Feasibility of Gene Therapy for Friedreich Ataxia Associated Cardiomyopathy in Non-Human Primates: Evaluation of Delivery Route, Biodistribution and Expression Following AAVRh.10-hFXN Administration

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Disclosure : BB, HP, RGC and MG have equity in Adverum Biotechnologies Inc.

BACKGROUND

Friedreich ataxia (FA) is a rare autosomal recessive disease (prevalence 1-9/100,000) characterized mainly by spinocerebellar and sensory ataxia, as well as hypertrophic cardiomyopathy which is the main cause of premature death in FA patients. To date, no treatment exists for stopping or slowing the progression of FA cardiomyopathy.

Most of FA patients (95%) are affected by a non-coding mutation in the first intron of the *Fxn* gene, characterized by the expansion of triple-nucleotide repeats (GAA) and resulting in the transcription silencing of *Fxn* and FXN deficiency (between 5 to 30% of normal protein level).
Frataxin (FXN) is a ubiquitous mitochondrial protein, involved in iron-sulfur cluster biosynthesis, which are inorganic cofactors important to many enzymes, proteins and biological functions. FXN deficiency results in mitochondrial and bioenergetic impairment, iron metabolism dysregulation and mitochondria overload, and eventually cellular dysfunction and/or cell death. FXN deficiency affects more severely cardiomyocytes, sensory neurons and neurons in the cerebellum and dentate nucleus.
Importantly, the parents of FA patients are most often asymptomatic heterozygote carriers, expressing around half the normal FXN protein level. This suggest that a partial increase of the cellular level in FXN could be therapeutic.

Previously, we demonstrated the therapeutic proof-of-concept of AAV-mediated cardiac gene therapy in the *Mck Fxn* knock-out mouse model (*Perdomini and Belbellaa et al 2014 Nat Med*). In this *Mck* model, we demonstrated that the re-expression of FXN could prevent and reverse the cardiac phenotype, which was driven by the normalization of the Fe-S biosynthesis and activity of the Fe-S enzymes, the correction of iron metabolism and mitochondria bioenergetics.

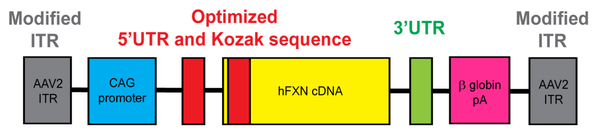
In a dose response study, we have demonstrated subsequently the correlation between cardiac vector biodistribution and the amplitude of the functional rescue (Belbellaa et al 2018 Hum Mol Gen). Full correction of the heart function and hypertrophy is achieved when half the cardiomyocytes are transduced with AAVRh.10-hFXN-HA vector and express FXN levels as low as 57% the endogenous level.

More recently, we showed that FXN overexpression is well tolerated when below 10-20-fold the normal endogenous level in WT and *Mck* mice, but toxic for mitochondria and cardiomyocytes when higher (*Belbellaa et al 2019 manuscript submitted*). All together, these studies have defined the therapeutic thresholds and index for cardiac gene therapy in FA mouse models.

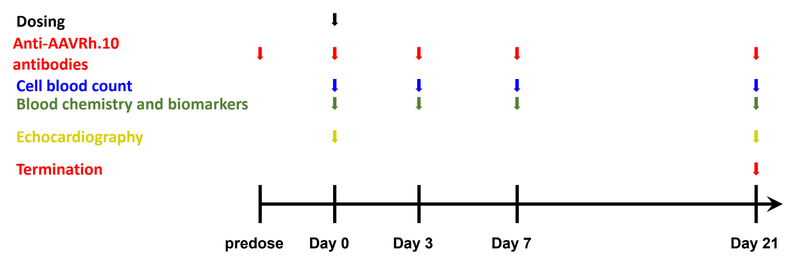
OBJECTIVES AND STUDY DESIGN

Objectives: To determine the optimal route and dose of administration of AAVRh.10-hFXN vector, in adult NHP, which results in heart vector biodistribution and expression in the heart, at levels predicted to be therapeutic.

Vector design: Optimization of the vector construct at the transcription and translation levels to accommodate for the lower vector expression in NHP compared to mouse models. The AAVRh.10-hFXN was manufacture in HEK293 system using the triple transfection method.



