearts.

Methods and Results: Mice at 65 days of age underwent PET scan using 11C-Palmitate and 18FDG as tracers for FAO or glucose utilization respectively. FRDA KO mice (loss of FRDA gene in sarcomeric tissues driven by MCK-Cre transgene) were significantly lower ($p = 0.012$) in palmitate utilization rate (12.2/min, ±0.17, n=3) vs controls (13.7/min, ±0.20, n=2). In contrast, FRDA KO mice were significantly higher ($p = 0.047$) in glycolytic rate (34.7 ml/g·min, ±2.34, n=2) than controls (22.29 ml/g·min, ±1.55, n=2). Expression of mitochondrial proteins involved in FAO (PPARa, LCAD, MCAD) was not significantly different between groups, but hexokinase (glucose metabolism) protein expression was significantly higher in FRDA KO mouse heart ($p < 0.05$). In parallel with these studies, adult patients with FRDA (n=10), or adult controls (n=5), underwent echo, serum biomarker analysis, and PET scan with 11C-Palmitate and 18FDG. Partial multivariate analysis indicated that the FRDA patients had impaired ability to metabolize fatty acids and greater glucose utilization than controls. Interestingly, serum fatty acid binding protein 3 (cardiac FABP3) was significantly elevated in the FRDA patients ($p=0.001$), as were inflammatory markers IL-6 ($p=0.006$), ICAM-1 ($p=0.001$), and MIP-1a (0.013). Pearson's correlation coefficient between cTnI and FABP3 was 0.632. There was no significant difference in systolic function by echo, nor did GAA repeat correlate significantly vs controls for markers or PET scan.

Conclusions: FRDA patients appear to have impaired ability to utilize fatty acids for energy in heart, and this is confirmed using the FRDA KO mouse. Early biomarker analysis suggests that inflammatory and fatty acid biomarkers have significant associations, and may be informative in longitudinal FRDA population studies. The clinical implications of these findings are that FRDA patients may respond poorly to physiologic stress, and therefore require additional metabolic support.

Biological and clinical characteristics of the European Friedreich Ataxia Consortium for Translational Studies (EFACTS): cross-sectional analysis of baseline data

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Background Friedreich ataxia (FRDA) is a rare autosomal recessive neurodegenerative disorder. We report cross-sectional baseline data to establish the biological and clinical characteristics for the first prospective FRDA pan-European database registry.
Methods within the European Friedreich Ataxia Consortium for Translational Studies (EFACTS), we assessed a large cohort of genetically confirmed FRDA patients. The primary outcome measure was the Scale for the Assessment and Rating of Ataxia (SARA), secondary outcome parameters were the Inventory of Non-Ataxia Signs (INAS), the performance-based coordination test Spinocerebellar Ataxia Functional Index (SCAFI), the neurocognitive verbal fluency and quality of life measures such as activities of daily living (ADL) and EQ-5D. The FRDA cohort was subdivided into three age-of-onset groups: early-onset (≤ 14 years), intermediate-onset (15 to 24 years), and late-onset (≥ 25 years), which were compared with respect to clinical characteristics and outcome measures. Linear regression analysis was used to estimate an annual rate of decline of clinical outcome measures based on disease duration. Findings We enrolled 592 genetically confirmed FRDA patients between 15-Sep-2010 and 30-Apr-2013 at eleven study sites. Age of onset was inversely correlated with the number of GAA repeats predicting a 2.3-year-earlier onset rate with every 100 GAA repeats added on the smaller repeat allele. The commonest reported symptom at disease onset was postural instability (78%), followed by scoliosis (25%) and falls (20%). Subgroup analysis showed differences in almost all measures such as SARA, SCAFI, INAS as well as ADL, with more severe impairments in early-onset patients compared to intermediate or late onset. SARA, SCAFI and INAS strongly correlated with clinical and functional measures, while verbal fluency performance showed small to medium correlations. Regression analyses revealed an estimated annual rate of worsening in SARA in early-onset (1.04±0.13 points) and intermediate-onset patients (1.17±0.22 points), which was almost twice as high as in the late-onset group (0.56±0.10 points). This pattern was also observed for ADL. The most frequently reported non-neurological symptoms were cardiac and endocrinological impairment. Interpretation The cross-sectional EFACTS baseline analysis demonstrates that earlier disease onset in FRDA patients is associated with higher GAA repeat lengths and a more rapid disease progression. The differential estimated progression rates of ataxia symptoms related to age of onset and phenotype findings have implications for clinical trial designs, for which SARA and ADL might be in particular suitable to monitor disease progression in FRDA. Outlook The 12-month longitudinal data are currently analysed and will also be presented.
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The views of individuals with, and parents of individuals with Friedreich ataxia regarding pre-symptomatic testing of minors

