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Review

Friedreich's ataxia: Past, present and future

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ABSTRACT

Friedreich's ataxia (FRDA) is an autosomal recessive inherited disorder characterized by progressive gait and limb ataxia, dysarthria, areflexia, loss of vibratory and position sense, and a progressive motor weakness of central origin. Additional features include hypertrophic cardiomyopathy and diabetes. Large GAA repeat expansions in the first intron of the *FXN* gene are the most common mutation underlying FRDA. Patients show severely reduced levels of a *FXN*-encoded mitochondrial protein called frataxin. Frataxin deficiency is associated with abnormalities of iron metabolism: decreased iron–sulfur cluster (ISC) biogenesis, accumulation of iron in mitochondria and depletion in the cytosol, enhanced cellular iron uptake. Some models have also shown reduced heme synthesis. Evidence for oxidative stress has been reported. Respiratory chain dysfunction aggravates oxidative stress by increasing leakage of electrons and the formation of superoxide. *In vitro* studies have demonstrated that Frataxin deficient cells not only generate more free radicals, but also show a reduced capacity to mobilize antioxidant defenses. The search for experimental drugs increasing the amount of frataxin is a very active and timely area of investigation. In cellular and in animal model systems, the replacement of frataxin function seems to alleviate the symptoms or even completely reverse the phenotype. Therefore, drugs increasing the amount of frataxin are attractive candidates for novel therapies. This review will discuss recent findings on FRDA pathogenesis, frataxin function, new treatments, as well as recent animal and cellular models. Controversial aspects are also discussed.

Contents

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1. Clinical features and pathogenesis of Friedreich's ataxia

1.1. *Clinical features*

Friedreich's ataxia (FRDA) was initially described in 1863 by Dr. Nikolaus Friedreich. The major clinical and pathological features of this inherited ataxia are the following: age of onset around puberty, degenerative atrophy of the posterior columns of the spinal cord contributing to progressive ataxia, sensory loss and muscle weakness. Additional features are scoliosis, foot deformity and cardiac symptoms (Table 1). The mutated gene in FRDA (*FXN*) was mapped to chromosome region 9q13 (Chamberlain et al., 1988). Most FRDA patients carry unstable GAA trinucleotide repeat expansions on both alleles within the first intron of the *FXN* gene. Rarely, patients have one GAA expansion on one allele and a loss-of-function mutation on the other allele (Campuzano et al., 1996; Cossée et al., 2000). The identification of the gene has been followed by the demonstration that the GAA repeat expansion generates an abnormal conformation of DNA, with a consequent decrease in the transcription of the *FXN* gene (Campuzano et al., 1996). Frataxin is localized in the mitochondria, but its full function remains unclear. Frataxin might be involved in iron homeostasis (iron–sulfur clusters and heme synthesis), iron storage and detoxification, respiratory control, and resistance to oxidative stress (Rötig et al., 1997; Babcock et al., 1997; Mühlenhoff et al., 2002; Lesuisse et al., 2003; Bulteau et al., 2004). A link to oxidative stress is suggested by the similarity of phenotypes between FRDA and ataxia with vitamin E deficiency (AVED).

Table 1 – Clinical features of Friedreich's ataxia.

Progressive ataxia (legs, arms, and speech)
Areflexia (up-going toe sign)
Dysarthria
Atrophy of the spinal cord (MRI)
Loss of position and vibratory sense
Extensor plantar responses
Heart disease (abnormalities on ECG)
Vision loss
Eye movements (fixation instability)
Hearing loss
Foot deformity
Scoliosis
Diabetes

1.2. *Pathophysiology*

Friedreich's ataxia neuropathology involves spinal cord, peripheral nerves and cerebellum (Pandolfo, 2009). The first site of neurodegeneration is the dorsal root ganglia (DRG) (Fig. 1), with loss of large sensory neurons and posterior columns, followed by degeneration of the corticospinal and spinocerebellar tracts of the spinal cords. Frataxin deficiency also leads to axonal neuropathy with a progressive reduction of large myelinated fibers (Rizzuto et al., 1981; Ouvrier et al., 1982; Said et al., 1986; Morral et al., 2010). Dentate nuclei in the cerebellum are also affected (Fig. 2).

DRG cells are the parent cell bodies of sural nerve fibers and axons in dorsal spinal roots (DSR). *In vivo*, myelin deficit in patients is progressive in the sural nerves (Ouvrier et al., 1982; Morral et al., 2010), whereas myelination appears almost normal in the DSR (Koeppen et al., 2009). Interestingly, dorsal roots and sensory nerves display normal axons counts per unit area (Koeppen et al., 2009), suggesting that the difference in the myelination status may be due to the exclusive origin of dorsal root Schwann cells from boundary cap cells as compared to those in peripheral nerves. Thus, we can speculate that boundary cap-derived Schwann cells may be more efficient in

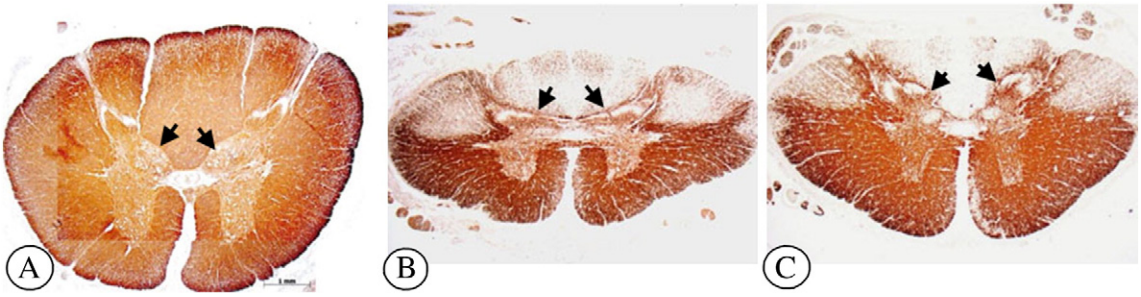

Fig. 1 – Size comparison of thoracic spinal cord in a normal control and patients with FRDA. (A) Normal thoracic spinal cord; (B–C) FRDA. The spinal cord areas are greatly reduced in FRDA, irrespective of age of FRDA onset. Fiber loss in dorsal columns, spinocerebellar, and corticospinal tracts is also comparable. Immuno-stain for MBP. The illustration of the normal spinal cord in (A) was made from three overlapping microphotographs. Bars, 1 mm (Adapted with permission from Koeppen et al., 2010).

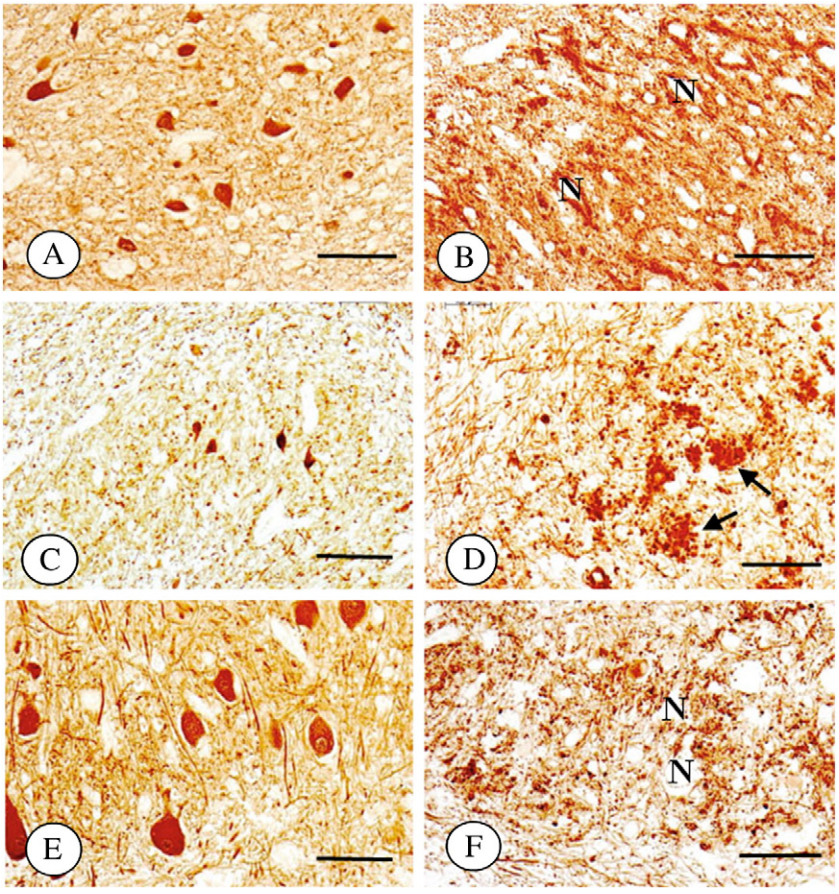

Fig. 2 – Progressive atrophy of the DN in FRDA. (A and B), DN in a 19-year-old man with fatal cardiomyopathy but minimal ataxia, and death after a disease duration of 4 years; (C and D) DN of a 24-year-old man with cardiomyopathy and advanced neurological disability after a disease duration of 14 years. (E and F) normal control. (A), (C), and (E), immunocytochemistry of NSE; (B), (D), and (F) immunocytochemistry of GAD. In the case with rapidly fatal cardiomyopathy ([A]–[B]), numerous neurons are present in the DN though they appear smaller than normal (E). GABA-ergic terminals (B) are preserved, and grumose degeneration is absent. The voids labeled by "N" in (B) and (F) indicate the location of unstained nerve cells. In the patient with comparable age (C and D) but a much longer course, only a few small NSE-reactive neurons remain (C). GAD-immunocytochemistry shows much less reaction product in the neuropil, but many clusters of grumose degeneration are present (arrows in [D]). The DN of the patient with the more serious neurological phenotype (C and D) differs greatly from the normal control (E and F) and the short-term survivor (A and B). GAA trinucleotide repeats: (A and B), 1153/841; (C and D), 1050/700. Bars, 50 μm (Adapted with permission from Koeppen et al., 2010).

myelinating axons than other Schwann cells. However, this is still controversial and need further evaluations. Impairments in Schwann cells in sural nerves could also be explained as a deficit in the interaction between these cells and axons in FRDA patients. This hypothesis is partly supported by *in vitro* evidence that frataxin knock-down severely affects Schwann cells viability and proliferation, while no effects are observed on cultured DRG cells (Lu et al., 2009). Together, these experiments suggest a primary involvement of Schwann cells, with a secondary involvement of flammogens secreted by Schwann cells which should affect DRG cells. However, there is no clear evidence of an inflammatory component in the mechanisms inducing cell death *in vivo* (Koeppen et al., 2009) adding a further question mark to the controversy.

