Stable isotopes and LC–MS for monitoring metabolic disturbances in Friedreich’s ataxia platelets

**Background:** Friedreich’s ataxia (FRDA) is an autosomal recessive disease with metabolic abnormalities that have been proposed to play an important role in the resulting neurodegeneration and cardiomyopathy. The inability to access the highly affected neuronal and cardiac tissues has hampered metabolic evaluation and biomarker development. **Methods:** Employment of a LC–MS-based method to determine whether platelets isolated from patients with FRDA exhibit differentiable metabolism compared with healthy controls. **Results:** Isotopologue analysis showed a marked decrease in glucose incorporation with a concomitant increase in palmitate-derived acyl-CoA thioesters in FRDA platelets compared with controls. **Conclusion:** Our findings demonstrate that platelets can be used as a surrogate tissue for in vivo biomarker studies to monitor new therapeutic approaches for the treatment of FRDA.

**Friedreich’s ataxia** (FRDA) affects one in 29,000 Caucasians making it the most frequently inherited ataxia [1]. It is an autosomal recessive genetic disorder, with a mean ± SD age at onset of 10 ± 7.4 years, and no known effective treatment [2]. The most affected tissues in FRDA are the heart, dorsal root ganglia, posterior columns of the spinal cord and the corticospinal tracts [1,3]. Most FRDA individuals develop dysarthria and are wheelchair-bound by 15.5 ± 7.4 years (mean age ± SD) after onset of the disease [1]. The genetic basis of most FRDA cases involves a nucleotide expansion in the FXN gene due to triplicate guanine–adenine–adenine (GAA) repeats in intron 1 [4]. This leads to a deficiency in the protein **frataxin**, which is thought to be involved in assembling iron-sulfur protein clusters [5]. A small minority of FRDA cases (~1–3%) carry a compound heterozygous GAA expansion on one allele of the frataxin gene with a point mutation or deletion on the other allele [1,6]. All of the mutations that have been discovered so far are in the 81–210 region of frataxin (L101S; D122Y; G130V; I40F; R165C; W173G; L182F; L198R) [7–11]. The mature biologically active form of frataxin arises from mitochondrial processing of the 23.1 kDa precursor (1–210) frataxin. Transfer of the precursor (1–210) from the cytosol into the mitochondria is facilitated by a mitochondrial targeting sequence, which is present at amino acids 1–20. Processing then occurs through an initial cleavage at glycine-41 by MPP, which removes the targeting sequence [12]. The resulting intermediate 18.8 kDa (42–201) frataxin is then cleaved by MPP at lysine-80 to give the 14.3 KDa mature (81–210) frataxin [13]. This means that the missense mutations in frataxin (101–198 region) do not prevent mitochondrial targeting. However, the mutations result in complex biochemical consequences that play a role in determining the severity of FRDA [14–16]. In contrast, the intronic triplet GAA repeats on both alleles of the FXN gene that occurs in most FRDA patients results in silencing of the gene together with significantly reduced expression of frataxin protein that has no missense mutations.

A range of cellular processes depend on iron-sulfur clusters for proper biological functions [17], such as the electron-transferring components of the mitochondrial electron transport chain and the Krebs cycle enzyme aconitase [18]. Frataxin localization to the mitochondria suggests that altered
could have a positive synergistic effect. In addition to antioxidants, it seems likely that supplementation of highly consumed metabolic precursors might go unnoticed with absolute quantification as the only indicator of the metabolic state of a system. Isotopologues provide insight into the metabolic state of a system for inherited metabolic diseases such as FRDA if these pathways are utilized in platelets. Furthermore, isolated platelets allow for more complex metabolic challenges than are possible in other in vivo assays.

Here, we have tested two isotopic tracers ([13C6]-glucose and [13C16]-palmitate) that indicate metabolic variations in FRDA platelets by analysis of isotopic enrichment in various acyl-CoA thioesters spanning multiple metabolic pathways using methodology that was validated in normal volunteers (Figure 1) (24). Glycolysis results in the incorporation of [13C6]-glucose directly into Krebs cycle metabolites through its conversion to [13C2]-acetyl-CoA, which then proceeds through the Krebs cycle (Figure 2). Conversely, [13C16]-palmitate is converted primarily into acetyl-CoA, β-hydroxybutyryl (HB)-CoA and 3-hydroxy-3-methylglutaryl (HMG)-CoA during short-term incubations in platelets (Figure 2). We report the use of isotopologue analysis for the characterization of [13C6]-glucose and [13C16]-palmitate incorporation into acetyl-CoA thioesters in the platelets of patients with FRDA, unaffected controls and the utility of this methodology as a biomarker of disease severity for FRDA. Furthermore, we propose this system as a potential model for tracking therapeutic response in FRDA.

