Effects of tocotrienol supplementation in Friedreich's ataxia: A model of oxidative stress pathology

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Short Title: Effects of tocotrienol supplementation in Friedreich's ataxia

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Abstract

Friedreich's Ataxia (FRDA) is an autosomal recessive disorder characterized by impaired mitochondrial function, resulting in oxidative stress. In this study, we aimed at evaluating whether tocotrienol, a phytonutrient that diffuses easily in tissues with saturated fatty layers, could complement the current treatment with idebenone, a quinone analogue with antioxidant properties. *Methods*. Five young FRDA patients received a low dose tocotrienol supplementation (5 mg/kg/day), while not discontinuing idebenone treatment. Several oxidative stress markers and of biological parameters related to oxidative stress were evaluated at time of initiation of treatment and two and twelve months post-treatment. Some oxidative stress-related parameters and some inflammation indices were altered in FRDA patients taking idebenone alone and tended to normal values following tocotrienol supplementation; likewise, a cardiac magnetic resonance study showed some improvement following 1-year tocotrienol treatment. The pathway by which tocotrienol affects the Nrf2 modulation of hepcidin gene expression, a peptide involved in iron handling and in inflammatory responses, is viewed in the light of the disruption of the iron intracellular distribution and of the Nrf2 anergy characterizing FRDA. This study provides a suitable model to analyze the efficacy of therapeutic strategies able to counteract the oxidative stress in FRDA, and paves the way to long-term clinical studies.

Keywords: Friedreich's Ataxia; Tocotrienol; Oxidative stress markers; Inflammation; Lipidomics; Hepcidin.

Impact Statement

Oxidative stress is involved in the pathogenesis of Friedreich's Ataxia (FRDA), a neurodegenerative disease caused by the decreased expression of frataxin. To date no cure is available for FRDA patients. In some countries, FRDA patients assume idebedone in order to counteract the effects of frataxin deficiency. We demonstrate that idebenone treatment alone is not able to abrogate oxidative stress in FRDA patients, whereas the combined treatment with tocotrienols might be

more efficient and perhaps produce clinical improvement. In fact, a decrease in oxidative stress and inflammation markers can be seen after two months and is more pronounced after one year of treatment. This is, in our opinion, valuable information for clinicians, since idebenone is the treatment of choice for FRDA patients in some countries.

Introduction

Friedreich's Ataxia (FRDA, OMIM #229300) is one of the most common hereditary ataxias, with a clinical onset around puberty. It is an inherited autosomal recessive disorder characterized by the degenerative atrophy of the posterior columns of the spinal cord, leading to progressive neurologic disability, sensory loss, and muscle weakness. Hypertrophic cardiomyopathy is rather common, affecting 45–63% of FRDA patients and can cause premature death. In some patients, diabetes mellitus, scoliosis, and foot deformity are also observed[1,2] .

FRDA is caused by the partial deficiency of frataxin, a 210-amino acid protein, which localizes in the mitochondria. Most patients are homozygous for an unstable GAA trinucleotide expansion in the first intron of the gene, FXN, located on chromosome 9q13[3]. The expanded GAA repeats cause a decrease in transcription, owing to an abnormal chromatin conformation. Frataxin function is somewhat unclear, although there is general agreement that it is involved in cellular iron homeostasis; in particular, it participates in the mitochondrial synthesis of iron-sulfur containing proteins and in heme synthesis[4]. Its deficiency leads to multiple enzyme deficits, mitochondrial dysfunction, deficit of ATP production and oxidative damage[5-7]. In addition, in vitro studies with Schwann cell lines which underwent FXN knockdown[8] showed that frataxin deficiency causes a strong activation of inflammatory pathways. Numerous studies have pointed out a hypersensitivity to oxidative stress (OS) in cell models and FRDA patients and some oxidation biomarkers in urine and blood samples from FRDA patients[9-13]. OS is a major cause of neuron degeneration and death, although it is unclear whether, in FRDA patients' cells, OS is a consequence of the observed iron accumulation in mitochondria, coupled to an iron cytoplasmic depletion (for an in-depth

discussion, see reviews[14, 15]. Some authors have suggested that frataxin deficiency is linked to oxidative stress condition mainly because Nrf2-driven responses appear to be impaired[16]. In addition, altered lipid metabolism is considered a hallmark of FRDA pathophysiology. The mechanisms by which the lipid metabolism is impaired in FRDA remains uncertain. It has been suggested that frataxin deficiency and the consequent dysfunction of the mitochondrial respiratory chain lead to the alteration of main pathways involved in fatty acid synthesis and degradation[17]. Based upon these considerations, we suggest that FRDA patients might benefit of treatment with antioxidants. Italian FRDA patients are currently treated with idebenone, a short chain quinone analogue, which acts as a free-radical scavenger; the rationale for treatment is based on the consideration that mitochondrial energy shortage and oxidative stress are critical factors for FRDA in neuronal cells[18,19]. Notwithstanding the therapeutic benefits of idebenone in improving neurological functions[20] and in the prevention of cardiopathy[21], patients still experience a progressive worsening of their condition. The present study was devised in order to understand whether idebenone is effective in abating OS and whether patients might better benefit of a combined therapy with another antioxidant, tocotrienol.

