

# Clinical Evidence for Variegated Silencing in Patients With Friedreich Ataxia

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## Abstract

### Background and Objectives

Friedreich ataxia (FRDA) is a neurodegenerative disease caused by a GAA triplet repeat (GAA-TR) expansion in intron 1 of the *FXN* gene. Patients have 100–1,300 GAA triplets compared with less than 30 in healthy controls. The GAA-TR expansion leads to *FXN* silencing, and consequent frataxin protein deficiency results in progressive ataxia, scoliosis, cardiomyopathy, and diabetes. The overt heterogeneity in age at onset and disease severity is explained partly by the length of the GAA-TR, in which shorter repeats correlate with milder disease. Evidence of variegated silencing in FRDA suggests that patients with shorter repeats retain a significant proportion of cells with *FXN* genes that have escaped GAA-TR expansion–induced silencing, explaining the less severe frataxin deficiency in this subpopulation. In *ex vivo* experiments, the proportion of spared cells negatively correlates with GAA-TR length until it plateaus at 500 triplets, an indication that the maximal number of silenced cells has been reached. In this study, we assessed whether an analogous ceiling effect occurs in severity of clinical features of FRDA by analyzing clinical outcome data.

### Methods

The FRDA Clinical Outcome Measures Study database was used for a cross-sectional analysis of 1,000 patients with FRDA. Frataxin levels were determined by lateral flow immunoassays.

### Results

The length of the GAA-TR in our cohort predicted frataxin level ( $R^2 = 0.38$ ,  $p < 0.0001$ ) and age at onset ( $R^2 = 0.46$ ,  $p < 0.0001$ ) but only with GAA-TRs with  $\leq 700$  triplets. Age and disease duration predicted performance on clinical outcome measures, and such predictions in linear regression models statistically improved in the subcohort of patients with  $>700$  GAA triplets. The prevalence of cardiomyopathy and scoliosis increased as GAA-TR length increased up to 700 GAA triplets where prevalence plateaued.

### Discussion

Our data suggest that there is a ceiling effect on the clinical consequences of GAA-TR length in FRDA, as would be predicted by variegated silencing. Patients with GAA-TRs of  $>700$  triplets represent a subgroup in which the severity of clinical manifestations based on GAA-TR length have reached maximal levels and therefore display limited clinical variability in disease progression.

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Friedreich ataxia (FRDA), an autosomal recessive neurodegenerative disorder, is most commonly caused by homozygosity for a guanine-adenine-adenine triplet repeat (GAA-TR) expansion in intron 1 of the *FXN* gene.<sup>1</sup> Patients have 100–1,300 GAA repeats compared with <30 in non-FRDA individuals. The expanded repeat induces epigenetic silencing of the *FXN* gene, leading to frataxin protein deficiency and the clinical manifestations of progressive ataxia, cardiomyopathy, scoliosis, and diabetes. Patients most commonly present with ataxia in their early teen years, but age at onset and severity of symptoms are heterogenous in FRDA, a phenomenon that is partially explained by the length of the GAA-TR.<sup>2,3</sup> Longer GAA-TRs typically are associated with lower cellular frataxin levels, earlier age at onset, and more severe disease.<sup>2-4</sup>

Historically, it has been presumed that in FRDA, the GAA-TR is present in every cell, and therefore, *FXN* is silenced to the same degree in every cell, resulting in all cells equally expressing 5%–30% of non-FRDA frataxin levels based on GAA-TR length. By contrast, a recent model of variegated gene silencing in FRDA revealed that the pattern of *FXN* silencing is instead analogous to position effect variegation (PEV). A phenomenon originally described in *Drosophila* spp., PEV occurs when a gene is located abnormally close to a source of repressive chromatin, resulting in epigenetic silencing of the gene, but only in a proportion of cells. The resulting phenotype in *Drosophila* spp. is a mosaic compound eye containing both white and red eyes due to variegated silencing of the red pigment expressing gene.<sup>5</sup> In a fluorescent reporter cell model, the GAA-TR itself acts as a source of heterochromatin that spreads bidirectionally resulting in gene silencing. As in PEV, the spreading is more extensive in some cells, resulting in stochastic pattern of gene silencing within a population of cells where some cells escape silencing.<sup>6</sup> Recently, a study based on ex vivo analysis of peripheral blood mononuclear cells (PBMCs) from patients with FRDA further supported the PEV model of silencing in FRDA.<sup>7,8</sup> High-resolution epigenetic mapping revealed that nearly all *FXN* genes in cells of patients with FRDA are silenced and express <5% *FXN*. Only a small subpopulation of *FXN* genes escape silencing (and express approximately 100% *FXN*), giving an overall apparent average of 5%–30% residual *FXN* levels in a given population of cells. Patients with shorter GAA-TRs have a higher proportion of spared cells, explaining their lesser degree of frataxin deficiency (approximately 30% residual *FXN*) and milder disease.

