Development of a novel Frataxin (FXN) variant as a protein replacement therapy candidate for Friedreich Ataxia.

The main objective of this project is to develop a novel candidate for protein replacement therapy to treat Friedreich's ataxia (FA), with improved distribution to the central nervous system (CNS).

Recently, an FXN analogue, nomlabofusp (CTI-1601), consisting of a cell-penetrating peptide (CPP) and the FXN sequence, has been evaluated in Phase I (NCT04176991) and Phase II (NCT06447025) clinical trials. [1,2] The results of the first clinical trial showed higher amounts of FXN in skin, buccal cells, and blood. [1] However, there is still no data on its ability to cross the BBB, its effect on tissues most affected by the disease, and whether it alters other metabolic pathways. Although clinical development was halted due to problems in preclinical trials in primates, a Phase II clinical trial has recently been initiated.[2] The CPP selected in nomlabofusp is TAT, an arginine-rich peptide derived from the human immunodeficiency virus transactivator protein.[3, 4] This peptide has been used to internalize a multitude of different compounds, such as nanoparticles, small molecules, and even proteins.[5, 6] Although TAT has been described as capable of crossing the BBB,[7] its use has some drawbacks. On the one hand, TAT-modified cargoes are internalized primarily via endocytosis, ending up in lysosomal structures that lead to their degradation. On the other hand, once in the cytoplasm, TAT has a tropism for the nucleus, which can hinder the distribution of cargoes to other organelles such as the mitochondria.

Our initial hypothesis is that modifying FXN with an optimized blood-brain barrier (BBB) shuttle peptide will improve the efficacy of a replacement therapy treatment for Friedreich's ataxia. This will allow for better distribution of FXN to the affected tissues and, therefore, a reduction in the dose of protein delivered, minimizing potential side effects. To develop this hypothesis, we propose the use of peptidomimetic "A," developed at IRB Barcelona. This compound has been shown to increase protein transport and a monoclonal antibody, both in vitro and in several mouse models, and there are several groups of patents protecting its use. **Recently, a formulation aimed at treating a paediatric tumour based on this compound has just obtained orphan drug designation from the European Medicines Agency.**[(a-d)]

In this project, **we propose the binding of human frataxin (FXN1-210) to peptide A** through a disulfide bond, allowing the protein to be released into the cell cytoplasm so it can access the mitochondria to carry out its function. Furthermore, we also propose to analyse its biodistribution in different tissues of the mouse model of the disease (FXN^{I151F})[8]. The development of these general objectives will be carried out through the following specific tasks:

1. **Recombinant expression of the FXN1-210 protein**. Dr. Sánchez Navarro's laboratory already has experience in obtaining and purifying this protein.

2. Modification of the FXN1-210 protein with peptide A. Dr. Sánchez Navarro's laboratory has participated in the development of this peptide and has experience using it.

3. Characterization of the in vitro activity of the prepared proteins in relevant models of the disease. Prof. Joaquim Ros's laboratory has extensive experience using patient-derived cultures and has a characterized animal model of the disease [8]. Experiments will be

conducted to evaluate the amount and the effect of FXN-A protein on processes related to FXN metabolism. For example, the effect on nuclear factor erythroid 2-related factor 2 (Nrf2) and mitochondrial complexes I (CI) and II (CII) of the oxidative phosphorylation (OXPHOS) system will be assessed. The quantification of these markers provides information on the amount of FXN delivered and whether it is being processed within the cell. Nrf2 is a transcriptional regulator of pathways involved in the regulation of reactive oxygen species and plays an important role in the development of the disease due to its potential interaction with FXN and its inefficient activation in the case of FXN deficiency. The CI and CII complexes of OXPHOS play an essential role in mitochondrial respiration, which is impaired in FXN deficiency. Furthermore, metabolic activity and mitochondrial function assays using SeaHorse® technology allow for non-invasive characterization of the energy metabolism of cultured cells. This allows for the determination of parameters such as oxygen consumption (OCR), ATP-related respiration, maximum respiration, and reserve respiratory capacity. The group also has cell models of cardiomyocytes and dorsal root ganglia (DRG) neurons, which are highly affected in FA patients.

4. Characterization of transport across the BBB using *in vitro* models. These experiments will be carried out in Dr. Sánchez Navarro's laboratory, which has developed a BBB transport model.

5. Analysis of frataxin increase in tissues of the mouse model of the disease (FXN^{I151F}). Dorsal root ganglia, heart, brain, and cerebellum will be analysed. Dr. J. Ros's group has experience isolating and quantifying frataxin in isolated mouse tissues.[8]

TIMELINE

		Year 1			
		T1	T2	T3	T4
Aim 1	**Obtaining FXN**				
	Recombinant FXN expression				
	Synthesis of A				
Aim 2	**Obtention of A-FXN**				
	Conjugation of A and FXN1				
Aim 3	**Characterization of A-FXN activity *in vitro***				
	in vitro assays				
Aim 4	***In vitro* Evaluation of transport BBB models**				
	In vitro assays of transport across the BBB				
Aim 5	**Analysis of frataxin increase in tissues FA mouse model**				
	Tissue collection and analysis of frataxin levels by western blot				

(a) Inventors: E. Giralt, M. Teixidó, B. Oller-Salvia; Actively transported and protease-resistant peptides as BB shuttles and shuttle-cargo constructs. Priority date: 14/07/2014; Nº WO2015001015A1; Licensed to the company Gate2Brain;
(b) Inventors: M. Sánchez, M. Teixidó, E. Giralt. BBB-shuttle site-specific modification of antibody-based entities able to cross the Blood-Brain Barrier. Priority date: 01/08/2022; WO/2024/028282;
(c) Inventors: M. Sánchez, M. Teixidó, N. Gené, E. Giralt, A. Montero. Peptidic conjugates of SN38 useful in the treatment of cancer. Priority date: 28/09/2020; WO2022064052A1. Licensed to the company Gate2Brain.[1]
(d) https://www.irsjd.org/es/actualidad/noticias/1006/gate2brain-obtiene-la-designacion-de-medicamento-huerfano-de-la-EMA-para-su-tratamiento-pionero-en-cancer-pediatrico

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(2) Jive: An Open Label Extension Study to Assess the Long-Term Safety, Efficacy, Pharmacodynamics, Pharmacokinetics, and Tolerability of Subcutaneous CTI-1601 in Subjects With Friedreich's Ataxia. February 15, 2024. https://clinicaltrials.gov/study/NCT06447025.

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