Tocotrienols are natural forms of vitamin E, which are found in several natural sources, including palm oil, rice bran oil, coconut oil, cocoa butter, barley and wheat germ[22]. Compared with tocopherols, tocotrienols have unsaturated isoprenoid side chain with three double bonds. , which increases their efficiency in diffusing in tissues with saturated fatty layers[22,23] and enhances their *in vitro* and *in vivo* antioxidant, anticancer, neuroprotective and cardioprotective activities[24,25]. Tocotrienols have quite different qualities compared to tocopherols: they are characterized by a distinct metabolism and different routes of tissue delivery and storage[26] and by their ‘‘chain-breaking’’ ability to neutralize peroxyl radicals through the formation of phenoxyl radicals, which are relatively stable Tocotrienols also attenuate oxidative-nitrosative stress and the inflammatory cascade in experimental models of alcoholic and diabetic neuropathy[24]. In FRDA patients,

tocotrienol leads to a specific and significant increase of FXN-3 expression, a minor, functionally active, FXN isoform[27]. Tocotrienols increase, in vitro, transcription of IKBKAP gene, mutated in the neurodegenerative genetic disorder Familial dysautonomia, , leading to an increase in the correctly spliced transcript and normal protein[28]. Moreover, tocotrienols possess in vitro antifibrotic properties[29], and recently, the mechanisms by which δ-Tocotrienol reduces inflammation by inhibiting TNF-α-induced activation of NF-κB and LPS-stimulated IL-6 have been elucidated[30]. Finally, tocotrienols are able to modulate the sensitivity to ferroptosis by inhibiting lipid peroxidation[31]. Ferroptosis is a form of regulated iron-dependent oxidative cell death, recently described in FRDA fibroblasts[32].

Recently, EPI-743 (alpha-tocotrienol quinone; vatiquinone), a last-generation tocotrienol variant, was tested in FRDA patients and found to improve neurological and disease progression parameters following a 24-month treatment[33].

Here we report data from a pilot study carried out with five FRDA patients, taking idebenone and a tocotrienol mixture (5 mg/kg/day) for one year, where biomarkers related to OS were compared with data obtained from age- and sex-matched healthy controls. Patients' blood samples were collected of initiation of treatment and two and twelve months of post-treatment, however, due to a freezer failure some biological evaluations could not be carried out for the twelve-month treatment. Patients were also studied by conventional and advanced Cardiac Magnetic Resonance (CMR) techniques, including a CMR cardiac study, completed by Gadolinium perfusion late enhancement evaluation[34]. Improvements observed in OS biomarkers and, to a lesser extent, in clinical outcomes suggested that adjunctive treatment with tocotrienols might be worthy of a study on a wider scale.

Materials and Methods

Patients and treatment

Seven FRDA patients and seven healthy volunteers of similar age were enrolled by the Child Neurology and Psychiatry Department of the IRCCS Istituto delle Scienze Neurologiche of Bologna. FRDA diagnosis was performed according to Harding diagnostic criteria[35] and confirmed by genetic testing, which showed that all FRDA patients were homozygous for GAA triplet expansion in the FXN gene. The level of clinical disability was quantified according to ICARS scale[36]. Data are summarized in Table 1.

All patients were treated with idebenone (5 mg/kg body weight /day) throughout the study. FRDA patients 1-5 shown in Table 1 assumed tocotrienol (5 mg/kg body weight/day) for twelve months. A venous blood sample was obtained before the initiation of tocotrienol ("FRDA"), following 2 months ("FRDA post-2 months") and following the full term of treatment ("FRDA post-1 year"). Biomarkers were compared in seven age-and-sex matched healthy control subjects ("CTR"). Controls were not affected by any neurological or psychiatric disease, did not take antioxidant and/or vitamin supplements or medications potentially affecting oxidative status. The Ethical Committee of Regional Health Service approved the study (1635- 08092011).

At the beginning of the study and following 1 year of treatment with tocotrienols, patients were evaluated according to the ICARS scale and assessed with Conventional Cerebral Magnetic Resonance Imaging (MRI), MRI study of cerebral protons (^{1}HMRS), Muscle Phosphorus Spectroscopy Study (^{31}PMRS), Diffusion-Weighted Imaging (DWI), Diffusion Tensor Imaging (DTI), and Cardiac Magnetic Resonance Imaging (CMR). For details, please see ref. 34.