Magnetic resonance imaging (MRI) of the cervical spinal cord in FRDA patients shows degeneration of the posterior and lateral columns (Mascalchi et al., 1994). Neuronal degeneration in the dorsal columns leads to the loss of position and vibration senses. Loss of myelinated fibers and gliosis is characteristic of these regions of the spinal cord (Hughes et al., 1968; Lamarche et al., 1982; Koeppen et al., 2009). Interestingly, the density of small myelinated fibers is generally normal (Said et al., 1986) or only slightly affected (Zouari et al., 1998), with the fine unmyelinated fibers remaining preserved. Onion-bulb complexes may also be observed (Rizzuto et al., 1981; Barreira et al., 1999). Sensory nerve action potentials (SNAPs) loss can be observed electro-physiologically in peripheral nerves, in association with a moderate decrease in nerve conduction velocities (Zouari et al., 1998). This explains the loss of deep tendon reflexes in FRDA patients. The sensory (proprioceptive) component of ataxia in FRDA patients is due to Clarke's column degeneration, with atrophy in the spinocerebellar tracts. Atrophy is also observed in the corticospinal motor tracts, mostly in the distal portions, suggesting a "dying back" degenerative process (Said et al., 1986). Degeneration of the corticospinal tracts results in muscle weakness and extensor plantar responses. In the cerebellum, dentate nuclei are severely affected, but cortex remains normal until Purkinje cell loss (Koeppen et al., 2007). Thus, cerebellar outflow is compromised, adding the cerebellar component of ataxia in FRDA. Additionally, heart, pancreas and skeleton are also affected. The majority of FRDA patients present hypertrophic cardiomyopathy in which ventricular and inter-ventricular septum walls are thickened (Fig. 3). Iron deposits in the myocardium have also been reported (Sanchez-Casis et al., 1976; Lamarche et al., 1993) (Fig. 3). Diabetes results of a combination of peripheral insulin resistance and insulin

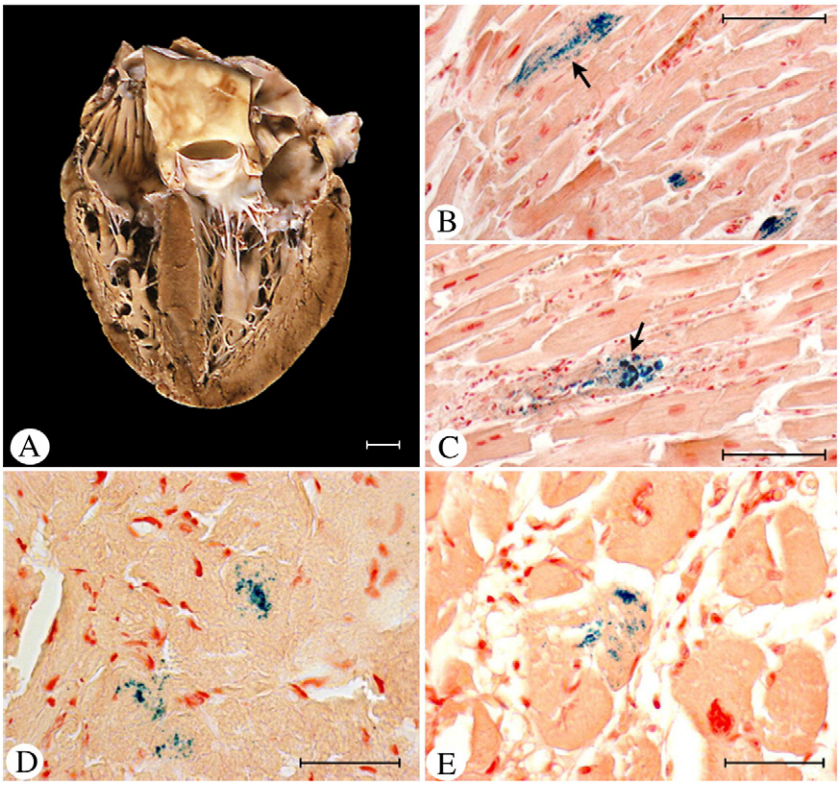

Fig. 3 – Gross pathology of an FRDA heart and light microscopy of iron-reactive granules in sarcoplasm and endomysium. (A) Gross specimen. Left and right ventricular walls are greatly thickened. The myocardium is discolored, lacking the normal dark and homogeneous appearance of normal heart. (B–E) Iron histochemistry. The stain shows finely granular reaction product that lie parallel to the long axis of cardiomyocytes (arrow in B). A cluster of much larger iron-positive granules lies adjacent to or within a necrotic muscle fiber (arrow in C). (D–E) Iron histochemistry of an endocardial biopsy of an FRDA patient at the age of 9 years (D) and a section of the autopsy specimens at the age of 26 years (E). Both sections display iron-positive granules in cardiac muscle fibers, and the frequency of iron-reactive fibers among all cardiomyocytes is similar. Sections illustrated in B–E were counterstained with Brazilin. Markers: A, 1 cm; B–C, 100 μm; D–E, 50 μm (Adapted with permission from Michael et al., 2006).

deficiency. Islet beta cells are lost in absence of signs of autoimmune attack usually associated with type I diabetes (Schoenle et al., 1989).

2. FXN gene

2.1. Structure

FXN gene is composed of seven exons (exons 1–4, 5a, 5b and 6). The 5′ end plus the first exon of the gene encloses an unmethylated CpG island containing rare restriction sites. The major *FXN* transcript (1.3 kb) is composed of five exons, from 1 to 5a, encoding a 210-amino acid protein called frataxin (isoform A). Alternative transcripts contain exon 5b instead of 5a, in presence or not of the non-coding exon 6 (Campuzano et al., 1996) (isoforms B and B1). Most conserved frataxin domains are encoded by exons 4 and 5a. A third transcript is generated by alternative splicing at intron 4 (isoform A1). This transcript, which is less abundant than isoform A, is present in brain, cerebellum, spinal cord, heart and skeletal muscle (Pianese et al., 2002). However, no functional data have been reported regarding the transcripts A1 and B.

The promoter is located in the 1255-bp region extending 5′ from the human *FXN* open reading frame. TATA sequences have not been found in this region, but repetitive retro-elements (*Alu*Jb, *Alu*Y and L2) and mammalian-wide interspersed repeats (MIR) are present. The *Alu* and MIR elements function as enhancers for the promoter (Greene et al., 2005). Further, an E-box element is located within the first intron and can modulate promoter activity (Greene et al., 2007). No differences result between the promoter sequences between FRDA and healthy people. The regulation of the human *FXN* gene is not yet completely known. However, since E-box sequence binds helix–loop–helix family transcription factors, a possible candidate could be the muscle-specific factor (Mt) (Greene et al., 2007). *FXN* expression may also be iron-regulated if we consider that the iron chelator deferoxamine significantly reduces frataxin expression and that addition of ferric ammonium citrate or hemin increases frataxin expression (Sarsero et al., 2003; Li et al., 2008). Moreover, promoter-luciferase constructs cell lines show an iron-dependent regulation of frataxin transcription (Li et al., 2008). Recently two transcription factors, SRF and TFAP2, as well as an intronic element encompassing EGR3-like sequence have been identified. These factors seem to work together in regulating *FXN* gene (Li et al., 2010). In mouse, the *fxn* gene is regulated by the hypoxia-inducible factor 2α (HIF-2α, *Epas1* gene) (Oktay et al., 2007). A reduction in frataxin amount (about 50%) in the liver of the $Epas^{-/-}$ knockout mice has also observed. However, no data are available about a role of HIF-2α in humans.

In conclusion, the elucidation of the mechanisms of frataxin regulation is still a critical point for the investigators. Today, we have no clear demonstration of transcription factors that could directly bind to frataxin promoter and/or of regulatory factors that could enhance or repress frataxin transcription in human. Many hypotheses have been postulated for animal and cellular models. Due to the numerous differences between the mouse and human phenotypes, and due to the difficulty for reproducing a mouse model recapitulating FRDA clinical features, the possibility that different regulatory systems exist among the different species should also be considered.

2.2. GAA triplet repeat expansion

Most FRDA patients have GAA trinucleotide repeat expansions in the first intron of the *FXN* gene. Shorter GAA repeats are present in normal chromosomes and generally carry nine GAA triplets. Alleles with interrupted GAA repeats are generally stable, unlike uninterrupted repeats which undergo hyper-expansion (Cossée et al., 1997; Montermini et al., 1997a; Schöls et al., 1997). The threshold length for instability and expansion is likely between 26 and 44 uninterrupted GAA repeats (Sharma et al., 2002; Pollard et al., 2004). Alleles carrying more than 44 uninterrupted repeats are already associated with FRDA symptoms (Sharma et al., 2004), but GAA expansions in most FRDA patient contain 500–1000 repeats, and up to 1700 (Campuzano et al., 1996; Dürr et al., 1996; Filla et al., 1996; Sharma et al., 2004). Paternal transmission is generally accompanied by a contraction of the repeats whereas maternal transmission may result in expansion or contraction (De Michele et al., 1998; Delatycki et al., 1998). Reversal to a normal length allele is very rare (Bidichandani et al., 1999; Sharma et al., 2002).

Expanded GAA triplet repeats show extensive instability in cultured cells, in peripheral blood leukocytes, in the central nervous system, in the DRG, the spinal cord and the heart (Sharma et al., 2002; De Biase et al., 2007); leading to somatic mosaicism. DRG are highly sensitive to frataxin deficiency (Simon et al., 2004). In DRG, somatic instability starts after early embryonic development and continues after birth, resulting in progressive, age-dependent accumulation of larger GAA triplet repeat expansions (De Biase et al., 2007), suggesting a contribution to disease progression. This pathology is not found in other regions of the central nervous system.