Patients & methods
Participants
Ten FRDA patients and ten controls were entered into the study. There were no significant differences between the two groups in their mean ages (32.0 ± 7.8 [SD] for FRDA, 31.4 ± 10.3 [SD] for control) nor the mean ± SD GAA repeat length among patients (476 ± 149.8). The GAA repeat lengths indicated that the FRDA was of moderate severity (Table 1). Blood samples were obtained from the participants who were enrolled in an ongoing Friedreich’s Ataxia Research Alliance study at the Children’s Hospital of Philadelphia (IRB # 01–002609).

Reagents
Glucose, [13C6]-glucose, sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate (NaHCO3), calcium chloride (CaCl2), magnesium chloride (MgCl2), 5-sulfosalicylic acid (SSA), ammonium acetate and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (MO, USA). [13C16]-palmitate was purchased from Cambridge Isotope Laboratories, Inc.

Key terms

Friedreich’s ataxia: The genetic basis of 98% of Friedreich’s ataxia cases involves a nucleotide expansion in the FXN gene due to triplicate GAA repeats in intron 1. The remaining cases arise from point mutations in the FXN gene.

Frataxin: Human frataxin is a 23 kDa protein that is synthesized on ribosomes in the cytoplasm. It has a mitochondrial targeting sequence, which is converted to the mature 14 kDa protein by a protease in the mitochondrial matrix. Frataxin is required for iron-sulfur cluster protein assembly (such as aconitase) in the mitochondria.

Platelets: Blood cells that are released from megakaryocytes which play an important role in hemostasis. In this study, human platelets were utilized in an ex vivo assay for studying metabolism as a surrogate for tissues that were not accessible.

Isotopologues: Identical molecules that have different isotopic compositions such as [13C6]CH3CH2CH3, [13C6]CH2CH2CH3 and [13C8]CH3CH2CH3 isotopologues.

CoA: An intracellular thiol derived from cysteamine, pantothenate and adenosine triphosphate that is found in both eukaryotes and prokaryotes. CoA forms thioester derivatives with short, medium or long chain fatty acids, which are involved in numerous metabolic processes such as acetylation of histone proteins.
Acid-citrate-dextrose Vacutainer® tubes (8.5 ml; Sol A) were purchased from BD Biosciences (NJ, USA). All solvents for LC–MS were Optima grade purchased from Fisher Scientific (PA, USA).

Platelet isolation & treatment
Whole blood was drawn into 8.5 ml acid-citrate-dextrose Vacutainer tubes, transferred to 15-ml polypropylene tubes and spun at 175 × g for 15 min with no brakes as described previously [24]. The upper platelet-rich layer was then transferred to a new tube and spun at 400 × g for 10 min. The resulting platelet pellet was resuspended in 1 ml prewarmed Tyrode's solution (139 mM NaCl, 3 mM KCl, 17 mM NaHCO₃, 3 mM CaCl₂, and 1 mM MgCl₂) at 37°C containing 5 mM [¹³C₆]-glucose or 5 mM glucose with 100 μM [¹³C₁₆]-palmitate and transferred to a 1.5 ml microcentrifuge tube. Samples used for isotopic correction were treated with Tyrode's containing only 5 mM glucose. Suspended platelets were incubated with tracers for 1 h at 37°C prior to quenching and acyl-CoA extraction.