In the variegated silencing model in PBMCs, the proportion of spared cells decreases with increasing GAA-TR length until it plateaus at 500 triplets, at which the maximum of both number of silenced cells and severity of *FXN* deficiency has been reached. If such an effect occurs in affected tissues in patients, there should be a similar ceiling effect of GAA-TR length on clinical severity. Patients with GAA-TR levels greater than this ceiling would constitute a subpopulation with relatively similar frataxin levels and potentially more homogeneous clinical features when controlling for disease duration and age. Based on the variegated silencing model

described in PBMCs, we aimed to determine whether the clinical features of FRDA reach a similar ceiling in which severity of symptoms reaches a maximum and worsens minimally past a certain GAA-TR length. This would refine methods for preclinical data analysis, design of clinical trials, and determining prognosis for clinical care. In this study, we have tested the variegated silencing model in a clinical data set from a large natural history study in FRDA.<sup>7-10</sup>

## Methods

### FRDA Clinical Outcome Measures Study Data Set

We used a cross-section of data from the FRDA Clinical Outcome Measures Study (FACOMS), a natural history study established to collect medical history and track progression of symptoms of patients with FRDA over time; its features have been previously reported.<sup>9-12</sup> The data used from the most recent visit included GAA-TR repeat length (n = 1,000), age (n = 990), age at onset (n = 1,000), frataxin level in whole blood (n = 498), clinical outcome measures (9-hole peg test, n = 1,000; timed 25-foot walk, n = 1,000; modified Friedreich ataxia rating scale neurologic score, n = 988; and activities of daily living questionnaire scores, n = 921), and a history of diagnosis of cardiomyopathy, scoliosis, and diabetes. Inclusion criteria for this study were GAA-TR length and age at onset (n = 1,000 patients with FRDA). Frataxin level in whole blood was measured by lateral flow immunoassays.<sup>13-15</sup> Frataxin data were averaged across 1–5 replicates from each participant. The frataxin assay used here should detect both mitochondrial frataxin and the recently characterized extramitochondrial frataxin, frataxin-E.<sup>16</sup> Linear regression analysis via StataSE17 software determined correlation coefficients (R<sup>2</sup>) and significance (p) values.

### Standard Protocol Approvals, Registrations, and Patient Consents

Full consent from all participants and institutional review board approval were obtained for the FACOMS data collection study from which the data for this article were sourced. Consent forms and institutional review board documentation are kept on file.

### Data Availability

The FACOMS data set used in this study is available through The Critical Path Institute Friedreich's Ataxia Integrated Clinical Database.<sup>17</sup>

## Results

Triplet-repeat expansions with >700 GAAs do not significantly contribute to linear clinicopathologic correlations in FRDA.

In this cohort, patients with FRDA had a median GAA1 (shorter allele) length of 690 ± 235 triplets and median age at onset of 11 ± 9 years (Table 1). Linear correlations of

**Table 1** Patient Demographics

Data point	
Female, %; n = 1,000	50.4
GAA1, <sup>a</sup> median ± SD (range); n = 1,000	690 ± 235 (18–1,320)
GAA2, <sup>a</sup> median ± SD (range); n = 1,002	930 ± 217 (99–1,733)
Age <sup>b</sup> , median ± SD (range); n = 990	26.5 ± 15.1 (7–85)
AoO <sup>b</sup> , median ± SD (range); n = 1,000	11 ± 9.4 (1–75)
Whole blood frataxin level, <sup>c</sup> median ± SD (range); n = 498	20.9 ± 16.3 (1.5–109)

<sup>a</sup> Number of triplets.<sup>b</sup> In years.<sup>c</sup> % of non-Friedreich ataxia control level.