In vitro, GAA expanded repeats form unusual structures (presumably a triplex) for sequences containing 79 and 100 GAA repeats, but not for 45 repeats (Bidichandani et al., 1999). Moreover, long tracts of GAA·TTC (150 and 270 repeats) can form "sticky DNA", which results from intra-molecular association of triplexes (Sakamoto et al., 1999; Le Proust et al., 2000; Heidenfelder et al., 2003). Accordingly, the transcription silencing occurring in FRDA has been attributed to the formation of intra-molecular triplexes and sticky DNA, or persistent RNA·DNA hybrids (Wells, 2008). Thus, the proposed molecular mechanism involves the sequestration of RNA polymerase by its direct binding to the complex DNA structure (Sakamoto et al., 2001). Further, transcription of a GAA·TTC template (88 repeats) using T7 DNA polymerase has showed that the polymerase pauses at the distal end of the repeat (Grabczyk and Usdin, 2000) and that this is tightly linked to RNA·DNA hybrid formation (Grabczyk et al., 2007). *In vitro*, RNA polymerase is arrested by triplex structures, preventing transcription elongation.

Epigenetic studies in the *FXN* promoter and intron regions flanking the GAA repeat expansions have revealed marks of condensed heterochromatin, indicating that an epigenetic mechanism may be ultimately responsible of *FXN* silencing *in*

vivo. These marks include increased methylation of specific CpG sites in FRDA lymphoblasts, peripheral blood, brain and heart tissues as well as reduction of histone H3 and H4 acetylation levels and increased histone H3 lysine 9 (H3K9) trimethylation (Greene et al., 2007; Herman et al., 2006; Castaldo et al., 2008; Al-Mahdawi et al., 2008). Histone hypoacetylation was not observed in the promoter region. However, recent studies show that marks of active chromatin are reduced in the promoter region in patients' cells. Precisely, the initiating form of RNA polymerase II and histone H3K4 trimethylation, a chromatin mark tightly linked to transcription initiation, were found to be reduced on both FRDA alleles. In addition, a mark of transcription elongation, trimethylated H3K36, shows a reduced rate of accumulation downstream of the repeat (Kumari et al., 2011). These data suggest that repeat expansion reduces both transcription initiation and elongation in FRDA cells. The non-B structures adopted by long tracts of GAA repeats may likely generate heterochromatin-dependent and-independent gene silencing.

DNA replication, recombination and repair are also affected by triplex and sticky DNA structures. In *Escherichia coli* and *Saccharomyces cerevisiae* the presence of a GAA repeat leads to attenuation of replication, occurrence of small slippage events and large contractions (Heidenfelder et al., 2003; Krasilnikova and Mirkin, 2004; Pollard et al., 2004). The somatic instability observed in post-mitotic neurons suggests that other mechanisms than replication, such as transcription and post-replicative DNA repair, could be responsible for the triplet repeat expansions observed in FRDA patients.

A recent study in human cells has shown that transcription through the repeat tracts is a major contributor for expansions (Ditch et al., 2009). Other uninterrupted GAA repeat sequences up to $(GAA)_{44}$ have been found in the human and mouse genomes, but only *FXN* GAA repeats of the same size show a high mutation load, suggesting that somatic instability is locus-specific (Rindler et al., 2006).

Progressive GAA repeat expansions in neurons are probably a major contributor to disease progression. The hypothesis of a transcription-driven expansion is fascinating and could partially explain the expansion bias of GAA repeats in the post-mitotic neurons of FRDA patients. DRG are the most vulnerable neurons to frataxin down-regulation and express the highest levels of frataxin. Accordingly, we can speculate that increased gene expression and low cell turn-over could promote the progression of the GAA repeat, which should reduce FXN mRNA levels, either directly or via a chromatin remodeling, thus causing cell death and neuronal degeneration *in vivo*.

2.3. *Genotype/phenotype correlation and point mutations*

A direct correlation between the length of GAA repeat expansions and the presence and timing of several features of FRDA is observed in patients. An inverse correlation has been found between the size of the smaller expansion and both the age of onset and rate of disease progression (time until patients become wheelchair-bound). Cardiomyopathy is more frequent in patients with large expansions. By contrast, diabetes is not associated with GAA repeat number, but appears at late stages of the disease (Dürr et al., 1996; Filla et al., 1996; Montermini et al., 1997a,b; Delatycki et al., 1999). However, insulin sensitivity is inversely correlated with the number of GAA repeats (Coppola et al., 2009). Furthermore, severity of dysarthria, skeletal deformities, optic atrophy and hearing loss and sensory neuropathy directly correlate to GAA expansion size (Santoro et al., 1999). Loss of large myelinated fibers directly correlates with the duration of the disease and inversely correlates with the GAA repeat. Moreover, the methylation of two CpG sites in the genome is directly correlated with the size of the smaller allele and is indirectly correlated with the age of onset in FRDA (Castaldo et al., 2008). Residual levels of frataxin may vary according to the expansion and cell type. In peripheral blood leukocytes, they range from 5 to 30% of normal values (Gellera et al., 2007). In conclusion, the clinical variability observed among individuals with almost identical number of repeats could be due to mitotic instability (Montermini et al., 1997a,b) or mitochondrial haplotype (Giacchetti et al., 2004).

Rarely, gene defects found in FRDA patients are loss-of-function mutations (nonsense, missense, frameshift, splice-site and a deletion) (Campuzano et al., 1996; Cossée et al., 1999). Patients carrying these mutations are compound heterozygotes with an expanded GAA repeat on the other allele. *Fxn* deletion in mice results in embryonic lethally (Cossée et al., 2000), suggesting that null mutations in humans might result in a very severe phenotype. Half of these mutations lead to the absence or a truncated form of frataxin, but some results in a translated protein lacking the mitochondrial targeting. Further mutations lead to aberrant splicing and predicted exon skipping. Aberrantly spliced mRNA may be unstable and rapidly degraded (Gellera et al., 2007). Only one deletion has been reported (Zühlke et al., 2004), probably leading to a truncated frataxin. Most common mutations in patients affect the ATG translation start codon, G130V and I154F. In particular, evidence of founder events for the M1I (Zühlke et al., 2004) and G130V (Delatycki et al., 1999) mutations has been documented. Generally, phenotypic features associated to loss of function mutations are those of typical FRDA. Some atypical features, such as less intense dysarthria and more severe optic pallor, have been reported with a greater frequency (Cossée et al., 1999). Among other mutations, the G130V mutation has been associated with significant vision deficits. Patients show a more severe optic atrophy and sensory neuropathy, without major ataxia (Diehl et al., 2010). Regarding the R165 mutation, it presents a phenotype that differs from the classic FRDA by the increased tone in lower limbs, the lack of dysarthria, the significant motor neuropathy and optic atrophy (McCormack et al., 2000).

In conclusion, point mutations resulting in complete loss of function are associated with a more severe phenotype. In all cases, the size of the GAA expansion in the other allele may modulate the effect of the mutation.

3. Cellular and animal models

Development of valuable animal and cellular models to study FRDA pathogenesis and test potential therapeutics has been difficult (Puccio, 2009). Frataxin knock-out mice show lethality

at the embryonic stage. Viable mouse models are muscle and neuron-specific conditional mutants recapitulating many of the symptoms or pathophysiology of FRDA. The tamoxifen-inducible neuron-specific prion mutant presents a slow progressive neurological phenotype similar to patients. Further, the homozygous knock-in mouse (*fxn*-GAA)$_{230}$ repeat shows a 25% decrease in frataxin expression (Rai et al., 2008), while the knock-in-knock-out mice (*fxn*-/230GAA) express 25–30% of wild-type frataxin levels. These mice manifest no pathology. They exhibit minor motor abnormalities, and changes in gene expression in the CNS, heart, muscle and liver. Moreover, two transgenic mouse lines carrying the human FXN have been generated by crossing the frataxin KO mice, in order to create a mutant only expressing human frataxin mRNA and protein with a homozygous frataxin KO background. The phenotype of the expanded GAA repeat transgenic (Y8 mice) shows decreased aconitase activity, oxidative stress, and develops progressive neurodegenerative and cardiac pathological phenotypes (Al-Mahdawi et al., 2006). Coordination deficits are also present, together with a progressive decrease in locomotor activity. These animals also recapitulate the epigenetic changes observed in FRDA cells. However, frataxin levels are close to wild-type levels in many tissues, as the expanded repeat is relatively small (around 200 triplets). The relatively severe phenotype is due to the fact that human frataxin only partially complements mouse frataxin. Drosophila and *C. elegans* models have been created on the basis of RNAi technology. These models show variable phenotypes according to the timing and severity of frataxin down-regulation and are of great interest for studies on genetic modifiers (Vázquez-Manrique et al., 2006).

Cellular models include cells derived from human FRDA patients, cells derived from animal models, and cells that have been engineered to modify frataxin expression. The available cells from patients are fibroblasts, lymphoblastoid cell lines, and primary lymphocytes. The problem with these cells is that they do not constitute tissues affected by FRDA. Recently, induced pluripotent stem (iPS) cell lines were generated from skin fibroblasts of FRDA patients. (FA-iPS) cell lines maintain the GAA repeat expansion and the reduced FXN mRNA expression. Furthermore, these cells are pluripotent and form teratoma when injected into nude mice. Authors have demonstrated that following *in vitro* differentiation the FA-iPS cells give rise to peripheral neurons and cardiomyocytes (Liu et al., 2010). Another group reported that iPS preserve FXN gene repression and global gene expression signatures reflecting the human disease. GAA/TTC repeats exhibit repeat instability, with expansion and/or contraction with changes in length between generations. Moreover, the mismatch repair enzyme MSH2 is highly expressed in pluripotent cells and occupies FXN intron 1 (Ku et al., 2010). Novel "humanized" mouse cell models based on FRDA point mutations have also been developed (Calmels et al., 2009). These cell lines express a human frataxin cDNA carrying pathogenic FRDA missense mutations and reproduce many biochemical aspects of the phenotype associated with FRDA, without the GAA repeat expansions.

Therefore, new cellular and animal models are needed to investigate the pathogenesis and physiopathology of FRDA, and to test new treatments.