Acyl-CoA extraction
The extraction and LC–MS analysis have been described in detail previously [24,34]. Briefly, treated platelets were pelleted by centrifugation at 1000 × g for 2 min and resuspended in 1 ml of ice-cold 10% TCA followed by pulse-sonication for 30 s on ice using a sonic dismembranator (Fisher, MA, USA), followed by a 10 min centrifugation at 15,000 × g. The supernatant was transferred to a fresh tube, and the pellet was discarded. The supernatant was purified by SPE as follows: Oasis HLB 1 cm³ (30 mg) SPE columns (Waters, MA, USA) were conditioned with 1 ml of methanol followed by 1 ml of water. The collected supernatant was applied, washed with 1 ml of water and finally eluted using three subsequent applications of 0.5 ml of methanol containing 25 mM ammonium acetate. Eluted compounds were evaporated to dryness under nitrogen and resuspended in 50 μl of 5% SSA in water. Injections of 20 μl were made for LC–MS/MS analysis.

LC–MS
Acyl-CoAs were separated using a reversed-phase HPLC (Phenomenex, CA, USA) Luna C18 column (2.0 × 150 mm, particle size 5 μm) with 5 mM ammonium acetate in water as solvent A, 5 mM ammonium acetate in 95:5 ACN/water (v/v) as solvent B and 80:20:0.1 (v/v/v) ACN/water/formic acid as solvent C as previously described [24,27]. Gradient conditions were as follows: 2% B for 1.5 min, increased to 25% B over 3.5 min, increased to 100% B in 0.5 min and held for 8.5 min, washed with 100% C for 5 min, before equilibration for 5 min. The flow rate was 200 μl/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (AB SCIEX, MA, USA) in the positive electrospray (ESI) mode. Samples (20 μl) were injected using a Leap CTC autosampler (CTC Analytics, Zwingen, Switzerland) where they were maintained at 4°C, and data were analyzed with Analyst 1.4.1 software. The column effluent was processed by an API 4000 triple-quadrupole mass spectrometer (AB SCIEX, MA, USA) in the positive electrospray (ESI) mode. Samples (20 μl) were injected using a Leap CTC autosampler (CTC Analytics, Zwingen, Switzerland) where they were maintained at 4°C, and data were analyzed with Analyst 1.4.1 software.
was diverted to the mass spectrometer from 8 to 18 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), nitrogen as curtain gas (15 units), ion source gas 1 (eight units), gas 2 (15 units) and collision-induced dissociation (CID) gas (five units). The ESI probe temperature was 450°C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV and the collision exit potential was 15 V for all analytes reported. The LC–SRM/MS transitions that were used are shown in Supplementary Table 1 and typical chromatograms showing separation of the acyl-CoAs and their isotopologues is shown in Figure 3. Peak areas of all isotopologues where \([^{13}C]\)-labeling of the acyl moiety is possible were utilized to determine the percentage isotopic enrichments, which are reflective of incorporation into downstream metabolites. Label-free cells were used to generate an isotopologue-enrichment matrix (also called a correction matrix) for acetyl-CoA, succinyl-CoA, βHB-CoA and HMG-CoA. An isotopologue array generated for each sample was multiplied by the inverse of the correction matrix determined experimentally from the label-free cells to determine the absolute enrichment of each isotopologue as described by Fernandez et al. [35]. This approach reduces the underestimation of isotopic labeling in highly labeled substrates and is amenable to a correction matrix generated from label-free experimental controls as were used in this study. It also addresses the issue of errors that could arise from analysis of isotopologues with low S/Ns. This is because the label free control will contain the lowest signal for peaks derived from natural isotopic abundance and thus it is possible to estimate the LOQ for the lowest abundance isotopologue of interest [36]. \([^{13}C]\)-incorporation has been presented as a percentage of the unlabeled isotopologue for each acyl-CoA that was analyzed (Figures 4 & 5).

Statistical analysis

Descriptive statistics were calculated. Unless otherwise specified, data have been expressed as means ± SD.
Normality of the data was examined by Shapiro–Wilk test. Comparison of the labeling between patients with FRDA and unaffected controls were made by a two sample $t$-test with equal variance assumption (or unequal variance when appropriate). All tests were two-sided. To evaluate the combination of $M + 2$ labeling from $^{[13C_6]}$-glucose for acetyl-CoA and $M + 2$ labeling from $^{[13C_{16}]}$-palmitate for $\beta$HB-CoA, a logistic regression analysis was performed. Sensitivity and specificity were computed at each possible cut-off value based on the predictive score estimated from the logistic model, and a receiver operating characteristic (ROC) curve was generated. The area under the ROC curve was computed to measure the ability of the combined biomarkers in correctly classifying those with and without FRDA. A higher AUC value for the ROC indicated better discrimination. Statistical analyses were conducted using GraphPad Prism v 6.0 for Mac OS X and STATA v13.0 for Windows.