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Friedreich ataxia (FRDA) is an autosomal recessive neurodegenerative disorder characterised by variable age of onset, with no treatment proven to alter its natural history. Siblings of individuals with FRDA have a 1 in 4 risk of developing the condition, raising issues around genetic testing of asymptomatic minors. Currently, there is a lack of professional consensus and limited empirical evidence to support provision or refusal of pre-symptomatic testing for FRDA. This exploratory study aimed to ascertain the opinions of individuals with FRDA and parents of individuals with FRDA regarding pre-symptomatic testing of minors for the condition. A qualitative research approach using semi-structured interviews and thematic analysis was employed. Interviews with ten individuals with FRDA, and ten parents of individuals with FRDA were conducted, recorded, transcribed and analysed. Four key findings emerged. First, a number of arguments for and against testing minors were identified. Second, strong support existed from parents about the parental right to test their at-risk immature children, however individuals with FRDA were of mixed opinions. Third, most participants feel it is not the clinician's role to make a final decision about whether testing occurs. Finally, a specific issue of concern regarding testing was what and when to tell at-risk children about the test result. The findings from this study highlight the dilemma of how to manage the desires of some individuals and families affected by FRDA to access testing, when there is a lack of professional consensus due to differing opinions regarding autonomy, confidentiality and risk of harm. Further empirical research regarding the impact of such testing and the views of at-risk individuals and clinicians is required so an appropriate framework for dealing with this contentious issue is developed.

Clinical trials and trial design

Reversing *FXN* gene silencing *in vivo* in humans -towards a disease-modifying therapy?

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Background: Friedreich's ataxia is a progressive degenerative disorder caused by deficiency of the frataxin protein. Expanded GAA repeats within intron 1 of the frataxin (FXN) gene lead to its heterochromatinisation and transcriptional silencing (Saveliev et al, Nature 2003). Preclinical studies have shown that the histone deacetylase inhibitor nicotinamide (vitamin B3) can remodel the pathological heterochromatin and upregulate expression of FXN (Chan et al, HMG, 2013). We aimed to assess the epigenetic and neurological effects and safety of high-dose nicotinamide in patients with Friedreich's ataxia. In this exploratory, open-label, dose-escalation study in the UK, male and female patients (aged 18 years or older) with Friedreich's ataxia were given single doses (phase 1) and repeated daily doses of 2—8 g oral nicotinamide for 5 days (phase 2) and 8 weeks (phase 3). Doses were gradually escalated during phases 1 and 2, with individual maximum tolerated doses used in phase 3. The primary outcome was the upregulation of frataxin expression. We also assessed the safety and tolerability of nicotinamide, used chromatin immunoprecipitation to investigate changes in chromatin structure at the FXN gene locus, and assessed the effect of nicotinamide treatment on clinical scales for ataxia (Libri et al Lancet 2014). In addition we have: 1) performed novel behaviourmetric analysis in order to develop non-invasive objective measures of both the existing clinical scales and activities of daily living; 2) developed a novel closed-loop coordination paradigm for fMRI analysis and 3) developed novel image techniques for assessing the dynamics of FXN expression.

Results: Nicotinamide was generally well tolerated; the main adverse event was nausea, which in most cases was mild, dose-related, and resolved spontaneously or after dose reduction, use of antinausea drugs, or both. Phase 1 showed a dose-response relation for proportional change in frataxin protein concentration from baseline to 8 h post-dose, which increased with increasing dose (p=0·0004). Bayesian analysis predicted that 3·8 g would result in a 1·5-times increase and 7·5 g in a doubling of frataxin protein concentration. Phases 2 and 3 showed that daily dosing at 3·5—6 g resulted in a sustained and significant (p=0·0001) upregulation of frataxin expression, which was accompanied by a reduction in heterochromatin modifications at the FXN locus. Clinical measures showed no significant changes. Nicotinamide was associated with a sustained improvement in frataxin concentrations towards those seen in asymptomatic carriers during 8 weeks of daily dosing. Further investigation of the long-term clinical benefits of nicotinamide and its ability to ameliorate frataxin deficiency in Friedreich's ataxia is warranted. To this end the novel behaviourmetric, fMRI and single cell analysis of FXN expression methodologies are being developed to accurately measure progression in this disease and response to therapy (MS in prep).

Invited Speaker: Massimo Pandolfo (University Libre de Bruxelles, Belgium)

Clinical trials in Friedreich's ataxia.

