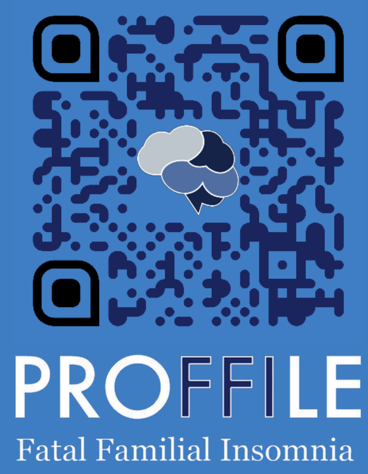

PRODROMAL BIOMARKERS IN FATAL FAMILIAL INSOMNIA: A LONGITUDINAL STUDY IN HUMANS AND MICE

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Research questions and aims

Fatal Familial Insomnia (FFI) is a rare genetic prion disease characterized by a progressive sleep disturbance, leading to a complete inability to achieve restorative sleep. It typically manifests in mid-adulthood, with a rapid decline in cognitive and physical functions, invariably leading to death. FFI is caused by a point mutation in the *PRNP* gene encoding the cellular prion protein (PrP^C), resulting in the substitution of asparagine with aspartic acid at codon 178 (D178N) in association with methionine at polymorphic site M129V. Molecular testing can identify individuals at risk of FFI decades in advance of symptoms onset. This makes such a subgroup of individuals particularly suited for the preventive therapies. However, the highly variable age of disease onset and the lack of defined prodromal biomarkers make it challenging to design efficacious preventive trials and to monitor the progression of the disease. **The overall aim of this project is to identify reliable biological markers predictive of disease onset and progression in carriers of the FFI mutation**. Brain-derived proteins, such as neurofilament light chain (NfL), tau and misfolded PrP^C (prion), have been proposed as prodromal biomarkers, but their validity remains to be established. In this project we aim to:

(1) establish a large collection of longitudinal biological samples from carriers of the FFI mutation and non-carrier relatives, through the collaboration with patient associations;
(2) test potential biomarkers in easily accessible biological fluids, such as urine and plasma;
(3) carry out proteomic and metabolomic studies on extracellular vesicles (EVs) of brain origin to identify new potential biomarkers;
(4) develop a new animal model of FFI and correlate the biomarker levels with the progression of behavioral deficits and neuropathological changes.

This poster illustrates the development of a sensitive protein misfolding cyclic amplification (PMCA) protocol to detect FFI prions in urine and plasma.