Experimental design: Three groups of adult cynomolgus monkey, seronegative for AAVRh.10, received the vector either by saphenous-intravenous (i.v.) infusion (~$6x10^{12}$vg/kg, n=3), retrograde coronary infusion (RCI) through the coronary sinus ($1x10^{13}$vg/kg, n=3) or directly by multiple epicardial injections (Ep) (2 sites; $2x10^{12}$vg each in 65-125µL volume, n=3).



BIOANALYTICALS

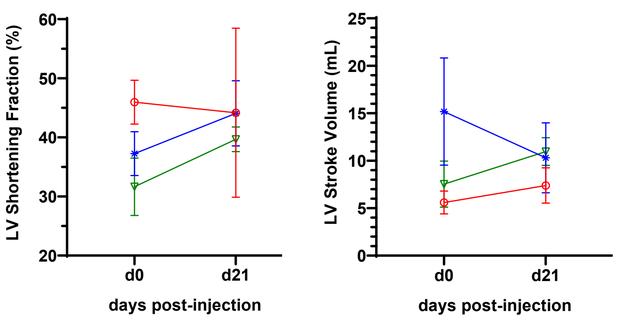
Vector biodistribution analyzed by qPCR
- Heart: left and right ventricles , septum, left and right atria
- Peripheral and major organs

For the i.v. injected group only, FXN protein levels in the heart
- ELISA assay to quantify FXN level
- Comparison to normal mouse, NHP and human levels
- WB analysis of FXN forms, i.e precursor, intermediary and mature

RESULTS

In-life assessment of tolerability for three routes of vector administration

No functional cardiotoxicity following AAVRh.10-hFXN vector administration

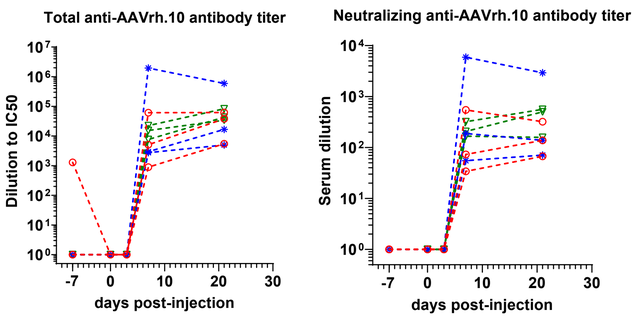


Echocardiography analysis of left ventricle (LV):

- LV shortening fraction and stroke volume. Data reported as mean±SD.
- Similar results for LV diameters at end-systole/diastole, septum thickness, LV anterior wall thickness (data not shown)

Normal body weight, heart rate, blood pressure.

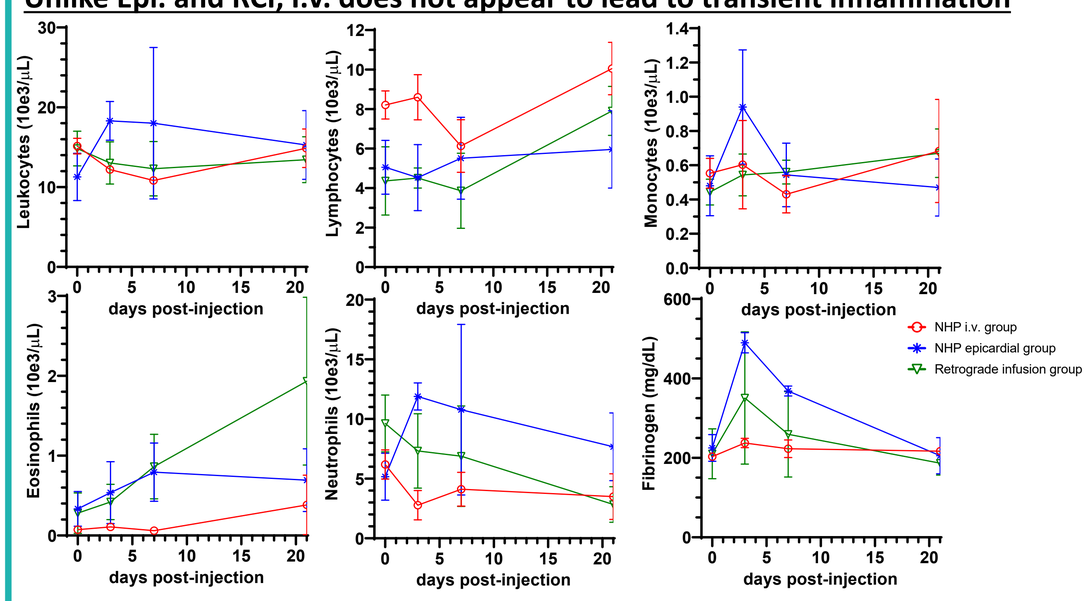
Similar humoral response against AAVRh.10 for all delivery routes