Tocotrienol

The toxicity level of tocotrienols for humans is presently undetermined, however, they are deemed to be safe as for long-term use in nutrition. The no-observed-adverse-effect level (NOAEL) in rats is estimated at 120–130 mg/kg body weight/day[37] or even more (303 mg/kg/day for males, and 472 mg/kg body weight/day for females)[38]. No adverse effects were observed in humans with consumption of 240 mg/kg body weight/day for 18-24 months[39]. The dose administered in the

present study was 5 mg/kg body weight /day, i.e. well below that reported by Tomeo et al.[39]. Moreover, it was demonstrated by Patel et al.[40] that 400mg/day of tocotrienol oral supplementation, a dose similar to the one used in this study, was sufficient to detect the presence of tocotrienol isoforms in blood, heart and brain after 80 days, 155 days (range 30-443) and 261 days (range 78-672 days) respectively.

No adverse effects were reported due to tocotrienol consumption. The tocotrienol mixture was developed by Ambrosialab s.r.l., a spin-off company of the Ferrara University, Italy. The mixture is mainly constituted by a Palm Oil (Elaeis Guineensis) phytocomplex, prepared as soft gel capsule formulation under the name OXI-3 (internal ref. name ALAB103), whose composition is reported in Table 2, containing tocotrienols and tocopherols in the enantiomerically pure natural form. Dose, composition of tocotrienol mix, and time of treatment used in this study were in the same ranges as those reported in ref. 40.

Blood sample treatment

Fifteen milliliters of blood were drawn and collected in a test tube containing EDTA. An aliquot of whole blood (1 mL) was used for lipidomic analysis; the remaining part was used to obtain the blood components. Following centrifugation of blood at 1000 x g for 10 minutes, plasma was collected, aliquoted in sterile tubes and stored at -20°C. Peripheral blood mononuclear cells (PBMCs) and erythrocytes were obtained by density gradient centrifugation with Ficoll (Histopaque 1077, Sigma, St.Louis, Mo) after dilution (1:1) with sterile Phosphate Buffered Saline (PBS). PBMCs were collected, lysed in 1 mL Trizol® Reagent (Invitrogen, Milan, Italy) and stored at -80°C for subsequent RNA extraction.

GSH and GSSG analysis

High-Performance Capillary Electrophoresis was used to analyze the levels of glutathione (GSH) and glutathione disulphide (GSSG) in plasma samples according to Raggi et al.[41] and to Kong et

al.[42]. The equipment included an automated Agilent 3D CE Instrument (Palo Alto, Ca) electrophoresis system with a DAD detector (operated at 200 nm) and controlled by a personal computer.

Briefly, plasma samples were deproteinized with acetronitrile, then analyzed with transient pseudo-isotachophoresis. An uncoated fused silica capillary (20 cm x 75 µm internal diameter) was used. Runs were carried out with 300 µM phosphate buffer at pH 7.4, with 10 kV separation voltage. Samples were pressure injected for 40 s, so that the sample length reached 25% of the effective capillary length, producing a 15-20 fold increase in sensitivity.

Carbonyl group evaluation

Plasma sample preparation

Plasma albumin/IgG-depletion was performed according to the protocol described in Guidi et al. [43] by using the Blue Albumin and IgG Depletion kit (PROTBA-1KT, Sigma-Aldrich, France) following the manufacturer's instructions. The albumin/IgG depleted plasma samples were stored at -20°C before use. Protein concentration was measured using the Bradford kit (Bio-Rad Laboratories, Hercules, CA).

Two-dimensional ~~polyacrylamide~~ gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis was performed under the same conditions described previously[43]. Briefly, protein plasma samples were diluited in 125 µL of a lysis buffer containing 8 M urea, 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM dithiothreitol (DTT) and 0.2% of appropriate IPG buffer (GE Healthcare, USA). For each subject, at least three samples were run in order to assess biological and analytical variation. Immobilized non linear pH gradient strips (pH 4-7; 7 cm length IPG strips; GE Healthcare, USA) were rehydrated at 16°C with lysis buffer with 0.2% carrier ampholyte. Isoelectrofocusing (IEF) was performed using the Ettan™ IPGphor™ system (GE Healthcare, USA) at 16°C following electrical conditions described previously[43]. Before the second dimension run, the IPG strips were incubated

with two different equilibrating buffer solutions. The second dimension was applied on 9-16% polyacrylamide linear gradient gels at 40 mA/gel and 10°C until the dye reached the lower end of the gel. The Coomassie gels were stained with colloidal blue Coomassie G-250.

