Frataxin Levels predict long-term Clinical Progression in Friedreich Ataxia

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**RUNNING HEAD: Clinical outcomes and Frataxin**

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Abstract –

Main Text -

# Abstract

**Background** - Novel therapeutics entering clinical trials are employing various strategies to increase or augment frataxin (FXN) protein levels. More precise knowledge on the direct impact on clinical outcomes can increase FXN utility as a biomarker, i.e., reasonable likely to predict clinical benefit. This will significantly accelerate the development of such therapies and can further support to establish dose levels.

Methods - We utilized four frataxin (FXN) datasets, stemming from two FA natural history studies, two peripheral tissues, two different assays and assessing two FXN isoforms. Blood samples from FACHILD (a pediatric natural history study) were analyzed using a new LCMS methodology (FXN-E from erythrocytes and mature FXN). Additionally, FACOMS samples (blood and buccal swabs) were analyzed using a lateral flow immunoassay. Protein levels were intercorrelated with age of onset and GAA1, compared between controls, carriers, and patients, as well as between clinical severity groups. Eventually predictive capacity of direct patient function was evaluated.

**Results** - Data from 87 FACHILD patients, and 428/532 FACOMS participants (blood samples/buccal swabs) were available. Patients showed relative frataxin levels of 15%-25%, while carriers had 50%-80% of control levels. Grouped by clinical severity, patient levels were consistent between datasets, e.g. typical onset patients (AOO 8-14y) had 160% of early onset patients (AOO <7y). FXN levels predicted both age at loss of ambulation and long-term progression slopes in the Upright Stability Score (Section E of the modified Friedreich’s Ataxia Rating Scale). Although the FACHILD dataset had only about 20% of FACOMS patients, the LCMS-derived FXN levels predicted function with similar significance.

**Discussion -** This work provides proof for the direct correlation of FXN levels with long-term patient function. In addition, relative FXN levels between clinical subgroups, as well as carriers and controls provide important guidance for drug development.

**Conclusion -** This work marks a significant step forward for the use of FXN levels as a clinical biomarker.

# Introduction

Friedreich ataxia is an autosome recessive neurodegenerative disorder cause by deficiency of frataxin, a mitochondrial protein involved in iron sulfur cluster synthesis (1-3). Clinically, FRDA manifests as progressive gait ataxia, loss of hand coordination, dysarthria, and cardiomyopathy, leading to an average age of death in the late 30s (4-5). Ninety percent of mutant alleles contain an expanded GAA repeat in intron 1 of the *FXN* gene that silences transcription, with the length of the GAA repeat predicting age of onset and other markers of clinical severity (1-3). The remaining 4 % of pathogenic alleles give rise to non-functional frataxin, creating a unified pathophysiological mechanism in which frataxin deficiency leads to downstream mitochondrial dysfunction as the cause of the disease (6-7). Frataxin levels in tissue provide a biomarker of clinical status, as they correlate with GAA repat length and age of onset, and results from clinical measures accounting for disease duration or age (8-19).

Many clinical trials have addressed the mitochondrial dysfunction, with one agent, the NRF2 activator (omaveloxolone) being approved (20-21). However, new therapies should ideally address the cause of disorder, deficiency of functional frataxin, through protein replacement, gene therapy, gene editing, or epigenetic approaches(22-32). Development of such therapies would be markedly aided by ability to assess frataxin levels in disease relevant tissues, allowing frataxin levels to be used as a pharmacodynamic biomarker in early trials to establish dose and as a monitoring biomarker in efficacy trials as a surrogate endpoint. A variety of approaches have been used to measure frataxin, including immune assays as well as more recently developed mass spectrometry-based methods (8-19). While such measures predict clinical outcomes to a moderate degree, their value in predicting future progression or change in disease status is unknown. In the present study, we have used serial analysis of clinical measures from the large FACOMS and FACHILD natural history studies in conjunction with expanded cohorts in which frataxin has been measured to assess the ability of frataxin measurement to predict long term progression in FRDA (33-38).

# **Methods**

## Datasets & Assays

We utilized four frataxin (FXN) datasets, available from two FRDA natural history studies: The FACOMS and FACHILD cohorts.(37-38) and FACOMS (33-36) FXN levels were available from two peripheral tissues and two different assays, and for two FXN isoforms (8-13). Blood samples from FACHILD were analyzed using a new LCMS technology( 12), generating values for both mature FXN (FXN-M, found in most cells) and FXN-E (found almost exclusively in erythrocytes). FACOMS samples (blood and buccal swabs) were analyzed using a lateral flow (‘dipstick’) immunoassay, (Lazaropoulos *et al.* 2015). The present data include expanded samples sizes from those previously published (n=87 pediatric patients in FACHILD, n=428 blood samples and 532 buccal swabs in FACOMS). Results are reported as absolute values (ng/ml) for LCMS assays and relative values ( % control) for lateral flow assays (8-13).