Longitudinal analysis of serum titer for total anti-AAVRh.10 antibodies and neutralizing antibodies.

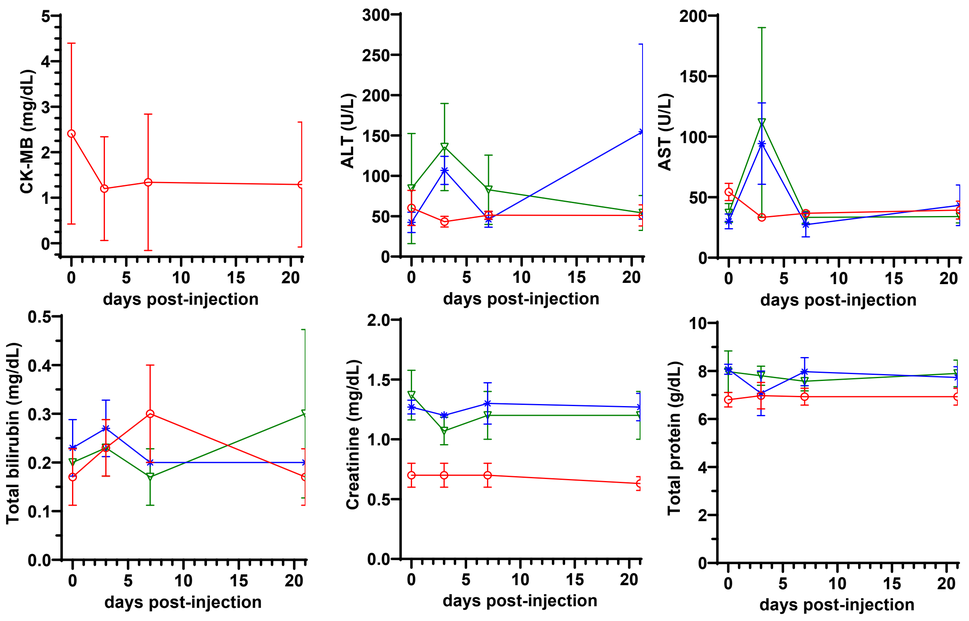
Each animal is represented by a separate curve.

Unlike Epi. and RCI, I.V. does not appear to lead to transient inflammation



Longitudinal analysis of total blood cell counts, hematocrit, coagulation and blood chemistry (Data reported as mean±SD).
- Retrograde and epicardial injections results in transient increase in inflammatory cells and fibrinogen blood concentrations.
- For all routes, normal hematocrit (MCV, MHC, MCHC, platelets), coagulation (APTT, PT) and blood chemistry (K^+, Cl^-, Ca^{2+}, $PO4^{-3}$).

Biomarkers of heart, liver and kidney injury and metabolism



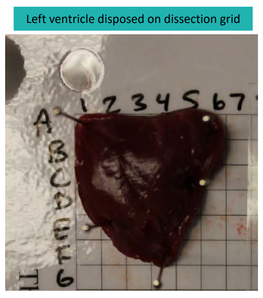
Analysis of blood biomarkers indicative of heart, liver and kidney injury and metabolism anomalies (Data reported as mean±SD).
- Biomarkers of heart and muscle injury (CK-MB, TnT-I) were only measured in i.v. group and are normal
- Biomarkers of liver injury, ALT and AST, transiently increased in epicardial and retrograde groups, but not for γ-GT, ALP, ALB, Glbn
- Biomarkers of kidney injury (creatinine, total protein, urea-nitrogen) normal in all groups
- Normal blood concentration of glucose, triglycerides, cholesterol.