4. Frataxin protein

4.1. *Frataxin structure*

Frataxins are small proteins (100–220 amino acids) highly conserved among eukaryotes and some prokaryotes. Eukaryotic frataxins are localized to the mitochondrial matrix (Vázquez-Manrique et al., 2006; Llorens et al., 2007). Structures have been determined in solution for the human isoform A (Musco et al., 2000), the *E. coli* homolog CyaY and the mature yeast homologue Yfh1. Crystal structures are also available (Dhe-Paganon et al., 2000). The frataxin fold is unique and consists of a large, twisted, six-stranded β-antiparallel sheet, flanked by N- and C-terminal α helices (α1 and α2), with no main surface cavity (Fig. 4). Frataxin presents a patch of negatively charged residues on the helical plane, which could be involved in iron binding (Adinolfi et al., 2002). On the other side, the β sheet surface is mostly uncharged and may be responsible for protein–protein interactions. Most of the residues affected by FRDA mutations are in the neutral surface. Frataxin is translated by cytoplasmic ribosomes (Saint-Georges et al., 2008), then imported into the mitochondria (Koutnikova et al., 1997), where it is proteolytically cleaved in a two-step process to generate the mature protein (Branda et al., 1999; Cavadini et al., 2000) (Fig. 5). In yeast and humans, frataxin maturation depends on the mitochondrial processing peptidase (MPP) (Branda et al., 1999; Cavadini et al.,

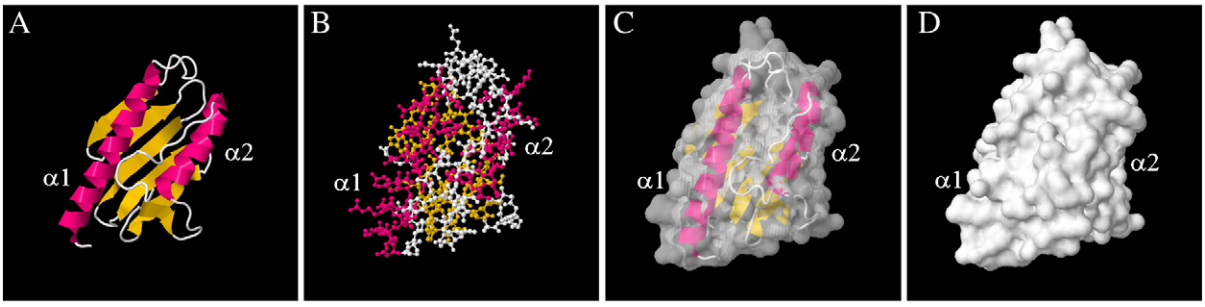

Fig. 4 – Frataxin structure. Frataxin structure consists of six-stranded β-antiparallel sheet (yellow), flanked by N- and C-terminal α helices (α1 and α2 in pink). Structure is based on PyMOL rendering of PDB 1ekg (Jmol). A–B) Frataxin structure, C–D) Frataxin molecular surface.

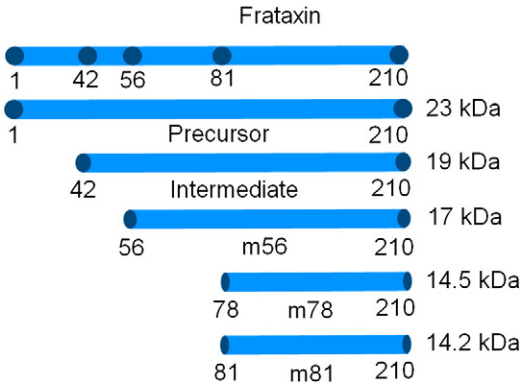

Fig. 5 – Representation of different frataxin forms. The precursor (aa 1–210), the intermediate (aa 42–210) and the three described mature forms: m56 (aa56–210), m78 (aa78–210) and m81 (aa 81–210).

2000; Gordon et al., 2001). Further, the N-terminus of mouse frataxin also interacts with the β-subunit of MPP (Koutnikova et al., 1998). Frataxin contains two MPP cleavage consensus sequences: domain I, residues 1–20 and domain II, and residues 21–51. In Yfh1, deletion of domain II leads to loss of the mitochondrial targeting signal (Knight et al., 1998; Voisine et al., 2000). In yeast, the Ssc1 Hsp70 family ATPase, constituent of the import motor component of the Translocase of the Inner Mitochondrial membrane (TIM), is crucial for Yfh1 transport across the inner membrane, since the *ssc1-3* mutants cannot process Yfh1 to the intermediate or mature forms (Voisine et al., 2000). Recombinant frataxin starting at amino acid 81 (m81-FXN) co-migrates with endogenous frataxin, resulting the essential functional form *in vivo* (Schmucker et al., 2011). Furthermore, it rescues aconitase activity deficiency (Condò et al., 2007) and the lethal phenotype in frataxin-deficient murine fibroblasts (Schmucker et al., 2008). However, longer intermediate forms can be produced when normal processing is impaired and in some normal cells. The functional role remains controversial. Possibly, the two-step cleavage by MPP has also regulatory functions. In humans, frataxin precursor is rapidly cleaved to generate the intermediate form; the second cleavage is slower and limits the overall rate of mature frataxin production in rat liver mitochondria (Cavadini et al., 2000). Probably mitochondrial proteins modulate the processing of the intermediate form.

4.2. *Cellular function of frataxin*

In *S. Cerevisiae* (Δ*yfh1*) cells, frataxin deficiency shows severe growth deficit, reduced rate of respiration, loss of mtDNA, high sensitivity to oxidants, mitochondrial iron accumulation with low cytosolic iron, and hyper-activation of the high affinity iron transport system in the plasma membrane (Babcock et al., 1997; Foury and Cazzalini, 1997; Koutnikova et al., 1997; Wilson and Roof, 1997). Reintroduction of Yfh1 in the Δ*yfh1* mutant shifts iron from mitochondria to cytosol (Radisky et al., 1999), suggesting that frataxin is important for mitochondrial integrity/function and iron metabolism. The role of frataxin in iron homeostasis is supported by the evidence of iron deposits in the heart of FRDA patients (Fig. 2), along with deficiencies in mitochondrial complexes I, II, and III, and aconitases (Lamarche et al., 1993; Rötig et al., 1997). These enzymes all have iron–sulfur clusters (ISCs) in their active sites, and are highly sensitive to reactive oxygen species (ROS). Alteration in iron homeostasis caused by frataxin deficiency suggests that an increase in mitochondrial iron and ROS production by Fenton reaction leads to inactivation of ISCs, mtDNA damage and hypersensitivity to oxidative stress (Babcock et al., 1997; Rötig et al., 1997). However, in yeast, the use of iron-depleted medium does not fully prevent aconitase deficiency (Foury, 1999). Furthermore, conditional KO mice show ISC enzyme deficiencies in the frataxin depleted tissues before iron accumulation in mitochondria (Puccio et al., 2001). These findings have led to the hypothesis that frataxin has a function in the biogenesis of ISCs (Mühlenhoff et al., 2002) (Fig. 5). At the same time, mitochondrial iron accumulation is a common feature of impaired ISC synthesis, providing an explanation of the occurrence in frataxin deficiency.

The exact role of frataxin in ISC biogenesis and the possibility that it is involved in additional cellular processes in mitochondria and in the cytosol are still controversial. One important controversy relates to the functional role *in vivo* – if any – of iron-containing oligo and polymers that frataxins can form *in vitro* in the presence of excess iron, Yfh1 doing much better than mammalian frataxins (Adamec et al., 2000).

4.3. *Fe–S cluster assembly*

Multiple functional deficits in proteins containing ISCs occur when frataxin is depleted in eukaryotic cells (Rötig et al., 1997; Wilson and Roof, 1997; Cossée et al., 2000; Anderson et al., 2005; Busi et al., 2006; Long et al., 2008), although these are not obvious in bacteria (Li et al., 1999). ISCs are ensembles of two or more iron atoms bridged by sulfide centers. Iron sulfur proteins (ISPs) in both prokaryotes and eukaryotes contain ISCs, which are essential for their function, structure and stability. Most biological ISCs are of the [2Fe–2S] and the [4Fe–4S] types. ISPs are found in different cell compartments, in mitochondria, and include several subunits of respiratory complexes I, II and III and a complex I assembly factor (NUBPL), aconitase, a ferredoxin, ferrochelatase, the molybdenum cofactor synthesis enzyme MOCS1A and the membrane associate protein of unknown function MitoNEET. In the cytosol and in the nucleus ISPs are involved in different biological processes, including iron metabolism (IRP1), metabolic pathways, signaling and DNA repair.

In higher eukaryotes, ISC biogenesis takes place in mitochondria and in the cytosol, but cytosolic ISC synthesis appears to depend upon mitochondrial integrity and upon the export of a still unknown component (Fig. 6). Mitochondrial ISC biogenesis in higher eukaryotes involves many factors (Ye and Rouault, 2010): the scaffold protein ISCU; the sulfur donor NFS1; the small protein ISD11; the ferredoxin FDX1 and the ferredoxin reductase FDXR; and the putative alternative scaffold NFU. Clusters transfer from ISCU to the apoproteins to form holo-ISC proteins requiring a further set of factors. Today, evidence supports a role of frataxin in mitochondrial ISC biogenesis. The similarity between the phenotype of frataxin deficient yeast and yeast depleted of other ISC biogenesis factors has been the first