Means and methods

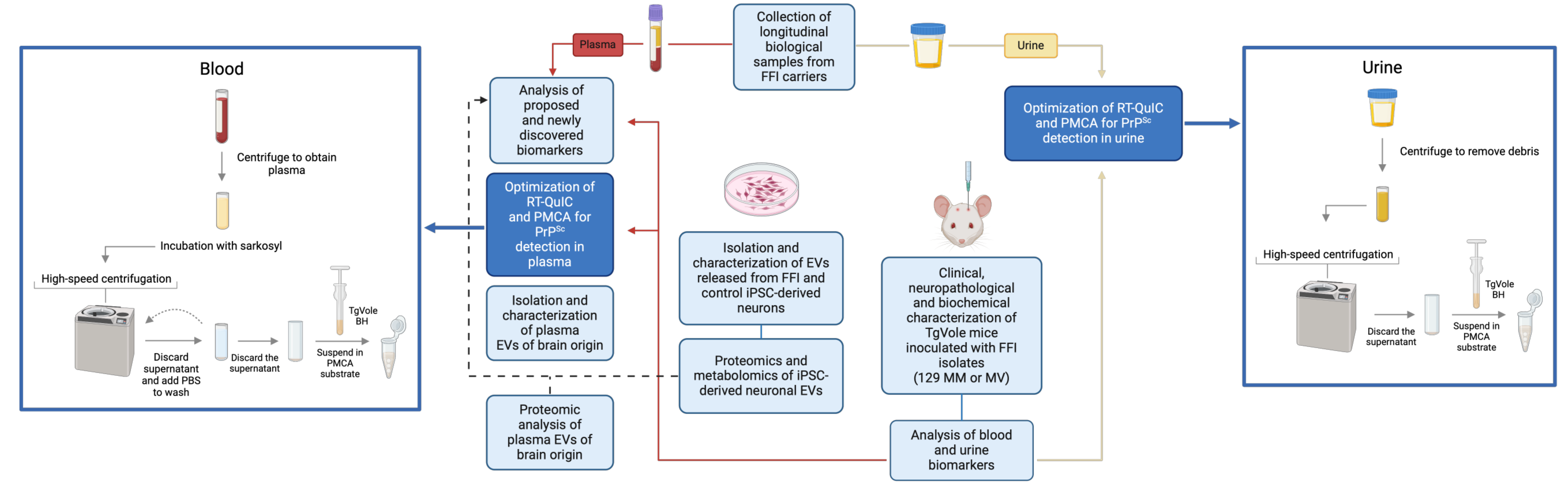

Figure 1: Graphical representation of the prinicpal methods implemented by the consortium. The main methods described show the PMCA optimization that is detailed below.

PMCA protocol optimization for the detection of prions in urine and blood: Before testing samples collected from FFI mutation carriers, the PMCA technique was optimized to detect FFI prions in both urine and plasma. The parietal cortex of two FFI brains, either with MM or MV polymorphisms at *PRNP* codon 129 (FFI_129MM and FFI_129MV), was collected along with the frontal cortex of a patient with Alzheimer's disease (AD) with the MM polymorphism at codon 129 (AD_129MM), as negative control. Brains were homogenized (BH) at 10% weight/volume in lysis buffer and 50 µL were serially diluted from 10^{-3} to 10^{-12} in 450 µL of PBS 1X.

Urine and blood samples were collected from a healthy subject. Urine were centrifuged at 2500 g for 15 minutes to remove debris, while the blood at 2000 g for 10 minutes to obtain the plasma. For the urine analysis, 100 µL of each FFI-BH or AD-BH were serially diluted from 10^{-3} to 10^{-12} in 900 µL of urine; while for plasma analysis 50 µL of each FFI-BH or AD-BH were serially diluted in 450 µL of plasma and each sample was also incubated with 450 µL of sarkosyl 20% for 10 minutes. Dilutions in urine and plasma were then processed with high-speed centrifugation at 47.000 g for 1 hour at 4 °C and then the supernatant was removed. The pellet was directly resuspended in 90 µL of substrate and transferred to 0.2 mL PCR tubes. All the PMCA experiments were performed using the BH of transgenic mice expressing the bank vole PrP^C with methionine at codon 109 (TgVole 1X) as substrate. Samples were then supplemented with three teflon beads and 0.05% digitonin, then subjected to PMCA analysis. Each PMCA cycle consisted of 29 minutes and 40 seconds of incubation at 37°C followed by a 20 seconds pulse of sonication for a total of 24 hours for each round.

Outcomes and key results

1. Protocol optimization

The amount of prions in FFI is much lower than other prion diseases and their detection is overall extremely challenging. Therefore, this poster will focus on the optimization of the PMCA for the detection of prions in two easily accessible peripheral tissues, urine and plasma. The protocol was set up by diluting FFI brain extracts into both of these matrices. All the FFI_129MM dilutions performed in PBS were successfully amplified already from the first round of PMCA, while the FFI_129MV dilutions at the second round of PMCA. The dilutions of AD_129MM BH remained all negative (Figure 2). Also for the dilutions performed in urine, the two FFI_BH were successfully amplified within the first two rounds of PMCA, while the AD_129MM BH dilutions remained always negative (Figure 3). All the FFI-BH dilutions in plasma were successfully amplified in the first round of PMCA, while the AD_129MM BH dilutions remained always negative (Figure 4). By means of PMCA, we were able to achieve a detection for FFI-prions up to the 10^{-12} dilution in all the tested tissues.