Advances in understanding the pathogenesis of Friedreich's ataxia (FRDA) have led to insights that allowed the development of new therapeutic strategies for treating the disease. Some of these strategies aim to increase frataxin levels at least to those of asymptomatic heterozygous carriers. These include approaches to alleviate the epigenetic transcriptional silencing of the frataxin (FXN) gene by the expanded GAA repeats as well as approaches to induce frataxin expression regardless of the presence of the expansion mutation. Epigenetic silencing can be overcome by using histone deacetylase inhibitors (HDACi) acting on class I (benzamides) or class III HDACs (nicotinamide). A number of compounds upregulate frataxin expression in model systems, regardless of the presence of a GAA repeat. These include molecules as disparate as GLP-1 agonists, gamma interferon, dicionine, hEPO, and others, which very likely act by different mechanisms. Specific ubiquitin-competing molecules can prevent frataxin degradation in model systems. Frataxin protein replacement by using tat-frataxin and other modified frataxins is another currently investigated approach. Gene therapy with AAV vectors expressing frataxin has been shown to be effective in a mouse model of FRDA cardiomyopathy and is being intensively investigated for the neurological component of the disease.

Other potential therapies target pathogenic processes triggered by frataxin deficiency, as mitochondrial dysfunction, altered iron metabolism, and oxidative damage.

Some of the proposed therapeutics are new molecules, not previously used in humans, others are currently approved medications for other diseases that may have FRDA as a new indication. New drugs must go through a complete pre-clinical and clinical development.

Early efficacy studies must then be performed, following the golden standard of the randomized controlled trial (RCT). Unfortunately, so far there is no positive RCT in FRDA. While lack of efficacy of the tested therapeutics is a likely reason for missed efficacy endpoints, trial design is also critical. Efficacy endpoints may consist first in appropriately validated biomarkers, then in clinical parameters shown to be sensitive to disease progression. Choice of endpoints and trial design should aim to maximize efficiency, in order to identify those therapeutics that deserve further study in a reasonable time frame and without involving too many subjects. FRDA is a rare disease and it is relentlessly, but slowly progressive, so sample size and trial duration are critical. Ongoing large natural history studies in America, Australia and Europe (CCRN, EFACTS) are providing essential guidance for trial design. Harmonization of data collection is also very important to allow comparisons and meta-analyses. For this purpose, FRDA has been included in the NIH Common Data Element (CDE) project, leading to the identification and publication of sets of parameters to be collected in future clinical trials.

An upcoming clinical trial testing the safety and efficacy of a stabilized polyunsaturated fatty acid in Friedreich's ataxia

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Polyunsaturated fatty acids (PUFAs) are susceptible to an accelerating damage cascade from an autocatalytic, free radical chain reaction. Damaged lipid end products (e.g. 4-hydroxy-nonenal and others) from this process have been associated with mitochondrial dysfunction and a host of age-related degenerative diseases, including Friedreich Ataxia (FRDA). Cells in multiple models of FRDA, when treated with a stabilized lipid mimetic of the normal dietary PUFAs in mitochondrial membranes, show stunning reversal of lipid peroxidation damage, increased cell viability, and improved mitochondrial function. The mechanism of action of the drug, a stabilized form of the essential fat linoleic acid, is believed to be down-regulation of PUFA autoxidation initiated by hydrogen abstraction from susceptible, bis-allylic sites of mitochondrial membrane PUFAs. Replacement of the bis-allylic hydrogen atoms with deuterium atoms (D-PUFAs) arrests PUFA autoxidation in vitro and in vivo due to the kinetic isotope effect. Unlike antioxidants, which are typically consumed as they quench lipid peroxidaiton products, D-PUFAs are not used up in the process of inhibiting lipid peroxidation, and don't suffer from the distribution and diffusion limitations of antioxidant approaches.

Surprisingly, cells from yeast, murine, and human (primary FRDA patient cells) treated with a mixture of approximately only 20% isotope-reinforced D-PUFA in a background of normal PUFAs are fully protected from lipid autoxidation-mediated cell killing. The findings also show mitigation of mitochondrial dysfunction and increased cell viability. As a minor perturbation on naturally occurring GRAS fats, D-PUFA drugs enjoy all the active transport in an out of tissues and mitochondria that evolved over decades to ensure critical PUFA molecules were replaced when damaged, and were granted an accelerated pathway into human testing by the US FDA. A trial in the orphan neurodegenerative disease, Friedreich Ataxia, is planned. Orally fed rodent models in other degenerative diseases and PK studies of the drug confirm efficacy in difficult to reach brain and retina tissues, and IND-enabling toxicity studies showed no signs of drug related adverse findings in any parameter tested.