**Results**

**Glucose labeling**

There was extensive labeling in the isotopologue of acetyl-CoA 2 Da higher in mass ($M + 2$) than the endogenous acetyl-CoA ($M + 0$) in platelets treated with $^{[13C_6]}$-glucose (5 mM) that were obtained from both FRDA and unaffected controls (Figure 4A). This resulted from the action of pyruvate dehydrogenase on pyruvate derived from $^{[13C_6]}$-glucose (Figure 2). FRDA platelets exhibited significantly reduced $M + 2$ labeling in acetyl-CoA (mean $\pm$ SD: 7.0 $\pm$ 3.9%; $p < 0.05$) when compared with acetyl-CoA labeling (12.3 $\pm$ 5.5%) in control platelets (Figure 4A & E). This finding is consistent with decreased utilization of $^{[13C_6]}$-glucose for the pyruvate dehydrogenase-mediated formation of acetyl-CoA (Figure 2). The FRDA platelets also showed decreased $M + 2$ labeling of succinyl-CoA (6.3 $\pm$ 6.1%; $p < 0.05$) when compared with control platelets ($10.8 \pm 2.3\%$) (Figure 4B & E). These levels of incorporation were very similar to those observed for acetyl-CoA (Figure 4A) as might be expected from its role as a Krebs cycle intermediate (Figure 2). The formation of $\beta$HB-CoA and HMG-CoA can arise through two different pathways. The first pathway involves thiolase-mediated formation of acetoacetyl-CoA followed by the 3-hydroxy-CoA dehydrogenase-mediated metabolism of acetoacetyl-CoA to $\beta$HB-CoA or HMG-CoA synthase-mediated metabolism of acetoacetyl-CoA to HMG-CoA (Figure 2). The second pathway, which we recently demonstrated to occur in neuronal cells [27], involves conversion of palmitate to butyryl-CoA, which is then further metabolized to $\beta$HB-CoA and HMG-CoA (Figure 2). The first pathway should lead to the formation of $M + 2$ isotopologues of $\beta$HB-CoA and HMG-CoA from $^{[13C_6]}$-glucose; whereas the second pathway should only be observed in these short-term incubations through the formation of $M + 4$ isotopologues from the metabolism of $^{[13C]}$-labeled palmitate (Figure 2). In keeping with these concepts, both $\beta$HB-CoA and HMG-CoA exhibited substantial labeling in their $M + 2$ isotopologues after a 1 h incubation with $^{[13C_6]}$-glucose (Figure 4C & D). The $M + 2$ labeling of HMG-CoA in both FRDA (10.3 $\pm$ 7.3%) and control (16.7 $\pm$ 4.8%) platelets (Figure 4D) was significantly higher than that observed for acetyl-CoA in FRDA (7.0 $\pm$ 3.9%) and control (12.3 $\pm$ 5.5%) platelets (Figure 4A). This suggested that the HMG-CoA pool size was smaller than the other analytes and so

| Labeled precursor | Variable                  | Controls SD | FRDA patients SD | p-value  |
|-------------------|---------------------------|-------------|------------------|----------|
| Subjects enrolled |                          | 10 NA       | 10 NA            | NA       |
| Male (%)          | 60 NA                     | 40 NA       | NA               | NA       |
| Mean age          | 31.4 10.3                 | 32 7.8      | 0.880            |
| Mean GAA repeat length | <30 NA               | 476 NA      | 150 <0.001       |
| $^{[13C_6]}$-glucose | Acetyl-CoA M+2 (%)     | 12.3 5.5    | 7.0 3.9          | <0.020   |
|                    | Succinyl-CoA M+2 (%)     | 10.8 2.3    | 6.3 6.1          | 0.040    |
|                    | $\beta$HB-CoA M+2 (%)    | 8.5 2.9     | 5.1 2.4          | 0.010    |
|                    | HMG-CoA M+2 (%)          | 16.7 4.8    | 10.3 7.3         | 0.090    |
| $^{[13C_6]}$-palmitate | $\beta$HB-CoA M+2 (%) | 14.9 2.2    | 21.6 4.4         | <0.001   |
|                    | $\beta$HB-CoA M+4 (%)    | 18.9 6.1    | 18.3 6.9         | <0.001   |
|                    | HMG-CoA M+2 (%)          | 23.4 4.9    | 29.8 12.8        | 0.150    |
|                    | HMG-CoA M+4 (%)          | 4.8 2.6     | 13.3 4.9         | <0.001   |