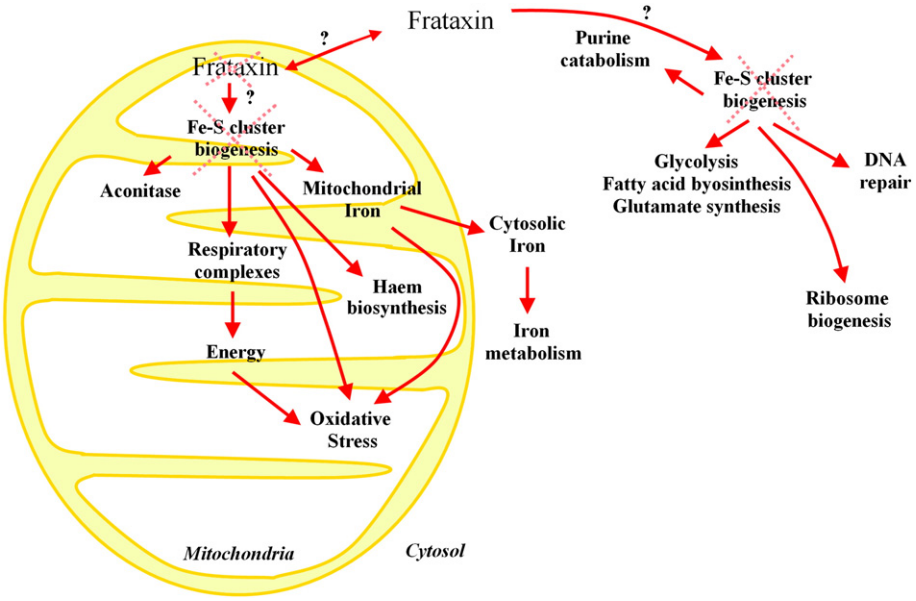

Fig. 6 – Iron-sulfur cluster deficit and cellular dysfunctions. Iron–sulfur (Fe–S) cluster biogenesis deficit has been associated to reduction in Fe–S proteins, such as aconitase, respiratory chain complexes I, II and III functions, haem biosynthesis, increased oxidative stress and altered iron metabolism within the mitochondria. Further, dysregulation of energy metabolism, fatty acid and glutamate biosynthesis, glycolysis, DNA repair, ribosome biogenesis and purine catabolism. Interestingly, models lacking frataxin recapitulate the pathological phenotype associated to Fe–S cluster biogenesis deficit, suggesting a major role for frataxin in these processes.

indirect evidence in this regard (Fig. 5). The observation of interactions with the NFS1/ISCU complex (Gerber et al., 2003; Mühlenhoff et al., 2002; Yoon and Cowan, 2003) has further supported this hypothesis. However, the identification of the functionally relevant form of frataxin and the confirmation of its exclusive sub-cellular localization in mitochondria is still a critical aspect (Schmucker and Puccio, 2010). The m81-FXN is clearly the most abundant isoform to interact with the ISC assembly factors. M42-FXN is considered by some authors as just a processing intermediate, but by others as a functionally relevant isoform because of its capacity to oligomerize. Clearly, this is crucial to establish if oligomerization is a property only shown by incompletely processed frataxin. Another controversy relates to the presence of a cytosolic pool of frataxin. Some observations have suggested that frataxin may participate in the assembly of cytosolic and nuclear ISC (Acquaviva et al., 2005), but others have proposed a specific pro-survival function of cytosolic frataxin (Condò et al., 2007).

Human frataxin interact with ISCU in presence of iron (Huang et al., 2008). Recently, the main endogenous interactors of m81-FXN have been shown to be ISCU, NFS1 and ISD11. Precisely, m81-FXN interacts with the preformed core complex, rather than with the individual components (Schmucker et al., 2011). By contrast, prokaryotic frataxin (CyaY) do not seem to interact with the isolated ISCU homolog (IscU), but rather with the NSF1 homolog IscS (Layer et al., 2006; Adinolfi et al., 2009). In yeast, both the frataxin (Yfh1) and the NSF1–ISD11 homologs (Nfs1–Isd11) can directly bind to the scaffold Isu1 (the ISCU homolog) in an iron dependent way (Gerber et al., 2003). The interaction seems to require Yfh1 oligomerization (Li et al., 2009). However, the crucial question is the following: why these interactions take place and what is the primary function of frataxin? Frataxin has been suggested to act either as a chaperone which delivers iron to the NSF1/ISCU complex (Yoon and Cowan, 2003) or as a scavenger (Cavadini et al., 2002) which prevents iron excesses in mitochondria and keep it available for key-acceptors. These hypotheses are based on the functional capacity of frataxin to bind iron. More recently, it has been shown that CyaY inhibits ISC formation on the scaffold protein IscU. This occurs without inhibiting desulfurase enzymatic activity and suggests a role for frataxin as a gatekeeper of ISC formation (Adinolfi et al., 2009). Clearly, these results have to be reconciled with the *in vivo* finding of decreased ISC biogenesis in frataxin deficiency. In any case, frataxin should play an important but not essential role in this process, since ISC biogenesis can occur in the absence of frataxin, even though at a reduced level (Duby et al., 2002).

Other functions have also been proposed for frataxin. In particular, it has been suggested that it can act as an iron chaperone in converting the oxidative damaged (3Fe–4S) cluster into the active (4Fe–4S) cluster of aconitase (Bulteau et al., 2004). Interaction of frataxin with aconitase, in the presence of citrate, would protect the cluster from oxidation, reducing the risk of enzyme inactivation.

4.4. *Iron homeostasis*

In Δ*Yfh1* yeast cells iron accumulates in mitochondria with a relative depletion in the cytosol (Babcock et al., 1997; Foury and Cazzalini, 1997; Wilson and Roof, 1997). The high-affinity iron transport system, normally suppressed in high iron

condition, is overexpressed in Δ*Yfh1* by the activation of the iron-sensitive transcription factor *Aft1* (Babcock et al., 1997). In yeast, *Aft1* activates the uptake system when cytosolic iron is low. However, in condition of frataxin deficiency, this model reacts as if iron is depleted, when in fact iron is exceeding inside mitochondria. Clearly, mitochondria cannot use iron for biosynthetic processes (ISC synthesis), thus the metal participates in redox chemistries and/or precipitates within the organelle, generating a signal to the iron-sensing mechanism of a need of iron to synthesize more ISCs. The nature of the latter signal is still unknown. It could involve the suppression of iron export out of mitochondria when too few ISCs are made, resulting in low cytosolic iron (Pandolfo, 2002). This model is supported by evidences that in yeast defective ISC synthesis causes the same alterations of iron homeostasis as observed in Δ*Yfh1* (Philpott and Protchenko, 2008; Rouault and Tong, 2008).

In upper eukaryotes iron homeostasis mostly takes place at the post-transcriptional level through the binding of regulatory proteins to the Iron Responsive Element (IRE) in the 5′ or 3′ untranslated regions of specific mRNAs (Muckenthaler et al., 2008). Two cytosolic proteins, IRP1 and IRP2, bind IREs when iron is low in their compartment. IRP1 is a cytosolic aconitase containing a cubane [4Fe–4S] cluster when iron levels are high. Its cluster loses one iron atom when iron levels decrease, leading to loss of aconitase activity and stimulation of IRE-binding activity. IRP2 has a similar structure to IRP1, but no enzymatic activity and is degraded through the ubiquitine–proteasome system when iron levels are high. When iron is low, IRP2 escapes proteolytic degradation and binds to IREs. IRP2 is thought to be the main regulator of iron metabolism in most tissues (Fig. 6). IRP binding to a 5′ IRE prevents mRNA translation, binding to a 3′ IRE stabilizes the mRNA and enhances translation. Proteins involved in iron uptake (as transferrin receptor, TfR) or iron storage (as ferritin) have a 3′ IREs or 5′ IRE in their mRNAs. Accordingly, when frataxin is low or absent (as in conditional KO models) or in FRDA patients' fibroblasts (Li et al., 2008), IRP1 becomes activated. In addition, the levels of TfR1 and the mitochondrial iron importer mitoferrin-2 are increased, with a decrease of the cell membrane iron exporter ferroportin 1 (Huang et al., 2009) was observed (Fig. 7).

Furthermore, no mitochondrial iron overload has been detected in patients' cells and in the complete KO (Cossée et al., 2000). Nevertheless, the embryonic lethality might have prevented iron accumulation. However, intra-mitochondrial iron deposits have clearly been detected in the heart of 10-week-old MCK (heart and muscle) conditional KO mice (Puccio et al., 2001) and in the humanized YAC transgenic mouse model (Al-Mahdawi et al., 2006). It should be kept in mind that, in both cases this is a late event which follows the appearance of ISC enzyme deficiencies.

In FRDA patients, iron mostly accumulates in the heart (Michael et al., 2006), liver and spleen. Iron deposits cannot be histologically detected in the DRG, spinal cord, skeletal muscle, cerebellum, peripheral nerve or pancreas (Bradley et al., 2000; Koeppen et al., 2007). Dentate nuclei analysis has shown no difference in total iron and ferritin between patients and controls in cerebellar sections (Koeppen et al., 2007). However, increase in iron levels have been detected in the

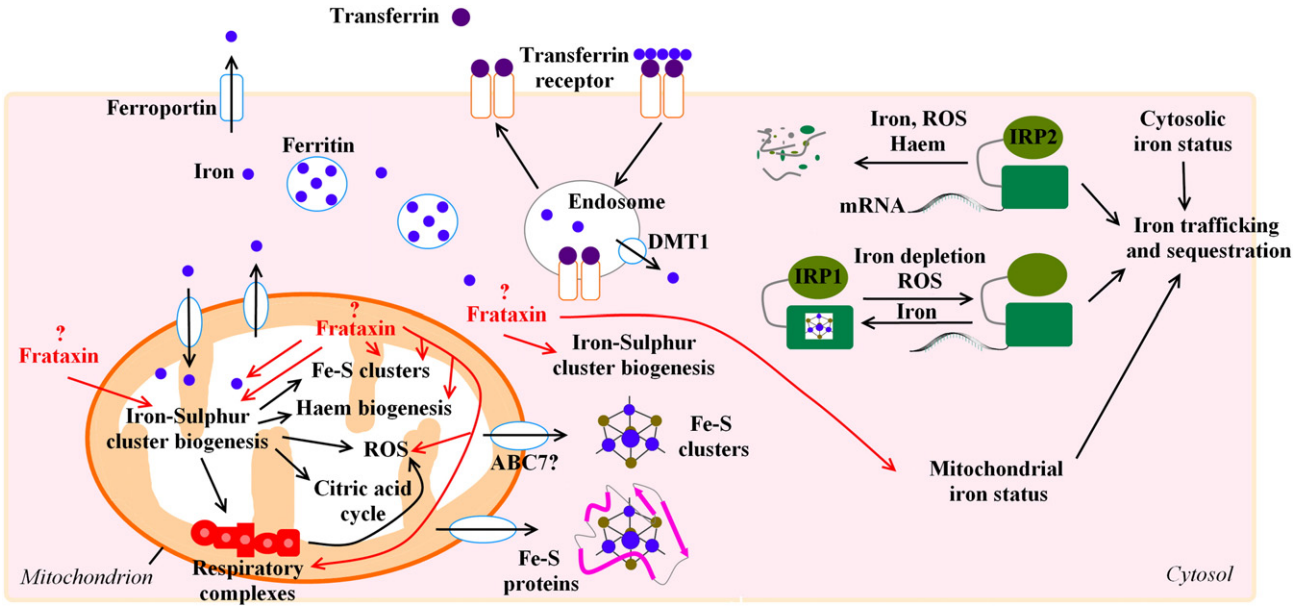

Fig. 7 – Iron homeostasis and possible frataxin function. Extracellular transferrin proteins bind the dimeric transferrin receptor and are internalized by endocytosis. Iron is transferred into the cytosol by the divalent metal transporter-1 (DMT1) and can pass to mitochondria. In the cytosol of mammalian cells, the iron regulatory protein-1 (IRP1) and the iron-dependent degradation of IRP2 are the sensors of intracellular iron levels. In healthy animals IRP1 (4Fe–4S) functions as a cytosolic aconitase. IRP2 regulates transferrin receptor, ferritin, DMT1 and ferroportin levels binding to IREs elements in their mRNAs. Frataxin has been suggested (?) to participate in iron–sulfur cluster biogenesis, iron homeostasis, ROS production, respiratory chain function, and haem biogenesis.