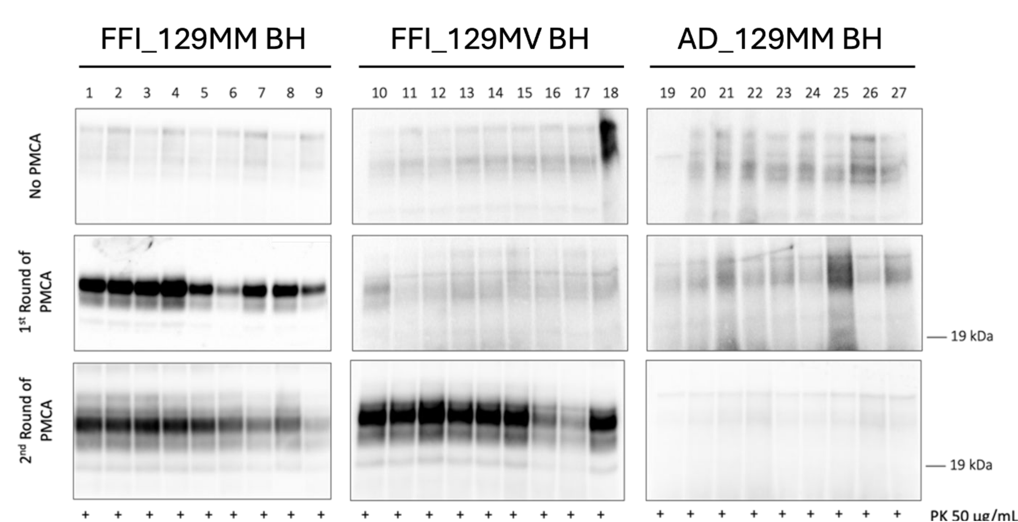

Figure 2: Western blot of the PMCA products spiked in PBS. 1-9 are the FFI_129MM BH diluted from 10^{-4} to 10^{-12}; 10-18 are the FFI_129MV BH diluted from 10^{-4} to 10^{-12}; 19-27 are the AD_129MM BH diluted from 10^{-4} to 10^{-12}.

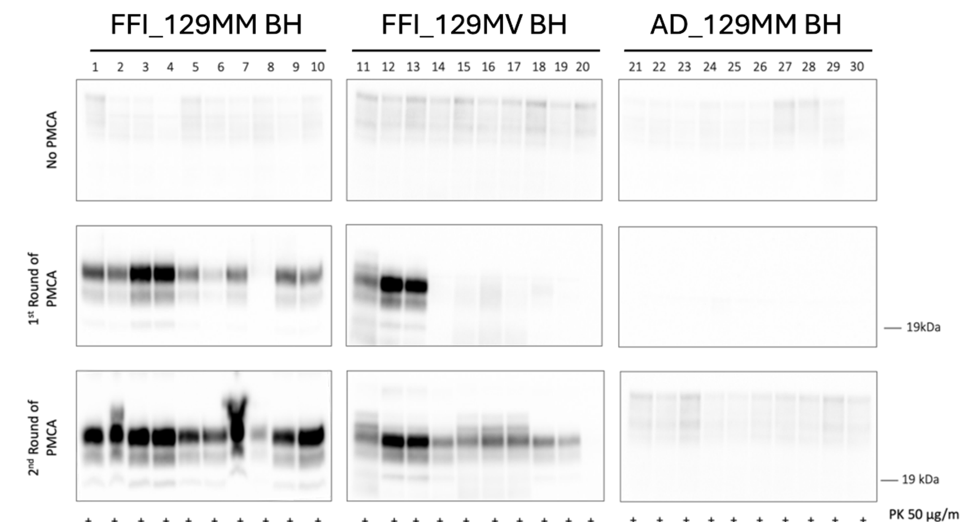

Figure 3: Western blot of the PMCA products spiked in urine. 1-10 are the FFI_129MM BH diluted from 10^{-4} to 10^{-12}; 11-20 are the FFI_129MV BH diluted from 10^{-4} to 10^{-12}; 21-30 are the AD_129MM BH diluted from 10^{-4} to 10^{-12}.