$\beta$HB: $\beta$-hydroxybutyryl; FRDA: Friedreich’s ataxia; HMG: 3-hydroxy-3-methylglutaryl; NA: Not applicable.
Figure 3. Representative chromatograms of the acyl-CoA thioesters analyzed in this study after incubating normal platelets with 5.5 mM [13C5]-glucose. (A) HMG-CoA M + 0, M + 2, M + 4, and M + 6. (B) Succinyl-CoA M + 0, M + 2 and M + 4. (C) βHB-CoA M + 0, M + 2, and M + 4. (D) Acetyl-CoA M + 0 and M + 2.

βHB: β-Hydroxybutyryl; HMG: 3-Hydroxy-3-methylglutaryl.

would be more readily labeled due to the lower concentration of unlabeled metabolite. M + 2 labeling in FRDA platelets from [13C6]-glucose for both βHB-CoA (5.1 ± 2.4%; p < 0.01) and HMG-CoA (10.3 ± 7.3%; p < 0.05) was significantly lower than the M + 2 labeling of βHB-CoA (8.5 ± 2.9%) and HMG-CoA (16.7 ± 4.8%) in control platelets (Figure 4C–E).

Palmitate labeling
To determine if fatty acid metabolism is affected in FRDA, platelets were treated with 100 μM [13C16]-palmitate for 1 h (Figure 1). This also made it possible to monitor the butyryl-CoA pathway for the formation of M + 4 isotopologues of βHB-CoA and HMG-CoA (Figure 2). Analysis of [13C]-isotopic enrichment in acyl-CoAs revealed that labeling from [13C16]-palmitate into the Krebs cycle occurred as we had shown previously [24]. In control platelets, the M + 2 incorporation into acetyl-CoA was 22.0 ± 6.0% and for succinyl-CoA was 20.6 ± 6.3%. However, unlike incorporation from [13C6]-glucose into these Krebs cycle acyl-CoA thioesters there was no significant difference in incorporation of [13C16]-palmitate between FRDA and control platelets (data not shown). Direct
thiolase-mediated formation of HMG-CoA from acetyl-CoA (Figure 2) resulted in substantial incorporation of [13C6]-palmitate into M + 2 for both βHB-CoA (14.9 ± 2.2%) and HMG-CoA (23.4 ± 4.9%) in control platelets (Figure 5A & B). In contrast to the [13C6]-glucose results, there was a significant increase rather than a decrease in M + 2 labeling from [13C6]-palmitate for βHB-CoA (21.6 ± 4.4%; p < 0.001) compared with controls (Figure 5A & C). There was also increased incorporation of [13C6]-palmitate into M + 2 of HMG-CoA (29.8 ± 12.8%), although this did not reach statistical significance when compared with controls (23.4 ± 4.9%) (Figure 5B). There was also significant incorporation of [13C6]-palmitate into M + 4 of βHB-CoA (18.5 ± 6.9%) and HMG-CoA (4.8 ± 2.6%) in control platelets (Figure 5B & C), through the second

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**Figure 4. [13C6]-glucose labeling was decreased in Friedreich’s ataxia platelets.** (A) Acetyl-CoA. (B) Succinyl-CoA. (C) βHB-CoA. (D) HMG-CoA. (E) Boxplots depicting significantly different enrichments from glucose. Mean percent labeling plotted with SD, *p < 0.05 for ten patients with FRDA and ten controls (students unpaired t-test). βHB: β-hydroxybutyryl; FRDA: Friedreich’s ataxia; HMG: 3-hydroxy-3-methylglutaryl.
pathway involving intermediate formation of \([^{13}C_4]\)-butyryl-CoA (Figure 2). Interestingly, incorporation of \([^{13}C_{16}]\)-palmitate into M + 4 for HMG-CoA in FRDA platelets (13.3 ± 4.9%; p < 0.001) was significantly increased when compared with controls (4.8 ± 2.6%) (Figure 5B & C). However, the incorporation of \([^{13}C_{16}]\)-palmitate into M + 4 of \(\betaHB\)-CoA in FRDA platelets (18.5 ± 6.9%) was almost identical with that observed for controls (Figure 5A). This unexpected finding suggests that there is compartmentalization of some of the metabolites and that the pool sizes of these individual compartments dictate the actual incorporation.