Two frataxin (FXN) datasets were available from two Friedreich's ataxia (FRDA) natural history studies (**Table 1**). All datasets included controls, carriers and patients.

Data analysis: In each dataset, we also compared FXN levels between controls, carriers and patients and correlated frataxin values with length of the repeat expansion, (GAA1), age of onset AOO and FXN levels. Predictive capabilities of Frataxin levels were compared with the same values from Age of Onset, and the shorter triplet repeat expansion (GAA1).

To examine the relationship between long-term clinical progression and FXN levels, two approaches were used (FXN levels were always analyzed on a log scale):

1. Age at loss of ambulation (LoA) was analyzed using cox proportional hazard models.
2. Progression of mFARS and Upright Stability was modeled using linear mixed-effects models within the FA-COMS and FA-CHILD natural history studies.

# Results

Data from 87 FACHILD patients, xxx FACOMS patients ( assayed by mass spectrometry), and 428/532 FACOMS participants (blood samples/buccal swabs assayed by lateral flow assay) were available. In general, the demographics of the present cohorts match those from previous large studies (33-38).

As shown in previous studies using smaller versions of the present cohorts, patients showed mean relative frataxin levels of 15%-25%, while carriers had 50%-80% of control levels (Supplementary Figure 1) Even though the FACHILD cohort was more restricted than the FACOMS cohorts, frataxin values were similar. For mass spectroscopic evaluation, mean absolute values in patients were xxx, with frataxin E values being slightly higher than mature frataxin values in blood. Similarly, by lateral flow assay in the FACOMS cohort blood values were slightly higher than buccal cell values. As seen in previous analyses with smaller versions of these cohorts, frataxin values in FRDA patients correlated with GAA length and age of onset (Table 1) . Correlations were generally high er for blood than for buccal cells, but similar between Isoform E and mature frataxin among the FACOSM cohort. Differences between clinical subgroups of FA (early, typical, intermediate and late onset) were similar between both FACHILD and FACOMS (Figure 1; Table 2) with typical onset patients (AOO 8-14y) having 160% of early onset patients (AOO <7y). Despite the much smaller dataset (~20% of FACOMS), FACHILD LCMS FXN level correlations were still significant . Results were similar when restricted to those in whom sufficient clinical data was available to allow modeling of progression slopes (Supp Table 2)

Clinical severity *(these parts need a discussion of the restrictions you place on the analysis)*

We also investigated whether frataxin levels predicted clinical progress ion by examining effects on time to LOA and on progression of the mFARS. Frataxin levels in each sample in each cohort correlated with age at loss of ambulation when viewed as linear correlations (Figure 2) or when separated by frataxin level quartiles (Figure 3). such predictions were best using the levels form the FACOMS blood cohort, Using Cox hazard models, frataxin levels predicted loss of ambulation similarly to AOO and GAA repeat length using both the FACHILD isoform E level or the FACOMS blood frataxin level (Table 3). Similar results were found with or without censoring (Supp table 4).

We also investigated if frataxin levels could predict speed of progression as assessed by the slope of mFARS progression. Examining the slope in ambulatory subset (and excluding subjects carrying non-GAA alleles), FXN levels predicted both age at loss of ambulation and long-term progression slopes in the Upright Stability Score (Section E of the modified Friedreich’s Ataxia Rating Scale). Although the FACHILD dataset had only about 20% of FACOMS patients, the LCMS-derived FXN levels predicted function with similar significance. both the FACHILD mature levels and the FACOMS blood levels predicted speed of progression, demonstrating the effect of frataxin on clinical features of FRDA.