I.V. injected NHP: AAVRh.10 vector biodistribution analyzed by Taqman qPCR

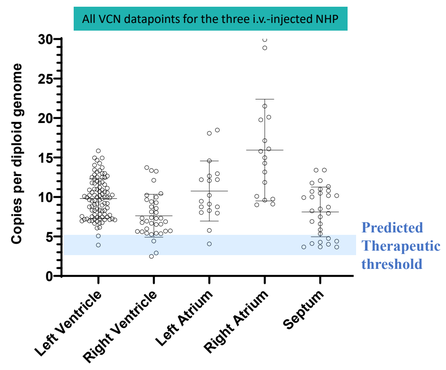
Mapping of the vector distribution in the heart

NHP hearts were dissected into five anatomical subregions: left and right atria and ventricles, and septum.
Each subregion was further subsampled into 1cm square tissue pieces, as illustrated below..
The vector copies number (VCN) was quantified by qPCR, in a representative numbers of samples, and graphed below.

Left ventricle disposed on dissection grid



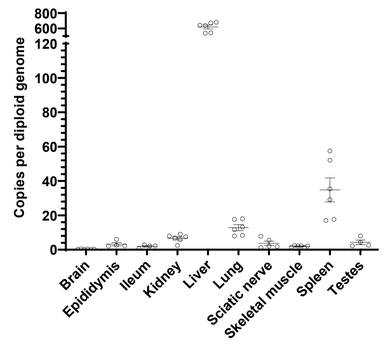
All VCN datapoints for the three i.v.-injected NHP



Peripheral organs biodistribution

The VCN was quantified by qPCR in major organs of interest.

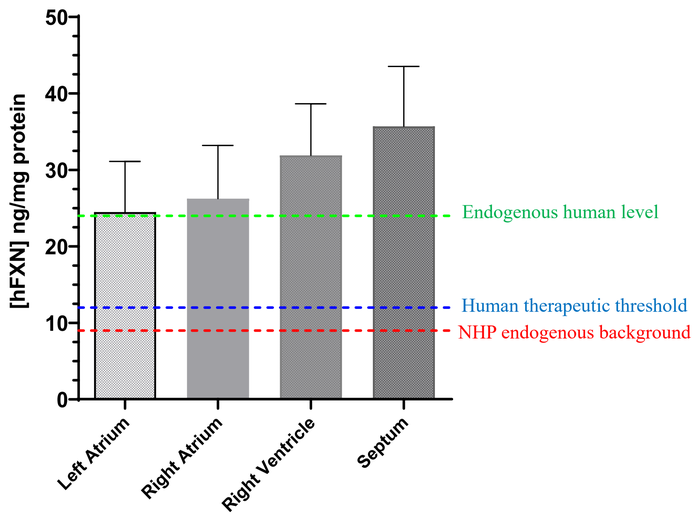
All VCN datapoints for the three i.v.-injected NHP



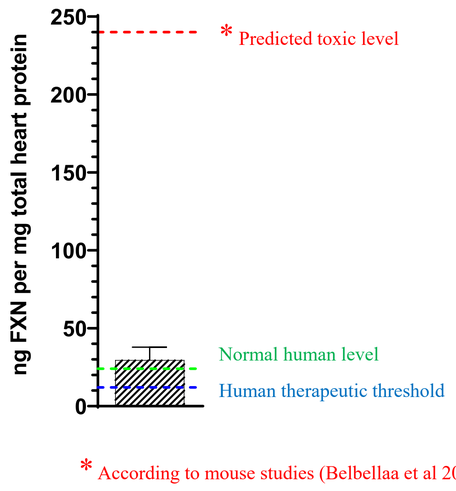
AAVRh.10-hFXN vector DNA was detected throughout the heart of all i.v.-injected NHP, at levels ranging mostly between 5 to 20 vector copies per diploid genome.
This is well beyond the predicted therapeutic threshold identified in the proof-of-concept and dose-response mouse studies.
However, i.v. delivery of AAVRh.10-hFXN does not result in meaningful vector biodistribution in the brain, where FXN deficiency leads to the ataxia phenotype.