Derivatization of protein carbonyls and DNP immunostaining

Protein carbonyls analysis was assessed according to the protocol described previously[43]. In particular, the IPG strips (oxyblot) were incubated in 2 N HCl with 10 mM DNPH (2,4-dinitrophenylhydrazine, Sigma-Aldrich, France) at 25°C for 20 min and then washed to remove the excess DNPH. DNPH-Stained IPG strips were used for the second dimension and, subsequently, the plasma proteins were blotted into a PVDF membrane as described previously[44,45]. The PVDF membranes were incubated overnight at 4°C with the DNP IgG antibody (D9781, Sigma-Aldrich, France) in PBS buffer containing 3% non-fat dry milk (Bio-Rad Laboratories), then incubated with a goat anti-rabbit IgG/HRP conjugate (1:3000) secondary antibody for 1 h at room temperature. Spot detection was carried out using the Immobilon Western Chemiluminescent AP substrate (Millipore) according to manufacturer's instructions.

Image acquisition and analysis

Two dimensional Coomassie-stained gels and oxyblots were scanned using an Epson expression 1680 PRO scanner; the acquired images were analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare, USA). For each subject, a minimum of three technical replicates were performed and only the spots present in all the replicates were taken into consideration for subsequent analysis. The intensity of carbonylated spots on the oxyblots was normalized to the Comassie gels as previously reported[43]. The normalized oxyblots obtained from all patients before therapy were then compared with the oxyblots obtained from patients after therapy (2 months and 1 year) and from healthy controls.

Oxygen Radical Absorbance Capacity (ORAC) assay.

Total antioxidant activity was evaluated by the ORAC assay according to a method modified by us [see Pessina et al.[46], and references quoted therein]. In the final reaction mixture (0.2 mL total volume), plasma samples were initially mixed with fluorescein sodium salt (85 nM). The reaction was initiated by the addition of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH), a peroxyl radical generator which targets the fluorescein. A calibration curve (10, 20, 30, 40, 50 µM) was made with a standard solution of Trolox, a water-soluble analogue of vitamin E. The fluorescence intensity was monitored at 37 °C, every 5 min for 30 min using Fluoroskan Ascent FL® (Thermo Fisher Scientific, Inc. Waltham, MA) (excitation at 485 nm, emission at 538 nm). The ORAC values, obtained by subtracting the areas under the quenching curve of fluorescein of the blank from that of each sample, were expressed as Trolox equivalents (TE), pH = 7.4. All the experiments were performed with three replicates.

Lipidomic analysis

Lipidomic analysis was performed as reported previously [47]. Briefly, plasma was separated from the whole blood by centrifugation; erythrocytes were lysed and then centrifuged in order to obtain plasma membranes. The extraction of phospholipids from red blood cell membranes was performed according to the method of Bligh and Dyer[48]. The total phospholipid fraction was incubated with KOH/MeOH solution (0.5 M) for 10 min at room temperature, and fatty acid methyl esters were subsequently extracted with n-hexane. Following gas chromatography, all fatty acids and their isomers were identified by comparison with commercially available standard markers and with a custom-made library of geometrical trans MUFA and PUFA obtained by thiyl radical-catalysed reaction of naturally occurring lipids[49]. The amount of individual fatty acids in erythrocyte membranes was expressed as percentages of the total fatty acids identified. In particular, we calculated the percentages of the main saturated and unsaturated residues of phospholipids, the sums of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA n-3 and PUFA n-6) fatty acid residues, and some indicative ratios (SFA/MUFA; Palmitoleic acid-

C16:1/ Palmitic acid-C16:0; Oleic acid-C18:1/ Stearic acid-C18:0).

The Peroxidation Index (PI)[50] was determined from the percentages of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic fatty acids according to the following formula:

PI = (% Monoenoic x 0.025) + (% Dienoic x 1) + (% Trienoic x 2) + (% Tetraenoic x 4)

+ (% Pentaenoic x 6) + (% Hexaenoic x 8).

The Unsaturation index (UI)[50], also known as the index of hydrogen deficiency, was calculated from the number of double bonds per number of unsaturation of each fatty acid, according to the following equation:

UI = (% Monoenoic x 1) + (% Dienoic x 2) + (% Trienoic x 3) + (% Tetraenoic x 4)

+ (% Pentaenoic x 5) + (% Hexaenoic x 6).

RNA separation and quality control

Total RNA was obtained from the Trizol® lysates following the manufacturer's instructions[51]. RNA quality was assessed as described previously [27]. Briefly, the integrity of RNA was confirmed by visualization of the 28S and 18S band sharpness. For removal of genomic contaminating DNA from RNA samples, a RNase-free Deoxiribonuclease I (DNase I) (Amplification Grade DNase I, Sigma, St. Louis, Mo) treatment was performed. After treatment, the absence of genomic DNA was confirmed by PCR analysis with specific primers for HSP70 promoter (Table S1). RNA concentration was measured by spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, England). Equal amounts of total RNA were reverse-transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas International Inc., Thermo Fisher, Burlington, Ontario) following the manufacturer's instructions.