Correlation with GAA repeat length
The decrease in M + 2 acetyl-CoA labeling from \([^{13}C_6]\)-glucose in FRDA platelets showed a negative correlation (\(r^2 = 0.39\); \(r = -0.625\); \(p = 0.054\)) with GAA repeat length (Figure 6A), a genetic marker of disease severity for FRDA [37]. Analogously, the increased labeling into \(\betaHB\)-CoA (M + 2) from \([^{13}C_{16}]\)-palmitate demonstrated a positive correlation (\(r^2 = 0.51\); \(r = 0.717\); \(p = 0.020\)) with GAA repeat length (Figure 6B). Importantly, neither of these markers correlated with patient age (data not shown).

Sensitivity & specificity
Generation of a ROC curve by combining decreased M + 2 labeling into acetyl-CoA from \([^{13}C_6]\)-glucose and increased M + 2 labeling into \(\betaHB\)-CoA from \([^{13}C_{16}]\)-palmitate revealed an AUC of 0.89 (95% CI: 0.73–1.00) (Figure 7). This combination of biomarkers gave high sensitivity and specificity (> 80%) under a wide range of scenarios as a result of the precision and accuracy with which the isotopologue analyses could be conducted.
sensitivity of 80% and specificity of 100% were observed at the optimal cut-off value for the current samples. These data provide confidence that the methodology will provide a useful biomarker reflecting the biochemical abnormalities of FRDA and the potential response of dysfunctional metabolic pathways to therapy.

Discussion
In the present study, we have shown that FRDA platelets exhibit differential mitochondrial metabolism when compared with healthy controls. Many biomarker studies focus on absolute quantification of analytes to monitor phenotypic differences. A critical shortcoming of such approaches is the need for external normalization, which often introduces a significant degree of variability. While LC–MS analysis utilizing stable isotope internal standards controls for variability introduced during sample processing and analysis [23], either volume, cell count, protein quantity or factor normalization is required for biological normalization. Isotopologue analysis of samples treated with isotopic tracers circumvents the need for external standardization because the relative levels of isotopic incorporation are normalized to the total analyte signal within a given sample. Thus, the ability to conduct isotopologue assays, which do not require external normalization, simplifies the workflow and removes steps that can introduce high levels of variation. Working with isolated platelets reduces the potential of diluting the isotopic purity of a tracer by highly abundant and variable endogenous metabolites such as blood glucose. There is no evidence that 5.5 mM glucose or 100 μM palmitic acid can activate human platelets, which could have possibly confounded the metabolic studies. In addition, supplementation of human subjects with palmitic acid has no effect on platelet aggregation [38]. Furthermore, no platelet activation was observed during our 1 h incubations with labeled glucose or palmitate. Of note, the platelet metabolic biomarker approach is particularly relevant to promising strategies that systemically increase frataxin levels as it remains to be determined, although isocitrate dehydrogenase, an important Krebs cycle enzyme, is succinylated [46]. The decreased glucose M + 2 labeling of acetyl-CoA in FRDA platelets (Figure 4A) also resulted in decreased incorporation of glucose into βHB-CoA (Figure 5A) and HMG-CoA (Figure 5B), important intermediates in the formation of D-β-hydroxybutyrate (ketone body), sterols and prenylated proteins. It appears that the increased β-oxidation of fatty acids can overcome these defects but cannot compensate for the reduction in acetyl-CoA and succinyl-CoA formation. Based on our results, multiple metabolic pathways dysregulated in FRDA can be monitored using platelets from affected patients as biomarkers. Two of the isotopologue biomarkers, percent M + 2 labeling of acetyl-CoA from [13C6]-glucose and percent M + 2 labeling of β-hydroxybutyryl-CoA from [13C16]-palmitate. The AUC is 0.89 (95% CI: 0.73–1.00). The equation of the ROC curve is 41.74 x (% M + 2 of acetyl-CoA) – 8.67 x (% M + 2 of β-hydroxybutyryl-CoA).