I.V. injected NHP: cardiac FXN protein level analyzed with ELISA assay

Average FXN protein tissue concentration ([FXN]) throughout the heart



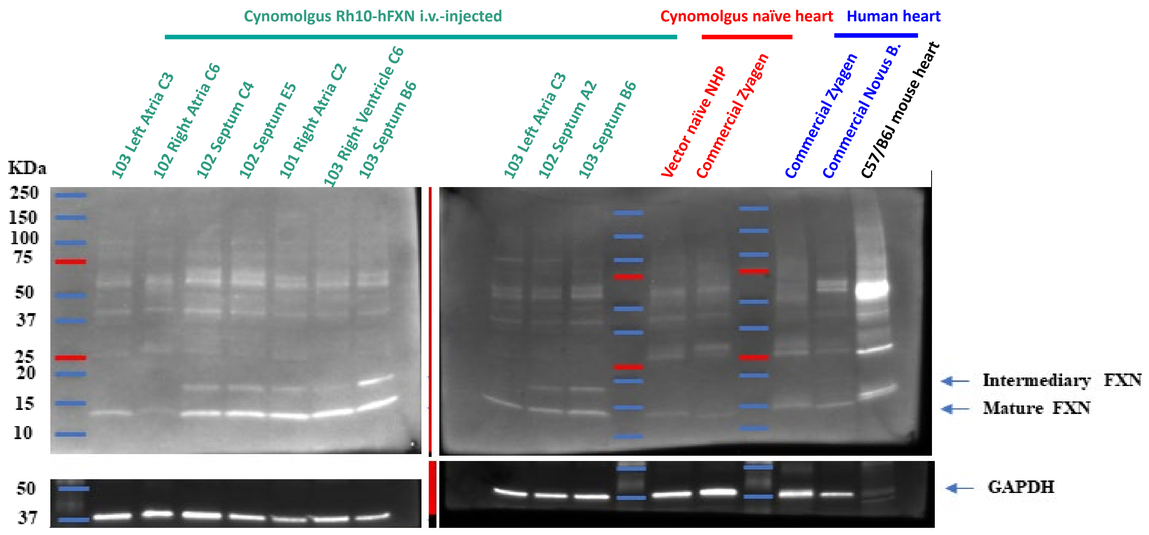
Average [FXN] in NHP heart and toxic levels



ELISA assay analysis of FXN tissue concentration ([hFXN]) from total heart proteins extracts, normalized to total protein concentration and reported as mean±SD.
Commercial human heart total protein: Zyagen HT-801 and Novus Biologicals NB820-59217. Commercial cynomolgus heart total protein: Zyagen, KT-801
Partial cross-reactivity with endogenous monkey FXN, with background level at 9ng/mg in naïve heart samples.
Detectable increase of FXN protein level throughout the heart, at levels close to human normal level and far below the predicted toxic overexpression levels.

I.V. injected NHP: hFXN protein mitochondrial import and processing

Western-blot analysis of FXN expression and processing from intermediary to mature form



4-20% SDS-PAGE, 15µg total protein per well, immunoblotting anti-FXN (IGBMC, 4F9 monoclonal, 1/10,000) and anti-GAPDH (SantaCruz, Rabbit polyclonal 1/3,000).
NHP i.v. injected with AAVRh.10-hFXN display detectable increase of FXN protein levels, with some accumulation of FXN intermediary form but not precursor.
Normal endogenous level of FXN in the heart of naïve NHP is similar to human heart, while heart from C57/B6J WT mice display much higher levels of FXN.

CONCLUSIONS

- **AAVRh.10-hFXN intravenous (i.v.) injection in adult cynomolgus monkey appears to be well tolerated at 21 days post-administration.**
- **I.V. administration safety profile at 21 days seems overall preferable to retrograde coronary or epicardial injection routes.**
- **I.V. dose of $6x10^{12}$vg/kg achieved widespread vector distribution in the heart, at levels predicted to be therapeutic, but not in the CNS.**
- **Increased FXN level correctly targeted to the mitochondria throughout the heart, at safe levels close to normal human level.**
- **Overall, i.v. administration of AAVRh.10-hFXN at the dose of ~$6x10^{12}$vg/kg would be the route and dose of choice to develop future gene therapy protocol to address Friedreich ataxia cardiomyopathy.**