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qPCR) was performed in triplicate in a BioRad CFX96 real-time thermal cycler using the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA). Data were normalized to the housekeeping genes Beta-Actin and GADPH. The primer sequences are reported in Table S1. Primers were chosen with the help of the *PRIMER3* and *AMPLIFY* free software; whenever possible, primers were designed so as to span an exon–exon junction and they were obtained from Sigma-Genosys (Sigma-Aldrich, St. Louis, Mo). Melting curve analysis followed by agarose gel electrophoresis confirmed primer specificity of PCR products. Data were analyzed with the $2^{-\Delta\Delta CT}$ method[52], taking into account the efficiency of the real-time PCR reaction[53] which was between 95% and 105%. The software package CFX Manager™ (Bio-Rad Laboratories) and qbase plus (http://www.biogazelle.com/) were used for data calculation and statistical analysis. Data were expressed as fold change with respect to controls ± confidence interval.

Statistical analysis

The Shapiro-Wilk test was applied to assess normalcy of the data. Depending on data distribution, non-parametric (Kruskal-Wallis) or parametric (ANOVA) tests were used for assessing statistical significance of results. Benjamini and Hochberg False Discovery Rate (FDR) was applied to correct *p*-values thus obtained. In order to compare 2 sets of data, non-parametric (Wilcoxon-Mann-Whitney) or parametric (two-tailed unpaired Student's *t*) tests were used. For all analysis, statistical significance was set at $p \leq 0.05$.

Results

Patient's outcomes

The five patients with FRDA participating in the study constituted a group with heterogeneous clinical and genetic parameters. The neurological evaluation, represented by the total score on the ICARS scale and repeated after one year of treatment with tocotrienols, showed a slightly worsening trend, in accordance with the slow progressive trend generally observed for FRDA.

Among the instrumental examinations carried out, the only one to demonstrate slight variations after one year of treatment was the Cardiac Magnetic Resonance Study (CMR). In fact, the tests carried out after one year of treatment showed a trend to a slight reduction in the extent of the myocardial mass characterized by myocardial damage (positive to late enhancement), which seemed to vary in a directly proportional relationship with the cardiac mass, and more specifically with hypertrophy. However, this figure was not statistically significant, most likely due to the small number of patients studied (Table 3).

GSH and GSSG content of plasma

The mean GSH content of FRDA patients' plasma was 2.5 ± 0.97 μM, a value not significantly different from that of healthy controls. However, patients were found to have a much higher (almost 9 fold) plasma concentration of GSSG, therefore their GSH/GSSG ratio was significantly lower than in controls (Figure 1). The two-month tocotrienol treatment caused a marked and significant increase of the GSH/GSSG ratio, due to both an increase in GSH and a decrease in GSSG content. It should be noted that GSH/GSSG ratio, a useful indicator of the plasma oxidative stress, is more sensible than the absolute amounts of GSH and GSSG[54].

Carbonyl groups

Carbonyl groups (*i.e.* aldehyde or ketone groups) are useful markers of metal-catalyzed protein oxidation. They are formed through the oxidation of some amino acids[55] as a consequence of OS. For each patient, we obtained a pattern of total plasma proteins stained in the 2-DE gels with Coomassie and a pattern of oxidized proteins recognized by western blot analysis. For each plasma protein sample, 2-D-oxyblots were compared with Coomassie-stained 2-D-gels for normalization purpose and for evaluation of the oxidation levels.

Figure 2 A shows representative images of the oxyblots obtained from the plasma; Figure 2 B shows the corresponding Coomassie-stained gels indicating that the same amount of protein had been separated in the different gels. Experimental reproducibility was verified by performing

several replicates of random samples. The profiles of the five FRDA patients were remarkably similar as for the total amount of oxidized proteins as well as for the presence of proteins with the same apparent molecular weight. About 400 protein spots were identified in the Coomassie-stained 2-D gels, as shown in the representative gel (Figure 2 B). Overall, the pattern of protein spots was similar in the plasma samples of normal subjects, of FRDA patients before therapy and of the same patients after two-month and one-year tocotrienol treatment. However, the comparison of the 2-D electrophoresis gels from normal control subjects and from FRDA patients showed that excess carbonyl groups are clearly present in the patient samples; moreover, the decrease of carbonylated proteins upon two-month tocotrienol treatment and their almost complete clearance upon one-year therapy was evident.

In Figure 2 A, six different spots in the oxyblot are marked, which greatly decrease in carbonylation level with tocotrienol treatment. The intensity of these carbonylated spots was expressed as the Mean ± SD of normalized percentage of oxyblot spot volumes, calculated according to the formula reported in ref. 43 and reported in the histogram of Figure 3. Since oxidation does not appear to affect all proteins at the same extent, the identification by means of MALDI-TOF analysis of these carbonylated proteins is currently under study.

The decrease in anti-DNP reactive proteins was already significant following 2-month tocotrienol treatment (p=0.0498) and even more marked following 1-year tocotrienol treatment (p=0.0004). After 1-year tocotrienol, the amount of carbonylated proteins, though highly reduced, was still higher in plasma from FRDA patients than in controls (p=0.04324).