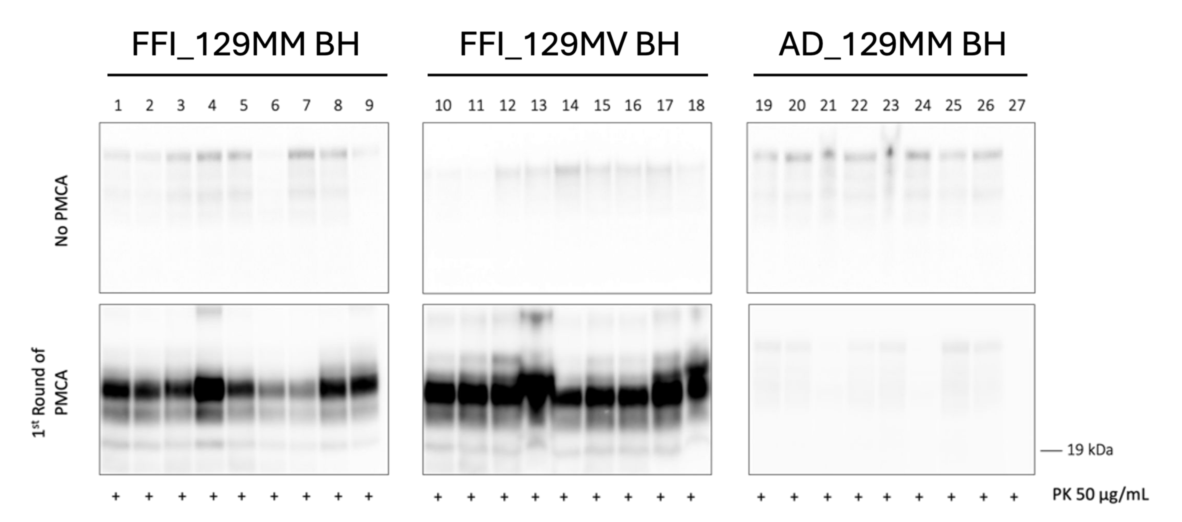

Figure 4: Western blot of the PMCA products spiked in plasma (high-speed centrifugation). 1-9 are the FFI_129MM BH diluted from 10^{-4} to 10^{-12}; 10-18 are the FFI_129MV BH diluted from 10^{-4} to 10^{-12}; 19-27 are the AD_129MM BH diluted from 10^{-4} to 10^{-12}.

2. Urine analyses

Once the protocol was optimized, we performed PMCA analysis of urine and plasma samples collected from FFI mutation carriers and healthy controls. Regarding the urine analyses, we tested samples collected from a total of n=15 mutation carriers at different stages of the disease and from a total of n=17 healthy subjects. After 3 rounds of PMCA, 2 out of the 15 urine samples collected from the mutation carriers tested positive, one from a patient in the clinical phase of the disease and one from a patient that was still asymptomatic. All the urine collected from the healthy subjects remained negative for all the rounds of PMCA.