GAA1 length with frataxin level and age at onset agree with historical observations across the entire cohort (Figure 1A:  $R^2 = 0.38$ ,  $p < 0.0001$ ; B:  $R^2 = 0.46$ ,  $p < 0.0001$ ).<sup>2,3,9–12</sup> However, stratifying the data based on GAA1 repeat length using continuously increasing bins of GAA size (i.e.,  $\leq 200$  GAAs,  $\leq 300$  GAAs,  $\leq 400$  GAAs...) revealed that GAAs of  $\leq 700$  contributed most to such correlations. The correlation coefficient improved for these measures as patients with longer GAA1 lengths were added until the improvement plateaued at 700 GAAs. Similarly, creating decreasing bins of GAA-TR length (i.e.,  $>900$  GAAs,  $>800$  GAAs,  $>700$  GAAs...) identified no correlation of GAA-TR length and either frataxin level or age at onset until GAA-TR lengths of less than 700 were included; the strength of the correlation continually increased as bins with shorter GAA-TRs were included. This indicates a ceiling effect at 700 triplets greater than which inclusion of participants with  $>700$  triplets does not add any significant variability in frataxin level or age at onset (Figure 1, C and D). A correlation comparing frataxin level with age at onset (both downstream consequences of the GAA repeat length) also showed some evidence of a ceiling effect, albeit a less obvious ceiling than the one produced by the GAA length itself (eFigure A and B, [links.lww.com/NXG/A528](https://links.lww.com/NXG/A528)), indicating that the ceiling effect is produced by the upstream GAA-TR length, not frataxin level. To rule out that the increase in correlation coefficients was not only due to an increase in sample size as more patients were added to the correlation model, we used a rolling analysis in which increasing numbers of participants were included at different mean GAA-TR lengths (eFigure 2, [links.lww.com/NXG/A528](https://links.lww.com/NXG/A528)). In this model, increasing numbers of participants improved  $R^2$  values, but improvement again plateaued as mean GAA values increased. In addition, cohorts of individuals with high mean GAA lengths had extremely low  $R^2$  values even at large number of participants. This illustrates

a ceiling effect at a GAA length of 700 on frataxin level and age at onset.

## Patients With $>700$ GAA Triplets Demonstrate Less Heterogeneity in Progression

We then assessed the effect of GAA-TR length on heterogeneity of disease course by examining the relationship of clinical outcome measures with disease duration in patients with GAA-TRs longer than 700 triplets and in the entire cohort. In all instances, the correlation coefficient improved when analysis was restricted to patients with GAA-TRs longer than 700 triplets (Figure 2, A–D, Table 2). Using age as a predictor of clinical outcome measures gave similar results (Table 2). Overall, the change in clinical outcome measures over time was more homogeneous in the subgroup of patients with GAA-TRs longer than 700 than across the entire cohort or in the subcohort of individuals with less than 700.