Oxygen Radical Absorbance Capacity (ORAC)

In Figure 4, the ORAC values of controls and of FRDA patients are compared. Tocotrienol treatment for two-months did not increase the plasma oxygen radical absorbance capacity of FRDA patients, whereas one-year-long supplementation was able to increase significantly (p=0.0454) ORAC values.

Lipidomics

Lipidomic analysis was carried out on only four out of five FRDA patients and on healthy controls; control values were comparable with those of healthy subjects with mean age (25 ± 10 years) or younger[56]. Red blood cell membrane fatty acid composition is representative of that of other important body tissues, such as brain[57], liver, heart and kidney[58].

The complete analysis is reported in Table S2, while Figure 5 shows some relevant results. In particular, patients displayed an excess (p=0.025) of the saturated lipid stearic acid (18:0), which, remarkably, decreased to normal levels as a consequence of the two-months tocotrienol treatment (p=0.044). However, the statistical significance of ANOVA for stearic acid was not maintained after False Discovery Rate correction. The increase of stearic acid could be due to the *de novo* synthesis of saturated fatty acids, which is the first step in the regeneration of the lipid pools after OS. Two indices of inflammation were found to be increased in FRDA patients, namely arachidonic acid (20:4), a precursor of a number of inflammation mediators, which tends to decrease towards normal levels with tocotrienol treatment and the ω6/ω3 ratio, an index of the balance between the prevalently inflammatory omega-6 pathway and the anti-inflammatory omega-3 pathway[59]. The arachidonic acid content can be correlated with the inflammatory status since, when released from the membranes, arachidonic acid initiates the eicosanoid cascade[59]. Worth nothing, the Peroxidation Index was found to be similar in controls and in FRDA patients. This value depends on amount of PUFA residues and reflects the propensity to peroxidation.

Gene expression of some antioxidant enzymes and inflammation molecules

Gene expression was evaluated by qPCR in leukocyte extracts, in order to study the expression of a number of antioxidant enzymes not otherwise evaluable. The reaction efficiency was between 0.95 and 1.05 for all genes and both the melting curve and the gel electrophoresis showed that a single product was amplified in the PCR, thus demonstrating that the primers had been correctly chosen. Figure 6 shows the results of the gene expression study of the following

genes: Thioredoxin Reductase 1, (TXNRD1); Nuclear Factor, Erythroid 2 like 2 (NRF-2); Superoxide Dismutase-1 (SOD-1), Superoxide Dismutase-2 (SOD-2), Catalase (CAT), Glutathione Peroxidase-1 (GPX-1), Glutathione Reductase (GSR), Hepcidin (HAMP), Tumor Necrosis Factor-alpha (TNF-α), Interleukin-6 (IL-6).

The mRNA expression of TNXRD1, a selenoprotein thioredoxin reductase, and of Nrf2, the major regulator of cellular responses to OS, were significantly upregulated in FRDA patients, both before and after two month tocotrienol treatment. The gene expression analysis of the antioxidant enzymes examined does not show significant variations in the comparison between control and FRDA subjects, although a trend for an increased expression in SOD-1 and GSR can be perceived. Following tocotrienol supplementation, a trend of decreased mRNA expression was observed. Moreover, we evaluated the gene expression of GST (Glutathione S-Transferase), which may be expressed independently of Nrf2 activation. Data (not shown) do not show any significant difference between groups.

The expression of HAMP, a peptide hormone that regulates iron homeostasis, was increased in FRDA patients. Such increase was significantly higher after two month-tocotrienol supplementation. The expression of two inflammatory genes, TNF-α and IL-6, was increased in FRDA patients compared to controls; such increase was less enhanced in two-month tocotrienol supplemented patients.

Limitations

This study has several limitations, including that some evaluations were carried out only for the two-month intermediate time, since a freezer failure destroyed some samples obtained at time 12 months, and that this was a pilot study, involving only a small number of patients (N=5). However, it is worth noting that other studies involving the evaluation of therapies in Friedreich's ataxia had comparable sample size[60]. Moreover, in accordance with other results, gene expression data support the involvement of oxidative stress and inflammation in FRDA patients and the

positive effects of tocotrienol supplementation, but, due to the insufficient amount of the PBMCs we could recover, we were not able to confirm these results by protein expression analysis. In any case, the ensuing discussion is meant to show that the present study provided new information that can be valuable for the scientific community.

Discussion

Owing to the severe disruption of Fe-S cluster assembly in key mitochondrial enzymes caused by frataxin deficiency, there is little doubt that significant levels of ROS are generated in FRDA patient's cells during mitochondrial activity, damaging key cellular structures and activities and causing apoptosis in the more metabolically active cells, such as neurons and cardiomyocytes. This undoubtedly qualifies FRDA as an OS-related disease[9] and suggests that counteracting OS generation or OS-induced damages would rescue sensitive tissues[61].