ROC: Receiver operating characteristic.

The decreased conversion of glucose to acetyl-CoA (Figure 4A) is consistent with studies that have shown diminished pyruvate oxidation in FRDA [39]. The lack of a compensatory pathway to acetyl-CoA from fatty acid β-oxidation suggests that decreased histone acetylation could occur in FRDA through decreased cofactor availability similar to our recent observation in tumor cells [40]. This finding is also consistent with epigenetic silencing of frataxin expression that occurs in FRDA [41] as a result of the GAA expansion [4,42], which results in increased DNA [43,44] and histone methylation [45] at

Figure 7. Receiver operating characteristic curve generated by combining the two biomarkers shown in Figure 6. Percent M + 2 labeling of acetyl-CoA from [13C6]-glucose and percent M + 2 labeling of β-hydroxybutyryl-CoA from [13C16]-palmitate. The AUC is 0.89 (95% CI: 0.73–1.00). The equation of the ROC curve is 41.74 x (% M + 2 of acetyl-CoA) – 8.67 x (% M + 2 of β-hydroxybutyryl-CoA).

ROC analysis

Area under ROC curve = 0.89

1 – specificity

Sensitivity

0.00 0.25 0.50 0.75 1.00

0.00 0.25 0.50 0.75 1.00

Figure 6. Percent M + 2 labeling of acetyl-CoA from [13C6]-glucose and percent M + 2 labeling of β-hydroxybutyryl-CoA from [13C16]-palmitate. The AUC is 0.89 (95% CI: 0.73–1.00). The equation of the ROC curve is 41.74 x (% M + 2 of acetyl-CoA) – 8.67 x (% M + 2 of β-hydroxybutyryl-CoA).

ROC: Receiver operating characteristic.

β-oxidation of fatty acids results in the removal of two carbon adjacent atoms and results in the formation of one molecule of FADH2, together with one molecule of NADH [27]. The fatty acid-derived FADH2 and NADH can then be used to maintain an electro-
chemical gradient across the inner mitochondrial membrane, which is necessary for the production of ATP. Therefore, the increased lipid metabolism observed in the FRDA could help maintain cellular homeostasis in times of mitochondrial dysfunction. Taken together, these results suggest that FRDA platelets have a diminished capacity for oxidative phosphorylation, as decreased glucose labeling into acetyl-CoA occurs in response to pharmacologic inhibition of mitochondrial complex I \[26\]. Cell culture studies have shown increased \(\beta\)-oxidation of lipids in response to diminished complex I activity \[27\], supporting the notion that lipid breakdown (to provide reducing equivalents) could be important during times of mitochondrial dysfunction. It is also consistent with the loss of mitochondrial complex I function that has been identified in FRDA \[18\]. Here, we have shown that there is a shift in metabolism toward production of \(\beta\)HB-CoA and HMG-CoA from palmitate in FRDA platelets.

Cardiac muscle is highly dependent on fatty acids for its energy needs \[47\], and would therefore likely be one of the most susceptible tissues to alterations in lipid metabolism in FRDA. Cardiovascular events \[1,3\] are among the most serious life-limiting conditions in patients with FRDA. Therefore, pharmacologic or dietary interventions to help amend such metabolic abnormalities could have therapeutic benefits. For example, medium-chain fatty acids (MCFAs) are able to enter mitochondria independently of the carnitine shuttle system \[48\], making them readily available in times of metabolic stress. In addition to increased lipid oxidation, complex I inhibition also induces a dramatic decrease in medium-chain acyl-CoAs \[27\]. Upregulation of the medium-chain acyl-CoA dehydrogenase has been implicated in FRDA \[49\], suggesting a possible connection with MCFA metabolism. It remains to be seen if MCFAs play a defining role in energy metabolism in FRDA, as other metabolic pathways are likely influenced in FRDA.