dentate nucleus of FRDA patients' cerebellum at MRI analysis (Waldvogel et al., 1999). This controversial finding could be explained by the fact that MRI analysis could also detect iron in a different status, such as ferritin and hemosiderin, thus resulting in increased signal of the metal in this region. Additionally, serum iron and ferritin result normal in patients' samples (Wilson et al., 1998). Cultured cells from FRDA patients or mouse models show a modest increase of mitochondrial iron (Wong et al., 1999; Tan et al., 2001; Calmels et al., 2009).Changes in intracellular iron distribution probably occur in most cell types when frataxin is low or absent. Iron accumulates in mitochondria at long term in some tissues. However, the pathogenic role of mitochondrial iron accumulation is supported by a ROS reduction and the increase in the activity of ISC enzymes with expression of mitochondrial ferritin or after addition of iron chelators (Kakhlon et al., 2008; Campanella et al., 2009).

Finally, no IRE is found in frataxin mRNA and *FXN* iron-mediated regulation is likely transcriptional and involves still unknown factor(s). *FXN* expression increases with high cytosolic iron and is reduced by the iron chelator deferoxamine, which removes iron from the cytosol (Li et al., 2008). Iron-dependent expression of *YFH1* has also been observed in yeast cells (Santos et al., 2004; Seguin et al., 2009). These observations suggest that cytosolic iron depletion may be critical for frataxin expression.

4.5. *Iron binding properties*

Frataxins from yeast, humans and bacteria bind iron in specific conditions (Huang et al., 2008). They generally bind ferrous iron as monomers. In the presence of iron excess, they oligomerize or aggregate (Adinolfi et al., 2002). Yfh1 in particular can self-assemble *in vitro* in an iron-dependent manner, suggesting a role in iron storage (Gakh et al., 2002). This process requires the presence of a large excess of Fe(II) (Adamec et al., 2000; Gakh et al., 2002), which is converted into Fe(III) during oligomerization (Park et al., 2002; Park et al., 2003). Whether these oligomers and multimers have physiological function is still unresolved. Interestingly, a frataxin construct unable to oligomerize can completely rescue the phenotype of Δ*Yfh1* yeast (Aloria et al., 2004) suggesting a non-physiological role of these oligomers.

Human frataxin overproduced in *E. coli* also assembles into homo-oligomers that bind iron (Cavadini et al., 2002) and have ferroxidase activity (O'Neill et al., 2005). In human, frataxin oligomerization can occur in an iron-independent manner (Adinolfi et al., 2002; Cavadini et al., 2002) and requires an N-terminal portion of the protein that is present in m42-FXN but absent in m81-FXN (O'Neill et al., 2005). Whether this property – which implies a physiological role for m42-FXN – has relevance *in vivo* is still unsettled. Supporters of a physiological role of frataxin oligo- and multimers propose that they detoxify iron in excess in the mitochondria, thus serving as antioxidant (Gakh et al., 2006; Gakh et al., 2008; Li et al., 2009).

Frataxin monomers from all species bind iron, probably due to the conserved exposed acidic residues in the two α-helices (Bencze et al., 2006), though this kind of metal binding site is unusual. Human frataxin binds up to seven Fe(II) atoms, with low affinity (Yoon and Cowan, 2003). Based on these findings, frataxin has been proposed to be a chaperone for Fe(II) in mitochondria, preventing its engagement in ROS-producing redox reactions and in ISC biogenesis (Yoon and Cowan, 2003; Yoon and Cowan, 2004; Wang and Craig, 2008). An iron donor role for frataxin has also been proposed for heme synthesis, through direct interaction with ferrochelatase (Yoon and Cowan, 2003). However, the fact that frataxin is a low abundance protein and the iron concentration in the mitochondrial matrix greatly exceeds its iron-binding capacity argues against a role of frataxin as mitochondrial iron chaperone (Seguin et al., 2009).

In conclusion, frataxin definitely plays a role in ISC biogenesis and interacts with the components of its machinery, but so far its role has not been conclusively defined and no strong evidence supports that it is the iron donor.

4.6. *Cellular antioxidants defenses*

Frataxin deficiency has been linked to increased oxidative stress (Al-Mahdawi et al., 2006; Busi et al., 2006; Vázquez-Manrique et al., 2006), which may play a central role in the disease. Increased levels of biomarkers of oxidative stress, such as the 8-hydroxy-2′-deoxyguanosine (DNA oxidative damage) and plasma malondialdehyde (lipid peroxidation) have been found in urine and blood from FRDA patients (Emond et al., 2000; Schulz et al., 2000; Bradley et al., 2004). Free radical production is increased in cultured cells with low frataxin (Santos et al., 2001; Santos et al., 2004; Irazusta et al., 2006; Napoli et al., 2006; Seguin et al., 2009). In addition, fibroblasts from FRDA patients are sensitive to oxidants (Chantrel-Groussard et al., 2001; Jiralerspong et al., 2001), suggesting that even non-affected cell types may be in a chronic oxidative stress state. Also, there is evidence of altered glutathione homeostasis in FRDA (Napoli et al., 2006; Auchère et al., 2008). Reduction in free glutathione levels and increase in the glutathione bound to hemoglobin in erythrocytes have been observed in blood samples from FRDA patients (Piemonte et al., 2001). Furthermore, Yfh1 depleted yeast shows remodeling of glutathione dependent defense systems as well (Auchère et al., 2008).

However, some findings argue against the role in oxidative stress. In patients, the increase in urinary 8-hydroxy-2′-deoxyguanosine could not be confirmed in an independent study (Di Prospero et al., 2007). In addition, the levels of urinary F_2-isoprostanes in FRDA patients were not different from controls (Myers et al., 2008). Moreover, in the heart–muscle (MCK) conditional KO mice, no sign of oxidative damage have been detected even in case of advanced disease (Seznec et al., 2004). It can be argued that a biomarker level can fluctuate and there may be technical issues in its determination. The complete shutdown of the respiratory chain, expected to occur when frataxin is completely absent (in the conditional KO mouse), might also prevent ROS formation.

About 90% of ROS come from mitochondrial respiration (Herrero et al., 2008; Winterbourn and Hampton, 2008). Excess $O2.^-$ can inactivate (4Fe–4S) cluster-containing enzymes, releasing iron and thereby increasing the free, redox-active iron pool, and can also react with nitric oxide (NO) to generate deleterious peroxynitrite ($ONOO^-$). Superoxide disposal is carried out by the superoxide dismutase enzymes (SODs), which convert $O2.^-$ into hydrogen peroxide (H_2O_2). Detoxification of H_2O_2 then involves the action of diverse scavenging enzymes such as catalases, glutathione peroxidases (GPXs) or peroxiredoxins. Iron, directly

mobilized by $O2.^-$, and H_2O_2, generated by SOD, can engage in the Fenton reaction ($Fe(II)+H_2O_2 \rightarrow Fe(III)+HO^-+HO.$), to produce the highly reactive, and toxic, hydroxyl radical (HO.). The Fenton reaction may be enhanced in FRDA because of excess mitochondrial iron and may contribute to pathogenesis.

In general, the cellular response to oxidative stress involves the induction of detoxifying enzymes such as SODs and GPXs (Calabrese et al., 2005). Under normal physiological conditions, the rate of ROS and reactive nitrogen species (RNS) production is compensated by the activity of scavenging enzymes and small antioxidant molecules (α-tocopherol, ascorbic acid and glutathione), an imbalance resulting in increased oxidative stress. An alteration of this capacity to mobilize antioxidant defenses occurs in FRDA and may further increase oxidative damage. This abnormality was first observed in fibroblasts from FRDA patients, which do not upregulate SODs in response to oxidative stress induced by H_2O_2 and iron (Paupe et al., 2009; Marmolino et al., 2010). The mechanism underlying this impaired response is unclear. A recent study has suggested that impaired nuclear translocation of the transcription factor Nrf2 may underlay the lack of oxidative stress response in FRDA cells (Paupe et al., 2009). Impaired Nrf2 activation in FRDA has been attributed to oxidative stress-caused changes in the glutathione pools, resulting in increased actin glutathionylation and altered cytoskeletal dynamics. Although Nrf2 may play a role in the impairment of antioxidant responses shown by FRDA cells, it is unlikely the sole culprit, because it has only a limited affect on SODs (whose induction is clearly defective in FRDA cells). Recently, we have shown that down-regulation of the transcriptional coactivator PGC-1α may play a critical role in the loss of antioxidant defenses occurring in FRDA fibroblasts (Marmolino et al., 2010). Furthermore, we have shown that upregulation of PGC-1α restore SOD levels both *in vitro* and *in vivo*.