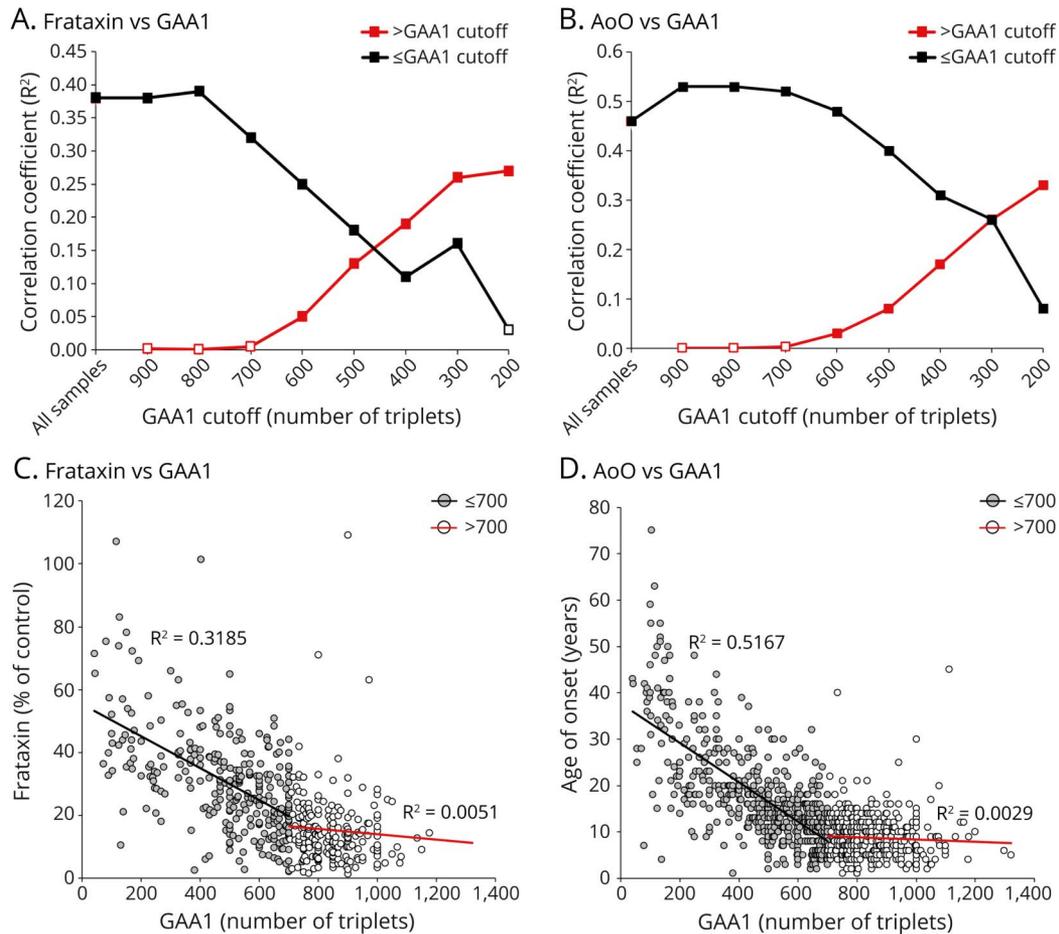
Surprisingly, not all clinical features of FRDA reflected a ceiling effect at 700 repeats. In this cohort, the prevalence of cardiomyopathy, scoliosis, and diabetes was 59%, 81%, and 7%, respectively (Figure 3, A–C). For cardiomyopathy and scoliosis, the prevalence increased as GAA-TR length increased up to 700 GAA triplets, where it plateaued, a phenomenon that was not altered with longer disease duration (eFigure 3 A, B, [links.lww.com/NXG/A528](https://links.lww.com/NXG/A528)). However, the prevalence of diabetes was similar when assessed across the entire cohort or when stratified by GAA repeat length. An increased prevalence of diabetes was seen in patients with longer repeats only when data were analyzed over time (Figure 3C, eFigure C, [links.lww.com/NXG/A528](https://links.lww.com/NXG/A528)).

## Discussion

This study demonstrates that severity of clinical features of FRDA generally increases with increasing GAA-TR length up to 700 triplets where a plateau occurs, creating a ceiling effect. The subcohort of patients with GAA-TRs of  $>700$  triplets show limited clinical variability in disease progression due to GAA-TR length. Our data further support the variegated silencing model by demonstrating that a ceiling effect exists in GAA-TR length on clinically measured frataxin levels and with severity and prevalence of clinical features of FRDA. Such a ceiling effect of frataxin levels on age at onset was far less prominent, consistent with the model that the GAA-TR is the causal event of the ceiling effect rather than the degree of frataxin loss, which is likely ultimately determined by the GAA repeat length and other upstream factors.

One of the main analytical challenges in FRDA is understanding the heterogeneity among patients. Many clinical studies have used GAA-TR as a continuous variable for analysis of clinical features and biomarker results.<sup>18–24</sup> However, the relatively linear effects of GAA-TR length are

**Figure 1** GAA1 Length Is Predictive of Blood Frataxin Level and Age of Onset Only in Sub-cohorts of Patients With Less Than 700 GAA Triplets



Linear regression analysis comparing GAA1 length with blood frataxin level (A) and age of onset (AoO) (B) in the entire cohort (All samples) and for subgroups stratified by GAA1 length. Correlation coefficient ( $R^2$ ) values are plotted for each subgroup. Black data points represent results for patients with less than or equal to the GAA1 cutoffs listed on the X-axis (e.g., less than or equal to 200 triplets, 300 triplets). Red data points represent results for patients with greater than the GAA1 cutoffs listed on the X-axis (e.g., greater than 200 triplets, 300 triplets). Filled data points indicate  $p < 0.01$ ; open data points indicate n.s. Linear regression plots comparing GAA1 length with blood frataxin level (C) and AoO (D) for the entire cohort stratified by GAA1 less than or equal to 700 triplets (gray circles, black fit line) or more than 700 triplets (white circles, red fit line).