In order to evaluate the efficacy of antioxidant treatments, the appropriate OS markers should be assessed taking into account the peculiarities of FRDA pathobiology. For instance, it has been reported that FRDA patients exhibit increased levels of plasma malondialdehyde[9], a highly reactive compound generated by radical attack to polyunsaturated fatty acids, while other markers of radical damage, such as urinary 8-oxo-2'-deoxyguanosine (8-oxodG), were not consistently found[10]. Similarly, since the Nrf2 system appears to be impaired in FRDA[16], it should not come as a surprise that OS failed to increase the expression of antioxidant genes regulated by Nrf2, whereas TNXRD1, which is upstream rather than downstream from Nrf2[62], and Nrf2 itself, is upregulated in FRDA patients, even if they are treated with idebenone.

Discussing into the detail the individual data,

-The GSH/GSSG ratio, a relevant index of ROS detoxifying ability, was found to be markedly reduced in the plasma of FRDA patients, but tended to normal values following the two-month treatment with tocotrienols.

-Carbonyl groups, an index of OS-induced damage to cell proteins, were markedly increased in FRDA patients compared to healthy subjects, but displayed a reduction by about 30% after two-month tocotrienol treatment and by about 66% following one-year tocotrienol supplementation. Notably, oxidation did not appear to affect all proteins at the same extent, and it would be of interest to identify these through further analyses.

-Plasmatic ORAC displayed a reduction in FRDA patients and was restored to normality only following a one-year tocotrienol supplementation, suggesting that this is not a very sensitive indicator of the OS affecting the whole organism.

- Changes in membrane fatty acid composition, when examined by functional lipidomics tools[63], were suggestive of the presence of both OS and inflammation in FRDA patients, because of the combined increase in stearic acid, arachidonic acid and the ω6/ω3 ratio; noteworthy, stearic acid is related to the production of TNF-alpha, a mediator of inflammatory processes[64]. The two-month tocotrienol supplementation favored the decrease of these indices. It can also be hypothesized that the patients' lipid membrane profile would have displayed a further normalization, should it be evaluated following a longer anti-oxidant treatment. It is worth mentioning that no dietary treatment of patients regarding the essential fatty acid (EFA) intake was made during treatment, thus suggesting that the observed specific fatty acid changes can be likely ascribed to the antioxidant supplementation. Moreover, our results suggest further investigations on the association of tocotrienol supplementation with a diet containing a specific EFA balance.

- Gene expression of antioxidant enzymes, as already pointed out, can be understood by taking into account the inability of chronically stressed cells to activate the Nrf2 driven response[16], which controls the level of SOD1, SOD2, catalase, GPX1, and GSR, among others. On the other hand, TXNRD1 and Nrf2 were upregulated in FRDA patients.

Notwithstanding the limited number of subjects in this study, the data examined so far clearly show that i) FRDA patients are affected by OS, despite long-term idebenone treatment: a result

which accounts for the limited efficacy of idebenone in counteracting the progression of the disease; ii) add-on tocotrienol treatment considerably reduces OS biomarkers even when administered at very low doses and for two months only; moreover, data suggest that a longer tocotrienol treatment may further decrease the OS biomarkers. Since we only evaluated the add-on treatment, we are unable to make any statement about the presence or absence of synergistic effects. On the clinical side, our results suggest that the combined treatment with idebenone and low tocotrienol doses may be beneficial. In effect, one should bear in mind that the efficacy of idebenone in delaying FRDA progression is supported by a relatively weak clinical evidence[65] and has not been previously investigated using OS biomarker studies. Our data support the finding that FRDA patients show increased inflammatory biomarkers, in accordance with the observation by Nachum et al.[66], who described the transcriptional upregulation of inflammatory innate immune response genes. This conclusion is based on the functional lipidomic analysis and on the upregulation of two cytokine mRNAs, TNF-α and IL-6; these indices were less pronounced following the two-month tocotrienol supplementation. Although we unfortunately were unable to assess the data relative to the one-year tocotrienol treatment, this study does show clear relationship between OS and inflammation.

Finally, this study examined for the first time the expression of HAMP mRNA in FRDA PBMCs. For healthy controls, HAMP gene expression showed a trend towards increase, which became highly significant after the two month-tocotrienol supplementation. HAMP is a key regulator of iron homeostasis, both at the systemic and at the cell level[67] and may be involved in the iron dysregulation occurring in cells of FRDA patients[68]. Here, the increase of HAMP expression should be viewed at the light of its upregulation in inflammation, where HAMP plays a double role in preserving the macrophage iron pool and in downregulating the levels of TNF-α and IL-6 expression[69]. An Antioxidant Response Element has been identified in the promoter of the murine HAMP gene[70], suggesting that HAMP might be a pivot in the Nrf2-regulated battery of genes acting

in OS responses, iron toxicity control and anti-inflammatory activities. The increase of HAMP expression following the two-months tocotrienol administration can be tentatively explained by a role for tocotrienols as Nrf2 activators, in a manner similar to that of Vitamins A and C and small polyphenols[71,72] or by their ability to decreas OS, with consequent reduction of the Nrf2 anergy.