The lack in the response to oxidative stress in frataxin deficient cells is clearly a secondary effect of frataxin down-regulation. PGC1a down-regulation in FRDA, a major regulator of the detoxifying enzymes activation in neurons, provides a link between the lack in the activation of this response and frataxin down-regulation. The similarity between the PGC1a knockout model and the FRDA mitochondrial phenotype, plus the evidence of a direct regulation between frataxin and PGC1a, strongly suggest a key role of this pathway in the pathogenesis of the disease. The scientific evidence that increasing PGC1a expression restores the response to oxidative stress in FRDA opens the door to a potential target for therapy. However, further studies are needed to clarify the founder event in this complex process.

4.7. *Mitochondrial DNA alterations*

Loss of mitochondrial DNA occurs in Δ*yfh1* yeast cells (Babcock et al., 1997; Foury and Cazzalini, 1997; Wilson and Roof, 1997; Karthikeyan et al., 2003). Moreover, in low frataxin conditions, loss of mtDNA occurs later, suggesting a progressive accumulation of damage. Accordingly, frataxin depletion is associated with mtDNA lesions in tissues from FRDA patients (Houshmand et al., 2006; Heidari et al., 2009) such as, skeletal muscle, heart, cerebellum and DRG. Mutations in mtDNA impair oxidative phosphorylation and may contribute to FRDA pathology.

4.8. *Alterations in nuclear DNA*

The Δ*yfh1* yeast model presents chromosomal DNA damage. These mutants show higher levels of illegitimate mating, spontaneous recombination and mutation rate. Frataxin deficiency also enhances sensitivity to the DNA-alkylating methyl methanesulfonate and to the replication inhibitor hydroxyurea (Karthikeyan et al., 2002). The DNA damage-inducible (DIN1) gene is upregulated in Δ*yfh1* cells, and is reduced in presence of antioxidant drugs, suggesting a ROS-induced nuclear damage mechanism. Moreover, Glutathione peroxidase (GPX1) deletion in Δ*yfh1* cells leads to increased nuclear mutation rate. Increased mitochondrial H_2O_2 has been first proposed as a possible mechanism for nuclear DNA damage (Karthikeyan et al., 2002). Subsequently, nuclear genome instability due to mitochondrial dysfunction has been linked to impaired biogenesis of ISPs (Veatch et al., 2009). Loss of mtDNA in yeast is associated with cell-cycle arrest, reduction in the inner mitochondrial membrane electrochemical potential, intracellular iron accumulation, increase in oxidative damage and impaired mitochondrial and nuclear/cytosolic ISC assembly.

Probably, these findings should be extended to FRDA, in which impaired ISC biogenesis may be the primary event, followed by disrupted iron homeostasis, oxidative stress and mtDNA damage.

5. Therapeutics approaches for the treatment of FRDA

The identification of the mutation underlying FRDA, the increase of our knowledge of frataxin function and pathogenesis, allowed us to develop several therapeutic approaches for FRDA. Current strategies aim to increase frataxin expression or to intervene in the pathogenetic cascade downstream of frataxin deficiency. Future approaches may also include gene or protein replacement and cellular therapies. Here, current therapeutics update and expectations will be discussed (Table 2).

5.1. *Antioxidants and oxidative phosphorylation*

Frataxin deficient cells presumably generate more free radicals and are more sensitive to oxidative damage, suggesting antioxidants as a therapeutic approach in FRDA. Quinone antioxidants, *i.e.* CoQ and vitamin E derivatives, are the molecules which have been studied with details in FRDA. Treatment with a combination of CoQ10 and vitamin E improves mitochondrial function and reduced oxidative stress. A four-year open-label trial of 10 patients treated with vitamin E (2100 IU/day) and CoQ10 (400 mg/day) has shown improvement in bioenergetic aspects in cardiac and skeletal muscle, without clear benefit on cardiomyopathy (Hart et al., 2005).

Idebenone is a short-chain analog of CoQ, which penetrates membranes and enters mitochondria much more efficiently than CoQ10. It reduces membrane lipids peroxidation and carries electrons from complexes I/II to complex III and rescue

Table 2 – Therapeutic approaches in Friedreich's ataxia.

Drug–Idebenone (*Santhera Pharmaceuticals*)
Phase III – (Catena®) – United States
Phase III – Idebenone – Europe
Drug — A0001 (*Endo Pharmaceuticals*)
Phase II – A0001 – United States
Drug — Egb-761 (*Ipsen*)
Phase II – EGb 761 (Tanakan®) – France
Drug — Pioglitazone (Takeda *Pharmaceuticals*)
Phase II – Pioglitazone – *France*
Drug — EPI-743 (*Edison Pharmaceuticals*)
Phase I — EPI-743
Drug — Resveratrol
Phase II – Resveratrol – Australia
Drug — Deferiprone (*Apopharma*)
Phase II — Deferiprone
Drug — Erythropoietin (EPO)
Phase II – EPO – Austria, Italy
Drug — Lu AA24493 carbamylated EPO (*Lundbeck*)
Phase II – Lu AA24493 or cEPO – Germany, Austria and Italy
Drug — Histone Deacetylase inhibitors (HDACi) (*RepliGen*)
Pre-clinical — HDACi
Drug — Varenicline (Chantix®) (*Pfizer*)
Phase II – (Chantix®) – United States
TAT-Frataxin
Pre-clinical

CoQ deficient cells (Meier and Buyse, 2009). Idebenone is reduced by ROS and releases electrons to the respiratory chain to be restored in its quinone form, promoting OXPHOS. In addition, it decreases lipoperoxidation in heart homogenates from patients with valvular stenosis (Rustin et al., 1999). Treatment with 5 mg/kg/day idebenone up to nine months in an open-non-placebo controlled clinical study has been associated with a decrease in heart size in three FRDA patients with hypertrophic cardiomyopathy (Rustin et al., 1999). However, this effect on the heart disease remains controversial because it has not been confirmed in controlled clinical trials (Artuch et al., 2002; Meier and Buyse, 2009; Lynch et al., 2010). A double-blind randomized placebo-controlled study has shown that 450 mg/day or 900 mg/day of idebenone does not change the cardiac status in FRDA patients, as compared to a placebo (Lagedrost et al., 2011). Authors concluded that idebenone has no effect on cardiac structure or function measured by echocardiography over 6-months treatment period (Lagedrost et al., 2011). Another double-blind-placebo-controlled study has shown a limited effect on cardiomyopathy (Mariotti et al., 2003). To interpret these controversial results we should consider some important aspects: first, the recruitment and selection of patients should follow strict and precise parameters, such as the degree of heart disease and the administration of previous pharmacological treatments; second, the choice of the methods for the evaluation of cardiac dysfunction is important to avoid bias in the interpretation of the results. Concerning this aspect, cardiac resonance imaging (MRI) may better detect changes in ventricular size and mass as compared to echocardiography that is more user-dependent. The sensitivity of the analytical method used could partially explain the differences observed between different clinical reports. Moreover, all randomized controlled trials using idebenone have shown no significant benefit on neurological symptoms, such as ataxia, in FRDA patients (Mariotti et al., 2003; Kearney et al., 2009; Meier and Buyse, 2009; Lynch et al., 2010). However, one study has reported that idebenone treatment at early stages of the disease seems to reduce the progression of cerebellar manifestations (Artuch et al., 2002). Furthermore, the effect of idebenone on oxidative markers has also been evaluated. An open-label trial in FRDA patients has shown a decrease of 8-hydroxy-2′-deoxyguanosine levels after eight weeks of treatment with idebenone (5 mg/kg/day) (Schulz et al., 2000). However, a monocentric double-blind placebo-controlled study on FRDA adolescents treated for six months with idebenone (5, 15, and 45 mg/kg/day) has not revealed any difference in the urinary levels of 8-hydroxy-2′-deoxyguanosine (Di Prospero et al., 2007). Again, this effect should be carefully evaluated and further investigated, because of the technical difficulties in evaluating a marker of oxidative stress.

At this time we can conclude that idebenone does not affect disease progression and the neurologic status in patients as compared to placebo, but may be still be helpful in selected patients for cardiomyopathy and a general feeling of energetic well being.

MitoQ is a CoQ10 analog targeting mitochondria. It protects FRDA fibroblasts from endogenous oxidative stress (Jauslin et al., 2003). However, clinical trials have not been carried out, mostly because MitoQ does not exchange electrons in the respiratory chain, and cannot be regenerated and stimulate OXPHOS.

EPI-A0001, is an α-tocopherol-ubiquinone hybrid with antioxidant properties, probably stimulating the OXPHOS *in vitro*, although no publication on this topic is currently available. A phase 2 clinical trial is ongoing.

5.2. *Iron chelating strategy*

Iron chelation is the current treatment of systemic iron overload diseases. However, its use in neurological disorders with an iron component is controversial (Richardson et al., 2001; Kalinowski and Richardson, 2005). The main point of discussion is the absence of systemic iron overload, but rather localized deposits, as in Parkinson and Alzheimer patients. In FRDA, iron is in excess in mitochondria and is relatively depleted in the cytosol. Iron deposits mostly occur in the heart, without systemic iron overload. Accordingly, in patients' serum iron and ferritin levels are normal, while transferrin receptor is increased (Wilson et al., 2000; Wilson, 2006). However, impaired ISC biogenesis and iron involvement in frataxin regulation open the discussion on the use of drugs that decreases iron availability, possibly with further deleterious effects in FRDA. Conversely, drugs that do not totally deplete iron, but only redistribute it from overloaded (mitochondria) to depleted (cytosol) could be potentially beneficial. Today, the challenge is the design of membrane permeable molecules reaching the brain and redistributing iron from mitochondria without compromising cellular availability (Richardson, 2003).

Desferoxamine is an iron chelator with a very poor membrane penetration and cannot be given orally. It chelates iron in the extracellular compartment and cytosol, promoting cellular iron depletion. Desferoxamine reduces Fe(II) toxicity

on mitochondrial complex II in FRDA heart homogenates, decreases markedly aconitase activities and down-regulates frataxin (Rustin et al., 1999; Li et al., 2008). Thus, it cannot be used in FRDA.