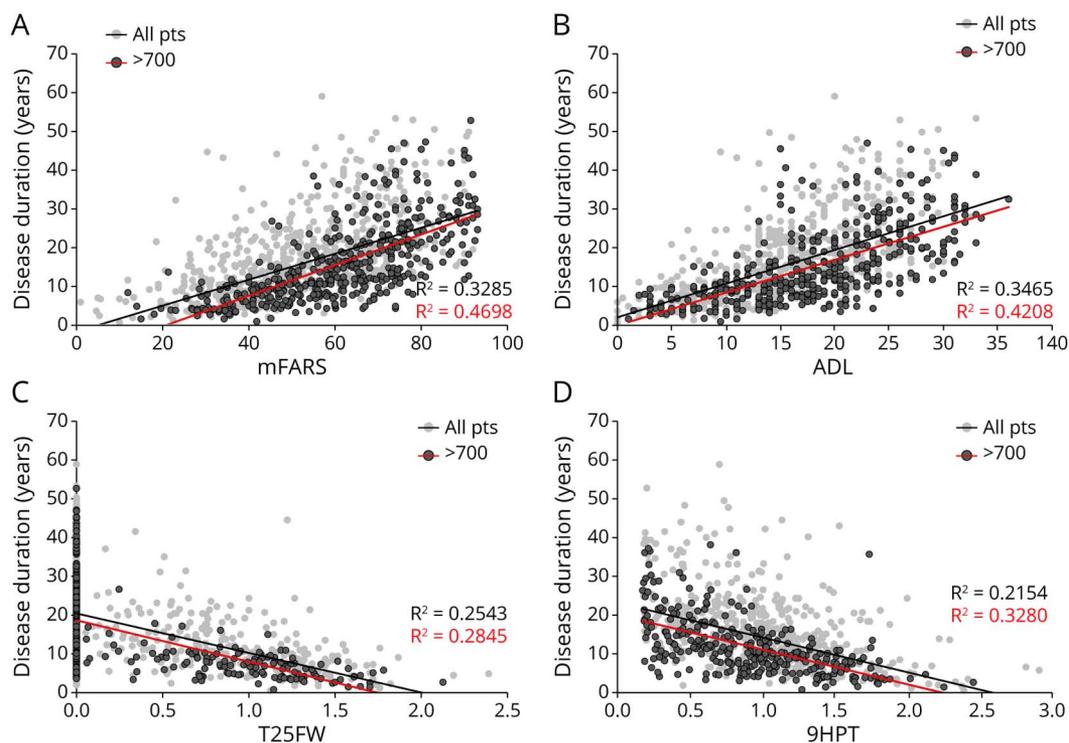
confined to lengths less than 700. Because disease progression in patients with GAA-TRs of >700 triplets is relatively homogeneous, it may be reasonable to stratify data above and below this cutoff point in both preclinical studies and clinical trials.

The present findings also may have specific implications for different therapies or modifiers of the FRDA phenotype. The primary cause of FRDA is frataxin deficiency, which varies in severity based on the GAA-TR length; the severity of FRDA also reflects variation in downstream events or unknown variables among patients. Based on the present results and the molecular concept of variegated silencing,<sup>8</sup> agents that alter gene silencing should produce the greatest effects on clinical status or frataxin levels in participants with GAA-TR lengths of less than 700. By contrast, participants with GAA-TRs of greater than 700 might prove most useful in assessing agents acting on disease aspects other than gene

silencing because this group has the greatest homogeneity in frataxin levels. This matches the results in the MOXIe trial for omaveloxolone, an agent not thought to alter frataxin levels. When patient data were stratified by GAA-TRs (greater than or less than 675 triplets), the subcohort with  $\geq 675$  triplets responded better to omaveloxolone and with less interindividual variability than patients with <675 triplets.<sup>25</sup>

The prevalence of cardiomyopathy and scoliosis was higher in patients with >700 triplets but did not change throughout disease progression for any subgroup analyzed in this study. The prevalence of diabetes was also higher in patients with >700 GAA triplets but differed from cardiomyopathy and diabetes in that it increases with disease duration. When analyzed as a function of disease duration, we noted a paradoxically higher prevalence of diabetes in patients with  $\leq 700$  triplets compared with that of either the

**Figure 2** Predictions of Neurological Dysfunction by Disease Duration Are Better in the Sub-cohort of Patients With >700 GAA Triplets Compared to the Entire Cohort