Author Contributions Statement

FF, SM and MM participated in the design of the study; CZ, SM and MM acquired funds; AB, AP, PMA, AG, AM, TG, CF, FB, FF and SV performed experiments, interpreted and analyzed the data; AB, PMA, CZ and MM wrote and edited the original draft; all authors reviewed and approved the manuscript.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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Figures Legends

Figure 1. GSH/GSSG ratio in plasma derived from healthy control subjects (CTR) and FRDA patients before (FRDA) and after two month (FRDA Post-2 Months) tocotrienol supplementation. Data are presented as Mean ± SD. ANOVA (p=0.0089) and two-tailed Student's t-test were applied. Significance was maintained after p value False Discovery Rate (FDR) correction. Significance: (*) $p<0.05$.

Figure 2. Representative images of two-dimensional electrophoresis gels and oxyblots of plasma from one healthy control subject (CTR) and one Friedreich Ataxia patient before (FRDA) and after two month (FRDA Post-2 Months) or one-year (FRDA Post-1 Year) tocotrienol supplementation. The pH range was 4 to 7. *Panel A*. Carbonylated proteins were detected by Western blot using a primary antibody directed against DNPH-derivatized carbonyl groups (see methods). Labeled spots represent the spots which showed a higher variation in carbonylation level. *Panel B*. Total proteins were visualized by colloidal blue Coomassie G-250 staining. The Coomassie-stained gels

correspond to the oxyblots shown in panel A.

Figure 3. Levels of plasma protein carbonylation in healthy control subjects (CTR) and Friedreich Ataxia patients before (FRDA) and following two-month (FRDA Post-2 Months) or one-year (FRDA Post-1 Year) add-on tocotrienol supplementation. Values are expressed as Means ± SD of percentage oxyblot spot volumes normalized to the Coomassie-stained spot volumes (%V, where V= integration of OD over the spot area and where % V= V single spot/V total spots. Significance was evaluated by ANOVA (p<0.00001) and maintained after p value False Discovery Rate (FDR) correction. Stars show statistical significance for relevant two-tailed Student's t test comparisons: (*) p<0.05; (**) p <0.01; p (***) <0.001.

Figure 4. Oxygen Radical Absorbance Capacity (ORAC) was analyzed in plasma derived from healthy control subjects (CTR) and FRDA patients before (FRDA) and after two-month (FRDA Post-2 Months) and one-year (FRDA Post-1 Year) tocotrienol supplementation. Data are expressed as µM Trolox/L (Mean ± SD). Significance was evaluated by ANOVA (p=0.0007) and maintained after p value False Discovery Rate (FDR) correction. Stars show statistical significance for relevant Student's two-tailed t test comparisons: (*) p<0.05¸(**) p<0.01.

Figure 5. Relevant lipidomic parameters (Mean ± SD) from healthy control volunteers (CTR) and from FRDA patients before (FRDA) and after two month (FRDA Post-2 Months) tocotrienol supplementation. The content of individual fatty acids in erythrocyte membranes is expressed as percentages of the total fatty acids identified. Significance was evaluated by ANOVA; significance was not maintained for stearic acid after p value False Discovery Rate (FDR) correction. Stars show statistical significance for relevant Student's two-tailed t test comparisons: (*) p<0.05; (**) p<0.01.

Figure 6. Messenger RNA expression of antioxidant and inflammation genes in mononuclear white blood cell extracts from healthy control subjects (CTR) and FRDA patients before (FRDA) and after (FRDA Post-2 Months) two months of tocotrienol by qPCR. Thioredoxin Reductase 1, (TNXRD1); Nuclear Factor, Erythroid 2 like 2 (NRF-2); Superoxide Dismutase-1 (SOD-1); Superoxide Dismutase-2 (SOD-2); Catalase (CAT); Glutathione Peroxidase (GPX-1); Glutathione Reductase (GSR); Hepcidin (HAMP); Tumor Necrosis Factor-alpha (TNF-α); Interleukin-6 (IL-6). Data were normalized with two housekeeping genes, Beta-Actin and GAPDH. For each gene, the normalized expression value of CTR was set to 1, and the other gene expression data were reported to that sample. Data are

expressed as Fold Change ± confidence interval (CI). Parametric analysis was applied for all genes except for GPX-1, HAMP and TNF-α, which were analyzed by non-parametric methods. Significance was maintained after *p* value FDR correction, except for SOD-1. Stars show statistical significance for relevant comparisons: (*) $p<0.05$; (**) $p<0.01$; p (***) <0.001.