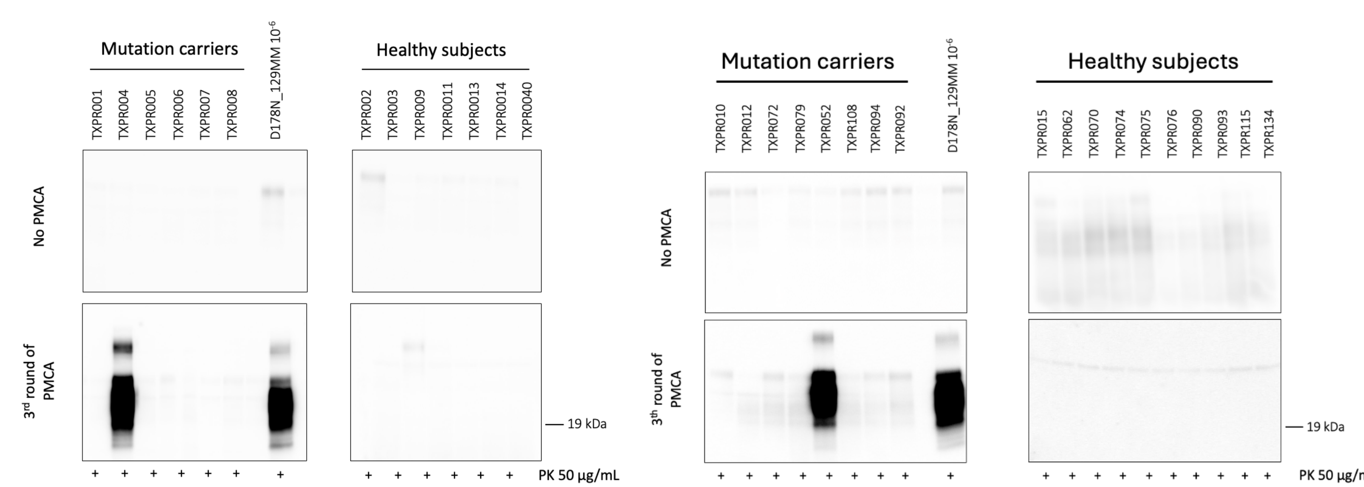

Figure 5: Western blot of the FFI mutation carriers urine analyzed by PMCA. On the left panel there is the first set of urine samples of both mutation carriers and healthy controls analyzed by PMCA, while on the right part the second set of urine. The 10^{-6} dilution of the FFI-BH was used as positive control in all the experiments.

3. Plasma analyses

Moreover, we were able to analyze one plasma sample collected from a patient in an advanced disease stage along with two samples from healthy donors. The FFI plasma yielded a positive result as well, already from the second round of PMCA, with the plasma samples collected from healthy subjects remaining negative.

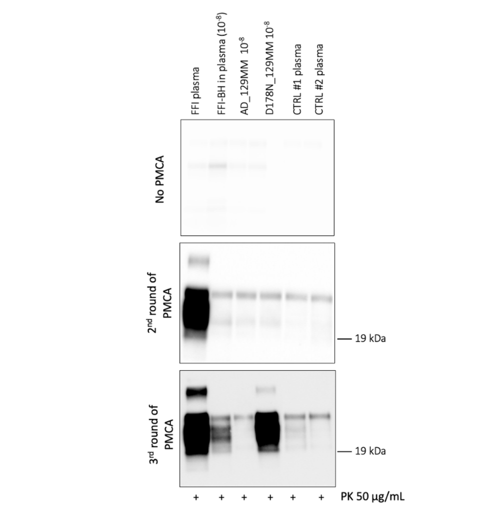

Figure 6: Western blot of the FFI patient plasma analyzed by PMCA. The 10^{-6} dilutions of the FFI-BH spiked in healthy plasma and directly added to the substrate were used as positive control. The 10^{-6} dilution of the AD-BH was used also as negative control.

Impact of the work and future steps

The collection of different longitudinal plasma and blood samples has enabled numerous studies and analyses. Further assessment of the specificity and sensitivity of the candidate biomarkers is planned. The PMCA technique, in particular, enabled for the first time the detection of small amounts of FFI prions spiked in biological matrices as urine and plasma, without any interference and maintaining a high sensitivity. Additional urine and plasma samples will be analyzed by PMCA to better assess the robustness of our results and their validity. Detecting prions in both urine and plasma samples is a critical step towards achieving the project's ultimate goal of identifying a reliable and specific biomarker of the disease. PMCA will then be further optimized for testing additional peripheral tissues from mutation carriers at different disease stages. This technique will also be employed to test the longitudinal urine and blood samples collected from the FFI mouse model that has been already successfully characterized.

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