Linear regression analysis comparing disease duration with clinical outcome measures: modified Friedreich ataxia rating scale (mFARS) (A), activities of daily living (ADL) (B), timed 25-foot walk (T25FW) (C), and 9-hole peg test (9HPT) (D). Light gray data points and black fit line=results for the entire cohort (All pts). Dark gray data points and red fit line = results for patients with greater than 700 triplets. Top  $R^2$  value (black text) is for the entire cohort, bottom  $R^2$  value (red text) is for the sub-cohort of patients with >700.

entire cohort or patients with >700 triplets when the analysis was restricted to patients with disease durations of less than 10 years. This may reflect the smaller sample size in the subcohort of patients with disease duration <10 years (average disease duration in this cohort =  $17 \pm 11$  years).

While the present data support variegated silencing as a mechanism for the observed ceiling effects in clinical features, there are both alternative explanations and differences between this study and the previous ex vivo study. Previous ex vivo data on variegated silencing in FRDA<sup>8</sup> found the GAA-TR ceiling cutoff length to be 500 triplets, which differs slightly from our finding of 700 triplets. The discrepancy could reflect differences in sample sizes between the 2 studies (approximately 70 patients vs ~1,000 patients), differences in GAA repeat lengths or mechanisms of silencing between different tissues, or the relative granularity of data between the studies. The ex vivo study used DNA methylation measured at single DNA strand level to give highly accurate and precise molecular results. This study used clinical data that are inherently more variable. Although the ex vivo study had fewer participants, the data were less variable. The difference in analysis of GAA-TR lengths could also contribute to the differing results

between studies. GAA-TRs were measured in house for the ex vivo study, but for this study, they were obtained from genetic testing results produced by different laboratories around the country during diagnosis for each patient, possibly introducing error from site-to-site variability. Frataxin transcript levels in the ex vivo study were measured in PBMCs with RT-qPCR. In this study, frataxin protein levels were measured in whole blood—a method now known to be measuring both mitochondrial and extramitochondrial frataxin (frataxin-E), which is found in high abundance in erythrocytes. The function of frataxin-E and its importance to FRDA clinical features is unknown. Its mere presence, however, adds an extra variable in this study compared with the ex vivo study.

The clinical data presented in this study cannot alone implicate variegated silencing in FRDA, but function to bolster previous molecular data. The ceiling effects seen on disease features are analogous to the ceiling effects seen on the number of silenced *FXN* genes in populations of FRDA cells. A complementary and convincing clinical observation is the fact that some patients with FRDA with short GAA-TRs overlap with asymptomatic carriers and even controls in frataxin levels (see range of frataxin level in Figure 1) but still develop the disease, while carriers with even

**Table 2** The Predictive Value of Disease Duration and Age on Measures of Disease Progression in FRDA Is Highest in Patients With >700 GAA Triplets

Comparison with mFARS		R <sup>2</sup>	p Value	Comparison with ADL		R <sup>2</sup>	p Value
<b>Disease duration</b>	All pts (n = 996)	0.33	<0.0001	Disease duration	All pts (n = 925)	0.35	<0.0001
	GAA1 ≤700 (n = 561)	0.33	<0.0001	GAA1 ≤700 (n = 525)	0.34	<0.0001	
	GAA1 >700 (n = 435)	0.47	<0.0001	GAA1 >700 (n = 400)	0.42	<0.0001	
<b>Age</b>	All pts (n = 996)	0.07	<0.0001	Age	All pts (n = 925)	0.1	<0.0001
	GAA1 ≤700 (n = 561)	0.07	<0.0001	GAA1 ≤700 (n = 525)	0.11	<0.0001	
	GAA1 >700 (n = 435)	0.33	<0.0001	GAA1 >700 (n = 400)	0.26	<0.0001	
Comparison with T25FW		R <sup>2</sup>	p Value	Comparison with 9HPT		R <sup>2</sup>	p Value
<b>Disease duration</b>	All pts (n = 373)	0.23	<0.0001	Disease duration	All pts (n = 765)	0.22	<0.0001
	GAA1 ≤700 (n = 240)	0.23	<0.0001	GAA1 ≤700 (n = 464)	0.22	<0.0001	
	GAA1 >700 (n = 133)	0.33	<0.0001	GAA1 >700 (n = 301)	0.33	<0.0001	
<b>Age</b>	All pts (n = 373)	0.13	<0.0001	Age	All pts (n = 765)	0.04	<0.0001
	GAA1 ≤700 (n = 240)	0.16	<0.0001	GAA1 ≤700 (n = 464)	0.04	<0.0001	
	GAA1 >700 (n = 133)	0.13	<0.0001	GAA1 >700 (n = 301)	0.2	<0.0001	

Linear regressions comparing measures of neurological progression (modified Friedreich ataxia rating scale [mFARS], timed 25-foot walk [T25FW], activities of daily living [ADL], and 9-hole peg test [9HPT]) to disease duration and age for the entire cohort (All pts), only patients with GAA1 ≤700 triplets, and only patients with GAA1 >700 triplets.