The iron chelator deferiprone can be administered orally. It rapidly distributes in the CNS, crossing membranes, and penetrating within mitochondria to remove iron excess. Deferiprone has a low affinity for iron, thus becoming an iron donor for biologically relevant molecules like transferrin. Further, it has less tendency to cause overall iron depletion and may also redistribute iron between overloaded and depleted compartments (Sohn et al., 2008). A pilot study in nine FRDA adolescents treated with deferiprone (20 to 30 mg/kg/day) for six months has shown reduced iron accumulation in the dentate nucleus (assessment with MRI) and improvement of ataxia (Boddaert et al., 2007). The molecule was well tolerated, although four patients had adverse events and a worsening of ataxia occurred at higher doses. The risk of agranulocytosis remains a threat of deferiprone treatment. *In vitro* treatment of human frataxin-depleted HEK293 T-Rex cells with deferiprone (50 μM) restores mitochondrial redox potential, reduces ROS, prevents apoptosis and increases aconitase activity, without affecting frataxin levels (Kakhlon et al., 2008). However, deferiprone (150 μM) in fibroblasts reversibly inhibits aconitase activity and cellular growth (Goncalves et al., 2008) as well as frataxin levels, suggesting that concentrations should be carefully considered. Accordingly, high doses of deferiprone have worsened ataxia in a pilot study and a phase 2 trial has also interrupted in the high dose group (60 mg/kg/day) for the same reason (results not yet available). This particular aspect should be carefully considered.

Toxicological and dose–response studies should clearly provide safety information. Additional studies are needed to clarify if the effect on frataxin levels is direct or indirect (post-transcriptional), even if this effect is likely secondary to iron depletion and inhibition of ISC enzymes.

In conclusion, iron chelation remains an interesting approach. However the choice of the dose remains a critical point for clinical trials since high doses are associated with deleterious effects in patients.

5.3. *Histone deacetylase inhibitors*

Histone deacetylases (HDACs) inhibitors (HDACi) have been proposed to counteract the chromatin-condensing effect of the GAA repeat expansions and to restore frataxin expression in FRDA. HDACs modulate acetylation levels in histones, non-histones chromosomal proteins and other cellular targets. HDACi revert silent heterochromatin to an active chromatin conformation with both positive and negative effects on gene expression (Di Prospero and Fischbeck, 2005; Riessland et al., 2006). Sodium butyrate was the first HDACi to increase frataxin expression in a frataxin reporter construct *in vitro* (Sarsero et al., 2003). However, most HDACi have a modest or no effect in upregulating frataxin in FRDA cells. In 2006, a study has shown that the HDACi BML-210 powerfully increases frataxin expression (Herman et al., 2006). In the same paper, the authors have synthesized a family of compounds with similar structures to BML-210. They have identified highly effective molecules for upregulating frataxin. These pimelic diphenylamide molecules directly interact with class I HDACs (particularly HDAC3) with specific properties of slow-on slow-off kinetics. Further, treatment of FRDA lymphoblastoid cells with the BML-210 derivate 4b increases frataxin transcription and revert histones H3 and H4 hypoacetylation in the chromatin regions upstream and downstream the GAA repeat, without affecting H3K9 trimethylation, a hallmark of heterochromatin (Herman et al., 2006). A similar compound, 106 (N1-(2-aminophenyl)-N7-p-tolylheptanediamide) increased histone acetylation in FRDA mice brain and normalized frataxin expression (Rai et al., 2008). Moreover, gene expression profiling in FRDA animals (brain, cerebellum, and heart) showed changes going toward the normalization of dysregulated genes after the treatment when compared to wild-type. Recently, two new pimelic diphenylamides HDACi have been synthesized (136 and 109) and tested in FRDA patients' lymphocytes and in mouse models. 109 results the most effective in up-regulating frataxin expression and is a possible candidate for clinical studies. Interestingly, the pharmacological effect of this compound on histone acetylation and frataxin levels largely outlasts the persistence of the molecule in the tissue, thus suggesting that intermittent dosing of the drug may be effective in FRDA (Rai et al., 2010).

The possibility of side effects by the use of these drugs for neurological diseases such as FRDA still requires more detailed studies. *In vivo* toxicological and pharmacological reports are urgently required before starting any clinical trial. The possibility of using HDACi molecules to restore the transcriptional deficit at the FXN gene in patients should take in consideration their positive and negative effects on both activating and inactivating gene transcription. Further, the possibility that elevating transcription levels from the FXN promoter could exacerbate the GAA expansion and further aggravate the pathological phenotype should not be excluded.

5.4. *Human recombinant erythropoietin*

Erythropoietin (EPO) controls erythropoiesis and primarily acts on erythrocyte precursors in the bone marrow. Moreover, it crosses the blood brain barrier. EPO receptors (EPOR) are also expressed in other tissues and organs, including the nervous system. Recombinant human EPO (Rhu-EPO) has been reported to increase frataxin protein levels in different FRDA cell lines, such as lymphocytes, cardiomyocytes and cardiac fibroblasts, and P19-derived neuronal-like cells (Sturm et al., 2005). The effect is exclusively associated with on frataxin protein without affecting mRNA, suggesting a possible effect on the stabilization of the transcript and/or on protein maturation (Acquaviva et al., 2008). An open-label study of FRDA patients treated with rhu-EPO (5000 IU) for 8 weeks has shown improvement of ataxia scores, associated with a reduction in oxidative stress markers and serum peroxides (Boesch et al., 2007; Boesch et al., 2008). Frataxin protein levels in patients' leukocytes increase (~27%) with high variability and some non-responders. Phlebotomies are also necessary in some cases. Further, the EPO derivate (carbamylated EPO) devoid of erythropoietic activity, show neuroprotective properties associated with mild increase in frataxin protein *in vitro* (Sturm et al., 2010). The molecule is now being clinically

tested, showing fewer side effects. Recently, a study of FRDA patients treated with a single subcutaneous dose of Epoetin alpha (600 IU/kg) followed from a 3 months later dose of 1200 IU/kg has shown a frataxin increase at 3 and 6 months. The treatment does not affect hematocrit, cardiac function, and neurological scale. Phlebotomies are not required, indicating lack of erythropoietic effect (Saccà et al., 2011).

The effect of EPO on frataxin expression is clearly post-transcriptional. However, the precise mechanism underlying this pathway and the possible cofactors involved are unknown. The mechanism possibly involves the activation of upstream proteins targeting frataxin maturation and transport machinery within the cytosol and or mitochondria, thus increasing frataxin cellular availability.

5.5. *Peroxisome proliferator-activated receptor-γ agonists*

PPAR-γ agonists are commonly used for the treatment of diabetes mellitus, but represent also a promising therapeutic strategy for other diseases including neurodegenerative disorders with an inflammatory or mitochondrial component. PPAR-γ agonists induce mitochondrial biogenesis (PGC1) and neobiosynthesis of several mitochondrial proteins (Wu et al., 1999) via the recruitment of PGC1a, a master regulator of mitochondrial biogenesis. Recently, we have shown that a synthetic PPAR-γ agonist, azelaoyl-PAF (A-PAF) increases frataxin amount in primary fibroblasts from FRDA patients and in neuroblastoma cells (Marmolino et al., 2009). Furthermore, we have provided evidence that pioglitazone, a commercially available PPAR-γ agonist crossing the blood–brain barrier, restores *in vitro* and *in vivo* SOD2 levels in FRDA models (Marmolino et al., 2010) reducing oxidative stress via PGC1a activation.

PGC1α down-regulation is known to occur in case of insulin resistance and diabetes, along with reduced mitochondrial oxidative phosphorylation and lower response to oxidative stress. All these features are present in FRDA patients. Accordingly, the PPAR-γ/PGC1α pathway deficit in correlation with low levels of frataxin in FRDA may negatively contribute to the severity of the FRDA phenotype. However, the link between PGC1α and frataxin is not yet established.

Currently, a clinical trial with pioglitazone is ongoing in France. This trial was prompted by previous observation of Dr. Rustin that pioglitazone increases fatty acid oxidation and mitochondrial function, and decreases ROS accumulation and inflammation (Babady et al., 2007).

5.6. *Protein replacement therapy*

Transactivator of transcription (TAT) from the human immunodeficiency virus is a short peptide able to efficiently guide the delivery of fused proteins across cellular and intracellular membranes. TAT fusion proteins containing a mitochondrial targeting sequence can translocate through the mitochondrial membranes, with appropriate processing and persistence of the fusion protein within mitochondria (Del Gaizo and Payne, 2003; Del Gaizo et al., 2003). A TAT/h-frataxin fusion protein able to localize within the mitochondria is currently being developed. Data indicate that the protein is incorporated *in vitro* and correctly processed.

This approach is interesting even if many question marks on the real application in patients remain. However the idea of a synthetic frataxin precursor protein to be directly injected in patients and targeting the mitochondria, where it is processed to generate the mature form of the frataxin, is not so far.

6. Conclusions

The unstable GAA trinucleotide repeat expansion affecting the FXN gene in patients is a dynamic mutation. Today, FRDA is the only known disease caused by this mutation. The development of treatments affecting the biology of unstable trinucleotide repeats still need to be expanded in parallel with our knowledge in this field. In this sense, drugs that have shown a capacity in increasing cellular frataxin, like rhu-EPO or HDAC inhibitors, need to be further investigated regarding their tolerability and safety, as well as doses and timing of administration. Moreover, considering that GAA repeats can both contract and/or expand, drugs stimulating the contraction of the repeat should be more considered for the next future.

The mitochondrial component clearly plays a crucial role in the pathogenesis of FRDA. However, the key-unanswered question still remains: which are the functions of frataxin? Missing this answer explains why we lack knowledge on molecular aspects underlying the disease, such as the pathways leading to cell damage, death and oxidative stress. Since the discovery of the FXN gene, mitochondrial dysfunctions have been considered as the primary defect in FRDA patients. Today, we can clearly state that the impairment of Fe–S cluster biosynthesis, iron metabolism, and oxidative stress play a central role in the pathogenesis of the disease. Strategies for the treatment of specific symptoms underlying frataxin deficiency (*i.e.*, antioxidants and iron chelators) are already in phases II and III.

However, further studies on the physiological role of frataxin in cells, in particular in DRG and dentate nucleus neurons, are needed to provide an explanation of their increased sensibility to degeneration in affected patients.

In conclusion, with the development of cell lines and transgenic mouse models recapitulating the pathological phenotype observed in FRDA patients, the research community will accelerate the screening of potential therapeutic molecules and provide important hints on the mechanisms underlying frataxin gene silencing and functions.

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