The planned Phase 1b/2a trial in 33 patients dosed for 6 months is expected to start in early 2016, will include an ascending dose safety study, and will measure FARS and multiple other FRDA disease readouts.

Rationale and design of a clinical study of RTA 408 in patients with Friedreich's ataxia

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RTA 408 is a semi-synthetic triterpenoid that potently induces nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and suppresses NF-κB at low nanomolar concentrations. Through modulation of these transcription factors, RTA 408 regulates multiple genes that play both direct and indirect roles in the production of cellular energy within the mitochondria. Genetic induction of Nrf2, as well as pharmacologic induction with RTA 408 and related analogs, has been shown to increase mitochondrial function in preclinical and *ex vivo* systems, by increasing reducing equivalents, oxygen consumption, and ATP production.

A hallmark of Friedreich's ataxia is impairment of antioxidative defense mechanisms, which play a major role in disease progression. Studies have demonstrated that Nrf2 signaling is grossly impaired in patients with Friedreich's ataxia and likely contributes to oxidative stress and reduced ATP production. Clinically, these effects manifest as reduced exercise capacity, visual function, energy levels, and quality of life. Therefore, the ability of RTA 408 to activate Nrf2 and induce antioxidant target genes is hypothesized to affect these abnormal biochemical and clinical deficits in patients with Friedreich's ataxia.

This phase 2 study of the safety, efficacy, and pharmacodynamics of RTA 408 in the treatment of Friedreich's Ataxia (MOXIe; NCT02255435) is a two-part study. The primary efficacy endpoint is the time-averaged effect on peak work during maximal exercise testing following 12 weeks of treatment with RTA 408 as compared to placebo. The study will also explore changes in the modified Friedreich's ataxia rating scale (FARS) score and changes in patient reported outcomes.

The first part of this study is a randomized, placebo-controlled, double-blind, dose-escalation study to evaluate the safety, efficacy, pharmacokinetics and pharmacodynamics of RTA 408 at 2.5 mg, 5 mg, and 10 mg in 16 total patients. The second part of this study will be a randomized, placebo-controlled, double-blind, parallel study to evaluate the safety, efficacy and pharmacodynamics of up to two dose levels of RTA 408 in 24-36 patients with Friedreich's ataxia. Patients in Part 2 will be randomized 1:1:1 to receive RTA 408 2.5 mg, RTA 408 10 mg, or placebo (n=8-12 per treatment group) and will be stratified by peak work at baseline. All qualified patients enrolled in the study will follow similar schedules of assessments and study drug administration. Patients will self-administer study treatment once daily for 12 weeks. A data safety monitoring board will perform monthly reviews of data for safety throughout the study.

Repurposed dyclonine for Friedreich's ataxia therapy

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There is currently no approved therapy for Friedreich's ataxia in the US. Because there is often inertia for large pharma to develop drugs for rare disease, we screened a library of FDA-approved compounds to identify drugs which might be effective in Friedreich's ataxia. From microarray and RNAseq of DRG neurons, defects in iron-sulfur and antioxidant transcripts and enzymes was identified, associated with mitochondrial antioxidant activity, eg. thioredoxin reductase. Thus, Friedreich's cells were screened with multiple poisons of thiol antioxidant systems, and sensitivity to diamide was observed. Using diamide sensitivity as a screen, 1600 compounds in clinical use were screened for protection from diamide. Of these 1600, 40 were protective. Of the 40, 10 induced frataxin expression in Friedreich's patient cells. We identified the topical anesthetic dyclonine as protective. Dyclonine increased FXN transcript and FXN protein dose-dependently in FA cells and brains of animal models. Dyclonine also rescued FXN-dependent enzyme deficiencies in the iron-sulfur enzymes, aconitase and succinate dehydrogenase. Dyclonine induces the Nrf2 [nuclear factor (erythroid-derived 2)-like 2] transcription factor, which we show binds an upstream response element in the FXN locus. Additionally, dyclonine also inhibited the activity of histone methyltransferase G9a, known to methylate histone H3K9 to silence FA chromatin. Chronic dosing in a FA mouse model prevented a performance decline in balance beam studies. A human clinical proof-of-concept study was completed in eight FA patients dosed twice daily using a 1% dyclonine rinse for 1 week. Six of the eight patients showed an increase in buccal cell FXN levels, and fold induction was significantly correlated with disease severity. Dyclonine represents a novel therapeutic strategy that can potentially be repurposed for the treatment of FA.