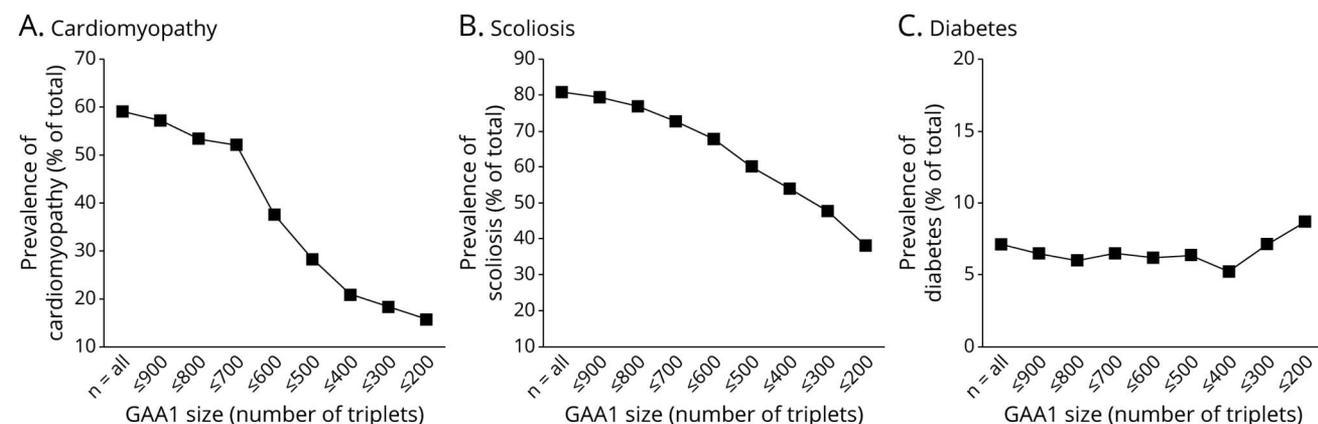
the lowest levels of frataxin are clinically normal. The variegated silencing model explains this conundrum; carriers have one fully functional *FXN* gene in every cell, while at most only half of the cells (based on molecular data from the ex vivo study) in patients with short GAA-TRs have a functional *FXN* gene. The severity (or presence) of disease features in FRDA is not dictated by relative frataxin deficiency alone but rather the relative proportion of cells/*FXN* genes that escape GAA-TR-mediated silencing. The proportion of

escaped cells reaches a minimum when GAA-TR length approaches 500–700 triplets, producing a ceiling effect on everything downstream (including clinical severity). The relative absence of a ceiling effect between frataxin levels and clinical severity suggests the mediator of the ceiling effect is in fact upstream from frataxin, i.e., the GAA-TR.

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The authors report no targeted funding.

**Figure 3** Prevalence of Cardiomyopathy and Scoliosis Increases in Patient Cohorts as GAA1 Length Increases Until It Plateaus at 700 GAA Triplets



Prevalence of cardiomyopathy (A), scoliosis (B), and diabetes (C) for the entire cohort (n = all) and for sub-cohorts stratified by GAA1 length.

## Disclosure

L.N. Rodden, Y.N. Dong, and D.R. Lynch report no disclosures relevant to the manuscript. Go to [Neurology.org/NG](https://www.neurology.org/NG) for full disclosures.

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<b>Christian Rummey, PhD</b>	Clinical Data Science GmbH, Basel, Switzerland	Drafting/revision of the article for content, including medical writing for content; analysis or interpretation of data
<b>Yi Na Dong, PhD</b>	Departments of Pediatrics and Neurology, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania	Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data
<b>David R. Lynch, MD, PhD</b>	Departments of Pediatrics and Neurology, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania	Drafting/revision of the article for content, including medical writing for content; study concept or design; analysis or interpretation of data

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