Discussion

The present study demonstrates that FXN levels directly correlate with long-term clinical outcomes in FRDA, providing further support for use of peripheral frataxin levels as a biomarker of clinical features of FRDA. FXN levels predicted age of onset, clinical status, time to loss of ambulation, and progression speed in multiple cohorts. This matches the proposed pathophysiology of FRDA with expanded GAA repeats in the *FXN* gene leading to silencing of frataxin mRNA production, which then decreases tissue levels of frataxin levels, which then give rise to the clinical and biochemical features of the disorder(1-3) . Thus, frataxin levels in peripheral tissue can accurately provide biomarkers of clinical processes in observational and therapeutic trials where appropriate. In contrast, measuring the length of the GAA repeat, while providing an index of clinical severity, does not change over time or in response to therapeutic intervention, and is confounded by somatic mosaicism and interruptions of unknown significance (1-3, 39-40) . Consequently, frataxin levels provide a more useful biomarker of disease status than assessment of the primary genetic defect, the expanded GAA repeat.

In the present data, although results agreed across different cohorts and techniques, the more recently developed mas spectrometric methods provided similar statistical significance with far fewer subjects. This suggests superior quantification with such methods, although some aspects of the differing procedures (such as tissue isolation) remain equally important in determining the fidelity of the assessment. For each cohort, we examined two types of frataxin: Isoforms M and E using mass spectrometry assays of FACHILD, and blood and buccal frataxin with lateral flow assays in FACOMS. As whole blood frataxin assessments contain much more Isoform E than Isoform M, the results from the two cohorts are probably parallel in many ways, with whole blood being analogous to isoform E and buccal cell results resembling mature frataxin. In each of the assessments except that for longitudinal slope, results from Isoform E / whole blood carried higher statistical associations that Isoform M buccal cell. This seems surprising, as frataxin E is not directly linked to the phenotype of disease, is not made in large amounts in affected tissues, and its retention in some patients carrying point mutations in the near N terminal region is associated with more severe phenotypes (9-10, 40-41). This paradoxical result most likely reflects the tight association between isoform E and mature form levels as both are controlled by GAA length. Isoform E is a soluble protein found almost exclusively in a simply isolated tissue (RBC). In contrast, mature frataxin is intramitochondrial, and thus may be associated more difficult extraction, leading to variability in assessment and thus less significant statistical associations. Understanding the extraction of frataxin from different tissues in individual studies is thus important for interpretation of the role of frataxin as a biomarker in such studies.

In the present study, the data show that frataxin levels as assayed as total levels functions as a biomarker of disease status. However, its use in clinical studies must be interpreted in the context of the cellular and tissue distribution of frataxin. Frataxin is ubiquitously expressed, but its levels vary across tissues, and selected transcription factors influence its expression(1-3). Still, the pathologic suppression of frataxin mRNA production by the expanded GAA repeat dominates control of frataxin expression in FRDA. Still, in some situations, other factors may alter expression of frataxin to some degree, possibly in a tissue specific manner, thus requiring interpretation of individual experimental paradigms.

In addition, frataxin itself is likely expressed in a mosaic, cell specific manner to some degree. In PBMC, the expression of frataxin reflects the number of alleles that unmethylated and thus are transcriptionally active, in any given cell being 0,1, or 2 . Cells with no active alleles make almost no frataxin, those with one active allele make about 50% of control , and 2 active alleles make normal levels. Thus the number of cells that active, not the amount of expression per cell, provides the major difference in overall expression between controls, carriers and FRDA patients, implying that cells in FRDA express frataxin in a mosaic pattern in cells. Such mosaicism occurs in PBMC but has not been investigated in other tissues. As the methylation pattern appears early in development, other processes may select cells of different frataxin levels over time. Interpretation of such processes (which are unknown at present) alters the meaning of tissue frataxin levels as measured as absolute values over time.

Furthermore, understanding the meaning of frataxin levels in therapeutic trials requires consideration of the mechanism by which such processes restores frataxin. Protein replacement and epigenetic activation generally restore frataxin similarly in all cells to which they distribute; such cells simply need to replace frataxin to the degree needed for the health of an individual cell. In contrast, virally delivered gene therapy and gene editing distribute unevenly in cells based on their transduction efficiency (in addition to differentially activating FXN based on promoter strength). Consequently, measurement of frataxin restoration must consider not only the overall level (as measured by assay described here) but also transduction efficiency in order to understand if target amounts and distribution of frataxin have been reached. Still, the present data show that peripheral measurements of tissue frataxin levels provide a marker of clinical status in FRDA in conjunction with immunohistochemistry or in situ hybridization that assess cellular distribution. Using measurements of frataxin levels in therapeutic trials should provide a useful surrogate marker in many situations.

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# Disclosures

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# Figure Legends

# Tables

SupplmentaryTable 12: Demographic Characteristics of the Analysis Populations in FACHILD and the comparative FACOMS cohort. Numbers after the scales indicate maximum possible score.

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