Patients with FRDA have variable numbers of GAA repeats in intron 1 of the frataxin gene, which causes epigenetic silencing that results in decreased expression of frataxin mRNA and as well as reduced frataxin protein expression \[42\]. One pathway of epigenetic silencing arises through upregulation of histone lysine methyltransferases resulting in increased methylation of specific lysine residues on histone tails (e.g., H3K9) and inhibition of frataxin gene transcription \[45\]. Coi-
Stable isotopes & LC–MS for monitoring metabolic disturbances in Friedreich’s ataxia platelets

Research Article

Executive summary

Materials & methods
• Platelets from Friedreich’s ataxia (FRDA) patients and healthy volunteers were isolated from whole blood and incubated with two [13C]-labeled metabolic tracers - [13C6]-glucose and [13C16]-palmitate.
• The incorporation [13C]-atoms into different platelet-derived short chain acyl-CoA thioesters isotopologues was determined.

Results
• Isotopologue analysis of acyl-CoA thioesters by LC–MS revealed a marked decrease in glucose incorporation into acyl-CoA thioesters in FRDA platelets compared with controls.
• There was concomitant increase in palmitate-derived acyl-CoA thioester isotopologues.
• The findings were consistent with a combination of decreased glycolysis and increased fatty acid β-oxidation known to occur in FRDA.
• Human platelets served as a surrogate tissue for in vivo biomarker studies.

Future perspective
• Human platelets coupled with stable isotopes and LC–MS will provide rigorous biomarkers that will be useful for monitoring new therapeutic approaches for the treatment of FRDA.

Future perspective
The current biomarkers of FRDA, which involve the quantification of the GAA triplicate repeats in the first intron of the FRX gene or the analysis mutations in the FRX gene have essentially 100% sensitivity and specificity for detection of the disease. Therefore, new biomarkers for the early detection of FRDA are not required. However, the genetic abnormalities in FRDA cannot be modified unless gene therapy is used to increase frataxin levels. This means that the ability to examine dysregulated metabolism in FRDA patients will be extremely useful for monitoring promising therapeutic strategies that systemically increase frataxin levels. The use of platelets coupled with stable isotopes and LC–MS will provide rigorous biomarkers to show that increased frataxin protein expression stimulated by conventional therapeutic strategies (such the use of HDAC inhibitors) can reverse the mitochondrial dysregulation in FRDA. Frataxin activity will be revealed through normalization of the Krebs cycle and related metabolic pathways in the platelets. We anticipate that this will be of great benefit in clinical trials testing agents that are designed to correct the defect in frataxin protein expression. Furthermore, the concept of using platelets as metabolic biomarkers is not limited to FRDA, but may be useful for disease detection for inborn errors of metabolism as well as for other diseases of mitochondrial dysfunction. Finally, the short half-life of platelets in the circulation (typically 100 h) makes it possible to evaluate biochemical effects over relatively small time scales, making this approach amenable to assessing the effectiveness of acute adaptations in response to therapeutic approaches.

Supplementary data
To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.15.118

Author contributions
All authors participated in the design of the study and in analysis of the results. AJ Worth, SS Basu, EC Deutsch and NW Snyder performed the platelet incubations and conducted the LC–SRM/MS experiments. W-T Hwang designed and conducted statistical analyses. AJ Worth, DR Lynch and IA Blair wrote the manuscript with significant input from all of the authors. All of the authors read and approved the final manuscript.

Financial & competing interests disclosure
The authors acknowledge the support of the Penn/CHOP Friedreich’s Ataxia Center of Excellence and NIH grants R21NS087343, P30ES013508 and T32ES019851. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Materials & methods
• Platelets from Friedreich’s ataxia (FRDA) patients and healthy volunteers were isolated from whole blood and incubated with two [13C]-labeled metabolic tracers - [13C6]-glucose and [13C16]-palmitate.
• The incorporation [13C]-atoms into different platelet-derived short chain acyl-CoA thioesters isotopologues was determined.

Results
• Isotopologue analysis of acyl-CoA thioesters by LC–MS revealed a marked decrease in glucose incorporation into acyl-CoA thioesters in FRDA platelets compared with controls.
• There was concomitant increase in palmitate-derived acyl-CoA thioester isotopologues.
• The findings were consistent with a combination of decreased glycolysis and increased fatty acid β-oxidation known to occur in FRDA.
• Human platelets served as a surrogate tissue for in vivo biomarker studies.

Future perspective
• Human platelets coupled with stable isotopes and LC–MS will provide rigorous biomarkers that will be useful for monitoring new therapeutic approaches for the treatment of FRDA.
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